The Journal of Experimental Biology 209, 3025-3042
Published by The Company of Biologists 2006
doi:10.1242/jeb.02304

The influence of collagen fiber orientation and other histocompositional characteristics on the mechanical properties of equine cortical bone

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Accepted 27 April 2006

Summary

This study examined relative influences of predominant collagen fiber orientation (CFO), mineralization (% ash), and other microstructural characteristics on the mechanical properties of equine cortical bone. Using strain-mode-specific (S-M-S) testing (compression testing of bone habitually loaded in compression; tension testing of bone habitually loaded in tension), the relative mechanical importance of CFO and other material characteristics were examined in equine third metacarpals (MC3s). This model was chosen since it had a consistent non-uniform strain distribution estimated by finite element analysis (FEA) near mid-diaphysis of a thoroughbred horse, net tension in the dorsal/lateral cortices and net compression in the palmar/medial cortices. Bone specimens from regions habitually loaded in tension or compression were: (1) tested to failure in both axial compression and tension in order to contrast S-M-S *vs* non-S-M-S behavior, and (2) analyzed for CFO, % ash, porosity, fractional area of secondary osteonal bone, osteon cross-sectional area, and population densities of secondary osteons and osteocyte lacunae. Multivariate multiple regression analyses revealed that in S-M-S compression testing, CFO most strongly influenced total energy (pre-yield elastic energy *plus* post-yield plastic energy); in S-M-S tension testing CFO most strongly influenced post-yield energy and total energy. CFO was less important in explaining S-M-S elastic modulus, and yield and ultimate stress. Therefore, in S-M-S loading CFO appears to be important in influencing energy absorption, whereas the other characteristics have a more dominant influence in elastic modulus, pre-yield behavior and strength. These data generally support the hypothesis that differentially affecting S-M-S energy absorption may be an important consequence of regional histocompositional heterogeneity in the equine MC3. Data inconsistent with the hypothesis, including the lack of highly longitudinal collagen in the dorsal-lateral 'tension' region, paradoxical histologic organization in some locations, and lack of significantly improved S-M-S properties in some locations, might reflect the absence of a similar habitual strain distribution in all bones. An alternative strain distribution based on *in vivo* strain measurements, without FEA, on non-Thoroughbreds showing net compression along the dorsal-palmar axis might be more characteristic of the habitual loading of some of the bones that we examined. In turn, some inconsistencies might also reflect the complex torsion/bending loading regime that the MC3 sustains when the animal undergoes a variety of gaits and activities, which may be representative of only a portion of our animals, again reflecting the possibility that not all of the bones examined had similar habitual loading histories.

Key words: collagen fiber orientation, osteon, equine third metacarpal, cortical bone, bone mechanical properties.

Introduction

In cortical bone, regional variations in predominant collagen fiber orientation (CFO) and other histocompositional (material) characteristics may represent biomechanically important adaptations related to specific strain modes of tension, compression, or shear. For example, regions habitually loaded in compression have relatively more oblique-to-transverse collagen compared to regions loaded in tension, which have relatively more longitudinal collagen (Carando et al., 1991; Kalmey and Lovejoy, 2002; Mason et al., 1995; Riggs et al., 1993a; Skedros, 2001; Skedros et al., 2004; Skedros and Kuo, 1999; Skedros et al., 1996). Standard material tests (conducted

typically in tension but not in the context of the local habitual loading mode) in bones from various species have shown that predominant CFO can significantly influence cortical bone stiffness and strength, supporting the suggestion that regional variations in this characteristic are generally mechanically important (Ascenzi, 1988; Batson et al., 2000; Currey, 2002; Evans and Vincentelli, 1969; Evans and Vincentelli, 1974; Martin and Boardman, 1993; Martin and Ishida, 1989; Reilly and Burstein, 1974; Riggs et al., 1993b).[1]

With few exceptions (e.g. Riggs et al., 1993b), these past studies did not examine the relative influences of predominant CFO, mineralization and other ultrastructural/microstructural variables on regional mechanical properties specifically in the more physiologic context of the presumed habitual loading mode, or 'strain-mode-specific' (S-M-S) loading (e.g. compression testing specimens from cortical regions habitually loaded in compression, or tension testing specimens from cortical regions habitually loaded in tension). Mechanical testing in this context is important since: (1) *in vivo* surface strain measurements have shown that many long bones receive a consistent direction of bending, which in most long bones occurs during the time of peak loading of stance phase in typical gait-related activities (Biewener, 1993; Biewener and Bertram, 1993; Biewener et al., 1986; Coleman et al., 2002; Fritton and Rubin, 2001; Indrekvam et al., 1991; Lanyon and Baggott, 1976; Lanyon et al., 1979; Lieberman et al., 2003) and (2) cortical bone is substantially stiffer and stronger, has different fatigue behavior and likely has greater toughness and/or energy absorption in compression than in tension or shear (Boyce et al., 1998; Burstein et al., 1976; Carter and Hayes, 1977; Jepsen et al., 2001; Norman et al., 1996; Pattin et al., 1996; Reilly and Currey, 2000; Turner et al., 2001). Because long bones must accommodate regional strain-mode-related disparities in mechanical requirements in order to ensure the beneficial aspects of strain produced by loading (e.g. nutrient delivery) (Martin, 2003), S-M-S mechanical testing may demonstrate a rather different role for CFO and other histocompositional characteristics than suggested by results of non-S-M-S testing (which typically emphasize pre-yield behavior). This is also suggested by studies showing that variations in collagen organization can significantly influence post-yield behavior (e.g. energy absorption) and the microdamage processes (e.g. microcrack formation, propagation and arrest) that correlate with fatigue life (Burr, 2002; Burstein et al., 1975; Gibson et al., 2006; Hiller et al., 2003; Riggs et al., 1993b; Shelton et al., 2000; Wang et al., 2001; Zioupos et al., 1999). From this perspective we hypothesized that mechanical testing of bone in the more physiologic context of S-M-S loading would show that, among several histocompositional characteristics, CFO is more important in influencing energy absorption and other aspects of post-yield behavior than was revealed by studies conducted in non-S-M-S contexts. In view of previous histocompositional studies of equine limb bones, expected CFO correlations in S-M-S test specimens include relatively more longitudinal CFO in habitual tension regions and relatively more oblique-to-transverse CFO in habitual compression regions (Mason et al., 1995; Skedros et al., 1996).

Using the equine third metacarpal (MC3), we examined the biomechanical significance of variations in CFO, mineralization and other microstructural characteristics in the context of S-M-S *vs* non-S-M-S testing. In general, we selected the equine MC3 because it has been the focus of studies dealing with its mechanical properties and biomechanical and pathophysiological (e.g. stress fractures) implications of its histocompositional organization (Hiller et al., 2003; Les et al., 1997; Martin and Boardman, 1993; Martin et al., 1997; Nunamaker, 2001; Reilly et al., 1997; Skedros et al., 2003a; Skedros et al., 1996; Stover et al., 1992). More specific reasons for selecting this bone included the fact that independent investigators have described its habitual strain distribution at mid-diaphysis using *in vivo* strain measurements (Biewener et al., 1983a; Biewener et al., 1983b; Gross et al., 1992). In the most recent of these studies (Gross et al., 1992), a finite element mesh and three equidistantly placed triple-rosette strain gauges were used to describe strain characteristics across an entire mid-diaphyseal cross-section of a thoroughbred horse's MC3. Their results showed that the neutral axis (where strains change from compression to tension) passed through the dorsal cortex, resulting in a narrow band of tension with the remainder of the cortex experiencing a wide range of compression strains (Fig. 1). Additionally, toward the end of the stance phase of the medium-speed gait examined, the neutral axis shifted significantly, placing the dorsal-lateral region in net tension. This strain distribution was also found in quarter horses when run at similar speeds (T. S. Gross and C. T. Rubin, personal communication). Although our hypotheses were formed prospectively in the context of this strain distribution, we also consider an alternative distribution reported by Biewener et al. (Biewener et al., 1983a; Biewener et al., 1983b). In this case a habitual tension-loaded region was not found; compression dominated along the dorsal-palmar axis (i.e. where the two gauges were placed), ranging from low compression in the dorsal aspect to high compression in the palmar aspect. As described below, considering both of these habitual strain distributions was important since the habitual loading histories of the bones that we used were obtained from non-thoroughbred horses, from which we could not determine details of their loading histories.

[1]Adaptation in cortical bone commonly refers to either: (1) changes in bone structure and/or material organization in response to loading conditions outside of a normal physiologic stress/strain range, distribution and/or duration (e.g. Biewener and Bertram, 1994; Currey, 2002; Lanyon et al., 1979; Martin et al., 1998; Schaffler et al., 1985; Woo et al., 1981), or (2) the presence of regional differences in structural and/or material organization that are strongly influenced by normal functional stimuli occurring during normal development within or between bones (e.g. Bertram and Swartz, 1991; Currey, 2002; Martin et al., 1998; Riggs et al., 1993a; Riggs et al., 1993b; Skedros et al., 2004; Skedros et al., 1996). In the present investigation, 'adaptations' are considered to be biomechanically relevant regional variations in cortical bone material organization that are produced by the modeling and remodeling processes during normal skeletal development. In addition to being mediated by genetic and epigenetic influences, which are heritable, these processes can be influenced by nonheritable ('extra-genetic') stimuli, such as regional variations in microdamage incidence.

