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New mtDNA and Isotopic Evidence on Late Pleistocene Cave Bears in the Balkans: the Case-study of Magura Cave, NW Bulgaria

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Abstract: Recent genetic studies have shed light on the phylogeography of cave bears; however, their paleoecology and their diet are still debated, and data from south-eastern Europe are still scarce. Magura Cave, in north-west Bulgaria, has delivered rich faunal assemblages from the Late Pleistocene. The chronology of the excavated area spans from ca. 35 kya to more than 50 kya; the oldest stratigraphic layers being associated with final Middle Palaeolithic tools. The fauna comprises herbivores and carnivores, and potentially different taxa of cave bears, the dental remains of which also showed different tooth morphotypes, suggesting the co-existence of different dietary adaptations. We investigated the mitochondrial DNA (mtDNA) lineages of the cave bears from Magura Cave as well as the stable carbon and nitrogen isotope composition of the faunal assemblage. Our data revealed that, regardless of the tooth morphotypes, only maternal lineages of *Ursus ingressus* were present in Magura Cave. Interestingly, one specimen with *Ursus arctos* mtDNA was also found, showing a clear carnivore diet. In contrast, the *U. ingressus* specimens had a predominantly herbivorous diet. The tooth morphotypes were associated with significantly different $\delta^{13}C$ values, suggesting different dietary adaptations.

Key words : Cave bear, aDNA, stable isotopes, $\delta^{13}C$, $\delta^{15}N$, $\delta^{18}O$, phylogeny, diet, morphology

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Introduction

Diversity and ecology of European cave bears

The cave bear is one of the most abundant and spectacular Late Pleistocene megafaunal species. Genetic and morphological evidence revealed that Late Pleistocene cave bears evolved from Middle Pleistocene *Ursus deningeri* and developed into at least three different lineages. *Ursus ingressus*, a possible synonym of *U. kanivetz* (see Spassov et al. 2017), inhabited south-eastern and central Europe as well as the Ural. *Ursus spelaeus* lived mainly in Western Europe, although its remains were also found in the Altai; it included three subspecies: *U.s. spelaeus*, *U.s. ladinicus* and *U.s. eremus* – the two last subspecies being smaller cave bear forms confined to the high alpine caves in Austria and Italy. The third group of large cave bears, *Ursus kudarensis* Baryshnikov, 1985, was found in the Caucasus and the Yana River region in Eastern Siberia (Stiller et al. 2014, Baca et al. 2016, Knapp 2019, Van Heteren & Figueirido 2019). Additionally, in England, the pre-Caucasus and the Balkans, other smaller forms of cave bears have been identified, i.e. *Ursus savini* and *Ursus rossicus* (or *U.s. rossicus*) (see Andrews 1922, Baryshnikov 2007, Spassov et al. 2017), the phylogenetic positions of which is still debated (Baryshnikov 2006, Spassov et al. 2017). From 50 thousand years ago (kya), the cave bear went through a slow loss of genetic diversity and its habitat range started contracting from east to west (Stiller et al. 2010, 2014). Several factors have been proposed to explain this decline, including climatic deterioration, reduced food resources, stronger competition for cave at a time of human population expansion and increased predation pressure (Knapp 2019, Terlato et al. 2019). The last populations of *U. spelaeus senso latu* (*s.l.*) probably survived in fragmented areas of suitable microclimatic conditions in Europe (Baca et al. 2016) until their eventual extinction around 24 kya (Terlato et al. 2019).

In the Balkan region, cave bear presence during the Late Pleistocene is attested in numerous caves but secure identification to the species level is often lacking. A mtDNA analysis performed on Late Pleistocene (ca. 50 and 40 kya) remains from the cave of Peştera cu Oase (Romania) revealed the presence of *U. ingressus* (Quilès et al. 2006, Stiller et al. 2014). In Serbia, previous studies of Late Pleistocene assemblages from 35 caves resulted in an early identification of "*U. spelaeus*"; however, the recent re-assessment of the assemblage from one of the caves allowed a new identification to *U. ingressus* (Cvetković & Dimitrijević 2014). In northern Greece, excavations at Loutra Arideas Cave delivered the remains of cave bears dated to *c.* 38 kya, identified as *U. ingressus* by morphological analysis (Dotsika et al. 2011). In Bulgaria (Fig. 1), the morphological analysis of macrofaunal remains from Kozarnika Cave showed the presence of *Ursus spelaeus s. l.* in the Late Pleistocene sequence (MIS

Fig. 1. Map of Bulgaria with the location of the caves mentioned in the text: 1. Magura. 2. Kozarnika. 3. Mishin Kamik. 4. Temnata.

6/7 – 45 kya) (Guadelli et al. 2005, Tables 1 and 2; Sirakov et al. 2010, 5, Table 1). The palaeontological study of the Middle Palaeolithic sequence from Temnata Cave revealed the presence of Ursidae identified as *Ursus deningeri* in the lower stratigraphic layers and as *Ursus spelaeus (s.l.)* in the upper layers (Guadelli & Delpech 2011: 50-51). At Mishin Kamik (Fig. 1), taxonomic identifications based on morphological analysis revealed the presence of *Ursus cf. ingressus* in the Late Pleistocene layers (Gurova et al. 2017, 2018).

Cave bear diet has been originally described to be highly reliant on plant foods (Kurten 1972, Bocherens et al. 1994, Mattson 1998, Rabeder et al. 2000, Van Heteren et al. 2016, Bocherens 2019) but more recent studies have proposed a more omnivorous diet (Pinto-Llona & Andrews 2003, Richards et al. 2008, Figueirido et al. 2009, Peigné et al. 2009, Robu et al. 2018). In particular, cave bears from two Romanian caves (Pestera cu Oase and Ursilor) have been argued to be omnivorous (Richards et al. 2008, Robu et al. 2013), although this hypothesis has been challenged and the interpretations remain contradictory (Bocherens et al. 2014, Bocherens 2015, Krajcarz et al. 2016). More recent studies have emphasized that cave bear diet was probably more flexible than originally thought, especially during the pre-dormancy period, and that it probably fluctuated depending on sex, age, season, altitude, local vegetation, or the presence of other bear species (Münzel et al. 2014, Robu et al. 2018, Peigné & Merceron 2019, Perez-Ramos et al. 2019, Ramirez-Pedrasa et al. 2019).

Previous diet studies have mainly focussed on northern and central Europe, while in southern Europe (Feranec et al. 2010, Ramirez-Pedrasa et al. 2019), notably in south-east Europe and the Carpathians, the ecology of the cave bear remains comparatively less documented. Studies already published concern Macedonia (Dotsika et al. 2011), the Romanian Carpathians (Richards et al. 2008, Robu et al. 2013, 2018), and a preliminary analysis performed on Magura Cave assemblage (Ivanova et al. 2013, 2016). The excavation at Magura Cave provided a rich faunal assemblage (NISP = 563) (Ivanova et al. 2016), amongst which a significant amount of cave bear remains. Combined mtDNA and morphological analysis carried out on two individuals suggested that the form that lived in Magura area between 36 and 50 kya was *Ursus ingressus* (see Ivanova et al. 2016). The palaeontological analysis further revealed the presence of different tooth morphotypes in the cave bears of Magura and suggested that this could be due, to one degree or another, to the presence (in some layers) of *U. spelaeus s. str.* (Ivanova et al. 2016).

In this study, a mtDNA analysis was carried out on a larger sample set with the aim to investigate further cave bear mtDNA biogeography and determine whether lineages other than *U. ingressus* were present in the Balkan region. The mtDNA results were compared with tooth morphotypes with the purpose to check whether these morphotypes corresponded to different taxa or were related to morphological polymorphism of the same species. An isotopic analysis using the stable carbon and nitrogen isotopes in bone collagen was performed on Magura Cave faunal assemblage to document young and adult cave bear diet, and compare dietary habits, tooth morphotypes and potential niche partitioning between the taxonomic groups identified by mtDNA analysis. The stable oxygen and carbon isotope compositions of tooth enamel carbonates of two herbivores were also used to help reconstruct the local palaeo-environment.

