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Bone 39 (2006) 143–151

BONE

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Dissociation of mineral and collagen orientations may differentially adapt compact bone for regional loading environments: Results from acoustic velocity measurements in deer calcanei

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Received 7 July 2005; revised 25 November 2005; accepted 7 December 2005
Available online 3 February 2006

Abstract

In limb bone diaphyses, it is hypothesized that collagen and extra-fibrillar mineral are aligned differently in relatively simple loading conditions (e.g., habitual longitudinal compression) when compared to complex or potentially deleterious strain environments (e.g., habitual shear or tension). These putative differences in collagen/mineral organization might be adaptations that enhance toughness and fatigue resistance by controlling the direction of microdamage propagation. This study examined relationships between the non-uniform strain distribution of wild deer calcanei and elastic anisotropy of cortical bone specimens in three preparations: (1) demineralized (collagen only), (2) deproteinized (mineral only), and (3) untreated. Using simulated functional loading, the following strain data were obtained from the dorsal "compression", plantar "tension", and medial and lateral ("neutral axis") cortices of one calcaneus of each of seven pairs: (1) peak strain magnitude, (2) prevalent/predominant strain mode (compression, tension, shear), and (3) principal strain orientation with respect to the bone's long axis. In the contralateral calcanei, elastic anisotropy ratios (ARs) were calculated using acoustic velocity (longitudinal and transverse) measurements from a pair of orthogonally sliced specimens (representing each of three preparation types) from each cortex. In a separate set of seven adult calcanei, predominant collagen fiber orientation (CFO) was measured using circularly polarized light (CPL) in the four cortical locations. Results showed that, in general, elastic anisotropy was significant in each region, with ARs being significantly different from isotropy (where AR = 1.0). Compared to CFO, mineral orientation more strongly influenced this anisotropy, which was most notable in the plantar "tension" cortex. High correlations (r values from −0.675 to −0.734, $P < 0.05$) were found between collagen anisotropy obtained from acoustic data when compared to the CPL data. Significant correlations of mineral and collagen anisotropy were also found between strain mode, magnitude, and orientation (all r values ~−0.750). The habitual compression, tension, and shear (neutral axis) regions also had different collagen/mineral organizations, which may be important in accommodating the well-known disparity in the mechanical properties of bone in these loading modes.

Keywords: Acoustic microscopy; Bone anisotropy; Bone mineral orientation; Collagen fiber orientation; Osteons

Introduction

In acoustic microscopic analyses (60 μm resolution) of osteonal bone from femora of skeletally mature dogs, Turner and co-workers [57] reported that the principal orientation of bone mineral was along the long axis of the bone, while bone collagen was aligned at a 30° angle to the long axis. This observation is important since the misalignment between the mineral and collagen suggests that: (1) a substantial percentage (~75% in many cases) of mineral is extra-fibrillar and has a different orientation from intra-fibrillar mineral [29,38] and (2) the alignment of extra-fibrillar mineral may be governed by external influences such as mechanical stresses, as opposed to the intimate relationship (e.g., van der Waals

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doi:10.1016/j.bone.2005.12.007

forces and ionic bonds) between collagen and intra-fibrillar mineral [9,10,25]. Additionally, these results imply that collagen and mineral orientations can be differentially modified as a means for adapting elastic anisotropy for regional mechanical demands [54]. For example, it is plausible that collagen and mineral are aligned differently in relatively simple loading conditions (e.g., habitually longitudinal compression) when compared to complex or potentially deleterious strain environments (e.g., habitual shear or tension). The probability that modifications such as these would be expected and are adaptive is supported by: (1) in vivo strain measurements from bones of various mammalian and avian species typically demonstrating that, during peak loading of controlled ambulation, loading conditions can be characterized as either habitual torsion (predominant/prevalent shear) [12,28,30] or directionally consistent bending that produces a habitual tension/compression/shear strain distribution [4,27,30] and (2) data showing that the mechanical properties, including fracture and microdamage mechanics, of cortical bone differ in tension, compression, and shear [8,20,40,55,58]. Consequently, differential modifications of preferred collagen and mineral orientations might be a means for adjusting matrix ultrastructural anisotropy in order to more optimally resist and/or accommodate the formation of fatigue microdamage in these common non-uniform or potentially deleterious strain environments [1,41].

