Combined influence of meteoric water and protein intake on hydrogen isotope values in archaeological human bone collagen

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ABSTRACT

Hydrogen isotopes in archaeological human bone collagen are poorly understood, but present an opportunity to add new depth to our understanding of ancient populations. The competing influences of meteoric water versus protein intake on human bone collagen hydrogen isotope values were examined through comparison with the well-understood proxies of hydroxyapatite oxygen and collagen nitrogen isotopes, respectively. Consideration of the data set as individual points compared to averaged pools of individuals in each of 11 archaeological sites suggested the latter partially eliminates inherent variability due to food choice or regional movement. Collagen hydrogen isotopes were moderately correlated with hydroxyapatite oxygen isotopes (R = 0.695, site averages) and collagen nitrogen isotopes (R = 0.562, site averages). Correlation improved with a multiple linear regression including both oxygen and nitrogen (R = 0.745, site averages). Correlation between meteoric water hydrogen and oxygen isotope values converted from hydroxyapatite and collagen values, respectively, yielded a slope well below the expected value of ~8 observed directly in meteoric water (i.e. the "meteoric water line"). Correlation between converted meteoric water hydrogen and the measured collagen non-exchangeable hydrogen isotope values showed a slope well below the expected value of 1.0. Theoretical meteoric water hydrogen isotope values and theoretical herbivorous collagen hydrogen isotope values were calculated based on previously established equations in order to construct a hypothetical framework free of trophic level influences. Deviations between actual values and these theoretical values correlated weakly with collagen nitrogen isotope values, suggesting that direct trophic level enrichment/depletion is not controlling the disparity between expected and measured values. The deviations are hypothetically caused by non-local food sources, and a decoupling of expected oxygen and hydrogen relationships as individuals consumed more meat and decreased in vivo non-essential amino acid production. This work presents a new model that facilitates understanding of the complex relationship between meteoric water and protein intake controls on hydrogen isotopes in omnivorous human populations that can potentially inform about past meteoric water values and amounts of animal protein consumption.

Keywords: bone, collagen, hydroxyapatite, hydrogen, oxygen, nitrogen, isotopes

1. INTRODUCTION

Stable isotope analysis of bones is relatively common in archaeology and paleontology to determine dietary components, provenance, migrations, climate proxies, metabolic functioning, and social demographics. Several decades of research have established a solid understanding of stable carbon, nitrogen, and oxygen isotope dynamics in archeological bone collagen and hydroxyapatite. Hydrogen isotopes in bone have been addressed only recently. The routing of hydrogen into bone collagen in particular is less well-understood, but presents new options for understanding archaeological remains.

Hydrogen isotopes have been examined more thoroughly in tissues which are similar to the collagen protein and can serve as basic comparisons. Keratin (i.e. feathers, claws, nails) has been studied most heavily, although blood, muscle, lipids, and other organ tissues have been examined as well (Chesson et al. 2009, Chesson et al. 2011, Hobson et al. 1999, Tuross et al. 2008, Wolf et al. 2011). Hydrogen is routed to keratin from both dietary food and drinking water, where the former pathway provides trophic information and the latter indicates latitudinal provenance (Bowen et al. 2005, Bowen et al. 2009, Ehleringer et al. 2008, O'Brien and Wooller 2007, Sellick et al. 2009). Where some studies suggest keratin hydrogen isotopes largely reflect drinking water isotope values (Hobson et al. 1999, Wolff et al. 2011), others suggest secondary dietary hydrogen input as well (Bowen et al. 2009, Ehleringer et al. 2008, Kirsanow and Tuross 2011, Pietsch at al. 2011). Bulk blood, muscle, lipid, and organ hydrogen isotope values reflect largely drinking water sources (Chesson et al. 2011, Hobson et al. 1999, Wolf et al. 2011), although dietary input may have some influence (Commerford et al. 1983). While this previous research provides background for understanding hydrogen isotopes in bone collagen, keratin and other tissues have a more rapid turnover, a fundamentally different structure, and potentially different hydrogen sources rendering them inadequate proxies for collagen.

Collagen is the primary protein in animal bones and includes hydrogen atoms bound to carbon, or bound within carboxyl, amide, and minimal sulfhydryl side-groups. These side-group hydrogen atoms are labile and exchange with hydrogen from other water sources. The more stable carbon-bound hydrogen atoms comprise a calculated fraction of 0.742-0.829 (majority 0.77-0.81) of all hydrogen atoms in collagen (Cormie et al. 1994b, Cormie et al. 1994c, Leyden et al. 2006, Sauer et al. 2009, Topalov et al. 2013) and are generally non-exchangeable with external water sources. The total (i.e. TOT) hydrogen isotope composition of bone collagen (i.e. COLL) can be represented as

$$\delta^2H_{COLL\text{-}TOT} = (1\text{-}f) * \delta^2H_{COLL\text{-}NEX} + f * \delta^2H_{COLL\text{-}EX}$$

where f represents fraction of exchangeable hydrogen (i.e. ~0.19-.23), $\delta^2H_{COLL\text{-}NEX}$ represents the isotope value of non-exchangeable hydrogen atoms, and $\delta^2H_{COLL\text{-}EX}$ represents the isotope value of exchangeable hydrogen atoms. Isotope values are in standard delta notation:

$$\delta X = [(R_{sample} - R_{standard}) / R_{standard}]$$

where R is the ratio (i.e. $^2H/^1H$), values are in parts per thousand (‰), and the standard is V-SMOW.

