A rodent model of caloric restriction using bone mass, microarchitecture, and stable isotope ratios: implications for revealing chronic food insufficiency in archaeological populations

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ABSTRACT
One important question with respect to past health and disease is the identification of patterns of caloric inadequacies. Given the substantial literature (animal and human) linking caloric inadequacy to reduced bone mass, microarchitectural deterioration, and changes in stable isotope values, we utilized a rodent model to examine whether integrating these data might help discern episodes of caloric insufficiency. Bone stable isotope values and bone morphometric data were analyzed from a sample of adult male rats in a controlled feeding study. Three-dimensional micro-computed tomography revealed substantial impacts to femoral bone mass and microarchitecture among calorie-restricted animals compared to controls, and we found significant correlations between those parameters and δ13C_Paste values. Results support consideration of caloric inadequacy in differential diagnoses of bone loss within archaeological populations, and suggest that similar relationships among stable isotope signatures and bone morphometric parameters delineated within past human populations may help illuminate periods of food insufficiency.

INTRODUCTION
The pursuit of additional and better evidence of malnutrition should be a major focus of biological anthropologists and bioarchaeologists (Ortner 2008). Both malnutrition (protein and protein-calorie) and starvation are known to cause bone loss by essentially decoupling bone formation and resorption processes (Arnaud-de-la-Rosa et al. 2011; Brickley and Ives 2008; Gonzalez-Reimers et al. 2007; Ortner 2003; Shires et al. 1980). Caloric restriction has been repeatedly shown to significantly suppress bone formation and increase (albeit more variably) bone resorption in humans (Legroux-Gerot et al. 2005; Reid 2002, 2008) and rodents (Devlin et al. 2010; Tatsumi et al. 2008). The link between caloric deprivation and bone loss is clearly illustrated by individuals suffering from anorexia nervosa, as low bone mass is the most common medical complication (Fazeli and Klibanski 2014).

Given the evidence from clinical and animal studies, it follows that chronic caloric insufficiency should be considered among the possible explanations when bone loss is identified in archaeological populations. Abnormal bone loss detected among archaeological skeletal samples, however, should be considered osteopenia, as osteoporosis is best diagnosed by the presence of fracture (Brickley and Agarwal 2003; Brickley and Ives 2008; Curate 2014; White and Armelagos 1997). Assuming intravitam and postmortem (diagenetic) changes can be differentiated and a diagnosis of osteopenia can be made, Ortner (2003) suggests the probability that severe malnutrition underlies bone loss increases when age at death among affected individuals can be confidently estimated to less than 40 years. Gonzalez-Reimers et al. (2007, 555) suggest that a high incidence of osteopenia in a population “raises the possibility of protein-calorie malnutrition as the underlying cause.” Indeed, Zaki, Hussein, and Abd El-Shafy El Banna (2009), cite the high prevalence of bone loss among male workers in ancient Egypt as a possible indicator of protein-calorie malnutrition, attributing bone loss to nutritional stress. Nevertheless, while nutritional hypotheses have been frequently offered to explain the presence of osteopenia in archaeological populations, specific micronutrient deficiencies—particularly calcium—are more commonly implicated (Curate 2014; Pfeiffer and Lazenby 1994), rather than macronutrient or caloric inadequacies.

Though skeletal remains might yield evidence consistent with chronic food shortage (Roberts and Manchester 2005), “few osteologists would be likely to
claim an ability to diagnose starvation per se from skeletal remains” (Wright and White 1996, 152), where starvation includes periods of chronic food insufficiency. Thus, while attributing bone loss specifically to a prolonged period of food insufficiency or starvation may not be possible (Ortner 2003), should inadequate caloric intake be deemed a possibility, other lines of evidence that can potentially provide complimentary data to support such hypotheses should be considered. Bone stable isotope ratios may be constructive towards this end. In recent years, stable isotope ratio analysis has moved beyond diet reconstruction to become part of the tool kit for assessing physiology, nutrition, and disease in both archaeological and living human (and animal) populations (Reitsema 2013; Scorrano et al. 2014). Although the use of stable isotopes to address questions in paleopathology represents a relatively nascent effort (Richards and Montgomery 2012), it has the potential to expand both our knowledge of disease processes as well as our understanding of the relationship between metabolic processes and tissue stable isotope signatures (Katzenberg 2000). Physiological factors are known to affect how stable isotopes are integrated into body tissues (including bone) and this isotopic variation can potentially shed light on important patterns germane to human health and nutritional status (Olsen et al. 2014; Reitsema 2013).

A growing body of both animal and human studies has implicated the physiological consequences of nutritional stress (e.g. poor diet quality, food rationing, fasting, starvation) as potentially significant sources of isotopic variation (Hedges and Reynard 2007; Scorrano et al. 2014; Sears, Hatch, and O’Brien 2009; Warinner and Tuross 2010). In particular, changes in body protein and lipid balance due to caloric insufficiency have been shown to significantly affect carbon and nitrogen stable isotope ratios in both animal and human tissues (Haubert et al. 2005). Food restriction studies across a variety of animal species (see Hatch 2012; McCue and Pollock 2008 for a review) have consistently reported $^{15}$N enrichment in tissue as body proteins are recycled, as well as $^{13}$C enrichment as isotopically–lighter lipid stores are utilized (Haubert et al. 2005). In fact, blood and urine $^{15}$N signatures are increasingly utilized as markers of nutritional stress in wild populations (e.g. Deschner et al. 2012; Kempster et al. 2007; Vogel et al. 2012). Several human observational studies have also correlated tissue $^{13}$C and $^{15}$N values with reduced food intake (Fuller et al. 2005; Mekota et al. 2006; Neuberger et al. 2013). For example, Mekota et al. (2006) demonstrated among anorexia patients during starvation and recovery phases that hair $^{15}$N values varied inversely with BMI, while $^{13}$C values were positively correlated. A recent study by Eerkens et al. (2017) applied similar analyses within a bioarchaeological context, where increases in $^{15}$N across serial hair samples taken from a mummified girl were interpreted as evidence of severe undernourishment or starvation.