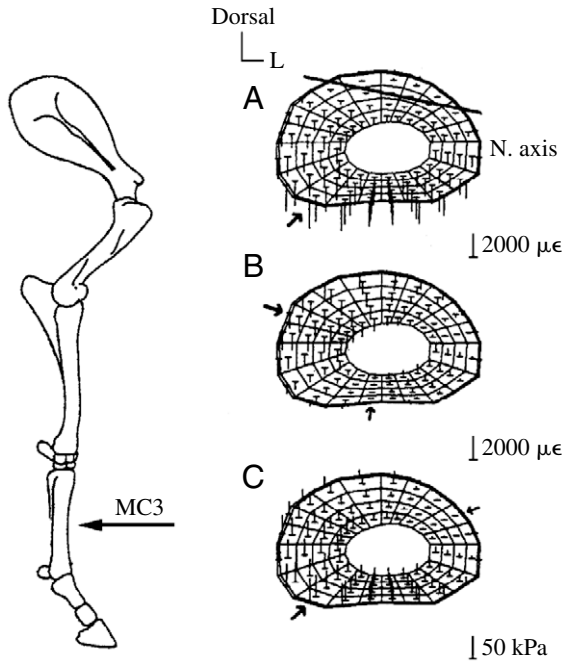

Fig. 1. Finite element model of the MC3 of a Thoroughbred. Left: Arrow indicates the mid-diaphyseal location of the third metacarpal. Right: Finite element meshes from Gross et al. (Gross et al., 1992) showing distribution of normal strain (A), shear strain (B) and strain energy density (C) acting on the mid-diaphyseal cross-section at the time of peak strain during the gait cycle [sites of maximum strain are noted by the large arrows, sites of least strain by the small arrows (B,C) or neutral axis (A)]. L, lateral. [Reprinted from *Journal of Biomechanics*, vol. 25, 'Characterizing bone strain distributions *in vivo* using three triple rosette strain gages,' pp. 1081-1087, with permission from Elsevier Science LTD, the Boulevard, Lanford Lane, Kidlington OX5 1GB, UK. This adaptation of the original figure (Gross et al., 1992) has already been used (Skedros et al., 2003a).]

Materials and methods

Right and left MC3s from ten skeletally mature horses (six quarter horses, two paint horses, two unknown), with no racing history or skeletal pathology, were dissected free of soft tissue, wrapped in saline-soaked towels, and stored in plastic bags at –20°C. We assumed no histological or material property differences between the MC3s of the left and right limbs. These bones, and the machined specimens obtained from them, were used in our previous study examining 'regional' safety factors (Skedros et al., 2003a). Mechanical test data from our previous study were also used in the present study.

Six 5 mm×5 mm×5 mm cubes for compression testing were cut from a 5 mm-thick transverse segment obtained immediately proximal to a mid-diaphyseal transection from one bone from each left-right MC3 pair. The cortical locations, the number of animals, and the number of test specimens obtained were as follows: dorsal-lateral (D-L, *N*=2; 20 total specimens), lateral (L, *N*=1; 10 total specimens), palmar-medial (P-M, *N*=2; 20 total specimens) and dorsal-medial (D-M, *N*=1; 10 total specimens) (Fig. 2A). In the context of the published strain distribution (Gross et al., 1992) and used previously (Skedros et al., 2003a), these locations represent areas that experience *in vivo* tensile strains, and a range of compression magnitudes [e.g. dorsal-medial (low) to palmar-medial (high)] (Fig. 1, Fig. 2A). This strain distribution was derived from *in vivo* strain data obtained from three triple-rosette gauges at equidistant locations and a two-dimensional finite-element mesh of a mid-diaphyseal MC3 of a 460 kg 5-year-old thoroughbred horse that was studied during a medium-speed trot (3.6 m s^{-1}). This study showed that toward the end of stance phase, the neutral axis shifted significantly, loading the lateral cortex in both tension and compression at different times during stance. Observations in racing horses imply that this shift might be greater at higher speeds, which

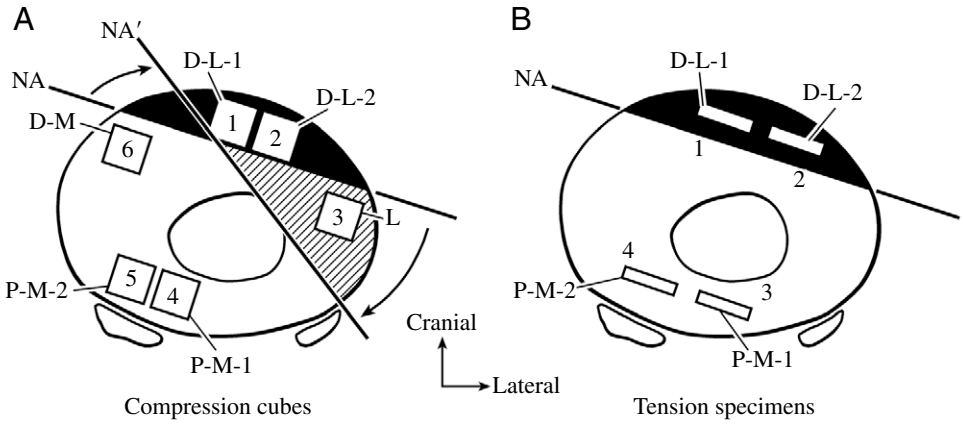

Fig. 2. Compression and tension specimen locations in the equine MC3. (A) Locations of the six compression cube specimens: dorsal-lateral (D-L, *N*=2), Lateral (L, *N*=1), palmar-medial (P-M, *N*=2), and dorsal-medial (D-M, *N*=1). Black regions denote habitual tension. White regions denote habitual compression. The lateral cortex may receive predominant tension at higher gait speeds due to shifting of the neutral axis (NA to NA′, hatched region). (B) Locations of the four tension dumbbell specimens: dorsal-lateral (D-L, *N*=2) and palmar-medial (P-M, *N*=2). [This adaptation of the original NA (Gross et al., 1992) has already been used (Skedros et al., 2003a) in a study examining 'regional' safety factors.]

places more of the lateral cortex in net tension (Nunamaker, 2001). In contrast, an independent study reported *in vivo* data obtained from two triple-rosette strain gauges at mid-diaphysis, with one located on the dorsal and one on the palmar cortex, with no finite element analysis, of three relatively smaller non-Thoroughbred horses (268, 281 and 291 kg) during gaits ranging from walking (1.3 m s^{-1}) to galloping (6.2 m s^{-1}) (Biewener et al., 1983a; Biewener et al., 1983b). These results showed the absence of net tension during the stance phase along the dorsal-palmar axis. Rather, these regions experienced net compression, and the magnitudes of the prevalent/predominant compression strains increased across the cortex from low in the dorsal region to high in the palmar region. The data from the present investigation are also considered in the context of this alternative strain distribution.

Mechanical testing was conducted on saline-moistened specimens that had equilibrated at room temperature (22°C), according to published methods (Riggs et al., 1993b). Although Riggs et al. used 8 mm×8 mm×8 mm cubes in compression testing of equine radii, we used 5 mm×5 mm×5 mm cubes because of narrower cortices in some of the MC3 locations (Fig. 3B).

From contralateral bones, a 50 mm-thick segment was sectioned transversely such that the midpoint was at 50% bone length. Rectangular slabs were milled to the proportions of the dumbbell-shaped tension specimens used in earlier work (Riggs et al., 1993b; Evans et al., 1992) (Fig. 3A). Two 'tension' (dorsal-lateral, D-L, cortex) and two 'compression' (palmar-medial, P-M, cortex) specimens were milled from each bone (Fig. 2B). However, only one tension specimen could be milled from the dorsal-lateral cortex of two bones with narrow cortices; this reduced the total number of specimens from 40 to 38. During milling, orientation of the long axes remained parallel to the longitudinal diaphyseal axis. Integrity of the specimens during the extraction and milling process included cutting and milling slowly under saline irrigation to minimize heating of the bone, saw blade and milling bit. Specimens were also examined under a dissecting (epi-fluorescence) microscope to ensure the absence of surface flaws.

Using an Instron Model 4303 (Canton, MA, USA) with a 25 kN load cell, cubic specimens were compressed to failure along the longitudinal diaphyseal axis unrestrained between parallel platens at a strain-controlled rate of 0.001 s^{-1}. Strain

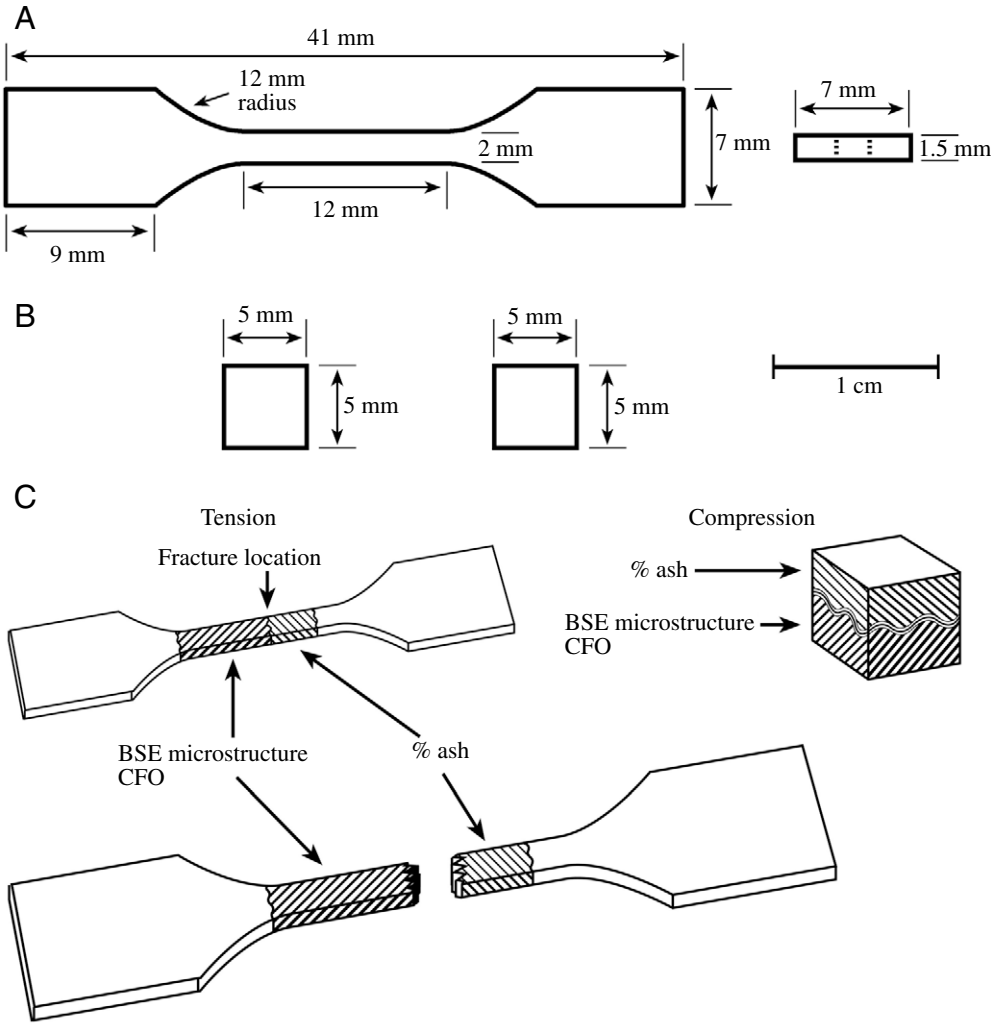

Fig. 3. Specimen geometry and locations of histocompositional analyses. (A) Tension (dumbbell-shaped) and (B) compression (cubic) specimens. In (C) the hatched areas indicate the locations where fragments were obtained for microstructural, CFO and % ash analyses. BSE denotes that backscattered electron imaging was used for analysis of microstructure. % Ash was determined on ~50–70% of the portion labeled '% ash' and indicated by the non-bolded hatched lines.

measurements were obtained from the measured crosshead displacement and corrected for machine compliance.

