Stable isotopes in modern ostrich eggshell: A calibration for paleoenvironmental applications in semi-arid regions of southern Africa

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Abstract—An isotopic study of modern ostrich eggshell (OES) is presented as a calibration for terrestrial paleoenvironmental applications. The stable carbon and nitrogen isotope fractionations of OES were determined for various organic and inorganic fractions of eggshell by measuring the isotopic ratios of modern OES samples collected from controlled settings (i.e., zoos and farms) and corresponding ostrich diet. These fractionations were used to evaluate the relationship between the isotope composition of OES laid by free-range birds living in South Africa and their environment. The carbon isotope composition of the total organic and inorganic fractions of OES was enriched by 2 and 16‰, respectively, relative to the diet. In natural settings, the δ13C values of both the organic and inorganic fractions of OES reflected that of ambient vegetation, with a noted dietary preference for C3 plants. The nitrogen isotope composition of the total organic fraction of OES was 3‰ enriched relative to the diet, and varied inversely with mean annual precipitation (MAP) in natural settings. A decrease in MAP of 100 mm was accompanied by an increase in δ15N values of approximately 1‰. The oxygen isotope composition of the inorganic fraction of the OES varied linearly with that of the drinking water in controlled settings. However, in natural settings, the δ18O of OES values were highly variable and are thought to be controlled primarily by the δ18O of ingested plant leaf-water. The stability of the isotopic signal in the organic fraction of OES through geologic time was evaluated through a series of heating experiments. The δ13C and δ15N values of the total organic fraction of heated OES increased by less than 0.6 and 0.2‰ for carbon and nitrogen, respectively, in spite of extensive diagenetic alteration and changes in the amino acid composition of the samples. The results of this study indicate that the stable carbon and nitrogen isotope composition of OES is relatively stable under experimental conditions used and may be used to derive a plethora of paleoenvironmental information, including changes in C3 and C4 vegetation and paleorainfall estimates.

Additionally, carbon isotopic analysis of individual amino acids (IAA) in the OES and corresponding diet were determined to elucidate information on isotopic fractionation during OES protein synthesis. The δ13C values of IAA in OES range over 12‰ and provide valuable information for future studies of (1) diagenesis in fossil OES samples and (2) comparative animal physiology, including the determination of digestive and feeding strategies of extant and extinct animals. Copyright © 1998 Elsevier Science Ltd

1 INTRODUCTION

Stable carbon, nitrogen, and oxygen isotopes in fossilized remains of animals provide valuable information on the animal’s diet and the environment in which it lived (reviewed in Ostrom and Fry, 1993; Koch et al., 1994; Koch, 1998). Carbon isotopes are typically used to determine the relative amounts of C3 and C4 plants consumed by herbivores (Vogel, 1978; DeNiro and Epstein, 1978; van der Merwe, 1986; Lee-Thorp et al., 1989; Vogel et al., 1990). Nitrogen isotopes can be used to determine trophic level (Minagawa and Wada, 1984; Sealy et al., 1987), and to estimate the degree of water- and or protein- stress of herbivores (Ambrose and DeNiro, 1986a; Hare et al., 1991; Ambrose, 1993). In some cases, the δ15N of herbivore collagen is inversely correlated with mean annual precipitation (Heaton, 1987; Sealy et al., 1987; Cormie and Schwarcz, 1996). Oxygen isotopes can be used as a proxy for ambient air temperature (Koch et al., 1989), or as a proxy for relative humidity (Ayliffe and Chivas, 1990). Successful paleodietary applications generally rely on (1) an understanding of the isotopic fractionation in the biominerallized studied, (2) an understanding of the stable isotope ecology and biophysiology of the extant animal (or nearby relative to the extinct animal), and (3) a proper evaluation of the preservation state of the sample.

Eggshell has long been a medium of great interest to paleoecologists due to its presence in much of the geologic record. Oxygen isotopes in modern avian and reptilian eggshell carbonate (1) have been correlated to the oxygen isotope composition of drinking water (Folinsbee et al., 1970; Erben et al., 1979; Sarkar et al., 1991; Schaffner and Swart, 1991) and (2) have been used as a means of interpreting oxygen isotopes in Mesozoic dinosaur eggshell (Folinsbee et al., 1970; Erben et al., 1979; Sarkar et al., 1991). Carbon isotopes in eggshell carbonate have been used to reconstruct the diets of living ostriches (von Schirnding et al., 1982) and extinct animals (Folinsbee et al., 1970; Erben et al., 1979; Sarkar et al., 1991; Stern et al., 1994). Procedures for extracting organic material from modern and fossil eggshell have recently been developed (Johnson, 1995) and have allowed for carbon and nitrogen isotopes in modern ostrich eggshell (OES) is presented as a calibration for terrestrial paleoenvironmental applications. The stable carbon and nitrogen isotope fractionations of OES were determined for various organic and inorganic fractions of eggshell by measuring the isotopic ratios of modern OES samples collected from controlled settings (i.e., zoos and farms) and corresponding ostrich diet. These fractionations were used to evaluate the relationship between the isotope composition of OES laid by free-range birds living in South Africa and their environment. The carbon isotope composition of the total organic and inorganic fractions of OES was enriched by 2 and 16‰, respectively, relative to the diet. In natural settings, the δ13C values of both the organic and inorganic fractions of OES reflected that of ambient vegetation, with a noted dietary preference for C3 plants. The nitrogen isotope composition of the total organic fraction of OES was 3‰ enriched relative to the diet, and varied inversely with mean annual precipitation (MAP) in natural settings. A decrease in MAP of 100 mm was accompanied by an increase in δ15N values of approximately 1‰. The oxygen isotope composition of the inorganic fraction of the OES varied linearly with that of the drinking water in controlled settings. However, in natural settings, the δ18O of OES values were highly variable and are thought to be controlled primarily by the δ18O of ingested plant leaf-water. The stability of the isotopic signal in the organic fraction of OES through geologic time was evaluated through a series of heating experiments. The δ13C and δ15N values of the total organic fraction of heated OES increased by less than 0.6 and 0.2‰ for carbon and nitrogen, respectively, in spite of extensive diagenetic alteration and changes in the amino acid composition of the samples. The results of this study indicate that the stable carbon and nitrogen isotope composition of OES is relatively stable under experimental conditions used and may be used to derive a plethora of paleoenvironmental information, including changes in C3 and C4 vegetation and paleorainfall estimates.