The $\delta^2H_{COLL\text{-}NEX}$ represents the isotope signal incorporated via water or dietary food and can be considered with a general conceptual framework:

$$\delta^2H_{COLL\text{-}NEX} = (\delta^2H_{ingested\ water} + \varepsilon_a) + (\delta^2H_{dietary\ amino\ acids} + \varepsilon_b).$$

The $\delta^2H_{ingested\ water}$ represents δ^2H of water taken into the body via food water or direct drinking and incorporated into amino acids synthesized in vivo during collagen construction (i.e. "non-essential" amino acids). The ε_a represents hydrogen isotope fractionation during this process. The $\delta^2H_{dietary\ amino\ acids}$ represents δ^2H of amino acids synthesized ex vivo and are incorporated directly from consumed dietary proteins (i.e. "essential" amino acids). The ε_b represents subsequent fractionation as these amino acids are incorporated into collagen, although this value is suspected to be minimal and constant within a given species. Cormie et al. (1994a), Cormie et al. (1994c) and Chesson et al. (2011) present a thorough review of factors contributing to bone collagen $\delta^2H_{ingested\ water}$, ε_a, $\delta^2H_{dietary\ amino\ acids}$, and ε_b;

additional insight is gained from detailed discussions of keratin hydrogen incorporation (Ehleringer et al. 2008, Bowen et al. 2009).

A relatively strong linear correlation between $\delta^2H_{ingested\ water}$ and $\delta^2H_{COLL\text{-}NEX}$ exists in strict herbivores obtaining all dietary fractions (i.e. amino acids, carbohydrates, water) from plants. Since leaf and stem δ^2H values reflect local precipitation δ^2H values, the herbivore $\delta^2H_{COLL\text{-}NEX}$ correlates with these local precipitation δ^2H values (Cormie et al. 1994a, Cormie et al. 1994c, Pietsch et al. 2011, Reynard and Hedges 2008). Hydrogen isotope values in herbivore bone collagen can be considered with the simpler representation of $\delta^2H_{COLL\text{-}NEX} = \delta^2H_{ingested\ water} + \varepsilon_a$. Carnivores tend to show an apparent trophic level effect where $\delta^2H_{COLL\text{-}NEX}$ deviates from the expected correlation with $\delta^2H_{ingested\ water}$ (Birchall et al. 2005, Pietsch et al. 2011, Reynard and Hedges 2008, Topalov et al. 2013, Tuross et al. 2008). This is due likely to the additional $\delta^2H_{dietary\ amino\ acids}$ variable which can show considerable range depending on the type and amount of animal protein consumed.

Humans present a complex case of omnivory. Limited research examining human collagen δ^2H values suggests a combination of ingested water and dietary input (Reynard and Hedges 2008), which agrees with limited data from other omnivorous mammals (Reynard and Hedges 2008, Tuross et al. 2008). As archaeological human remains are of high interest, examining human collagen δ^2H could provide another dimension by which to examine dietary input and ingestion of environmental water in a uniquely coupled pathway. It has the potential to contribute additional information to the study of geographic origin, migrations, and dietary choices or available foods.

This study uses the well-known relationships of bone nitrogen and oxygen with trophic structure and meteoric water, respectively, to explore these mechanisms' effects on $\delta^2H_{COLL\text{-}NEX}$. Nitrogen in collagen (i.e. $\delta^{15}N_{COLL}$) is represented in standard delta notation as indicated previously where R is $^{15}N/^{14}N$ and the standard is atmospheric air. The $\delta^{15}N_{COLL}$ increases approximately 3-4 ‰ with trophic level (Bocherens and Drucker 2003, DeNiro and Epstein 1981, Schoeninger and DeNiro 1984) providing a proxy for amount and type of dietary protein intake. Oxygen is found in the hydroxyapatite mineral fraction of bone in both the phosphate (i.e. PHOS) and carbonate (i.e. CARB) sites. Phosphate and carbonate oxygen isotopes (i.e. $\delta^{18}O_{PHOS}$ and $\delta^{18}O_{CARB}$) are represented in standard delta notation where R is $^{18}O/^{16}O$ and the standard is V-SMOW. Both $\delta^{18}O_{PHOS}$ and $\delta^{18}O_{CARB}$ correlate with drinking water isotopes (Bryant and Froehlich 1995, Daux et al. 2008, Kohn 1996, Longinelli 1984, Luz and Kolodny 1985, Luz et al. 1984) providing a proxy for geographic locality. The $\delta^{18}O$ and δ^2H values of meteoric water (i.e. MW) are strongly correlated according to the known meteoric water line: $\delta^2H_{MW} = 8 * \delta^{18}O_{MW} + 10$ (Craig 1961, Kendall and Coplen 2001). In the absence of dietary influence, the $\delta^2H_{COLL\text{-}NEX}$ is expected to correlate to $\delta^{18}O_{PHOS}$ and $\delta^{18}O_{CARB}$ with a similar slope to that of the meteoric water line. Deviations from this end member were compared to associated $\delta^{15}N_{COLL}$ values, and multiple linear regression models constructed to determine the combined relative influence of ingested water and dietary proteins on the $\delta^2H_{COLL\text{-}NEX}$ values. Combinations of δ^2H and $\delta^{18}O$ values in bone collagen have been used to examine herbivores, but this study adds to the sparser comparisons with $\delta^{15}N$, omnivores, and carnivores (Cormie et al. 1994a, Kirsanow and Tuross 2011, Kirsanow et al. 2008, Pietsch et al. 2011, Topalov et al. 2013, Tuross et al. 2008).

2. MATERIALS AND METHODS

2.1 Sample collection and preparation

Human remains were sampled from 11 North American archaeological sites primarily on the east coast with one southern site including individuals from Texas (Figure 1, Table 1, Supplementary Table S1). These sites were selected based on availability of samples, range of geographic localities, and range of potential protein consumption. The sites are primarily temperate regions with similar humidity

and temperature conditions. The exception is Glorieta Pass wherein the individuals hailed from the warmer dryer regions of Texas (Alberts 1984). Carbon, nitrogen, and oxygen isotope data for some samples were published previously in France et al. (2014) and France and Owsley (2015).