Tension tests were performed using an MTS® 858 Bionix testing machine (MTS® Systems Corporation, Eden Prairie, MN, USA) with a 20 kN load cell. Tensile specimens were held by identical grips that spanned the transverse breadth of the specimen ends. This allowed elongation along the anatomic longitudinal axis of the bone. Strain measurements were obtained using an MTS® 632.13F-20 extensometer attached to each specimen with rubber O-ring fasteners. Specimens were loaded to failure at a strain-controlled rate of 0.01 s^{-1}. The use of different strain rates in compression and tension was done so that the compression and tension test data from our previous study (Skedros et al., 2003a) could be compared to data previously reported (Riggs et al., 1993b) that also used these different strain rates (verified by personal communication, C. M. Riggs). Although these previous investigators did not provide a rationale for the use of these different strain rates, they both fall in a physiologic range. Additionally, Evans et al. have shown similar mechanical behaviors of equine MC3 cortical bone tested in tension at 0.01 s^{-1} and 0.001 s^{-1} strain rates (Evans et al., 1992).

Elastic modulus, yield stress and strain and ultimate stress and strain were determined for each specimen in both tension and compression testing, with the yield point defined at 0.2% strain offset (0.002 strain offset criterion) (Fig. 4B). Although this strain–offset yield criterion is arbitrary, it is commonly used to define material yield (Tommasini et al., 2005; Turner, 1989; Turner and Burr, 1993).

Microstructural analyses were conducted on fragments of the tested specimens, representing at least 50% of a transverse section of each specimen. Fragments were obtained from locations adjacent to the fracture site (Fig. 3C), embedded in methyl methacrylate (Emmanual et al., 1987) and ground and polished to a surface transverse to the longitudinal diaphyseal axis. This surface was then imaged in the backscattered electron (BSE) mode of a scanning electron microscope (Mason et al., 1995).

Depending on the cross-sectional area available for analysis, one or two high-resolution 50× BSE images (1.6×2.4 mm, Polaroid 52 film: Polaroid Corp., Waltham, MA, USA) were taken of the ground/polished surfaces of the fragments of each tested specimen. These images were used for standard stereological point-counting techniques (Parfitt, 1983; Russ, 1986), including analysis of: secondary osteon population density (On.N/T.Ar), fractional area of secondary osteon bone (On.Ar/T.Ar), estimated mean area per osteon (On.Ar), fractional area of porosity (porosity) and osteocyte lacuna population density (Ot.Lc.N/B.Ar) (see List of Abbreviations) (Skedros et al., 2000; Skedros et al., 1996). Secondary osteon counts included: (1) complete secondary osteons, (2) partially formed osteons, in which the entire circumference of the preceding resorption space was lined with new bone and (3) osteon fragments with a complete central canal. Central canals were considered part of the osteons. The On.N/T.Ar refers to the number of secondary osteons (On.N) per mm^2 of the total (T) image area (Ar), where T.Ar includes central canals but excludes primary vascular canals and Volkmann's canals. The On.Ar/T.Ar is the fraction of total bone area occupied by secondary osteon bone and is expressed as a percentage: (secondary bone area/total bone area)×100. Porosity is also expressed as a percentage and is defined as: (total porosity area/total bone area)×100. Porosity includes all vascular canals but excludes osteocyte lacunae and canaliculi. The On.Ar was calculated by dividing the total area of complete secondary osteons in each image by the total number of complete secondary osteons in each image. The Ot.Lc.N/B.Ar refers to the number of osteocyte lacunae per mm^2 of bone (B.Ar), where B.Ar excludes all non-lacunar porosity (e.g. primary vascular canals, Volkmann's canals and central canals of secondary osteons). Osteocyte lacuna population density is not meant to estimate the concentration of osteocytes since no differentiation could be made between living and dead osteocytes.

Mineral content (% ash) was determined by ashing a portion representing ~30% of each fractured specimen (Fig. 3C), and was calculated by dividing the mass of the ashed bone (M_{ab}) by the mass of the dried, defatted bone (M_{db}), and multiplying by 100 [$(M_{ab}/M_{db})\times100$] (Skedros et al., 1996).

Two 1 mm-thick sections, obtained from each of the embedded specimens used in the BSE-image microstructural analyses, were ultra-milled to 100±5 μm. In these sections, and depending on area of the specimen fragments available for analysis, predominant CFO was determined in 1–3 62× images using circularly polarized light, according to published methods (Boyde and Riggs, 1990; Skedros et al., 1996). Regional differences in CFO were quantified in terms of corresponding differences in the transmitted light intensity, where darker gray levels (lower numerical values) represent relatively more longitudinal CFO and brighter gray levels (higher numerical values) represent relatively more oblique-to-transverse CFO. Transmitted light intensity is referred to as weighted mean gray level (WMGL). The methods used to quantify regional CFO differences in cortical bone as differences in gray levels (Bromage et al., 2003; Skedros et al., 1996) have produced relative differences that are similar to the 'longitudinal structure index' described by Martin and co-workers (Martin and Burr, 1989; Martin et al., 1996a), and recently used by Takano et al. (Takano et al., 1999) and Kalmey and Lovejoy (Kalmey and Lovejoy, 2002).

A one-way ANOVA design with Fisher's PLSD test was used to assess regional variations. Analyses of the histocompositional characteristics were conducted on: (1) all sampled regions, (2) sampled regions from locations habitually loaded in compression (S-M-S compression), and (3) sampled regions habitually loaded in tension (S-M-S tension). Pearson's correlation coefficients (r values) were determined for comparisons of mechanical test results and histocompositional values. Multivariate multiple regression analyses were used to determine the percentage of variance explained by each histocompositional variable for each mechanical property based on R^2 values, allowing for the number of degrees of

freedom. Although formal analysis of statistical interactions could not be determined due to limitations in degrees of freedom, the Pearson correlation coefficients that are reported were examined in order to identify and discuss how these might reflect interactions between factors.

Forward stepwise multiple linear regression analyses were used to determine the statistical significance of each variable (i.e. histocompositional characteristic) in explaining variance (Martin and Boardman, 1993) (SAS v.8, 2002. Cary, NC, USA). Compression-tested specimens from the lateral cortex were excluded from regression analyses since this region might be adapted for the tensile strains that it could receive at higher speeds. In forward stepwise multiple regression analyses, all variables are independently considered in the first regression analysis, or step, which results in a ranking of the variables according to relative variance explained. During the second step, the top variable (i.e. highest variance explained) from the first regression is carried over, and all possible two-variable regressions containing this top variable are computed. Again, the two-combination model explaining the most variance is identified, thereby ranking the second most explanatory variable. The third step involves all possible three-variable models containing the first two explanatory variables. This process continues until all variables have been added to the model.

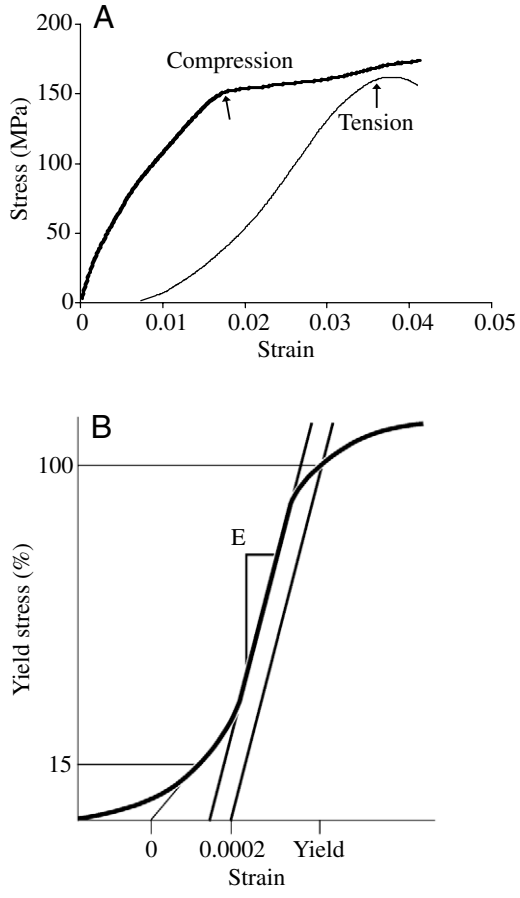

Fig. 4. Stress–strain curves. (A) Representative curves from strain-mode-specific (S-M-S) tests. The arrows indicate the yield points, which were defined using the 0.002 strain offset criterion (see B). Note that in B the tension curve has been offset toward the right so that it can be viewed clearly. (B) Illustration showing the 0.002 strain offset criterion, which is modified from Turner (Turner, 1989). This criterion is used because it helps to avoid the nonlinear 'toe' region of the stress–strain curve, which ends at about 15% of the yield stress in linearly elastic materials. E indicates the portion of the curve where elastic modulus was determined.

