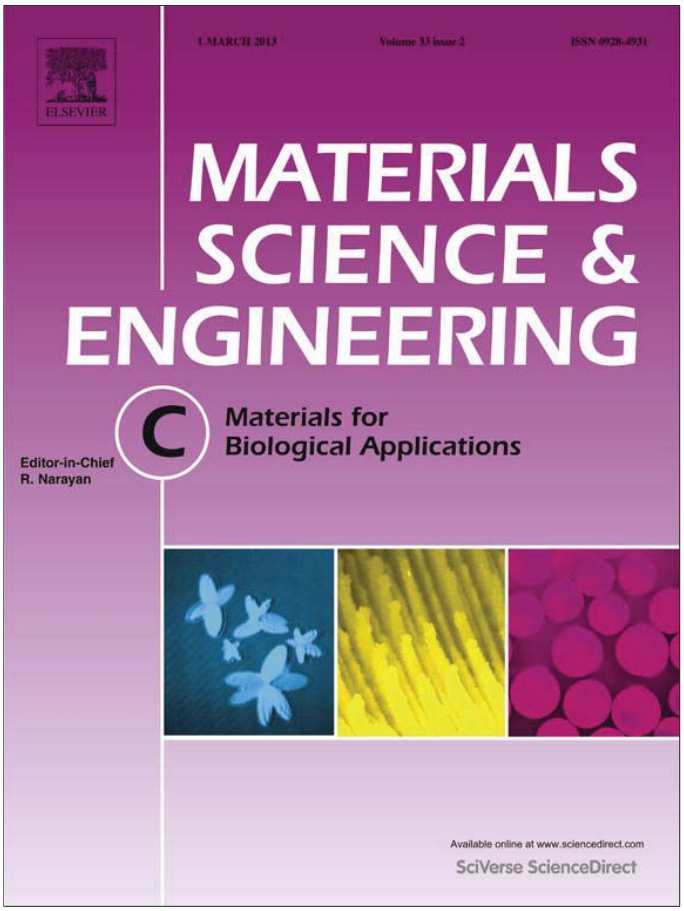

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Initial anisotropy in demineralized bovine cortical bone in compressive cyclic loading–unloading

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ABSTRACT

The mechanical properties of demineralized bovine cortical femur bone were investigated by cyclic loading–unloading compression in three anatomical directions (longitudinal, radial, transverse) within the physiological strain range. The loading responses in the radial and transverse directions were nearly linear up to 2% strain, while the response in longitudinal direction was strongly non-linear in that range. The unloading responses were non-linear for each anatomical direction, giving rise to overall loading–unloading hysteresis and cyclic dissipation of energy. The mechanical properties were observed to be anisotropic: the radial direction was found to be the most energy dissipative, while the longitudinal direction appeared to be the stiffest bone direction. The cyclic loading mostly affects the bone stiffness in the radial and transverse directions, while the longitudinal direction was found to be the least affected. These anisotropic properties can be attributed to the differences in collagen fibers alignment and different microstructural architecture in three different anatomical bone directions.

1. Introduction

Bone is a hierarchically structured composite material mainly composed of a biopolymer (mostly type-I collagen), a mineral phase (carbonated hydroxyapatite), and water. The internal structure and mechanical properties of bone have been investigated by many research groups, including seminal works of Currey [1,2]; Burstein et al. [3], and Rho et al. [4]. Bone is anisotropic biological material; therefore a significant effort was made in the past to investigate mechanical properties of bone in different anatomical directions (e.g., Reilly and Burstein [5]; Bonfield and Grynpas [6]; Hasegawa et al. [7]; Iyo et al. [8]; Macione et al. [9], Skedros et al. [10]).

