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Osteocyte Lacuna Population Densities in Sheep, Elk and Horse Calcanei

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Key Words
Artiodactyl calcaneus · Bone adaptation · Osteocyte · Osteocyte lacunae · Osteons

Abstract
Osteocytes, the most prevalent cell type in bone, appear to communicate via gap junctions. In limb-bone diaphyses, it has been hypothesized that these cellular networks have the capacity to monitor habitual strains, which can differ significantly between cortical locations of the same bone. Regional differences in microdamage associated with prevalent/predominant strain mode (tension, compression, or shear) and/or magnitude may represent an important 'variable' detected by this network. This hypothesis was indirectly addressed by examining bones subjected to habitual bending for correlations of osteocyte lacuna population densities (n/mm^2 bone area, Ot.Lc.N/B.Ar) with locations experiencing high and low strain, and/or prevalent/predominant tension, compression, and shear. We examined dorsal ('compression'), plantar ('tension'), and medial/lateral ('shear' or neutral axis) cortices of mid-diaphyseal sections of calcanei of adult sheep, elk, and horses. Ot.Lc.N/B.Ar data, quantified in backscattered electron images, were also evaluated in a context of various additional structural and material variables (e.g. % ash, cortical thickness, porosity, and secondary osteon population). Results showed significant differences in dorsal versus plantar comparisons with the highest Ot.Lc.N/B.Ar in dorsal cortices of sheep and elk ($p < 0.0001$); but this was a statistical trend in the equine calcanei ($p = 0.14$). There were no consistent transcortical (pericortical to endocortical) differences, and Ot.Lc.N/B.Ar in neutral axes was not consistently different from dorsal/plantar cortices. Correlations of Ot.Lc.N/B.Ar with structural and

Abbreviations used in this paper

Ar	area
CFO	predominant collagen fiber orientation
D	dorsal
En	endocortical aspect of the cortex
L	lateral
Lc-Lc	estimated lacuna-lacuna distance (μm)
M	medial
Mi	middle aspect of the cortex
MC3s	third metacarpals
On	osteons
On.Ar	mean area of complete secondary osteons
On.Ar/T.Ar	fractional area of secondary osteonal bone × 100 (%, includes central canals in total area)
On.N/T.Ar	secondary osteon population density (n)/total area (mm^2), includes central canals in total area
Ot.Lc.N/B.Ar	osteocyte lacuna population density (n)/bone area (mm^2), excludes central canals
Pe	pericortical aspect of the cortex
P	plantar
T	total

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material parameters were also poor and/or inconsistent within or between species. These results provide little or no evidence that the number of osteocyte lacunae has a functional role in mechanotransduction pathways that are typically considered in bone adaptation. Although dorsal/plantar differences may be adaptations for prevalent/predominant strain modes and/or associated microdamage, it is also plausible that they are strongly influenced by differences in the bone formation rates that produced the tissue in these locations.

Introduction

Results of a companion study of osteocyte lacuna population densities per bone area (Ot.Lc.N/B.Ar, n/mm^2) in multiple locations of mid-diaphyseal equine radii and third metacarpals (MC3s) showed poor correlations with many mechano-biological and morphological characteristics, including variations in strain magnitude/mode, marrow proximity, cortical thickness, mineral content (% ash), porosity, secondary osteon population densities, and mean osteon cross-sectional areas [Skedros et al., 2005]. These data also reject the hypothesis that osteocytes might be distributed in a manner reflecting their putative role as 'operational' networks that sense microdamage, which can differ between 'tension' and 'compression' areas in formation frequency and/or other characteristics (e.g. differences in microcrack size and shape). This is because differences in Ot.Lc.N/B.Ar, although statistically significant between the 'tension' and 'compression' areas, were *opposite* in these two equine bones (i.e. relatively higher Ot.Lc.N/B.Ar in the 'compression' area of equine radii, and relatively lower Ot.Lc.N/B.Ar in the 'compression' area of equine MC3s). However, it is possible that the habitual loading environment of the MC3s is sufficiently complex to obscure tension versus compression Ot.Lc.N/B.Ar differences. This complexity is most obvious in the equine MC3 when increased torsional stresses associated with fast gait speeds cause the neutral axis to rotate 30–45° [Gross et al., 1992; Skedros et al., 1996; Nunamaker, 2001]. It is possible that correlations of Ot.Lc.N/B.Ar with regional strain, histology and/or other morphologic or mechano-biological characteristics might be consistent and significant in other bones that are relatively more simply loaded. This possibility appears to be supported by data in calcanei of mature wild deer showing significantly greater Ot.Lc.N/B.Ar in the dorsal ('compression') cortex than the 'tension' (plantar) cortex [Skedros et al., 2004]. A goal of the present study was to determine if this relationship in deer calcanei and equine radii is consistent in similarly loaded regions of other bones. Additionally we further evaluate relationships of Ot.Lc.N/B.Ar with characteristics of habitual strain environments, and regional metabolism/remodeling-related activities. This was accomplished by examining Ot.Lc.N/B.Ar in calcanei of mature sheep, elk, and horses.

Cortices within the same mid-diaphyseal transverse cross-sections of these calcanei have marked structural and material heterogeneity that is correlated with a habitual dorsal/plantar ('compression/tension') strain distribution produced during functional loading typical of weight-bearing activities [Su et al., 1999] (fig. 1). It has been hypothesized that specific features of the non-uniform strain distributions of these bones are important causal influences in the attainment of this heterogeneous construction [Skedros et al., 2001a, 2004]. These features include, among others, strain mode (i.e. tension, compression, and shear) and the corresponding strain magnitude differences, where the highest absolute values of longitudinal strains are compressive and dominate in the dorsal cortex, and tensile strains dominate in the plantar cortex. Longitudinal strains are lower in the medial and lateral cortices; however, neutral axis regions *generally* undergo more prevalent/predominant shear strains [i.e., where shear strains are not generally predominated by longitudinal (i.e. normal strains) tension or compression] and maximal strains that are ~20–30° oblique to the long axis of the bone. [While shear strains exist throughout the bone cross-section, this is the prevalent strain mode along the neutral axis since bending loads, superimposed on torsional loading, would eclipse the shear strains in the compression/tension (dorsal/plantar) regions.] In adult mule deer calcanei the dorsal cortex is significantly thicker, and has greater population density of secondary osteons, smaller osteon cross-sectional areas, osteons with quasi-circular to circular shapes, more oblique-to-transverse collagen fiber orientation (CFO), more highly cross-linked collagen fibers, lower porosity, and lower fractional area of secondary bone compared to the plantar 'tension' cortex [Gunasekaran et al., 1991; Skedros et al., 1994a, 1997, 2001a, 2001b, 2004; Skedros and Brady, 2001]. In contrast to the dorsal and plantar cortices, the medial and lateral cortices ('neutral axis' regions) exhibit fewer osteons, which are also often oval in cross-section (suggesting oblique osteon orientation) with highly transverse CFO, suggesting adaptation to prevalent shear strains and obliquely oriented principal strains [Petrtýl et al., 1996; Su et al., 1999]. The organization of the plantar