Mechanical and chemical preparation methods followed France et al. (2014). Briefly, ~500 mg of solid bone cross section (majority cortical with traces of trabecular) was removed for collagen analysis using pliers or a rotary tool. This cross section yields a homogenized average isotope value across the final ~10-20 years of life. Approximately 50mg of powdered bone for phosphate and carbonate analysis was obtained by crushing with an agate mortar and pestle or using a rotary tool. Phosphates were extracted via dissolving mineral phases in hydrofluoric acid (2 M), buffering in ammonium hydroxide (20 %), and precipitating silver phosphate using a silver nitrate solution (2 M). Carbonates were isolated by eliminating organics with sodium hypochlorite (2-3 %) and eliminating secondary carbonates using acetic acid buffered with calcium acetate (pH ~4.5). Collagen extraction proceeded via sonication to remove sediments and labile salts, acidification (0.6 M HCl, 4 °C) to remove mineral phases, removal of humic and fulvic acids with sodium hydroxide (0.125 M), denaturing of the collagen pseudomorph in hydrochloric acid (0.03 M, 95 °C), and lyophilization.

2.2 Mass spectrometry and ATR-FTIR

All isotope ratios were measured on Thermo Delta V Advantage stable isotope mass spectrometers at the Smithsonian MCI Stable Isotope Mass Spectrometry Laboratory. Silver phosphates weighed into silver cups (~500 µg) were thermally decomposed (1450 °C) on a Thermo Temperature Conversion Elemental Analyzer (TCEA) coupled to a Conflo IV interface and measured for $\delta^{18}O_{PHOS}$ values. Carbonates (~4 mg) were acidified in 100 % phosphoric acid (SG > 1.92) at 25 °C for 24 hours on a Thermo GasBench II unit and measured for $\delta^{18}O_{CARB}$ values. Approximately 500 µg of collagen weighed into tin cups was combusted (1020 °C) on a Costech 4010 Elemental Analyzer coupled to a Conflo IV interface and measured for $\delta^{15}N_{COLL}$, weight % N, and weight % C values.

A separate portion of collagen (~350 µg) was weighed into silver cups for hydrogen isotope analysis. Open cups remained in ambient air for 72 hours to equilibrate exchangeable hydrogen atoms with local water vapor. Open cups were then placed in a vacuum oven at 60 °C for 72 hours to remove secondary adhered water molecules. The vacuum oven was vented with pure argon before cups were removed, quickly sealed, and loaded into a Costech zero-blank autosampler then flushed with ultra-pure helium. Exposure to atmosphere was <10 minutes. Samples were thermally decomposed on the TCEA with a chromium reactor column at 1100 °C (modified from Armbruster et al. 2006, Gehre et al. 2015, Kelly et al. 2001). Resulting H_2 gas was introduced to the mass spectrometer via a Conflo IV interface and measured for raw δ^2H values.

Attenuated total reflectance Fourier transform infrared spectroscopy (ATR-FTIR) was performed on bone powders using a Thermo Nicolet 6700 FTIR with Golden Gate ATR (diamond crystal, single bounce, 45°) equipped with a DTGS detector. Spectra were collected from 450 to 4,000 cm^{-1} for 128 scans at a resolution of 4 cm^{-1}. All baseline corrections and ratio calculations were performed using an automated program in TQAnalyst EZ version 8.

2.3 Data normalization

All data were normalized to international reference materials. The $\delta^{18}O_{VSMOW\text{-}SLAP}$ values of phosphates (i.e. $\delta^{18}O_{PHOS}$) were corrected against USGS 34 ($\delta^{18}O_{VSMOW\text{-}SLAP}$ = -27.9 ‰) and USGS 35 ($\delta^{18}O_{VSMOW\text{-}SLAP}$ = +57.5 ‰) nitrates. The $\delta^{18}O_{VPDB\text{-}LSVEC}$ values of carbonates (i.e. $\delta^{18}O_{CARB}$) were corrected against LSVEC ($\delta^{18}O_{VPDB\text{-}LSVEC}$ = -26.7 ‰) and NBS 19 ($\delta^{18}O_{VPDB\text{-}LSVEC}$ = -2.2 ‰) carbonates and converted to VSMOW values for easier comparison to $\delta^{18}O_{PHOS}$ values using Coplen et al. (2002). The $\delta^{15}N_{AIR}$ values of collagen (i.e. $\delta^{15}N_{COLL}$) were corrected against Urea_UIN3 and an acetanilide calibrated to USGS 40 ($\delta^{15}N_{AIR}$ = -4.52 ‰) and USGS 41 ($\delta^{15}N_{AIR}$ = +47.57 ‰) amino acids (Schimmelmann et al. 2009). Weight

% N and weight % C values were calibrated using a homogenous acetanilide standard. Errors associated with $\delta^{18}O_{PHOS}$ values are ±0.4 ‰ (1σ); $\delta^{18}O_{CARB}$ and $\delta^{15}N_{COLL}$ are ±0.2 ‰ (1σ); weight % N and weight % C are ±0.5 % (1σ).