The goal of this study is to further examine relationships between the strain distribution caused by simple dorsal–plantar (compression–tension) bending of an artiodactyl calcaneus and elastic anisotropy of bone (demineralized, deproteinized, and untreated). This investigation is also aimed at confirming that the misalignment of preferred collagen and mineral orientations is a potential means for differentially adapting a bone for one or more prevalent/predominant strain characteristic(s). The artiodactyl calcaneus has been described as a simply loaded "tension/compression" bone [47,48,52] (Fig. 1). In vivo and rigorous ex vivo (with up to seven stacked-rosette strain gauges on the bone) studies have shown that, during physiologic weight-bearing activities, the calcaneal diaphysis behaves like a short-cantilevered beam with longitudinal compression and tension strains predominating on opposing dorsal and plantar cortices, respectively [26,52]. This strain distribution is highly consistent, even with 5° medial and 5° lateral off-axis loading simulating twisting and turning during ambulation [51]. These results demonstrate that during >80% of stance phase there is a highly consistent distribution of net compression in the dorsal cortex and net tension in the plantar cortex. Recent studies have shown that artiodactyl calcanei have unusually heterogeneous structural and material organization between cortical regions of the same transverse cross-section [47,48]. For example, sub-adult and mature mule deer calcanei exhibit biomechanically significant regional differences in cortical thickness, mineral content (% ash), microstructure (e.g., porosity, new osteon remodeling events, and secondary osteon cross-sectional shape and population densities), and ultrastructure (e.g., predominant collagen fiber orientation and collagen molecular cross-links) [17,46,47]. In fact, to our knowledge, the differences in percent ash that occur between dorsal and plantar cortices of mature deer calcanei are the *largest* that have been described *within the same cross-section* of a mature limb bone of any vertebrate taxon. It has been speculated that these unusually conspicuous morphologic heterogeneities represent the outcome of adaptive bone modeling and remodeling responses to a long history of the functional dorsal–plantar compression–tension strain distribution [45]. Consequently, the artiodactyl calcaneus is being promoted as an experimental model for investigating the mechanisms that govern the modeling and remodeling processes that produce and maintain functionally adapted bone [48]. Using this model, the following specific hypotheses were analyzed in the present study:

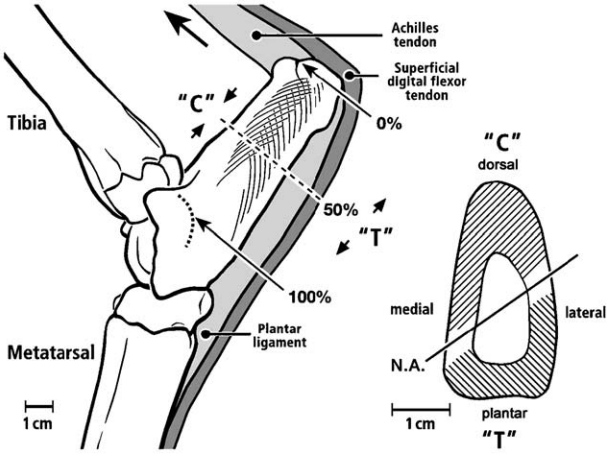

Fig. 1. Deer calcaneus in lateral view showing typical loading along the Achilles tendon (large arrow) producing compression ("C") and tension ("T") on dorsal and plantar cortices, respectively. The cross-section shows image locations and the oblique neutral axis (N.A.) that has been shown in an ex vivo study of mature deer calcanei [52].

(1) Mineral and collagen can be dissociated and therefore align differently to adapt elastic anisotropy for local mechanical demands.
(2) Mineral orientation is more influential than collagen orientation in affecting the longitudinal elastic anisotropy in cortical bone, therefore corroborating the data of Takano et al. [54].
(3) Regions of bone experiencing more obliquely oriented principal strains will have lower anisotropy ratios than regions with more longitudinally oriented principal strains.
(4) The acoustic anisotropy data will corroborate optical (circularly polarized light) data suggesting that cortical bone is capable of differentially adapting to a habitual strain mode (i.e., brighter birefringence in the dorsal "compression" cortex indicating predominantly oblique-to-transverse collagen fibers, and darker birefringence in the plantar "tension" cortex indicating predominantly longitudinal collagen fibers).

Materials and methods

Specimens

Paired calcanei were obtained from seven, wild, male, adult Rocky Mountain mule deer (*Odocoileus hemionus hemionus*) that were shot in their natural habitat during an October hunting season. The use of these animals was approved by the Institutional Animal Care and Utilization Committee (IACUC) at the University of Utah (for the Orthopaedic Bioengineering Laboratory) and received institutional approval at Orthopaedic Hospital, Los Angeles, California. As described below, one bone from each pair was used for ex vivo strain measurements, and the other bone was used for determining predominant mineral and collagen orientations using acoustic microscopy. From similarly sized male animals, seven additional unpaired calcanei were obtained for the analysis of predominant collagen fiber orientation using circularly polarized light (see below). None of the fourteen animals had evidence of skeletal pathology or advanced age (i.e., antler morphology or dental wear). At the time of collection, the periosteal ("velvet") covering of the antlers had been shed and antler growth was complete, suggesting that the relatively minor amount of appendicular cortical bone that had been resorbed for antler growth would have been replenished by this stage [3,21]. Maturity was confirmed by direct inspection showing co-ossification of the distal calcaneal growth plate (near the gastrocnemius tendon insertion).