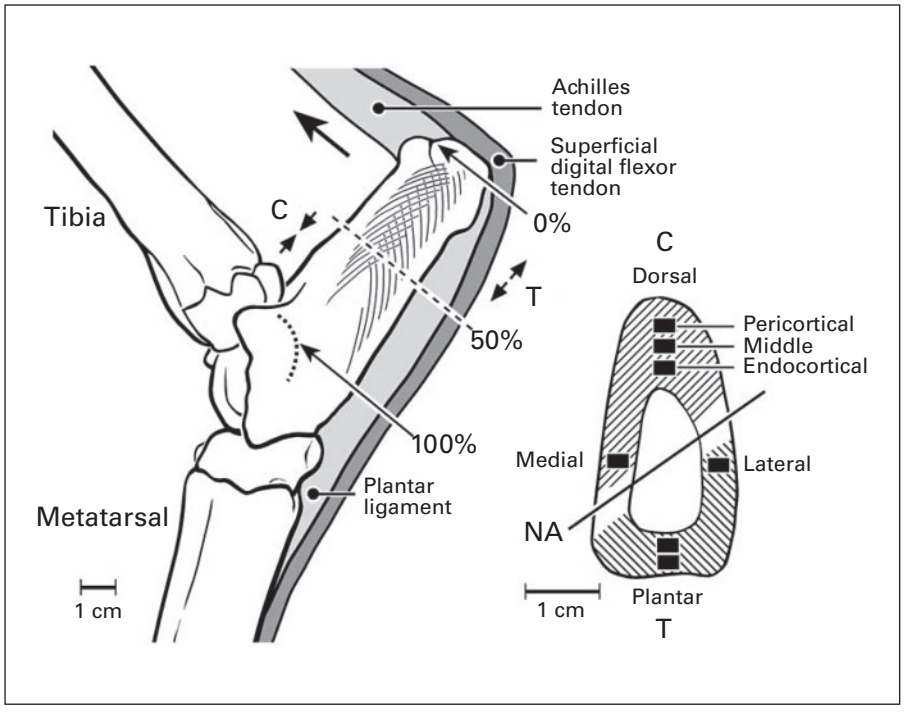

Fig. 1. Deer calcaneus in lateral view showing typical loading along the Achilles tendon (large arrow) producing compression (C) and tension (T) on dorsal and plantar cortices, respectively. This loading regime is similar in all of the calcanei. The cross-section shows image locations and the oblique neutral axis (NA), the location of which is based on empirical data derived from multiple triple stacked-rosette strain gauges as reported in an ex vivo study of functionally loaded sub-adult and adult deer calcanei [Su et al., 1999]. This study used multiple loading regimes to simulate a variety of functional loading conditions, including turning and running. The dorsal/plantar (compression/tension) strain distribution was found to be highly consistent in these various loading conditions, probably reflecting the high degree of constraint imposed by the talo-calcaneal articulation. It is not known if the neutral axis is similarly obliquely oriented in the calcanei of the other species.

'tension' cortex of deer calcanei is also distinct, with its increased resorption spaces, more longitudinal CFO, and larger, younger (lower mineralization), and more irregularly shaped secondary osteons – suggesting an increased remodeling rate compared to the other cortices[1], in addition to adaptation for tension strain mode (i.e. longitudinal CFO). Most of these differences are also present in calcanei of adult sheep, elk, and horses.

In view of these comprehensive data in deer calcanei, we hypothesized that sheep, elk, and horse calcanei would show: (1) strong correlation of spatial variations in osteocyte lacuna population densities (Ot.Lc.N/B.Ar) with remodeling activity and one or more of the myriad, potentially adaptable characteristics that are linked with bone remodeling (e.g. osteon population densities, porosity, mineral content); (2) strong correlation of spatial variations in Ot.Lc.N/B.Ar with the increased renewal activity or metabolic demands associated with the plantar ligament insertion (pericortical margin of the plantar cortex) and marrow proximity (endocortical regions); (3) increased Ot.Lc.N/B.Ar in the actively renewing plantar cortices or, by contrast, Ot.Lc.N/B.Ar will be highest in the dorsal cortex, reflecting the putative role of these cells in detecting microdamage differences between tension (also lower strain) and compression (also higher strain) environments [Skedros et al., 2005], and (4) increased Ot.Lc.N/B.Ar in endocortical regions (compared to other intra-cortical regions) of each cortex, perhaps in association with bioactive molecules from the nearby marrow, which may activate regional remodeling [Frost, 1998; Martin, 2000].

Correlations of Ot.Lc.N/B.Ar with regional variations in mean osteon cross-sectional areas were also examined. This inquiry stems from observations in a previous study of developing mule deer calcanei showing increased Ot.Lc.N/B.Ar and significantly reduced osteon cross-sectional areas in the dorsal cortex [Skedros et al., 2004]. Suggested mechanisms that might explain this relationship are also discussed.

[1] Although this interpretation seems most parsimonious in view of available data, it is constrained by the lack of dynamic measures of remodeling activity (e.g. fluorochrome labels). For example, it is plausible that small secondary osteons are indicative of a history of high activation frequency [Martin et al., 1998]. Resorption spaces only reflect the most recent remodeling events. Therefore, at one time the activation frequency may have actually been higher in the dorsal as opposed to the plantar cortex. Nevertheless, data available from the adult tissues examined in the present study suggest that even if the dorsal cortex at one time exhibited a higher activation frequency, the plantar cortex was more actively remodeling (higher resorption spaces and new remodeling events) at the time of necropsy.

