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Isotopic (C, O) variations of fossil enamel bioapatite caused by different preparation and measurement protocols: a case study of *Gigantopithecus* fauna

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Abstract Stable isotopic (C, O) analysis of fossil enamel bioapatite has been widely used in paleontological fields to reconstruct the paleoecology and paleoenvironment. It is common to compare the isotopic data of enamel bioapatite made by different pretreatment and measuring methods in different labs, without considering the isotopic variations possibly caused by different protocols. Here, we chose the same samples from *Gigantopithecus* fauna in the Longgu Cave (Longgudong), Hubei and remeasured their $\delta^{13}C$ and $\delta^{18}O$ values, which had been previously reported in Zhao et al. (2011) and Nelson (2014) with different pretreatment and measuring methods, in order to evaluate the effects of the above factors on the isotopic variability. The comparison among three isotopic dataset indicates that there did exist small isotopic variations on the $\delta^{13}C$ and $\delta^{18}O$ values. It seems that the $\delta^{13}C$ values were more influenced, probably due to differential practices to eliminate the diagenetic effects using varied chemicals and retaining reaction time during the process of bioapatite preparation. However, we should emphasize that the small isotopic variations observed here do not have produced substantial isotopic variance among fossil taxa and localities, providing the preliminarily theoretical foundation to make isotopic comparison directly. Even so, we still recommend that it is best to compare the isotopic data according to the same preparing and measuring protocols to remove the systematic errors or to re-measure samples again in different labs to calibrate the data.

Key words fossil enamel bioapatite, stable isotope analysis, bioapatite pretreatment, isotopic measurements

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1 Introduction

Stable isotope (C, O) analysis of fossil tooth enamel has been widely applied in paleontological research to reconstruct the paleoecology, paleoclimate and paleoenvironment of animals and hominins (Clementz, 2012). Bioapatite, the main inorganic component (97%) in enamel, is the hardest tissue in teeth and resistant to the diagenetic effects during the geological times. Chemically, enamel bioapatite is similar to hydroxyapatite crystal and consists of calcium, phosphate and hydroxyl ($Ca_{10}(PO_4)_6(OH)_2$) (Metcalfe et al., 2009). The carbonate can substitute for the phosphate and hydroxyl in hydroxyapatite and be called as structural carbonate generally. As there is a strong relationship between the isotope values of animals and their diet (Kohn, 1999), the carbon and oxygen isotope compositions of carbonates in enamel bioapatite are analyzed to reflect the information on diets and habitats of animals during the stage of tooth development (Clementz, 2012; Lee-Thorp and Sponheimer, 2014; Sponheimer and Lee-Thorp, 2014).

The teeth enamel is the hardest tissue with little organic matters (<2%) and high crystallinity in animals (Shin and Hedges, 2012). Therefore, it is always expected to be most resistant to suffering from the diagenetic effects (Clementz, 2012). However, some studies show that it is still possible for enamel to be contaminated during long-term deposits and that the chemical compositions might have been altered to some extents (Zazzo, 2014; Kendall et al., 2018; Price et al., 2019). Thus, various pretreatment methods have been proposed, trying to eliminate the potential contaminants. In general, sodium hypochlorite (NaClO), hydrogen peroxide (H_2O_2) have been suggested to get rid of the organic matters and acetic acid (CH_3COOH) to remove the potentially diagenetic carbonate (Koch et al., 1997; Snoeck and Pellegrini, 2015). But the protocols to utilize the chemicals such as the type, reaction time, concentration etc., are varied and have not reached a consensus yet. Several comparisons have been made to evaluate the chemical effects of the treatment methods on isotopic variations using modern and fossil enamel (Koch et al., 1997; Crowley and Wheatley, 2014; Snoeck and Pellegrini, 2015; Pellegrini and Snoeck, 2016; Skippington et al., 2019). However, it has been routine in paleontological research to directly compare the isotopic data of fossil animals produced in different pretreatment methods and in different labs, without considering the probably isotopic differences (Nelson, 2014; Bocherens et al., 2017; Stacklyn et al., 2017; Suraprasit et al., 2018). To date, the isotopic variations generated by diverse pretreatment methods and labs, using the same samples of vertebrate fossils, have always been neglected and not been investigated systematically yet.

