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Testing the Sample Preparation Method and Oxygen Isotopic Analysis of Tooth Enamel for the Reconstruction of the Birth Seasonality of Ovicaprines (the Case of Teeth from Istykskaya Cave, Eastern Pamir)

Cyclic variations of $\delta^{18}O$ along the growth line of M2 and M3 molars provide information about the seasonality of enamel formation and thereby about reproductive seasonality in animals, taking into account the time of tooth eruption and full enamel maturation. Determination of birth seasonality of small ruminants is relevant to the reconstruction of pastoralist strategies. Two peaks of reproductive activity per year are one of the most reliable indicators of human control of the small ruminants. As part of this work, for the first time in Russia, a method of sample preparation and analysis of oxygen isotope ratios in the tooth enamel of small ruminants is proposed. Traditionally, breeding seasonality was evaluated by the isotopic analysis of carbonates, the content of which in dental enamel is only 4 %. According to a new approach, oxygen from a sample is converted to gaseous CO by the interaction of enamel with carbon in a helium flow under 1300 °C, enabling one to measure $\delta^{18}O$ in all components of the dental enamel, including phosphates, which make up 90 % of enamel mass and are resistant to diagenesis. In this study, four sample preparation protocols depending on the degree of preservation of teeth and their age were tested: (A) cleaning and sampling of enamel, (B) treatment by H_2O_2, (C) treatment by NaOCl and CH_3COOH, and (D) extraction of Ag_3PO_4. Results show that for assessing seasonality breeding, it suffices to evaluate the lowest and the highest $\delta^{18}O$ along the tooth growth line. If the preservation of sample is good, minimal chemical treatment is enough to observe these extremes.

Keywords: *Isotopic analysis, oxygen, stable isotope ratio, farming, animal breeding, sheep, Central Asia.*

Introduction

The analysis of light stable isotopes (H, C, N, S) is widely applied in archaeological studies (Svyatko, 2016; Makarewicz, Sealy, 2015), mainly for the reconstruction of diet (Post, 2002; Reynard, Hedges, 2008) or mobility (Chala-Aldana et al., 2018) of ancient groups, as well as for the reconstruction of paleoclimate (Bocherens et al., 2011). In most cases, bone and teeth collagen is utilized for the isotopic analysis. In the 2010s, a new paradigm of isotopic studies in archaeology emerged: the analysis of stable isotopes of oxygen and carbon in small ruminants' dental enamel (Balasse et al., 2012). This tissue contains 96 % of hydroxyapatite ($Ca_{10}(PO_4)_6(OH)_2$) where hydroxyl groups and phosphate ions are partially replaced by carbonate ions. Hydroxyapatite contains ca 4 % calcium carbonate by weight (Ressler et al., 2021). As an object of an isotopic study, dental enamel has two important advantages: fair preservation for thousands of years (as hydroxyapatite is less prone to diagenetic processes than bone and dentine), and high time resolution of the information record. Thus, the analysis of sequential samples taken from the enamel/root junction to the tip provides for detecting cyclical seasonal fluctuations of ^{13}C and ^{18}O in dental enamel (Hermes et al., 2019; Ventresca Miller et al., 2020). The isotopic composition of carbon from enamel carbonates encodes the data on the contribution of plants with C3 and C4 types of photosynthesis to the animal's diet throughout the year (Zazzo et al., 2010). The isotopic composition of oxygen from dental enamel depends mainly on the isotopic compound of water absorbed directly from reservoirs and from plants. As such, it indirectly reflects climatic factors in the area where the animal has been grazing during the formation of enamel (Pederzani, Britton, 2019). The cyclic seasonal variation of $\delta^{18}O$ in dental enamel of ruminants depends on fluctuations of ambient temperature and precipitation during a year (Balasse, 2002).