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isotopic study of fossil eggshell proteins and individual amino acids (Johnson et al., 1993, 1997).

We present here an isotopic study of modern ostrich (Stru- thio camelus) eggshell (abbreviated hereafter as OES), dietary uptake, and environment as a means to evaluate the stable isotope composition of fossil OES in the context of paleoenvironmental change. OES is a relatively common component at archaeological settings in semi-arid regions across Africa through the Late Quaternary and has excellent potential for paleodietary and paleoenvironmental applications. The organic fraction of OES is comprised almost entirely of proteins and remains well preserved through diagenesis (Brooks et al., 1990; Miller et al., 1991, 1992), and the inorganic fraction of OES is made of calcite, the stable polymorph of calcium carbonate. Additionally, individual fragments of fossil OES can be dated through much of the late Quaternary (using radiocarbon, amino acid racemization, and uranium series), thereby insuring good chronologic control on the resultant paleoenvironmental data.

Isotopic offsets between the diet and various fractions of OES were determined by analysis of OES laid by ostriches living in controlled settings (e.g., zoos and commercial ostrich farms) and fed monotonous diets. These isotopic offsets, or fractionations, were used in OES laid by South African free-range ostriches to evaluate the relationship between stable isotopes and environment, including ambient vegetation, precipitation, temperature, and relative humidity. Prior to using stable isotopes in fossil material for paleodiet and paleoenvironmental research, it is necessary to provide proof of preservation of the original isotopic signal. Heating experiments designed to simulate diagenesis are typically used to access the effects of diagenetic alteration on geologic materials (cf. Hare and Mitterer, 1969; Hare and Hoering, 1977). In this study, heating experiments were used to evaluate the isotopic stability of the organic fraction in OES through protein decomposition reactions (cf. Miller et al., 1992). Ernst (1989) has used heating experiments to demonstrate the remarkable integrity of OES with respect to migration of organic material into or out of the system.

Finally, information on the isotopic fractionation associated with OES protein synthesis was derived from analysis of individual amino acids within OES and corresponding ostrich diet. The resultant isotopic data are compared to those derived from other vertebrate proteins extracted from animals subjected to controlled feeding experiments as well, and provide information needed for future studies of comparative biophysics in extant and extinct animals.

2. STABLE ISOTOPES IN ANIMAL TISSUE

2.1. Carbon

Plants that utilize the C3 (Calvin) and C4 (Hatch-Slack) photosynthetic pathways fractionate atmospheric carbon differently during the synthesis of plant carbohydrate. The mean $\delta^{13}C$ of C3 plant foliage is approximately $-26.5\%e$, and of C4 grasses is approximately $-12.6\%e$ (see reviews by O'Leary, 1988; Farquhar et al., 1989; Fogel and Cifuentes, 1993). Plants that use Crassulacean acid metabolism (CAM) employ a combination of the C3 and C4 photosynthetic pathways and have a carbon isotope composition intermediate between those of C3 and C4 plants (Kortschack et al., 1965; Osmond, 1978). During tissue synthesis of consumers, the isotopic composition of the diet (i.e., vegetation, for herbivores) is passed on and modified from its original value.

The amount of isotopic fractionation that occurs varies with tissue type and varies between classes of organic compounds. For example, plant and animal lipids are depleted in $^{13}C$ by approximately 2 to 5%e with respect to the carbohydrates and proteins of a sample (Park and Epstein, 1960; Abelson and Hoering, 1961; DeNiro and Epstein, 1978). Bone collagen is generally 5%e enriched relative to the diet (Vogel, 1978) and OES proteins are approximately 2%e enriched relative to the diet (von Schirnding et al., 1982).

Ostriches are opportunistic vegetarians utilizing a wide variety of plant species (Kok, 1980; Brown et al., 1982; Bertram, 1992). They have a slight preference for C3 shrubs and select against some vegetation, including CAM plants (Robinson and Seely, 1975; von Schirnding et al., 1982; Williams et al., 1993). Assuming negligible consumption of CAM plants, the stable isotope composition of the OES can be used to calculate the percentage of C3 and C4 plants consumed using a two-end member mixing model (after von Schirnding et al., 1982).

2.2. Nitrogen

Nitrogen isotopes in animal tissue reflect that of the diet, and in some cases, the physiological response of the animal to the environment. In herbivores, the isotopic composition of the diet is transferred to the herbivore with a 3 to 6%e enrichment in the bone collagen (Minagawa and Wada, 1984; Sealy et al., 1987). This isotopic enrichment (defined here as the trophic effect) results from fractionation associated with tissue formation, and is more pronounced among consumers from arid regions of South Africa (Heaton, 1987; Sealy et al., 1987). The $\delta^{15}N$ values of collagen are 3 to 4%e and often 6 to 8%e enriched relative to the plants under conditions of moderate and low rainfall, respectively.

