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Assessing the reliability of stable isotopes in fossil bone: a unique case study of prehistoric lung pathology.

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Abstract

Mammals at Ashfall Fossil Beds State Historical Park (NE Nebraska) offer a unique opportunity to test for the preservation of primary isotopic signatures in fossilized materials. At this site, large herbivores such as rhinoceroses, horses and camels are buried in volcanic ash sourced from an eruption of the Bruneau-Jarbridge caldera (Idaho/Nevada) ca. 11.8 million years ago. Most fossils from Ashfall display pathologic bone symptomatic of a lung disease (hypertrophic osteopathy or HPOA) likely related to inhalation of volcanic ash. In this study, we compare the stable oxygen isotopic composition ($\delta^{18}O$) of pathologic bone with that of normal cortical bone to determine if elevated body temperatures associated with HPOA can be reconstructed.

Our results show consistently lower $\delta^{18}O$ values in pathologic bone, suggesting it may have formed at a higher body temperature. While the direction of offset between normal and pathologic bone ($\Delta^{18}O_{N\text{-}P}$) is consistent, the magnitude of the offset is variable and sometimes larger than can be explained solely by elevated body temperature. A change in body water $\delta^{18}O$ related to physiology and/or HPOA could be an additional factor influencing $\Delta^{18}O_{N\text{-}P}$. For example, an increase in drinking water contribution to the body water reservoir could help to explain the observed $\delta^{18}O$ offset for animals whose behavioral response to disease includes increased water consumption. This interpretation is supported by the high number of individuals concentrated in and around a shallow paleo-water body at Ashfall. Isotopic exchange during diagenesis may be another variable affecting the magnitude of $\Delta^{18}O_{N\text{-}P}$, and can be examined further through intra-individual $\delta^{18}O$ comparisons of different skeletal tissues (e.g., enamel and bone, phosphate and carbonate) or through $\delta^{18}O$ comparisons between different members of the Ashfall fauna.

Keywords: oxygen isotopes, vertebrate paleontology, diagenesis, Ashfall Fossil Beds State Historical Park, *Teleoceras major*, *Procamelus*, *Protolabis*, *Pseudohipparion gratum*, *Pliohippius supremus*.

Introduction

The stable oxygen isotopic composition ($\delta^{18}O$) of fossil bone and tooth enamel has proven extremely valuable for understanding past climate and ecology of extinct species (Koch 1998; Kohn and Cerling 2002; Clementz 2012; Koch 2007). However, the fidelity of primary $\delta^{18}O$ values in fossils is frequently questioned, because skeletal materials are subject to possible chemical alteration through the process of fossilization. Despite decades of application to paleoclimatology and paleoecology, the degree to which $\delta^{18}O$ values are affected by fossilization remains poorly understood. Kohn and Cerling (2002) have suggested several indicators for isotopic preservation such as expected sample heterogeneity or homogeneity in comparisons of different taxa and biological and/or inorganic tissues, retention of expected isotopic differences between different sympatric animals, or the retention of biological fractionations between separate tissues from a single animal. However, while these can give good checks

on isotopic signal preservation, especially when used in conjunction with each other, no conclusive test for alteration of oxygen isotopes in fossils currently exists. While some studies have suggested that well-preserved bone PO_4 in Cretaceous Period dinosaurs still maintains original $\delta^{18}O$ values (Barrick et al, 1996), most $\delta^{18}O$ studies of bone are confined to Holocene/Pleistocene aged samples (Koch, 1998) and bone is generally considered to be a less reliable source for $\delta^{18}O$ values due to higher porosity and smaller crystal size of apatite which increase susceptibility of alteration (Kohn and Cerling 2002). In particular, bone that is spongy or shows any structural weakness is generally avoided and considered to be much more susceptible to diagenesis that would affect primary isotopic ratios (Kohn and Cerling, 2002).

Ashfall Fossil Beds State Historical Park (Orchard, NE) offers a unique opportunity to assess the integrity of $\delta^{18}O$ values in fossil bone, as well as to better understand the ecology and physiology of extinct mammals. Ashfall preserves fossilized remains of hundreds of mammals, including large herbivores such as rhinoceroses, horses, and camels, which are buried in volcanic ash that was carried east following eruption of the Bruneau-Jarbridge caldera (ID/NV) approximately 11.8 million years ago (Perkins and Nash, 2002). Nearly all of the fossils from Ashfall display areas of spongy, pathologic bone, which is symptomatic of a lung disease (Hypertrophic Osteopathy or HPOA) likely related to inhalation of volcanic ash. Other symptoms associated with HPOA include localized inflammation at the area of the abnormal bone growth, and sometimes fever (Cotchin, 1944; Kelly, 1984; Ali et al, 1979). Therefore, Ashfall mammals likely experienced elevated body temperatures in the week(s) or month(s) preceding their deaths, during precipitation of the pathologic bone.