Determining cyclic variation of $\delta^{18}O$ along the growth line of the molars, accounting for the timing of dental eruption, can point to the period of the full maturation of enamel and, thus, help in reconstructing the birth seasonality (Balasse et al., 2012). The main limitation of this technique is the presence of seasonality in the studied area: only in such cases the observation of cyclic $\delta^{18}O$ variation along the tooth growth line is possible. The second and third molars are utilized for determining the birth seasonality, as these are formed during the first 2.5 years of life in ovicaprines: M2 and M3 fully erupt at ≈1.3 and ≈2.4 years of life, respectively (Geiger et al., 2020). Thus, an isotopic analysis of oxygen along the tooth growth line provides high resolution information regarding the changing seasons during this period. The first molars begin to form before the birth of the animal and fully erupt at ≈0.5 years, while the terms of a complete eruption of the permanent incisors and premolars varies in the range of more than half a year. Thus, the latter are not suitable for determining the birth seasonality of ovicaprines (Silver, 1963).

Sample preparation is the most important stage of the isotopic analysis. It includes purification, sequential sampling, and multi-stage chemical processing. In all the few studies employing isotopic analysis of dental enamel of small ruminants, carbonates are analyzed, which comprise only 4 % of hydroxyapatite by weight. But for getting reliable results on $\delta^{18}O$ in dental enamel samples of varying age and preservation it seems more interesting to analyze the oxygen isotopes ratios in phosphates, as enamel consists of almost 90 % of phosphates. Also, oxygen in phosphate groups is more resistant to diagenesis processes than that in carbonates. There are only few studies on the isotopic analysis of phosphates extracted from sequentially sampled specimens of dental enamel of animals. Cyclic changes of $\delta^{18}O_p$ along the growth line of M2 and M3 of the bison (Bernard et al., 2009), M3 of the pronghorn (Fraser et al., 2021), and M2 of the horse (Fabre et al., 2011) were previously detected. But this method has never been applied for the reconstruction of the birth seasonality of small ruminants before. In this study, we performed an isotopic analysis of oxygen in enamel of second and third molars of the ancient ovicaprines from the archaeological layers of the Istykskaya Cave, as well as modern *Ovis aries* from the neighboring area. A modern technique based on the conversion of enamel oxygen (including that in phosphates comprising 90 % of the enamel samples by weight) into CO through the interaction of the sample with carbon in a helium flow at a temperature of 1300 °C. The results of the oxygen isotopic analysis of the dental samples prepared using various chemical protocols were compared as well.

Material and methods

Samples. Five teeth of ovicaprines from Eastern Pamir were studied. This region is characterized by a

sharply continental climate with short summers and long winters (minimum temperatures reach –47 °C). The mountain landscape is divided by wide rivers. The study area is located at the Istyk River, at an altitude of 4000 m above sea level, in the zone of cryophytic alpine and subalpine meadows. The area is populated mainly by nomadic Kyrgyz migrating three or four times during a year.

Teeth of the domestic sheep (nomadic type of herding) and wild-living ovicaprines were selected for testing the protocol of determining the birth seasonality of small ruminants. The wild ovicaprines' specimens were sampled from the Istykskaya Cave collection (Fig. 1): one from the upper part of the

Fig. 1. Location of Istykskaya Cave (*1*), a view on the Istyk River valley (*2*) and on the entrance to the cave (*3*).

first layer of recent excavations (1920–1520 cal BP, GV-02963), and two from the lower layers excavated by V.A. Zhukov (14–10 ka BP) (Chelovek…, 2021). The season of birth of the wild ovicaprines was late April–May (Fedosenko, Blank, 2001, 2005).

As the degree of contamination of the modern samples (specimens 1 and 2) and the ancient tooth from the upper cave layer (3) was minimal, these were only mechanically cleaned before taking enamel samples. But the two other specimens (4 and 5) were subjected to three different protocols of preparation, including chemical treatment, in order to compare their effectiveness for determining the birth seasonality of the animals.

Sample preparation. *Protocol A—cleaning and sampling of enamel.* This protocol was applied to all the specimens. An engraver (Dremel) and a set of cutters were used. The samples were taken from the lingual side of the tooth from the enamel/root junction to the tip, perpendicular to the tooth growth axis, with increments 1–4 mm and cutting depth ca 1 mm.