Non-exchangeable hydrogen isotope values (i.e. $\delta^2H_{COLL\text{-}NEX}$) were determined using three new collagen reference materials developed in parallel experiments with the USGS Reston Stable Isotope Laboratory. An Alaskan moose femur (AMF), Alaskan seal femur (ASF), and Minnesotan otter leg (MOL) were selected based on their observed raw differences in δ^2H values. Whole bone segments of these reference materials were degreased in sequential soaks of 2:1 chloroform:methanol. Collagen was extracted in bulk from the degreased reference bones according to procedures outlined above. The $\delta^2H_{COLL\text{-}NEX}$ and f values of AMF, ASF, and MOL were determined via methods in Qi and Coplen (2011). Briefly, the exchangeable hydrogen atoms in two identical sets of samples were equilibrated with waters of known and disparate isotope composition in separate vacuum dessicators. Samples were transferred to a vacuum oven and dried at 60 °C to remove secondary adhered water molecules. Samples were removed from the oven, quickly sealed into silver cups, placed in a zero-blank autosampler and flushed with ultra-pure helium. Samples were calibrated against VSMOW2 and SLAP2 water standards sealed in silver tubes on a TCEA with a chromium reactor column (Gehre et al. 2015). The fraction of exchangeable hydrogen (f_{AMF} = 0.136, f_{MOL} = 0.145, f_{ASF} = 0.147) was calculated by $f = [\delta^2H_{tot1} - \delta^2H_{tot2}] / [\delta^2H_{w1} - \delta^2H_{w2}]$ where δ^2H_{tot1} and δ^2H_{tot2} are the isotope values of total hydrogen in the collagen equilibrated with the two different waters. The $\delta^2H_{VSMOW\text{--}SLAP}$ of non-exchangeable hydrogen was calculated by isotope balance (AMF $\delta^2H_{COLL\text{-}NEX}$ = -73.4 ‰, MOL $\delta^2H_{COLL\text{-}NEX}$ = +18.3 ‰, ASF $\delta^2H_{COLL\text{-}NEX}$ = +164.9 ‰). The $\delta^2H_{COLL\text{-}NEX}$ in unknown samples was calculated using a 3-point linear calibration on the $\delta^2H_{COLL\text{-}NEX}$ for AMF, ASF, and MOL reference materials as per Wassenaar and Hobson (2003). Errors associated with $\delta^2H_{COLL\text{-}NEX}$ values are ±2.0 ‰ (1σ).

2.4 Examination of Diagenesis

Collagen preservation (i.e. post-mortem diagenesis) was examined using established protein and elemental abundance parameters (Table 2). Hydroxyapatite phosphate and carbonate were examined using ATR-FTIR peak height data (Table 2). Acceptable FTIR values for well-preserved hydroxyapatite are based on modern material in this study, previous ATR-FTIR data, and data converted from suggested offsets from more ubiquitous transmission FTIR methods. As phosphates are generally more resistant to post-mortem isotope alteration and more strongly correlated to drinking water compared to carbonates (Iacumin et al. 1996, Person et al. 1995, Person et al. 1996, Tuross et al. 1989), the $\delta^{18}O_{PHOS}$ values are used in subsequent statistical analysis and modeling.

2.5 Isotope value conversions and comparisons

Corrected $\delta^2H_{COLL\text{-}NEX}$, $\delta^{18}O_{PHOS}$, $\delta^{15}N_{COLL}$ were compared and considered via various conversions and regression models, both as individual points and by site averages. The $\delta^2H_{COLL\text{-}NEX}$ was converted to meteoric water δ^2H values using the equation of Reynard and Hedges (2008): $\delta^2H_{MW\text{-}COLL} = (\delta^2H_{COLL\text{-}NEX} - 71.9)/1.069$ (r = 0.957, SE = 16.2 ‰). This equation is based on data from humans, non-human herbivores, and omnivores. The $\delta^{18}O_{PHOS}$ values were converted to meteoric water $\delta^{18}O$ values using the equation of Longinelli (1984): $\delta^{18}O_{MW\text{-}P} = (\delta^{18}O_{PHOS} - 22.37)/0.64$ (r = 0.982, SE = 0.68 ‰). The $\delta^2H_{MW\text{-}COLL}$ values were compared to theoretical δ^2H_{MW} values calculated from the meteoric water line using the $\delta^{18}O_{MW\text{-}P}$ values: $\delta^2H_{MW\text{-}THEOR} = 8 * \delta^{18}O_{MW\text{-}P} + 10$. Differences between $\delta^2H_{MW\text{-}COLL}$ and $\delta^2H_{MW\text{-}THEOR}$were compared to $\delta^{15}N_{COLL}$ in an effort to discern trophic effects that may influence deviations from the meteoric water line. Cormie et al. (1994a) provide evidence of a well-correlated relationship (r = 0.917) between $\delta^2H_{COLL\text{-}NEX}$ and $\delta^{18}O_{PHOS}$ in strict herbivores from the same locality, thereby essentially eliminating trophic effects on the $\delta^2H_{COLL\text{-}NEX}$. This study used their equation to calculate a theoretical $\delta^2H_{COLL\text{-}NEX}$ based on $\delta^{18}O_{PHOS}$ assuming an end-member scenario of humans as strict herbivores: $\delta^2H_{HERB\text{-}}$

$_{THEOR}$ = 7.8 * $\delta^{18}O_{PHOS}$ -160 (r = 0.917, SE = 11.9 ‰). Differences between $\delta^{2}H_{COLL\text{-}NEX}$ and $\delta^{2}H_{HERB\text{-}THEOR}$ were plotted against $\delta^{15}N_{COLL}$ in another effort to determine a relationship between trophic effects of human omnivory on deviations from a pure meteoric water relationship between $\delta^{2}H_{COLL\text{-}NEX}$ and $\delta^{18}O_{PHOS}$. Finally, multiple linear regression of $\delta^{18}O_{PHOS}$ and $\delta^{15}N_{COLL}$ against $\delta^{2}H_{COLL\text{-}NEX}$ examined the concurrent influences of protein intake and meteoric water on hydrogen isotopes in bone collagen. All statistical analyses were run in SigmaPlot 14.0.