Despite abundant soft tissue evidence, as Reitsema (2013) points out, it is unclear how useful such isotope effects of nutritional stress would be when only mineralized tissues are available. Due to its comparatively slow turnover rate, bone should normally be “among the last tissues affected by short-term dietary change” (Katzenberg and Lovell 1999, 323), and its “time-averaged stable isotope signatures may not reveal episodic nutritional deprivation” (Reitsema 2013). Although the first and most widely cited controlled feeding study to demonstrate significant effects of severe nutritional stress on tissue stable isotope signatures (Hobson, Alisauskas, and Clark 1993) utilized bone tissue (avian), few subsequent animal studies have focused on mineralized tissues (e.g. Ambrose 2000). Few human studies have investigated possible correlations between the physiological consequences of disease or nutritional stress and changes in bone stable isotope signatures (but see Katzenberg and Lovell 1999; Olsen et al. 2014; Scorrano et al. 2014). Paleodiets and paleopathology studies necessarily rely on bone, and bone has the capacity to register variation in stable isotope ratios during times of disease and nutritional stress (Katzenberg and Lovell 1999), yet the possible physiological impacts of nutritional stress on isotopic dietary modeling (Warinner and Tuross 2010) and the potential for bone stable isotope signatures to serve as indicators of nutritional stress have not generally been considered with respect to archaeological populations (but see Arny-de-la-Rosa et al. 2011; Beaumont and Montgomery 2016; Scorrano et al. 2014; White and Armelagos 1997). The limited amount of relevant data from controlled hard tissue studies might explain much of this incongruity.

To address this dearth of data outlined above, we conducted a controlled feeding study in adult male rats to determine whether moderate, chronic food restriction (not “acute” starvation) could produce detectable and meaningful changes in $^{13}$C and $^{15}$N in adult mammalian bone – a tissue that arguably reflects long-term dietary signals (Robertson, Rowland, and Krigbaum 2014). Controlled feeding studies are often designed to maximize isotopic differences among tissues (Crowley et al. 2010) by employing severe restriction protocols (fasting/starvation) and focusing on rapidly growing individuals. As such, very few studies have investigated the effects of moderate, chronic food restriction on tissue stable isotope values (Kempster et al. 2007; Williams et al. 2007), particularly among adult animals. We chose to focus on a level of nutritional stress that would be reflective of conditions which any human population (past or present) might repeatedly experience. This is an important point, as humans possess physiological and cultural
flexibility with respect to diet, and human populations are therefore much more likely to suffer extended periods of moderate nutritional stress rather than severe, acute episodes of caloric insufficiency/starvation (Reitsema 2013).

Our published results demonstrated that moderate caloric restriction was evident in stable isotope ratios of bone – specifically, food-restricted animals exhibited a small, but statistically significant increase in $\delta^{15}N_{\text{collagen}}$ and a highly significant decrease in $\delta^{13}C_{\text{apatite}}$ compared to animals fed ad libitum (Robertson, Rowland, and Krigbaum 2014). The increase in $\delta^{15}N_{\text{collagen}}$ was deemed attributable to the recycling of body protein due to protein-calorie malnutrition, while the decrease in $\delta^{13}C_{\text{apatite}}$ was due to the utilization of less-enriched body fat stores to help meet energy needs. Although these results indicated this degree of caloric restriction does not appreciably undermine broad interpretations of dietary signals in adult mammalian bone, a significant amount of variability among individuals or groups was explained by marked differences in energy intake over variable timescales. The inverse relationship between $\delta^{13}C_{\text{apatite}}$ (decreased) and $\delta^{15}N_{\text{collagen}}$ (increased) observed suggests a more robust pattern is expected with more severe or prolonged restriction, potentially obfuscating interpretations of dietary composition and quality in some cases. The relationship between boneapatite and bone collagen $\delta^{13}C$ values further suggested this pattern could have utility as a proxy of food deprivation in certain contexts (Robertson, Rowland, and Krigbaum 2014).

In a recent study by Beaumont and Montgomery (2016), incremental changes to tooth dentine collagen $\delta^{13}C$ and $\delta^{15}N$ profiles (attributed to nutritional stress) were identified in a historical population where records from a period of famine (Great Irish Famine, 1845–1846) could be connected to the individual victims. As Reitsema (2013) notes and the above study demonstrates, the connections between stable isotope signatures and health represent a potentially useful bioarchaeological tool, particularly when interpreted in conjunction with data from both paleopathology and the archaeological record. With respect to the present study, both isotopic and morphometric analyses such as bone mass and quality measures can help address questions of individual health and disease in archaeological populations by providing evidence of dietary adequacy versus stress, however, combining both types of data is less routine. Considering the evidence from animal and human studies linking caloric inadequacy to reduced bone mass, compromised microarchitecture, and changes in isotopic values, the present study was designed to investigate the potential of combining bone stable isotope and bone morphometric data sets for revealing evidence of malnutrition – specifically caloric insufficiency.