In this paper, we re-measured the stable isotope values of the giant ape (*Gigantopithecus blacki*) fauna samples in our lab that were reported separately by Zhao et al. (2011) and Nelson (2014) and tried to find out the isotopic variations among them. Our aim was to discuss the factors to influence the isotopic fluctuations among different methods and labs and better understand the feasibility of isotopic comparisons in combination with isotopic data with various sources.

2 Materials and methods

2.1 Materials

The materials used in this study are the same as those in Zhao et al. (2011) and Nelson (2014) and the original numbers are listed in Table 1.

Twenty-one teeth from 7 taxa (Table 1) were sampled from Longgu Cave (Longgudong), Hubei Province for C and O isotope analysis. They included four bovids (*Leptobos* sp.), three deer (*Cervus* sp.), four tapirs (*Tapirus sinensis*), four rhinoceroses (*Rhinoceros sinensis*), one giant panda (*Ailuropoda wulingshanensis*), one hyena (*Pachycrocuta licenti*) and four giant apes (*Gigantopithecus blacki*). They were used for the following analyses. All the isotopic results listed below are expressed as $\delta^{13}C$ and $\delta^{18}O$ values relative to V-PDB.

2.2 Methods for enamel preparation and isotopic measurements

2.2.1 Method 1 from Zhao et al. (2011)

Firstly, the surface dirt and remained dentine of enamel samples were cleaned off and then the enamel was powdered. Secondly, the samples were soaked in 5% sodium hypochlorite for 12 h and then were rinsed with distilled water. Thirdly, the samples were soaked in 6% acetic acid for 12 h and then were rinsed with distilled water again to remove diagenetic carbonates. After that they were dried and collected. Finally, the CO_2 was extracted by H_3PO_4 method, reacting with 100% phosphoric acid for 12 h at 25 °C and analyzed by a Finnigan Mat 252 mass spectrometer at the Stable Isotope Laboratory in the State Key Laboratory of Lithospheric Evolution of Institute of Geology and Geophysics, Chinese Academy of Sciences (Zhao et al., 2006). Only the $\delta^{13}C$ values of samples were reported and listed here in Table 1. The analytical precision is better than 0.1‰.

2.2.2 Method 2 from Nelson (2014)

Firstly, the enamel was collected by drilling from the surface of same samples. Secondly, the enamel powders were soaked in 3% hydrogen peroxide (H_2O_2) for 15 min and rinsed by neutral water. After that, the samples were soaked in 0.1 M acetic acid for 15 min and then rinsed again. Finally, the samples reacted with anhydrous phosphoric acid for 17 min at (77±1)°C and were analyzed by a Finnigan MAT Kiel IV device coupled with a Finnigan Mat 253 mass spectrometer at Department of Earth and Environmental Sciences at the University of Michigan. The isotopic results (C, O) were calibrated by international isotopic standards (NBS18 and NBS19) and listed in Table 1. The analytical precisions of both isotopic values are better than 0.1‰.