Acoustic microscopy

One bone from each pair was cut multiple times so that three pairs of orthogonal slices ("specimens") were obtained from each of the dorsal, plantar, medial, and lateral cortices (Fig. 2). These specimens were cut to 400 μm thickness under continuous water irrigation using a low-speed diamond blade saw (Exact Technologies, Oklahoma City, OK). A subsequent thickness measurement that was used for the acoustic delay calculations (see below) was based on the average of three measurements made with a digital caliper (Mitutoyo™, Kanagawa, Japan) in the central portion of each specimen. The density (g/cm^3) of each specimen was determined using an analytical balance to measure wet and submerged weights (submersion in 100% ETOH). Wet weight was obtained after rinsing the specimen with 100% ETOH and using an absorbent towel to blot dry. Archimedes principle was used to calculate density as wet weight divided by volume [18].

Acoustic velocity measurements were made using a scanning acoustic microscope (UH3, Olympus). A 50 MHz lens (V-390, Panametrics, Waltham, MA) was used to transmit and receive acoustic waves in pulse echo mode. A digital oscilloscope (TDS 620, Tektronics Inc., Beaverton, OR) measured the delay time between acoustic waves reflected at the top of the specimen and waves reflected from the bottom of the specimen (Fig. 3). These methods are identical to those described by Hasegawa et al. [18].

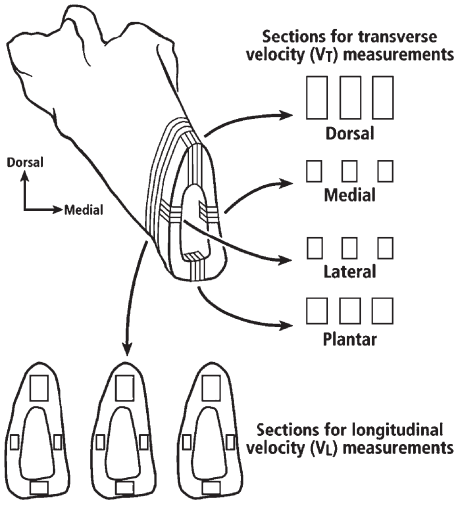

Fig. 2. Illustration showing locations (dorsal, plantar, medial, and lateral) on the deer calcaneus where multiple cuts were made to produce three pairs of orthogonal slices ("specimens"). Locations where longitudinal velocity (V_L) measurements were taken are indicated by rectangular boxes in the transverse sections at the lower left.

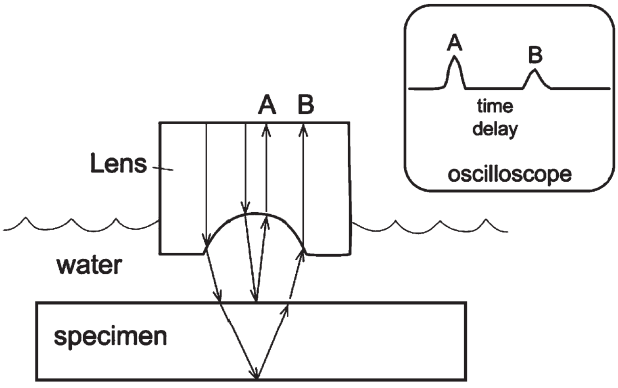

Fig. 3. Acoustic velocity measurement using an acoustic microscope. A 50 MHz transducer was used to generate acoustic waves in pulse echo mode. Delay time was measured on an oscilloscope between waves reflected at the surface of the specimen and those reflected from the bottom of the specimen. The acoustic velocity is calculated as twice the specimen thickness divided by the delay time.

Acoustic velocity was calculated as twice the specimen thickness divided by the delay time. Time delay was calculated as the average of measurements at five different sites within a 600 × 600 μm region of the central portion of each specimen. The precision of the velocity measurement was 0.3%–0.5%. The elastic coefficient (C) for the each specimen was obtained as the product of density (ρ) and acoustic velocity (v) squared ($C = \rho \cdot v^2$).

After acoustic velocity and specimen density measurements were made, one pair of specimens from each of the four cortical locations was placed in 10% ethylenediaminetetraacetic acid (EDTA) solution for a 72-h period. This procedure dissolved the mineral and left behind the collagen framework. Microradiography confirmed that no mineral crystals remained in these specimens. The "demineralized" specimens were rinsed in distilled water before the acoustic velocities were measured for bone collagen using the acoustic microscope as described above. Elastic coefficients for demineralized bone were also calculated as the product of density (re-measured for demineralized bone) and acoustic velocity squared.