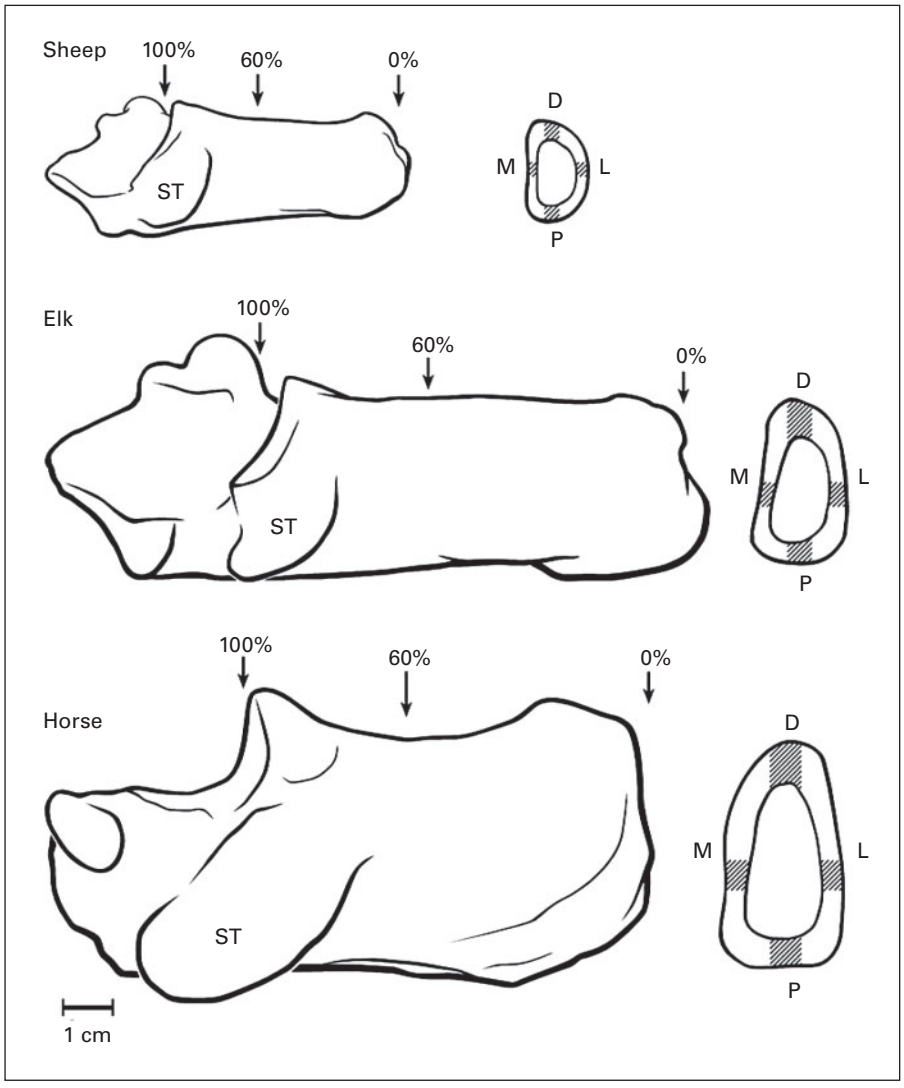

Fig. 2. Lateral view of calcanei of a sheep, elk, and horse: the cross-sections of each bone show the locations (in 'gray') where microscopic analyses were conducted. ST = Sustentaculum talus (at the medial aspect of each bone); 0% = distal 'end' of each bone.

Material and Methods

The Calcaneus Model, Specimens, Specimen Preparation and Analysis

Available in vivo strain gauge data suggest that, during functional loading, the artiodactyl (sheep) calcaneus (fig. 2) behaves like a short-cantilevered beam with longitudinal compression and tension strains predominating in opposing dorsal and plantar cortices, respectively [Lanyon, 1974; Su et al., 1999]. Corroborative ex vivo analyses in deer calcanei reported in detail in previous studies [Su, 1998; Su et al., 1999] include a variety of loading regimes to estimate strain distributions. These data were obtained in both subadult and adult deer, and used up to seven stacked triple-rosette strain gauges, including one beneath the plantar ligament. Off-axis loading (with the Achilles tendon loaded 5° medially to the sagittal plane of the bone and 5° lateral to the sagittal plane of the bone) has also been accomplished in order to estimate affects of turning and jumping during ambulation [Su, 1998]. These results demonstrate a highly consistent distribution of net compression in the dorsal cortex and net tension in the plantar cortex during ~80% of stance phase. There are biomechanical data also suggesting that in vivo strain distributions are similar at equivalent anatomical locations of sheep, deer, and horse calcanei [Badoux, 1987; Skedros et al., 1997].

One calcaneus was obtained from each of seven Standardbred horses with no history of racing or race training, seven wild-shot North American elk *(Cervus elaphus)*, and seven domesticated sheep (*Ovis aries*, breed is crossed Suffolk/Hampshire and Rambouillet; fig. 2). All bones were from adult animals that had been used in a previous study [Skedros et al., 1997]. The bones were randomly selected, resulting in an approximately even number of left and right bones. The elk were males, had shed the periosteal cover of their antlers, and were obtained from their natural habitat (northern Utah) in a fall hunting season. The equine calcanei were from mixed sexes that had been set to pasture. Although specific ages of several of the horses were not known, the animals were be-

tween 2 and 9 years old. The sheep were females and approximately 2 years old. The sheep and horses had been kept in large pastures. Skeletal maturity of all of the bones was confirmed by gross examination showing co-ossification of the sagitally cut distal calcaneal epiphyseal growth plate. None of the animals had evidence of skeletal disease and all were sacrificed for reasons other than limb lameness.

Unstained, undecalcified transverse segments, 4–5 mm thick, were cut from each calcaneus at 60% of shaft length. These segments were embedded in polymethyl methacrylate using conventional methods [Emmanual et al., 1987]. The proximal face of each segment was ground, polished, and prepared for backscattered electron imaging using a scanning electron microscope (JEOL, Peabody, Mass., USA) [Skedros et al., 1993a, 1993b].

One 50× BSE image representing 3.41 mm^2 (2.23 × 1.53 mm) was obtained in each transcortical 'region' [pericortical (Pe), middle cortical (Mi), endocortical (En)] of the dorsal cortices of each specimen. In many cases only two images could be obtained in the thinner plantar cortices. These images were taped together and subdivided into Pe, Mi, and En regions. The images excluded the circumferential lamellar bone in the dorsal and plantar cortices. One image was also taken within each medial and lateral 'neutral axis' cortex. Each backscattered electron micrograph was developed on high-resolution Polaroid film. Osteocyte lacunae were manually counted in these images by trained technicians and confirmed by the principal investigator who independently analyzed a large subset of images. The images were randomly assorted and the technicians were blinded to cortical location and to the hypotheses of the study. Intra- and interobserver error was below ±1.5%. In each image, the population density of osteocyte lacunae per bone area (Ot.Lc.N/B.Ar; n/mm^2) was calculated by dividing total number of lacunae by bone area (excluding central canals and other vascular canals and non-lacuna porosity). Lacunae with viable and non-viable osteocytes could not be determined.

In each image, a mean distance between osteocyte lacunae was also estimated. This was accomplished by using the formula shown in figure 3, which allowed for both estimates of two-dimensional and three-dimensional lacuna-lacuna (Lc-Lc) distances. This method produced similar values for distances between two neighboring lacunae reported by Weinbaum et al. [1994].

Additional characteristics were also used in correlation analyses. These characteristics, reported in previous studies of the same bones [Skedros et al., 1997], include: (1) secondary osteon population density (On.N/T.Ar), (2) fractional area of secondary bone (On.Ar/T.Ar), (3) porosity, (4) estimated mean cross-sectional area of osteons in each image (calculated from mean dorsal and plantar osteon diameters and assuming circularity) and minimum-to-maximum chord ratio which may indicate osteon obliquity with respect to the long axis of the bone, (5) population density of new remodeling events [number of resorption spaces and newly forming osteons per total (T) square millimeter area (Ar, n/mm^2, includes central canals in T.Ar)], (6) cortical thickness of the dorsal, plantar, medial, and lateral cortices, and (7) percentage of ash of adjacent dorsal, plantar, medial, and lateral segments.

Spatial heterogeneity of osteocyte lacunae was estimated by calculating the coefficient of variation for each intracortical region in each species. This was accomplished by dividing the standard deviation by the mean [Vashishth et al., 2000]. Spatial heterogeneity was examined: (1) in the context of strain mode and transcortical region (which correlates with strain magnitude and marrow proximity), and (2) for correlations with On.Ar/T.Ar, which would be expected if remodeling produces bone with different cell densities than the antecedent primary bone, and if there are differences in the amount of remodeling. Alternatively, remodeling may produce regional differences in osteocyte spatial heterogeneity if secondary osteon 'types' differ between cortical locations. This is plausible in these calcanei since cell densities are known to differ significantly between parallel-fibered secondary osteons (prevalent in the 'tension' cortices of these bones) and lamellar ('alternating') osteons (prevalent in the 'compression' cortices of these bones). (As discussed below, these osteon distinctions are consistent with the scheme of Marotti [1996, and pers. commun.].) Support for this possibility derives from a study where Ferretti et al. [1999] quantified significant differences in osteocyte densities in these two secondary osteon types in limb-bone cortices of adult animals of various species (frog, sheep, dog, cow, horse, and human).