2.2.3 Method 3 in this study

The samples available for isotopic analysis here were from the enamel powders prepared by Zhao et al. (2011). They were rinsed in distilled water again, freeze-dried, and grinded into powder. The CO_2 was prepared by H_3PO_4 method, reacting with ultrapure phosphoric acid (H_3PO_4) for 1 h at 80°C, and analyzed by an Isoprime-100 Isotope Ratio Mass Spectrometry

Table 1 Results of $\delta^{13}C$ and $\delta^{18}O$ values in Zhao et al. (2011), Nelson (2014) and this study (VPDB, ‰)

Number	Number (original)	Species	$\delta^{13}C$ (our study)	$\delta^{13}C$ (Nelson, 2014)	$\delta^{13}C$ (Zhao et al., 2011)	$\delta^{18}O$ (Nelson, 2014)	$\delta^{18}O$ (our study)
1	1	*Leptobos* sp.	–15.4	–14.4	–15.8	–9.7	–9.9
2	2	*Leptobos* sp.	–15.2	–14.5	–15.4	–9.3	–9.6
3	3	*Leptobos* sp.	–16.7	–15.8	–17.1	–9.3	–9.6
4	4	*Leptobos* sp.	–15.0	–14.3	–15.3	–7.2	–7.2
5	5	*Cervus* sp.	–16.5	–15.5	–16.8	–5.7	–5.7
6	6	*Cervus* sp.	–17.4	–16.5	–17.8	–7.6	–7.7
7	7	*Cervus* sp.	–14.9	–14.1	–15.5	–6.4	–6.2
8	8	*Tapirus sinensis*	–15.9	–15.0	–16.6	–10.4	–10.1
9	9	*Tapirus sinensis*	–17.1	–16.2	–17.7	–10.9	–10.8
10	10	*Tapirus sinensis*	–15.6	–15.1	–16.3	–9.2	–9.4
11	11	*Tapirus sinensis*	–15.8	–15.7	–16.1	–12.2	–11.3
12	12	*Rhinoceros sinensis*	–14.0	–13.8	–14.4	–9.7	–10.0
13	13	*Rhinoceros sinensis*	–15.6	–15.3	–15.8	–10.0	–10.4
14	14	*Rhinoceros sinensis*	–14.6	–14.1	–14.9	–6.4	–6.1
15	15	*Rhinoceros sinensis*	–15.5	–15.2	–15.8	–9.4	–9.1
16	16	*Pachycrocuta licenti*	–13.8	–13.7	–14.1	–9.8	–9.1
17	18	*Ailuropoda wulingshanensis*	–17.9	–16.7	–18.3	–7.7	–7.2
18	23	*Gigantopithecus blacki*	–16.7	–15.6	–17.2	–9.4	–9.5
19	24	*Gigantopithecus blacki*	–15.4	–15.4	–15.9	–9.2	–9.2
20	25	*Gigantopithecus blacki*	–17.4	–16.6	–18.2	–8.1	–8.7
21	26	*Gigantopithecus blacki*	–13.7	–12.1	–14.2	–10.5	–9.5

(IRMS) coupled with a multi-flow system at the Archaeological Stable Isotope Laboratory in Department of Archaeology and Anthropology, University of Chinese Academy of Sciences. The isotopic results (C, O) were calibrated by international isotopic standards (IAEA CO-8 and IAEA 603) and the measurement stability was monitored by the insertion of another international isotopic standard (NBS 18) into the sample list. The analytical precisions for both isotopic values are better than 0.2‰. The results were listed in Table 1.

3 Results

The isotopic results from published data (Zhao et al., 2011; Nelson, 2014) and our study are presented in the Table 1 and Fig. 1.