The remaining bone specimens were placed in 7% sodium hypochlorite for 72 h to remove organic matrix constituents, yielding "deproteinized" specimens. Deproteinization was confirmed by subsequent demineralization of the specimens, which produced no organic residue. The possibility that this treatment may have caused the dissolution of very poorly crystalline mineral crystallites seems unlikely in view of previous studies using X-ray diffraction and Fourier-transformed infrared spectroscopy that did not defect such mineral phases in untreated samples of the regions examined in the present study [5,17]. Acoustic velocities for the deproteinized specimens were measured using the acoustic microscope as described above. For the purposes of this study, the predominant 'orientation' of the mineral phase is considered to approximate the crystallographic c axis of the mineral, which generally parallels the long axis of the crystallite [43,60]. Limited data from previous studies also show that the c axes of mineral crystals are typically aligned with the longitudinal axis of a long bone [2,15,43,59].

Anisotropy ratios (ARs = C_L/C_T or v_L^2/v_T^2) were calculated by dividing the measured longitudinal (L, long axis of the bone) elastic coefficients by the elastic coefficients in an orthogonal (transverse, T) direction for each of the dorsal, plantar, medial, and lateral cortices.

Strain characteristics in contralateral bones

The bones that were contralateral to those used in the acoustic analyses had been used in a previous ex vivo study of strains produced during simulated functional loading [52]. The strain data used in correlation analyses conducted

in the present study were averaged from the seven bones used in this previous study and included: (1) principal strain magnitude in each cortical location and (2) principal strain orientation (i.e., the angle subtended by the principal strain and the bone's long axis) in each cortical location. Correlations were conducted using acoustic data from dorsal, plantar, medial, and lateral cortices of one bone compared to the averaged strain data from these cortices of its contralateral pair.

Predominant collagen fiber orientation (CFO), determined using circularly polarized light

The seven additional unpaired bones used in this part of the study had been examined previously in a study that quantified predominant collagen fiber orientation (CFO) in each of the four cortical locations [44]. The methods used to obtain CFO data included transversely sectioning the bones at mid-diaphysis to produce one 5-mm-thick segment (unstained, undemineralized, and undeproteinized), which was then embedded in methylmethacrylate [14] for microscopic analyses. Predominant CFO was based on gray levels in circularly polarized images of slices of the embedded segments that were ultramilled to 100 ± 5 μm [49]. Using this technology, higher gray levels (brighter birefringence) represent predominantly transverse-to-oblique "compression" collagen fibers, and lower gray level values represent predominantly longitudinal "tension" collagen fibers. Two 50× images (512 × 480 pixels; approximately 2.3 mm^2/image) were analyzed from each dorsal, plantar, medial, and lateral cortices in the regions corresponding to the locations where the acoustic measurements were made in the other sample of bones. This resolution is not sufficient to visualize collagen fibers or mineral crystallites individually. As in the acoustic measurements, care was taken to avoid the highly birefringent circumferential lamellar bone. In order to ensure minimal sampling error within cortical locations [22], pilot studies were conducted to confirm that the number of images selected in any given cortical region accurately accounted for over 95% of the local microstructural variation. The scatter in the mean gray level data among the imaged locations was on the order of ±5% in dorsal and plantar cortices and ±15% in medial and lateral cortices. The methods used to express regional CFO differences in cortical bone as differences in gray levels [49] have produced relative differences that are similar to the "longitudinal structure index" used by others [24,33,54].

Statistical analysis

Paired comparisons were assessed for statistical significance ($P < 0.05$) using a one-way ANOVA design with Fisher's PLSD post-hoc testing [50] (StatView version 5.0, SAS Institute Inc., Cary, NC, USA). Pearson correlation coefficients (r values) were used to evaluate comparisons between the strain, acoustic anisotropy, and circularly polarized light data.

Table 1
Acoustic anisotropy ratios (ARs) of dorsal, plantar, medial, and lateral cortices for untreated, demineralized, and deproteinized specimens

	Acoustic ratios			
	Dorsal ("compression")	Plantar ("tension")	Medial ("compression/shear")	Lateral ("tension/shear")
Untreated	1.34 [a]	1.55 [b]	1.25 [b]	1.30 [b]
Demineralized	1.13 [c]	1.22 [b]	1.10 [b]	1.13 [c]
Deproteinized	1.54 [b]	2.03 [b]	1.49 [b]	1.47 [b]

Superscripts indicate significant differences from isotropy (AR = 1).
Note that all show statistically significant difference or trend when compared to isotropy (AR = 1.0). Therefore, anisotropy is present in demineralized (collagen only) and deproteinized (mineral only) bone. However, there is stronger anisotropy in mineral, so mineral must be considered when analyzing the anisotropy of bone. Either acoustic microscopy is more sensitive to mineral or mineral is more closely aligned to the longitudinal or transverse sound wave.
[a] $0.01 < P \leq 0.05$.
[b] $P \leq 0.01$.
[c] $0.05 < P \leq 0.1$.