Protocol B—treatment by H_2O_2. In order to remove humic substances, enamel samples were placed in 1.5 ml centrifuge tubes (Eppendorf), then 1.0 ml of 30 % H_2O_2 solution was added, and the samples were left at room temperature and stirring in a shaker for 24 hours. After this, the samples were cleaned from the reaction products by sedimentation of enamel using a centrifuge (MiniSpin, Eppendorf), removal of the liquid fraction, adding 1 ml of distilled water, and redispersing.

Protocol C—treatment by NaOCl and CH_3COOH. The way of removing humic substances was different from Protocol B: 1.0 ml of 2 % (wt.) aqueous solution of sodium hydrochloride (NaOCl) was added. The subsequent stages of cleaning the specimens from the reaction products were the same as in Protocol B. "Soft" acidic treatment was carried out to remove exogenous carbonates: 1.0 ml of 10^{-2} M acetic acid solution was added, then the specimens were kept at room temperature and stirring for 4 hours. A solution with a pH of ca 4 was applied in order to avoid sample weight loss. After the acid treatment, the cleaning procedure was repeated until the pH of the wash water reached 5–6. Then the enamel samples were dried at 70 °C for 24 hours.

Protocol D—extraction of Ag_3PO_4. The specimens were first cleaned from organic matter according to Protocol B, and from exogenous carbonates following Protocol C. After this, the samples were kept in 1.5 ml of 20 % hydrogen fluoride solution for 24 hours, then the liquid fraction was separated by centrifugation, and the solutions were brought to pH 5–7 by adding a 20 % ammonia solution dropwise. For extracting silver phosphate, 0.8 ml of 2 M $AgNO_3$ was added dropwise to the solutions. The Ag_3PO_4 samples were washed seven times by successive centrifugation/redispersion in 1.5 ml of distilled water. They were then dried at 70 °C for 24 hours.

Isotopic analysis of samples. Sample powders were weighed on a high-precision analytical scale with a division value of 10^{-6} g (ME36S, Sartorius), and were sealed by 800–1000 μg portions in special silver capsules. Immediately before the analysis, the samples were placed in the autosampler of the FLASH 2000 HCNS-analyzer (Thermo Scientific). The $^{18}O/^{16}O$ stable isotopes analysis was carried out using this device and "Delta V Advantage" gas isotope mass spectrometer. In the pyrolysis mode of the HCNS analyzer, a corundum reactor with a glassy carbon insert filled with carbon granules was used. The IAEA-603 international standard was employed for the determination of the $^{18}O/^{16}O$ ratio: $\delta^{18}O_{VPDB} = -2.37$ ‰. The results were presented relative to calcite from belemnite rostra (PDB) from the Peedee Formation (South Carolina, USA) as follows: $\delta^{18}O_{VPDB} = (R_{exp}/R_{st}-1)\cdot 1000$, where R_{exp} and R_{st} are the $^{18}O/^{16}O$ ratios in the studied samples and the standard, respectively. Results are expressed in per mille (‰).

Results

Comparison of the $\delta^{18}O$ cyclic variations in enamel of *Ovis aries* and ovicaprine (Protocol A). The enamel samples from M2 and M3 of modern *Ovis aries* and M2 of wild ovicaprine 1.5–1.9 thousand years old display marked cyclic correlations between $\delta^{18}O_{VPDB}$ and the distance from the enamel/root junction (Fig. 2). Note that the X-axes in these plots are inverted in order to display the temporal change of $\delta^{18}O$, as teeth eruption and enamel formation progress from the tip to the enamel/root junction. Images of the teeth are placed above the plots: the enamel cuts correspond to $\delta^{18}O$ on the graphs. The cyclic curves of M2 and M3 of the modern animal are depicted with a shift of the area of superposition of parts of the teeth (Fig. 2, *a*) in order to demonstrate the areas formed at the same time. The shift reaches 16 mm. It is possible to estimate what difference in eruption time this distance corresponds to: the maximums of the curves are separated by 23 mm, while their minimums by 19.5 mm. The difference

