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INTRA-INDIVIDUAL VARIABILITY OF DENTAL ENAMEL $\delta^{13}C$ AND $\delta^{18}O$ VALUES IN LATE PLEISTOCENE CAVE HYENA AND CAVE BEAR FROM PERSPEKTYWICZNA CAVE (SOUTHERN POLAND)

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Abstract:

An important source of palaeoecological and palaeoenvironmental information is intra-specimen variability of isotopic composition of mammal tooth enamel. It reflects seasonal or behavioral changes in diet and climate occurring during a life of the animal. While well-known in ungulates, in carnivorans this variability is poorly recognized. However, carnivoran remains are amongst the most numerous in the Pleistocene fossil record of terrestrial mammals, so their isotopic signature should be of particular interest. The aim of the study was to verify if enamel of a fossil cave hyena (*Crocuta crocuta spelaea*) and a cave bear (*Ursus ingressus*) records any regular inter- or intra-tooth isotopic variability. We examined intra-individual variability of $\delta^{13}C$ and $\delta^{18}O$ values in permanent cheek teeth enamel of fossil cave hyena and cave bear from the site of the Perspektywiczna Cave (southern Poland). We conclude that the isotopic variability of the cave hyena is low, possibly because enamel mineralization took place when the animals still relied on a uniform milk diet. Only the lowermost parts of P_3 and P_4 enamel record a shift toward an adult diet. In the case of the cave bear, the sequence of enamel formation records periodic isotopic changes, possibly correlating with the first seasons of the animal life.

Key words: Pleistocene, MIS 3, stable isotopes, Perspektywiczna Cave, Kraków-Częstochowa Upland

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INTRODUCTION

Stable isotopic composition of fossil teeth enamel is widely applied in palaeoecological and palaeoenvironmental research (Bocherens and Drucker, 2013; DeNiro and Epstein, 1978; Koch, 2007; Pederzani and Britton, 2019). Among numerous isotopes which occur in the enamel mineral, the ratios of carbon and oxygen stable isotopes ($^{13}C/^{12}C$ and $^{18}O/^{16}O$, respectively) are most usually used. Presented as "delta" notations ($\delta^{13}C$ and $\delta^{18}O$), they found application in reconstructions of palaeoclimate, palaeodiet, canopy density, water sources, foraging altitude and niche partitioning (e.g., Bocherens *et al*., 1991, 1995, 2011; Levin *et al*., 2006; Reinhard *et al*., 1996; Sánchez Chillón *et al*., 1994; Shahack-Gross *et al*., 1999; Skrzypek *et al*., 2011; Tütken *et al*., 2007). Intra-specimen variability of isotopic composition of tooth enamel is an important source of palaeoecological and palaeoenvironmental information. This variability was found to be regular and is believed to follow the changes of the isotopic composition of food and water consumed by an animal during its life (Blumenthal *et al*., 2014; Bryant *et al*., 1996a, 1996b; Cerling and Sharp, 1996; Fricke and O'Neil, 1996; Passey and Cerling, 2002). The basic premise behind such a conclusion is that the enamel of different teeth is not formed and mineralized simultaneously, but represents different time frames of the animal life (Hillson, 2005). Moreover, the enamel of a single tooth is also mineralized non-simultaneously – its mineralization

starts at the top of the crown and continues downwards to the enamel-root junction (Fricke and O'Neil, 1996; Trayler and Kohn, 2017). The enamel is thus sequentially mineralized and gets its final isotopic signature some time after apposition, shortly before tooth eruption (Hillson, 2005; Klevezal, 1996; Trayler and Kohn, 2017).