Detailed examination of mechanical properties of the major bone constituents (mineral and protein) in different anatomical directions is important to better understand bone mechanical response. Hasegawa et al. [7] performed acoustic velocity measurements on dog femur in the longitudinal and transverse directions. The bone was demineralized by 10% ethylenediaminetetraacetic acid (EDTA) (100% protein), and deproteinized by 7% sodium hypochlorite (100% minerals). They concluded that the minerals play the major role in the anisotropic behavior of the whole bone, while the protein matrix is highly isotropic. Iyo et al. [8] examined the effect of mechanical anisotropy on the elastic modulus relaxation. They proposed the model for the relaxation of the elastic modulus of cortical bone which included a combination of two processes: a fast one, attributed to the relaxation of protein matrix, and a slow one, attributed to the mixture of protein and mineral matrices. Furthermore, they suggested that the slower process was responsible for the anisotropic behavior of bone. Skedros et al. [10] used acoustic microscopy to evaluate elastic modulus of untreated, demineralized and deproteinized cortical bone of wild deer calcanei. They found that anisotropy ratio (AR), calculated as ratio between longitudinal and transverse elastic coefficients, was significantly different from isotropy (where $AR = 1$) not only for untreated bone, but for demineralized and deproteinized bones as well, proving that not only untreated bone, but also its main constituents (mineral and protein phases alone) behave in an anisotropic manner.

Cyclic loading–unloading experiments on bone have been studied by many groups for both cortical and trabecular bones [11–14]. Keaveny et al. [11] performed compression loading up to 4% strain, followed by the immediate unloading to a zero stress level and reloading up to approximately same strain level on a human vertebral trabecular bone. They reported percent of elastic modulus and strength reduction and concluded that occasional overloading of bone can increase the probability of bone fracture because of the mechanical degradation of a trabecular network. Pattin et al. [12] measured the fatigue properties of human femoral cortical bone, investigating the changes in secant moduli and cyclic energy dissipation during the load-controlled experiments. They reported that loading in tension up to 2.5×10^{-3} and in compression up to 4×10^{-3} strain recovered to zero strain after unloading. Schaffler et al. [13] examined the fatigue properties of bovine cortical bone loaded up to strain magnitudes less than 2×10^{-3}. They found that bone can

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withstand several millions of cycles without fatigue failure, and, moreover, after initial stiffness degradation of about 6%, stiffness does not significantly changes. These findings suggest that physiological loading conditions within the average lifetime number of cycles do not result in fatigue failure. All experiments mentioned above were performed on samples in longitudinal direction. To the best of the authors' knowledge, there are no reports on cyclic loading–unloading experiments on demineralized cortical bone (pure bone protein matrix) in the three orthogonal anatomical directions.

Anisotropic behavior of demineralized cortical bone in compression was recently investigated by Novitskaya et al. [15], who have shown that bone protein matrix (completely demineralized bone) demonstrates anisotropic properties: the longitudinal direction was found to be the stiffest bone direction due to preferential collagen fiber orientation in this direction. The preferential orientation of collagen fibers and mineral crystals along bone growth direction is one of the main reasons for bone anisotropy. Landis et al. [16] investigated the ultrasound interaction between collagen and mineral crystals in chicken bone by high voltage electron microscopic tomography, and found that individual platelet-shaped mineral crystals were periodically arranged along collagen fibrils preferentially aligned along the longitudinal direction. Martin et al. [17,18] found out that longitudinal fiber orientation in compact bone greatly contributed to increased elastic modulus and strength in four-point bending tests. The current study expands these findings by reporting the results on strain-controlled cyclic loading–unloading compression tests on demineralized bovine cortical bone in three anatomical directions. Collagen based biomaterials are widely used to construct tissue engineering scaffolds and have found many applications from artificial bone substitutes [19] to artificial skin [20]. For each of these applications, the analysis of mechanical behavior of bone collagen under different loading conditions is of great importance. This research is of medical interest since many groups have recently investigated collagen sponge structure and properties for prospective bone substitutes [21–25].

2. Materials and methods

2.1. Sample preparation

Bovine femur bone samples were obtained from a local butcher. The slaughter age of the cattle was about 18 months. The bone was thoroughly cleaned with water. Samples were cut from the mid-diaphysis region. About 60 samples for compression testing (parallelepipeds 5 mm×5 mm×7.5 mm, 20 for the each anatomical direction) were prepared from close locations in order to minimize variations in density and mineral content. The samples were first roughly cut by handsaw and then by a diamond blade under the constant water irrigation with the prospective loading surfaces as parallel as possible. Samples were cut in all three anatomical directions (Fig. 1). The longitudinal direction was chosen to be parallel to the growth direction of the bone, the transverse direction was normal to the bone growth direction, and the radial one was orthogonal to both. Samples were stored in a refrigerator (T = 4 °C) until chemical procedure and testing were performed.

