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Source: Journal of Mammalogy, 93(2) : 368-380

Published By: American Society of Mammalogists

URL: <https://doi.org/10.1644/11-MAMM-S-179.1>

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## New insight from old bones: stable isotope analysis of fossil mammals

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Stable isotope analysis of fossil materials has become an increasingly important method for gathering dietary and environmental information from extinct species in terrestrial and aquatic ecosystems. The benefits of these analyses stem from the geochemical fingerprint that an animal's environment leaves in its bones, teeth, and tissues. Ongoing study of living mammals has found the stable isotopic composition of several light (hydrogen, carbon, nitrogen, oxygen, and sulfur) and even a few heavy (calcium and strontium) elements to be useful tracers of ecological and physiological information; many of these can be similarly applied to the study of fossil mammals. For instance, the carbon isotopic composition of an animal's tissues tracks that of the food it eats, whereas the oxygen isotopic compositions of the carbonate and phosphate in an animal's bones and teeth are primarily controlled by that of the surface water it drinks or the water in the food it ingests. These stable isotope proxies for diet and habitat information are independent of inferences based on morphological characters and thus provide a means of testing ecological interpretations drawn from the fossil record. As such, when well-preserved specimens are available, any dietary study of fossil species should seriously consider including this approach. To illustrate the potential benefits associated with applying these methods to paleontological research, a review of current work on the ecological and evolutionary history of fossil mammals through geochemical analysis is presented. After a brief introduction to issues associated with the preservation of stable isotopic information in soft and mineralized tissues, a series of case studies involving the application of stable isotope analysis to fossil mammal research is discussed. These studies were selected to highlight the versatility of this analytical method to paleontological research and are complemented by a discussion of new techniques and instrumentation in stable isotope analysis (e.g., laser ablation and compound-specific isotope ratio mass spectrometry, and calcium and clumped isotopes), which represent the latest advances in the extension of these geochemical tools to the paleontology of fossil mammals.

Key words: bioapatite, calcium isotopes, collagen, migration, paleodietary reconstruction, strontium isotopes

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DOI: 10.1644/11-MAMM-S-179.1

With the discovery of measurable natural variation in the stable isotopic composition of vertebrate fossil remains, paleontologists gained a valuable tool for studying fossil mammals from ancient marine and terrestrial communities. Because direct observation of extinct species within a community is not possible, stable isotope analysis has become an increasingly important tool for paleontologists interested in the paleoecology of ancient mammals (Cerling et al. 1997; Clementz et al. 2003b; Hoppe et al. 1999; MacFadden et al. 2004). Prior to the initial application of this analytical tool to archaeological (Van der Merwe and Vogel 1978; Vogel and Van der Merwe 1977) and subsequently paleontological (DeNiro and Epstein 1978; Ericson et al. 1981; Schoeninger and DeNiro 1982a, 1982b) research in the late 1970s and early 1980s, ecological interpretations of fossil mammals were primarily restricted to interpretations based on either examination of the morphology of the specimens or careful study of sedimentary environments in which fossils were deposited. Because morphological

structure is often strongly correlated with function, examination of these features, especially dentition and appendicular skeletal anatomy, can provide information on various ecological characters, including diet, trophic position, and ecological guild structure within fossil communities (Damuth and Janis 2005; Janis 1993; Meachen-Samuels and Van Valkenburgh 2009; Van Valkenburgh 1995; Van Valkenburgh et al. 2004). Likewise, the depositional history inferred from the sedimentary matrix surrounding the fossilized remains of mammals can provide information on habitat preferences, species associations, and climatic tolerances (Badgley and Behrensmeyer 1980; Behrensmeyer 1988; Boucot and Janis 1983; Zobaa et al. 2011). However, applying these methods to fossil remains is not always straightforward. For instance, fossil species may possess



morphological traits that are not present in extant species, making interpretation of their function and ecological significance through comparison with analogous structures in living species impossible. Likewise, the remains of an organism can be transported considerable distances from where the individual originally lived and died, biasing interpretations of habitat preferences of extinct species if based solely on association with sedimentary environments. Although prone to its own set of caveats, stable isotope analysis has proven to be an effective means of assessing the integrity of these other lines of evidence and, when used in combination with more traditional methods of paleontological inquiry, can offer a more rigorous and quantitative method for ecological interpretation that is independent from morphology- or phylogeny-based inference and covers a broad range of timescales and environments.

Stable isotope analysis is applied to the paleobiology of fossil mammals either to gain insight into the biology of the extinct species or to better understand the environmental conditions it experienced. Diet, habitat preferences, and physiology are the most commonly investigated aspects of fossil mammals sought through the application of stable isotope analysis. As noted in Ben-David and Flaherty (2012), isotopic differences among resources ingested by mammals (i.e., food and water) can serve as natural labels for these resources, which can then be identified by their incorporation into the tissues of a mammal. These labels allow paleobiologists to discriminate among potential diets and habitats for extinct species. In turn, these labels can provide information about environmental conditions of a region once biological factors affecting the fractionation and incorporation of the environmental signal into tissues of an animal (i.e., vital effects) are removed (Ben-David and Flaherty 2012; Martínez del Rio and Carleton 2012). The isotopic compositions of fossil remains are routinely used by archaeologists, paleoclimatologists, and paleoceanographers as proxies for temperature, precipitation, elevation, and salinity of past terrestrial and aquatic environments (Behrensmeyer et al. 2007; Fricke et al. 1998; Garzione et al. 2008; Koch et al. 1995). These studies have provided a wealth of isotopic data that can be exploited by paleobiologists to answer questions about the ecology of ancient mammals.