Intra-individual isotopic variability of enamel has been well-recognized in ungulates, whose teeth are large and therefore easy to sample, and likely record long time intervals. Numerous studies established the expected trends in present-day ungulates (Balasse, 2002; Britton *et al*., 2009; Chritz *et al*., 2009; Fricke *et al*., 1998; Kohn *et al*., 1996, 1998; Trayler and Kohn, 2017; Wang *et al*., 2008, Zazzo *et al*., 2010) and were followed by studies of fossil relatives (Bernard *et al*., 2009; Chritz *et al*., 2009; Fabre *et al*., 2011; Feranec *et al*., 2009; Gadbury *et al*., 2000; Julien *et al*., 2012; Krajcarz and Krajcarz, 2014a; Velivetskaya *et al*., 2011, 2016; Widga *et al*., 2010; Wiedemann *et al*., 1999). In carnivorans, the intra-specimen isotopic variability is poorly recognized. One of the few examples is a study of the *Smilodon* canines (Feranec, 2004), which are, however, unusual among carnivorans due to their long enamel profile. This lack of interest is an effect of difficulties in sampling caused by a relatively low height of the carnivoran tooth crowns and thin enamel. An additional reason is a short duration of tooth formation, which restricts the temporal range of the isotopic record within a tooth. On the other hand, carnivoran remains are common in the Pleistocene fossil record (Diedrich, 2012; Krajcarz and Krajcarz, 2014b; Kurtén, 2007a, 2007b; Stiller *et al*., 2014). Because of this, fossil remains of some carnivoran taxa, such as the cave bear or the cave hyena, are relatively easily obtainable. Better understanding the analytical and interpretational limitations of their usage for isotopic studies may prove advantageous for future palaeoecological research. Therefore in this paper, we aim to examine the potential of intra-specimen isotopic variability of cave bear and cave hyena tooth enamel.

Enamel formation in carnivorans

As opposed to ungulates, whose individual tooth enamel may form for as long as one or two years (e.g., Balasse, 2002; Bendrey *et al*., 2015; Fricke and O'Neil, 1996), in carnivorans the enamel is formed relatively quickly (Klevezal, 1996). In the extant spotted hyena the order of permanent mandibular cheek teeth eruption is deemed to be M_1-P_2-P_4-P_3, but the eruption spans of different teeth overlap each other (Kruuk, 1972; Slaughter *et al*., 1974; Van Horn *et al*., 2003). Mineralization most probably follows this order. According to studies of modern and fossil spotted hyenas (Jimenez *et al*., 2019; Stiner, 1994), the entire formation and mineralization of the cheek teeth lasts for several months only (Fig. 1). X-ray photography of around 2-months-old cub mandibles reveals no signs of permanent cheek teeth formation, while at 8–12 months most of the permanent teeth are already erupted (Jimenez *et al*., 2019), so their enamel is fully mineralized. The first completed tooth is M_1, while the last one is P_3.

In the cave bear, the formation of the mandibular cheek teeth follows the order M_1-P_4-M_2-M_3 (Andrews and Turner, 1992; Debeljak, 1996) (Fig. 1). The enamel of M_1 is already formed at 2–4 months of postnatal life (Veitschegger *et al*., 2019), but possibly not fully mineralized (Krajcarz and Krajcarz, 2014b). The M_1 erupts at around 4–5 months and then its mineralization is completed, while formation of the P_4, M_2 and M_3 enamel is started (Debeljak, 1996; Veitschegger *et al*., 2019). The mineralization of the P_4 and M_2 enamel is completed around 9 months, and of the M_3 enamel around 12 months.