2.2. Demineralization process

Bone samples were demineralized (DM) by aging in 0.6 N hydrochloric acid (HCl) at room temperature using the procedures outlined in Toroian et al. [26] and Chen et al. [27]. Although EDTA is frequently used to demineralize bone, we chose HCl because the process is much quicker and has been used successfully in previous works [26–29]. Acid solutions were changed daily. The whole process took 7 days. All solutions were quantitatively analyzed by inductively coupled plasma optical emission spectrometry (ICP-OES) to evaluate the Ca concentration. The completeness of demineralization was verified by the Ca absence in the solutions [29].

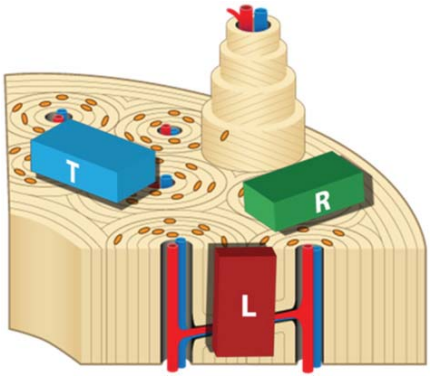

Fig. 1. Sample orientation for the three anatomical directions tested in bovine cortical bone. Samples are not shown to scale. Adapted from Ref. [15].

2.3. Compression testing

Three different sets of samples were prepared: 20 for the each anatomical direction. Specimens from all groups were submerged in Hank's balanced saline solution for 24 hours before testing, and were tested in the hydrated condition. Compression testing of DM samples was performed on universal testing machine equipped with 500 N load cell (Instron 3342 Single Column System, Norwood, MA) at a strain rate of 1×10^{-3} s^{-1}. An external deflectometer SATEC model I3540 (Epsilon Technology Corp., Jackson, WY) was used to measure the small samples displacement. Compression testing was performed in loading–unloading conditions: samples were loaded under strain-controlled loading until 1% compressive strain, then unloaded at the same rate until zero stress was reached. Three consecutive cycles up to 1% compressive strain, followed by unloading, were performed in all three anatomical directions. In addition, ten consecutive cycles up to 2% compressive strain were performed for samples in three anatomical directions. The strain levels of 1% and 2% were chosen because they are within the physiological strain region of soft biological tissues [30].

2.4. Structural characterization

Fracture surfaces of the specimens were investigated by scanning electron microscopy (SEM) using FEI-XL30 (FEI Company, Hillsboro, OR). All samples were subjected to critical point drying procedure with a purge time equal to 20 minutes, using the fully automatic critical point drier (Tousimis Autosamdri-815, Rockville, MD) before SEM imaging in order to avoid excessive shrinkage and deformation. For SEM imaging all samples were mounted on aluminum sample holders, and sputter-coated (Emitech K575X, Quorum Technologies Ltd., West Sussex, UK) with irridium for 8 seconds before imaging. Samples were observed at a 10 kV accelerating voltage.

2.5. Statistical analysis

One-way ANOVA analysis was performed to determine significant differences between the data for three anatomical bone directions. The criterion for statistical significance was $p<0.05$.

3. Results and discussion

It was previously shown that bone demineralization produced contiguous, stand-alone structure [26,28]. All microstructural features were well preserved by the demineralization process. Moreover, it

was shown that preferential orientation of collagen fibers plays an important role in bone mechanical response [15,28].

Fig. 2 shows SEM images of fracture surfaces of DM bone samples for longitudinal (Fig. 2a), radial (Fig. 2b), and transverse (Fig. 2c) directions for two different magnifications. The preferential alignment of demineralized collagen bundles (composed of several collagen fibrils) along the bone growth direction (shown by the arrow) is clearly seen from Fig. 2a. Due to our sample geometry, collagen fibrils were oriented perpendicular to the bone growth direction for both radial and transverse directions (Fig. 2b,c). This preferential orientation plays a crucial role in the DM cortical bone response during the compression.