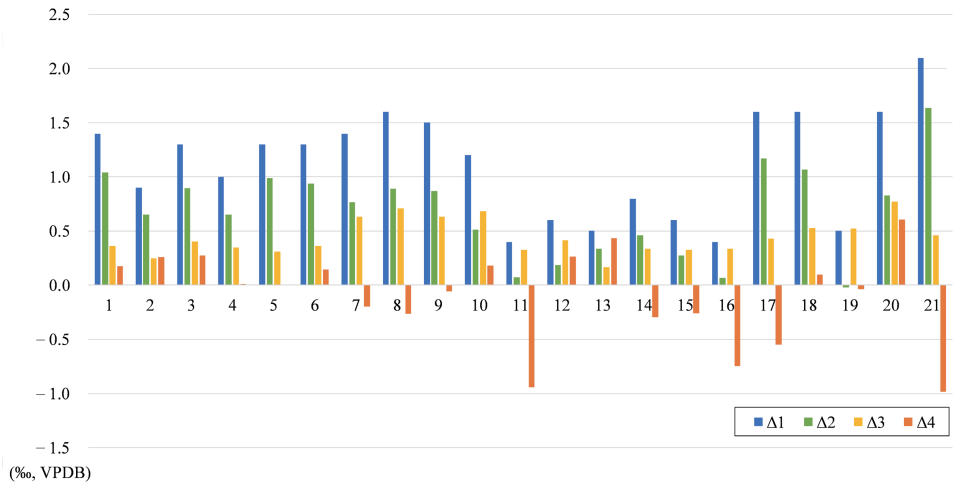

Fig. 1 The comparison of $\delta^{13}C$ and $\delta^{18}O$ values in Zhao et al. (2011), Nelson (2014) and this study ($\Delta1=\delta^{13}C_{Nelson}-\delta^{13}C_{Zhao}$; $\Delta2=\delta^{13}C_{Nelson}-\delta^{13}C_{Our\ Study}$; $\Delta3=\delta^{13}C_{Our\ Study}-\delta^{13}C_{Zhao}$; $\Delta4=\delta^{18}O_{Nelson}-\delta^{18}O_{Our\ study}$)

Although the samples for isotopic measurements were selected from the same teeth in three studies, there are still substantial differences among them in Table 1. In our study, the $\delta^{13}C$ values are –13.7‰ to –17.9‰, averaged by (–15.7 ±1.2)‰ (n=21), and the $\delta^{18}O$ values range from –5.7‰ to –11.3‰, averaged by (–8.9 ±1.6)‰ (n=21). The $\delta^{13}C$ values of Zhao et al., (2011) have a range from –14.1‰ to –18.3‰ and the mean value is (–16.2 ±1.3)‰ (n=21). The $\delta^{13}C$ values of Nelson (2014) range from –12.1‰ to –16.7‰ with the mean of (–15.0 ±1.1)‰ (n=21) and the $\delta^{18}O$ values are from –5.7‰ to –12.2‰ with the mean of (–9.0 ±1.6)‰ (n=21).

We made a histogram plot that described the isotopic differences among three studies. In Fig. 1, largest difference of $\delta^{13}C$ values with the mean of (1.1±0.5)‰ (n=21) is observed between Zhao et al. (2011) and Nelson (2014) while there is an intermediate difference of $\delta^{13}C$ values with the mean of (0.7±0.4)‰ (n=21) between our study and Nelson (2014). Smallest difference of $\delta^{13}C$ values between our study and Zhao et al. (2011), averaged by (0.4±0.2)‰ (n=21), is seen in Fig.1. Generally, there is a small difference of $\delta^{18}O$ values between our study

and Nelson (2014) with the mean of (0.3±0.3)‰.

Furthermore, the paired samples t-test results show that there are significant differences of the $\delta^{13}C$ values (P=0.000, P<0.05) between our study and Nelson (2014), between our study and Zhao et al. (2011) (P=0.000, P<0.05) and between Zhao et al. (2011) and Nelson (2014) (P=0.000, P<0.05). For $\delta^{18}O$ values, there is no significant difference (P=0.336, P>0.05) between our study and Nelson (2014).

4 Discussions

Our study in combination with two previous studies presents substantial isotopic variations in the same teeth, which is unexpected. Although Stacklyn et al. (2017) had mentioned that the discrepancy between Nelson (2014) and Zhao et al. (2011) could be caused by the differences in pretreatment. In our opinion, two main factors should be responsible for this phenomenon.