A multiple-comparison ANOVA model was used for statistical analysis. Pair-wise comparisons between cortical *locations* (D = dorsal, P = plantar, M = medial, L = lateral) and transcortical *regions* (endocortical, middle cortical and pericortical) of each species were assessed for statistical significance ($\alpha \leq 0.05$) using Fisher's post-hoc protected least-significance difference test [Sokal and

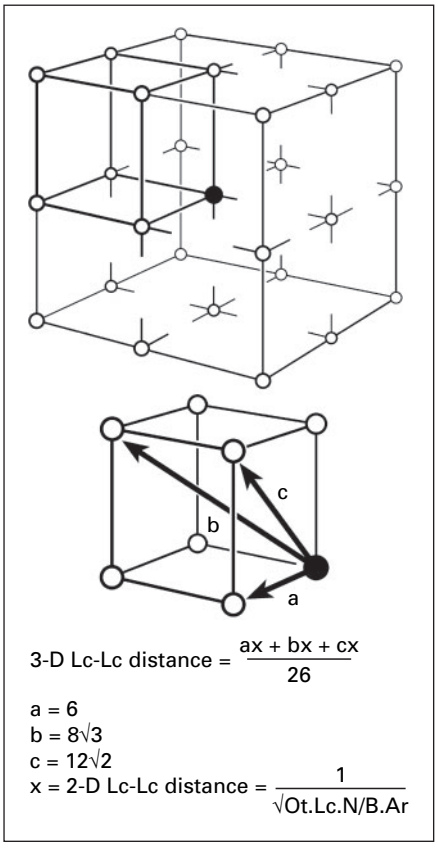

Fig. 3. Three-dimensional lattice used to estimate three-dimensional (3-D) mean osteocyte Lc-Lc distances from two-dimensional (2-D) Ot.Lc.N/B.Ar data. The equations used for these estimates are also shown.

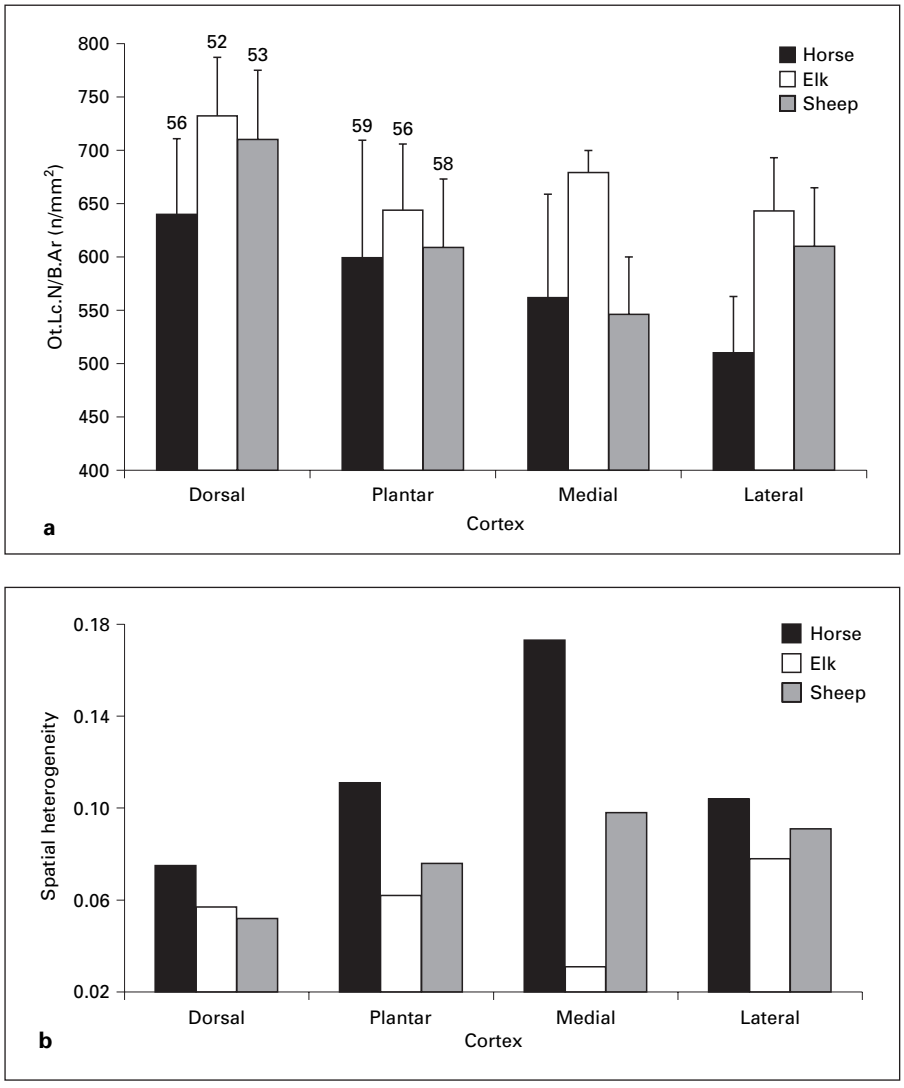

Fig. 4. Histograms of Ot.Lc.N/B.Ar (**a**) and spatial heterogeneity of osteocyte lacunae (**b**) in cortices of all three species. The numbers above the dorsal and plantar data bars in **a** are mean 3-D Lc-Lc distances in microns. In **a** the Ot.Lc.N/B.Ar data are shown as means and standard deviations.

Rohlf, 1995] (StatView, version 5.0, SAS Institute, Cary, N.C., USA). Pearson correlation coefficients (r values) for various comparisons were determined within each group.

Results

Equine (Horse) Calcanei

Mean osteocyte lacuna population densities (Ot.Lc.N/B.Ar; n/mm^2) for each of the cortical locations of each species are shown in figures 4a and 4b and table 1. In the equine calcanei, Ot.Lc.N/B.Ar is higher in the dorsal 'compression' cortex when compared to the plantar 'tension' cortex, but in contrast to sheep and elk (see below) this difference was not statistically significant ($p = 0.14$, table 2). The cortex-to-cortex comparisons showed significant differences or statistical tendencies in Ot.Lc.N/B.Ar in all but the plantar ('tension') versus medial cortex ($p \geq 0.3$).

Comparisons among intracortical regions (Pe vs. Mi vs. En, in D and P cortices) showed no statistically significant differences in the equine calcanei (table 3).

There were no statistically significant differences in the spatial heterogeneity of osteocyte lacunae between the four cortical locations (D, P, M, L; fig. 4b). These data reject the hypothesis that the prevalence of lamellar osteons in the 'compression' area would result in lower spatial heterogeneity. Similarly, there were no significant differences in transcortical spatial heterogeneity between the Pe, Mi, and En regions in all possible comparisons.