Magura Cave and its palaeo-environment (60-10 kya)

Magura Cave is located in western Stara Planina (Balkan Mountains), in north-western Bulgaria (43.7281, 22.5826 decimal degrees, 375 m a.s.l.) (Fig. 1). The cave formed *c.* 15 million years ago in Lower Cretaceous (Aptian) Urgonic limestones (Angelov et al. 1995). In 2011–2012, trench excavations were carried out and preliminary results were published. These focussed on two trenches (I and III) located in the "Triumphant hall", at 25 m from the entrance (Ivanova et al. 2012, 2013). Magura Cave was included in a larger project entitled "Late Pleistocene Environments and Human Adaptations to Climate Change in the Balkans" and a detailed paper, focussing on the significant results of a pluridisciplinary study, was published (Ivanova et al. 2016). The results of this research, which focussed on the same two trenches (Trench I – layers 1 to 45; Trench III – layers 3 to 10), provide a stratigraphical, chronological and palaeo-environmental framework to our study.

The cave bear findings and associated archaeological assemblages analysed in this paper belong predominantly to the Late Pleistocene and cover the period corresponding to the Middle to the Upper Palaeolithic ca. >60–12 kya (>50 to ca. 35 kya for the radiocarbon dated bear remains from Magura Cave) (Ivanova et al 2016). The stable isotope records from speleothem from SW Romania (Constantin et al. 2007) give an overview of the climatic fluctuations for this timespan in a region adjacent to Magura Cave. The δ^{18}O speleothem records suggest warming from 60 to 57 kya, a subsequent overall cooling trend up to 42 kya, followed by an extreme cold phase between 38 and 35 kya and a second one during the Last Glacial Maximum at 18 kya (Constantin et al. 2007).

During the period covering Marine Isotope Stage 3 (MIS 3 ~57–29 kya) and roughly corresponding to the possible age of the studied fossils (Ivanova et al 2016), the vegetation in low mountain ranges such as those where Magura Cave is situated, consisted of parkland boreal forests (with *Picea abies*, *Pinus sylvestris*, *P. cembra*, *Larix decidua*); the patches of boreal vegetation were accompanied by some temperate deciduous tree taxa (*Ulmus*, *Salix* and *Alnus*) (Feurdean et al. 2014). The pollen analysis of the hyena coprolites from layer 7-10, Trench III, confirmed this picture for the local vegetation surrounding Magura Cave (Ivanova et al. 2016). The high-resolution studies of dry loess steppe areas south of 45°N, where the study area was also situated, did not recognise millennial-scale vegetation fluctuations and this suggests that temperature and precipitation fluctuations during the MIS 3 were of relatively low magnitude (Feurdean et al. 2014).

Magura Cave bears

The excavation campaigns of 2011 and 2012 in Magura Cave delivered more than 1400 animal remains. Among the identified specimens (NISP = 563), almost 80 % belong to the family Ursidae. Their palaeontological analysis included the study of sex, age distribution and mortality patterns (Ivanova et al. 2016). Sex determination was performed on canines (n = 35) and revealed an almost equal representation of both males and females. The age distribution of the bears, estimated from tooth eruption and attrition, comprised all ontogenetic stages from cubs to senile individuals, which suggests a hibernation mortality pattern: the bears died of non-violent causes, likely starvation.

Fig. 2. First metatarsals of *Ursus ingressus*. a. Replica of FM 3449 (M3/1359), which was tested for mtDNA. b. FM 2921 (M1/283). Scale bars: 20 mm.

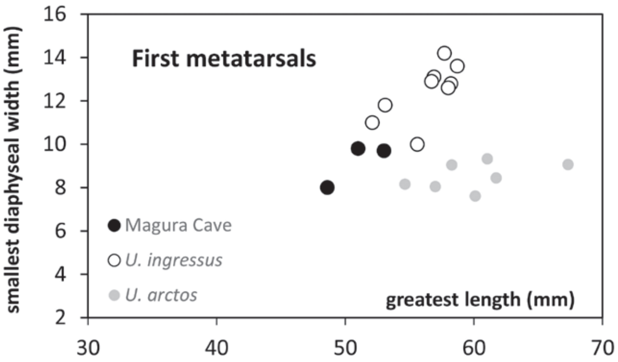

Fig. 3. Comparison of the greatest lengths and smallest diaphyseal widths of the small, convex, bear metatarsals from Magura Cave with those of *Ursus ingressus* and *U. arctos*. The *U. ingressus* data are mean values of samples from different caves, while for *U. arctos* and the metatarsals from the Magura Cave the data are those measured on the different specimens individually (cf. Suppl. Data 4 for the raw data and references).

The palaeontological analysis also included the study of metapodial morphology and tooth morphotypes, which revealed a high variability in the morphology of the bears of Magura Cave (Ivanova et al. 2016). The spectrum of tooth morphotypes observed at Magura Cave is quite wide and seems to cover the morphological features of both *U. spelaeus* and *U. ingressus*, suggesting *a priori* the presence of the two cave bear species at Magura (Ivanova et al. 2016). A preliminary mtDNA analysis (Ivanova et al. 2016) revealed that a specimen showing a characteristic *U. spelaeus* feature (B to D, Ivanova et al. 2016) was carrying *U. ingressus* mtDNA. All morphotypes identified in this study were grouped in either primitive (n = 8) or evolved types (n = 11), this distinction being based on the complexity of tooth shape and on tooth breadth. Both tooth morphotypes could be observed in one and the same layer but, to some degree, the primitive morphotypes were more frequent in the lowermost layers and the evolved ones in the uppermost. It has been suggested that the primitive morphology of the premolars could be related to an earlier stage of the *U. ingressus* evolution (Ivanova et al. 2016).

Among the metapodials uncovered during the excavation campaigns, four small, convex metapodials (three metatarsals from layer 25, Trench I and layer 8, Trench III, and one metacarpal from layer 25, Trench I) stood out with a more slender morphology than those of *U. spelaeus s.l.* and *U. ingressus.* However, these were yet with different proportions from *U. arctos* (Fig. 2), casting doubts on their specific identification. A mtDNA test performed on one of them (sample M3, first metatarsal, Fig. 2C) made it possible to assign the specimen to the *U. ingressus* clade (Ivanova et al. 2016). The morphometric comparison shows that the dimensions of the small metapodials are at the lowest limits of the *U. ingressus* first metapodials dispersion and most likely these remains belonged to very small female individuals (Fig. 3).

Materials and Methods

Sampling strategy

Sampling for genetic and isotopic analyses on the faunal assemblages excavated during the 2011 and 2012 campaigns (Ivanova et al. 2016) was performed in June 2014 at the National Museum of Natural History in Sofia. Maxillary and mandibular remains were primarily targeted for sampling the *Ursus* sp. specimens: a tooth was preferred for genetic analysis, while dense cortical bone was sampled for stable isotope analysis (Table 1, Suppl. Data 1 and 2); the only exception being a metapodial (sample M3) received in January 2015 for genetic analysis only. Whenever possible, information about the age at death, the tooth morphology, and metapodial size of the bear specimens was recorded for further comparison with mtDNA results and stable carbon and nitrogen isotope compositions.