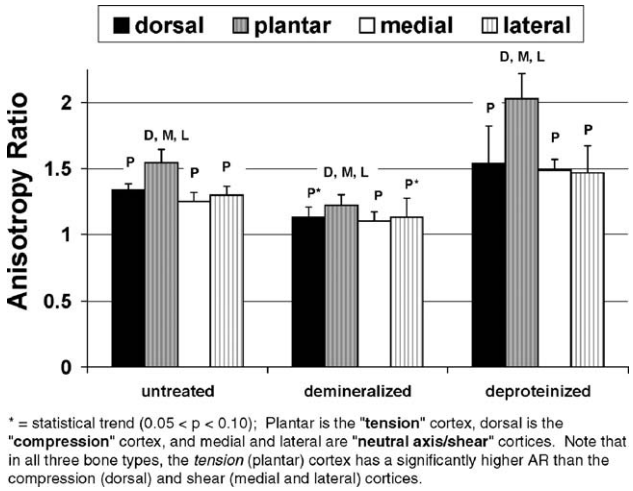

Fig. 4. Representation of cortical (dorsal, plantar, medial, and lateral) comparisons of anisotropy ratios for untreated, demineralized, and deproteinized specimens. Letters above the bar graph indicate statistical differences between cortices (e.g., D, M, and L means that the plantar (P) cortex shows a significant statistical different than the dorsal (D), medial (M), and lateral (L) cortices). The compression (dorsal cortex) and neutral axis (M and L cortices) regions are significantly different from the "T" region (plantar cortex) only in the untreated and deproteinized specimens.

Results

Acoustic data

Anisotropy ratios (ARs) for untreated, deproteinized (mineral only), and demineralized (collagen only) bone were significantly higher in the plantar "tension" cortex than in all other cortices ($P < 0.05$; demineralized bone showed statistical trends for plantar vs. dorsal, and plantar vs. lateral) (Table 1; Fig. 4). Particularly in deproteinized bone from the plantar cortex, the ratio of longitudinal stiffness to transverse stiffness is ~2:1 (AR ~ 2.0).

In demineralized specimens, all four cortices exhibited significantly lower ARs than untreated bone (ranging from 1.10 in the medial cortex to 1.22 in the plantar "tension" cortex; $P < 0.05$ for all cortices) (Table 1): demineralization decreased the average (of dorsal, plantar, medial, and lateral cortices) AR by ~19%, while deproteinization increased the average AR by ~20%. This illustrates a relatively greater influence of mineral orientation on longitudinal acoustic velocity. Despite the lower degree of anisotropy in demineralized bone, the untreated, demineralized, and deproteinized specimens from all cortices had ARs that were, or tended to be, statistically greater than isotropy (AR = 1.0) (Table 1).

Predominant collagen fiber orientation (CFO) data from circularly polarized images

Analysis of circularly polarized images of the ultramilled sections showed that the plantar "tension" cortex had

significantly more longitudinal CFO than the dorsal, medial, and lateral cortices (mean gray levels: plantar 101.2 ± 5.1; dorsal 135.4 ± 7.0; medial 153.8 ± 17.8; lateral 133.3 ± 25.7). The medial and lateral cortices were not significantly different.

Correlations

Predominant collagen fiber orientation (CFO), measured using circularly polarized light, was very highly inversely correlated with ARs (r values from −0.675 to −0.734, $P < 0.001$) measured in each of the three bone preparation types (Table 2). Predominant CFO also showed very high negative correlation with the longitudinal coefficient of elastic modulus (C_L) in demineralized bone ($r = -0.701$, $P < 0.001$) when compared to C_L in deproteinized and untreated specimens (r values ~0.220).

Strain magnitude and angle (i.e., principal strain orientation) correlated significantly with ARs. As the angle of the principal strain decreased and approached 0° (i.e., along the longitudinal axis of the bone), the AR of untreated, demineralized, and deproteinized bone increased (respectively: $r = -0.807$, $P < 0.001$; $r = -0.730$, $P < 0.001$; $r = -0.758$, $P < 0.001$) (Table 2). Additionally, ARs showed high positive correlations with strain magnitude (r values from 0.672 to 0.802, $P < 0.001$) (Table 2).