Figure1 Example of porous pathologic bone growth on top of normal cortical bone, on the mandible of an adult Teleoceras major.

The pathologic bone in the Ashfall fossils provides a way to test for clear oxygen isotopic patterns expected for bone that has not been diagenetically altered. The $\delta^{18}O$ of phosphate-bound oxygen in skeletal hydroxylapatite ($Ca_5(PO_4)_3OH$) is a function of two variables: 1) the $\delta^{18}O$ of the animals' body water, and 2) body temperature (Pucéat et al. 2010; Longinelli and Nuti, 1973), and can be represented by the following equation:

$$T(°C) = 124.6 - 4.52(\delta^{18}O_P - \delta^{18}O_W) \quad (1)$$

In most cases involving fossils, body water $\delta^{18}O$ is not known and body temperature can only be estimated to within several ºC based on modern mammal physiology. The Ashfall animals, in contrast, each possess both normal and pathologic bone that should have formed at different temperatures, but from a single body water reservoir (i.e., constant body water $\delta^{18}O$). In this study, we measured the $\delta^{18}O$ of normal and pathologic bone in order to see if their offset is consistent with equilibrium temperatures that

are persistently and reasonably higher for pathologic bone than for normal bone. This pattern would support the hypothesis that oxygen isotopes in bone are resistant to chemical alteration during fossilization.

Methods

Geological Setting

The skeletons of fossilized rhinoceroses, camels and horses examined in this study lie in a 0.3-3m ash bed within in the Cap Rock member of the Ash Hollow Formation, a ridge-forming sandstone with caliche in the Ogallala Group, in eastern Nebraska (Voorhies and Thomasson, 1979). The deposited ash was erupted from the Bruneau-Jarbridge caldera c. 11.8 Ma and carried eastward by winds before it was deposited at what is now Ashfall. The Ashfall ash was linked with the Bruneau-Jarbridge eruption by chemical comparison of glass composition in the distal ashes to proximal rhyolites and tuffs, using electron microprobe analyses, and X-ray fluorescence at the University of Utah (Perkins and Nash, 2002). The Bruneau-Jarbridge caldera eruptions produced an estimated volume of rhyolite greater than 7,000 km^3, with an overall volume of total ejecta including ash and tuff, of possibly greater than 10,000 km^3 (Bonnichsen et al., 2007; Perkins and Nash 2002).

Field Methods

A low-speed rotary drill with a 0.5 mm bit was used to collect ca. 2.0-3.0 mg samples of powdered hydroxylapatite from Ashfall fossils housed at the University of Nebraska State Museum (UNSM) in Lincoln, Nebraska. Low speed drilling was used to prevent potential isotopic fractionation caused by heating. Samples were collected from the mandible and tibia areas from camelid genera *Procamelus* and *Protolabis*, equid genera *Pseudohipparion, Pliohippus* (adult and juvenile), and *Dinohippus*, and rhinocerotid species *Teleoceras major* (adult and juvenile). In juvenile specimens, samples were collected from normal cortical bone, pathologic bone, and also porous but healthy infantile bone. Sampling was minimally destructive and did not affect diagnostic morphology of the fossils.

Laboratory Methods

We chemically prepared samples to convert the hydroxylapatite to Ag_3PO_4 using methods described in Matson and Fox (2010) and Basett et al. (2007), modified from O'Neil et al (1994). Approximately 300-800 µg of hydroxylapatite was measured into microcentrifuge tubes, and prepared with approximately 50 µL of 30% H_2O_2 to dissolve any remnant organics. The samples were then dissolved in 100 µL of 0.5M HNO_3 for 24 hours. 75 µL of 0.5 M KOH and 200 µL of 0.17 M KF were then added, and allowed to sit for 24 hours. The supernatant was then transferred to a clean microcentrifuge tube where 250 µL of silver amine (0.2 M Ag NO_3, 0.35 M NH_4NO_3, 0.74M NH_4OH) was added to precipitate crystals of $AgPO_4$. Precipitated crystals were rinsed five times, dried completely in an oven at approximately 50°C for over 24 hours, and then measured, and placed into silver packets. The $\delta^{18}O$ values were measured using an Isotope Ratio Mass Spectrometer at the Stable Isotope Laboratory at Boise State University.