Correlations exceeding r = |0.500| are shown in table 4; Ot.Lc.N/B.Ar did not exhibit any correlations exceeding this absolute r value in the equine calcanei (table 4). Spatial heterogeneity of osteocyte lacunae correlated with r > |0.5| only with On.Ar/T.Ar data from medial and lateral cortices. The estimated three-dimensional mean distance between two neighboring lacunae was 56 ± 3 μm in the dorsal and 59 ± 7 μm in the plantar cortex (p = 0.11; fig. 4a).

Cervine (Elk) Calcanei

In the cervine calcanei, Ot.Lc.N/B.Ar was significantly higher in the dorsal 'compression' cortex than in the plantar 'tension' cortex (p < 0.0001; fig. 4a, table 2). Significant differences were also found between the dorsal cortex and each of the medial and lateral cortices. There were no significant differences between the plantar, medial, and lateral cortices.

Table 1. Osteocyte lacuna population density data: cortex and transcortical region data (means ± standard deviations)

Cortex	Ot.Lc.N/B.Ar, n/mm^2		
	horse	elk	sheep
Dorsal	640 ± 71	732 ± 55	710 ± 65
Plantar	599 ± 110	644 ± 62	609 ± 64
Medial and lateral	536 ± 69	661 ± 34	578 ± 45
Medial	562 ± 97	679 ± 21	546 ± 54
Lateral	510 ± 53	643 ± 50	610 ± 55
Dorsal			
Pericortical	656 ± 59	760 ± 45	737 ± 61
Middle	660 ± 69	728 ± 14	705 ± 64
Endocortical	605 ± 79	709 ± 27	688 ± 69
Plantar			
Pericortical	624 ± 155	654 ± 103	574 ± 70
Middle	595 ± 86	637 ± 41	668 ± 21
Endocortical	580 ± 86	640 ± 24	611 ± 51

Statistically significant differences are shown in tables 2 and 3.

Table 2. One-way ANOVA (p values of paired comparisons) of Ot.Lc.N/B.Ar data: dorsal ('compression'), plantar ('tension'), medial, and lateral cortices

	Dorsal			Plantar	
	vs. plantar	vs. medial	vs. lateral	vs. medial	vs. lateral
Horse	0.14 (6.8%)[a]	*0.04* (12.2%)*	*0.001* (20.3%)*	0.3 (6.2%)	*0.02* (14.9%)*
Elk	*<0.0001* (13.7%)*	*0.03* (7.2%)*	*0.0003* (12.2%)*	0.13 (–5.4%)	>0.5 (0.2%)
Sheep	*<0.0001* (16.6%)*	*<0.0001* (23.1%)*	*0.0004* (14.1%)*	*0.02* (10.3%)*	>0.5 (–0.2%)

[a] Numbers in parentheses are the percent differences of the paired comparisons; percent differences are calculated as: [(compression – medial or lateral)/(compression)] × 100; [(tension – medial or lateral)/(tension)] × 100. Statistically significant values are italicized.

*p ≤ 0.05.

Table 3. One-way ANOVA (p values of paired comparisons) of regional Ot.Lc.N/B.Ar data

	Dorsal ('compression') regions		Plantar ('tension') regions	
	pericortical vs. middle	middle vs. endocortical	pericortical vs. middle	middle vs. endocortical
Horse	>0.5	0.16	>0.5	>0.5
Elk	0.3	>0.5	>0.5	>0.5
Sheep	0.4	>0.5	0.02	0.12

Table 4. Pearson's correlation coefficients > |0.5|

Location	Ot.Lc.N/B.Ar n/mm^2	Cortical thickness cm
Horse		
All	none	On.N/T.Ar (0.62)*
Dorsal, plantar	none	% ash (0.76)*
		On.N/T.Ar (0.72)*
		Rs.N/T.Ar (–0.51)
		porosity (–0.78)*
		On.Ar (–0.57)*
Dorsal	none	none
Plantar	none	none
Medial, lateral	none	none
Elk		
All	cortical thickness (0.63)*	Ot.Lc.N/B.Ar (0.63)*
Dorsal, plantar	% ash (0.63)*	% ash (0.90)*
	cortical thickness (0.82)*	On.N/T.Ar (0.77)*
		On.Ar/T.Ar (–0.60)*
		porosity (–0.65)*
		On.Ar (–0.66)*
		Ot.Lc.N/B.Ar (0.82)*
Dorsal	On.N/T.Ar (0.57)*	none
	On.Ar/T.Ar (0.65)*	
Plantar	On.N/T.Ar (–0.64)*	none
	On.Ar (0.72)*	
Medial, lateral	none	none
Sheep		
All	cortical thickness (0.70)*	Ot.Lc.N/B.Ar (0.70)*
Dorsal, plantar	On.Ar/T.Ar (–0.69)*	% ash (0.72)*
	porosity (–0.53)	On.Ar/T.Ar (–0.71)*
	On.Ar (–0.58)*	porosity (–0.77)*
	cortical thickness (0.65)*	Ot.Lc.N/B.Ar (0.65)*
Dorsal	On.Ar/T.Ar (–0.51)*	none
	On.Ar (–0.61)*	
Plantar	none	none
Medial, lateral	Rs.N/T.Ar (–0.68)*	On.N/T.Ar (–0.60)*
	porosity (–0.64)*	porosity (–0.53)
		On.Ar (0.67)*

*$p < 0.05$. Rs.N/T.Ar = Number of resorption spaces and newly forming osteons per T.Ar (n/mm^2, includes central canals in T.Ar).

Comparisons among intracortical regions (Pe vs. Mi vs. En, in D and P cortices) showed no statistically significant differences (table 3).

There were no statistically significant differences in the spatial heterogeneity of osteocyte lacunae between the four cortical locations (D, P, M, L) in the cervine calcanei (fig. 4b). These data reject the hypothesis that the prevalence of lamellar osteons in the 'compression' area would result in lower spatial heterogeneity. Similarly, there were no significant differences in transcortical spatial heterogeneity between the Pe, Mi, and En regions in all possible comparisons.

Correlation analyses of Ot.Lc.N/B.Ar with the other characteristics showed two high and several moderate correlations (table 4). Spatial heterogeneity of osteocyte lacunae showed several moderate-to-high correlations ($r > |0.5|$) with the microstructural variables (– indicates a negative correlation, * $p < 0.05$): On.Ar/T.AR (*), porosity, On.N/T.Ar (–, *), Rs.N/T.Ar in the dorsal cortex; porosity, On.N/T.Ar (–, *), On.Ar (*) in the plantar cortex; On.Ar/T.Ar in the medial and lateral cortices; On.N/T.Ar (–, *) in all cortices. The estimated mean three-dimensional distance between neighboring lacunae was 52 ± 2 μm in the dorsal and 56 ± 3 μm in the plantar cortex ($p < 0.0001$; fig. 4a).

Ovine (Sheep) Calcanei

In the ovine calcanei, Ot.Lc.N/B.Ar was significantly higher in the dorsal 'compression' cortex than in the plantar 'tension' cortex ($p < 0.0001$; fig. 4a, table 2). Significant differences were found in all other comparisons, except plantar ('tension') versus lateral ($p = 0.5$).