An inverse relationship has been determined for mean annual precipitation (MAP) and $\delta^{15}N$ values of herbivore collagen in South Africa (Schoeninger and DeNiro, 1984; Ambrose and DeNiro, 1986a, 1989; Heaton, 1987; Sealy et al., 1987) and North America (Cormie and Schwartz, 1996). Ambrose and DeNiro (1986a) and Ambrose (1991) attribute this relationship to renal concentration of urea in herbivores that are water stressed. Because nitrogen-rich, $^{15}N$-depleted urine is excreted from the body during periods of water-stress, the tissues of the herbivore become isotopically enriched. The $\delta^{15}N$ value of the animal is thus a question of isotopic mass balance. Sealy et al. (1987) consider the processes associated with protein synthesis in herbivores. In ruminants and hind-gut fermentors, for example, urea is recycled to the rumen to support microbial growth. The ammonia generated by the gut bacteria is eventually used for protein synthesis in the host animal. During periods of severe protein-stress (as is often the case in areas of low rainfall), the nitorgenous compounds are recycled such that nutrients are drawn from an increasingly enriched internal nitrogen pool.

It is beyond the scope of this paper to verify either of the aforementioned processes in ostriches; however, based on the ecology and physiology of ostriches, the $\delta^{15}N$ values of South African OES are expected to parallel that of South African
herbivore collagen and to vary inversely with MAP. Ostriches inhabit semi-arid and arid regions and are able to survive on small amounts of water often derived solely from plants (Skadhauge et al., 1984; Williams et al., 1993). They are hind-gut fermentors and can subsist on food of minimal nutritional value (Sampson, 1994). Though ostriches have been observed to consume small mammals and lizards on occasion (Sauer, 1970), meat proteins are thought to make an insignificant contribution to the isotopic composition of the total diet.

2.3. Oxygen

The oxygen isotope composition of a biomineral depends upon the (1) temperature of formation, (2) kinetic and equilibrium fractionations that occur during formation of the mineral, and (3) the isotopic composition of the animal’s body water (reviewed in Koch et al., 1989). The temperature of formation is constant for warm-blooded animals, and kinetic and equilibrium effects can often be estimated under controlled experiments. The body water of an animal depends upon the isotopic composition of ingested waters (drinking water, plant water, and metabolized water), respiratory gases, and the relative contributions of each to the biomineral.

The major component contributing to the ingested water of most nonobligate drinkers (those animals, such as ostriches, that do not need access to free-standing water to meet their physiological needs) is plant-leaf water. The $\delta^{18}O$ of the plant-leaf water increases with decreasing relative humidity (Foster, 1978; Ferhi and LeGuellec, 1977) and with increasing temperature (Gonfiantini et al., 1965; Farris and Strain, 1978; Burk and Stuiver, 1981; Flanagan et al., 1991). In Australia, where the $\delta^{18}O$ of rainwater is effectively constant, the $\delta^{18}O$ of kangaroo bone phosphate is inversely correlated to humidity (Ayliffe and Chivas, 1990). Because the $\delta^{18}O$ of rainwater across South Africa is relatively constant at $-3.0\%$ (Rozanski et al., 1993), an inverse relationship is expected to exist between the $\delta^{18}O$ values of OES and relative humidity.

2.4. Eggshell: An “Instantaneous” Signal of Diet

Isotopic turn-over rates in various tissues have been measured for a variety animals raised in captivity (Tieszen et al., 1983; Hare et al., 1991; Hobson and Clark, 1992a,b). The turn-over rate of collagen in Japanese quail is slow, with the half-life of carbon equal to 173 days (Hobson and Clark, 1992a). Conversely, the turn-over rate of eggshell laid by the Japanese quail is rapid, where the $\delta^{13}C$ and $\delta^{15}N$ values of eggshell proteins are in complete isotopic equilibrium with the diet within 4 days of the diet switch (Johnson, 1995). Isotopic fractionation (1) varies with tissue-type, animal size, metabolic rate, and body temperature (DeNiro and Epstein, 1978) and (2) varies with animals living in the wild vs. captivity (Nagy, 1987). We surmise that the isotopic turn-over rate of OES is similar to that of quail eggshell, and is on the order of 3 to 5 days.

In natural environments, ostriches are opportunistic breeders and typically lay 8 to 12 eggs over a 2 week period of time (Bertram, 1992). Egg-laying depends on the build-up of sufficient nutrient stores in the ostriches (Sauer and Sauer, 1966) and usually occurs a few months after the rainy season (Sinclair, 1978; Leuthold, 1977). Thus, the isotopic composition of OES represents a “snap-shot” of diet averaged over 3 to 5 days after the rainy season. Ostrich bone collagen represents an integration of dietary uptake over a couple of years.

3. Methods

Modern OES and corresponding ostrich food and water were collected from farms in the United States and South Africa (Table 1). OES laid by free-ranging ostriches were collected from seven sites across South Africa, the most extensively sampled site being at Kimberley (Tables 2 and 3). Heating experiments were performed on one OES sample (JAX A; Table 1). Separate pieces of JAX A were (1) buried in protosterilized quartz sand moistened with DI water, (2) sealed in pyrex tubes under atmosphere, and (3) isothermally heated at 143°C for up to 7 weeks (Table 4).