Results

We tested normal and pathologic bone of eighteen skeletons from genera *Teleoceras major, Procamelus, Protolabis, Pseudohipparion gratum, Dinohippus* and *Pliohippius supremus*. In juvenile skeletons, porous infantile bone samples were also collected (n=3). In some cases normal and pathologic bone samples were collected from different elements from the same individual skeletons (mandibles vs. tibiae), to look for variability between elements.

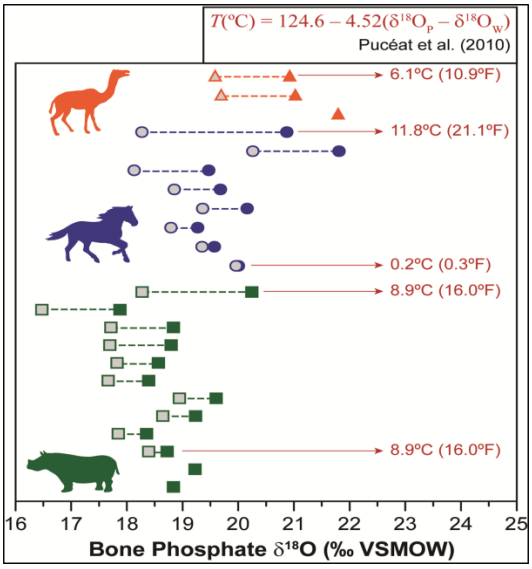

Figure 2 Bone phosphate $\delta^{18}O$ for pathologic and normal bone from this study. Red text indicates hypothetical maximum and minimum body temperature differences (ΔT) for each family, calculated using the offset between normal and pathologic bone $\delta^{18}O$ and assuming

A total of 10 normal/pathologic sample pairs were tested from rhinocerotids. The offset between normal and pathologic bone ($\Delta^{18}O_{N-P}$) in *Teleoceras major* varied between 0.34 – 1.97, with a mean $\Delta^{18}O_{N-P}$ of 0.99, yielding a change in temperature (ΔT) of 4.45ºC. The greatest offset, 1.97, suggests a change in temperature of 8.91ºC.

We tested bone phosphate from two camelid skeletons (*Procamelus* and *Protolabis*). The $\Delta^{18}O_{N-P}$ values in camelids clustered at 1.33 and 1.34, with an average ΔT of 6.03ºC.

The greatest magnitude and variability of $\Delta^{18}O_{N-P}$ values were seen in the equid samples. We tested a total of 8 healthy/pathologic pairs among equid skeletons, (3 *Pliohippus supremus, 1 Dinohippus, and 4 Pseudohipparion)*. The range of $\Delta^{18}O_{N-P}$ varied from 0.04 to 2.6, with a mean offset of 0.99, and a mean ΔT of 4.45ºC. However, some ΔT values were substantially higher, at 7.02ºC (*Dinohippus*) and 11.77 ºC (juvenile *Pliohippus supremus*).

Discussion

We see a consistent trend of lighter $\delta^{18}O$ in pathologic bone compared to normal bone from the same specimen. The magnitude of differences between these values in some samples is greater than can be explained solely by difference in body temperature using the phosphate-water fractionation equation (Pucéat et al, 2010), suggesting that change in body temperature (fever) related to HPOA cannot be the only contributing factor to the difference in isotope ratios. To consider what other factors could be responsible for this consistent offset, we explored the possibilities of contributions from several other sources.

Diagenesis

Diagenesis is a possible contributing factor to the offset between the healthy and pathologic bone. One possible explanation can be related to porosity. Increased porosity in pathologic bone could lead to increased oxygen isotope exchange during diagenesis, either through inorganic isotopic exchange with groundwater, or through organic isotope exchange by microbially-mediated removal of phosphate ions, as demonstrated by Zazzo et al. (2004). To test for the influence of diagenesis related to porosity we collected samples from three juvenile skeletons that still possessed porous infantile bone believed to have formed at normal body temperatures prior to the onset of HPOA, from areas where the skeleton had not yet developed into dense cortical bone. From these individuals we collected pathologic bone as well as healthy bone from both dense cortical areas and porous infantile bone areas. Results showed $\delta^{18}O$ of healthy infantile bone fell generally half-way between pathologic and normal bone (see Appendix). It is, however, inconclusive how much the $\delta^{18}O$ of infantile bone may have been shaped by changes in metabolism between when the individual was *in utero* and after it was born.