Table 2
Correlation matrix (r values)

	CFO	Strain Mag.	Strain Angle	AR untreated (mineral + collagen)	AR demineral. (collagen only)
A					
Strain Magnitude	−0.624[a]				
Strain Angle	0.525[a]	−0.627[a]			
AR untreated (mineral + collagen)	−0.722[a]	0.721[a]	−0.807[a]		
AR demineral. (collagen only)	−0.734[a]	0.802[a]	−0.730[a]	0.992[a]	
AR deprotein. (mineral only)	−0.675[a]	0.672[a]	−0.758[a]	0.974[a]	0.963[a]
B					
C_L untreated (mineral + collagen)	−0.219				
C_L demineral. (collagen only)	−0.701[a]				
C_L deprotein. (mineral only)	0.220				

(A) Correlation coefficients (r values) for comparisons of strain angle, strain magnitude, and predominant collagen fiber orientation (CFO) to anisotropy ratios (ARs) of untreated, demineralized, and deproteinized bone. (B) Correlation coefficients for comparisons of CFO to C_L (longitudinal elastic modulus coefficient) of untreated, demineralized, and deproteinized bone.
CFO = predominant collagen fiber orientation (CFO), which is expressed as mean gray levels; Strain Mag. = strain magnitude (with negative values for compression and positive values for tension); Strain angle = the angle that maximum principal strain deviates from the longitudinal axis of the bone; deprotein. = deproteinized (mineral only); demineral. = demineralized (collagen only); AR = anisotropy ratio; C_L = longitudinal coefficient of elastic modulus.
[a] $P < 0.001$.

Discussion

If the predominant orientation of collagen fibers directly influences mineral orientation, then it follows that collagen orientation would primarily affect intra-fibrillar mineral crystals (i.e., those that form in the "hole zones" between tropocollagen molecules, which are arranged in a staggered array to form the collagen fibril). In support of this idea, Christoffersen and Landis ([9] p. 442) noted that the hole zones appear to have inherent spatial constraint that could confine the orientation of intra-fibrillar mineral:

> Moreover, we suggest that the protein components and bonding sites within a hole zone become restricted in their degrees of freedom following the [mineral] nucleation events and that such a result causes increased limitation in position and orientation of bonding sites of the organic molecules composing the adjacent overlap zones …

In contrast to intra-fibrillar mineral, acoustic microscopy studies of Turner et al. [57] and Takano et al. [54] suggest that, in osteonal bone of canine femora, the predominant orientation of the *extra-fibrillar* mineral crystallites is strongly influenced by mechanical strain-related characteristics, including strain mode and/or magnitude. Results of the present study also suggest that strain mode, magnitude, and/or orientation (principal strain angle) might play a role in determining extra-fibrillar mineral orientation. In this perspective, Turner and co-workers [18,53,54,57] suggested the possibility that functional loading influences growth of extra-fibrillar mineral. This is implied by the observation that non-hydrostatic thermodynamics predict crystal growth in some inorganic minerals in a direction parallel to principal stress [36]. In many long bones, the c axes of mineral crystals should therefore generally be aligned with the longitudinal axis of the bone. There are limited data that support this hypothesis [2,15,43,59]. Load-induced changes in the spatial distribution of highly ordered (structural) interstitial water have been implicated as a possible mechanism for mediating regional variations in collagen/mineral alignments that emerge during developmental adaptation [61].

While the anisotropy ratio (AR) discussed in this study is a *mechanical* anisotropy ratio, we based our hypotheses on previous observations showing that this is strongly correlated with *structural* anisotropy in osteonal bone [18,54]. This exposes a potential limitation of our study; namely, since apatite is much stiffer than collagen [11], it may also be much more anisotropic mechanically. If one were to consider the hypothetical situation of a single collagen fibril lying next to a single mineral platelet (extra-fibrillar) with the same size and alignment of their long axis (i.e., having the same structural anisotropy), then this would be an example of two structures with the same structural anisotropy but different mechanical anisotropy. Since data needed for rigorously examining this issue are limited [11] and we do not know the mechanical anisotropy of mineral platelets, it is difficult to make definitive conclusions about the association between the structural anisotropy of mineral and collagen phases and the mechanical anisotropy of bone tissue. Previous acoustic microscopy studies

of demineralized turkey leg tendon (parallel-fibered collagen) demonstrate a mean AR value of 1.6 [52]. The AR of mineralized (untreated) turkey tendon is about 2.0 or ~25% greater than the AR for tendon collagen. These findings demonstrate that mineralization of highly organized collagen further reinforces the anisotropic structure and substantially increases the AR. For the deer calcaneal cortex, the ARs were increased by 14% (medial) to 27% (plantar) by the mineral phase. In the plantar cortex, the mineral reinforced the bone structure and increased the AR to a similar degree as in mineralized turkey tendon. This is not surprising since the plantar cortex is a habitual "tension" region that would be expected to have predominantly longitudinally oriented fibers similar to the turkey leg tendon. Consequently, the mineral may align with the collagen more completely in the plantar cortex. This construction is not present in the other three cortical locations of the deer calcanei. These data suggest a dissociation of mineral–collagen alignment in the compressive or shear regions of the cortex.