In an effort to eliminate other demographic factors as controlling influences on collagen hydrogen isotopes, the $\delta^{2}H_{COLL\text{-}NEX}$, $\delta^{18}O_{PHOS}$, and $\delta^{15}N_{COLL}$ values were compared with ancestry (i.e. African American or Caucasian), sex, estimated age, and socioeconomic status (i.e. lower, middle, upper, or military class). Socioeconomic status was assigned based on the context of the burial site. Sex and age were not available or were indeterminate for some individuals (Supplementary Table S1).

3. RESULTS

Samples adhering to the defined parameters for well-preserved collagen and hydroxyapatite were included in subsequent analyses. Table 3 includes all $\delta^{2}H$, $\delta^{18}O$, and $\delta^{15}N$ data pertinent to statistical analyses and modeling. Table 4 includes full statistical results from regressions. Yield data, C:N ratios, FTIR data, and calculated differences between isotope values are presented in the Supplementary Tables S2 and S3. Although $\delta^{18}O_{CARB}$ is not used in any subsequent analyses or modeling, it is included in Supplementary Table S3 to present a complete oxygen data set to the research community for future comparisons.

As a coarse examination, $\delta^{2}H_{COLL\text{-}NEX}$, $\delta^{18}O_{PHOS}$, and $\delta^{15}N_{COLL}$ values showed no significant differences between males and females nor between Caucasians and African Americans (two-tailed t-tests, all $p > 0.3$). No discernable correlation was observed between $\delta^{2}H_{COLL\text{-}NEX}$, $\delta^{18}O_{PHOS}$, and $\delta^{15}N_{COLL}$ values and minimum estimated age (all $R^2 < 0.12$). These demographic groupings included individuals from different regions. No single site yielded more than eight individuals with determinate age and sex, nor did any single site include both Caucasians and African Americans, thereby precluding a rigorous statistical examination of these factors while controlling for regional variation in meteoric water isotope values. Comparison of $\delta^{2}H_{COLL\text{-}NEX}$ between socioeconomic groups showed no significant differences (two-tailed t-test, all $p > 0.05$), with the exception of the lower class group versus the upper class group ($p = 0.00045$) and military group ($p = 0.020$).

The $\delta^{2}H_{COLL\text{-}NEX}$ for individual points varied from -15.8 to +26.2 ‰; site averages ranged from -2.8 to +17.8 ‰. The $\delta^{18}O_{PHOS}$ for individual points and site averages ranged from +15.8 to +20.3 ‰ and +16.5 to +20.0 ‰, respectively. The $\delta^{15}N_{COLL}$ for individual points and site averages ranged from +8.7 to +11.7 ‰ and +9.7 to +11.7 ‰, respectively. All regressions and correlations between $\delta^{2}H_{COLL\text{-}NEX}$, $\delta^{18}O_{PHOS}$, and $\delta^{15}N_{COLL}$ pass Shapiro-Wilk normality tests ($p > 0.05$). Linear regression showed a moderate correlation between individual $\delta^{2}H_{COLL\text{-}NEX}$ points and $\delta^{18}O_{PHOS}$ or $\delta^{15}N_{COLL}$, with slightly stronger correlation between site averages (Figure 2, Table 4).

Conversion to meteoric water values resulted in $\delta^{2}H_{MW\text{-}COLL}$ ranging from -82.0 to -42.8 ‰ for individual points and -69.8 to -50.6 ‰ for site averages. The $\delta^{18}O_{MW\text{-}P}$ for individual points and site averages ranged from -10.3 to -3.2 ‰ and -9.2 to -3.7 ‰, respectively. As these values, and subsequent $\delta^{18}O_{MW\text{-}P}$ values, were direct transformations of $\delta^{2}H_{COLL\text{-}NEX}$ and $\delta^{18}O_{PHOS}$, the correlations were similar with site averages showing a stronger correlation than individual points (Table 4). Both individual points and site averages showed slopes considerably lower than that expected from the known meteoric water line (Figure 3).

Conversion of $\delta^2H_{COLL\text{-}NEX}$ to theoretical values based on the meteoric water line resulted in $\delta^2H_{MW\text{-}THEOR}$ values ranging from -72.2 to -15.9 ‰ for individual points, and -63.6 to -19.7 ‰ for site averages. The $\delta^2H_{MW\text{-}COLL}$ and $\delta^2H_{MW\text{-}THEOR}$ values were compared to one another and showed moderate correlation (Figure 4, Table 4). Differences between these two values were compared to $\delta^{15}N_{COLL}$ (Figure 4). Correlation was very poor between $\delta^{15}N_{COLL}$ and the $\delta^2H_{MW\text{-}COLL}$-$\delta^2H_{MW\text{-}THEOR}$ difference for individual points (r = 0.0524) and site averages (r = 0.284).

Conversion of $\delta^2H_{COLL\text{-}NEX}$ to a theoretical value that assumes humans are strict herbivores resulted in $\delta^2H_{HERB\text{-}THEOR}$ values ranging from -36.8 to -1.7 ‰ for individual points and -31.4 to -4.0 ‰ for site averages. The $\delta^2H_{COLL\text{-}NEX}$ and $\delta^2H_{HERB\text{-}THEOR}$ values were compared to one another and showed moderate correlation (Figure 5, Table 4). Differences between these two values were compared to $\delta^{15}N_{COLL}$ (Figure 5). Correlation was very poor between $\delta^{15}N_{COLL}$ and the $\delta^2H_{COLL\text{-}NEX}$-$\delta^2H_{HERB\text{-}THEOR}$ difference for individual points (r = 0.176) and site averages (r = 0.0547).