Fig. 3 shows the representative stress–strain curves during cyclic loading–unloading compression of samples in three anatomical directions for the first three consecutive cycles. The stiffness in the longitudinal direction was significantly larger compared to the radial ($p=0.03$), and transverse ($p=0.01$) directions, while there was no significant difference in stiffness between radial and transverse directions ($p=0.24$). These results corroborate the findings of Novitskaya et al. [15], who reported the same trend for the bulk compression elastic modulus of DM cortical bone. Furthermore, the loading portion of the stress–strain curve for the longitudinal direction was strongly non-linear, while it was nearly linear for the other two directions. The unloading responses were non-linear for each anatomical direction, giving rise to overall loading–unloading hysteretic response. Additionally, a pronounced difference in dissipated energy (hysteresis area) between longitudinal/transverse and radial directions, particularly for the first cycle, was reported. These results support the conclusion of our previous study [15] on the importance of the periosteal sheath for the mechanical properties of bone in the radial direction. In this sheath, the mineralized collagen lamellae do not form an osteonal structure, but are oriented smoothly in the longitudinal direction, making a significant contribution to the mechanical properties in the radial bone direction. As a result, cortical bone was found to be more energy absorbent in the radial direction. The results of present study show that radial direction is the most energy dissipative for DM cortical bone as well, demonstrating that bone internal microstructure plays the crucial role in both untreated and DM cortical bone.

Table 1 summarizes the hydrated density, initial tangent modulus, secant modulus, and hysteresis areas for three consecutive cycles tested in three anatomical directions. The initial modulus was defined as a tangent modulus at the beginning of each cycle. The secant modulus of the ith cycle was defined as the ratio of the stress $\sigma^{(i)}$ at the end of the loading portion of that cycle and the recovered strain upon unloading during the previous cycle, i.e.,

$$E_s^{(i)} = \sigma^{(i)} / \left(\varepsilon_0 - \varepsilon_r^{(i-1)}\right), \quad (i = 1, 2, 3, \ldots),$$

where ε_0 is the maximum strain level during the loading (1% or 2% for this study) and $\varepsilon_r^{(0)} = 0$. The small gradual increase of the secant modulus with the number of cycles can be attributed to progressive collagen compaction and rearrangement of the microstructure during repeated compressive loading.

The secant moduli were progressively larger in consecutive cycles for all three anatomical directions (Table 1). Additionally, the secant modulus was more than twice lower than the initial (tangent) modulus for the longitudinal direction by the end of the third cycle, but there was a little difference between these two moduli for the radial and transverse directions. This means that DM bone was able to rearrange its internal structure in the radial and transverse directions to evenly support the stresses in three loading–unloading cycles, while it was unable to do so for the longitudinal direction, mainly because of collagen fibers preferential orientation in this particular direction. The preferential orientation of vascular channels in the longitudinal (bone growth) direction could be another reason for differences in mechanical response between longitudinal and radial/transverse directions. Previous studies on the internal microstructure of cortical bone by micro computed tomography (μ-CT) show that vascular channels are preferentially oriented in the longitudinal bone direction [31]. The microchannels deform differently under the longitudinal and radial/transverse loadings, giving its contribution into anisotropic response in cyclic loading–unloading compression. In addition, the relative change of secant modulus between the first two cycles was found to be greatest for the radial direction. The initial and secant moduli for three consecutive cycles were found to be the most consistent in the transverse bone direction. The strain offsets