In this study, we examine the relationship between the bone stable isotope signatures recently characterized in our rodent model of caloric restriction (Robertson, Rowland, and Krigbaum 2014) and the bone morphometric data from a subset of the same animals. Femora were scanned using micro-computed tomography ($\mu$CT) to determine whether the restriction protocol resulted in significant decreases in bone mass and changes in microarchitecture that might be congruent with previously reported (Robertson, Rowland, and Krigbaum 2014) differences in bone stable isotope ratios. Such congruent lines of evidence for caloric inadequacy in an adult mammalian model may suggest its utility for discerning episodes of food inadequacy in archaeological populations.

Based on earlier studies (e.g. Devlin et al. 2010; Tatsumi et al. 2008), we expected food-restricted animals to exhibit robust decreases in bone quantity and evidence of structural (microarchitectural) deterioration compared to controls. Although the impacts to bone stable isotope ratios previously reported were modest (particularly with respect to $\delta^{15}N_{\text{collagen}}$ values), we anticipated significant correlations with bone morphometric data. We hypothesized that higher $\delta^{15}N_{\text{collagen}}$ and lower $\delta^{13}C_{\text{apatite}}$ values (as among rationed animals) would correspond to lower bone quantity and structural measures, while lower $\delta^{15}N_{\text{collagen}}$ and higher $\delta^{13}C_{\text{apatite}}$ values (as among controls) would correspond to increased bone quantity and structural integrity measures.

**Materials and methods**

**Experimental design:** 26 male Sprague Dawley rats were obtained from Harlan laboratories (now Envigo; Indianapolis, IN) at 8 weeks of age and assigned to one of four groups balanced for initial body weight: 1) ad lib 90-day ($N = 6$), 2) rationed 90-day ($N = 7$), 3) ad lib 180-day ($N = 6$), and 4) rationed 180-day ($N = 7$). Rats were housed singly and received water ad libitum and Harlan-Teklad rodent diet #2918 per group assignment. Rationing began at 10 weeks of age (day 0), with restricted animals receiving a ration equivalent to 45% of the averaged daily ad libitum intakes (calculated weekly). At 90 or 180 days, animals were deeply anesthetized under isoflurane to facilitate the collection of blood samples for subsequent physiological analyses (including bone turnover assays) and ultimately to achieve euthanasia. Left and right femora were excised and frozen for later stable isotope and $\mu$CT analyses. While femora from the entire cohort ($N = 26$) were utilized for stable isotope ratio analysis (see Robertson, Rowland, and Krigbaum 2014), a subsample of 12 femora (3 per group) was scanned by $\mu$CT prior to isotopic analysis. To increase statistical power, all data (isotopic and morphometric) from the 90 and 180 day groups within each feeding condition were...
combined (i.e. \( N = 6 \) ad lib and \( N = 6 \) rationed), as statistical analyses of the entire cohort demonstrated no isotopic effects by time (Robertson, Rowland, and Krigbaum 2014). Additional protocol details (e.g. anatomical and physiological measures/assays) are described in Robertson, Rowland, and Krigbaum 2014.

\textit{µCT Analysis of Bone Morphometry:} A subsample of femora (\( N = 12; 6 \) per feeding condition) was scanned by \( \mu \)CT using a high-resolution Bruker Skyscan 1172 (Kontich, Belgium). Cancellous bone quantity and microarchitecture were assessed at the right distal femoral metaphysis and cortical bone morphometric data were collected at both the femoral diaphysis and the distal femur (metaphysis). The following parameters were used for image acquisition: 80kVP/120 \( \mu \)A with a 0.5 mm aluminum filter, 1k camera resolution, 19.2 \( \mu \)m voxel size, 0.7\(^\circ\) rotation step, and 180\(^\circ\) tomographic rotation. The cancellous region of interest (ROI) began 1.5 mm proximal to the distal femoral growth plate and encompassed 10% of the total femoral length. The cortical diaphysis ROI encompassed 5% of the total bone length surrounding the femoral midshaft and avoided the third trochanter. The distal cortical ROI began at 22.5% of the bone length from the distal femur and continued for 5% of the bone length. Cross-sectional images were reconstructed using a filtered back-projection algorithm (NRecon, Kontich, Belgium). 2D and 3D morphometric measurements were calculated using CTan software (version #1.13.1.1, Bruker Skyscan, Kontich, Belgium) and included: tissue (medullary) volume (TV, \( \text{mm}^3 \)), cancellous bone mass (Ct.B.M, \( \text{mm}^3 \)), percent cancellous bone volume (cBV/TV, \%), trabecular number (Tb.N, \#/mm), trabecular thickness (Tb.Th, mm), trabecular separation (Tb.Sp, mm), structure model index (SMI), and trabecular pattern factor (Tb.Pf, \( \text{mm}^{-1} \)) within the cancellous ROI. Measures within the femoral diaphysis and distal cortical ROIs included: total cross-sectional area inside the periosteal envelope (Tt.Ar, \( \text{mm}^2 \)), cortical bone area (Ct.Ar, \( \text{mm}^2 \)), medullary area (Ma.Ar, \( \text{mm}^2 \)), cortical area fraction (Ct.AR/Tt.Ar, \%), and average cortical thickness (Ct.Th, mm). Medullary volumetric (v) BMD (cancellous bone only) and cortical tissue mineral density (TMD) were also evaluated within the distal femoral metaphysis and diaphysis ROIs, respectively, following calibration with hydroxyapatite phantoms. The \( \mu \)CT methods and nomenclature used are standard in our laboratory (Yarrow et al. 2010).