Sampling was carried out with protective clothes and the tools were cleaned with bleach between each sample. In total, 10 *Ursus* sp. specimens were sampled from trench I (distributed over the layers 14, 24, 25 and 39) and 25 from trench III (distributed over the whole stratigraphical sequence, with the exception of the tephra layer 5). Herbivores and carnivores from trench I (layers 25, 26-27 and 29; n = 8) and trench III (layers 4, 6, 7, 9 and 10; n = 13) were sampled to reconstruct the ecosystem-specific isotopic background. Two herbivores teeth (M3-825 – an upper third molar (M^3) of *Equus germanicus* and M3-1072 – an upper second molar (M^2) of *Cervus elaphus*) were also sampled for sequential stable carbon and oxygen isotope analysis to provide insights into the seasonal variations in the local environment. Both teeth come from the layer 6 of trench III. A detailed description of the morphology, aDNA and stable isotope analysis protocols implemented in this study is provided in Suppl. Data 5.

aDNA analysis

The genetic analyses were performed in the dedicated ancient DNA facilities of the Laboratory of Forensic Genetics and Molecular Archaeology in Leuven (University of Leuven, Belgium). A series of precautions commonly described in literature (e.g. Gilbert et al. 2005) and already followed for the analyses of animal and human aDNA samples in the same facilities (e.g. Ivanova et al. 2016, Ottoni et al. 2017) were taken to avoid contamination. Three short fragments of the mitochondrial DNA control region were amplified in singleplex with primers CB2670a-H45 (fragment 1), L130-H177 (fragment 4) and L164-H221 (fragment 5) and PCR conditions described in Stiller et al. (2014). More details about the aDNA methods are available in Suppl. Data 5.

Isotopic analysis principles

Bone collagen has been extracted for the bulk analysis of stable carbon and nitrogen isotope compositions, while the tooth enamel of a horse and a red deer has been sequentially sampled for stable carbon and oxygen isotope analysis. Tooth enamel can provide a sequence of isotopic variations over several months to years of life of an animal depending on tooth development timing (e.g. Fricke & O'Neil 1996). In contrast, bone collagen is remodelled throughout an animal's lifespan and the isotopic values measured in a bulk sample represent averages over life (Tieszen et al. 1983).

Mammalian tooth enamel carbonates precipitate in isotopic equilibrium with body water, the $\delta^{18}O$ value of which reflects the stable oxygen isotope composition of ingested water, mainly drinking and plant water (Longinelli 1984, Luz et al. 1984, Iacumin et al. 1996). Both track the oxygen isotopic composition of meteoric water, which varies seasonally with ambient temperature at middle and high altitudes (Land et al. 1980, Longinelli 1984, Luz et al. 1984, Flanagan & Ehleringer 1991): higher $\delta^{18}O$ values are recorded in the warmest months, the lowest in the coldest months (Dansgaard 1964, Gat 1980). The main factor influencing $\delta^{13}C$ values in terrestrial environments is the plant photosynthetic pathway: modern C_3 plants display $\delta^{13}C$ values ranging from –37 to –20 ‰ (average: –27 ‰) (Kohn 2010). Other factors may influence plant $\delta^{13}C$ to a lesser extent, including plant growth stage, time of day, season, aridity, salinity, luminosity and recycling of atmospheric CO_2 (Lowdon & Dick 1974, Garten & Taylor 1992, Gleixner et al. 1993, Matus et al. 1995, Heaton 1999, Saranga et al. 1999). Plant nitrogen isotope composition depends on their ability to fix atmospheric N_2 (as in legumes) and is impacted by abiotic factors such as temperature, water availability, soil type, altitude, etc. (Peterson & Fry 1987, Ambrose 1991, Männel et al. 2007, Pardo & Nadelhoffer 2010). Along the food chain, an average stepwise enrichment of ca. 3 ‰ is observed in animal bone collagen $\delta^{15}N$ with increasing trophic level (Schoeninger & Deniro, 1984).

Results

aDNA results

Following the authentication criteria applied (Suppl. Data 5), DNA sequencing was successful in 12 of the 30 cave bear individuals analysed (40% success yield). Stable isotope analyses revealed low bone collagen preservation in four individuals from which the teeth were genetically analysed (Table 1, Suppl. Data 1), indicating, as expected, substantially different biomolecular diagenetic trajectories in teeth and bones.

Sequences of the three fragments targeted for DNA amplification were generated in 11 individuals. The Bayesian phylogenetic tree of 60 unique haplotypes of the three concatenated mtDNA fragments showed that the main group separation corresponding to the taxonomic units described previously with longer sequences was reproduced (taxa corresponding to *U. spelaeus, U. eremus*, *U. ingressus*, *U. kudarensis*, *U. rossicus / U. s. rossicus* from Ural Mountains, and *U. ladinicus*), and that the sequences of the cave bears from Magura Cave clustered with the *U. ingressus* clade (Fig. 4). Six different haplotypes were detected: a, b, c, d, e and f. All the *U. ingressus* sequences generated in this study were deposited in GenBank with the accession numbers MN580373–MN580383.

In sample MG16 one fragment (fragment 4 as

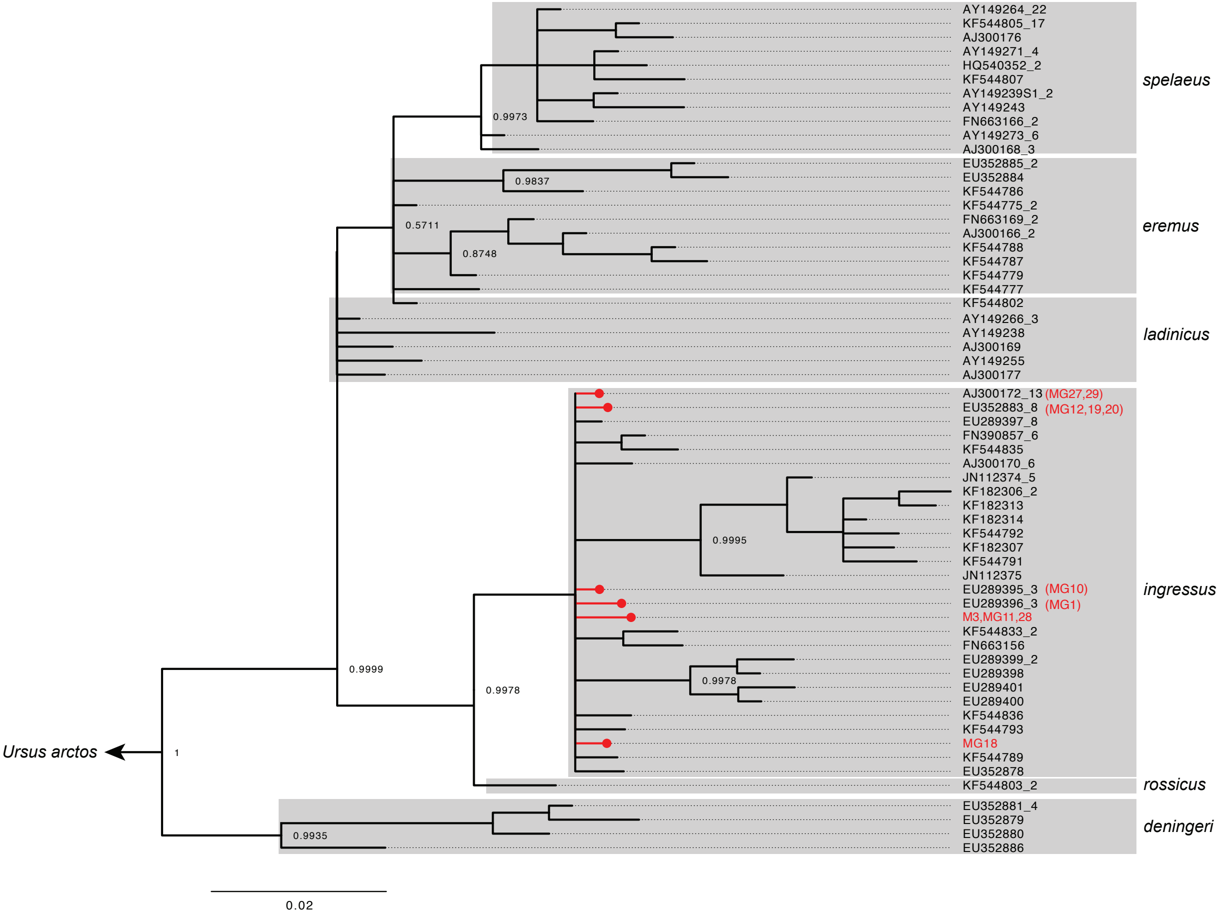

Fig. 4. Bayesian tree of 60 unique haplotypes of the concatenated mtDNA fragments 1, 4 and 5. Bear samples from this study are reported in red (in brackets, samples matching sequences from the literature). Node names are identified by the accession number of one sequence from the literature, followed by the total number of sequences matching the haplotype.