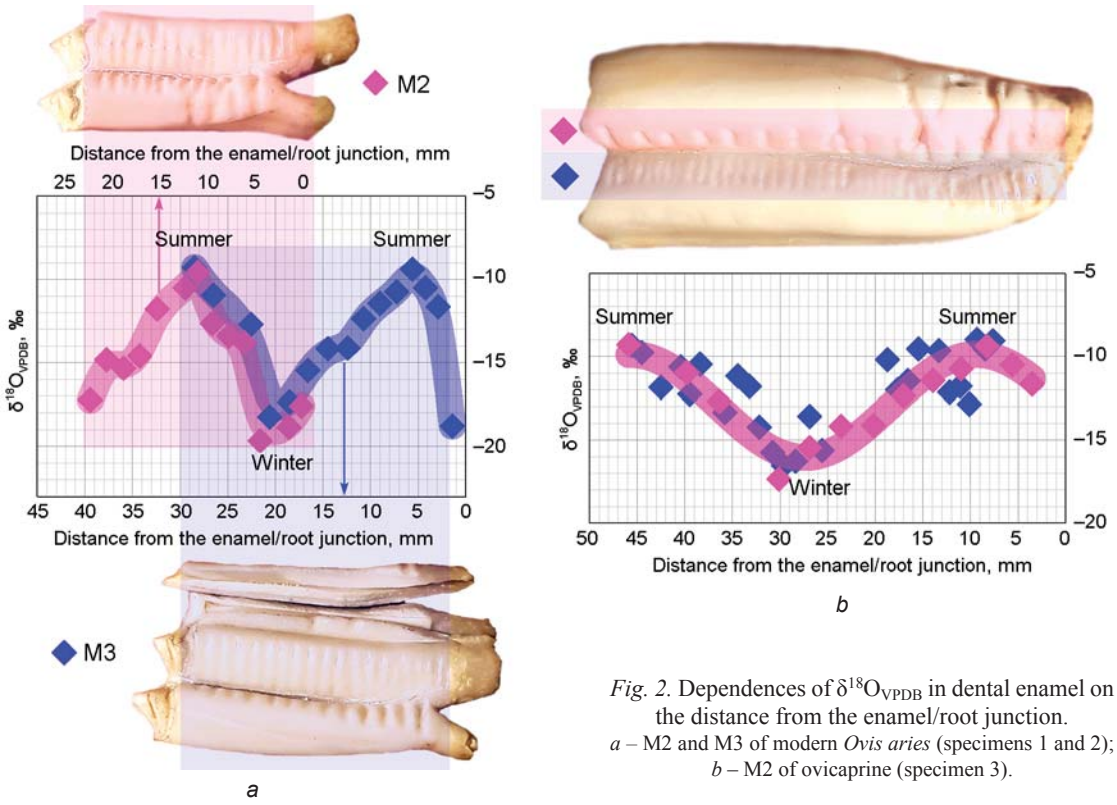

Fig. 2. Dependences of $\delta^{18}O_{VPDB}$ in dental enamel on the distance from the enamel/root junction.
a – M2 and M3 of modern *Ovis aries* (specimens 1 and 2); *b* – M2 of ovicaprine (specimen 3).

can be attributed to the disparity of growth rates during different seasons. Thus, the estimated rate of growth of the teeth is 1.6–1.9 mm/month, and the time lag between the M2 and M3 eruption in *Ovis aries* is 8.5–10.0 months, which corresponds to the literature (Silver, 1963).

We were able to detect the cyclic dependency for the relatively ancient ovicaprine's specimen (Fig. 2, *b*) without any chemical sample preparation, while such a treatment had been carried out in all the previous studies on the determination of $\delta^{18}O$ along the tooth growth line (Balasse, 2002; Balasse et al., 2012; Blaise, Balasse, 2011; Ventresca Miller et al., 2020). Chemical cleaning is an inevitable stage in the analysis of carbonates (employed in most studies on the small ruminants' domestication), since these comprise only ca 4 % of hydroxyapatite, and even subtle contamination with humic substances or/and exogenous carbonates can mask the cyclic correlations. The analysis of phosphate, which is typically employed in climate research, requires a multi-stage treatment for separating the purified Ag_3PO_4. In such studies it is necessary to obtain exact $\delta^{18}O$ specifically in phosphates mineralized during the animals' life, because any small admixture of exogenous substances and carbonates can distort the climate reconstructions.