\textit{Stable Isotope Analyses:} Femoral diaphyses were mechanically and chemically prepared to isolate bone collagen and apatite fractions (see Robertson, Rowland, and Krigbaum 2014). \( \delta^{13} \)C\textsubscript{collagen} and \( \delta^{15} \)N\textsubscript{collagen} was determined with a Delta V Advantage isotope ratio mass spectrometer coupled with a ConFlo IV interface and a Carlo Erba NA 1500 CNS Elemental Analyzer (Thermo Scientific, Sunnyvale, CA, USA). \( \delta^{13} \)C\textsubscript{apatite} was assayed with a Finnigan MAT 252 isotope ratio mass spectrometer coupled to a Kiel carbonate preparation device (Thermo Scientific, Sunnyvale, CA, USA).

\textit{Statistical Analyses:} \( T \)-tests were conducted to confirm that there were no significant differences in relevant anatomical or physiological parameters between the subset of animals chosen for \( \mu \)CT evaluation and the larger (original) experimental cohort. \( T \)-tests or Mann–Whitney rank sum tests (as appropriate) were used to determine whether differences in bone morphometry existed between the \textit{ad libitum} and rationed conditions within the subset. Relationships between stable isotope and bone morphometric data sets were evaluated using Pearson product moment correlations and post-hoc Holm–Bonferroni corrections. All analyses were performed using SigmaPlot (ver. 12.5) with a \( p \)-value less than 0.05 considered significant.

\textbf{Results}

\textit{Anatomical and Physiological Measures:} Anatomical and physiological data are presented in Table 1. The subset of animals (\( N = 12 \)) for which bone morphometric characteristics were examined is representative of the broader sample (\( N = 26 \); Robertson, Rowland, and Krigbaum 2014) from which it was taken. For all measures, subset and cohort group means were not significantly different (\( P = 0.390 \) to 1.00, \textit{t}-tests or Mann–Whitney rank sum tests as appropriate). Final body weights within the study subset (Table 1) differed substantially across feeding levels (\( P < 0.002 \)): rationed animals lost an average of 13% of their initial (day 0) body weight while those fed ad lib all attained weights in excess of 150% baseline (day 0) values. Maximal body lengths among control animals were approximately 14% longer than food-restricted animals (\( P < 0.001 \), Table 1). Control rats had heavier (~27%); \( P = 0.002 \) and longer (~11%; \( P = 0.002 \)) femora than rationed animals (Table 1).

Circulating concentrations of osteocalcin and tartrate-resistant acid phosphatase (TRACP 5b) were assayed via commercially available enzyme-linked immunosorbent assays (ELISA), as specific markers of bone formation and bone resorption, respectively. Mean values (subset) obtained for these measures indicate bone turnover was markedly reduced in calorie-restricted rats at time of sacrifice, as both osteocalcin and TRACP 5b levels were significantly (\( P < 0.001 \) and \( P = 0.004 \), respectively) lower in rationed animals compared to controls. Temporal data for these markers among the entire cohort (see Robertson, Rowland, and Krigbaum 2014) indicate the caloric restriction protocol severely inhibited the normal aging-induced
alterations in bone formation and caused a decoupling of bone formation and resorption processes, resulting in net bone loss among rationed animals. These observations are consistent with results of calorie-restriction studies in mice which indicated that reduced long bone mass is primarily attributable to decreased bone formation, although increases in bone resorption markers which decline with age have been documented (Devlin et al. 2010; Tatsumi et al. 2008).

**μCT characterization of cancellous morphometry**

Compared to those fed ad lib, mean medullary tissue volumes (TV) at the distal femoral ROI were significantly lower among calorie-restricted animals (P = 0.002, Table 2). As the femora of rationed animals were shorter (and lighter) than those of control animals (Table 1), some allometric difference in TV was expected. Cancellous bone volume (BV) among food-restricted animals was roughly one third that of controls (P < 0.001). μCT analysis indicated that percent cancellous bone volume (BV/TV) was also significantly (P < 0.001) lower among rationed animals compared to controls (Table 2). The large difference in BV/TV between feeding levels is particularly compelling, as it reveals a significant disparity in bone volume beyond that attributable to allometry. The 3D μCT images in Figure 1 (lower panel) illustrate the comparatively low cancellous bone volume observed among rationed animals.

The low BV/TV among rationed animals was characterized by a 47% decrease in trabecular number (P < 0.001), a 13% decrease in trabecular thickness (P < 0.001), and a 25% increase in trabecular separation (P = 0.027) compared to ad lib-fed (Table 2). These results indicate that our food restriction protocol elicited a much more robust effect on Tb.N than Tb.Th. Compared to humans, rodents appear to have thicker and fewer trabeculae, possibly because mechanically functional trabeculae are governed by a minimum thickness threshold (Barak, Lieberman, and Hublin 2013). Consequently, Barak and colleagues contend that differences in BV/TV in rodents are largely achieved by variation with Tb.N rather than with Tb.Th (the main factor underlying differences in BV/TV in humans); our results are consistent with this hypothesis.