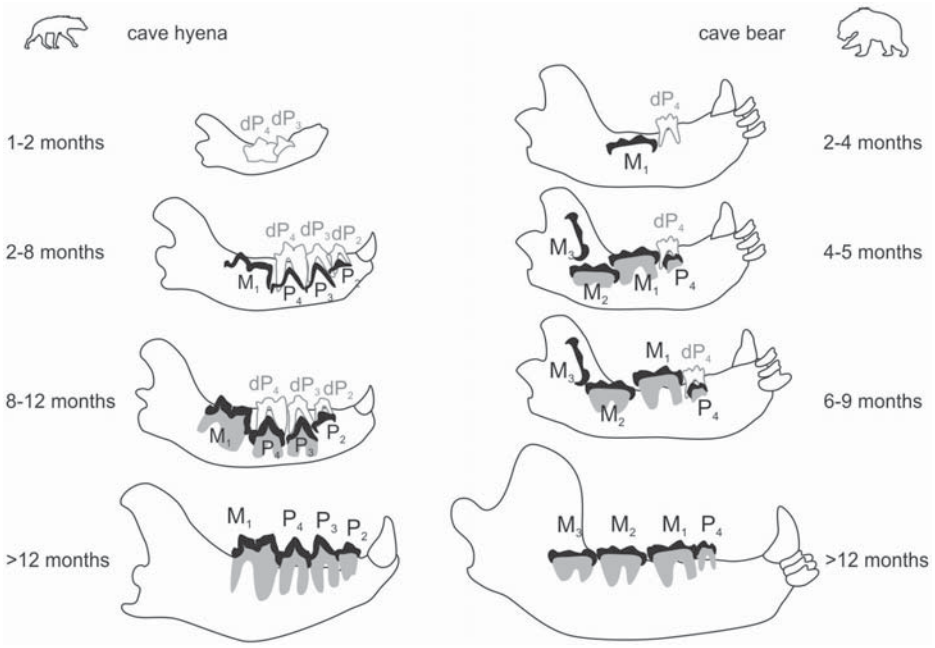

Fig. 1. Formation of enamel in cave hyena and cave bear (based on: Debeljak, 1996; Veitschegger *et al*., 2019; Jimenez *et al*., 2019; simplified), not to scale. Enamel and other tissues of cheek teeth are shown in black and grey, respectively.

Impact of nursing on the enamel isotopic signature

Because the enamel in carnivorans mineralizes at a very young age, when they still rely on mother's milk, nursing is one of the most important factors responsible for isotopic signature (Bocherens, 2004; Herrscher *et al*., 2017; Jenkins *et al*., 2001). The mother's milk, similarly to other fat-rich tissues, is depleted in ^{13}C (Bocherens *et al*., 1997; Grandal-d'Anglade *et al*., 2019; Nelson *et al*., 1998). Milk is also the main source of water for sucklings and it is ^{18}O-enriched compared to the water drunk by the mother (Tsutaya and Yoneda, 2015; Wright and Schwarcz, 1998). Therefore, the expected situation within the enamel affected by nursing is low $\delta^{13}C$ values and high $\delta^{18}O$ values. Shifting into higher $\delta^{13}C$ values and lower $\delta^{18}O$ values indicates that the animal begun relying on the adult diet.

Impact of hibernation on the enamel isotopic signature

By analogy to the extant brown and black bears (*U. arctos* and *U. americanus*), the adult cave bears are believed spent winters in the state of hibernation, during which neither ate nor drank, but instead burnt up their fat stores (Hissa, 1997; Nelson *et al*., 1983). This resulted in low $\delta^{13}C$ values, commonly noticed in cave bears (Bocherens, 2004; Bocherens *et al*., 1994). Females gave birth during winter hibernation and cubs likely spent their first winter suckling. The lactation together with hibernation is believed to be responsible for low $\delta^{13}C$ values in young cave bears (Bocherens *et al*., 1997; Grandal-d'Anglade *et al*., 2019; Nelson *et al*., 1998). During their first summer, the cubs likely started to feed on solid food but probably continued to suckle until their second winter, when milk again became the most important part of their diet (Grandal-d'Anglade *et al*., 2019; Lidén and Angerbjörn, 1999; Nelson *et al*., 1998). The isotopic effect of such dietary shifts would be periodical oscillation of stable isotope ratios of the body, which could be recorded in the enamel mineralized during that time (Krajcarz and Krajcarz, 2014b).

MATERIAL AND METHODS

Material

The material comprises three right dentary bones with cheek teeth. Two of the mandibles (W-3493 and W-4152) belong to *C. crocuta spelaea*, and the other specimen (W-17) belongs to *U. spelaeus* s.l., precisely *U. ingressus* (Gretzinger *et al*., 2019). The geological ages of the bones have been radiocarbon dated to 40,200 ± 1200 (W-17), 36,500 ± 800 (W-3493) and 34,700 ± 600 BP (W-4152). All of the bones were collected at the Perspektywiczna Cave, situated in the Częstochowa Upland, Olkusz County, southern Poland (for a summary of the site see: Krajcarz, 2016).

Sampling

The lingual enamel surface of each studied tooth was selected for sampling (the longest preserved and not damaged by wearing). Prior to the sampling, the surface was mechanically cleaned using a Dremel diamond-coated bit. We removed cementum, dental plaque and any mineralization if presented. Also, around 0.3–0.5 mm of the external enamel layer was removed, as this outer part was reported to be isotopically biased by later mineralization (Trayler and Kohn, 2017). The exposed surface was cleaned with demineralized water and dried. To avoid contamination during sampling, the entire tooth outside the sampling surface was covered with parafilm. Enamel samples were collected by careful drilling about 10 mg of powder (taking care to avoid sampling the dentine). Samples were taken in sequences reflecting the direction of enamel mineralization (from the tip of the crown toward the enamel-root junction). The sizes and positions of the sampling spots are shown (Fig. 2), and the full list of samples is provided (Table 1).