Table 1. Summary table recapitulating palaeontological, genetic and stable isotope analysis (SIA) data for each sampled bear specimen of Magura Cave, including collagen extraction yields in mg/g, carbon percentages (%C), nitrogen percentages (%N), carbon : nitrogen atomic ratios (C : N), and stable carbon ($\delta^{13}C_{VPDB}$) and nitrogen ($\delta^{15}N_{N2AIR}$) isotope compositions.

Trench	Layer	Zooarch_ID	Tooth morphotype	Estimated age	DNA_ ID	mtDNA	SIA_ID	Yield (mg/g)	%C	%N	C:N	δ¹³C	δ¹⁵N	Radiocarbon dating (y = years)
I	14	38	na	subad., ~4 yr.	MG25	-	MCI-cb8	<0	na	na	na	na	na	
I	14	19	evolved	na	MG28	*U. ingressus*-c	MCI-cb9	19.5	24.1	8.7	3.2	-20.5	3.0	direct ^{14}C dating not successful
I	24	124	evolved	adult	MG27	*U. ingressus*-f	MCI-cb1	102.7	30.0	10.8	3.2	-21.0	5.8	
I	24	113.3	na	2-3 months	na	na	MCI-cb10	25.2	35.5	12.8	3.2	-23.8	7.4	
I	25	3116 / 458-467	evolved	na	MG23	-	MCI-cb2	7.9	13.7	5.5	2.9	-20.8	2.9	OxA-29991: >50100 y BP
I	25	443-446	primitive?	adult/senile	MG24	-	MCI-cb6	34.3	33.9	12.5	3.2	-21.7	4.1	OxA-29991: >50100 y BP
I	25	484	na	na	MG26	-	MCI-cb5	55.2	41.7	15.3	3.2	-19.4	5.7	OxA-29991: >50100 y BP
I	25	274-275	evolved	adult	MG29	*U. ingressus*-f	MCI-cb7	46.4	38.7	14.3	3.2	-20.9	2.9	OxA-29991: >50100 y BP
I	25	433	na	na	na	na	MCI-cb3	8	5.7	1.8	3.6	-21.7	3.9	OxA-29991: >50100 y BP
I	39	976	evolved	18-20 months	MG22	-	MCI-cb4	5.9	4.7	1.3	4.3	-24.7	1.8	
III	4	562	na	adult	MG6	-	MCIII-cb17	<0	na	na	na	na	na	OxA-31009: 32750 ± 500 y BP / 38341-35765 y cal BP (95.4%)
III	4	534	primitive	na	MG10	*U. ingressus*-b	MCIII-cb8	22.4	42.8	15.7	3.2	-21.1	3.4	OxA-31009: 32750 ± 500 y BP / 38341-35765 y cal BP (95.4%)
III	4	555	na	adult?	MG16	*U. arctos*	MCIII-cb15	85.5	44.4	16.1	3.2	-18.3	8.2	RICH-22492: 33922 ± 496 y BP / 37600 - 34800 y BC (95.4%)
III	6	1085-1086	primitive	na	MG1	*U. ingressus*-a	MCIII-cb7	<0	na	na	na	na	na	
III	6	657-658	na	adult	MG7	-	MCIII-cb16	<0	na	na	na	na	na	
III	6	1025	na	adult	MG8	-	MCIII-cb20	<0	na	na	na	na	na	
III	6	1085/1086	na	6.5-7.5 months	na	na	MCIII-cb23	1.5	na	na	na	na	na	
III	7	1183	primitive	adult	MG11	*U. ingressus*-c	MCIII-cb9	35.5	41.9	15.5	3.2	-21.4	2.2	direct ^{14}C dating not successful
III	8	M3/1359	na	na	M3	*U. ingressus*-c	na	na	na	na	na	na	na	OxA-31115: >50000 y BP

Table 1. Continuation.

Trench	Layer	Zooarch_ID	Tooth morphotype	Estimated age	DNA_ID	mtDNA	SIA_ID	Yield (mg/g)	%C	%N	C:N	$\delta^{13}C$	$\delta^{15}N$	Radiocarbon dating (y = years)
III	8	1211-12	na	~6.5-7.5 months	na	na	MCIII-cb22	79.8	43.8	15.9	3.2	-22.2	5.3	OxA-31115: >50000 y BP
III	8	1349-50	na	6.5-7.5 months	na	na	MCIII-cb24	40.1	38.3	13.9	3.2	-21.9	4.8	OxA-31115: >50000 y BP
III	9	1389	primitive	na	MG9	-	MCIII-cb10	<0	na	na	na	na	na	
III	9	1433	evolved	adult	MG18	*U. ingressus*-e	MCIII-cb13	<0	na	na	na	na	na	
III	10	1482	primitive	na	MG2	-	MCIII-cb2	7.3	3.8	1.0	4.5	-19.7	3.4	
III	10	1497	na	~18 month	MG3	-	MCIII-cb21	<0	na	na	na	na	na	
III	10	1674	na	adult	MG4	-	MCIII-cb18	<0	na	na	na	na	na	
III	10	1559-1560	na	18-20 months	MG5	-	MCIII-cb14	<0	na	na	na	na	na	
III	10	1682-3	na	adult/senile	MG12	*U. ingressus*-d	MCIII-cb19	<0	na	na	na	na	na	
III	10	1692	primitive	adult	MG13	-	MCIII-cb6	<0	na	na	na	na	na	
III	10	1545-1546	evolved	adult/senile	MG14	-	MCIII-cb11	<0	na	na	na	na	na	
III	10	1511	evolved	18-20 months	MG15	-	MCIII-cb4	6.3	3.9	1.0	4.4	-27.4	5.9	
III	10	1659-1660	primitive	adult	MG17	-	MCIII-cb5	10.6	23.1	8.5	3.2	-22.0	7.5	
III	10	1565-6	evolved	adult/senile	MG19	*U. ingressus*-d	MCIII-cb1	<0	na	na	na	na	na	
III	10	1598	evolved?	adult/senile	MG20	*U. ingressus*-d	MCIII-cb12	<0	na	na	na	na	na	
III	10	1481	evolved	~18-24 months	MG21	-	MCIII-cb3	7.2	6.1	0.9	7.6	-10.3	4.3	

in Stiller et al. 2014) could not be amplified. Comparison with the dataset of sequences form the literature and a phylogenetic tree built on the two fragments sequenced (data not shown) made it possible to assign the mtDNA of MG16 to the *Ursus arctos* clade. This suggests that mismatches in the primer binding site, in particular of primer H177a (up to six mismatches, of which one at the 3'-end binding site), could be responsible for DNA amplification failure of fragment 4. Radiocarbon dating of the sample revealed an age of 34,800-37,600 years BP.

Isotopic results

Sequential sampling in the two herbivore teeth produced $\delta^{13}C$ and $\delta^{18}O$ values varying between –12.5 and –11.4 ‰, and between –10.1 and –4.8 ‰, respectively (Fig. 5; Suppl. Data 3). $\delta^{13}C$ intra-tooth variation amounts to 1.1 ‰ (from –12.5 to –11.4 ‰) in M3-825 and 0.4 ‰ (from –12.2 to –11.8 ‰) in M3-1072; $\delta^{18}O$ intra-tooth variation is 3.3 ‰ (from –10.1 to –6.8 ‰) in M3-825, and 5.1 ‰ (from –9.9 to –4.8 ‰) in M3-1072. In M3-825, the $\delta^{18}O$ sequence follows a sinusoidal pattern, with minima measured at ca. 45, 17, and 4 mm from the ERJ, and maxima at c. 27 and 9 mm from the ERJ. In M3-1072, the $\delta^{18}O$ sequence shows an increasing pattern, with the lowest values measured at the tip of the crown, and the highest values close to the ERJ.