It is also possible that differences in crystallite maturity and size and/or the presence of different mineral phases (e.g., poorly vs. highly crystalline hydroxyapatite or variations in the degree of carbonate substitution) could confound interpretations of structural anisotropy as inferred from mechanical anisotropy measurements [11]. However, previous studies using X-ray diffraction and Fourier-transformed infrared spectroscopy have shown that the same degree of mineral maturity/crystallinity and carbonate substitution of the mineral phase occurs between the cortical regions examined in the present study, in addition to the absence of other apatite or non-apatite mineral phases [5,17].

Although the deproteinized specimens showed high anisotropy in *all* of the calcaneal cortices, AR data in the plantar "tension" cortex suggest that predominant mineral orientation is more closely aligned with the longitudinal axis of the bone (AR = 2.0) in this region when compared to the other cortices (ARs from 1.47 to 1.54). This, in addition to exhibiting the highest AR in untreated bone (AR = 1.6), suggests that the ultrastructure of the plantar cortex is organized – primarily via the preferential orientation of mineral crystallites – in order to improve longitudinal tensile modulus (other possible important 'down-stream' mechanical effects of this mineral anisotropy are discussed below). In canine radii, Takano et al. [54] report similar findings showing higher ARs (primarily attributable to mineral orientation) in regions experiencing tensile strains when compared to compression and neutral axis regions.

In their acoustic microscopy studies of canine femora, Turner et al. [57] and Hasegawa et al. [19] reported that elastic anisotropy is more strongly influenced by mineral orientation than collagen orientation. Similarly, results of the present study support this hypothesis (#2) and demonstrate that in all cortices collagen orientation has much less influence on elastic anisotropy (for example, average ARs are approximately ~20% lower in demineralized (collagen only) bone than in deproteinized bone) (Table 1; Fig. 4). Nevertheless, demineralized specimens from all four cortices of these deer calcanei exhibit ARs that, although relatively "low" (ARs from 1.13 to 1.22), are significantly different from 1.0—consistent with the interpretation that this reflects the presence of a predominant collagen fiber orientation (CFO) [56]. However, Martin et al. [32] offers the following cautionary comments on the 'low' values for demineralized bone reported in the present and in previous studies:

> It is seen that (in studies of Turner et al. [57] and Turner and Burr [56]) the mineral phase is as anisotropic as the whole [untreated] bone but the organic [collagen only] phase is *more nearly isotropic* [ARs ~ 1.18]. These data need somehow to be squared with the idea, held for some time, that it is the orientation of bone's collagen fibers that produce its anisotropic qualities [56].

One limitation of the present study is that only two orthogonal sections were used for acoustic velocity measurements. Studies examining ARs in multiple angles in coronal, sagittal, and transverse planes are needed to rigorously determine where exactly peak values for collagen and mineral

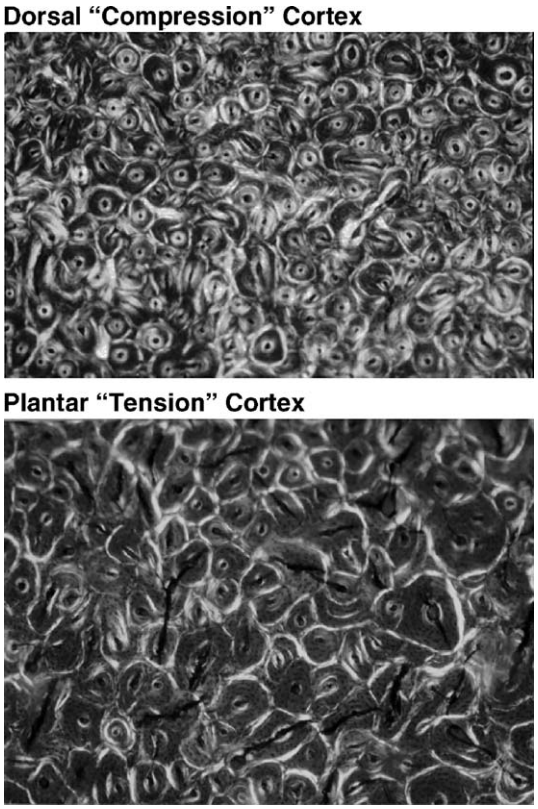

Fig. 5. Representative circularly polarized light images taken at the same magnification in the middle portion of the dorsal "compression" cortex (Top) and plantar "tension" cortex (Bottom) from a mid-transverse section of a skeletally mature mule deer calcaneus used in the present study. The tissue was unstained, undemineralized, and undeproteinized and was embedded in methylmethacrylate and ultramilled to 100 ± 5 μm. The dorsal cortex exhibits relatively greater numbers of lamellar osteons (i.e., those with alternately bright/dark birefringence), and the plantar cortex exhibits relatively greater numbers of parallel-fibered osteons (i.e., those with dark osteonal lamellae). This nomenclature for these secondary osteons (Haversian systems) is consistent with Marotti ([31], and personal communication). We suggest that a 'specific' collagen/mineral organization for accommodating differences in regional loading environments might be achieved by regional variations in the production of these, or other, different secondary osteon types.