Chemical pretreatment

In order to clear out the enamel of possible contamination, 30% H_2O_2 solution and 0.1M acetic acid (CH_3COOH) were used, the former to remove organic pollution and the latter to purge away exogenous carbonate. The reaction lasted for 24h and 48h, respectively, with centrifugation,

Table 1. List of samples analyzed in this study, weight yield after chemical pretreatment and isotopic results.

Taxon	Inv. no.	Tooth	Sample no.	Yield [%]	$\delta^{13}C$ (VPDB) [‰]	$\delta^{18}O$ (VPDB) [‰]
Ursus ingressus	W-17	P_4	1	73.7	-17.49	-5.58
		M_1	1	60.3	-18.21	-7.78
		M_2	1	89.2	-17.40	-5.67
		M_2	2	89.9	-17.07	-5.37
		M_3	1	65.1	-18.08	-7.17
		M_3	2	66.4	-17.98	-6.30
Crocuta crocuta	W-3493	P_2	1	63.2	-14.83	-10.10
		P_3	1	61.1	-15.58	-10.16
		P_3	2	60.7	-15.00	-10.32
		P_3	3	63.3	-14.43	-10.48
		P_4	1	62.7	-15.66	-10.09
		P_4	2	63.8	-14.51	-10.75
		M_1	1	77.2	-15.50	-9.43
		M_1	2	70.7	-14.95	-10.10
		M_1	3	60.1	-14.80	-10.06
Crocuta crocuta	W-4152	P_2	1	72.4	-14.98	-8.73
		P_3	1	66.8	-15.55	-8.74
		P_3	2	68.4	-14.12	-8.90
		P_4	1	68.4	-15.48	-9.02
		P_4	2	69.5	-14.99	-9.00
		M_1	1	71.7	-15.48	-8.70
		M_1	2	72.4	-15.59	-9.30
		M_1	3	66.2	-15.25	-8.77