Comparisons among intracortical regions (Pe vs. Mi vs. En, in D and P cortices) showed only one statistically significant difference in the ovine calcanei: Pe vs. Mi in the P 'tension' cortex (table 3).

There were no statistically significant differences in the spatial heterogeneity of osteocyte lacunae between the four cortical locations (D, P, M, L) in the ovine calcanei (fig. 4b). These data reject the hypothesis that the prevalence of lamellar osteons in the 'compression' area would result in lower spatial heterogeneity. Similarly, there were no significant differences in transcortical spatial heterogeneity between the Pe, Mi, and En regions in all possible comparisons.

Correlation analyses of Ot.Lc.N/B.Ar with the other characteristics showed two high and several moderate correlations (table 3). Spatial heterogeneity of osteocyte lacunae showed only a few moderate-to-high correlations ($r > |0.5|$) with some of the microstructural variables, but these were not statistically significant (– indicates a negative correlation): On.Ar/T.Ar (–), On.N/T.Ar (–) in the dorsal cortex; On.Ar/T.Ar, On.N/T.Ar, Rs.N/T.Ar in the plantar cortex. The estimated mean three-dimensional distance between neighboring lacunae was 53 ± 2 μm in the dorsal and 58 ± 3 μm in the plantar cortex ($p < 0.0001$; fig. 4a).

Interspecies Differences in Osteocyte Lacuna Densities

Interspecies differences are apparent in the statistically significant differences between them ($p < 0.01$; means ± SD of On.Ar/T.Ar data from all regions analyzed: sheep 640 ± 85, elk 681 ± 68, horse 599 ± 97).

Discussion

Results of this study typically showed inconsistent correlations between Ot.Lc.N/B.Ar and the various structural and material characteristics. Spatial heterogeneity of osteocyte lacunae was also inconsistently correlated with On.Ar/T.Ar. Also in conflict with one of our hypotheses (No. 3), the plantar cortices, where remodeling is more active, exhibited *lower* Ot.Lc.N/B.Ar than the dorsal cortices. This dorsal/plantar difference was statistically significant in sheep and elk calcanei, but was only a statistical tendency in the equine bones ($p = 0.14$). This lack of statistical significance may be the result of inadequate statistical power, and/or due to our inability to control the age range of the horses. This latter suggestion implies that age-related changes in lacuna density might increase regional variations (as shown in human bone by Vashishth et al. [2000]), hence making it difficult to discern significant differences with the sample size ($n = 7$). Another limitation of this study is our inability to determine the percentage of lacuna with viable osteocytes. However, our specimens were generally from relatively young adults, and our observations did not reveal regions with lacunae that were plugged with hypermineralized tissue, which has been associated with aging, ischemia, or necrosis [Frost, 1960; Currey, 1964; Kornblum and Kelly, 1964; Jowsey, 1966; Stout and Simmons, 1979; Parfitt, 1993]. In human bone, it has also been suggested that extensive death of osteocytes may not occur in osteons because of their younger bone age when compared to interstitial/primary bone [Qiu et al., 2003]. These observations, coupled with the estimated 'younger' ages of the animals used in the present study, suggest that there are a relatively small percentage of dead osteocytes.

In order to consider the possibility that the Ot.Lc.N/B.Ar differences shown between some of the cortical locations have significant mechano-biological implications, data from the present study are considered in the following contexts: (1) predominant strain mode, magnitude and putative associations with microdamage morphology incidence; (2) marrow proximity (endocortical regions), active renewal (plantar cortex) and ligament insertion proximity (pericortical region of plantar cortex); (3) issue constraints (primary vs. secondary bone) and spatial heterogeneity of osteocyte lacunae; (4) correlations with mean cross-sectional osteon areas and (5) cortical thickness and the concept of 'stressed volume'.

Strain-Mode- and Magnitude-Related Associations; Load Predictability and Habitual Bending

In all three species, strain mode was correlated with Ot.Lc.N/B.Ar – the dorsal 'compression' cortices having higher Ot.Lc.N/B.Ar than the plantar 'tension' cortices. Although a similar tension vs. compression pattern is reported in horse radii in a companion study [Skedros et al., 2005], it is not known if this pattern can be generalized to other similarly loaded bones. Additionally, in these equine radii the medial and lateral cortices, where longitudinal strains are presumably low and shear strains are prevalent/predominant, had lacuna densities that are similar to the plantar 'compression' cortices of these bones. The medial/lateral ('neutral axis') cortices of the calcanei had lowest Ot.Lc.N/B.Ar in only two of the species (table 1). Furthermore, the companion study showed unexpected (i.e. *opposite*) differences between 'tension' and 'compression' regions of equine third metacarpals (MC3s). But this result may be confounded by the comparatively more prevalent torsional loading of this bone, which may, while enhancing shear-related adaptations, tend to obscure or eliminate what might be considered obvious adaptations for tension and compression in bones that are relatively more simply loaded [Skedros et al., 2003c; Skedros and Hunt, 2004].

Although in the calcanei it was found that Ot.Lc.N/B.Ar also positively correlates with areas that receive higher strains (i.e. dorsal cortex) and lower densities were observed in areas that receive lower strains (i.e. plantar cortex), other findings are inconsistent with the hypothesis that Ot.Lc.N/B.Ar correlates with strain magnitudes. For example, as noted above, the neutral axes (i.e. medial and lateral cortices) did not consistently have the lowest Ot.Lc.N/B.Ar. Additionally pericortical-to-endocortical (high-to-low strain, respectively) strain-related variations in Ot.Lc.N/B.Ar were also not detected. (Absence of associations with marrow proximity and plantar ligament insertion are considered below.) The results of the companion study also did not reveal consistent relationships with transcortical variations in strain magnitude in equine radii and MC3s. These inconsistent correlations with strain mode and/or magnitude question the reliability of using Ot.Lc.N/B.Ar for inferring a history of habitual bending.

Microdamage Implications and Mechanical Relevance

In the companion study we considered the hypothesis of Vashishth et al. [2000] that regional/strain-related variations in microdamage incidence and/or other microdamage characteristics might require differences in the osteocyte 'networks', communicating via gap junctions, for localizing microdamage. In this context, regional variations in osteocyte densities, if present, might reflect enhancements in their capacity to detect the microdamage produced in different strain environments. Although there are data suggesting that this interpretation is plausible [Donahue, 2000; Tami et al., 2003], the rate of bone apposition during modeling and remodeling may more strongly influence regional osteocyte densities, suggesting that variations in Ot.Lc.N/B.Ar are epiphenomena in some cases. This possibility is discussed further in the companion study [Skedros et al., 2005]. However, a habitual non-uniform strain distribution must be accommodated by what we have called 'strain-mode-specific' adaptations of the local bone matrix, since failure to do so could result in deleterious increases in microdamage [Skedros et al., 2003a]. It has been hypothesized that deleterious increases are avoided by modeling/remodeling-mediated enhancements in fatigue resistance, toughness and/or energy absorption (e.g. by introducing cement lines and/or reorienting predominant CFO) [Skedros et al., 2003a, 2004]. Among various modifiable material characteristics, predominant CFO appears to be most consistently associated with a locally prevalent/predominant strain mode in various bones that have been studied [Mason et al., 1995; Skedros et al., 1996, 2000, 2003c, 2004]. In turn, studies of cortical bone organization in developing mule deer calcanei suggest that Ot.Lc.N/B.Ar and predominant CFO are not correlated [Skedros et al., 2004].