Results

Mechanical tests

Representative stress/strain curves and scatter plots are shown in Figs 4 and 5, and results of mechanical test data and *P*-values for regional comparisons are shown in Table 1. The following results describing strain-mode-specific (S-M-S) tests are considered in the context of the strain distribution of Gross et al. (Gross et al., 1992) (Fig. 1). The alternative strain distribution of Biewener et al. (Biewener et al., 1983a; Biewener et al., 1983b) is considered in the Discussion.

During tensile testing, premature catastrophic failure (failure at low stress) and/or failure outside the region between the extensometer clips resulted in seven specimen exclusions from the dorsal-lateral region and two specimen exclusions from the palmar-medial cortex; this reduced the sample size from 19 to 12 in the dorsal-lateral cortex and from 19 to 17 in the palmar-medial cortex. No failures occurred at the gripped ends. Three compression specimens from the lateral cortex were also excluded for premature catastrophic failure (failure at low stress); this reduced the sample size from 10 to 7.

When considering all tests, elastic modulus values were on the order of 35% higher in tension testing than in compression testing regardless of their location in the bone (i.e. when considering all S-M-S and non-S-M-S data). Yield stress was

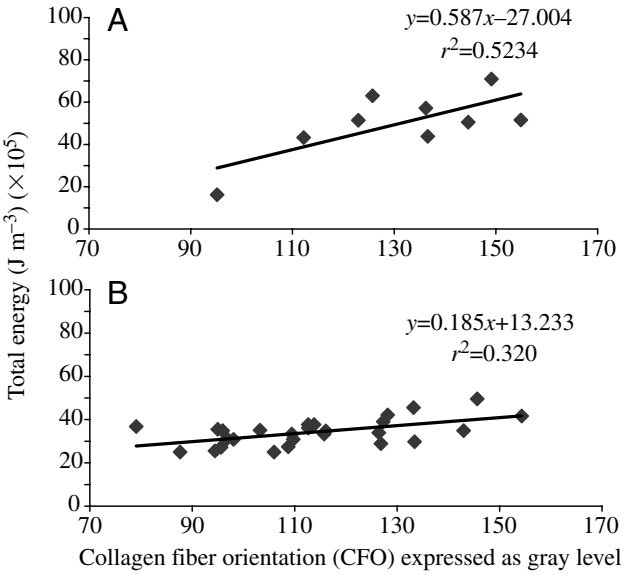

Fig. 5. Two representative scatter plots for strain-mode-specific (S-M-S) tension (A) and compression (B) testing. The equations represent the least-squares regression lines.

lower in S-M-S tension testing (148.59 MPa, dorsal-lateral) than in S-M-S compression testing (167.03 MPa, mean of palmar-medial and dorsal-medial) (P>0.2 for dorsal-lateral in tension *vs* palmar-medial in compression; P<0.001 for dorsal-lateral in tension *vs* dorsal-medial in compression). Ultimate stress of the tension-tested dorsal-lateral cortex (161.20 MPa) was significantly lower than the compression-tested dorsal-medial cortex (191.54 MPa) (P<0.0001), and only slightly lower than the compression-tested palmar-medial cortex (170.28 MPa) (P>0.14).

Regardless of location in the bone, pre-yield (elastic) energy values were on the order of 60% lower in tension tests, and post-yield (plastic) energy values were on the order of 70% lower in compression tests. Values of pre-yield (elastic) energy and post-yield (plastic) energy show significant S-M-S differences between the tension-tested dorsal-lateral cortex, and the compression-tested palmar-medial and dorsal-medial cortices (P<0.0001 for pre-yield energy; P<0.01 for post-yield energy).

The results from the S-M-S testing of both the dorsal-lateral and palmar-medial cortices show that in a number of ways the bone material is unexpectedly 'weaker' in S-M-S testing than non-S-M-S testing. For example, the dorsal-lateral cortex had lower pre-yield energy and yield and ultimate stresses in S-M-S tension testing when compared to non-S-M-S compression testing. Additionally, the palmar-medial cortex had lower post-yield and total energy, ultimate strain and elastic modulus in S-M-S compression testing when compared to non-S-M-S tension testing.

Histocompositional analyses

When considering data in a non-S-M-S perspective, there were generally no significant On.N/T.Ar, On.Ar/T.Ar, On.Ar, Ot.Lc.N/B.Ar, or porosity differences in either tension-tested or compression-tested specimens, and mineral content (% ash) variations were minimal (<1.5%) (Table 2). Predominant CFO values were also similar in tension-tested specimens (P>0.5) even though they are presumed to come from habitual 'tension' or 'compression' regions (Table 2, rows A and B). Among compression-tested specimens, there were also no significant differences in predominant CFO between the dorsal-lateral 'tension' and dorsal-medial 'compression' cortices (P>0.5), but each of these locations had significantly more oblique-to-transverse collagen than the lateral cortex (P<0.01). This difference, representing gray levels ranging from 132 to 94, has been shown to be significant in affecting stiffness and strength in material testing of cranial 'tension' and caudal 'compression' cortices of equine radii (Riggs et al., 1993b).

Correlation matrices suggest that some variables (especially predominant CFO and On.N/T.Ar (and/or On.Ar/T.Ar)) can be interdependent (Table 3A–D). Two unexpected findings in the histocompositional data included: (1) the dorsal-lateral 'tension' specimens tested in tension and compression had significantly different histocomposition in two out of seven characteristics (On.N/T.Ar, On.Ar/T.Ar; rows A and C in Table 2), and (2) the palmar-medial 'compression' specimens

Table 1. *Summary of mechanical properties in cortices of equine MC3s*

			Energy [(J m^{-3})×10^5]			Yield		Ultimate		Elastic
Test mode	Cortical region	*N*	Pre-yield	Post-yield	Total	Stress (MPa)	Strain (%)	Stress (MPa)	Strain (%)	modulus (GPa)
Tension										
A	Dorsal-Lateral (T)	12	14.93 (2.26)^{C,D,E,F}	37.51 (12.96)^{C,D,E,F}	52.44 (14.38)^{D,E}	148.59 (15.45)^{C,F}	1.00 (0.07)^{C,D,E,F}	161.20 (17.73)^{C,F}	3.38 (0.79)^{D,F}	17.81 (1.86)^{C,D,E,F}
B	Palmar-Medial (C)	17	15.22 (3.27)^{C,D,E,F}	42.30 (14.82)^{C,D,E,F}	57.52 (14.95)^{D,E,F}	148.07 (17.36)^{C,F}	1.02 (0.15)^{C,D,E,F}	163.85 (17.02)^{C,F}	3.73 (0.84)^{D,E,F}	17.26 (2.21)^{C,D,E,F}
Compression										
C	Dorsal-Lateral (T)	20	29.89 (6.97)^{A,B,D,E}	18.26 (16.39)^{A,B}	48.14 (35.44)^{D}	172.04 (20.79)^{A,B,D,E}	1.76 (0.50)^{A,B,D,E,F}	182.87 (16.54)^{A,B,D}	2.89 (2.50)	11.88 (3.04)^{A,B,F}
D	Palmar-Medial (C)	20	22.78 (4.14)^{A,B,C,F}	10.29 (6.22)^{A,B}	33.08 (5.28)^{A,B,C}	157.71 (23.25)^{C,F}	1.45 (0.15)^{A,B,C}	170.28 (15.13)^{C,F}	2.11 (0.49)^{A,B}	12.98 (2.56)^{A,B}
E	Lateral (C) (C,T?)	7	21.21 (6.36)^{A,B,C,F}	11.54 (11.12)^{A,B}	32.75 (9.64)^{A,B}	146.52 (40.70)^{C,F}	1.44 (0.22)^{A,B,C}	168.82 (13.37)^{F}	2.25 (0.84)^{B}	12.04 (3.87)^{A,B}
F	Dorsal-Medial (C)	10	28.39 (3.66)^{A,B,D,E}	8.04 (7.22)^{A,B}	36.44 (6.94)^{B}	185.68 (23.85)^{A,B,D,E}	1.53 (0.14)^{A,B,C}	191.54 (21.72)^{A,B,D,E}	2.00 (0.57)^{A,B}	14.31 (2.76)^{A,B,C}

Values are means (± s.d.).
Most mechanical properties have been reported previously (Skedros et al., 2003a).
N=number of specimens.
(T)=Specimen obtained from region subject to habitually prevalent/predominant tensile strains.
(C)=Specimen obtained from region subject to habitually prevalent/predominant compressive strains.
The lateral cortex receives predominant tension at higher gait speeds.
$^{A\text{-}F}$Significantly different (P<0.05) from test mode/region indicated by superscripted letter.

Table 2. *Regional comparisons of On.N/T.Ar, On.Ar/T.Ar, On.Ar, porosity, % ash, predominant CFO and Ot.Lc.N/B.Ar*

Test mode	Cortical region	*N*	On.N/T.Ar ($N\ mm^{-2}$)	On.Ar/T.Ar×100 (%)	On.Ar ($mm^2\ osteon^{-1}$)	Porosity×100 (%)	Ash (%)	CFO (WMGL)	Ot.Lc.N/B.Ar ($N\ mm^2$)
Tension									
A	Dorsal-Lateral (T)	14	19.38 (5.93)^{C,D}	44.83 (11.71)^{C,D}	0.024 (0.005)	4.62 (2.33)	72.05 (0.77)*^{D}	132.17 (17.75)^{D,E}	619.85 (59.04)
B	Palmar-Medial (C)	14	20.19 (5.99)^{C,D}	48.55 (11.82)^{C,D}	0.024 (0.004)	5.70 (2.00)^{D,E}	71.61 (1.08)*	133.21 (23.03)^{D,E}	658.92 (82.94)^{C,E,F}
Compression									
C	Dorsal-Lateral (T)	20	13.80 (8.06)^{A,B}	32.63 (20.17)^{A,B}	0.024 (0.006)	5.28 (2.34)	71.79 (1.02)^{D}	121.93 (19.88)**^{,E}	604.38 (76.69)^{B}
D	Palmar-Medial (C)	20	13.57 (6.43)^{A,B}	29.09 (16.86)^{A,B}	0.022 (0.007)^{F}	3.95 (1.77)^{B}	71.02 (1.17)^{A,C,F}	109.73 (18.00)**^{,A,B}	612.62 (60.14)
E	Lateral (C,T?)†	10	14.70 (6.91)	35.84 (20.10)	0.024 (0.005)	3.91 (1.24)^{B}	71.70 (1.17)	94.36 (11.78)^{‡,A,B,C,F}	564.03 (83.26)^{B}
F	Dorsal-Medial (C)	10	16.06 (7.29)	40.56 (19.24)	0.026 (0.005)^{D}	5.30 (2.80)	72.06 (1.03)^{D}	121.50 (18.36)^{†,E}	578.62 (74.25)^{B}

Values are means and (s.d.), *N*=number of specimens.
(T), specimen obtained from region subject to habitually prevalent/predominant tensile strains; (C), specimen obtained from region subject to habitually prevalent/predominant compressive strains.
†This region receives predominant tension at higher gait speeds.
*N=19; **N=18; $^{\dagger}N$=9; $^{\ddagger}N$=8.
$^{A–F}$Significantly different (P<0.05) from test mode/region indicated by superscripted letter.

tested in tension and compression had significantly different histocomposition in four out of seven characteristics (On.N/T.Ar, On.Ar/T.Ar, porosity, CFO; rows B and D in Table 2). Additionally, in each location these variations were present in 50% to 70% of the bone pairs.