But when it comes to determining the birth seasonality of animals, the only aim is to fix the cyclic variations along the molars' growth line. It is also important to establish the distance between the enamel/root junction and the extremes corresponding to winter and summer months of the enamel formation. The $\delta^{18}O$ value for the isotopic analysis of an unpurified sample consists of the ratio of oxygen isotopes in phosphates, endogenous carbonates, as well as exogenous organic and inorganic substances. As dental enamel is 90 % phosphate, minor contamination of the specimen from the upper level of Istykskaya Cave did not obstruct fixing a sinusoidal $\delta^{18}O$ fluctuation along the growth line of the molar.

Maximums of the curves correspond to the warmest summer month—July, while minimums to the coldest winter month—January (Balasse et al., 2012). Enamel formation of the second molar of domestic sheep begins around 2 months after birth (Blaise, Balasse, 2011), while its maturation and hence stabilization of the isotopic composition of hydroxyapatite occurs with a shift of ca 5 months in respect to the start of the formation (Balasse et al., 2012). To determine the season of birth of an animal, not only the relative $\delta^{18}O$ value at the tooth tip (minimum or maximum of the curve) is required, but also the shift should be taken into account. In our

case, the curve suggests that the animal (specimens 1 and 2) was born in autumn, as tip of the tooth shows the $\delta^{18}O$ minimum. Unfortunately, there have been no detailed researches on the time of the start of enamel formation in wild ovicaprines so far. But their lambing term is reliably known: early spring. Thus, the curve obtained for specimen 3 suggests that the enamel formation in wild ovicaprines takes 2–3 months, since the maturation of the tissue on the tip of the tooth falls on the warmest period of the year.

Comparison of the cyclic $\delta^{18}O$ variations in enamel of molars of the ancient ovicaprine specimens (sample preparation protocols B, C, D). Specimens 4 and 5 were prepared for the isotopic analysis following the protocols B, C, and D, as contamination of the teeth was substantial due to more than 10,000 years of deposition in the soil. The positions of $\delta^{18}O$ minimums and maximums in respect to the distance from the enamel/root junction are identical for the samples treated according to different protocols (Fig. 3). The areas of sampling for different protocols are highlighted in the image of the M3. The results of the measurements for the specimens subsequently treated with NaClO and CH_3COOH (Protocol C) are presented in relative units, since our first analyzes were carried out without sticking to the international standards.

The shapes of the cyclic $\delta^{18}O$ curves for the specimens subjected to different types of chemical treatment are identical if the samples were taken from the same section of the tooth: all the curves for the M2 (samples from the upper section), blue and green curves for the M3 (also samples from the upper section). The $\delta^{18}O_{VPDB}$ values for phosphates from the specimens after the H_2O_2 treatment were 1–3 ‰ higher than for phosphates extracted from enamel of the respective areas of the M2. The samples taken from different sections of the M3 display a divergence in the shapes of the curves while maintaining the location of the maximums and minimums.