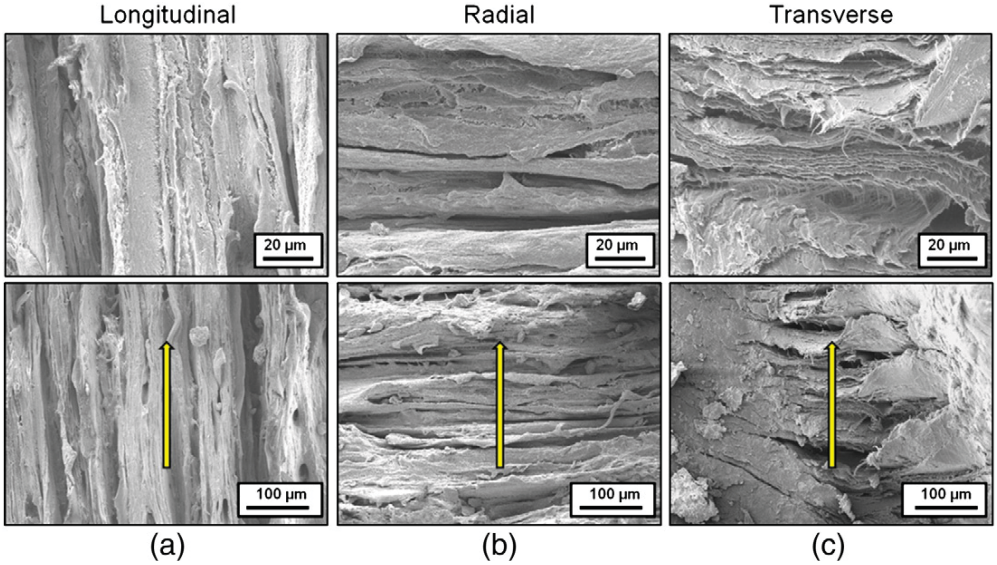

Fig. 2. SEM images of fracture surfaces of demineralized cortical bone showing collagen fibrils alignment for (a) longitudinal, (b) radial, and (c) transverse directions. Bone growth direction shown by the arrows.

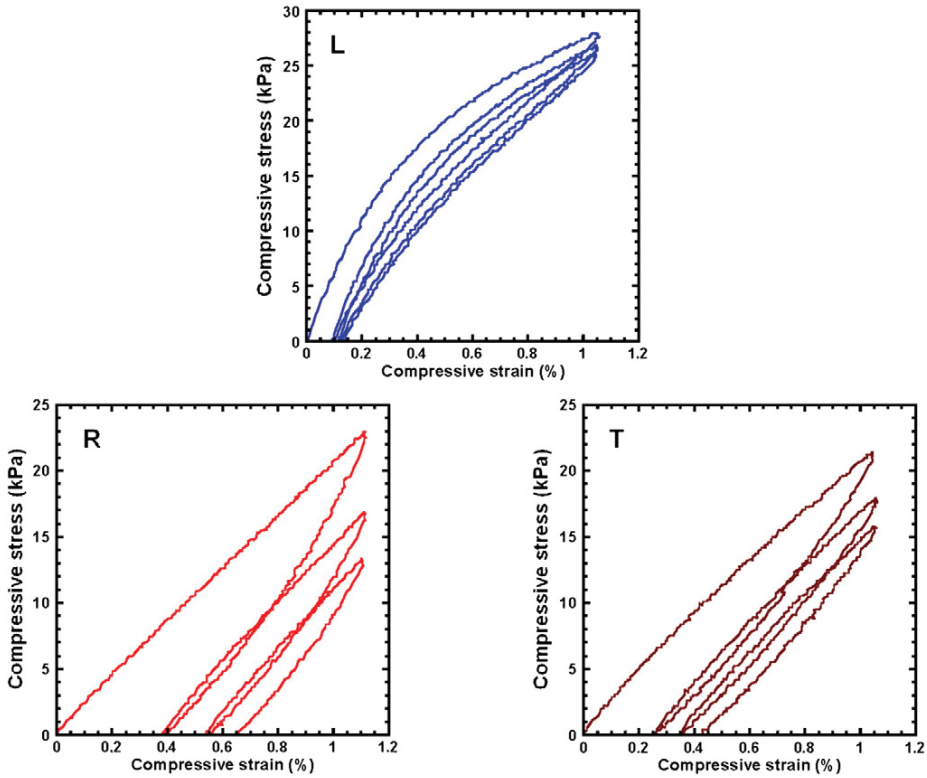

Fig. 3. Representative stress–strain curves during cyclic loading–unloading compression of demineralized samples in three anatomical directions, showing first three consecutive cycles. L = longitudinal, R = radial, T = transverse.