Trabecular bone stability is dependent upon the amount of bone tissue present, along with trabecular microarchitecture (which includes both 3-dimensional orientation and connectedness of trabeculae; Hahn et al. 1992). Trabecular pattern factor (Tb.Pf) is an inverse measure of trabecular network connectivity, which describes the relative convexity or concavity of the total bone surface. A large number of concave surfaces indicates a well-connected trabecular lattice (low Tb.Pf values), whereas numerous convex surfaces (high Tb.Pf values) represent the presence of isolated, disconnected structures (struts) and a weaker cancellous network (Hahn et al. 1992). Thus, the high Tb.Pf values among rationed animals compared to controls (P < 0.001, Table 2) suggest substantial degradation of trabecular structure during the course of the restriction period. SMI is a morphometric parameter which quantifies the character of trabecular bone structure in terms of plate-like vs rod-like elements (Hildebrand and Rüegsegger 1997), where perfect plates and rods are designated as 0 and 3, respectively (Bouxsein et al. 2010). A transition from plate-like to rod-like elements characterizes the osteoporotic deterioration of cancellous bone structure (Hildebrand and Rüegsegger 1997). Mean SMI values differed significantly across feeding levels, with higher values among rationed animals reflecting a greater prevalence of rod-like elements and (presumably) weaker cancellous structure than the ad lib-fed sample (P < 0.001, Table 2).

As medullary volumetric bone mineral density (vBMD) relates to the amount of bone within a mixed bone/soft tissue region (i.e. cancellous bone),
this parameter does not provide information about the material density of the bone itself. Differences in mean vBMD values across feeding conditions, not surprisingly, echoed the significant differences found in BV/TV (described above). Mean vBMD for ad lib-fed animals was nearly double that of rationed animals ($P < 0.001$). Collectively, the data gleaned from bone turnover assays, BMD evaluation, and the assessment of multiple trabecular morphometric parameters clearly indicate a substantial loss of cancellous bone and a deterioration of trabecular integrity (microarchitecture) among calorie-restricted animals compared to controls.

**µCT characterization of cortical morphometry**

Differences in cortical parameters across feeding conditions were less robust in comparison to trabecular disparities. This was expected, as cancellous bone is metabolically more active and generally exhibits much quicker turnover than cortical bone (Parfitt 2003). Consequently, initial bone loss should be more severe within trabecular bone (Grynpas 2003; Ives and Brickley 2005). Additionally, the lack of a well-developed Haversian remodeling system in rats (Lelovas et al. 2008) limits the comparability of cortical bone loss between humans and rodents. Nevertheless, significant differences between rationed and control animals were identified (at both ROIs) for most cortical parameters examined.

Rationed animals demonstrated lower mean total cross-sectional area (Tt.Ar) than controls at both ROIs (Table 3), approaching significance at the distal femur ($P = 0.065$), and significant at the diaphysis ($P = 0.002$). As with TV, mean Tt.Ar values among treatment groups varied allometrically reflected by differences in femur length and mass (Table 1). Cortical bone area (Ct.Ar) among food-restricted animals was approximately 25% less than those fed ad lib ($P < 0.001$) at both bone regions (Table 3). Medullary (marrow) area (Ma.Ar) did not differ across treatments at either ROI. Although the differences were modest, cortical area fraction (Ct.Ar/Tt.Ar) at both cortical bone areas was significantly lower among rationed animals compared to controls ($P = 0.041$ and $P = 0.012$, Table 3). The significant difference in Ct.Ar/Tt.Ar between feeding levels (like that of BV/TV) represents a non-allometric disparity. Average cortical thickness (Ct.Th) among food-restricted animals was approximately 17% lower than ad lib-fed ($P = 0.003$) at the femoral diaphysis and approximately 12% lower ($P < 0.001$) at the distal femur (Table 3). The 3D µCT images in Figure 1 (top panel) illustrate the difference in cortical thickness across feeding levels.

Although µCT has been mainly used to investigate bone structure, it can also be used to estimate cortical TMD (Bouxsein et al. 2010). TMD is a density measurement which excludes surrounding soft tissue and is thus confined to within the volume of calcified bone tissue. In contrast to BMD, the assessment of cortical bone TMD provides information regarding the material density of the bone itself. Mean TMD values (femoral diaphysis only) for ad lib-fed and rationed animals were nearly identical (1.279 vs 1.281 g/cm$^3$). These data suggest that differences in morphometric parameters across feeding levels are the result of less bone rather than the presence of poorly mineralized bone among calorie-restricted animals.

**Stable isotope analyses**

Stable isotope analysis of the original ($N = 26$) femoral sample revealed a small but significant difference in $\delta^{15}N_{\text{collagen}}$ between feeding levels ($P = 0.028$), where food-restricted groups were $^{15}$N enriched compared to controls ($5.45 \pm 0.03$ % vs $5.33 \pm 0.03$ %). The
higher $\delta^{15}$N of the rationed animals is consistent with numerous controlled food restriction studies across a variety of species, tissues, and protocols, and probably reflects body tissue proteolysis and amino acid recycling resulting from protein-calorie stress–induced negative nitrogen balance (Robertson, Rowland, and Krigbaum 2014). This difference in $\delta^{15}$N$_{collagen}$ across treatments within the original cohort, however, was no longer significant after re-analysis (t-test) to include only the subset ($N = 12$) of animals utilized for μCT (5.45 ± 0.07 ‰ vs 5.36 ± 0.04 ‰, $P = 0.329$).