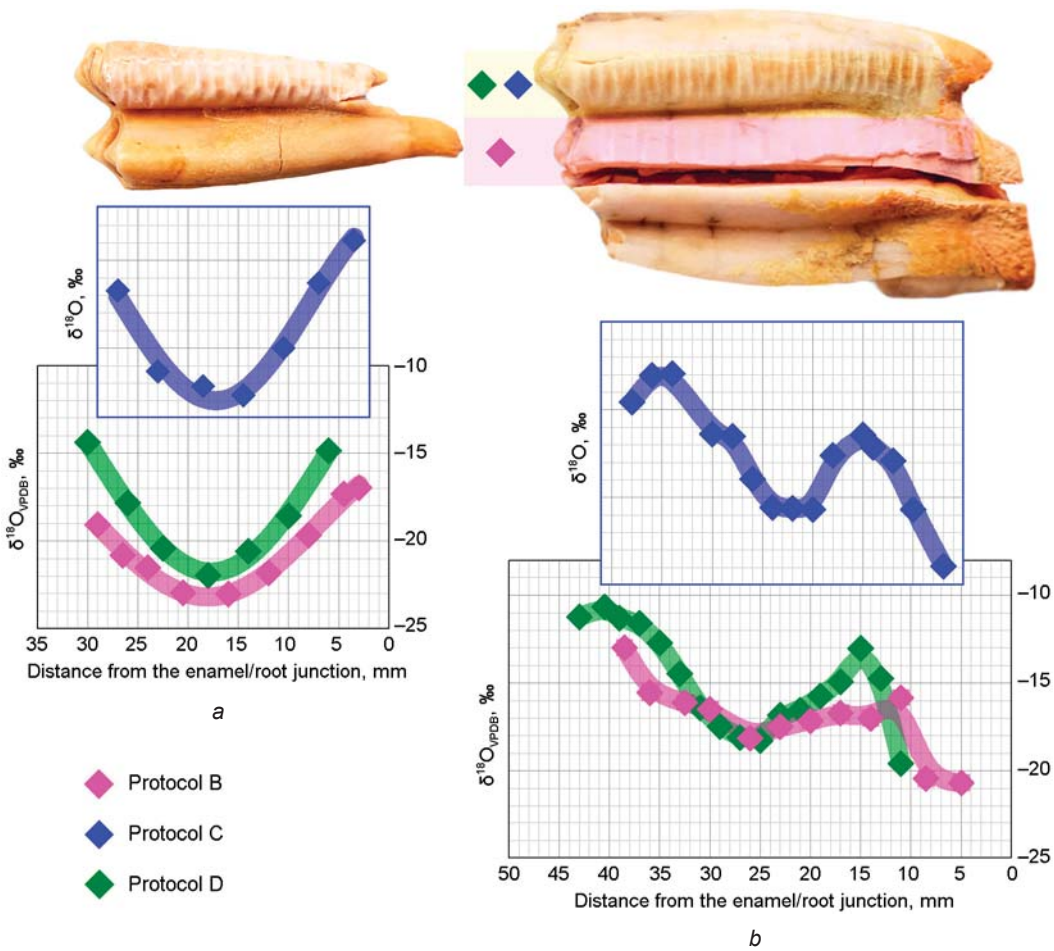

Fig. 3. Dependences of $\delta^{18}O_{VPDB}$ in molar enamel of ovicaprines on the distance from the enamel/root junction after applying different protocols of chemical treatment.
a – M2 (specimen 4); *b* – M3 (specimen 5).

The results for the ovicaprine's second molar confirm that the animal was born in spring, since the maturation of enamel at the tooth tip falls on a warm period. In this case, this process likely occurred during the 3rd to 5th months of the animal's life.

Conclusions

This study outlines a technique of oxygen isotopic analysis of tooth enamel for determining the birth seasonality of ovicaprines. The method is based on the conversion of oxygen of all the enamel components into CO in pyrolysis mode directly in the elemental analyzer. Its advantage as compared to the traditional analysis of carbonates is the high mass content (≈ 96 %) of phosphates in hydroxyapatites and their high resistance to diagenesis processes. Using this technique, it is possible to analyze more than 100 samples a day, and it requires less than 1 mg of the analyzed tissue. It is ideal for oxygen isotopic analysis of hydroxyapatite/phosphates of enamel sampled subsequently as thin strips (1 mm) along the growth line of teeth of small ruminants.

If the aim of the researcher is only to determine the seasonality of animals' birth but not to make paleoecological reconstruction, the information on the position of $\delta^{18}O$ minimums and maximums along the growth line is sufficient. For well-preserved teeth, the extremes can be detected after a minimal chemical treatment with H_2O_2, or even mechanical cleaning only. The method of Ag_3PO_4 extraction is prospective for working with teeth heavily contaminated with exogenous carbonates.

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