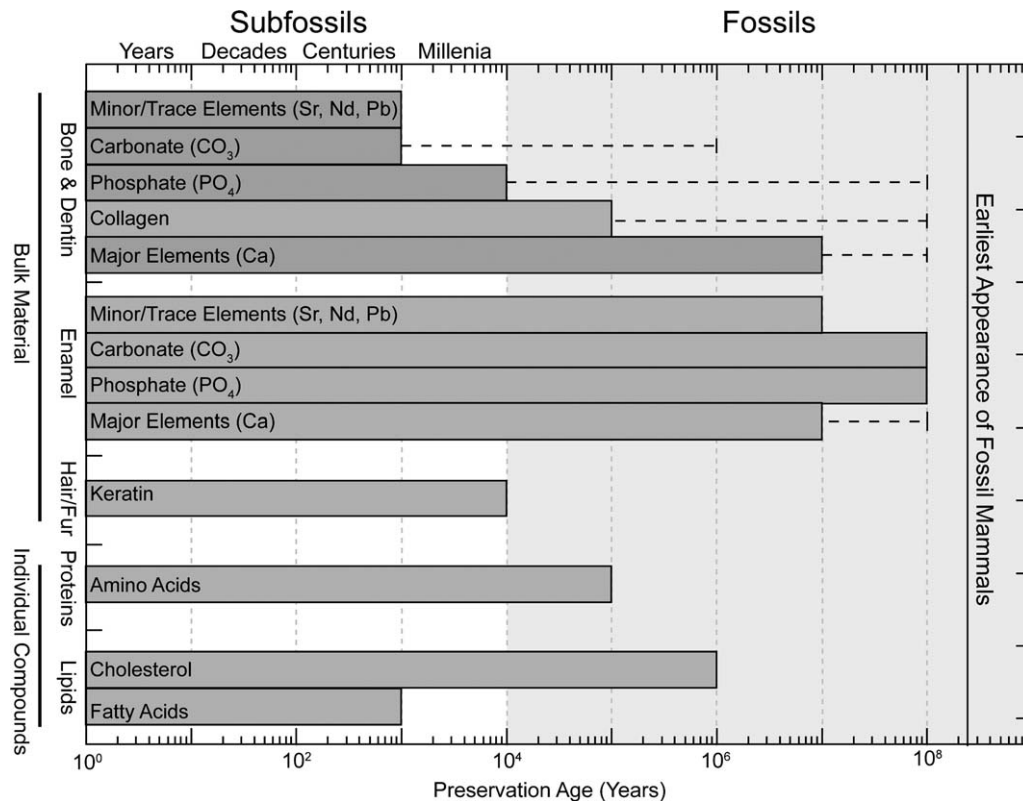
Here, I will provide a review of how paleobiologists have exploited the isotopic composition of fossil remains to answer questions about the ecology and evolution of mammals. These techniques can and have been applied to similar questions within archaeology; however, because the scope of this paper is fossil mammals (nonhuman), I have restricted the content of this review to purely paleontological examples. After a brief introduction into the preservation potential of soft and mineralized tissues in the fossil record, I will discuss a series of case studies that exemplify the ways stable isotope analysis has been applied to paleobiological research of fossil mammals. Because the popularity of this technique has increased since its 1st application to paleobiology nearly 40 years ago, these examples represent only a small sampling of the research that has or is currently being conducted in this field. For more information on this topic, excellent reviews of

different aspects of this research are provided by Koch (1998, 2007) and Kohn and Cerling (2002).

### PRESERVATION OF MAMMAL REMAINS IN THE FOSSIL RECORD

For geologically young fossils ( $<100 \times 10^3$  years), both the inorganic and organic components of the skeleton are commonly available for stable isotope analysis (Fig. 1) and can be extremely informative when measured in tandem (Clementz et al. 2009). The stable isotopic compositions of carbon ( $\delta^{13}\text{C}$ ), hydrogen ( $\delta\text{D}$ ), nitrogen ( $\delta^{15}\text{N}$ ), oxygen ( $\delta^{18}\text{O}$ ), and sulfur ( $\delta^{34}\text{S}$ ) all have been measured from fossil collagen, as well as carbon and nitrogen isotopic compositions from individual amino acids within the collagen matrix (Fogel and Tuross 2003; Styring et al. 2010), making it a suitable substrate for multiple lines of ecological and physiological inquiry. Preservation of original isotopic information in bone and dentin proteins (e.g., collagen) and isolated organic compounds should be assessed before inferring ecological information. For collagen, the most commonly used indexes of preservation quality are the total yield of collagen, the concentrations and ratio of atomic or molar carbon to nitrogen ( $\text{C:N}_{\text{atomic}}$ ) in collagen, and amino acid analysis (Ambrose 1990; Tuross et al. 1988; van Klinken 1999). Based on these criteria, well-preserved collagen typically constitutes  $>1\%$  by weight (wt %) of fossil bone (fresh bone is approximately 22 wt % collagen), is composed of about 35 wt % carbon and 11–16 wt % nitrogen, and has a  $\text{C:N}_{\text{atomic}}$  ratio between 2.9 and 3.6. Collagen yields with  $<1$  wt % and carbon contents  $<30$  wt % are indicative of significant degradation, which may be large enough to affect the isotopic composition of bulk collagen (Ambrose 1990; van Klinken 1999). When the carbon content of collagen is much higher ( $>>35$  wt %), contamination from exogenous sources (e.g., soil humic matter) may be responsible, which can also affect isotopic composition, making these samples unsuitable for analysis. Determination of the relative abundances of amino acids in bulk collagen also is informative because each may degrade at different rates, creating a composition very different from the original collagen. Because the isotopic compositions of amino acids vary greatly, changes in the relative proportions of these amino acids can further complicate isotopic analysis. For collagen yields that fall within an acceptable range (20.0–1.0 wt %), amino acid abundances and profiles do not appear to vary much from expectations for fresh collagen, so this appears to only affect specimens that are severely degraded ( $<0.5$  wt % collagen—van Klinken 1999).

Although unique examples of soft tissue preservation of Pleistocene-aged and possibly Cretaceous-aged remains are known (Kosintev et al. 2010; Schwarz et al. 2009; Schweitzer et al. 2002, 2007a, 2007b), the rapid degradation of organic remains shortly after death often excludes most tissues from stable isotope analysis. Loss of most organics from early Pleistocene-aged or older fossil remains ( $>100$  thousand years) means paleontologists are often limited to using



**FIG. 1.**—Age range over which different organic and mineralized tissues in mammal remains are likely to be preserved. Bars denote the expected age range for preservation, whereas horizontal dashed lines represent maximum age of preservation under exceptional conditions. The figure is based on information in Koch (2007:table 5.2). Shaded field separates fossil material ( $>10^4$  years) from subfossil material ( $<10^4$  years). Vertical black line to the right marks the 1st appearance of mammals in the fossil record (approximately  $2.25 \times 10^8$  years—Lucas and Luo 1993). Present is located at the far left of the x-axis.

bioapatite, specifically tooth enamel, for stable isotope analysis. The high crystal density, low organic content ( $<5$  wt %), and large crystal size of enamel increase its resistance to diagenetic alteration, a process that involves the exchange of original biogenic material with pre- or postburial environmental fluids and is aided by the microbial breakdown of organic matter in skeletal remains (Koch et al. 1997; Lee-Thorp and van der Merwe 1987; Wang and Cerling 1994; Zazzo et al. 2004). Within enamel, stable isotope analysis has been performed on several elements: the oxygen within phosphate (PO<sub>4</sub>), which is thought to be more resistant to exchange with fluids; the carbon and oxygen of carbonate (CO<sub>3</sub>) that is structurally integrated into the enamel mineral lattice (2.0–4.0 wt %); and calcium and strontium (Sr), which are major and trace elements, respectively, within bioapatite. Of these, stable isotope analysis of carbonate in enamel is most often measured, because the chemical preparation and analysis of this component is easier, less time consuming, and provides isotopes of 2 elements (C and O) for interpretation rather than just 1 as in phosphate (O).