Prior to isotopic and amino acid analysis, the inner and outer surface layers of all OES samples were removed by mechanical abrasion followed by a 30% acid leach with 2N HCl. This pretreatment is designed to remove any surficial contamination and to isolate the core of the eggshell for chemical analysis. The total organic fraction (also called the total hydrolyzate) of OES was prepared for isotopic and amino acid analysis by dissolving approximately 50 mg of OES in an excess of 7 N HCl (0.02 mL/mg OES). The dissolved OES was sealed under an N$_2$ atmosphere and heated at 153°C for 20 min to hydrolyze.
the proteins and polypeptides. Samples were desalted using HF (1.25 mL/mg OES) which resulted in occlusion of approximately 5% of nonfractionated amino acids in the precipitated CaF$_2$ (Johnson, 1995). The total organic fraction was either transferred to quartz tubes for isotopic analysis or analyzed for amino acid composition by ion-exchange chromatography (after Hare et al., 1985).

Ostrich bone collagen was isolated from whole bone samples by decalcification in 0.5 M EDTA, followed by extensive rinsing with DI water, and a total lipid extraction using a methanol:chloroform mixture of 3:1 (V:V). Ostrich food was desalted with DI water, dried, and ground to a coarse powder.

All organic samples (i.e., OES, bone collagen, and food) were combusted in quartz tubes under vacuum in the presence of native copper (1 to 2 g) and copper oxide (2 to 3 g) (cf. Bebout and Fogel, 1992). The resultant CO$_2$ and N$_2$ gases were cryogenically distilled prior to isotopic analysis. The inorganic fraction of OES was finely ground and reacted with 100% H$_3$PO$_4$ in side-arm reaction vessels at 50°C (after McCrea, 1950). The resultant CO$_2$ was cryogonically distilled prior to isotopic analysis.

The isotopic analysis of all samples (except the waters) were performed at the Geophysical Laboratory using a Finnigan MAT 252 isotope ratio mass spectrometer (IRMS) for CO$_2$, and a modified double-focusing Du Pont 491 IRMS for N$_2$. The analytical precision of these instruments is $\pm 0.02\%$ for C, N, and O isotopes. The external precision is approximately 0.2% for C, N, and O isotopes in the total organic and inorganic fractions of OES. All isotopic data reported relative to PDB (carb), air (nitrogen), and SMOW (oxygen) (Johnson, 1995).

Oxygen isotope analysis of the water samples were measured at INSTAAR, University of Colorado, using the VG (Series II) SIRA and

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<th>Sample Location</th>
<th>Latitude °S</th>
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<th>MAP mm/yr</th>
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Table 3: Site locations and climate data where modern OES were collected. Mean annual precipitation (MAP), mean annual temperature (MAT), and mean annual relative humidity (MARH) data from MET stations denoted in parentheses after sample location (WWD, 1994). (* = Carbon isotope composition of ostrich collagen determined at Orighstadt corrected to OES by subtracting 3%).

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Table 4: Stable isotope and amino acid results of OES (JAX A) heating experiments, where N.D. = not detected, * = all amino acids except arginine (and NH$_3$), and # = all amino acids except aspartate, threonine, serine, cysteine, methionine, arginine (and NH$_3$).
a shaker bench to equilibrate the waters with CO$_2$ of known isotopic composition. The reproducibility on this technique is $\pm 0.1\%e$ for $\delta^18$O measurements.

The carbon isotope composition of N-TFAA isopropyl derivatives of individual amino acids (IAA) comprising the total organic fraction of OES and corresponding diet were determined by gas chromatography/combustion/IRMS (GC/C/IRMS) at the Geophysical Laboratory. Because the isotopic fractionation introduced during amino acid derivatization cannot be explained by mass balance considerations alone, the effective isotopic composition of the carbon added during derivatization was determined for each amino acid and accounted for using stoichiometric mass balance equations (cf. Siller et al., 1991). The running conditions of the GC were as follows: column = HP Ultra-1 (25 m $\times$ 0.32 mm $\times$ 0.52 $\mu$m); injector temperature = 220°C; $T_0 = 75$°C (2 min hold); $T_1 = 90$°C at 4°C/min (2 min hold); $T_2 = 190$°C at 4°C/min; $T_3 = 280$°C at 25°C/min (2 min hold). The external precision for amino acids in the total organic fraction of OES is between 0.3 and 1.0$\%e$, depending on the amino acid analyzed (Johnson, 1995).

4. RESULTS AND DISCUSSION

4.1. Carbon Isotopes and Vegetation

The carbon isotope offset between the ostrich food and the total organic fraction of OES samples collected from the three controlled settings is 1.5 $\pm$ 0.8$\%e$, where the OES samples are isotopically enriched relative to the diet (Table 1). The carbon isotope offset between the food and the inorganic fraction of the OES samples is 16.2 $\pm$ 0.5$\%e$, where the OES are enriched relative to the diet (Table 1). These carbon isotope fractionations are consistent with earlier studies of OES (von Schirnding et al., 1982).

In natural settings, such as Kimberley, the $\delta^{13}C$ of multiple OES and ostrich bone fragments reveal the same information about ostrich diets and ambient vegetation (Table 2). The $\delta^{13}C$ values of the total organic fraction of the OES collected at Kimberley is $-18.6 \pm 3.4$ $%e$ ($n = 7$), and of the ostrich bone collagen was $-16.9 \pm 1.8$ $%e$ ($n = 5$). Based on a 2$%e$ fractionation between diet and the total organic fraction of OES, and using a two endmember mixing model of $C_3$ and $C_4$ plants, the average $\delta^{13}C_{OES}$ value corresponds to a diet comprised of 60% ($\pm$20%) $C_3$ plants and 40% ($\pm$20%) $C_4$ plants. Similarly, assuming a 5$%e$ carbon isotope fractionation for collagen formation (after DeNiro and Epstein, 1978), the average $\delta^{13}C_{collagen}$ corresponds to a diet of 70% ($\pm$10%) $C_3$ plants and 30% ($\pm$10%) $C_4$ plants.