Among the 34 bear specimens sampled for bone collagen analysis, 14 – eight from Trench I, six from Trench III – provided reliable isotopic data (Suppl. Data 1 and 4; DeNiro 1985, Ambrose 1990, Van Klinken 1999). The results range between –23.8 and –18.3 ‰ for $\delta^{13}C$, and between 2.2 and 11.1 ‰ for $\delta^{15}N$ (Fig.6A, Suppl. Data 2). Considering all species together, specimens from trench I provided $\delta^{13}C$ values varying between –23.8 and –19.2 ‰ and $\delta^{15}N$ values between 2.9 and 8.3 ‰. Specimens from trench III display $\delta^{13}C$ values comprised between –22.2 and – 18.3 ‰ and $\delta^{15}N$ values between 2.2 and 11.1 ‰ (Fig. 6B). There are no significant differences in the C (t-test, $p = 0.14$) and N (Mann-Whitney test, $p = 0.08$) isotopic ratios between the two trenches. Similarly, there is no significant difference in $\delta^{13}C$ and $\delta^{15}N$ values between the layers that provided enough data for statistical comparison (namely layers 4, 6, 7, and 10-25; Fig. 6C).

Herbivores' isotopic values fall between –21.4 and –19.0 ‰ for $\delta^{13}C$ and between 2.7 and 7.9 ‰ for $\delta^{15}N$ (Fig. 6B, Suppl. Data 2). Carnivores' isotopic values lie between –19.6 and –18.8 ‰ for $\delta^{13}C$ and between 8.3 and 11.1 ‰ for $\delta^{15}N$ (Fig. 6B, Suppl. Data 2). Among *Ursus* sp., adult specimens display values between –22.0 and –18.3 ‰ for $\delta^{13}C$, and between 2.2 and 8.2 ‰ for $\delta^{15}N$ (Fig 6A). The specimens with a primitive tooth morphotype have $\delta^{13}C$ and $\delta^{15}N$ values averaging –21.6 ± 0.4 ‰ and 5.2 ± 2.3 ‰, respectively; while bears with an evolved tooth morphotype display mean $\delta^{13}C$ and $\delta^{15}N$ values of –20.8 ± 0.3 ‰ and 3.9 ± 1.7 ‰, respectively. The carbon isotopic ratios significantly differ between the two tooth morphotypes (t-test, $p = 0.0355$). One *Ursus* sp. (MCIII-cb15 = MG16) stands out with both the highest $\delta^{13}C$ and $\delta^{15}N$ values (–18.3; 8.2). In addition, three cubs, two aged 6.5-7.5 months and one 2-3 months, provided values between –23.8 and –21.9 ‰ for $\delta^{13}C$ and between 4.8 and 7.4 ‰ for $\delta^{15}N$, both the lowest $\delta^{13}C$ and the highest $\delta^{15}N$ values being displayed by the youngest cub (Fig 6A, Suppl. Data 1).

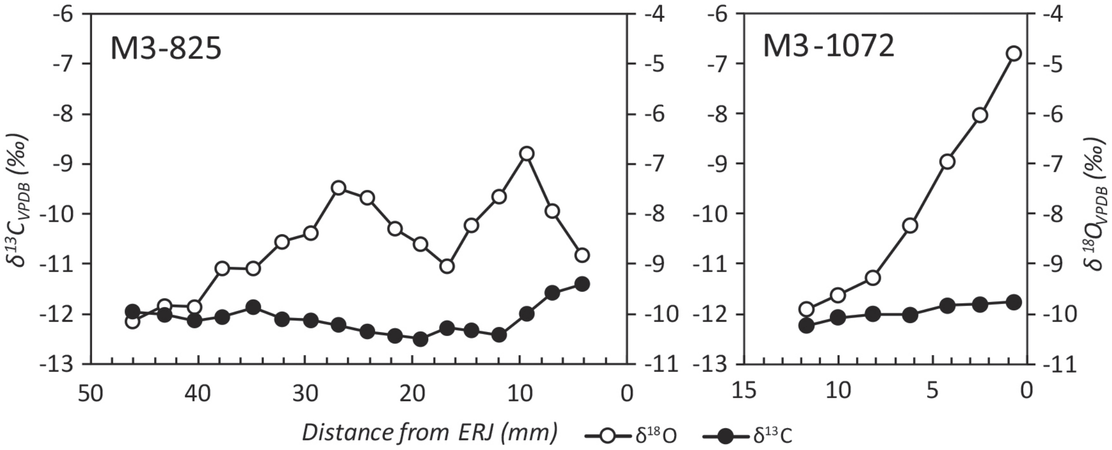

Fig. 5. Stable carbon ($\delta^{13}C$) and oxygen ($\delta^{18}O$) isotope compositions measured in an upper third molar (M^3) of *Equus germanicus* (M3-825, layer 6 of trench III) and an upper second molar (M^2) of *Cervus elaphus* (M3-1072, layer 6 of trench III).