Multiple linear regression produced better predictive ability for both individual points and site averages, where the latter again showed better correlation (Table 4). The highest predictive power lies in a linear combination of both $\delta^{18}O_{PHOS}$ and $\delta^{15}N_{COLL}$.

4. DISCUSSION

Hydrogen isotopes in human collagen present a complex combination of influences. Before discussing the influences of meteoric water and protein intake on the $\delta^2H_{COLL\text{-}NEX}$ values, it is worth considering potential confounding factors in the oxygen and nitrogen proxy variables. These may include inter-regional movement of individuals, demographic factors, individual health status, variable isotope baselines of food, and non-local food sources.

Movement between regions and cities did occur in historic times. While the δ^2H_{MW} and $\delta^{18}O_{MW}$ values should still co-vary in all locations, the available food (and presumably its inherent δ^2H values) would change. However, the sites included in this study likely represent local populations with a common set of food resources. Several (Foscue Plantation, Hilleary Cemetery, Walton Family Cemetery, Woodville Cemetery) are established family plots including individuals that most likely lived and worked in the local area for significant portions of their lives. The variability in $\delta^{18}O_{MW\text{-}P}$, an indicator of regional origin, for each of these sites is <1.0 ‰ (1σ), supporting the idea that these individuals did not spend significant portions of their lives elsewhere. Three sites (A.P. Hill, Pettus, Robinson Cemetery) are slave populations that were not free to move around as they pleased; these sites show individual $\delta^{18}O_{MW\text{-}P}$ variability of <1.2 ‰ (1σ). Three northern urban sites (FABC, Parkway Gravel, Trinity Catholic Church) are church cemeteries that historically represent local communities. Although the urban setting might suggest higher likelihood for mobility, the $\delta^{18}O_{MW\text{-}P}$ variability is <0.6 ‰ (1σ) for each of these sites. The Glorieta Pass site is constituted entirely of a military unit that historic records show mustered out of Texas. These individuals show a $\delta^{18}O_{MW\text{-}P}$ variability of 1.3 ‰ (1σ), which is the expected range in Texas.

Comparison of $\delta^2H_{COLL\text{-}NEX}$, $\delta^{18}O_{PHOS}$, and $\delta^{15}N_{COLL}$ values with demographic factors showed no statistically significant connections between ancestry, sex, and estimated age. France et al. (2014) found that ancestry was in fact most strongly predicted by collagen carbon isotope values rather than oxygen or nitrogen. Comparison of $\delta^2H_{COLL\text{-}NEX}$ to social class showed significant differences between the lower class group versus the upper class and military groups. However, the sole military group in this test is the southernmost site, while lower class individuals were found in northern locations. This introduces regional controls on drinking water isotopes as a potential factor and precludes the conclusion that social class is the prevailing component in this observed isotopic difference between the lower class and military groups. The lower and upper class group contained individuals from similar regions which does

impose some control on regional variability in meteoric water isotope values. In some populations, social class is apparently linked to food availability and food choice, which in turn is a rough proxy for nutritional status (France et al. 2014, Yoder 2012). As protein intake, and consequently essential amino acid intake, are linked to food availability and choice, it bears consideration that social class may influence the $\delta^2H_{COLL\text{-}NEX}$ values in so much as social class influences the type of food consumed. However, social class is only moderately predictable using a combination of carbon, nitrogen and oxygen isotope values and does not appear to be the prevailing factor controlling stable isotope distributions in these humans (France et al. 2014). Therefore, the potential influence of social class on $\delta^2H_{COLL\text{-}NEX}$ values does not preclude examination of meteoric water and protein intake on said values.

The $\delta^{15}N_{COLL}$ values can be complicated by health issues. The $\delta^{15}N_{COLL}$ values can reflect extreme circumstances including disease, infection, and pregnancy (Beaumont et al. 2013, 2015, D'Ortenzio et al. 2015, Fuller et al. 2004, Scorrano et al 2014). Physiological examination of the remains in this study suggests no obvious presence of these factors, with the exception of two elderly individuals (31FOSCUE-ECU-4 and 44JC33-PETTUS-270) who exhibit osteopenia (Barca and Owsley 2014). Neither individual has outlying $\delta^{15}N_{COLL}$ values which suggests this particular condition did not significantly affect their nitrogen isotope values.

The baseline $\delta^{15}N$ value of plants can vary somewhat by region and incorporation of nitrogen isotopes into bone collagen can be affected by climate. Mammals in hotter drier climates will sometimes exhibit ^{15}N enrichment compared to counterparts in cooler wetter climates (Ambrose 1991, Cormie and Schwarcz 1994, Fizet et al. 1995, Sealy et al. 1987). However, the individuals in this study's sites do not conform to this pattern. The larger data set of France et al. (2014), which includes most of the sites in the current study, shows no correlation between human $\delta^{15}N_{COLL}$ and $\delta^{18}O_{MW\text{-}P}$ ($r = 0.0892$). The exception is Walton Cemetery in Connecticut, the most northern and coolest site. The human remains from this site do show the most negative $\delta^{15}N_{COLL}$ values, but the Walton Cemetery site average (+9.7 ‰) is only 0.6 ‰ less than the average of all sites combined (+10.3 ‰). Regional climates and health issues may still exhibit some control over $\delta^{15}N_{COLL}$ values, but it appears to be minor in the remains sampled here. The dominant controlling factor is more likely to be dietary input as discussed below.

Although 18th and 19th century humans consumed a much more localized diet than modern humans, they still consumed some percentage of non-local food and could exercise choice in their dietary selections, unlike non-human mammals considered in previous studies. The more common imports of the time included cocoas, coffee, tea, rum, molasses and sugar from the West Indies and other regions; rice from Asia; wine from Europe and other regions; fresh produce and some meat from other United States regions. However, broad interregional and international food trade did not become widespread until the late nineteenth century (Bruegel 2002, Nützenadel and Trentmann 2008, Perren 2006).