In contrast to the calculated plant composition of the ostrich diets, the flora at Kimberley does not reflect a dominance of $C_3$ vegetation. The area sampled for OES and ostrich bones is dominated by $C_4$ grasses with some $C_3$ trees and shrubs. Thus, the $\delta^{13}C$ values of OES and ostrich bone measured in this study are biased by a dietary preference for $C_3$ plants (in agreement with von Schirnding et al., 1982).

Despite the preference of ostriches for $C_3$ browse, there are differences in the $\delta^{13}C$ values of the South African OES samples which may be explained by regional differences in $C_3$ and $C_4$ grass distributions. Samples of OES from some of the interior sites (i.e., Kimberley and Orighstad) are approximately 5$%e$ enriched in $^{13}C$ relative to the samples from the southwestern coastal sites (Posberg and Cape Point) and imply increased consumption of $C_4$ grasses at two of the interior sites (Fig. 1; Table 3). These results parallel the $C_3$ and $C_4$ grass distributions across South Africa, where $C_3$ grasses (in addition to $C_3$ trees and shrubs) dominate in the southwestern Cape province and $C_4$ grasses dominate the interior (Vogel et al., 1978; Ellis et al., 1980). Based on these results, the $\delta^{13}C$ of modern OES appears to be tracking large scale changes in $C_3$ and $C_4$ vegetation.

4.2. Nitrogen Isotopes, Ambient Vegetation, and Rainfall

The nitrogen isotope offset between the ostrich diet and the total organic fraction of OES samples collected from the three controlled settings average 3.0 $\pm$ 0.4$%e$, where the OES samples are isotopically enriched relative to the diet (Table 1). This nitrogen isotope fractionation is the same as that between diet and bone collagen in successively higher trophic levels (Ambrose and DeNiro; 1986b) and indicates that similar nitrogen isotopic fractionations occur between OES and bone during synthesis of organic matter.

In natural settings, such as Kimberley, the $\delta^{15}N$ values of the OES and bone collagen samples imply that each incorporate similar dietary information. The $\delta^{15}N$ values of the total organic fraction of OES collected from ostriches living at Kimberley average 11.9 $\pm$ 3.1$%e$ ($n = 7$), whereas ostrich bone collagen average 12.5 $\pm$ 1.8$%e$ ($n = 5$) (Table 2). The $\delta^{15}N$ values of vegetation consumed by ostriches at Kimberley must have averaged between 9 and 10$%e$. Although no plants were measured from the site, these values are not unreasonable for semi-arid regions (Guy et al., 1980; Seemann and Critchley, 1985; Heaton, 1987).

Despite differences in isotopic turnover times for OES and bone collagen, it is remarkable that the two tissues incorporate
The increased isotopic variability in OES indicates that the dietary variability over 3 to 5 days (as measured in OES) is greater than the dietary variability averaged over several years (as measured in ostrich bone). Because all OES and bones were collected between 1988 and 1993, the increased isotopic variability in OES may result from (1) variable diets and vegetation during the egg-laying season of each year or (2) variable diets and vegetation during the egg-laying season over the five year collection period. It will never be possible to distinguish between these two possibilities in the geologic record, as the age resolution will never be good enough. Thus, isotopic variability of ±3‰ is to be expected for the similarly aged fossil material.

There is an 11‰ range in δ15N values of OES collected across South Africa (Table 3). The δ15N values of the OES samples correlate inversely with MAP, where an increase in δ15N values corresponds to a decrease in MAP of approximately 100 mm (Fig. 2). The relationship between nitrogen isotopes in OES and MAP is in direct agreement with that measured for South African herbivore bone collagen (Heaton, 1987; Sealy et al., 1987) (Fig. 2), which suggests that the animal’s physiological response to aridity is reflected in the isotopic composition of organic matter of OES, as well as herbivore bone collagen. The strength of the relationship between δ15N values of OES and MAP implies that there is excellent potential for constructing paleoprecipitation amounts from well-preserved fossil OES.

4.3. Oxygen Isotopes, Drinking Water, and Climate

There is a positive, linear correlation between the δ18O of drinking water and the δ18O of OES laid by ostriches across South Africa (Table 3). The δ18O of OES samples is intermixed with that of MAP, where an increase in δ18O values corresponds to a decrease in MAP of approximately 3‰ (Fig. 3). The relationship between oxygen isotopes in δ18O of OES and MAP implies that the bulk of the ostrich body water is derived from drinking water, as such, each can be used successfully in the geologic record for paleodiets. The isotopic results of OES laid by free-range ostriches living across South Africa, however, deviate significantly from this linear trend (Fig. 3). This is particularly evident at Kimberley where despite the consistency of δ18O values measured in various surface waters available for consumption (average δ18O = −3.1 ± 0.7‰; n = 5), the δ18O values of OES are highly variable (average δ18O = 36.9 ± 3.4‰; n = 7) (Table 2). The isotopic variability measured in the OES at Kimberley implies that the bulk of the ostrich body water is derived from an alternate water source (e.g.,plant leaf-water) or that other factors contribute to the δ18O of OES laid by free-range birds.