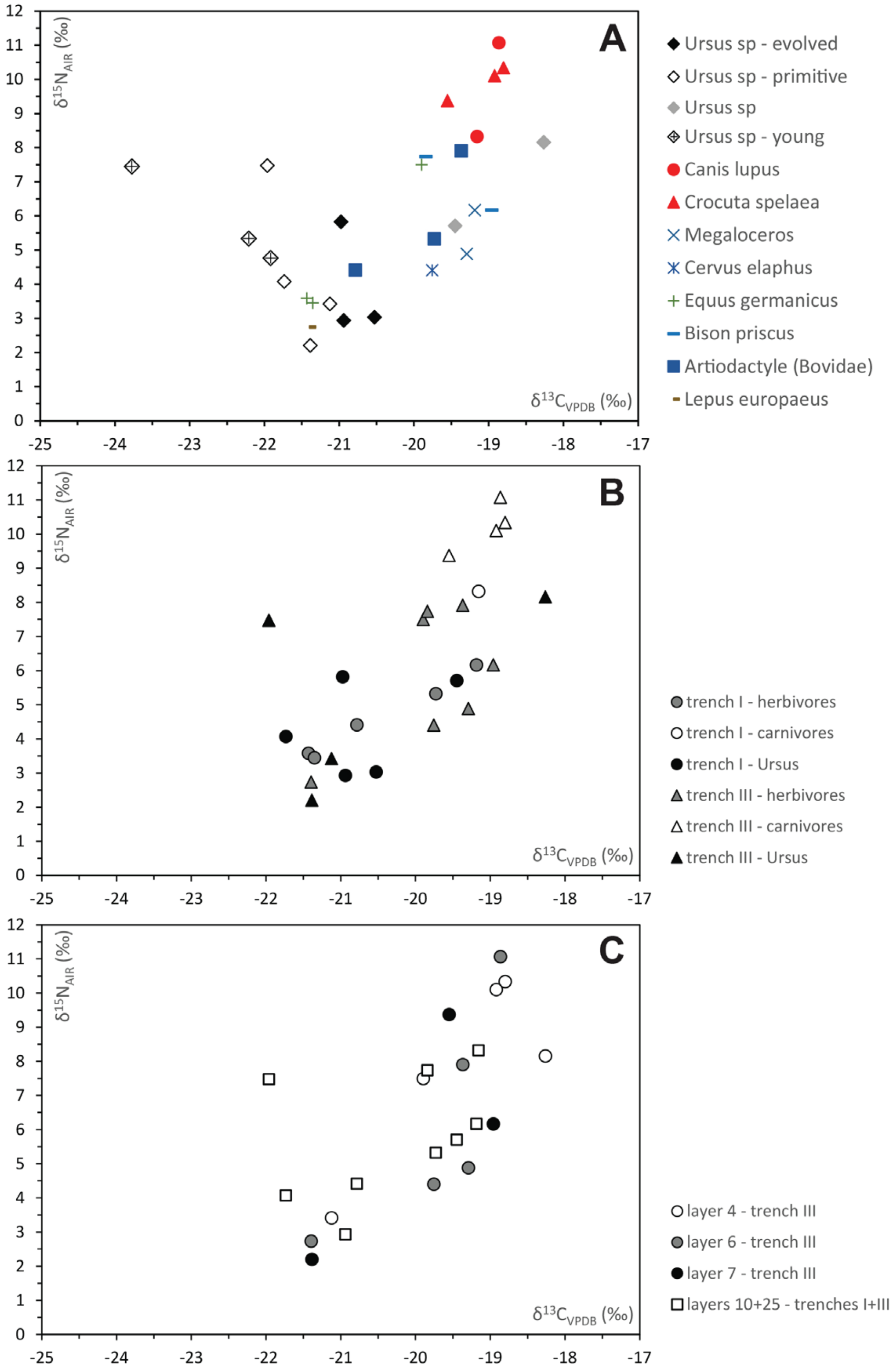

Fig. 6. Stable carbon ($\delta^{13}C$) and nitrogen ($\delta^{15}N$) isotope values measured in the animal bone samples from Magura Cave: A. results displayed per taxon; B. results displayed per trench and broad taxonomic groups (herbivores, carnivores, bears); C. results displayed per layers (only layers that provided the highest NISPs are included).

Discussion

Magura Cave bear mtDNA and morphological diversity

A preliminary aDNA analysis revealed the presence of *U. ingressus* in the faunal assemblage of Magura Cave (Ivanova et al. 2016). This identification was confirmed by the present study, which identified *U. ingressus* maternal lineages among all the cave bear specimens investigated. Of the six haplotypes identified, four (named here a, b, d and f, see Table 1) have already been found in cave bears from central to southeast Europe. The geographic distribution range of these haplotypes includes Austria, Romania, Ukraine, Croatia and Slovenia (Hofreiter et al. 2002, Richards 2008, Stiller et al. 2010). In agreement with previous results, this suggests a distribution of *U. ingressus* in southeastern Europe (i.e. the Balkans) during the Late Pleistocene (Stiller et al. 2014).

Two new *U. ingressus* haplotypes were identified (named here e and c). The haplotype e was found in Trench III layer 9 (MG18), and the haplotype c in trench III layers 8 (M3) and 7 (MG11), and in Trench I layer 14 (MG28). The M3 sample was taken from a small, convex metapodial, while samples from MG11 and MG28 were taken from right and left M^2s, respectively. While MG11 tooth (older than 39.3 kya) was assigned to a primitive morphotype, MG28 tooth (likely dating closer to 39.3 kya) was identified as carrying an evolved morphotype. If the earlier dating of MG28 compared with MG11 is confirmed, this finding will support the hypothesis of the primitive morphotypes being an earlier stage of the *U. ingressus* evolution (Ivanova et al. 2016). Comparing the MG11 M^2 with other *U. ingressus* M^2s from the site (Fig. 8), it appears that the former is comparatively small. To a lesser extent, this applies also to MG28 (Fig. 8). It is therefore possible that the cave bears carrying the haplotype c are characterised by a small body size, although more specimens are needed to confirm this hypothesis.

One tooth possessed *U. arctos* mtDNA (sample MG16). The poor preservation of its occlusal surface, which is seriously eroded and abraded (Fig. 7A, 7B), had prevented the species identification of this specimen by palaeontological analysis. This tooth is also quite large – its maximal length and width are estimated to 42 and 22 mm, respectively, but fall in the range of the largest Late Pleistocene brown bears of the European continent (Fig. 8; Baryshnikov 2007). The presence of *U. arctos* in Magura suggests that cave bears and brown bears may have coexisted in the Balkan Mountains (sample MG16-*arctos*, dated to 36.8-39.6 kya, and e.g. MG10-*ingressus* dated to 35.7-38.4 kya), providing chances of admixture episodes, as observed in a recent analysis of cave bear and brown bear genomes (Barlow et al. 2018).

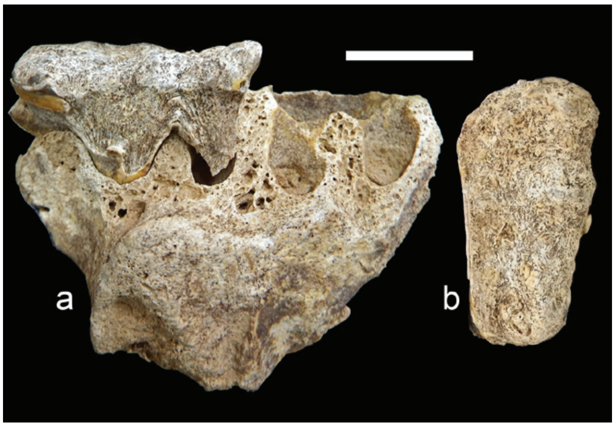

Fig. 7. Tooth FM 3448 (M3/555) of *Ursus arctos*, Trench III. A. Lingual view, with maxillary fragment. B. Occlusal view.

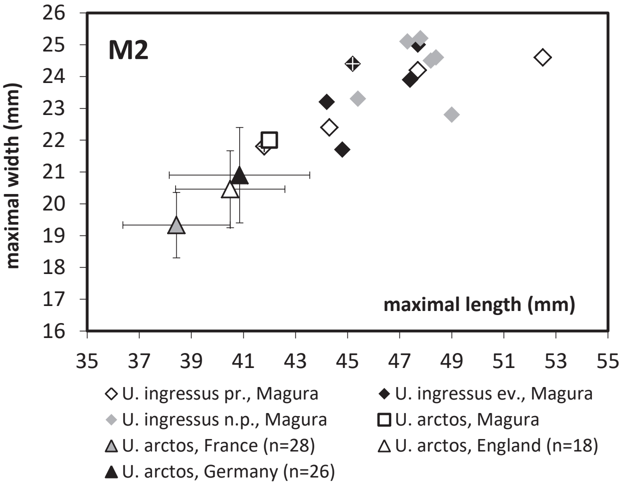

Fig. 8. Maximum lengths and widths of the second upper molars (M2) of Magura Cave bears (*U. ingressus* and *U. arctos*) compared with those of Late Pleistocene *U. arctos* from Europe (Baryshnikov 2007). The crosses within symbols identify *U. ingressus* specimens bearing haplotype c (pr. = primitive morphotype; ev. = evolved tooth morphotype; n.p. = specimens with not preserved tooth occlusal surface).

Magura Cave palaeo-environment inferred from herbivores stable isotope results

Sequential sampling performed in the teeth of two herbivores (a horse and a deer) from the layer 6 of trench III provides insights into the seasonal climatic and environmental conditions before 39.3 kya, dating of the Tephra layer 5. The δ^{18}O sequence measured in Magura Cave horse third molar shows a clear sinusoidal pattern, which can be interpreted as seasonal variations. The $\delta^{18}O$ sequence measured in Magura Cave *Cervus elaphus*' M^2 shows a similar pattern of variation as in red deer from Late Pleistocene sites in Italy. For the latter, intra-tooth variation amounts to 3.8 ‰, while for Magura Cave red deer, it is around 5 ‰, which suggests a more continental climate in Bulgaria compared to Italy, hence indicating that palaeoclimatic gradients were similar to those under actual climatic conditions.

There is only little intra-tooth variation in the $\delta^{13}C$ signal (maximum 1.1 ‰, in *Equus germanicus*). The absolute average value (–12 ‰) is consistent with consumption of C_3 vegetation. The absence of seasonal pattern in the $\delta^{13}C$ values of both herbivores could indicate seasonal change in dietary habit: they could have fed on plants or in environments characterised by low $\delta^{13}C$ values (e.g., under forest canopy or in wet areas) in summer, and on plants or in areas characterised by high $\delta^{13}C$ values (e.g., open air / dry environment) in winter, which would have resulted in an attenuated $\delta^{13}C$ seasonal signal (Heaton 1999, Bonafini et al. 2013).