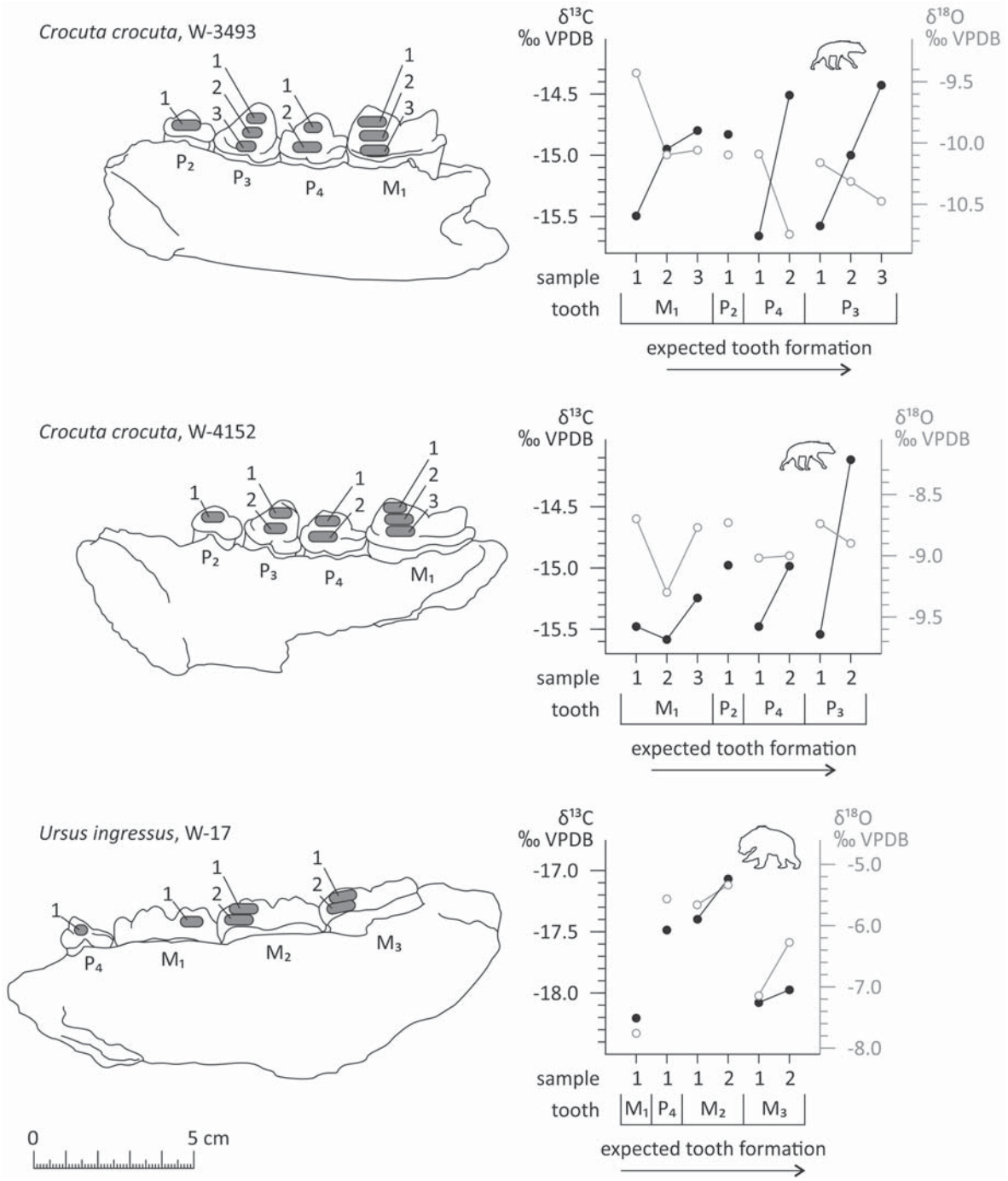

Fig. 2. The location of samples in the studied specimens and obtained isotopic results, arranged according to the order of expected tooth formation. The open circles are used for $\delta^{18}O$ and black dots for $\delta^{13}C$.

decantation and rinsing with deionized water several times after each step. An enamel sample of the recent cattle (*Bos taurus*) was selected as an internal standard and subjected to the same procedure.

Isotopic measurement

Carbon isotopic analysis was conducted with a GasBench II apparatus combined with a Thermo MAT 253 mass spectrometer in the Continuous Flow system, with standard analytical procedures being applied, in the Stable Isotopes Lab of the Institute of Geological Sciences, Polish Academy of Sciences (Warsaw, Poland). CO_2 was derived from the samples by reaction with orthophosphoric acid (H_3PO_4). Balancing time was 18h, balancing temperature 70°C. Oxygen isotopic analysis was conducted with the same apparatus, with balancing time 18h and balancing temperature 32°C. Laboratory standards were NBS19, NBS18 and LSVEC. The equation for the δ values, show-

ing a deviation from the values established as an internationally accepted standard, is presented as $\delta(‰) = [(R_{sample} - R_{standard})/R_{standard}] \times 1000$ (Tütken *et al*., 2007), with R being the ratio of heavy to light isotope ($^{18}O/^{16}O$ or $^{13}C/^{12}C$). The $\delta^{13}C$ and $\delta^{18}O$ values were reported as relative to the VPDB standard.

RESULTS

Results of isotopic analyses of the samples are presented (Table 1). The $\delta^{13}C$ values fall in the ranges of -18.21 to -17.07‰ (specimen W-17), -15.66 to -14.43‰ (specimen W-3493) and -15.59 to -14.12‰ (specimen W-4152). Therefore, the differences between the highest and the lowest values reach 1.14, 1.23 and 1.47‰ respectively. The ranges of $\delta^{18}O$ values are -7.78 to -5.37‰ (W-17), -10.75 to -9.43‰ (W-3493) and -9.30 to -8.70‰ (W-4152). Thus, the differences between the highest and the lowest value equal 2.41, 1.32 and 0.60‰. The linear correlation between $\delta^{13}C$ and $\delta^{18}O$ values is distinct in the cave bear, while weak in the case of the hyena specimen W-3493 and lacking in the hyena specimen W-4152 (Fig. 3).

The $\delta^{13}C$ and $\delta^{18}O$ values obtained for the internal lab standard (sample PC-Bos-1) were situated close to the centre of the distribution established by previous analyses of this standard (Table 2). This suggests that the chemical pretreatment was conducted properly and the results are reliable.