Plants commonly consumed by ostriches range in water content from between 37 and 70% (Williams et al., 1993). The δ18O of plant leaf water can vary by as much as 14‰ over a 12 hour period of time (Sternberg et al., 1986) and is controlled by the isotopic composition of the source waters (precipitation, soil and ground) modified by uptake and evaporative transpiration. Because ostrich feeding habits are variable throughout this linear trend (Fig. 3). This is particularly evident at Kimberley where despite the consistency of δ18O values measured in various surface waters available for consumption (average δ18O = −3.1 ± 0.7‰; n = 5), the δ18O values of OES are highly variable (average δ18O = 36.9 ± 3.4‰; n = 7) (Table 2). The isotopic variability measured in the OES at Kimberley implies that the bulk of the ostrich body water is derived from an alternate water source (e.g., plant leaf-water) or that other factors contribute to the δ18O of OES laid by free-range birds.

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Climatic factors such as relative humidity and temperature affect the δ18O values of the plant’s source water and the degree of evaporative transpiration that occurs. Low relative humidity and high temperatures increase evaporative enrichment in source waters and increase evapotranspiration in plants, which should consequently be reflected as isotopically enriched OES. However, there is no significant linear correlation between the average δ18O of the South African OES and mean annual temperature (MAT) at the collection site (δ18O_{OES} =

Fig. 2. The δ15N values of the total organic fraction of South African OES and plants vs. mean annual precipitation (MAP) at the collection site (MAP after WWD, 1994). Linear regressions fit to the OES data (equation included) and to the plant data (from Heaton, 1987). OES collection sites abbreviated as in caption for Fig. 1. Two fields are identified: one encompasses all OES data (shaded box) and one encompasses South African herbivore collagen (dashed box; after Sealy et al., 1987).

Fig. 3. The δ18O values of OES vs. the δ18O of drinking water for samples collected from controlled settings (with linear regression fit to the data). Free range samples plotted against the average δ18O of surface waters measured at Kimberley (−3.1 ± 0.7‰; n = 5), or plotted against the average δ18O of precipitation (−3.0‰; from Rozanski et al., 1993) for all other collection sites. OES collection sites abbreviated as in caption for Fig. 1.
There is some significance in the inverse linear relationship between the average δ18O of South Africa OES and mean annual relative humidity (MARH) (δ18O$_{OES}$ = −0.2 * MARH + 46.1; $R^2 = 0.55$), which may be strengthened if additional data are incorporated into the analysis.

We surmise that in some settings, factors other than climate are partially responsible for the δ18O values of OES laid by free-range ostriches. The physiological response of ostriches to humidity, for example, may differ under different environmental conditions. Thus, caution must be used in reconstructing paleoclimates from the δ18O values of fossil eggshell, particularly those laid by extinct species. At best, provided there is independent control on available moisture it may be possible to reconstruct qualitative changes in paleotemperature from the δ18O of fossil eggshell (cf. Johnson et al., 1997).

### 4.4. Isotopic Stability Through Diagenesis

Protein diagenesis is measured by the amino acid concentrations and the amount of isoleucine epimerization that has occurred in the heated OES samples (Table 4). Isoleucine epimerization is represented as the D-alloisoleucine/L-isoleucine (A/I) value, where A/I approximates 0.0 in modern samples and increases with time until epimeric equilibrium is reached at A/I values of 1.1. Under these experimental conditions, epimeric equilibrium is reached at approximately 250 hours of heating (Fig. 4a) and is roughly equivalent to 1 million years at 10°C.

The carbon isotope composition of the total organic fraction in the heated OES exhibits only a slight increase (0.6‰) from 0 to 500 hours of heating (Fig. 4b). Changes in the amino acid mass balance of the samples alone cannot be used to explain the enrichment in δ13C values of the total organic fraction (Table 4). Some of the most unstable amino acids (e.g., threonine, serine, and aspartate) are major components to modern OES and are also the most isotopically enriched [serine and threonine (measured by Hare et al., 1991) and aspartate (measured in this study)]. Decomposition and loss of these unstable amino acids would drive the remaining organic matter towards isotopic depletion. We propose that the enrichment of δ13C values in heated OES may be explained by (1) loss of isotopically depleted organic material and/or (2) isotopic fractionation of the total organic fraction during heating.

Regardless of the mechanisms, the changes observed in the δ13C are relatively small and the nitrogen isotope composition of the organic fraction in the OES exhibits no consistent trend (Fig. 4c). The changes in δ13C and δ15N are barely greater than the ± 0.2‰ errors associated with sample preparation. Moreover, the total carbon isotope offset (<1‰) is small relative to the variability measured for a specific ecosystem, and to an environment undergoing changes in $C_3$ and $C_4$ vegetation distributions (ca. 13‰). This indicates that the isotopic changes induced by diagenesis are far less than the isotopic signal we hope to capture to reconstruct past environmental changes.

In addition to heating experiments, the carbon isotope offset between the inorganic and the total organic fractions (referred to as $\Delta^{13}C$, where $\Delta^{13}C = \delta^{13}C_{\text{inorg}} - \delta^{13}C_{\text{org}}$) of the modern eggs was measured and evaluated as a means of detecting diagenesis in fossil OES. Consistency in the $\Delta^{13}C$ value implies minimal exchange with the environment (cf. von Schirnding et al., 1982).