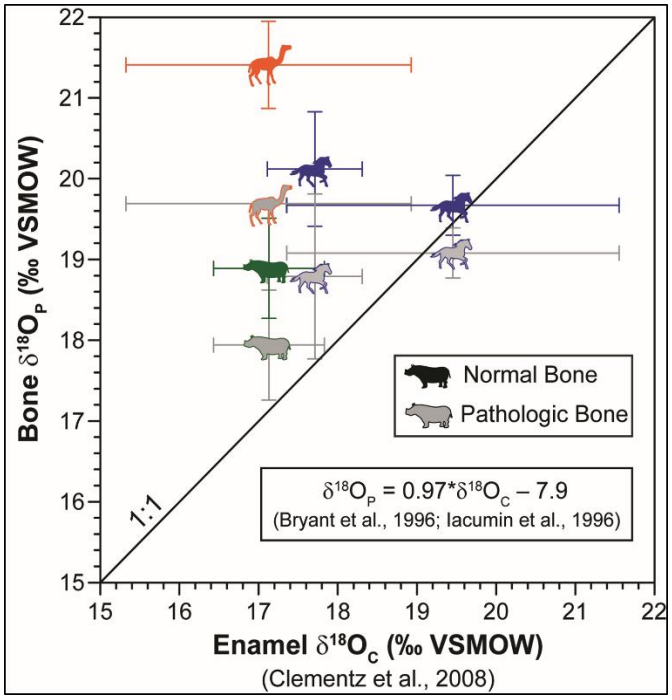

Figure 3 Bone phosphate $\delta^{18}O$ plotted against enamel carbonate δ18O values for Ashfall mammals reported by Clementz et al. (2008). Error bars represent ± 1σ for multiple (n = 2–12) individuals.

The possible effects of diagenesis on $\delta^{18}O$ values were also examined by comparing $\delta^{18}O$ results from our study of bone phosphate in Ashfall specimens to published enamel carbonate $\delta^{18}O$ ($\delta^{18}O_c$) values from animals also at Ashfall (Clementz et al., 2008). The $\delta^{18}O$ of enamel is generally considered more reliable than bone $\delta^{18}O$ due to the hardness, and large crystal size of the enamel apatite, which reduces the risk of alteration through diagenesis (Kohn and Cerling, 2002; Koch 1998, 2007).

We plotted this study's bone $\delta^{18}O$ values against the enamel $\delta^{18}O_c$ values from Clementz et al. (2008), corrected for the expected offset between phosphate and carbonate studies using linear regression for the combined data set from Bryant et al. (1996) and Iacumin et al., (1996):

$$\partial^{18}O_P = 0.97 * \partial^{18}O_C - 7.9 \quad (2)$$

Based on this comparison, bone $\delta^{18}O$ values are consistently higher than enamel $\delta^{18}O_c$ (Fig. 3).

Bone phosphate is more likely to be diagenetically altered via microbially-mediated organic processes that replace phosphate ions in the crystal lattice, and this reaction is suggested to happen within weeks of bone deposition (Zazzo et al., 2004). We assumed reasonable ranges of values for ground water $\delta^{18}O$ and ground temperature, and, assuming that microbially-mediated isotope exchange was another equilibrium process, we then computed likely changes in $\delta^{18}O$ values for replaced phosphates in the bone (Fig.4)

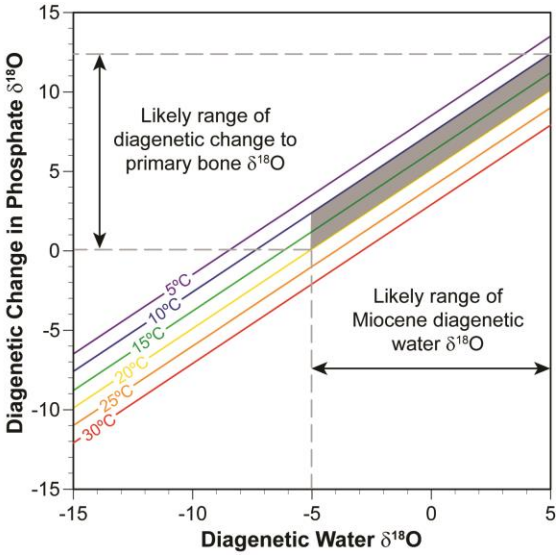

Figure 4 Diagenetic water $\delta^{18}O$ plotted against changes in phosphate $\delta^{18}O$ at different temperatures. Likely ranges for diagenetic temperature and water $\delta^{18}O$ (shaded box) indicate that diagenesis should likely result in a positive shift in bone $\delta^{18}O$.