Regression analyses

Results of stepwise regression analyses show that in S-M-S compression tests (Table 4A), CFO was the most important histocompositional variable in explaining total energy, and second-most important in pre-yield and post-yield energy absorption. In S-M-S tension testing (Table 4B), CFO was most important in explaining post-yield energy and total energy. Compared to energy absorption data, CFO was less important in explaining elastic modulus, and yield and ultimate stress in S-M-S tension and compression tests. Porosity in S-M-S tension tests, and % ash and Ot.Lc.N/B.Ar in S-M-S compression tests best explained variability in these three mechanical parameters. For example, in S-M-S compression Ot.Lc.N/B.Ar was the first or second most important explanatory variable in pre-yield energy, elastic modulus, yield stress and ultimate stress. Porosity was the first or second most important in explaining all six S-M-S tension tests. In contrast, % ash was the first or second most important explanatory variable in four of the six S-M-S compression tests.

In S-M-S compression tests, only 34–70% of the total variance is explained by all seven variables in the measured mechanical parameters. In contrast, the same variables explained comparatively greater variance in S-M-S tension tests.

Discussion

Among the parameters examined, predominant collagen fiber orientation (CFO) most strongly influenced total energy in strain-mode-specific (S-M-S) compression testing, explaining 32% of variance (P<0.01), and post-yield energy and total energy in S-M-S tension testing, explaining 52–54% of variance (P<0.03). In contrast, non-S-M-S tests showed that the variability in total energy in compression explained by predominant CFO was much less (19%, P<0.01), and in non-S-M-S tension testing, post-yield energy and total energy were best explained by porosity (13% and 17%, P>0.05) (data not shown). Furthermore, in S-M-S testing CFO explained less variability in elastic modulus and yield stress than porosity, % ash, and osteocyte lacuna population density (Ot.Lc.N/B.Ar). Although consistent with the hypothesis that collagen organization most strongly influences post-yield behavior, which the data showed was most significant in tension, the results of the S-M-S tests are novel in the context of past studies that have not examined relative influences of histocompositional characteristics in this more physiologic context. For example, in a previous study Martin and Ishida investigated the relative importance of CFO, porosity, mineralization and apparent density [i.e. bone mass per bulk volume (or ‘volume fraction’ of bone)] on tensile strength of

bovine cortical bone in non-S-M-S tests (Martin and Ishida, 1989). They showed that CFO was consistently the best predictor of tensile strength. Using bovine bone, Martin and Boardman demonstrated that among CFO, porosity, mineralization and histologic type (plexiform, osteonal, or mixed), CFO ranked highly as a predictor of bending properties (Martin and Boardman, 1993). Such studies support a conventional view that CFO primarily affects strength- or stiffness-related material properties of cortical bone (Ascenzi, 1988; Evans and Vincentelli, 1974; Reilly and Burstein, 1974;

Table 3. *Pearson correlation coefficients (r values) for histocompositional characteristics*

	CFO	On.N/T.Ar	On.Ar/T.Ar	On.Ar	Porosity	% Ash	Ot.Lc.N/B.Ar
(A) Strain-mode-specific compression testing							
CFO	1.000						
On.N/T.Ar	0.614[a]	1.000					
On.Ar/T.Ar	0.668[a]	0.897[a]	1.000				
On.Ar	0.278	–0.043	0.321	1.000			
Porosity	0.112	–0.052	0.103	0.291	1.000		
% Ash	–0.130	–0.001	-0.002	0.050	–0.095	1.000	
Ot.Lc.N/B.Ar	–0.325	–0.480[a]	–0.465[a]	–0.032	0.022	0.027	1.000
(B) Strain-mode-specific tension testing							
CFO	1.000						
On.N/T.Ar	0.607[b]	1.000					
On.Ar/T.Ar	0.673[a]	0.863[a]	1.000				
On.Ar	–0.265	–0.730[a]	–0.324	1.000			
Porosity	–0.181	0.369	0.275	-0.347	1.000		
% Ash	–0.366	–0.504	–0.435	0.315	–0.125	1.000	
Ot.Lc.N/B.Ar	–0.177	0.122	–0.140	–0.300	0.291	–0.305	1.000
(C) All compression-tested specimens							
CFO	1.000						
On.N/T.Ar	0.497[a]	1.000					
On.Ar/T.Ar	0.560 [a]	0.912[a]	1.000				
On.Ar	0.273[b]	-0.057	–0.271[b]	1.000			
Porosity	0.300[b]	0.054	0.130	0.137	1.000		
% Ash	–0.235	–0.011	–0.056	–0.033	–0.191	1.000	
Ot.Lc.N/B.Ar	–0.150	–0.280[b]	–0.397[a]	–0.197	0.174	0.058	1.000
(D) All tension-tested specimens							
CFO	1.000						
On.N/T.Ar	0.447[b]	1.000					
On.Ar/T.Ar	0.465[b]	0.839[a]	1.000				
On.Ar	–0.186	–0.590[a]	-0.102	1.000			
Porosity	–0.111	0.171	0.171	–0.114	1.000		
% Ash	0.105	–0.318	–0.363	0.099	–0.016	1.000	
Ot.Lc.N/B.Ar	–0.315	–0.302	–0.343	0.032	0.235	–0.074	1.000

Note: The analysis in A excludes the lateral cortex.
[a]$P<0.01$; [b]$P<0.05$.

Vincentelli and Evans, 1971). However, as noted, these and other studies have sustained this view without examining mechanical behavior in the context of S-M-S loading. Additionally, these previous studies have typically examined non-equine bone, and the extent to which inter-species comparisons can be made between mechanical properties of bovine and equine limb-bone tissue is not well understood (Martin et al., 1997).

Limitations of the present study may include our prospective preference, and hence selection bias, for the habitual strain distribution derived from the finite element analyses of Gross et al. (Gross et al., 1992). However, the alternative strain distribution of Biewener et al. (Biewener et al., 1983a; Biewener et al., 1983b) is considered below. Examining cortical bone of animals from which *in vivo* strains and finite element analysis were obtained would have strengthened our comparisons. The importance of this limitation is reduced by the observation that the strain distribution obtained by Gross et al. (Gross et al., 1992) is highly consistent in quarter horses and Thoroughbreds when run at similar speeds (T. S. Gross and C. T. Rubin, personal communication). Additional limitations are revealed by the results of the multiple regression analyses for S-M-S testing showing that the histocompositional characteristics in tension tests explain a much greater percentage of variance than in compression tests (e.g. in S-M-S compression testing, Ot.Lc.N/B.Ar ranks first in pre-yield energy absorption, but explains only 18% of variance). Predictive power in compression tests may be improved by further increasing sample size and/or the area/volume of each specimen analyzed, and by considering additional histocompositional characteristics such as

Table 4. *Multiple regression analysis of habitual compression regions tested in compression (A) and habitual tension regions tested in tension (B) (mechanical properties vs histocompositional parameters)*

Observed mechanical property	*N*	Best explaining variable	Proportion of variance explained (%)	Best second variable	Proportion of variance explained by both variables (%)	Best third variable	Proportion of variance explained by all three variables (%)
(A) S-M-S compression							
Pre-yield energy	27	Ot.Lc.N/B.Ar (r=–0.42)	18 (P<0.03)	CFO (r=0.41)	26 (P<0.03)	% Ash (r=0.22)	33 (P<0.03)
Post-yield energy	27	% Ash (r=–0.26)	7 (N/S)	CFO (r=0.21)	11 (P<0.04)	Ot.Lc.N/B.Ar (r=0.16)	18 (N/S)
Total energy*	27	CFO (r=0.57)	32 (P<0.01)	On.Ar (r=0.26)	34 (P<0.01)	% Ash (r=–0.11)	36 (P<0.02)
Elastic modulus	27	% Ash (r=0.45)	20 (P<0.02)	Ot.Lc.N/B.Ar (r=–0.40)	37 (P<0.01)	Porosity (r=–0.31)	44 (P<0.01)
Yield stress	27	Ot.Lc.N/B.Ar (r=–0.50)	25 (P<0.01)	% Ash (r=0.40)	42 (P<0.01)	Porosity (r=–0.29)	48 (P<0.01)
Ultimate stress	27	% Ash (r=0.51)	26 (P<0.01)	Ot.Lc.N/B.Ar (r=–0.49)	52 (P<0.001)	Porosity (r=–0.31)	58 (P<0.001)
*Total energy = pre- + post-yield energy. Note: This analysis excludes the lateral cortex.							
(B) S-M-S tension							
Pre-yield energy	9	Porosity (r=–0.74)	54 (P<0.03)	% Ash (r=–0.28)	64 (P<0.05)	On.Ar (r=0.61)	75 (*N/S*)
Post-yield energy	9	CFO (r=0.73)	54 (P<0.03)	Porosity (r=–0.62)	75 (P<0.02)	% Ash (r=–0.43)	82 (P<0.03)
Total energy*	9	CFO (r=0.72)	52 (P<0.03)	Porosity (r=–0.67)	79 (P<0.01)	% Ash (r=–0.44)	87 (P<0.02)
Elastic modulus	9	Porosity (r=–0.76)	57 (P<0.02)	Ot.Lc.N/B.Ar (r=–0.73)	68 (P<0.04)	On.Ar (r=0.68)	83 (P<0.03)
Yield stress	9	Porosity (r=–0.83)	69 (P<0.01)	On.Ar (r=0.71)	78 (P<0.02)	CFO (r=0.45)	91 (P<0.01)
Ultimate stress	9	Porosity (r=–0.82)	68 (P<0.01)	CFO (r=0.51)	78 (P<0.02)	On.Ar (r=0.63)	88 (P<0.02)
*Total energy = pre- + post-yield energy.							

collagen cross-links, mineral crystal size and orientation, population densities of specific secondary osteon 'types' (e.g. 'hoop' osteons, which can influence fatigue resistance by modifying osteon pullout), and microscopic mineralization heterogeneity (e.g. percentages of compliant (younger) *vs* less compliant (older) osteons or interstitial bone) (Bailey et al., 1999; Boskey et al., 1999; Burr, 2002; Gibson et al., 2006; Hiller et al., 2003; Martin et al., 1996b; Turner et al., 1995; Yeni et al., 2001; Zioupos and Currey, 1998).