$\delta^{13}$C$_{apatite}$ within the original cohort also varied significantly across feeding levels, but this difference was more robust than that observed with $\delta^{15}$N$_{collagen}$. Rationed animals demonstrated significantly lower $\delta^{13}$C$_{apatite}$ compared to ad lib-fed animals ($-12.87 \pm 0.04$ ‰ vs $-12.57 \pm 0.04$ ‰, $P < 0.001$). Because the $\delta^{13}$C of lipids are characteristically lower than those of carbohydrates and proteins within an organism, and body fat is thus depleted in $^{13}$C compared to other tissues (Lee-Thorp, Sealey, and van der Merwe 1989; Neuberger et al. 2013), the lower $\delta^{13}$C$_{apatite}$ among the calorie-restricted animals is likely attributable to the utilization of body fat stores to meet energy needs (Robertson, Rowland, and Krigbaum 2014). The difference in $\delta^{13}$C$_{apatite}$ between rationed and ad lib-fed animals was significant within the 12-animal subset ($-12.85 \pm 0.04$ ‰ vs $-12.60 \pm 0.03$ ‰, $P < 0.001$).

$\delta^{13}$C$_{collagen}$ values did not differ across feeding levels within the original cohort (and were not evaluated for the μCT subset). The assimilation of $^{13}$C-depleted body fat should decrease $\delta^{13}$C$_{apatite}$ without affecting $\delta^{13}$C$_{collagen}$ values due to dietary routing (Crowley et al. 2010). Static $\delta^{13}$C$_{collagen}$ values recorded across feeding levels suggest that macronutrient routing was not altered as a result of caloric restriction (Robertson, Rowland, and Krigbaum 2014).

### Discussion

#### General

While a substantial body of work aimed at discerning past diets using stable isotope ratio analysis has accumulated in the past few decades, remarkably few studies (e.g. Scorrano et al. 2014) have specifically compared stable isotope and paleopathological data (Richards and Montgomery 2012). Further, although numerous studies have investigated bone loss among archaeological populations, only a handful (e.g. Arnay-de-la-Rosa et al. 2011) have explicitly evaluated potential relationships between bone stable isotope signatures and bone loss. For example, in their study of

### Table 3. μCT characterization of cortical morphometry at femoral diaphysis and femoral metaphysis for ad libitum-fed (Ad lib) and food-restricted (Ration) rats

| Parameter                  | Ad lib (N = 6) | Ration (N = 6) | t-test   | Mann-Whitney |
|---------------------------|---------------|---------------|----------|--------------|
| **Femoral Diaphysis**     |               |               |          |              |
| TLAr (mm$^2$)             | 12.78 ± 0.44  | 10.41 ± 0.11  | (<0.001) | 0.002        |
| ClAr (mm$^2$)             | 8.01 ± 0.32   | 6.05 ± 0.12   | (<0.001) |              |
| Ma.Ar (mm$^2$)            | 4.77 ± 0.30   | 4.36 ± 0.13   | (n.s.)   |              |
| Cl.Ar/TL.Ar (%)           | 0.627 ± 0.016 | 0.582 ± 0.011 | 0.041    |              |
| Ct.Th (mm)                | 0.813 ± 0.023 | 0.677 ± 0.016 | 0.003    |              |
| **Distal Femoral Metaphysis** |          |               |          |              |
| TL.Ar (mm$^2$)            | 16.70 ± 0.66  | 14.70 ± 0.22  | (0.016)  | 0.065        |
| Cl.Ar (mm$^2$)            | 7.22 ± 0.20   | 5.50 ± 0.11   | (<0.001) |              |
| Ma.Ar (mm$^2$)            | 9.48 ± 0.66   | 9.20 ± 0.29   | (n.s.)   |              |
| Cl.Ar/TL.Ar (%)           | 0.435 ± 0.020 | 0.375 ± 0.011 | 0.012    |              |
| Ct.Th (mm)                | 0.547 ± 0.019 | 0.447 ± 0.010 | (<0.001)|              |

Group means ± SEM are reported. Values at femoral diaphysis are followed by values at distal femur (metaphysis). Abbreviations: TL.Ar = total cross-sectional area; Cl.Ar = cortical bone area; Ma.Ar = medullary area; Cl.Ar/TL.Ar = cortical area fraction; Ct.Th = average cortical thickness. P-values indicate significant differences between feeding levels; n.s. = non-significant.

#### Relationships between stable isotope and bone morphometric data sets

As $\delta^{15}$N$_{collagen}$ between rationed and ad lib-fed animals did not differ significantly among the 12-animal subset, there was, of course, no possibility of any significant relationship between these values and any bone morphometric data collected from these animals. In contrast, unadjusted Pearson correlations revealed significant relationships between $\delta^{13}$C$_{apatite}$ values and all trabecular measures, as well as select cortical parameters.

Strong positive correlations between $\delta^{13}$C$_{apatite}$ values and BV ($r = 0.832$, $P = 0.0008$), BV/TV% ($r = 0.863$, $P = 0.0003$), Tb.N ($r = 0.878$, $P = 0.0002$), BMD ($r = 0.876$, $P = 0.0002$) and Tb.Th ($r = 0.759$, $P = 0.004$), as well as robust negative correlations with Tb.Pf ($r = -0.866$, $P = 0.0003$) and SMI ($r = -0.874$, $P = 0.0002$), serve to illuminate the concordant effects of the restriction protocol among these data sets. Less robust correlations between $\delta^{13}$C$_{apatite}$ values and TV ($r = 0.6363$, $P = 0.026$) and Tb.Sp ($r = -0.607$, $P = 0.036$), although not significant after post-hoc Holm–Bonferroni correction, provide additional support for the concomitant effects of feeding level on bone stable isotope ratios and morphometric values.