Since isotope exchange via organic processes in diagenesis likely occurred shortly after death and bone deposition, ground water $\delta^{18}O$ values were assumed for a reasonable range during the Miocene (between -5‰ and 5‰ VSMOW). Ground temperatures were considered to be reasonable between 10 and 20ºC. Using these boundary parameters, we project that diagenesis would likely alter $\delta^{18}O$ by making values higher. This would explain the higher values of bone phosphate $\delta^{18}O$ compared to enamel, but would not explain the large offsets between pathologic relative to normal bone (higher values in the more porous or susceptible bone from diagenesis would decrease the amount of offset, not increase it).

Change in Body Water

Other explanations for the wide offset in $\delta^{18}O$ values between normal and pathologic bone could explained by changes in body water $\delta^{18}O$. We initially assumed that change in body water had not occurred between the formation of healthy and pathologic bone, but the large $\Delta^{18}O_{N\text{-}P}$ values may require this variable to change as well. Possible explanations for changes in body water include physiological or behavioral responses related to the HPOA.

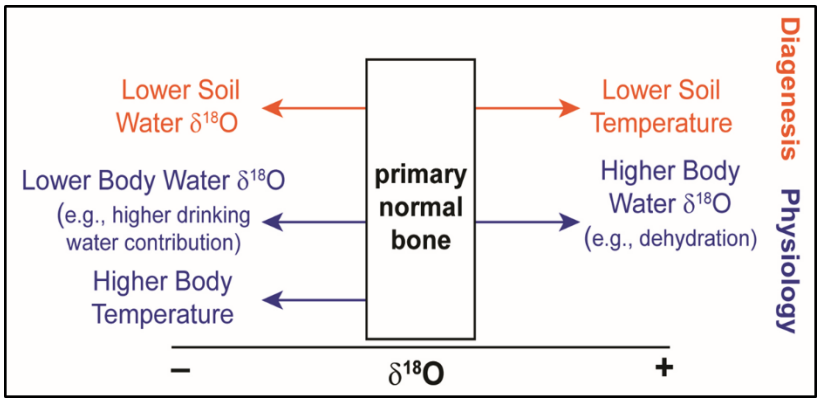

Figure 5 Direction of change for hypothetical influences of the $\delta^{18}O$ of pathologic and/or altered bone related to disease processes and/or

Dehydration caused by sickness or fever could make body water $\delta^{18}O$ values higher. This would make the offset between normal and pathologic bone smaller, not larger, similar to the projected effect of diagenesis. However, increased drinking water input associated with illness (a behavior frequently seen in modern herd mammals such as cattle, Voorhies, pers. comm) could result in lower body water $\delta^{18}O$, and could have contributed to the large offsets between normal and pathologic bone $\delta^{18}O$. That the animals buried at Ashfall engaged in this behavioral response to illness is evinced by the presence of such a large number of skeletons (n>200) buried at the Ashfall Fossil Beds, an area interpreted to be a small seasonal watering hole based upon the fauna and sedimentology (Voorhies and Thomasson, 1979).

Other Contributing Factors

Wider ranges of body temperature variation related to taxonomic/physiological variability contribute an additional variable. For example, modern camels and rhinoceroses have wider ranges of daily body temperatures than other many other mammals (Schmidt-Nielsen, 1964; San Diego zoo, web). Some of the large ΔT values seen in the camelids and *Teleoceras* could be reasonable values for their genera. Additionally confounding factors include the metabolic changes associated with juvenile versus adult animals, and the possible effect that these changes may have on $\delta^{18}O$.

Conclusion

The fossil remains buried at Ashfall Fossil Beds State Park offer a unique opportunity to test for reliability of oxygen isotopes in bone. The consistent offset between healthy and pathologic bone in the animals at Ashfall that died from hypertrophic osteopathy suggests that some portion of the isotopic record is still preserved. The magnitude in $\Delta^{18}O_{N\text{-}P}$ in some individuals, however, is not explainable by temperature variation alone. Another factor is likely needed to account for high $\Delta^{18}O_{N\text{-}P}$ we observe. Bone phosphate $\delta^{18}O$ compared to enamel carbonate $\delta^{18}O_c$ from mammals also at Ashfall showed higher bone phosphate values, which agrees with projected values for altered $\delta^{18}O$ from diagenesis. Diagenesis is therefore not projected to have increased the $\Delta^{18}O_{N\text{-}P}$. Changes in body water related to physiological or behavioral responses during progression of the disease could have altered the $\delta^{18}O$ of the pathologic bone. Increased contribution of drinking water to body water reservoir could account for lower $\delta^{18}O$ in the pathologic bone.