Recent studies indicate that regional variations in some histocompositional characteristics (e.g. variations in collagen content/structure), which can substantially affect cortical bone toughness, are not necessarily linked with, nor explain, a relatively small percentage of variance in the tissue's mineral density, porosity, elastic modulus, yield strength and ultimate strength (Currey et al., 1996; McCalden et al., 1993; Reilly et al., 1997; Wang et al., 2001). From this perspective, additional characteristics from the residual portions of our tested specimens will be used in future studies that examine these possibilities. This is not unexpected since many of the material characteristics are interdependent (e.g. On.N/T.Ar, On.Ar and On.Ar/T.Ar are all produced by the remodeling process). Important interactions were also detected in our results, and are illustrated by the high correlations shown in Tables 3A–D (especially between CFO and On.N/T.Ar and CFO and On.Ar/T.Ar in S-M-S tests).

Strain-mode-specific tension testing in the dorsal-lateral 'tension' region showed unexpectedly more oblique-to-transverse CFO (i.e. the higher WMGLs typically associated with compression) than the other regions similarly tested. An explanation for this finding may be that the strain distribution of Gross et al. (Gross et al., 1992) does not apply for the majority of the bones from the non-Thoroughbreds that we examined. Although some of our animals might have experienced tension strains that were of magnitudes/durations that were adequate for evoking the 'expected' collagen adaptation in the dorsal/lateral cortices (i.e. lower WMGL, representing more longitudinally oriented CFO) (Carando et al., 1991; Kalmey and Lovejoy, 2002; Mason et al., 1995; Riggs et al., 1993a; Skedros et al., 2003a; Skedros et al., 2004; Skedros et al., 1996), it is possible that the majority of our horses did not. In turn, the strain distributions across the mid-diaphyseal MC3s of our non-Thoroughbreds may have experienced net compression as described by Biewener et al. (Biewener et al., 1983a; Biewener et al., 1983b). This would be expected to produce relatively higher WMGLs (i.e. relatively more oblique-to-transverse CFO) as a consequence of the habitual compression. If the strain distribution of Biewener et al. (Biewener et al., 1983a; Biewener et al., 1983b) is actually more applicable to our sample of bones, then this helps to explain why the dorsal-lateral cortex exhibited CFO more indicative of a compression environment.

Paradoxes and inconsistencies: Dissimilar histocomposition in similar locations

It is difficult to explain why the dorsal-lateral specimens tested in tension and compression (Table 2, rows A and C) and the palmar-medial specimens tested in tension and compression (Table 2 rows B and D) did not have similar histologic characteristics. In other words, specimens from essentially the same location in the MC3 should have similar histologic organization. Dissimilar organization occurred in two out of seven characteristics (On.N/T.Ar, On.Ar/T.Ar) for the dorsal-lateral cortex, and four out of seven characteristics (On.N/T.Ar, On.Ar/T.Ar, porosity, CFO) for the palmar-medial cortex. These 'paradoxes' were not infrequent, occurring in 50% to 70% of the ten left-right bone pairs for each of the six cases noted. The relatively small sampling volumes, especially for the dumbbell specimens used for tension testing, might influence these data. It is possible that inadequate sampling was further skewed by natural variation within the locations examined, which could be influenced by the laterality of gait ('handedness') that has been shown in galloping horses (Deuel and Lawrence, 1987). But the random selection of bones for compression *vs* tension testing would be expected to minimize this possibility since roughly equal left and right bones were used for each testing mode.

We suggest that it is more likely that these paradoxical and inconsistent findings reflect the possibility that some of our bones had habitual strain distributions similar to that described by Biewener et al. (Biewener et al., 1983a; Biewener et al., 1983b), and others had habitual strain distributions similar to that described by Gross et al. (Gross et al., 1992). It is also possible that some may have had habitual loading conditions that were intermediate with respect to these two loading regimes. Additionally, the shifting neutral axis shown by Gross et al. represents significant torsion, which can obscure 'expected' strain-mode-related osteonal or CFO patterns in limb bones that also receive bending. For example in bones that receive habitual torsion superimposed on bending (e.g. turkey ulnae and sheep tibiae), regional CFO variations and secondary osteon distributions that might be expected between the 'tension' and 'compression' regions of these relatively complexly loaded bones do not occur; shear stresses may have 'priority' in evoking adaptation when compared to these other strain modes (Skedros, 2001; Skedros and Hunt, 2004). Hence, we have speculated that the relatively uniform oblique/transverse CFO across mid-diaphyseal cross-sections of these bones *is* an adaptation for prevalent/predominant shear (Skedros and Hunt, 2004). If these interpretations are correct in whole or in part, the paradoxical findings reported in the present study may similarly represent relatively heterogeneous regional histologic organization that correlates with relatively heterogeneous loading histories in the sample studied. Indirect support for this possibility is further suggested by the observation that equine radii from various breeds show highly consistent regional histologic organization that correlates with habitual tension- and compression-loaded (cranial/caudal) cortices (Riggs et al., 1993a; Riggs et al., 1993b; Mason et al., 1995). In contrast to the equine MC3, the spatially consistent structure/function relationships in the equine radius are also correlated with this bone's longitudinal

curvature and oblong cross-sectional geometry, which are synergistic in highly constraining bending in the cranial-caudal direction (Bertram and Biewener, 1988; Skedros et al., 1996). Similar to our findings in equine MC3s, unexpected or paradoxical regional CFO patterns in mid-diaphyses of human femora have been reported in a study where it was hypothesized that habitual medial-lateral (compression) to anterior-lateral (tension) bending in this region would be of sufficient intensity/duration to evoke corresponding S-M-S CFO patterns (Goldman et al., 2003). Although the strain environment at the mid-diaphyseal femur has never been measured experimentally, *in vitro* strain measurements on femora loaded in simulated single-legged stance contradict this idea of a habitual medial-lateral bending moment by showing increased inter-specimen variability of the strain distribution and reduction in the magnitude of the bending moment at this location (the medial-lateral bending moment is substantially greater in the subtrochanteric area) (Cristofolini et al., 1996; Oh and Harris, 1978). Consequently, the relatively complex loading environment (torsion/bending) of the femoral mid-diaphysis might explain why 'expected' tension/compression lateral/medial CFO differences are typically absent (Goldman et al., 2003; Portigliatti Barbos et al., 1984; Portigliatti Barbos et al., 1987), but are present in the proximal diaphysis and subtrochanteric regions (Skedros et al., 1999) where a habitual medial-lateral (compression-tension) bending moment has been shown *in vivo* during walking at peak loading of stance phase (Aamodt et al., 1997). Our explanation for these relatively heterogeneous histologic findings in mid-diaphyseal human femora is similar to that given for the present study; heterogeneous loading histories, especially if the bones studied received varying amounts of torsion and bending, would *not* be expected to produce clear regional patterns of histologic organization.

Clearly these unexpected/paradoxical results in previous studies, and those reported herein, suggest that the MC3s from the mixed breeds that we examined do not represent the 'clean' model that we had anticipated. Studies investigating the etiology of these paradoxes are warranted, especially since they may help to elucidate how activity-related differences in loading conditions among the individuals examined – a possibility that is typically not considered, even in rigorous studies (e.g. Tommasini et al., 2005) – might influence the pathogenesis of stress fractures in horses and humans (Burr, 1987; Milgrom, 2001; Nunamaker, 2001). We speculate that investigation of bones from the same breed of horses that also share similar histories of physical activities will yield greater statistical power for testing the hypothesis of S-M-S osteonal or CFO adaptation. The possibility that the mixed breeds and probable various ambulatory activities among our horses can explain their histologic paradoxes is supported by the relatively uniform regional histologic organization of the Thoroughbred MC3s that has been reported by Martin, Gibson and co-workers [(Gibson et al., 2006) and table 1 therein].

When considered in the context of the strain distribution of Gross et al. (Gross et al., 1992) (Fig. 1), our results from S-M-S testing of both the dorsal-lateral and palmar-medial cortices also show that in a number of ways the bone material is 'weaker' in S-M-S testing than non-S-M-S testing (e.g. dorsal-lateral: lower pre-yield energy and yield and ultimate stresses in S-M-S tension testing; palmar-medial: lower post-yield and total energy and ultimate strain and elastic modulus in S-M-S compression testing). If the different cortices of the equine MC3 were 'adapted' to their habitual strain environments, then it would be reasonable to expect that the values of the mechanical properties examined would be higher under S-M-S testing. In view of these results, the hypothesis of S-M-S adaptation is not strongly supported by our data. However, when considering the alternative strain distribution of Biewener et al. (Biewener et al., 1983a; Biewener et al., 1983b), which considers the dorsal region as being habitually loaded in net compression, the mechanical properties are not unexpectedly deficient (e.g. in compression the values in the dorsal-lateral cortex are relatively high for pre-yield energy, yield stress and ultimate stress).