For bioapatite, isotopic alteration can occur through 5 processes (Koch 2007). The most obvious of these is the postmortem precipitation of secondary minerals on or around bioapatite crystals in the fossil remains. Typically, this occurs following burial as soil or groundwater passes through pore

spaces within skeletal elements, but it can occur before burial in semiarid or arid environments when soil moisture is pulled up through bones exposed on the surface (Trueman et al. 2004). Similarly, ions freely available from the burial environment may be adsorbed onto the surface of bioapatite crystals. This alteration may affect both modern and fossil materials, but can be removed through controlled chemical leaching in the laboratory in preparation for analysis. Over longer timescales, bioapatite may be altered more extensively through solid-state diffusion; ion or atom exchange within the crystal lattice (most problematic for bone and dentin due to the high surface-to-volume ratio of these crystals); and dissolution, reprecipitation, and recrystallization. Alteration resulting from these 3 processes may be impossible to correct.

Methods used to evaluate the extent of diagenetic alteration were compiled by Kohn and Cerling (2002). These methods include assessing the extent of isotopic heterogeneity or homogeneity among specimens from a single deposit; exploiting ecological and associated isotopic differences among sympatric species; retention of expected inter-tissue differences in isotopic composition from a single specimen; changes in bioapatite crystallinity through alteration; comparison with isotopic composition of surrounding sediments and cements; and retention of expected correlation between chemical components of the same tissue (e.g., bioapatite



PO<sub>4</sub> and CO<sub>3</sub>). These authors conclude that enamel, especially the phosphate component of enamel, is most resistant to alteration and all other bioapatites, especially bone, should be considered suspect in specimens from the late Pleistocene or earlier. However, microbial degradation of organic remains can facilitate or enhance the alteration of the oxygen isotopic composition of phosphate in bioapatite (Zazzo et al. 2004). Although the low organic content of enamel means it would be less susceptible to this process, close association with soft tissues early in the decay process (e.g., organic matter in bone or dentin) could impact tooth enamel for those species with thin enamel caps or small teeth through changes in pH and chemical conditions associated with microbial degradation of this organic matter. Thus, no bioapatite should be considered immune from the effects of alteration, and the isotopic integrity of all materials should be assessed following the methods listed by Kohn and Cerling (2002) before making any interpretations.

### STABLE ISOTOPE APPLICATIONS TO FOSSIL MAMMAL PALEOBIOLOGY

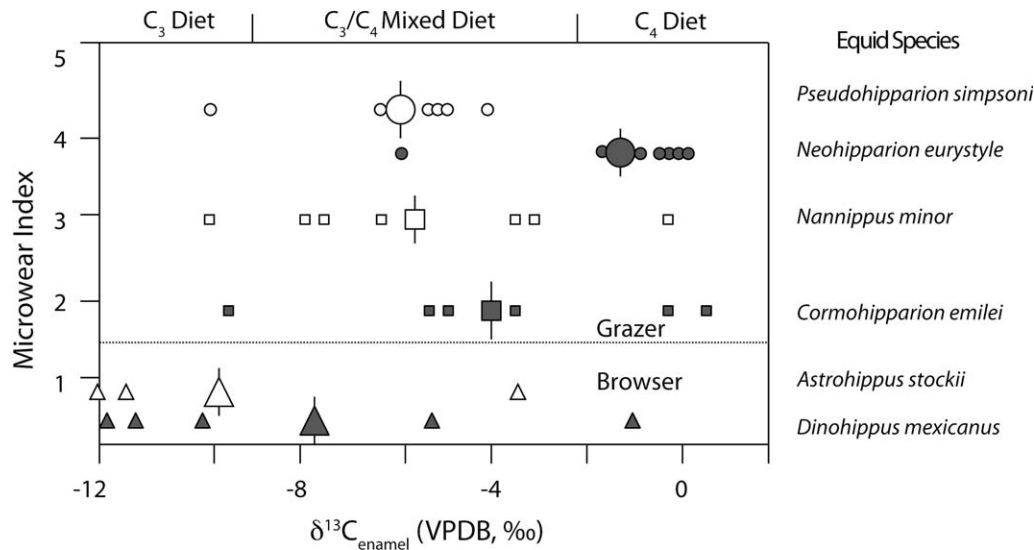
Stable isotope analysis is most commonly applied to the study of fossil mammals as a proxy for paleodietary information. Paleontologists have taken advantage of naturally occurring differences in the stable isotopic composition of various food resources, which are most often derived from primary producers at the base of the food web. Variation in physiology (i.e., C<sub>3</sub>, C<sub>4</sub>, and crassulacean acid metabolism [CAM] photosynthetic pathways), uptake of isotopically distinct materials and nutrients (e.g., atmospheric CO<sub>2</sub>, respired CO<sub>2</sub>, and HCO<sub>3</sub><sup>−</sup>), and environmental conditions can all affect the isotopic composition of different producers and the consumers that eat them, providing a label for particular diet types or foraging habits. A thorough discussion of the relationship between these isotopic labels in diet and mammalian tissues is presented by Ben-David and Flaherty (2012) and Martínez del Río and Carleton (2012). Here, I will present a few examples of how these relationships, which are based on studies of modern mammals, have been applied to the fossil record.

The large carbon isotopic difference between C<sub>3</sub> and C<sub>4</sub> primary producers has provided 1 of the most widely used and broadly applied dietary tracers in paleobiological study (Bocherens et al. 1996; Cerling et al. 1997, 1998; Fox and Koch 2004; Franz-Odenaal et al. 2002; Koch et al. 1998, 2004; Latorre et al. 1997; MacFadden and Cerling 1996; MacFadden et al. 1996; Wang et al. 1994; Zazzo et al. 2000). In low and midlatitude grasslands where C<sub>4</sub> grasses are the dominant grass type today,  $\delta^{13}\text{C}$  values of herbivore enamel record a dramatic increase in consumption of C<sub>4</sub> grasses during the late Miocene (protracted rise from  $8 \times 10^6$  to  $3 \times 10^6$  years ago—Cerling et al. 1997; Edwards et al. 2010; Tipple and Pagani 2007). These mammal fossils provide the initial evidence for appearances of C<sub>4</sub> grass in the past because macrofossils of the actual grasses are rare (Nambudiri et al.

1978; Thomasson et al. 1986) and pollen and phytoliths of C<sub>4</sub> grasses are indistinguishable from those for C<sub>3</sub> grasses (Stromberg 2004). Thus, stable isotope analysis of tooth enamel from ungulates inferred to have been grazers based on their high-crowned, or hypsodont, dentition provides a novel means for constraining the availability and prevalence of C<sub>4</sub> grasses in herbivore diets. However, work with extant equids suggests this proxy may not be suitable for identifying the earliest presence of C<sub>4</sub> grasses in the fossil record (Hoppe et al. 2004).