While some of the factors discussed above may have a minor influence on the isotope values, none appear to be a dominant or significant factor in this study. However, it is worth bearing such factors in mind in future examinations of human $\delta^2H_{COLL\text{-}NEX}$ since the body of published data is currently small and the understanding of how demographics and health affect hydrogen routing in humans is poorly understood. Considering data as individual points will include wider variation while averaging data by site eliminates some of the variability due to food choice, food availability, and lifetime movement. The $\delta^{18}O$ and $\delta^{15}N$ values are likely to be reliable proxies for meteoric water influence and protein intake, respectively. The $\delta^{18}O_{PHOS}$ and $\delta^{15}N_{COLL}$ values showed moderate correlation to $\delta^2H_{COLL\text{-}NEX}$ values. Better correlation through multiple linear regression suggests both are coupled to $\delta^2H_{COLL\text{-}NEX}$ with ~56 % of the variation in $\delta^2H_{COLL\text{-}NEX}$ related to a combination of $\delta^{18}O_{PHOS}$ and $\delta^{15}N_{COLL}$ (from $r = 0.745$). A larger data set of pooled individuals (i.e. site averages) might facilitate a model whereby a reasonable approximation of any one variable can be determined from a combination of the other two.

Likewise, calculations of $\delta^2H_{MW\text{-}COLL}$ and $\delta^{18}O_{MW\text{-}P}$ can be performed once $\delta^2H_{COLL\text{-}NEX}$ or $\delta^{18}O_{PHOS}$ are known. This holds potential for reconstructing historic meteoric water isotope values from archaeological remains when faunal remains are unavailable, and subsequently examining geographic origins, migration, and movement.

Deviation of the $\delta^2H_{MW\text{-}COLL}$ and $\delta^{18}O_{MW\text{-}P}$ relationship from the expected meteoric water line suggests minor input of non-local food sources and metabolic decoupling of the δ^2H-$\delta^{18}O$ relationship. The observed slope of this relationship (i.e. 2.1 for individual points, 2.5 for site averages) was well below the expected value of 8.0. Regression of $\delta^2H_{MW\text{-}COLL}$ against $\delta^2H_{MW\text{-}THEOR}$ produces a slope well below 1.0. Since $\delta^2H_{MW\text{-}THEOR}$ represents a theoretically expected value for δ^2H_{MW} based on the meteoric water line, a slope <1.0 indicates the δ^2H incorporated into bone collagen is more negative than it should be if controlled strictly by regional meteoric water. This may indicate that dietary amino acids entering the body via protein consumption had an inherently depleted $\delta^2H_{COLL\text{-}NEX}$ value. Local herbivorous protein sources (i.e. deer, cow, sheep, etc.) foddered on local vegetation should conform to the meteoric water line where the slope of $\delta^2H_{COLL\text{-}NEX}$ versus $\delta^{18}O_{PHOS}$ is approximately 8.0, and the slope of $\delta^2H_{COLL\text{-}NEX}$ versus δ^2H_{MW} is approximately 1.0 (Cormie et al. 1994a, Cormie et al. 1994c, Pietsch et al. 2011, Reynard and Hedges 2008, Topalov et al. 2013). In this study, the deviation from these expected slopes may be due to inclusion of protein sources imported from northern latitudes, or from local animals foddered on imported northern grains. This would result in a depleted $\delta^2H_{dietary\ amino\ acid}$ input into the human body, while maintaining the local $\delta^{18}O_{MW\text{-}P}$ signature, thereby decreasing the $\delta^2H_{COLL\text{-}NEX}$ versus δ^2H_{MW} slope to <1.0. Bowen et al. (2009) and Kirsanow and Tuross (2011) also noted that amount of local versus non-local dietary input can affect expected correlations between δ^2H and $\delta^{18}O$ in human keratin and collagen. While the influence of imported and non-local foods on isotope values cannot be completely discounted, it is likely to be a relatively minor factor, as discussed above.

Differences between $\delta^2H_{MW\text{-}COLL}$ and $\delta^2H_{MW\text{-}THEOR}$ did not correlate well with $\delta^{15}N_{COLL}$, nor did differences between $\delta^2H_{COLL\text{-}NEX}$ and $\delta^2H_{HERB\text{-}THEOR}$. The $\delta^2H_{MW\text{-}THEOR}$ and $\delta^2H_{HERB\text{-}THEOR}$ represent expected values for meteoric water-controlled hydrogen isotope values without trophic level fractionations. Nitrogen isotopes show a very systematic enrichment during increased meat consumption where ^{14}N is preferentially excreted with urea thereby leaving the body enriched in ^{15}N (Sutoh 1987). If a similar mechanism fractionated hydrogen isotopes during incorporation into collagen, one would expect $\delta^2H_{MW\text{-}COLL}$-$\delta^2H_{MW\text{-}THEOR}$ differences and $\delta^2H_{COLL\text{-}NEX}$-$\delta^2H_{HERB\text{-}THEOR}$ differences to correlate with $\delta^{15}N_{COLL}$. The noticeable lack of correlation suggests some other metabolic mechanism at work.