An alternative explanation for the emergence of what might have a greater influence upon bone adaptation than tension or compression, includes the possibility that these variations are correlated with the variable presence of shear stresses, which might have greater priority than tension or compression in evoking adaptation. This idea is supported by data showing that the mechanical properties of bone material are generally superior in compression and tension when compared to shear (Boyce et al., 1998; Burstein et al., 1976; Carter and Hayes, 1977; Jepsen et al., 2001; Norman et al., 1996; Pattin et al., 1996; Reilly and Currey, 2000; Turner et al., 2001). Spatial/temporal variations in shear strain magnitudes across mid-diaphyseal equine MC3s, which are indicative of torsion, have been reported (Biewener et al., 1983b): (1) shear stress is greater in the dorsal than in the palmar MC3 during walking, (2) shear stresses decrease during slow and fast trotting and (3) shear stresses increase again dorsally during cantering. Examining a 'shear-resistance priority hypothesis' might be one avenue of research that could help to clarify some of the paradoxes and inconsistencies found in the present study [see McMahon et al. (McMahon et al., 1995) where a 'tension-resistance priority hypothesis' is considered). Advanced mechanical testing methods that more closely mirror physiologic loading conditions and mimic natural damage accumulation should also prove useful for determining if histocompositional variations between regions of the same bone represent mechanical adaptation (Gibson et al., 2006; Tommasini et al., 2005).

Influences of Ot.Lc.N/B.Ar in S-M-S and non-S-M-S tests

Results of an analytical study (Yeni et al., 2001) suggest that Ot.Lc.N/B.Ar can significantly influence the apparent stiffness of the bone matrix ('matrix' refers to hard tissue containing lacunae without microstructural pores such as central canals); however, the differences were small within the physiologic Ot.Lc.N/B.Ar range. Nevertheless, these data

challenge conventional wisdom that osteocyte lacunae are inconsequential in influencing the mechanical behavior of cortical bone (Martin, 1984). Osteocyte lacunae may also become stress-concentrators for microdamage formation (Currey, 1962; Kim et al., 2004; McCreadie and Hollister, 1997; Reilly, 2000), especially with age or excessive exercise, and osteocytes may be the mechanosensors of microdamage (Lanyon, 1993; Martin, 2003) influencing their repair. Thus decreasing their concentrations may enhance a bone's fatigue life, hinder stress and fragility fractures, and/or modify other aspects of mechanical behavior (Dunstan et al., 1993; Fyhrie and Vashishth, 2000; Martin, 1984; Martin, 2002; Reilly, 2000; Vashishth et al., 2000; Yeni et al., 2001).

Results of the present study show that Ot.Lc.N/B.Ar can have important influences on bone mechanical properties in S-M-S testing. For example, in S-M-S compression tests, Ot.Lc.N/B.Ar was first or second most important in explaining observed variation in stiffness (elastic modulus), yield and ultimate stress, and pre-yield (elastic) energy absorption. Our studies of S-M-S compression testing of the dorsal 'compression' cortex of deer calcanei show that Ot.Lc.N/B.Ar is similarly important: third most important in explaining variance in all six of the same mechanical parameters that were evaluated (Skedros et al., 2003c). Also, in both deer calcanei and equine MC3s the correlations of Ot.Lc.N/B.Ar with S-M-S compressive stiffness, and yield and ultimate stress are negative, suggesting that osteocyte lacunae may have a significant role as stress-concentrators in these *ex vivo* compression tests. Additional investigations are necessary to determine the role that the relative percentage of lacunae with and without viable osteocytes, and the corresponding density of the cellular network may play in influencing bone mechanical properties, especially in the context of influencing the propagation, arrest and targeted repair of microdamage (Da Costa Gómez et al., 2005; Vashishth et al., 2000). Fatigue testing and more rigorous 'formal' fracture toughness testing[2] may also reveal important, more physiologically relevant roles for Ot.Lc.N/B.Ar in the mechanical behavior of cortical bone that are not evident in the present study.

[2]In this study we distinguish between 'informal' and 'formal' methods for assessing energy absorption (also referred to as 'toughness'; see definition below). 'Informal' methods, such as employed in the present study, measure energy absorption as the area under the stress/strain curve obtained in typical material tests. 'Formal' tests of 'work-of-fracture' or 'toughness' include measures of energy required to propagate a crack through specimens that are specifically machined to control crack formation and the direction of crack propagation (e.g. Brown et al., 2000; Currey, 2002; Norman et al., 1996; Turner et al., 2001). Toughness refers to the amount of energy required to fracture a specimen. The more the overall amount of energy consumed, the 'tougher' the material (Zioupos and Currey, 1998). Because toughness, more rigorously defined, reflects the ability to resist crack growth and withstand brittle fracture from an applied single load, it is a function of applied stress, size of material flaws, and specimen composition and geometric thickness. This contrasts with energy absorption, a measure of work of fracture. An example in terms of mineral content would be a highly mineralized material that absorbs a large amount of energy to failure and typically brittle failure (very little energy absorbed post-yield) *vs* a less mineralized, less stiff material which, although it may or may not absorb a high amount of energy to failure, undergoes a greater degree of post-yield strain, thereby exhibiting fracture toughness.

High elastic moduli in tension: adaptation or inherent material property?

Regardless of the habitual strain mode, elastic moduli in tension testing were ~35% higher than in compression, which are also consistent with results of S-M-S and non-S-M-S testing of cranial 'tension' and caudal 'compression' cortices of mature equine radii at mid-diaphysis (Riggs et al., 1993b). Using bone from various species, such increases in elastic moduli are positively correlated with bending strength (Currey, 1999a). In turn, it is suggested that this relationship may help resist failure in the equine MC3 should accidental/atypical loading conditions occur (e.g. tension in a habitually compressed cortex) (Batson et al., 2000; Reilly et al., 1997). The alterative scenario (compression in a habitually tensed cortex) might be comparatively less deleterious since bone has higher yield in compression. This is also supported by data from the radius of a Thoroughbred horse, showing that there are different thresholds for microdamage formation in tension *vs* compression: tension microcracks appeared at lower strains than compression microcracks (Reilly and Currey, 1999).

In bone from MC3 diaphyses of eight Thoroughbred horses subjected to 4 months of race training, elastic modulus and bending strength are well correlated with impact strength or work (the area under the load-deformation curve to failure, per unit of cross-sectional area of the specimen) (Reilly et al., 1997). It was concluded that although elastic modulus is a good indicator of bending strength, it is not a good indicator of toughness (work). The possibility that there is a 'division of labor' for predominant CFO *vs* the other histocompositional characteristics in this context is supported by our results: predominant CFO is generally more influential in energy absorption and post-yield behavior, while the other histocompositional characteristics have a more dominant influence in elastic modulus, pre-yield behavior and strength. The possibility that CFO and/or other aspects of collagen content/structure are mechanically adaptive in this context is supported by results of past investigations (Burstein et al., 1975; Shelton et al., 2000; Wang et al., 2001; Zioupos et al., 1999). For example, in human bone, Wang et al. (Wang et al., 2001) showed that the percentage of denatured collagen compared to the total collagen content is significantly related to failure energy and fracture toughness (work), supporting the idea that collagen in bone is a primary arrestor of microcracks (Burr, 2002; Sobelman et al., 2004).

Regardless of habitual strain mode, values of post-yield energy were approximately 2–3 times higher in tension than in compression, while values of pre-yield energy were approximately 50–100% higher in compression than in tension. The higher elastic modulus we observed in tension-tested specimens may therefore also serve to offset a corresponding lower yield and ultimate stress. This could, in turn, lead to higher total energy absorption values for tensile specimens by increasing pre-yield energy absorption. This disparity may

reflect an arrangement aimed at keeping habitual tensile and compressive strains at the same proportion of their yield point (Currey, 1984). In the equine radius, it has been suggested that similar differences may reflect a propensity to maintain uniform 'regional' safety factors throughout the bone (Riggs et al., 1993b), but this interpretation has been challenged by data from our study of equine MC3s when considered in the context of the strain distribution of Gross et al. (Gross et al., 1992; Skedros et al., 2003a). Reconsidering this issue in the context of the strain distribution of Biewener et al. (Biewener et al., 1983a; Biewener et al., 1983b) is warranted.

Additional possible reasons for the high modulus values in tension, compared to compression, can be gleaned from considering the results of tension and bending tests of cortical bone specimens from various species reported by Currey in the perspective of bending of the whole bone (Currey, 1999b). Currey showed that because the stress at a particular strain is nearly proportional to Young's elastic modulus, for a similar yield strain, a high Young's modulus would be associated with a high yield stress and therefore a high bending moment when the specimen yields. If, furthermore, tissue modifications that enhance energy dissipation (e.g. by osteonal or interlamellar debonding) allow more post-yield strain, then the bending moment increases further, increasing the apparent bending strength. This is a context where S-M-S histocompositional variations across a bone's cross-section might be important. In turn, loading in bending results in a much smaller proportion of the volume of the structure being raised to high stresses than would be the case for pure tension loading of a machined specimen, and this reduces the likelihood of a weak part of the specimen being loading to failure (Currey, 1999b; Taylor, 2000). Hence, the high elastic modulus in tension locations shown in Table 1 might enhance performance in bending, and this might be a general characteristic of bone material (i.e. regardless of habitual strain mode). In other words, high tensile elastic modulus in this perspective might simply be a consequence/constraint of the material properties of bone and not a product of developmental adaptation. Similarly, the higher post-yield tension energy absorption, and higher pre-yield compression energy absorption, regardless of location in the bone, might also reflect an inherent material property of bone.