In the study by Hoppe et al. (2004), isotopic compositions of carbon and oxygen of tooth enamel from modern feral horses were measured from 2 locations: the C<sub>3</sub> grasslands of eastern Oregon (100% C<sub>3</sub> grass species) and the C<sub>4</sub>-dominated grasslands of New Mexico (>95% C<sub>4</sub> grass species). Horses were selected because of their morphological adaptations for grass-based diets (e.g., high-crowned teeth) and their long fossil record in North America (about  $55 \times 10^6$  years ago), a point that has made them widely exploited within isotopic studies of fossil mammals. Based on morphological characters, equids are commonly viewed as grazers, which would make them an ideal group to use as proxy for the abundance and type (C<sub>3</sub> compared to C<sub>4</sub>) of grasses in the past. Careful examination of fecal samples from these populations showed that whereas isotopic values for tooth enamel and feces were in good agreement with the dominant grass types of the regions (100% C<sub>3</sub> in Oregon and 85% C<sub>4</sub> in New Mexico), the actual abundance of grass in the diet was lower (95% grass in Oregon and 75% grass in New Mexico—Hoppe et al. 2004). These results suggest that estimations of proportion of C<sub>3</sub> to C<sub>4</sub> grasses based solely on  $\delta^{13}\text{C}$  values from fossil equid tooth enamel could seriously underestimate (or possibly overestimate—see Fox and Koch 2000) the true abundance of C<sub>4</sub> grasses. Analysis of whole communities of fossil ungulates, which would improve the odds of sampling consumers with purely grass-based diets in combination with other methods more reflective of relative abundances of grass types and less prone to bias based on herbivore dietary preferences (e.g., carbon isotope analysis of pedogenic carbonates), might be the one way to get past this limitation.

One of the primary advantages of applying stable isotope analysis to infer dietary preferences for extinct mammals is that it allows researchers to make these interpretations independent of morphology. As noted above, work with extant mammal species has shown that dietary preferences can vary considerably among species, even when morphological characters suggest highly restricted diets. A similar but more extreme finding was made by paleontologists working in latest Miocene- to Pliocene-aged fossil deposits of Florida (MacFadden et al. 1999), which have produced fossils from 6 sympatric species of equids. All possessed hypsodont dentition and were initially interpreted as grazers. Enamel  $\delta^{13}\text{C}$  values for these species in combination with examination of the microscopic abrasion and attrition of the occlusal surface of the cheek teeth by food, food-borne grit, and tooth-on-tooth contact (i.e., microwear), however, revealed that the diets of



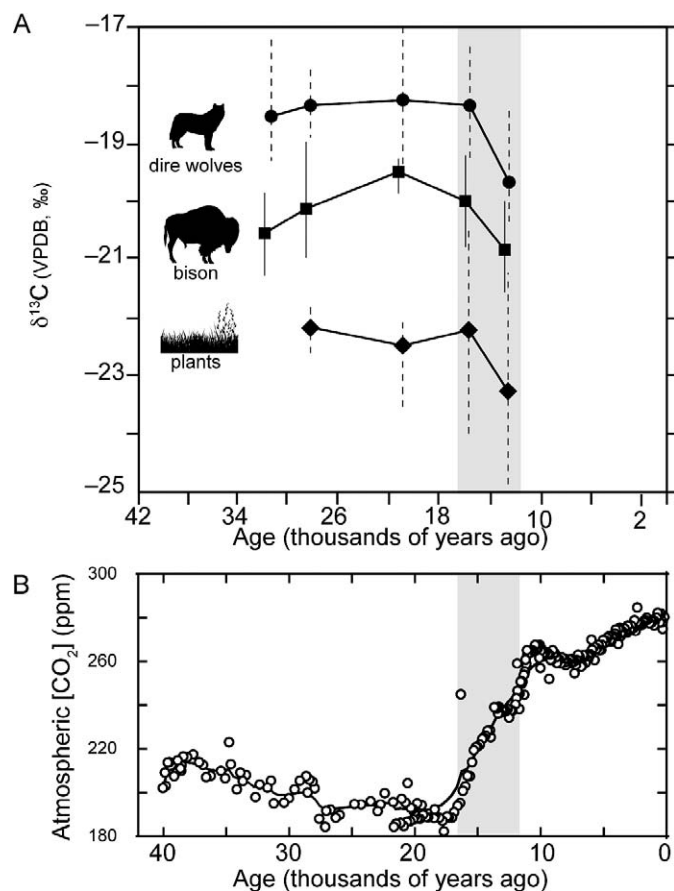
**FIG. 2.**—Individual (small symbols) and mean (large symbols) enamel  $\delta^{13}\text{C}$  values plotted against mean microwear index for 6 sympatric equid species from the late Miocene of Florida (MacFadden et al. 1999). Symbol shape (circles, squares, and triangles) and color (white and gray) are used to differentiate values for species that lie close together. Vertical error bars represent  $\pm 1$  SD for mean microwear, which is calculated as the ratio of scratches to pits per unit area ( $0.5\text{ mm}^2$ ) on the occlusal surface of a tooth. Carbon isotope values are referenced to the international standard Vienna Pee Dee Belemnite (VPDB).

these equids were much more diverse (Fig. 2). Microwear for 4 species was consistent with a grass-based diet, but enamel  $\delta^{13}\text{C}$  values showed that the diet for only 1 species (*Neohipparion eurystyle*) was primarily  $\text{C}_4$  grasses, whereas those for the other 3 species included some  $\text{C}_3$  grass or browse as well. Most surprising was the discovery of 2 hypsodont species (*Astrohippus stockii* and *Dinohippus mexicanus*) with microwear and enamel  $\delta^{13}\text{C}$  values indicative of a diet of  $\text{C}_3$  browse and little to no grass. Prior to these findings, paleobiologists assumed that the presence of high-crowned teeth in a fossil species was strong evidence of a grass-based diet and this connection had become a paradigm of paleodietary and ecomorphological research. These findings showed that this model was not appropriate in all situations and provided the best example of how stable isotope analysis could benefit paleobiological research.