Data from mechanical tests suggest that the dorsal-plantar differences in predominant CFO and Ot.Lc.N/B.Ar that occur in mule deer calcanei are mechanically adaptive. For example, predominantly longitudinal CFO enhances material properties of the plantar cortex in tensile loading (i.e. its habitual loading mode) [Skedros et al., 2003b]. By contrast, Ot.Lc.N/B.Ar and osteonal characteristics (e.g. mean area and shape of secondary osteons, and On.N/Ar) relatively more strongly influence material properties of the dorsal cortex in compressive loading (i.e. its habitual loading mode). This difference may reflect fundamental differences in microdamage formation/morphology in these two loading modes. Support for this possibility is suggested by Reilly's [2000] data in rat ulnae and her overview of previous studies suggesting that osteocyte lacunae can influence microdamage formation differently in specific strain modes [Reilly, 2000, p. 1131]:

"Until recently, most research on damage created in tension concerned microcracks easily visible under bright-field light microscopy [Huja et al., 1999]. These types of microcracks have been called 'linear' by some authors [Vashishth et al., 2000]. They have been shown to form preferentially at cement lines and interlamellar boundaries [Carter and Hayes, 1976; Schaffler et al., 1989; Courtney et al., 1996; Vashishth et al., 1997] where one might expect planes of weakness. Microdamage created in torsion [i.e., prevalent shear] has also been shown to form at planes of weakness [Jepsen et al., 1999]. In previous microdamage investigations only *compression* cracking has been shown to be influenced by (osteocyte) lacunae [Carter and Hayes, 1976]."

Thus, habitual *compression* environments may be where modifications in Ot.Lc.N/B.Ar are most beneficial. This suggestion has been corroborated in recent mechanical testing studies of equine MC3 diaphyseal cortices loaded in strain-mode-specific tension and compression [Skedros et al., 2003d].

Marrow Proximity and Active Renewal, and Ligament Insertion Proximity

Contrary to one of our hypotheses (No. 2), there were no correlations between Ot.Lc.N/B.Ar and marrow proximity (i.e. endocortical regions). In turn, there was also no correlation between Ot.Lc.N/B.Ar and active renewal (i.e. population densities of new remodeling events) in the plantar cortex compared to the other cortical locations. However, the endocortical regions examined in this study may not be analogous to the area of active renewal that can occur along the endocortical regions of limb bones of humans with osteoporosis or advanced age [Smith and Walker, 1964; Atkinson, 1965; Ruff and Hayes, 1988]. This is because, according to criteria used to define the endocortical region in the present study, larger vascular spaces were avoided – along endosteal margins of osteoporotic or aged bones such 'larger' vascular spaces are prevalent [Amtmann, 1971; Feik et al., 2000; Bousson et al., 2001].

The absence of significant Ot.Lc.N/B.Ar differences between the endocortical region and other regions of the plantar cortex in all species also rejects the hypothesis that increased osteocyte densities are associated with the insertion of the plantar ligament. But this does not preclude the possibility that the generally more active osteonal re-

newal in the plantar cortex is a product of biomechanical stimuli associated with this insertion, including enhanced blood supply and associated fluid-flow dynamics, and lower ambient strains [Skedros et al., 1994b, 2001b].

Tissue Constraints (Primary vs. Secondary Bone) and Spatial Heterogeneity of Osteocytes

When all cortical locations were considered, none of the species showed correlations between Ot.Lc.N/B.Ar and On.Ar/T.Ar. However, the variation in primary versus secondary bone may not have been sufficient for detecting differences in these calcanei – in all three species the majority of the sampled areas were predominately secondary osteonal bone (typically >70% of the imaged area) [Skedros et al., 1997]. The possibility that broader variations in the relative percentages of secondary bone might show strong correlations with Ot.Lc.N/B.Ar is suggested by Remaggi et al. [1998] in their histomorphometric study of mature limb-bone diaphyseal cortices in various species (frog, chick, rabbit, cow, horse, dog, and human). They found that (p. 152):

"… the shape, size and density of osteocyte bodies and cytoplasmic processes … strictly depend on the spatial organization of collagen fibers and not on the time of bone deposition (both primary and secondary bone may be composed of woven-fibered, parallel-fibered or lamellar bone); …"[2]

In a follow-up study, Ferretti et al. [1999, p. 127] found that the distribution of osteocytes is more regular in lamellar bone, but Ot.Lc.N/B.Ar is higher in parallel-fibered bone. However, this is opposite of what we found in the deer calcanei: *higher* Ot.Lc.N/B.Ar in dorsal cortex where there are *lamellar* osteons, and *lower* Ot.Lc.N/B.Ar in plantar cortex where there are *parallel-fibered* osteons.

Results of the present study also did not show consistent dorsal versus plantar differences in spatial heterogeneity of osteocyte lacunae (fig. 4b). This parameter was examined since regional differences in the coefficient of variation of osteocyte lacunae would indicate increased heterogeneity in the spatial organization of osteons. For example, Vashishth et al. [2000] showed that this coefficient of variation increased linearly with age in human femoral cortical bone. In turn, the clustering of osteocytes that this represents could adversely affect the sensitivity of osteocyte networks within regions (e.g. some regions failing to detect a microcrack) [Qiu et al., 2005]. This theoretical possibility, however, cannot be implied by the results of the regional analyses conducted in present study, including the plantar cortices where the rate might be expected to show the highest regional spatial heterogeneity. Furthermore, spatial variations in Ot.Lc.N/B.Ar also did not consistently correlate with any of the other regional characteristics of the spatially heterogeneous morphologic organization. This is not surprising since these characteristics are also not strongly correlated with the local differences in strain magnitude or the prevalence/predominance of a specific strain mode [Skedros, 2001].

Although in each species there were statistically significant regional differences in Lc–Lc, the range was only on the order of 3–5 μm. These small differences are probably not biomechanically significant. This is consistent with suggestions of the companion study [Skedros et al., 2005] wherein we hypothesized that a relatively broad range of osteocyte densities reported in various species primarily reflects constraints imposed by corresponding histology/growth rates and/or other species-related differences. Consequently, the minimal requirements of a communication network are probably readily satisfied at cell densities that are lower than those required for other mechano-biological demands. As discussed in the companion study, it is also possible that there are differences in cell level adaptations (e.g. cell matrix tethering) between populations of osteocytes that modify their responsiveness in significantly different regional strain environments.

Significant differences in Ot.Lc.N/B.Ar were found between species. Factors that influence these interspecies variations might be most important in affecting osteocyte lacuna density. For example, some factors might include animal activity, animal age, or degree of osteocyte death. One limitation of this study is, as noted, our inability to control for these factors.