orientation occur in artiodactyl calcanei. Acoustic microscopy methods that should be considered for more precisely determining predominant mineral and collagen orientations in bone include those of Turner et al. [57]. These investigators used parallel-piped specimens of cortical bone that were prepared so that acoustic velocities could be measured at 10° increments from 0° (longitudinal) to 90° (transverse). Additionally, orientation distribution analyses of collagen and mineral obtained using advanced high-resolution analysis techniques will help to determine if there are crystallographic *c* axes or collagen fiber orientations that are subsidiary to predominant orientations (e.g., perpendicular or oblique to the long axis of the bone) and how subsidiary and predominant collagen/mineral alignments might influence local mechanical properties. In these contexts, the high spatial resolution of synchrotron micro-focus X-ray beam analyses (λ = 0.78 Å) holds promise for future studies. Using this technology, studies of bovine osteonal bone have shown that predominant mineral orientations can be associated with considerably more complex orientation patterns than revealed by previous studies that have used techniques with lower spatial resolution [60].

In the context of hypothesis #3, correlation analyses conducted in the present study show that in all preparation types the orientation of the principal strains highly negatively correlated with ARs (Table 2). These results suggest that as the angle of principal strain approaches 0° (along the bone's longitudinal axis) the ARs increase for the three preparation types, reflecting the probability that the predominant mineral and collagen orientations are generally more closely aligned along the bone's longitudinal axis than the transverse axis. Furthermore, strain magnitude correlated moderately/highly and positively with ARs of each preparation type, suggesting that regions of bone experiencing greater maximum strain magnitudes might be constructed in order to enhance longitudinal elastic modulus (Table 2). When untreated osteonal bone is viewed in circularly polarized light, higher mean gray levels (brighter birefringence) are thought to represent predominant transverse-to-oblique ("compression") collagen fibers, and lower (darker) gray level values are thought to represent predominantly longitudinal ("tension") collagen fibers [7,34,42,49] (Fig. 5). In the present study, results of correlation analyses showed that, as ARs increase, mean gray levels decrease (that is, collagen is relatively more longitudinal with respect to the long axis of the bone). This is consistent with our previous data from the analysis of circularly polarized light images showing significant regional variations in predominant CFO in deer and sheep calcanei, with the plantar "tension" cortices having more longitudinally oriented collagen fibers [44,45]. Thus, these acoustic data, in addition to those shown in Fig. 4, corroborate hypothesis #4.

In future studies, caution must be exercised when comparing ARs between regions with different histomorphology. For example, this is important when evaluating osteonal vs. fibrolamellar bone, which often typifies comparisons between human and some non-human bones, respectively (e.g., some birds, small mammals, and some bovines (especially younger animals)) [10,12,39]. Future studies are also needed to clarify the meanings of the terms collagen and mineral "dissociation" or "misalignment" since they do not necessarily mean the absence of a "typical" angular relationship between predominant collagen and mineral orientations. For example, the angle subtended by collagen and mineral might have an optimal range in a "tension" region (e.g., 0°–20°) of bone cortex, which might be different from the optimal range in a habitual "shear" or "compression" region (e.g., 20°–40°). It may be that these differences in matrix ultrastructural anisotropy have important mechanical down-stream effects that are aimed at controlling microdamage formation and/or propagation, which is fundamentally important for ensuring adequate toughness and fatigue resistance in cortical bone [13,35,37]. Differential strain-mode-related cortical bone adaptations are expected in view of data showing that: (1) microdamage accumulation can be very different when bone is loaded in compression vs. tension vs. shear (e.g., the length, shape, and/or three-dimensional propagation of a microcrack can differ significantly in these three loading modes) [6,16] and (2) bone ultrastructure can influence the formation of these strain-mode-related microdamage characteristics [1,10,23].

Acknowledgments

The authors are grateful for the technical work of Milena Zirovich and thank Dr. Kent Bachus of the University of Utah Bioengineering Research Laboratory and Dr. Roy Bloebaum for support of this research project and Drs. Harlan Amstutz and Pat Campbell for the gracious use of equipment at the Joint Replacement Institute of Orthopaedic Hospital, Los Angeles. This research was supported by the Salt Lake City, Utah, Department of Veterans Affairs Medical Center Medical Research Funds, the Utah Bone and Joint Center, Salt Lake City, Utah, the Doctor's Education and Research Fund (DERF) of Orthopaedic Hospital, Los Angeles, California and an Orthopaedic Research and Education Grant (OREF; #01-024).

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