The average $\Delta^{13}C$ value of the OES samples collected in captivity and in the wild is 14.7 ± 1.3‰ ($n = 16$) (Tables 1 and 2) (in agreement with von Schirnding et al., 1982). There is a strong linear correlation between the $\delta^{13}C$ of the inorganic and total organic fractions ($y = 15.1 + 1.0x; r^2 = 0.93$) (Fig. 5) implying the $\Delta^{13}C$ values are relatively consistent, regardless of dietary input. Fossil OES samples with $\Delta^{13}C$ values of 15 ± 2‰ are presumed to have undergone minimal diagenesis or exchange with the environment.

If ostriches rely on increasing amounts of meat proteins, the $\Delta^{13}C$ value may differ from 15‰ (cf. Lee-Thorp et al., 1989). Thus, $\Delta^{13}C$ values should be used in conjunction with other criteria (e.g., physical appearance and amino acid composition of the fossil OES) for evaluating the state of preservation of a fossil sample. In general, well-preserved OES will be cream-colored, glossy, and relatively fresh looking. Routine amino acid analysis using ion-exchange chromatography is recommended because it is relatively inexpensive, rapid, and effective at identifying samples that may be diagenetically altered (cf. Wehmiller, 1993; Miller et al., 1992).
4.5. Isotopes of Individual Amino Acids in Ostrich Eggshell

Isotopic analysis of individual amino acids (IAA) in modern samples provides the necessary background information for diagenetic and comparative biochemical studies in the fossil record (cf. Macko and Engel, 1991; Ostrom and Fry, 1993; Engel et al., 1994). Additionally, an understanding of the fractionation processes in living animals allows for comparative isotopic studies in the fossil record of extinct animals.

Of the nine amino acids analyzed in modern OES, the $\delta^{13}C$ values of nonessential amino acids (glycine, proline, glutamate, and aspartate) are more positive than the $\delta^{13}C$ composition of essential amino acids (phenylalanine, isoleucine, leucine, and valine) (Table 5; Fig. 6a). The acidic amino acids (glutamate and aspartate) are enriched in $^{13}C$ because of the presence of the extra carboxyl group (Abelson and Hoering, 1961). The neutral amino acids (isoleucine, leucine, and valine) are relatively depleted in $^{13}C$ (Macko et al., 1987) and may reflect the fractionation associated with the enzymatic formation of acetyl-CoA, a major metabolic intermediate to lipid synthesis (DeNiro and Epstein, 1977). Additionally, the $\delta^{13}C$ values of three out of four of the essential amino acids (phenylalanine, leucine, and valine) in the diet differ from the $\delta^{13}C$ of these amino acids in the OES proteins, implying that partial modification occurs during catabolism of these compounds.

We have restricted our discussion of IAA to a comparison between OES proteins and previously published data on pig collagen (Hare and Estep, 1983; Hare et al., 1991) because pigs and ostriches are both vertebrates with complex digestive systems. The reader is referred elsewhere for isotopic fractionation in simple heterotrophic and autotrophic organisms (Abelson and Herring, 1961; Macko and Estep, 1984; Macko et al., 1987), in mollusk shells (Serban et al., 1988), in extraterrestrial samples (Engel et al., 1990), and during diagenesis (Bada et al., 1989; Silfer et al., 1992).

The pattern of isotopic labeling of IAA in OES proteins is in general agreement with that measured in pig collagen (Fig. 6b). For the amino acids that can be compared, the difference in $\delta^{13}C$ of the isotopically heaviest (glycine) and lightest (valine) amino acid for OES samples is approximately 12‰, and for pig collagen is approximately 11‰ (Hare et al., 1991). The range in $\delta^{13}C$ values of IAA comprising OES makes it important to quantify the amino acid composition of every sample whose total organic fraction is analyzed for isotopes.

Proline in pig collagen is isotopically depleted relative to proline in OES (Fig. 6b). Hydroxylated proline (i.e., hydroxyproline) is a major constituent of collagen and a minor constituent of OES (Brooks et al., 1990). We surmise that hydroxylation favors isotopically enriched proline, thereby leaving a relatively depleted pool of proline in the pig collagen. Serine and threonine are significantly enriched amino acids in collagen relative to all other amino acids, and a similar relationship is hypothesized for threonine and serine in OES.

Table 5. The $\delta^{13}C$ composition of amino acids in modern OES and corresponding diet from Jacksonville, Florida

<table>
<thead>
<tr>
<th>Amino Acid</th>
<th>OES</th>
<th>Food</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glycine</td>
<td>-18.7</td>
<td>-16.2</td>
</tr>
<tr>
<td>Proline</td>
<td>-19.0</td>
<td>-23.7</td>
</tr>
<tr>
<td>Glutamate</td>
<td>-20.9</td>
<td>-22.3</td>
</tr>
<tr>
<td>Aspartate</td>
<td>-23.9</td>
<td>-17.6</td>
</tr>
<tr>
<td>Alanine</td>
<td>-26.3</td>
<td>-13.4</td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>-26.4</td>
<td>-28.4</td>
</tr>
<tr>
<td>Isoleucine</td>
<td>-26.8</td>
<td>-27.6</td>
</tr>
<tr>
<td>Leucine</td>
<td>-30.2</td>
<td>-28.2</td>
</tr>
<tr>
<td>Valine</td>
<td>-30.6</td>
<td>-28.5</td>
</tr>
</tbody>
</table>

Fig. 5. The $\delta^{13}C$ values of the total organic fraction vs. the inorganic fraction of OES collected in captivity and in the wild, where the linear fit to the data is described by the equation and the bold line. White boxes designate the range of $\delta^{13}C$ values expected for OES generated from a diet comprised of 100% C3 plants and 100% C4 plants. All data fit in between these boxes (i.e., in the shaded region) and indicate consistency in the isotopic offset regardless of dietary input.