Subtle linkages between consumer and producer isotopic values also have been exploited to examine how the environmental conditions experienced by mammal communities have shifted over time (Bump et al. 2007). The carbon isotopic composition of primary producers can fluctuate in response to changes in the growth environment (e.g., light intensity,  $[\text{CO}_2]$ , and water availability) and, if these changes are sustained over long stretches of time, can result in a distinct isotopic shift that can be passed on to consumers foraging within the community. Because herbivorous and carnivorous mammals sample multiple plant and prey types, respectively, over the course of their lifetimes, the isotopic composition of their tissues maintains a running average of the baseline isotopic composition of a food web. The spatial and temporal integration of this isotopic information increases with trophic level, ultimately reducing variation, and improving the signal-to-noise ratio of isotopic, and therefore

environmental, change within a community. Bump et al. (2007) demonstrated this in their examination of  $\delta^{13}\text{C}$  values for primary producers (cellulose from pine [*Pinus flexilis*] needles and juniper [*Juniperus*] wood), herbivores (bone collagen from bison [*Bison antiquus*]), and carnivores (bone collagen from dire wolves [*Canis dirus*]) from the Great Basin and La Brea tar pit over the period  $12\text{--}30 \times 10^3$  years ago (Fig. 3A). These isotopic records were then compared by Bump et al. (2007) to temporal changes in atmospheric  $[\text{CO}_2]$  over the same time interval that had been recovered from ice core records (Fig. 3B), which document a significant increase in  $[\text{CO}_2]$  after the Last Glacial Maximum. Increased atmospheric  $[\text{CO}_2]$  provides a greater carbon pool for primary producers to use during photosynthesis, which in turn enables them to more strongly discriminate against the heavier isotope of carbon ( $^{13}\text{C}$ ). As a result, primary producer  $\delta^{13}\text{C}$  values would be expected to drop during periods of elevated atmospheric  $[\text{CO}_2]$ . This effect is evident in the findings of Bump et al. (2007), where mean  $\delta^{13}\text{C}$  values for producers and consumers show a significant decrease in  $\delta^{13}\text{C}$  values at  $15\text{--}12 \times 10^3$  years ago (Fig. 3A), which corresponds with the interval of increasing  $[\text{CO}_2]$ . These results demonstrate how environmental perturbations experienced at the base of the food web can be propagated up through higher trophic levels. In addition, reduced variation in the isotopic signal of the carnivores included in this study suggests that these consumers may be better proxies for this type of information than organisms that are more routinely sampled (i.e., fossil ungulates). This implies that predator-trap deposits such as the tar pits at La Brea may provide more ecosystem-level information than previously thought.

In addition to assessing isotopic differences at the ecosystem or species level, stable isotope analysis of tooth



**FIG. 3.**—Correlation between change in organism  $\delta^{13}\text{C}$  values and change in atmospheric  $\text{CO}_2$  concentration. A comparison is made of A) mean carbon isotope data for collagen from late Pleistocene dire wolves and bison (approximately  $10\text{--}35 \times 10^3$  years ago) and for plant material from La Brea tar pits and Great Basin packrat middens, with B) variation in atmospheric concentrations of  $\text{CO}_2$  over the last  $40 \times 10^3$  years (redrawn from Bump et al. 2007). Solid and dashed vertical lines denote the range in individual isotopic values for specimens of each taxon sampled within each time interval. Measurements of past  $\text{CO}_2$  concentrations were compiled from ice-core-derived data (Ahn and Brook 2007, 2008; Barnola 1987; Monnin et al. 2001). Note the significant drop in producer and consumer  $\delta^{13}\text{C}$  values that corresponds with the rapid rise in  $[\text{CO}_2]$  (in parts per million) during the Late Glacial Maximum ( $12\text{--}15 \times 10^3$  years ago). Carbon isotope values are referenced to the international standard Vienna PeeDee Belemnite (VPDB).

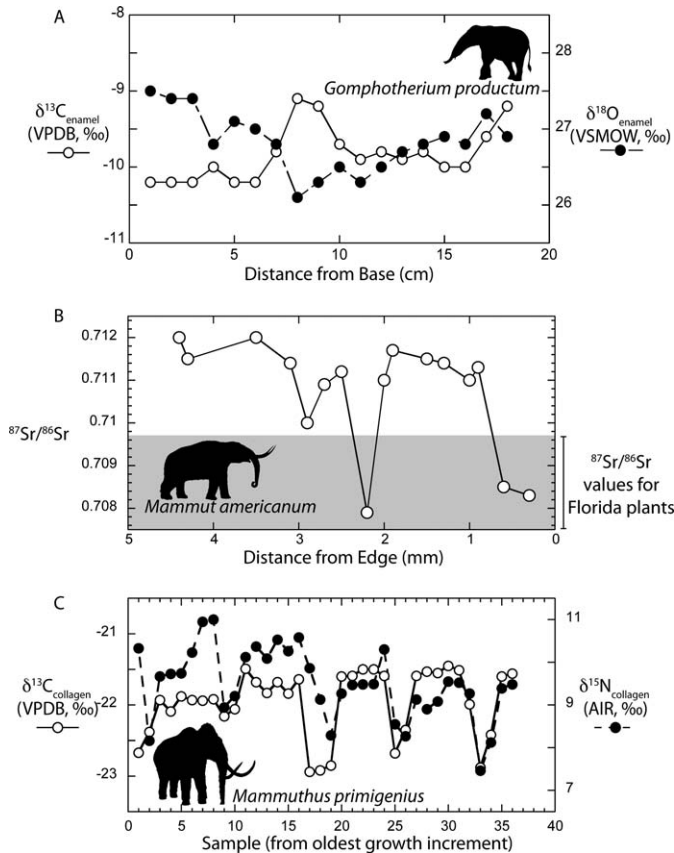
enamel can be applied to questions of biological or ecological change within a single individual through the process of serial sampling (Fricke and O'Neil 1996; Higgins and MacFadden 2004; Koch et al. 1989; Kohn et al. 1998; Passey and Cerling 2002; Zazzo et al. 2010). The usefulness of enamel stems from its formation via accretion along the tooth surface over a limited duration of time during an animal's life. Once formed, the stable isotope composition of enamel remains fixed (i.e., enamel is no longer metabolized by the body), providing a nearly continuous stable isotope record that may cover a period of months to years and can be retained for millions of years after fossilization (Lee-Thorp and van der Merwe 1987; Wang and Cerling 1994). Sequential sampling of distinct

enamel layers within teeth can provide information on dietary and habitat change over the course of an individual's development from juvenile to adult as well as seasonal variation in these ecological parameters later in the animal's life. Preservation of these temporal differences in isotopic values in fossilized tooth enamel has an added benefit for paleontologists in that the differences also provide a means to assess the quality of preservation. As noted above, extensive alteration of fossil materials tends to homogenize stable isotopic values among and within specimens (Kohn and Cerling 2002). Preservation of strong, temporal oscillations in stable isotopic values of fossilized teeth can therefore be used as another check on the isotopic integrity of fossil specimens.

Three studies of fossil proboscideans, that is elephants and their extinct relatives (Fox and Fisher 2004; Hoppe et al. 1999; Rountrey et al. 2007), highlight how serial sampling of tooth material can be used to answer very different questions about the ecology and life history of these animals (Fig. 4). Proboscideans represent one of the best groups of mammals to examine with this technique because of the large size of their teeth, which means that growth layers accreted within them will be easily identifiable, and the modification of the incisors into large, ever-growing tusks, which can record large amounts of information over the lifetime of an individual. These physical characters evolved very early within this order (earliest appearance: late Paleocene, approximately  $60 \times 10^6$  years ago), which means that serial sampling can be used to examine the ecological history of this group early on in its evolutionary history.