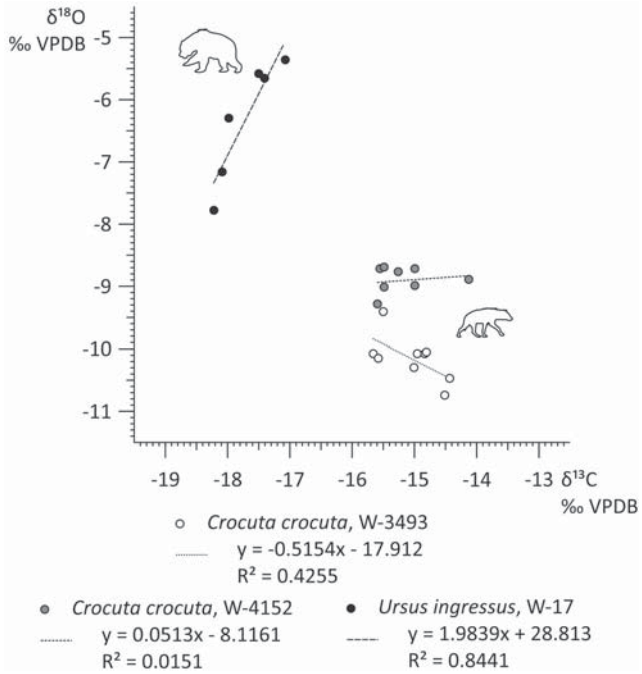

Fig. 3. $\delta^{13}C/\delta^{18}O$ plot for the studied specimens. The linear correlation between $\delta^{13}C$ and $\delta^{18}O$ is provided separately for each specimen with the coefficient of determination (R^2).

Table 2. The results for internal lab standard: sample PC-Bos-1 (this study) and the previous measurements.

Sample	$\delta^{13}C$ (VPDB) [‰]	$\delta^{18}O$ (VPDB) [‰]
PC-Bos-1	-12.24	-9.86
IS-1	-12.28	-10.39
IS-2	-11.99	-10.67
IS-3	-12.17	-10.48
IS-4	-12.29	-10.79
IS-5	-12.23	-10.65
IS-6	-12.21	-10.66
Bos 4 CH	-12.40	-10.12
Bos 3	-12.13	-10.11
Bos 4	-12.24	-9.50
Bos 5	-12.31	-9.66
Bos 6	-12.35	-8.59
Bos 7	-12.35	-8.92
Bos 8	-12.39	-8.97
Bos 9	-12.24	-9.70

DISCUSSION

Inter- and intra-tooth variability in the cave hyena

Both specimens of the cave hyena exhibit distinct trends in both $\delta^{13}C$ and $\delta^{18}O$ values in M_1, P_4 and P_3 (Fig. 2). Each of these teeth shows an increase of $\delta^{13}C$ values during the tooth formation, starting from an equated level between -15.5 and -15.7‰ VPDB near the apex of the crown. This suggests that all these teeth started to form at the same time. Moreover, the two individuals had almost the same diet during that phase of their life. Mineralization of this portion of enamel happens when hyenas are 2–8 months old and rely exclusively on milk diet (Jimenez *et al*., 2019; Stiner, 1994) (Fig. 1). This is consequent with relatively low $\delta^{13}C$ values and high $\delta^{18}O$ values characteristic for milk (Bocherens *et al*., 1997; Grandal-d'Anglade *et al*., 2019; Nelson *et al*., 1998; Tsutaya and Yoneda 2015; Wright and Schwarcz, 1998).