This could have resulted from the different methods to prepare the enamel bioapatite. It should be noted that the type, concentration and reacting time of chemicals used in the preparation procedure are of great difference between Nelson (2014) and Zhao et al. (2011). Nelson (2014) used 3% hydrogen peroxide for 15 min and 0.1 M acetic acid for 15 min while Zhao et al. (2011) used 5% sodium hypochlorite for 12 h and 6% acetic acid for 12 h.

Hydrogen peroxide and sodium hypochlorite are the most common chemicals in bioapatite pretreatment for removing organics in enamel (Snoeck and Pellegrini, 2015). However, both of them are suggestive of some problems. For example, NaOCl can adsorb exogenous carbonates from the external circumstances and change the contents of $\%CO_2$ in bioapatite which may not be totally eliminated in the following step of acid treatment and possibly affect the isotopic values measured (Crowley and Wheatley, 2014). In addition, utilization of NaOCl rather than H_2O_2 might reduce the yields of biogenic carbonate and isotopic results could be less reproducible (Gilg et al., 2004). On the other hand, H_2O_2, acidic, would cause the carbonate dissolution and change the inner structure of bioapatite possibly, which may trigger the isotopic changes as well (Snoeck and Pellegrini, 2015; Pellegrini and Snoeck, 2016). What's more, H_2O_2 is supposed to be insufficient to remove organics even at 80°C (Snoeck and Pellegrini, 2015). In reality, the adoption of NaClO is more than H_2O_2 as it is suggested that most of the exogenous carbonate produced by NaClO can be removed in the addition of acetic acid afterwards and it is a more efficient chemical to remove organics (Snoeck and Pellegrini, 2015; Pellegrini and Snoeck, 2016).

Acetic acid is widely used to remove exogenous carbonate. If the bioapatite is exposed to more concentrated acids and for longer treatment times, the recrystallization could have occurred, which leads to lower $\delta^{13}C$ and higher $\delta^{18}O$ values (Kohn et al., 1997; Garvie-Lok et al., 2004). Further, the recent study claims that the long treatment time of acetic acid might affect the isotopic integrity (Skippington et al., 2019). Nevertheless, other study argued that those isotopic differences caused by different treatment times were slight (Yoder and Bartelink,

2010). In general, no consensus on the acid concentration and reacting time has been made yet.

Summarizing the above, the adoption of chemicals and determination of acid concentration and reacting time in different pretreatment approaches can influence the isotopic values somehow. Thus, the significant difference of $\delta^{13}C$ values among Zhao et al. (2011), Nelson (2014) and our study could be likely caused by the above factor. The smaller difference of $\delta^{13}C$ values is observed between our study and Zhao et al. (2011) than between our study and Nelson (2014), as the samples in our study are just the same as the tooth powder prepared in Zhao et al. (2011). On the other hand, it should be noted that the minor variations of $\delta^{18}O$ values between Nelson (2014) and our study suggest that the preparation methods do not have important influence on the $\delta^{18}O$ values in enamel bioapatite.

Another important factor, the inter-laboratory differences for isotopic measurements, cannot be ignored. Those can include the measuring conditions, isotopic standards, data calibration method and so on, which also result in considerable variability of isotope data (Roberts et al., 2018; Demény et al., 2019). For example, the $\delta^{18}O$ values of enamel can be influenced by reaction conditions of generating CO_2 such as temperature and phosphoric acid concentration (Demény et al., 2019). Recent study (Ma et al., 2019) found there existed relatively moderate variations of isotopic data of Asian elephant fauna during the Late Pleistocene between our lab and the lab at the University of Tubingen, Δ=0.42‰ for $\delta^{13}C$ and Δ=0.06‰ for $\delta^{18}O$ values respectively. Therefore, this possibility cannot be ruled out to interpret the isotopic differences among two previous studies and our study. Small variations of $\delta^{18}O$ values in our lab and Tubingen lab (Ma et al. 2019) as well as in Nelson (2014) and our study might imply that oxygen isotope ratios are much less influenced than carbon isotope ratios in different labs. In contrast, recent study (Chesson et al., 2019) alleged that the oxygen isotope ratios changed significantly rather than carbon isotope ratios in a parallel measurement of the same samples in different labs. Obviously, the conditions for isotopic measurements need to be examined in the near future to understand the isotopic variations and calibrate the isotopic data.