Rather than a systematic enrichment or depletion of hydrogen isotopes during collagen formation, this data may indicate a threshold of meat consumption exists beyond which the expected δ^2H-$\delta^{18}O$ relationships decouple. Pietsch et al. (2011) demonstrated that pure carnivores drinking very little water show virtually no correlation between δ^2H and $\delta^{18}O$ in hair keratin, nor do keratin and meteoric water δ^2H and $\delta^{18}O$ values correlate. Cormie et al. (1994a), Cormie et al. (1994c), Reynard and Hedges (2008), and Pietsch et al. (2011) demonstrated that strict herbivores show the opposite with strong correlations between bone δ^2H and $\delta^{18}O$ values and strong correlations between meteoric water δ^2H and $\delta^{18}O$ values. Data from omnivorous humans in this study fall somewhere in between these two end members. This supports the conclusions of Pietsch et al. (2011) that a threshold of meat consumption may exist above which animals obtain the majority of their amino acids from consumed meat and have minimal need to produce amino acids in vivo. Without in vivo amino acid production, meteoric water hydrogen will not be incorporated into $\delta^2H_{COLL\text{-}NEX}$ values either through direct water consumption or via plant tissues. The primary factor contributing to $\delta^2H_{COLL\text{-}NEX}$ will then become the baseline δ^2H value of the meat itself which has already cycled through several metabolic processes and associated fractionations. The more meat consumed, the more the δ^2H-$\delta^{18}O$ relationships deviate from the expected meteoric water line. Thus the overall $\delta^2H_{COLL\text{-}NEX}$ will show some correlation with a proxy

for meat amount consumed (i.e. $\delta^{15}N_{COLL}$), while relationships between $\delta^{2}H_{COLL\text{-}NEX}$ and $\delta^{18}O_{PHOS}$ (and calculated values based on these factors) will decouple.

This opens the question as to where the threshold lies. From the previously introduced general conceptual model, one may consider the $\delta^{2}H_{COLL\text{-}NEX}$ for mammals as follows:

Herbivores: $\delta^{2}H_{COLL\text{-}NEX} = \delta^{2}H_{ingested\ water} + \varepsilon_a$

Omnivores: $\delta^{2}H_{COLL\text{-}NEX} = (\delta^{2}H_{ingested\ water} + \varepsilon_a) + (\delta^{2}H_{dietary\ amino\ acids} + \varepsilon_b)$

Carnivores: $\delta^{2}H_{COLL\text{-}NEX} = \delta^{2}H_{dietary\ amino\ acids} + \varepsilon_b$

Herbivores and carnivores are presented here as extreme end members, which is oversimplified. Herbivores do obtain some essential amino acids from plants, but these amino acids generally have similar $\delta^{2}H$ values to local meteoric water. Most carnivores will ingest some meteoric water, albeit in very limited quantity, or it may enter the body already incorporated into food (i.e. meat). Nonetheless, the point at which one end member grades into another is unknown, but potentially useful information. Future work in feeding studies with gradations of consumed meat amount may be able to use deviations from the expected meteoric water line $\delta^{2}H$-$\delta^{18}O$ relationships to determine percentage of meat in a person's diet. The slope between $\delta^{2}H$ and $\delta^{18}O$ in keratin or collagen appears to decrease from ~8.0 to ~0.0 as animals move from pure localized herbivory to pure carnivory; likewise the slope between $\delta^{2}H$ in collagen or keratin and meteoric or drinking water $\delta^{2}H$ appears to decrease from ~1.0 to ~0.0 between herbivores and carnivores (Bowen et al. 2009, Cormie et al. 1994a, Cormie et al. 1994c, Ehleringer et al. 2008, Pietsch et al. 2011, Reynard and Hedges 2008, Topalov et al. 2013). More data is needed for bone collagen in particular. The ability to estimate percentage meat consumption would add new depth to understanding ancient lifestyles, food choice, and food availability in historic and archaeological populations.

5. CONCLUSIONS

Hydrogen isotopes in human bone collagen represent a combination of drinking water and dietary input. Using phosphate oxygen isotopes and collagen nitrogen isotopes as meteoric water and protein intake proxies, respectively, one can estimate the collagen hydrogen isotope values. This is contingent upon pooling multiple individuals from a given site to eliminate inherent variability from food choice, food availability, and movement between locations. Relationships between meteoric water oxygen isotope values and bone collagen hydrogen isotope values show deviation from the expected meteoric water line. This is hypothetically due to the minor inclusion of non-local food sources and a decoupling of oxygen/hydrogen relationships as individuals reduce in vivo amino acid production due to increased meat consumption. Further examination of this decoupling could determine percentage of dietary meat, thereby adding valuable new information into archaeological dietary studies. Currently nitrogen isotope data can provide only relative amounts of meat consumption between individuals or populations. Incorporating collagen hydrogen isotopes may facilitate absolute determinations of meat consumption, as well as adding depth to our understanding of locality and movement within ancient populations.

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FIGURE CAPTIONS

Figure 1 – Site map showing burial locations. Note individuals buried at Glorieta Pass, New Mexico, were a military unit of enlisted Texas soldiers.

Figure 2 – (A) Phosphate oxygen isotope values versus collagen non-exchangeable hydrogen isotope values. (B) Nitrogen isotope values from collagen versus non-exchangeable hydrogen isotope values from collagen. Enlarged symbols indicate site averages.

Figure 3 – Collagen hydrogen isotope values converted to meteoric water values versus phosphate oxygen isotope values converted to meteoric water values. Enlarged symbols indicate site averages.

Figure 4 – (A) Collagen non-exchangeable hydrogen isotope values converted to meteoric water values versus theoretical meteoric water hydrogen isotope values based on the meteoric water line. (B) Difference between converted values and theoretical values versus collagen nitrogen isotope values. Enlarged symbols indicate site averages.

Figure 5 – (A) Measured collagen non-exchangeable hydrogen isotope values versus theoretical hydrogen isotope values based on pure herbivory. (B) Difference between measured values and theoretical values versus collagen nitrogen isotope values. Enlarged symbols indicate site averages.

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