Serial samples of enamel from the upper tusks of 17 individuals of the proboscidean *Gomphotherium productum* from across the Great Plains of North America were analyzed by Fox and Fisher (2004) to determine the feeding ecology of this species and constrain the environmental conditions it experienced during the middle to late Miocene (about  $15\text{--}8 \times 10^6$  years ago; Fig. 4A). Unlike living elephants, which have tusks composed solely of dentin, tusks of *G. productum* and other gomphotheres maintained a band of enamel that ran along the lateral margin of the tusk, making them suitable for stable isotope analysis at this timescale. A distance of 2.5–4.5 cm was sampled along each tusk, which corresponds to a maximum of 1 year of the individual's life (Fox 2000). Enamel  $\delta^{13}\text{C}$  profiles along the tusk for each individual indicated a diet consisting of  $\text{C}_3$  vegetation, either all browse or a mix of browse and  $\text{C}_3$  grasses. These values cluster at the upper extreme for a  $\text{C}_3$  consumer (assuming an enamel to diet isotope discrimination of  $14.1\text{‰} \pm 0.5\text{‰}$ —Cerling and Harris 1999), indicating that these individuals foraged in partially open, possibly arid conditions and would have favored woodlands rather than deep forests. Variation in  $\delta^{13}\text{C}$  values along the tusk was minor, which suggests that diets did not vary much seasonally, or at least that this variation could not be determined by stable isotope analysis. Oxygen isotope values also varied little along the tusk (approximately 1.5‰), but did cycle from high to low and back to high  $\delta^{18}\text{O}$  values, most likely reflecting seasonal changes in local precipitation





**FIG. 4.**—Stable isotope values for serially sampled teeth (tusks and molars) from 3 extinct proboscidean species, showing A) late Miocene gomphothere *Gomphotherium productum* (tusk enamel  $\delta^{13}\text{C}$  and  $\delta^{18}\text{O}$  values—Fox and Fisher 2004); B) late Pleistocene mastodon *Mammuthus americanum* (molar enamel  $^{87}\text{Sr}/^{86}\text{Sr}$  values—Hoppe et al. 1999); and C) a juvenile late Pleistocene mammoth *Mammuthus primigenius* (tusk dentin collagen  $\delta^{13}\text{C}$  and  $\delta^{15}\text{N}$  values—Rountrey et al. 2007). Solid and dashed lines connect sequential samples taken from incremental growth layers of enamel or dentin in the tusk or molar. In panel B, gray shaded region marks expected range in  $^{87}\text{Sr}/^{86}\text{Sr}$  values for consumers foraging in Florida. Carbon, nitrogen, and oxygen isotope values are referenced to the international standards Vienna Pee Dee Belemnite (VPDB), atmospheric air (AIR), and Vienna Standard Mean Ocean Water (VSMOW), respectively.

and temperature (high  $\delta^{18}\text{O}$  values = warm-season rains; low  $\delta^{18}\text{O}$  = cool-season rains). These changes imply that seasonal changes in precipitation did not correspond with seasonal changes in availability of  $\text{C}_3$  vegetation. Lack of significant differences or an apparent trend in  $\delta^{13}\text{C}$  and  $\delta^{18}\text{O}$  values among individuals of *G. productum* sampled from different times and locations implies that environmental conditions in the Great Plains did not change or degrade significantly from  $15$  to  $8 \times 10^6$  years ago, and the woodland habitats preferred by *G. productum* were available throughout this time interval.

Spatial variation in the isotopic composition of prey species (Hobson and Schell 1998; Schell et al. 1998) or precipitation (Chamberlain et al. 1997; Marra et al. 1998) has been exploited to track the movements of living marine and terrestrial animals, and a similar approach can be employed

to track the movements of fossil mammals in the past. Serial sampling of a much younger (latest Pleistocene) proboscidean species was performed by Hoppe et al. (1999) to determine how range size and migration patterns of late Pleistocene proboscideans in North America may have been impacted by changing environmental conditions at the end of the Pleistocene (Fig. 4B). Using geographic variation in the ratio of strontium isotopic ( $^{87}\text{Sr}/^{86}\text{Sr}$ ) composition of local bedrock, soils, and plants, Hoppe et al. (1999) created an isotopic map of the southeastern United States (Florida and Georgia). Unlike other isotope systems, the mass difference between strontium isotopes ( $^{87}\text{Sr}$  and  $^{86}\text{Sr}$ ) is too small relative to the atomic mass of the element to permit measurable biological fractionation (Price et al. 1985). Thus, the strontium isotopic composition of producers and consumers tracks that of the local soils and bedrocks where they live without any variation due to trophic level (see Ben-David and Flaherty 2012 for more information). However, movement between regions with distinct  $^{87}\text{Sr}/^{86}\text{Sr}$  values would result in oscillations in the values recorded in accreted tissues (i.e., tooth enamel), and could then be used to track the seasonal movements of individuals between these areas. Serial sampling of tooth enamel from the mastodon *Mammuthus americanum* showed significant variation in the  $^{87}\text{Sr}/^{86}\text{Sr}$  values along the crown of the tooth (0.7078–0.7121). The majority of these values exceeded the range of measured  $^{87}\text{Sr}/^{86}\text{Sr}$  values for plants sampled from Florida, suggesting these animals were moving considerable distances (approximately 120 to approximately 300 km 1 way) during the year and may have been migrating from coastal areas to upland regions in central Georgia. This implies that home ranges for these animals were quite large and may have been significantly greater than those of living elephants (Cerling et al. 2006; Thomas et al. 2008), although calculations of these home ranges from historical observations and current field studies are most likely underestimates, given restrictions in accessible habitat today.

In addition to evidence of seasonal diets and migration, serial sampling of accreted biogenic materials from proboscideans also has been used to infer other life-history information, such as the timing of nursing and weaning (Rountrey et al. 2007). Consumption of milk by young mammals leaves a distinct isotopic label in the  $\delta^{13}\text{C}$ ,  $\delta^{15}\text{N}$ , and  $\delta^{18}\text{O}$  values of accreted tissues (Hobson and Sease 1998; Newsome et al. 2006; Wright and Schwarcz 1998), because they are effectively feeding 1 trophic level above that of their mother and therefore go through an additional isotopic discrimination. For collagen in tooth dentin,  $\delta^{15}\text{N}$  values best reflect this effect as the isotopic discrimination factor with trophic level is quite large at about 3.0‰ (Schoeninger et al. 1983). Examination of the  $\delta^{15}\text{N}$  profiles from the tusk of a juvenile woolly mammoth (*Mammuthus primigenius*) by Rountrey et al. (2007) shows a cyclical pattern that is consistent with seasonal shifts in its diet and that of its mother (as reflected in her milk; Fig. 4C). This pattern is mirrored in the  $\delta^{13}\text{C}$  values as well. However, the  $\delta^{15}\text{N}$  values also show a steady drop in maximum values during each cycle,



which the authors have interpreted as reflecting a steady decrease in the contribution of maternal milk to the diet of the juvenile. This suggests a prolonged weaning period for mammoth calves, which, based on counting the number of cycles recorded in the tusk, would have occurred over a period of  $\geq 4$  years. Given that the tip of this specimen was lost, the authors estimate that about 1–1.5 years are missing from the record, which would suggest weaning occurred over a period of at least 5 years. The weaning period for living elephants is typically shorter (approximately 3.5 years), but maternal investment in the calf can be extended to as long as 5.6 years under stressful conditions (Lee and Moss 1986). The interpretation of a prolonged period of weaning ( $\geq 5$  years) is consistent with environmental reconstructions for this time period (late Pleistocene) and location (Wrangel Island, Siberia). Harsh climatic conditions at the end of the last ice age may have required female mammoths to expend more energy and time rearing each calf, which would have reduced the number of calves that could be produced within the lifetime of a female. This reduction in fitness may have increased the sensitivity of this species to predation, increasing their susceptibility to extinction from intense hunting pressure.