Correlations with Mean Cross-Sectional Osteon Areas

It was hypothesized that Ot.Lc.N/B.Ar would be associated with osteon size (i.e. high Ot.Lc.N/B.Ar and small osteon cross-sectional areas). This seems to be supported in the dorsal and plantar cortices of each species since, compared to the plantar cortices, the dorsal cortices have: (1) significantly lower mean cross-sectional osteon areas and significantly higher On.N/T.Ar [Skedros et al.,

[2] This reflects Marotti and co-worker's [Marotti, 1990, 1996; Marotti and Muglia, 1992; Ferretti et al., 1999] scheme of osteon morphologies that, contrary to the classical view that ascribes a lamellar structure to all secondary osteons, describes the existence of three differently structured secondary osteons, namely, woven fibered, parallel-fibered and lamellar. In other words, all three types of bone tissue may be present inside secondary osteons.

1997], and (2) higher Ot.Lc.N/B.Ar (present study). An explanation supporting the possibility that these differences in Ot.Lc.N/B.Ar are biomechanically relevant and might influence local osteon size and/or population density is that spatial variations in load intensity (e.g. strain magnitude variations) cause differences in osteo*blast* recruitment and/or percent survival. In turn, the relatively higher densities of the osteocytes that remain in some areas could have a repressive effect on subsequent osteo*clast* activity. A high-stress-related repressive effect on osteoclast vigor has been offered as an explanation for the smaller diameter osteons in femora and tibiae between groups of Pleistocene hominids compared to recent populations [Abbott et al., 1996].

Bioactive non-collagenous proteins within bone matrix may help explain relationships that exist between Ot.Lc.N/B.Ar, osteon size, and other features of the local histology. For example, studies in 6-month-old rat tibiae loaded in vivo have shown that levels of transforming growth factor β (TGF-β) increase with loading and become incorporated in the matrix [Raab-Cullen et al., 1994]. Mice and rat cell culture experiments suggest that TGF-β suppresses osteoclastic vigor and differentiation [Heino et al., 2002]. In turn this may stimulate osteoblastic vigor, increasing osteoblast proliferation and matrix formation [Baylink et al., 1993; Geiser et al., 1998; Bonewald, 2002]. Studies in mice femoral diaphyses have also shown that the overexpression of TGF-β increases the rate of osteoblast differentiation, subsequently increasing osteocyte population density [Erlebacher et al., 1998; Hamrick et al., 2004]. Extrapolating these data to larger mammals, Susan Pfeiffer [pers. commun.] has hypothesized that suppressed osteoclastic vigor, mediated by TGF-β, could lead to the production of secondary osteons with reduced cross-sectional areas. The possibility that this can be extrapolated from small mammals (that lack secondary osteons) to larger mammals (that have secondary osteons) is indirectly supported by recent ex vivo studies of cortical bone from adult human tibiae [Yeni et al., 2004] demonstrating that mechanical loading can mobilize TGF-β from the mineralized matrix. However, this relationship may not be generally applicable since inconsistent relationships between mean osteon cross-sectional areas and prevalent/predominant strain mode or magnitude have been observed in tibiae of sheep, radii of sheep and horses, and MC3s of horses [Martin et al., 1996; Skedros et al., 1996; Skedros, 2001, and unpubl. data].

Data in the companion study of equine MC3s and radii suggest that circumferential strain gradients, which are highest in neutral axis regions, correlate with regionally increased Ot.Lc.N/B.Ar. This correlation was not found in the present study. A possible explanation for this may be that in some instances cell populations are constrained by growth rate and histologic organization more so than by associated strain gradients. For example, the smaller and more numerous osteons in the cranial cortices compared to the larger, oval, and less numerous osteons in the medial and lateral cortices may reflect stronger influences associated with tissue construction on osteoblast recruitment/osteocyte survival than putative influences of local strain gradients. An additional confounding variable in the calcanei examined in this study, which is not present in equine MC3s and radii, includes the perturbation of fluid flow dynamics that might be associated with the plantar ligament – thus rendering comparisons difficult with other bones that do not exhibit a similar enthesis.

Cortical Thickness and the Concept of 'Stressed Volume'

We had also speculated that Ot.Lc.N/B.Ar would positively correlate with cortical thickness, possibly reflecting a requirement for detecting higher prevalence of microdamage in larger 'stressed volumes' [Skedros et al., 2005]. This interpretation, if correct, might be supported in calcanei of elk and sheep when all cortices were considered ($r = 0.63$ and 0.70, respectively). However, this correlation was <0.4 in the equine calcanei. This issue is also confounded by the presence of cancellous bone in the marrow cavity, which tends to be more prevalent in the cross-sections of the equine calcanei when compared to the section locations in the other two species (fig. 2).

Distinguishing Osteocyte Cell Bodies from Dendrites in Affecting Bone Matrix Modification

In view of other investigators' findings that have shown at least a portion of remodeling is targeted to the repair of microdamage, it is possible that other mechanotransduction pathways might predominate, leading to the remodeling and osteocyte distributions observed in the bones of the present study. These include innervation of bone, and changes in the dendritic processes of osteocytes within canaliculi that are not associated with osteocyte death or changes in osteocyte lacunar density [Colopy et al., 2004; Burt-Pichat et al., 2005; Szczesniak et al., 2005]. For example, using in vivo loading of rat ulnae, Colopy et al. [2004] report that remodeling ('quasi-remodeling' in the rat) distant from linear microcracks may be targeted by osteocytes with disrupted cell processes rather

than osteocytes with disruption of the cell body. Their results showed that while matrix injury and disruption of dendritic cell processes appear at least partially reversible, disruption of osteocyte cell bodies does not. These results suggest that cell dendrites can have important biomechanical functions that might not be recognized when only cell bodies (hence osteocyte lacunae) are considered. This bespeaks potentially important, but controversial [Parfitt, 1977], aspects of osteocyte physiology – osteocytes are thought to have the capacity to modify the matrix of bone [Belanger et al., 1967], and the architecture of canaliculi containing the dendritic cell processes of osteocytes is known to be quite dynamic [Palumbo et al., 2004] and to potentially change over time [Okada et al., 2002].

Summary and Conclusions

Results of this study showed significant differences or tendencies (elk) in comparisons of plantar 'tension' vs. dorsal 'compression' cortices, with highest Ot.Lc.N/B.Ar in dorsal cortices of all species. Although these relatively small differences might be adaptations for prevalent/predominant strain characteristics and/or associated microdamage, it is also plausible that they are strongly influenced by corresponding differences in the bone formation rates that produced the tissue in these locations. Bone growth rates and other factors (e.g. animal activity and age) might help to explain the interspecies differences. Consequently, the results of this study provide little or no evidence that the number of osteocyte lacunae has a functional role in mechanotransduction pathways that are typically considered in bone adaptation. Lack of consistent correlations among Ot.Lc.N/B.Ar and the structural and material characteristics also reject the possibility that regional variations in Ot.Lc.N/B.Ar are mechanically 'synergistic' with the significant regional differences in mineral content, porosity, On.N/T.Ar, and cross-sectional osteon area, for example, that have been described within the same cross-sections of these bones. Additionally, regional variations in Ot.Lc.N/B.Ar do not appear sufficiently reliable for inferring a history of habitual bending. The mechanisms that govern the production and maintenance of species/site-specific histogenesis might be the most important factors governing the distribution of these cells. In turn, these tissue level 'adaptations' appear to require strain and fatigue-damage sensors, which may be the function of osteocyte networks.

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