As differences in cortical morphometry across feeding conditions were generally less pronounced than differences in trabecular morphometry, few significant relationships between $\delta^{13}$C$_{apatite}$ values and cortical measures were identified, none of which were significant post-hoc. For both cortical ROIs (femoral diaphysis and metaphysis), neither Cl.Ar/TL.Ar nor Ct.Th were significantly correlated with $\delta^{13}$C$_{apatite}$ values and both Tt.Ar and Ct.Ar at the femoral diaphysis ($r = 0.659$, $P = 0.02$ and $r = 0.625$, $P = 0.03$, respectively) and the distal femoral metaphysis ($r = 0.625$, $P = 0.03$ and $r = 0.644$, $P = 0.024$, respectively) were identified, but were not significant after post-hoc (Holm–Bonferroni) correction.
X-group Nubian mummies, White and Armelagos (1997) examined the relationship between osteopenia and stable isotope ratios. Females with osteopenia (particularly those estimated be in their third and fifth decades) exhibited significantly elevated $\delta^{15}$N_{collagen} compared to those females in their sample with normal bone mass. This increase in $\delta^{15}$N_{collagen} was attributed to differences in urea nitrogen excretion, although the authors cite multiple factors which could potentially explain this correlation between osteopenia and $\delta^{15}$N. In fact, with respect to the premenopausal women in the population, protein-calorie malnutrition was specifically suggested, as the clinical features characterizing the osteopenia, determined histologically, were consistent with those of juvenile osteopenia - a condition often associated with this type of dietary stress.

More recently, Armay-de-la-Rosa et al. (2011) conducted a paleonutritional and paleodietary assessment of individuals buried at Las Cañadas del Teide (Tenerife, Canary Islands) which included both chemical and histomorphometric bone analyses. Although significant differences in stable isotope ratios detected between individuals buried before and after the Spanish conquest were ultimately attributed to dietary differences, low trabecular bone mass (compared to modern controls) among several individuals within the pre-conquest group prompted the researchers to consider the possibility that malnutrition might, in part, underlie differences in stable isotope signatures among these groups. Though this "dietary stress" hypothesis was not supported (no relationship between trabecular bone mass and stable isotope ratios was observed), this study represents one of the few attempts to expressly integrate bone histomorphometric and stable isotope data as possible evidence for food insufficiency.

The paucity of such comparative studies may partly stem from concerns regarding their utility, given inherent physiological correlates which underlie signal detection and amplitude among different types of data. Whether and to what extent both bone mass/microarchitecture and stable isotope ratios might be affected by an episode of caloric inadequacy is, we suggest, presumably influenced by the severity and duration of such an event. Controlled feeding studies using various animal models indicate there are, in fact, "threshold" levels of nutritional stress below which bone isotopic changes are inconsequential (e.g. Ambrose 2000; Kempster et al. 2007), and above which changes become detectable (e.g. Hobson, Alisauskas, and Clark 1993; Robertson, Rowland, and Krigbaum 2014). Though their nature and scope may differ from those governing isotopic changes, such thresholds should similarly apply to changes among bone morphometric parameters. Threshold disparities across the two types of measures may partly explain why the impacts to bone mass and microarchitecture reported in this study were generally much more considerable than those registered isotopically.

In addition to the level or degree of nutritional stress, there are also temporal differentials to consider. For example, individuals experiencing conditions of extreme (i.e. acute) starvation would likely die before any difference in bone stable isotope signatures could be detected (Richards and Montgomery 2012). In contrast, the results of our study demonstrate that effects of moderate, chronic caloric restriction can be recorded concurrently in both bone stable isotope signatures and bone morphometric measures. Differences in signal magnitude, both within and between the two data sets, however, ostensibly reflect aspects of "temporal" variability related to bone turnover. Indeed, while the restriction protocol negatively affected both cancellous and cortical bone morphometry, more severe declines among trabecular measures were documented because cancellous bone turns over more quickly than cortical bone. Further, bone loss and structural deterioration likely accumulated more quickly than alterations to stable isotope ratios in part because bone morphometric parameters are influenced immediately and additively by the imbalance between bone formation and bone resorption processes, while stable isotope ratios reflect time-averaged signatures that are essentially limited by the rate of new bone formation (which is markedly suppressed under conditions of caloric insufficiency).

Diagenetic concerns may also account for the dearth of studies focused on distinguishing relationships between stable isotope ratios and bone loss in past human populations. As the mineral portion of bone is vulnerable to both chemical and structural postmortem alterations, diagenetic affects to bone apatite ($\delta^{13}$C_{apatite}) are important considerations to assess for any archaeological sample studied with these questions in mind (e.g. Brickley and Agarwal 2003; Mays 2000). As Schultz (2003) cautions, microscopic examination of thin ground sections is often required to differentiate features of intravital and postmortem bone loss and successfully interpret porotic bone structures. Although such post-depositional processes are not an issue in a controlled study such as ours, the application of $\mu$CT/image analysis to archaeological bone would help to address these concerns, as this method characterizes actual bone microstructure and various microarchitectural measures in addition to BMD. The use of $\mu$CT for examining archaeological bone offers other advantages as well. For example, unlike the more commonly utilized method for assessing bone loss within past populations (dual energy X-ray absorptiometry or DEXA), $\mu$CT is not influenced by the absence of soft tissue. In fact, no special sample preparation is required, the procedure is non-destructive (Rühli et al. 2007), and specialized instruments are increasingly available at larger research institutions.
Thus, as Brickley and Agarwal (2003) suggest, µCT should provide an ideal, non-invasive means to quantify bone loss and microarchitecture in archaeological populations.