### FUTURE DIRECTIONS AND APPLICATIONS

The application of stable isotope analysis to paleontological research is primarily driven by new advances in techniques and instrumentation. As the precision and sensitivity improve for isotope ratio mass spectrometers, smaller sample amounts are measurable and smaller isotopic differences in mass can be assessed, which opens up entire new isotope systems and fossil materials for study. Here, I list a few recent developments in stable isotope analysis as applied to paleobiology and emphasize those that have the most promise for future fossil mammal research.

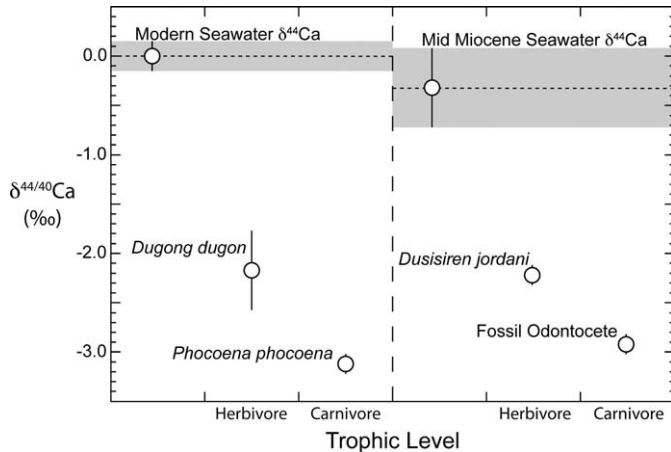
An established technique that has only recently gained interest in the paleontological community is laser ablation of samples (Cerling and Sharp 1996; Passey and Cerling 2006; Sponheimer et al. 2006). In this method, the surface of the sample is heated rapidly using a thermal laser, which creates a series of small pits on the surface and produces  $\text{CO}_2$  by thermal breakdown of the carbonate and phosphate components in the enamel (Cerling and Sharp 1996). A major benefit of this method is that it is less destructive than traditional methods of isotopic sampling, which involve low-precision drilling of tooth surfaces. Laser ablation also requires much smaller quantities of enamel to yield enough  $\text{CO}_2$  for each analysis ( $<1.0$  mg compared to  $>5.0$  mg for traditional sampling methods). This reduction in sample size makes fossilized teeth from small mammals (e.g., rodents and insectivores) available for analysis and opens up a whole new level of isotopic research within ancient communities. Likewise, extremely rare and scientifically significant specimens for which paleodietary information is vital (e.g., early hominines—Sponheimer et al. 2006) can now be considered for isotopic analysis as well. Caveats for this method include

reduced accuracy in  $\delta^{18}\text{O}$  analysis relative to conventional methods and significant isotopic fractionation associated with gas–surface interactions during analysis of large teeth, which experience a greater blank effect than small teeth as a result of their enhanced adsorption of residual  $\text{CO}_2$  from previous laser ablations on their outer surface (Passey and Cerling 2006). Even with these concerns, this method offers considerable advantages for paleobiologists working with small or rare specimens.

Although most applications to paleontology have relied on analysis of whole tissues (soft or mineralized), there is growing interest in analyzing individual organic compounds that may be retained within fossilized remains. Compound-specific stable isotope analysis is increasingly used to analyze modern samples (O'Brien et al. 1998; Popp et al. 2007) as well as ancient human remains (Fogel and Tuross 2003) but has yet to be applied extensively to the field of paleontology (Clementz et al. 2000; CoBabe and Pratt 1995; Stott et al. 1997). Compounds of interest could include individual amino acids, which can be separated from bone collagen, and lipid molecules (e.g., fatty acids and sterols), which adhere to the outer surface of pore spaces and channels in bones and teeth or have been entrained in bioapatite during biomineralization (CoBabe and Pratt 1995). Exceptional cases of preservation of fatty acids, amino acids, and sterols within fossilized bones and teeth have been reported, and the stable isotopic composition of these compounds have been determined and used to infer ecological information from these specimens (Clementz et al. 2003c; Evershed et al. 1995; Stott et al. 1997).

Through careful treatment and chemical extraction, organic molecules can be isolated from fossils and analyzed using gas or liquid chromatography linked to isotope ratio mass spectrometers. Individual amino acids are thought to be primarily restricted to relatively young fossil materials ( $<10^5$  years), whereas lipids have been successfully recovered from much older ( $>10^6$  years ago) remains of fossil invertebrates (CoBabe and Pratt 1995) and fossil vertebrates (Clementz et al. 2000, 2003c). Analyses of these compounds can be beneficial to paleontological studies because the isotopic compositions of these compounds provide a means of discriminating between protein and carbohydrate contribution to omnivore paleodiets (as evident from analysis of individual amino acids), and can be used to identify unique diet sources through identification and analysis of distinct biomarkers and essential components that consumers are incapable of producing on their own and must therefore solely come from diet (lipids and individual amino acids—Evershed et al. 1995). This method of analysis significantly expands the potential paleodietary information that can be extracted from a single specimen.

From a paleontological standpoint, a further benefit of this method is that many of these compounds may be produced by only a small number of organisms, which reduces the chances of contamination by external sources and provides a means for assessing isotopic integrity. For instance, cholesterol is a steroidal lipid that is not produced in significant quantities by plants, microbes, or most fungi, but is found in relatively high



**FIG. 5.**—Comparison of calcium isotope values ( $\delta^{44}\text{Ca}$ ) for tooth enamel from modern and fossil marine mammals (Clementz et al. 2003a) relative to that of present-day and Miocene-aged seawater (Griffith et al. 2008). Enamel values for modern marine consumers (herbivore: sirenian *Dugong dugon*; carnivore: cetacean *Phocoena phocoena*) and Miocene-aged fossils of marine consumers (herbivore: sirenian *Dusisiren jordani*; carnivore: unidentified small odontocete) were initially reported in Clementz et al. (2003a). Each symbol represents a single specimen and vertical bars represent analytical error associated with a single series of analyses on an instrument.

abundance in vertebrate remains. As long as potential contamination from handling is minimized, cholesterol extracted from fossil remains should be original and should not have been introduced postmortem. The stable isotopes of carbon have been the primary target of analysis for most of these compounds, but hydrogen isotope analysis of lipids (i.e., fatty acids and sterols) as well as analysis of hydrogen, nitrogen, oxygen, and sulfur isotopes in amino acids also is possible. Continued interest in this area will, we hope, promote further exploration of the utility of these other isotope systems for paleoecological information.