Further Research

One way to further test for diagenetic effects would include testing for $\delta^{18}O$ of carbonates from the same bone samples collected for this study. Additionally, testing the crystallinity index of the healthy juvenile bone versus the pathologic bone would provide a way to quantify the difference in porosity between the two, and this could be compared to the $\Delta^{18}O_{J\text{-}P}$ values and $\Delta^{18}O_{N\text{-}J}$, to see if the differences can be totally accounted for by diagenesis.

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Appendix

Sample ID	*Taxon*	Element	Normal bone $\delta^{18}O$	Pathologic bone $\delta^{18}O$	Juvenile bone $\delta^{18}O$	$\Delta^{18}O_{N-P}$	ΔT °C
HPOD 01	*Procamelus*	Mandible	21.79	-	-	-	-
HPOD 02	*Procamelus*	Mandible	21.02	19.69	-	1.33	6.0
HPOD 03	*Protolabis*	Mandible	20.92	19.58	-	1.34	6.1
HPOD 15	*Teleoceras major*	Rib	18.79	17.69	-	1.10	4.97
HPOD 17	*Teleoceras major*	Rib	19.22	-	-	-	-
HPOD 18	*Teleoceras major*	Tibia	18.83	17.71	-	1.12	5.06
HPOD 19	*Teleoceras major*	Tibia	18.56	17.82	-	0.74	3.34
HPOD 20	*Teleoceras major*	Tibia	18.39	17.66	-	0.73	3.30
HPOD 21	*Pseudohipparion*	Mandible	19.68	18.85	-	0.83	3.77
HPOD 22	*Pseudohipparion*	Mandible	19.27	18.79	-	0.48	2.15
HPOD 23	*Pseudohipparion*	Mandible	19.57	19.35	-	0.22	1.01
HPOD 24	*Pseudohipparion*	Mandible	20.16	19.36	-	0.80	3.63
HPOD 25	*Teleoceras major*	Mandible	18.35	17.84	-	0.51	2.31
HPOD 26	*Teleoceras major*	Tibia	-	-	17.71	-	-
HPOD 27	*Teleoceras major*	Tibia	18.72	18.39	-	0.34	1.53
HPOD 29	*Teleoceras major*	Mandible	17.87	16.47	-	1.40	6.32
HPOD 30	*Teleoceras major*	Tibia	20.24	18.27	19.82	1.97	8.91
HPOD 31	*Teleoceras major*	Mandible	19.23	18.64	-	0.59	2.68
HPOD 33	*Teleoceras major*	Mandible	19.60	18.94	19.36	0.66	2.99
HPOD 34	*Pliohippus supremus*	Mandible	20.87	18.27	-	2.60	11.77
HPOD 35	*Pliohippus sp.*	Tibia	19.47	18.13	-	1.35	6.08
HPOD 36	*Pliohippus sp.*	Mandible	20.00	19.96	-	0.04	0.18
HPOD 37	*Dinohippus sp.*	Mandible	21.81	20.26	21.28	1.55	7.02

Table 1. δ18O results for mammal bone phosphate.

	Pathologic Bone Mean $\delta^{18}O$*	Normal Bone Mean $\delta^{18}O$*	Mean $\delta^{18}O$* offset between normal and pathologic bone	Mean ΔT value or "fever" (°C)
All (n=20)	18.58	19.57	0.99	4.45
Teleoceras major (n = 10)	17.94	18.86	0.92	4.14
Horses, all (n=8)	19.12	20.10	0.99	4.45
Pseudohipparion gratum (n=4)	19.08	19.67	0.58	2.64
Pliohippus supremus (n=3)	18.79	20.12	1.33	6.01
Dinohippus (n=1)	20.26	21.81	1.55	7.02
Camels, all (n=2)	19.64	20.97	1.34	6.03
Procamelus (n=1)	19.69	21.02	1.33	6.01
Protolabis heterodontus (n=1)	19.58	20.92	1.34	6.06
Juveniles, all (n=3)	18.78	20.06	1.27	5.75
Adults, all (n=17)	18.47	19.31	0.83	3.72

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