Fig. 6. (a) The $\delta^{13}C$ of amino acids in modern OES and corresponding diet from Jacksonville, Florida, USA. The essential amino acids (those not manufactured by the animal) are boxed in the diagram. Error bars of ±1‰ are routine and are obscured by the size of the data points. (b) The $\delta^{13}C$ of amino acids in modern pig collagen and corresponding diet (from Hare et al., 1991).
The isotopic fractionation between amino acids in the diet and OES are distinctly different from the fractionation between the amino acids in the diet and pig collagen (Hare et al., 1991) (Figs. 6a,b). Most of the amino acids in the OES proteins are isotopically the same or depleted relative to the diet, and all of the amino acids in pig collagen are the same or isotopically enriched relative to the diet. This implies different carbon sources or assimilation pathways during OES and bone protein synthesis.

The reason for the differences in isotopic fractionation between diet and protein amino acids of OES and pig collagen may relate to the different digestive systems of both animals. Ostriches have two caeca (blind intestines approximately 80 cm in length) in the hind-gut which provide chambers for microbial fermentation (McLelland, 1979; Skadhauge et al., 1984; Noble, 1991; Williams et al., 1993), whereas pigs have a digestive system similar to humans (Stevens, 1988). The varied carbon isotope fractionation between dietary amino acids and OES amino acids suggests assimilation of an isotopically heterogeneous pool of compounds. Isotopic fractionation resulting from nutrient decomposition by anaerobic bacteria in the hind-gut may act to shuffle the resulting compounds into a heterogeneous mixture with respect to the diet. In pigs, however, a more direct transfer of compounds appears to take place during protein and amino acid catabolism.

5. CONCLUSIONS

Studies of ostriches living in captivity and natural settings demonstrate that the isotopic composition of OES reflects ostrich diet and environment. The $\delta^{13}C$ values of modern OES reflect the percentage of $C_3$ and $C_4$ plants in natural settings overprinted by a feeding bias towards $C_3$ vegetation. The $\delta^{15}N$ values of modern OES are inversely correlated with MAP, where a decrease in MAP of 100 mm/yr is accompanied by an increase in $\delta^{15}N$ values of approximately 1‰. The $\delta^{18}O$ values of modern OES laid for free-range birds are thought to be controlled by plant leaf-water fractionation processes combined with physiological responses of ostriches to their environment.

Based on experiments simulating diagenesis, isotopic integrity is preserved in the total organic fraction of fossil OES samples. Thus, there is great potential for using OES to track changes in environment and climate in semi-arid regions through geologic time. Carbon isotopes can be used to track regional changes in $C_3$ and $C_4$ paleovegetation. Because the distribution of $C_3$ and $C_4$ grasses in South Africa depends on the seasonal rainfall patterns, isotopic analysis of OES collected over large spatial and temporal regions provides the opportunity to evaluate changes in season of precipitation through geologic time. Nitrogen isotopes have the potential to provide reliable estimates of MAP in semi-arid and arid regions, and in areas where other proxy records of paleoprecipitation often do not exist. Caution must be used when interpreting $\delta^{18}O$ data of fossil eggshell in light of paleotemperature or paleohumidity. Oxygen isotopes are only useful for deriving paleoclimatic information when coupled with independent proxies for paleotemperature and paleohumidity.

The organic fraction of ostrich bone and OES records similar dietary information, implying that both can be used to reconstruct paleo(diets in the fossil record. Such comparative studies are useful for geological applications because of potential differences in availability of fossil material at a given site. Isotopic analysis of individual amino amino acids (IAA) demonstrates differences in carbon isotope fractionations during synthesis of proteins in animals with different digestive physiologies. Modern studies such as these provide some of the ground-work needed for comparative studies of animal physiology in the geologic record. For example, isotopic analysis of fossil samples with indigenous amino acids may yield information on the digestive strategies of extinct animals.

Because of the variable isotopic composition of individual amino acids, isotopic analysis of the total organic fraction of any fossil material, including eggshell, must be accompanied by quantification of its organic constituents. Amino acid analysis of each fossil eggshell fragment analyzed for stable isotopes is recommended for the following three reasons: (1) to identify samples that have been subject to anomalous thermal histories (i.e., burning) (cf. Miller et al., 1992), (2) to provide amino acid racemization ages (after Brooks et al., 1990; Miller et al., 1992), and (3) to quantify the organic constituents of the samples.

Studies of Tertiary ratite eggshell and Cretaceous dinosaur eggshell indicate that indigenous amino acids may be preserved in eggshell for tens of millions of years. These eggshells have significantly different amino acid distributions relative to Quaternary OES and relative to each other. Because there is a $\pm12^\circ$ range in amino acids comprising modern OES, the total organic fraction of Tertiary and Cretaceous eggshell can be seriously biased by the presence or absence of certain amino acids. Thus, paleodietary and paleoenvironmental reconstructions using stable isotopes in the organic fraction of all eggshell, including dinosaur eggshell must be supported by consistent amino acid compositions in the samples analyzed.

Finally, caution should be used in drawing paleoenvironmental conclusions from small sets of samples. The isotopic variability as measured by the standard deviation of the average of modern OES collected from natural settings can be as high as $\pm3%$ for carbon, nitrogen, and oxygen isotope analysis (e.g., Kimberley site). This isotopic variability results either from varied feeding strategies of ostriches occupying the same ecosystem or seasonal/annual differences in the availability of various food and water sources. Therefore, average isotope values should be obtained by measuring at least three, and preferably between five and ten fossil OES fragments from a site, and from each geologic age.

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