Biological fractionation of stable calcium isotopes ( $^{40}\text{Ca}$ ,  $^{42}\text{Ca}$ ,  $^{43}\text{Ca}$ ,  $^{44}\text{Ca}$ ,  $^{46}\text{Ca}$ , and  $^{48}\text{Ca}$ ) was first identified by Skulan et al. (1997) as part of a study in which they sampled an assortment of modern terrestrial and marine organisms, both vertebrates and invertebrates, and noted a significant drop in  $\delta^{44}\text{Ca}$  values with increasing trophic level. Since this initial observation, application of  $\delta^{44}\text{Ca}$  analysis to biological and paleobiological research has primarily focused on the calcium isotope composition of marine microfossils, which can serve as a proxy for temperature or calcium cycling in the ocean over long timescales (Bohm et al. 2006; De La Rocha and DePaolo 2000; Gussone et al. 2005; Sime et al. 2005; Zhu and Macdougall 1998). However, a few studies also have explored the way this isotope system can be applied to paleodietary interpretations of organisms within ancient ecosystems, specifically as a proxy for trophic level information (Clementz et al. 2003a; Reynard et al. 2010; Skulan and DePaolo 1999; Skulan et al. 1997).

Work on modern and fossil marine mammals by Clementz et al. (2003a) supported earlier results by Skulan et al. (1997), which showed a significant drop in  $\delta^{44}\text{Ca}$  values with trophic level (Fig. 5). However, the authors acknowledged that this

drop also could reflect differences in prey type, separating consumers that foraged on soft-bodied prey or vegetation from those that consumed vertebrate prey. Because biological fractionation of calcium isotopes relative to diet is mainly restricted to mineralization (Skulan and DePaolo 1999), soft-tissue  $\delta^{44}\text{Ca}$  values show little offset relative to that of diet, which implies that unless consumers ingest significant quantities of the hard parts from their prey, consumer tissues should show little to no fractionation with trophic level. This interpretation is supported by Reynard et al. (2010), who examined bones from archaeological remains of domesticated species, humans, and a few wild species of mammalian carnivores and herbivores and found little to no correlation between  $\delta^{44}\text{Ca}$  values and trophic level. Because consumption of skeletal remains of prey species by predators (including humans) was minimal, lack of fractionation with trophic level could reflect this difference between soft and mineralized tissues in prey species. Although a complete understanding of the factors controlling the calcium isotope composition of mammal tissues is needed,  $\delta^{44}\text{Ca}$  values in fossil mammals still hold considerable promise as a paleodietary proxy for extinct species in deep time ( $>10^6$  years ago).

Clumped isotope analysis may represent the most novel and recent development in geochemical analysis of fossil mammal remains (Eagle et al. 2010). Clumped isotope values ( $\Delta_{47}$ ) are defined as the difference between the measured abundance of the  $\text{CO}_2$  molecules of mass 47 (mostly  $^{13}\text{C}^{18}\text{O}^{16}\text{O}$ , but a small amount of  $^{12}\text{C}^{18}\text{O}^{17}\text{O}$  and  $^{13}\text{C}^{17}\text{O}^{17}\text{O}$ ) and the expected abundance for the molecule of that mass assuming a stochastic distribution (Huntington et al. 2009). Within carbonates and the carbonate component of other minerals (e.g., bioapatite), the tendency of heavy isotopes of carbon ( $^{13}\text{C}$ ) and oxygen ( $^{18}\text{O}$ ) to form bonds, or “clump,” with each other is strongly affected by the temperature of mineralization, but not by the isotopic composition of the system (for more detailed information on this method see Eiler et al. [2008] and Huntington et al. [2009]). This creates a natural thermometer that can be broadly applied within geological research, including the estimation of body temperature in extinct vertebrates. Eagle et al. (2010) evaluated the fidelity of this method by analyzing fossilized enamel and dentin from late Pleistocene-aged mammoth teeth recovered from the Rhine River valley and the North Sea. Enamel  $\Delta_{47}$  values for mammoth teeth from each site corresponded to estimated body temperatures and were statistically indistinguishable (Rhine River:  $39.1^\circ\text{C} \pm 2.8^\circ\text{C}$ ; North Sea:  $36.8^\circ\text{C} \pm 1.3^\circ\text{C}$ ) and within close agreement to average body temperatures for extant large mammals (approximately  $37^\circ\text{C}$ ). This technique holds promise as a paleothermometer for extinct species as well as providing an additional way to evaluate the isotopic integrity of fossil materials. However, current application to most paleobiological research is restricted by the considerable amounts of sample (100–200 mg) and time (3–4 h per sample) required for each analysis, which is significantly greater than the amounts of sample (1–2 mg) and time (approximately 10 min) typically used for traditional isotope measurements of bioapatites. Further refinement of this

technique is necessary before it can be used extensively in vertebrate paleontology.

## CONCLUSIONS

Paleontologists have undoubtedly benefited from stable isotope analysis of fossil mammal remains. In addition to providing information on paleodiets of extinct organisms, this technique also has offered insight into seasonal movements, habitat preferences, physiology, and life histories for species, all of which complement and extend the information available from other more traditional methods of paleontological research (e.g., morphology and depositional setting). Continued study of modern systems by ecologists (and some paleontologists) has further improved the utility of these proxies by providing much needed baseline information on the factors that influence isotopic composition of mammalian tissues. New methods of analysis (compound-specific isotope ratio mass spectrometry and laser ablation) and isotope systems (calcium isotopes and  $\Delta_{47}$  values) are expanding the range of applications within this field as well as increasing the types and number of fossil specimens appropriate for this type of analysis.

## ACKNOWLEDGMENTS

The content of this paper benefited greatly from countless discussions with colleagues in paleontology, ecology, and geochemistry, with special thanks to P. L. Koch, D. L. Fox, K. L. Fox-Dobbs, G. J. Bowen, J. C. Zachos, B. J. MacFadden, N. L. Tuross, and T. E. Cerling as well as the long list of cited authors who provided the material for this review. J. P. Whiteman, M. Ben-David, D. L. Fox, and an anonymous reviewer provided helpful comments, which significantly improved the composition of this paper. Support for the research and writing of this paper was provided by a grant from the National Science Foundation (EAR 0847413).

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*Special Feature Editor was Barbara H. Blake.*