**Limitations**

The small size of the femoral subset utilized for µCT is an important limitation of this study. As this evaluation of bone morphometry was designed as a pilot study, group size was necessarily restricted. Had the femora from the entire cohort been scanned prior to processing for stable isotopic assays, the statistical power of our analyses would have been more robust. With group sizes approximately doubled, additional morphometric parameters may have demonstrated significant differences between feeding levels, and possible correlations between $\delta^{13}\text{C}_{\text{cholesterol}}$ values and bone morphometric data might have been assessed. Regardless, statistical differences were noted among groups (ad lib vs. restricted) for all cancellous structural variables assessed and for many cortical bone outcomes.

A lack of dietary quality can be as important as a lack of calories, and the two often co-occur (Roberts and Manchester 2005). As we did not provide calcium supplementation to the rationed animals, and adequate calcium intake is essential for maintaining bone health, we must address the possibility that the differences in bone volume and microstructure between ad lib-fed and rationed groups might be related to calcium deficiency. Because the diet utilized in our study is designed to support gestation, lactation, and growth (vendor data), it contains very high levels of micronutrients, including 1% calcium (Ca). Since rodent diets providing 0.5% Ca are widely recommended (Devlin et al. 2010; Seto et al. 1999), and bone parameters (e.g. mass, biomechanical properties) appear unimpaired at 0.3% Ca (Hunt et al. 2008), the rationed animals in our study should have ingested levels of calcium sufficient for proper bone maintenance. Further, in a recent caloric restriction study in mice, calcium supplementation failed to reverse the reduction in bone mass induced by the restriction protocol (Tatsumi et al. 2008). Importantly, decreased bone mass resulting from food deprivation has been repeatedly characterized as osteoporotic rather than osteomalacic (Shires et al. 1980). Comparable mean TMD values between ad lib-fed and rationed groups in our study are in line with this characterization, as they point to decreased bone mass rather than the presence of poorly mineralized bone among calorie-restricted animals.

Although physical activity levels influence bone microarchitecture in rodents and reduced activity precedes age-induced bone loss (Hamrick et al. 2006), we did not quantitatively evaluate activity levels during the course of this study. We find it unlikely, however, that our calorie restriction protocol negatively influenced spontaneous physical activity, as studies indicate both humans (Martin et al. 2007) and non-human primates (Yamada et al. 2013, 2017) undergoing 4–6 months of caloric restriction (without malnutrition) have similar or higher spontaneous activity levels compared to controls undergoing caloric maintenance.

Finally, though the laboratory rat is by far the preferred animal model in osteoporosis research, there are limits to its congruity with the human condition. The difference most germane to this study is the lack of a well-developed Haversian remodeling system in the rat (Lelovas et al. 2008) which presumably limits the comparability of cortical bone loss among species. For example, periosteal bone gain and endocortical bone loss primarily dictate cortical bone remodeling in the rat skeleton (Lelovas et al. 2008). As such, cortical bone loss in our rodent model of caloric restriction differs mechanistically from human cortical bone loss in response to caloric restriction. Additionally, differences in bone mass and microarchitecture were observed within the temporal constraints of the study because rat bone turns over more quickly than human bone; a longer time frame would presumably be required for similar outcomes to be detected within a human sample.

**Future directions**

Although the femora for the entire original cohort have all been utilized, we are currently processing humeral samples for compound specific stable isotope analyses. $\delta^{13}\text{C}_{\text{cholesterol}}$ values of bone cholesterol compliment those of collagen and apatite (Stott et al. 1999). For example, like $\delta^{13}\text{C}_{\text{apatite}}$, $\delta^{13}\text{C}_{\text{cholesterol}}$ have been shown to reflect whole diet, however, unlike $\delta^{13}\text{C}_{\text{apatite}}$, $\delta^{13}\text{C}_{\text{cholesterol}}$ are unlikely to be affected by diagenetic alteration (Jim, Ambrose, and Evershed 2004). Additionally, as the turnover rate of cholesterol is faster than that of collagen, cholesterol analyses offer a means of investigating $\delta^{13}\text{C}$ within shorter time scales (Stott et al. 1999). Isotopic analysis of bone cholesterol could potentially offer support for the data reported here, and help better clarify its application to human bone tissue.

**Conclusions**

The results of our study clearly demonstrate that the food restriction protocol employed in this animal model markedly decreased bone mass and bone structural integrity as determined by µCT. These data are consistent with other literature (human and animal) linking food restriction and bone loss, thereby offering further support for the incorporation of caloric inadequacy within differential diagnoses of bone loss in archaeological populations. Results also indicate that
moderate, chronic caloric insufficiency can concurrently impact both bone morphometric and isotopic data (albeit to different degrees) under time-limited conditions. Where significant differences in stable isotope ratios between ad lib-fed and rationed animals were previously defined (i.e. $\delta^{13}$C$_{apatite}$), significant correlations between these and numerous bone morphometric parameters were identified. Despite the limitations outlined above, the congruent data revealed in this rodent model of caloric restriction suggest that similar relationships between bone stable isotope ratios and bone morphometric data delineated within archaeological populations may have the potential to help discern periods of food insufficiency in the past. Although more work needs to be done to further characterize such relationships, our results suggest they could represent a potentially powerful tool, particularly when interpreted in combination with other paleopathological, archaeological, and historical data, and should thus be routinely evaluated.

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