The isotopic signals measured in the bone collagen of Magura Cave herbivores provide further insights into the local environment (Fig. 5, Suppl. Data 2), and over the whole stratigraphical sequence under study (>50 to c. 35 kya). The upper layers (above tephra layer 5, trench III) coincide with warm and mild periods (interstadials GIS8 – c. 38.5-36 kya, and GIS7 - 35-34 kya, according to the Greenland ice cores), which means probably favourable conditions were providing good availability of vegetation to feed on. It is noteworthy that in trench III *Bos/Bison* and *Equus* are characterised by higher nitrogen isotope values compared to Cervidae. This could be due to niche partitioning between species within a same ecosystem, and/or to different species grazing in different ecosystems. For example, open landscape vegetation displays higher $\delta^{13}C$ values compared to plants growing under canopy (Heaton 1999, Bonafini et al. 2013), while wet environments are characterised by lower plant $\delta^{13}C$ (Heaton 1999) and higher plant $\delta^{15}N$ (Codron et al. 2005).

The hypothesis of different species grazing in different ecosystems would indicate that the landscape around Magura Cave was a mosaic of biotopes and would be in accordance with the known ecological niches of the megafauna. The presence of a mosaic landscape around Magura Cave is further supported by the results of the palaeoenvironmental analysis based on pollen preserved in hyaena coprolites, amphibian remains and herpetofaunal assemblage. This analysis revealed the existence of steppe vegetation dominated by grasses, and including shrubs or smaller heliophilous trees as well as boreal woodland patches and wet and aquatic environments (Ivanova et al. 2016).

Cub diet and weaning

The cave bear is thought to have had a reproductive cycle similar to that of extant bears, with parturition and the first 2-3 months of maternal care occurring during hibernation, when bear metabolism is based on the adipose tissue accumulated before denning (Hellgren 1998, Lidén & Angerbjörn 1999). As a result, bear milk is one of the richest in fat among terrestrial mammals. The high fat content of bear milk (up to 36 % in polar bears, see Hellgren 1998), together with the known ^{13}C-depletion of fat compared to other animal tissues (e.g., 7.9 ‰ on average compared to bone collagen; Tieszen et al. 1983, Tieszen & Boutton 1989), results in very low $\delta^{13}C$ values in the cubs. On the opposite, the consumption of milk by the young is known to induce an increase of approximately 2-3 ‰ in collagen $\delta^{15}N$ values compared to the mother (Fuller et al. 2006), offset which is further increased due to the influence of hibernation on the mother's $\delta^{15}N$ blood (Bocherens 2019).

At Magura Cave, cubs aged 2-3 months and 6.5-7 months at death (Table 1, Fig. 9, Suppl. Data 1) means they probably died during their first and before their second winter, respectively (Lidén & Angerbjörn 1999, Veitschegger et al. 2019). The 2-3 month cub has $\delta^{13}C$ values ca. 3 ‰ lower and

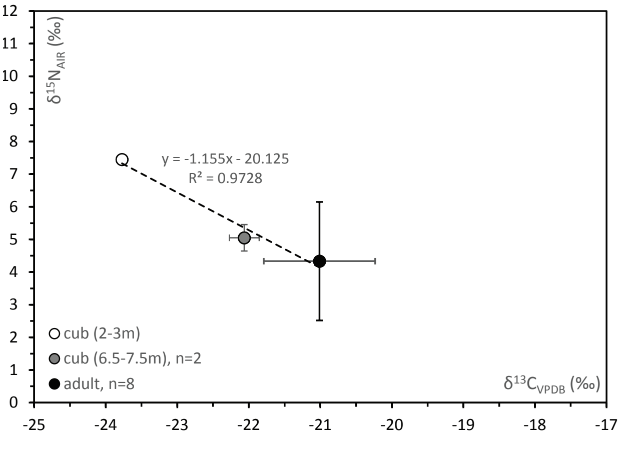

Fig. 9. Averaged carbon ($\delta^{13}C$) and nitrogen ($\delta^{15}N$) isotopic values of the cave bear displayed per age category: 2-3 month old cub (n=1), 6.5-7.5 month old cub (n=2) and adults (n=8).

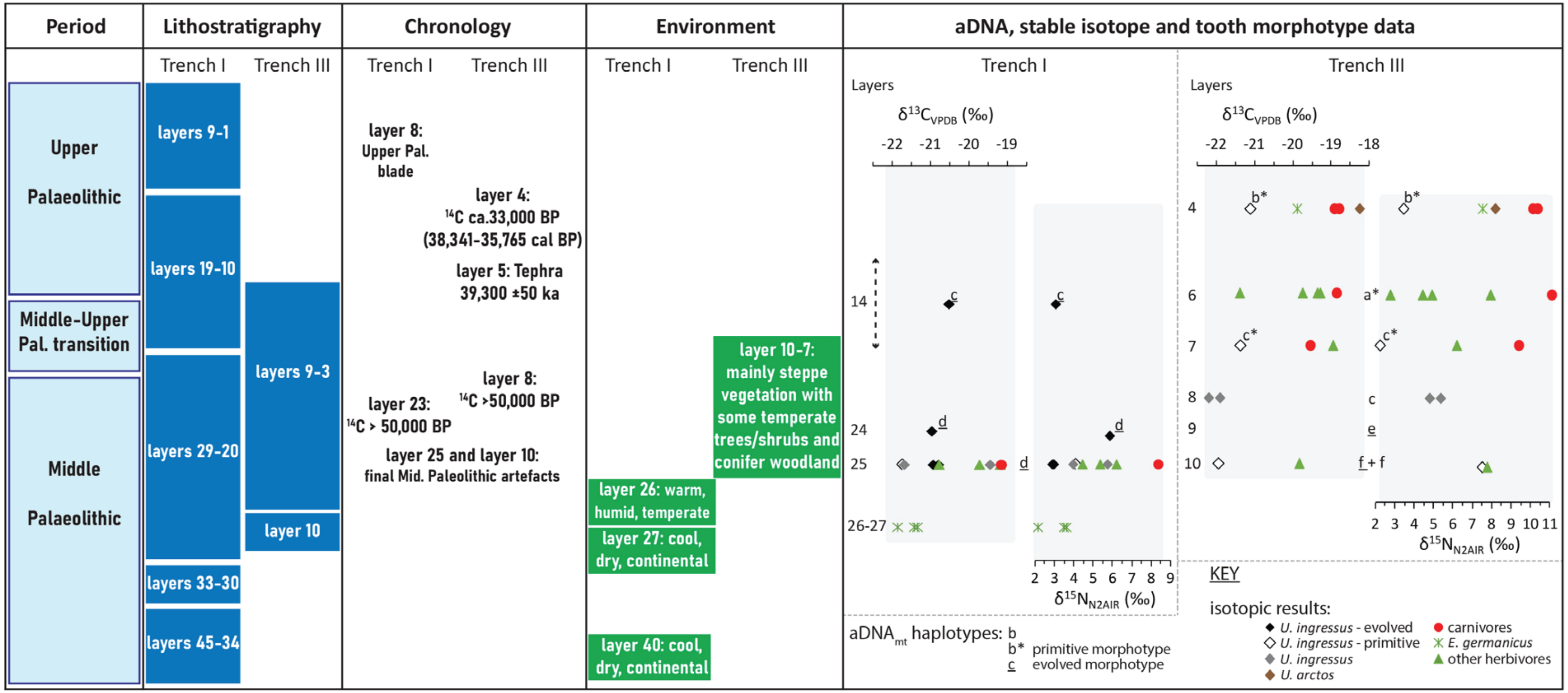

Fig. 10. Synthetic chart showing the different paleo-environmental proxies, dates and tentative stratigraphic correlations between Trenches I and III at Magura Cave as published in Ivanova et al. (2016), and updated with the results of mtDNA, isotopic and tooth morphotype analyses performed on Magura Cave bears.