CFO and secondary osteon population densities

Interactions, associations and adaptation

In S-M-S compression testing, predominant CFO was a relatively important explanatory variable in total energy absorption, but relatively less important in pre-yield and post-yield energy absorption. In contrast, in S-M-S tension tests, predominant CFO was relatively important in post-yield and total energy absorption, but also was less important in pre-yield energy absorption. These differences, if adaptive, may reflect the complex interactions of modulus, strength and energy absorption (Currey, 1999a; Zioupos et al., 1999). It is possible that the S-M-S tensile modulus, which is not greatly influenced by predominant CFO, may play a much stronger role in tension pre-yield energy than the compression modulus does in compression pre-yield energy. Clarifying the interactions between these variables may be an avenue for future studies.

Les, Stover and co-workers (Les et al., 2002; Stover et al., 1995) reported indirect evidence of biomechanically significant S-M-S material adaptation in the equine MC3; the dorsal cortex is significantly less ductile and has greater fatigue life than the palmar cortex. Stover et al. attributed these differences to regional variations in osteonal pullout (Stover et al., 1995), but did not examine possible regional CFO variations, which may be the product of osteonal remodeling. This possibility is suggested by the observations of highly transverse collagen in the most peripheral aspects of osteons (i.e. 'hoop' osteons) in the dorsal and dorsal-lateral cortex of equine MC3s (Martin et al., 1996b; Skedros et al., 1996; Skedros et al., 2006), which significantly enhance fatigue resistance by modifying osteon pull-out strength, and can be beneficial by dissipating energy in fatigue loading (Gibson et al., 2006; Hiller et al., 2003). In the present study, the possibility of the existence of a biomechanically important CFO/remodeling association is supported by the positive correlations between On.N/T.Ar and predominant CFO in regions tested in S-M-S loading (r=0.614 in compression; r=0.607 in tension) (Table 3A,B). Region-predominant CFO and On.N/T.Ar have also been shown to be strongly correlated in 'tension' and 'compression' cortices of other bones including ovine, cervine and equine calcanei, and ovine and equine radii; the 'compression' cortex usually has increased On.N/T.Ar and more highly oblique-to-transverse CFO (Mason et al., 1995; Riggs et al., 1993a; Skedros, 2001; Skedros et al., 2004). Although comparisons of bone histocompositional organization between species and between different locations within the same animal or bone can be subject to the confounding influences of growth rates and histologic/developmental constraints (Chinsamy-Turan, 2005; Currey, 2003; McMahon et al., 1995; Skedros and Hunt, 2004), studies of various bones of various species that exhibit osteonal remodeling generally show that regional histocompositional variations (e.g. CFO, On.N/T.Ar, On.Ar/T.Ar, On.Ar) can modify local mechanical properties (e.g. strength, fatigue and impact resistance, energy absorbed to failure, fracture toughness) (Batson et al., 2000; Carter and Hayes, 1976; Corondan and Haworth, 1986; Evans and Bang, 1967; Evans and Vincentelli, 1974; Hiller et al., 2003; Moyle et al., 1978; Reilly et al., 1997; Saha and Hayes, 1977; Shelton et al., 2000; Skedros et al., 2003b; Yeni et al., 1997). One challenge posed by these data is to determine if one or more of these characteristics is strongly sensitive and specific for interpreting a bone's loading history. A growing body of data supports predominant CFO as pre-eminent in this regard in the context of a spectrum of habitual loading complexity, from simple bending (e.g. artiodactyl calcanei) to multiaxial or complex/torsional loading (e.g. equine MC3s, sheep tibiae and turkey ulnae) (Skedros, 2001; Skedros and Hunt, 2004; Skedros et al., 2004; Skedros et al., 1996).

Regional histocompositional adaptation, microdamage formation and stress fracture risk

The idea that regional adaptations would be expected in the equine MC3 is supported by the high incidence of two major disorders involving the dorsal MC3 cortex in race horses: 'bucked shins' and stress fractures (Norwood, 1978; Stover et al., 1993). In these maladies, it has been suggested that osteonal remodeling does not keep pace with microdamage production in prevalent/predominant 'tension' *vs* 'compression' regions (Nunamaker, 2001; Stover et al., 1993). In horses that sustain habitual dorsal/palmar variations in net tension/compression, respectively, corresponding variations in predominant CFO and/or On.N/T.Ar may represent adaptations that differentially enhance energy absorption, minimizing regional disparities in fatigue behavior. This may be an important selective advantage of the regional variations in predominant CFO and/or On.N/T.Ar that have been reported between the 'tension' and 'compression' regions of other mammalian bones subject to habitual bending (Carando et al., 1991; Kalmey and Lovejoy, 2002; Lanyon et al., 1979; Mason et al., 1995; Skedros, 2001; Skedros et al., 1999).

However, the majority of stress fractures in the equine MC3 occur in the dorsal-lateral cortex (Nunamaker, 2001). Such fractures are more common in Thoroughbreds, which probably reflects their relatively fast racing speeds and lower cross-sectional moments of area. Relationships between cross-sectional geometry, cortical robusticity (e.g. ratio of cortical area to total cross-sectional area), and tissue mechanical properties produced by variations in histocomposition, may be important considerations in this context (Tommasini et al., 2005) but were not considered in the present study. Nevertheless, in all racing breeds it is not entirely clear why microcracks tend to form more frequently in the dorsal-lateral cortex. Nunamaker has suggested that the shift from prevalent tension in the dorsal cortex during race *training* at lower speeds to compression at higher racing speeds may provide an explanation (Nunamaker, 2001). He suggests that tension-related adaptations produced by modeling and remodeling activities (specific remodeling-mediated adaptations were not stated) would not be expected to accommodate compression strains produced at greater speeds: 'It seems obvious that bone that models and remodels for tensile forces on the dorsal aspect of M3III [MC3] will be poorly adapted for the large compressive strains that are seen during racing' [p. 213 (Nunamaker, 2001)]. In turn, when the tension-adapted dorsal cortex receives more prevalent, high magnitude compression strains microcrack formation is enhanced. This probability is supported by Reilly and Currey, who showed that microdamage formed in compression is highly detrimental to tensile mechanical properties (Reilly and Currey, 2000). In tension *vs* compression, microdamage not only occurs at different thresholds (more readily forms in tension), but can exhibit different morphologic characteristics (e.g. length, shape and orientation) (Boyce et al., 1998; George and Vashishth, 2005; Joo et al., 2004; Muir et al., 1999; Reilly and Currey, 1999; Reilly et al., 1997). In MC3s from horses run at higher speeds, the probability that microdamage formation might also occur at different strain thresholds in different regions might be especially important for understanding the material organization of the dorsal-lateral region since it experiences a greater range of strain by being loaded in tension and compression when compared to the palmar-medial and dorsal-medial cortices. Also, the prevalence of shear strains, which can be more deleterious to mechanical properties than tensile strains, probably increase in the vicinity of the dorsal-lateral cortex during racing, since the neutral axis (where shear strains tend to be greatest, Fig. 1B) traverses this region at these speeds (Nunamaker, 2001). In view of these possibilities, an MC3 sufficiently exposed to sub-racing speeds would be expected to become adapted primarily for tension in the dorsal/lateral cortices. However, if higher speeds are more consistently sustained, the MC3 may become primarily adapted for compression and shear in this region. As we have suggested above, the fact that the mechanical test results reported in the present study are not strongly consistent with either of these interpretations may reflect the possibility that not all of our bones experienced dorsal-palmar bending of sufficient duration/intensity to evoke what we had considered would be 'expected' tension-compression S-M-S adaptations.

Conclusions

Despite a number of limitations and difficulties arising from this complex study, several issues are clear. (1) Predominant CFO appears to be most strongly correlated with post-yield energy absorption, especially in tension. (2) These data generally support the hypothesis that differentially affecting S-M-S energy absorption may be an important consequence of regional histocompositional heterogeneity in the equine MC3. (3) Data inconsistent with this hypothesis (e.g. lack of highly longitudinal collagen in the dorsal-lateral 'tension' region, paradoxical histologic organization in some locations, and lack of significantly improved S-M-S properties in some locations) might reflect the absence of a similar habitual strain distribution in all bones. In contrast to a history of habitual dorsal/lateral-to-palmar/medial bending, an alternative strain distribution showing net compression along the dorsal-palmar axis might be more characteristic of the habitual loading of some of the bones that were used in the present study. In turn, some of the inconsistencies that were found might also reflect the complex loading regime that the MC3 sustains when the animal undergoes a variety of gaits and activities, which may be representative of only a portion of our animals. Nevertheless, the paradoxical/inconsistent histologic findings reported in this study are useful for drawing attention to problems that can be encountered even when using models that are based on rigorous measurements of seemingly simple strain environments. In turn, ensuring detailed knowledge of the loading histories of the animals and bones studied would enhance future studies that attempt to decipher the causal mechanisms that mediate developmental structure–function relationships.

List of abbreviations

% ash	percent ash (mineral content determined by ashing the bone)
BSE	backscattered electron
CFO	collagen fiber orientation (expressed as a WMGL, see below)
D	dorsal
FEA	finite element analysis
L	lateral
M	medial
M_{ab}	mass of ashed bone
MC3	equine third metacarpal
M_{db}	mass of dried defatted bone
On.Ar	area (Ar) of an individual secondary osteon (including the area of the central canal)
On.Ar/T.Ar	fractional area of secondary osteonal bone ×100 (%; includes central canals in T.Ar)
On.N	number of secondary osteons
On.N/T.Ar	secondary osteon population density per total (T) mm^2 area (Ar) (no. mm^{-2}, includes central canals in T.Ar, but excludes primary vascular canals, Volkmann's canals and osteocyte lacunae)
Ot.Lc.N/B.Ar	ostecyte lacunae per bone area (B.Ar) (no./mm^2, excludes central canals and primary vascular channels in B.Ar)
P	palmar
S-M-S	strain-mode-specific
WMGL	weighted mean gray level (used for expressing differences in predominant CFO)

This work was supported by the Department of Veterans Affairs Medical Research Funds, Salt Lake City, UT, USA, the University of Southern California Department of Orthopaedics, Los Angeles, CA, USA and the Utah Bone and Joint Center, Salt Lake City, UT, USA. The authors thank Mark C. Nelson and Scott M. Sorenson for their contributions in completing this study, Kerry Matz for illustrations, Krista Jensen for statistical consultation, and Ken Hunt and Cameron Bevan for technical support.

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