The increase of $\delta^{13}C$ values is recorded in the younger parts of the M_1, P_4 and P_3 enamel, and in the P_2 enamel. The most extreme value is recorded in the lower part of the P_3 enamel in the W-4152 specimen (Fig. 2). This likely reflects the higher amount of ^{13}C-enriched food, an effect of a turning into the adult diet. This is also confirmed by the simultaneous decrease of $\delta^{18}O$ values, probably caused by drinking more water instead of milk. The negative correlation between $\delta^{13}C$ and $\delta^{18}O$ values in the W-3493 specimen, although weak (Fig. 3), stays in accordance with the expected change during the milk-to-adult diet turnover. A possible chronological sequence of the formation of the isotopic composition of the enamel is shown in Fig. 4. Noteworthy, the $\delta^{18}O$ record differs between the studied specimens. While W-3493 records the decrease of $\delta^{18}O$ values downwards the crown profiles, in W-4152 there is a low $\delta^{18}O$ variability and rather irregular $\delta^{18}O$ distribution within particular teeth as well as between different teeth. Also, the correlation between $\delta^{13}C$ and $\delta^{18}O$ values is lacking in this specimen (Fig. 3). This can be interpreted as the result of different modes

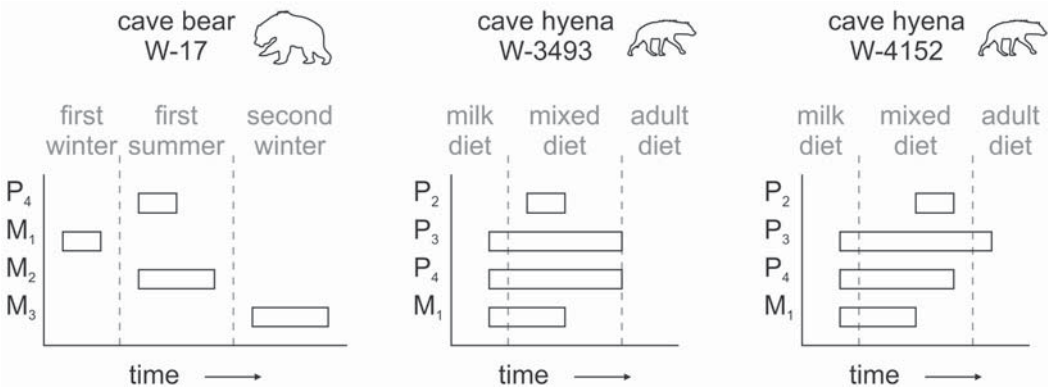

Fig. 4. Estimated relative time of enamel mineralization in the studied specimens, as inferred from their isotopic signature.

of milk-to-adult diet shift among individuals, and may also indicate different individual use of water sources.

Inter- and intra-tooth variability in the cave bear

In the studied specimen, both $\delta^{13}C$ and $\delta^{18}O$ signatures record periodic changes, starting from low values in M_1, the tooth which has been formed and possibly mineralized first (Fig. 2). In P_4 and M_2 the values are high, followed by low values again in the last-formed M_3. This likely records the seasonal changes. The sequence M_1-P_4-M_2 could be interpreted as representing a change in the isotopic composition of mother's milk. The increasing $\delta^{13}C$ values likely reflect the increasing reliance of the nursing mother on her summer diet, instead of using her winter fat storage. Similarly, the increasing $\delta^{18}O$ values reflect a change in the isotopic composition of the meteoric water that the mother was drinking, which provides a base for the isotopic composition of milk. It cannot be excluded that the elevated $\delta^{13}C$ and $\delta^{18}O$ signature of P_4 and M_2 may be additionally influenced by a self-reliant adult-like diet of the cub during the warm season. The values received for M_3 may be a result of a gradual return of the cold conditions. The isotopic signature of M_3 may have also been influenced by the altered metabolism during the cub's hibernation.

CONCLUSIONS

We reported the results showing intra-individual $\delta^{13}C$ and $\delta^{18}O$ variability in the Pleistocene cave hyena and cave bear. A limitation of the applied method when dealing with carnivoran cheek teeth is a difficulty in obtaining many samples from one tooth. This is caused by the relatively small height of the teeth and their thin enamel, in comparison to the teeth of large ungulates. Moreover, as the eruption periods of particular teeth overlap, it would be problematic to chronologically correlate the samples taken from different teeth in order to reconstruct the life histories of the examined specimens more precisely. Our results allow, to estimate the relative age of those stages of enamel mineralization during which the enamel obtained its final isotopic signature. We confirmed the expected uniform isotopic signature of the cave hyena's enamel, except of the basal parts of P_3 and P_4. In the case of the cave bear, it seems that each tooth records the milk-dependent diet, possibly additionally influenced by a self-reliant adult-like diet of the cub during the warm season in P_4 and M_2. Thus the observed periodic changes reflect well the seasonal changes in the diet of the nursing mother.

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