δ^{15}N values ca. 3 ‰ higher than the averages for adult cave bears, which is in good agreement with what has been observed at other caves (Robu et al. 2018, Bocherens 2019). In the 6.5-7.5 month-old cubs, both isotopic signals are closer to the averages for the adult cave bears, although offset of +0.7 ‰ and –1.1 ‰ separate averaged δ^{15}N and δ^{13}C values respectively, which means that the cave bear cubs were already consuming solid food but were not fully weaned. The use of a simple two-end-member mixing model allows to estimate the amount of milk consumed by the 6.5-7.5 month-old cubs to 39 % (based on δ^{13}C) and 23 % (based on δ^{15}N). This estimation suggests cubs were suckling during their first winter but already consumed solid food during their first summer and autumn (Lidén & Angerbjörn 1999, Peigné et al. 2009).

Cave bear diet at Magura

Most of the sampled adult bear specimens of Magura Cave display δ^{15}N values, which are overlapping with those of herbivores (respective range of δ^{15}N values are 2.2 to 7.5 ‰ and 2.1 to 7.9 ‰; Fig.6A). The only exception is the specimen MG16, which has a δ^{15}N value (8.2 ‰) above herbivores and a high δ^{13}C value (–18.3 ‰), placing it closer to carnivorous species (Fig. 6A) and suggests a more omnivorous or carnivorous diet. This is in accordance with the mtDNA results identifying it as *Ursus arctos*. In comparison, the adult *U. ingressus* bears of Magura Cave had a predominantly herbivorous diet, likely sustained by favourable climatic and environmental conditions around the cave, which ensured good availability of plant resources. The diet of *U. ingressus* might not have excluded seasonal dietary flexibility and omnivory (Peigné et al. 2009, Peigné & Merceron 2019, Ramirez-Pedraza et al. 2019). The presence of brown bear and cave bear in a same layer (4, trench III) may suggest a niche partitioning between the two species – as previously observed in Austria at Ramesch and Gamssulzen Caves (Bocherens et al. 2011, Bocherens 2015), or in the Swabian Jura (Bocherens et al. 2014).

The range of δ^{13}C values measured in the adult cave bears (–19.4 to –22.0 ‰) corresponds to animals with a dominant consumption of C_3 plants, in accordance with the carbon isotope signal measured in Magura Cave herbivores (Fig. 6A). Within this range, inter-individual variability can be explained, at least partially, by cave bears preferentially feeding in one of the different environments that could be found in the mosaic landscape surrounding Magura Cave (Ivanova et al. 2016, section 6.2). Furthermore, statistical comparison between tooth morphotypes reveals that the evolved morphotype δ^{13}C mean (n=3, layers 14, 24 and 25 – trench I) is significantly higher than the primitive morphotypes (n=4, layers 4, 7 and 10 – trench III – and 25 – trench I; Fig. 6A, t-test, p = 0.0177); however, the two morphotypes do not significantly differ in δ^{15}N value (Fig. 6A, t-test, p = 0.808). As different climatic conditions, different levels of herbivory/omnivory or feeding at different altitudes would impact both δ^{13}C and δ^{15}N values of the cave bear collagen (Männel et al. 2007, Krajcarz et al. 2016), these factors can be excluded to explain the different δ^{13}C values.

Cave bear feeding behaviour, especially the type of food plant selected, could explain the difference in δ^{13}C values observed between tooth morphotypes. At Gamssulzen (Totes Gebirge, Austria), Münzel et al. (2014) observed the existence of two dietary groups within the *U. ingressus* population, based on enamel δ^{13}C values that were inversely correlated with different microwear scratch densities. Higher scratch densities have been linked to increased consumption of grasses, sedges, fruits, and decreased consumption of gritty, hard plant food (such as roots) or leaves (Merceron et al. 2004). Both fruits and roots are ^{13}C-enriched compared to green aboveground C_3 plant parts (Badeck et al. 2005, Codron et al. 2005). A higher consumption of hard plant food by bears displaying the evolved tooth morphotypes could be the cause of their higher δ^{13}C values compared to the primitive morphotypes, and would be in accordance with the hypothesis of the evolved tooth morphotype being an adaptation to the consumption of tougher plant foods (Grandal-D'anglade & Lopez-Gonzalez 2004). Considering the hypothesis of the primitive morphotypes being an earlier stage of the *U. ingressus* evolution (section 6.1), this observation could be the evidence for a dietary specialisation or adaptation of the *ingressus* clade towards the consumption of tougher plant foods through time (>39.3 kya to 38-35 kya), although more specimens would be needed to confirm this trend.

Conclusion

The phylogeny of the cave bear clade is quite complex and its understanding has quickly changed in the recent years, leading to the establishment of new species and subspecies. In papers published in the early 2000s, the mention of *Ursus spelaeus sensu lato* could refer as well to *U. ingressus* as to any of the *U. spelaeus* subspecies. For the first time, an analysis combining morphometry, mtDNA and stable isotope compositions has been performed on the Late Pleistocene (>60 kya – 35 kya) cave bear of Magura Cave in Bulgaria. Despite a restricted sample set (n = 12 successful mtDNA analyses), the study confirms the presence of *U. ingressus* in the adjacent areas south of the Carpathians and enriches our knowledge of *U. ingressus* genetic and morphological diversity with the discovery of two new haplotypes. It also reveals the coexistence of *U. ingressus* and *U. arctos* in the later layers (ca. 39-35 kya).

The paleo-environmental and isotopic analyses revealed that the environment in which Magura Cave bears lived was a mosaic landscape, which provided a variety of plant-feeding resources. The isotopic analysis confirmed a predominantly herbivorous diet for *U. ingressus*, although a flexible dietary behaviour could not be excluded. The cross-comparison of multidisciplinary data – tooth morphotypes, stratigraphy, mtDNA and stable isotope ratios – showed a possible diachronic dietary adaptation. The cave bear clade spread over a large geographical range and a diversity of environments. It also went through a rapid evolution and speciation process that remains to be fully understood. It is possible that this diversification across time and space explains the sometimes contradictive conclusions drawn about cave bear behaviour and diet. Our results, together with morphometric reassessment in other caves of south-eastern Europe, calls for a revision of the cave bear assemblages in the Balkan region to clarify cave bear ecology and phylogeography. The use of inter-disciplinary approaches and the reassessment of old assemblages can help address cave bear diversity and understand their behaviour, their physiology and their evolution through time with more fineness.

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Supplementary Materials

Supplementary Data 1-4 – link: http://www.acta-zoologica-bulgarica.eu/002379_SM_1_2_3_4

SD1: Synthetic table recapitulating the available palaeontological, genetic and stable isotope analysis (SIA) data for each bear specimen of Magura Cave, including collagen extraction yields in mg/g, carbon percentages (%C), nitrogen percentages (%N), carbon : nitrogen atomic ratios (C : N), and stable carbon (δ^{13}CVPDB) and nitrogen (δ^{15}NN2AIR) isotope compositions.

SD2: Results of the collagen extraction and stable isotope analysis (SIA) of the animal bone remains from Magura Cave, including collagen extraction yields in mg/g, carbon percentages (%C), nitrogen percentages (%N), carbon : nitrogen atomic ratios (C : N), and stable carbon (δ^{13}CVPDB) and nitrogen (δ^{15}NN2AIR) isotope compositions.

SD3: Stable carbon (δ^{13}CVPDB) and oxygen (δ^{18}OVPDB) isotope ratios measured in an upper third molar (M3) of Equus germanicus (M3-825, layer 6 of trench III), and an upper second molar (M2) of Cervus elaphus (M3-1072, layer 6 of trench III) from Magura Cave (dist. is the distance between each sample groove drilled in the enamel and the enamel-root junction of the tooth measured in mm).

SD4: List of the measurements (greatest lengths and smallest diaphyseal widths) used to draw Fig. 3.

Supplementary Data 5 – link: http://www.acta-zoologica-bulgarica.eu/002379_SM_5

SD5: Materials and Methods (additional data)

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