All in all, our study finds considerably isotopic variations among two previous studies and our study, targeting the same teeth of *Gigantopithecus* fauna. This could be derived from two factors, pretreatment methods and labs for isotopic measurements. To be strict, the direct isotopic comparison among studies using different preparation approaches and labs is theoretically impossible, as the substantial fluctuations of isotopic data could have possibly led to mis-explanation. In reality, the reasonable isotopic range, *i.e.*, the minimum meaningful differences (MMD), are supposed to be 1.2‰ and 3.1‰ in bone bioapatite and 0.6‰, 1.6‰ in enamel bioapatite of $\delta^{13}C$ and $\delta^{18}O$ values respectively (Pestle et al., 2014; Chesson et al., 2019). The isotopic differences observed here are roughly located within the above range, indicating that the isotopic uncertainty caused by the different methods of pretreatment and measurements might have had minor effects on isotopic interpretation.

Given the popularity of direct isotopic comparison and no consensus on pretreatment

methods and/or measuring conditions in international fields, we recommend some measures should be taken in the future to avoid the uncertainty of comparing the isotopic data as much as possible. Firstly, more experiments need to be undertaken for better understanding the mechanism on the effects of preparation procedure on isotopic variations. Secondly, repeat measurements of enamel bioapatite in different labs can dramatically eliminate the inner-lab isotopic differences and calibrate the possible systematic errors if it is applicable when comparing the isotopic data previously produced elsewhere.

5 Conclusion

In conclusion, a comparison was conducted to evaluate the differences in the isotopic values of the enamel bioapatite from the same *Gigantopithecus* fauna pretreated by different pretreatment protocols and labs. The results show there are larger differences in the $\delta^{13}C$ values than in the $\delta^{18}O$ values among previous studies and our study. The two factors, pretreatment methods and lab measurements, could account for the above phenomenon mainly. However, the direct isotopic comparison among different studies seems still applicable, thanks to the minor isotopic variations observed here. We encourage that more similar studies should be undertaken to better understand the isotopic deviations caused by the above two factors.

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前处理与测试条件差异对化石牙釉质羟磷灰石稳定同位素数据的影响：以步氏巨猿动物群为例

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摘要：牙釉质羟磷灰石的稳定同位素分析被广泛应用于古生物学研究之中，以重建古生态和古环境信息。在对不同研究中的同位素结果进行对比分析时，往往会忽略不同实验室、不同前处理方法可能引发的数据误差。为了探讨这些因素对牙釉质羟磷灰石同位素值的影

响，重新测量了湖北省龙骨洞步氏巨猿动物群动物牙釉质样本的碳氧稳定同位素值，该批样本曾使用不同的前处理和实验方法进行过测试(Zhao et al., 2011; Nelson, 2014)。研究结果显示，重测的数据与Zhao et al. (2011)、Nelson (2014)发表的数据结果均存在一定差异。前处理方法与实验室测试差异都会造成牙釉质碳、氧稳定同位素结果的偏差。相较氧同位素而言，碳同位素值会更容易被前处理过程中反应试剂、反应时间等的不同所影响。但上述因素所导致的数据差异较小，不会对后续的分析产生实质性影响。本研究为直接对比不同来源牙釉质同位素值的可行性提供了初步的理论支持。建议为减少由于样品前处理和实验测试方案引发的数据误差，获得更加精确的研究结果，应尽可能采用同样的前处理与测试方案，多进行实验室间数据校正对比分析。

关键词：牙釉质羟磷灰石，稳定同位素分析，羟磷灰石前处理，同位素数据测量

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