(the residual strain upon unloading) reached equilibrium after the first three loading–unloading cycles for longitudinal direction, while there were progressively larger strain offsets for both radial and transverse directions (Fig. 3). Additional experiments for the strain-controlled cyclic loading–unloading compression up to ten cycles were performed to verify this assumption. Fig. 4 shows representative stress–strain curves along with stress–time, and strain–time curves for the experiments with the strain levels up to 2%. The difference between areas of hysteresis loops for cycles after the third cycle was negligibly small for the longitudinal direction, while the hysteresis loops gradually became smaller up to tenth cycle for the radial and transverse directions. These findings clearly demonstrate the anisotropic behavior of DM cortical bone in cyclic loading–unloading compression. The difference between the radial and transverse directions is a consequence of the existence of the periosteal sheath with a different microstructure in the radial direction. Mineralized collagen lamellae do not form osteonal structures in the sheath, making the internal microstructure of radially oriented samples nonuniform (containing both osteonal part and peroisteal sheath part), in contrast to rather uniform transversely oriented (containing only osteonal part) samples [15].

Table 1
Hydrated density, initial tangent modulus, secant modulus, and hysteresis area for demineralized cortical bone under loading–unloading compression for different anatomical directions. Initial tangent modulus, secant modulus, and hysteresis area are presented for three consecutive cycles, shown in Fig. 3. L = longitudinal ($N = 10$), R = radial ($N = 10$), T = transverse ($N = 10$).

Bone direction		L	R	T
Density (g/cm^3)		1.21 ± 0.02	1.18 ± 0.02	1.19 ± 0.01
Initial tangent modulus, $E^{(i)}$ (MPa)	1st cycle	5.1 ± 0.9	3.6 ± 0.5	2.3 ± 0.2
	2nd cycle	5.2 ± 0.5	4.4 ± 0.5	2.5 ± 0.2
	3rd cycle	5.3 ± 0.5	4.5 ± 0.5	2.6 ± 0.2
Secant modulus, E_s (MPa)	1st cycle	2.1 ± 0.4	2.6 ± 0.4	1.8 ± 0.1
	2nd cycle	2.2 ± 0.4	3.5 ± 0.6	2.0 ± 0.2
	3rd cycle	2.3 ± 0.5	3.6 ± 0.6	2.1 ± 0.2
Hysteresis area (mJ/m^3)	1st cycle	3.7 ± 0.4	10.1 ± 2.5	3.2 ± 0.8
	2nd cycle	2.3 ± 0.4	2.3 ± 0.6	1.7 ± 0.2
	3rd cycle	2.0 ± 0.2	1.3 ± 0.4	1.2 ± 0.2

The stresses versus time curves are different for all three anatomical directions (Fig. 4). The areas of the consecutive cycles are almost the same for the longitudinal direction, but are progressively smaller for the radial and transverse directions. Moreover, from the strain–time curves it can be seen that strain accumulation is larger for the radial and transverse directions, while it is smallest in the longitudinal direction.

The main difference between cortical bone behavior reported by Jepsen et al. [14] and DM cortical bone behavior from the present study, is that for DM cortical bone the initial tangent modulus at the beginning of each cycle remains nearly the same for all three anatomical directions, while it drops significantly (about 30%) for untreated cortical bone. This supports the conclusion that DM cortical bone does not experience large stiffness degradation during the first 10 cycles of compressive cyclic loading within the physiological strain region.

It is worth mentioning that, after sufficient amount of time, the samples for all three anatomical directions returned back to their original shape and volume (in water at zero load) after the testing, proving that DM cortical bone did not experience permanent or plastic deformation during first 10 cycles of loading–unloading in the considered strain range.

Fig. 5 shows the variation of the secant/tangent moduli ratio ($E_s^{(i)}/E^{(i)}$), versus the cycle number (i) for three anatomical directions. The results in

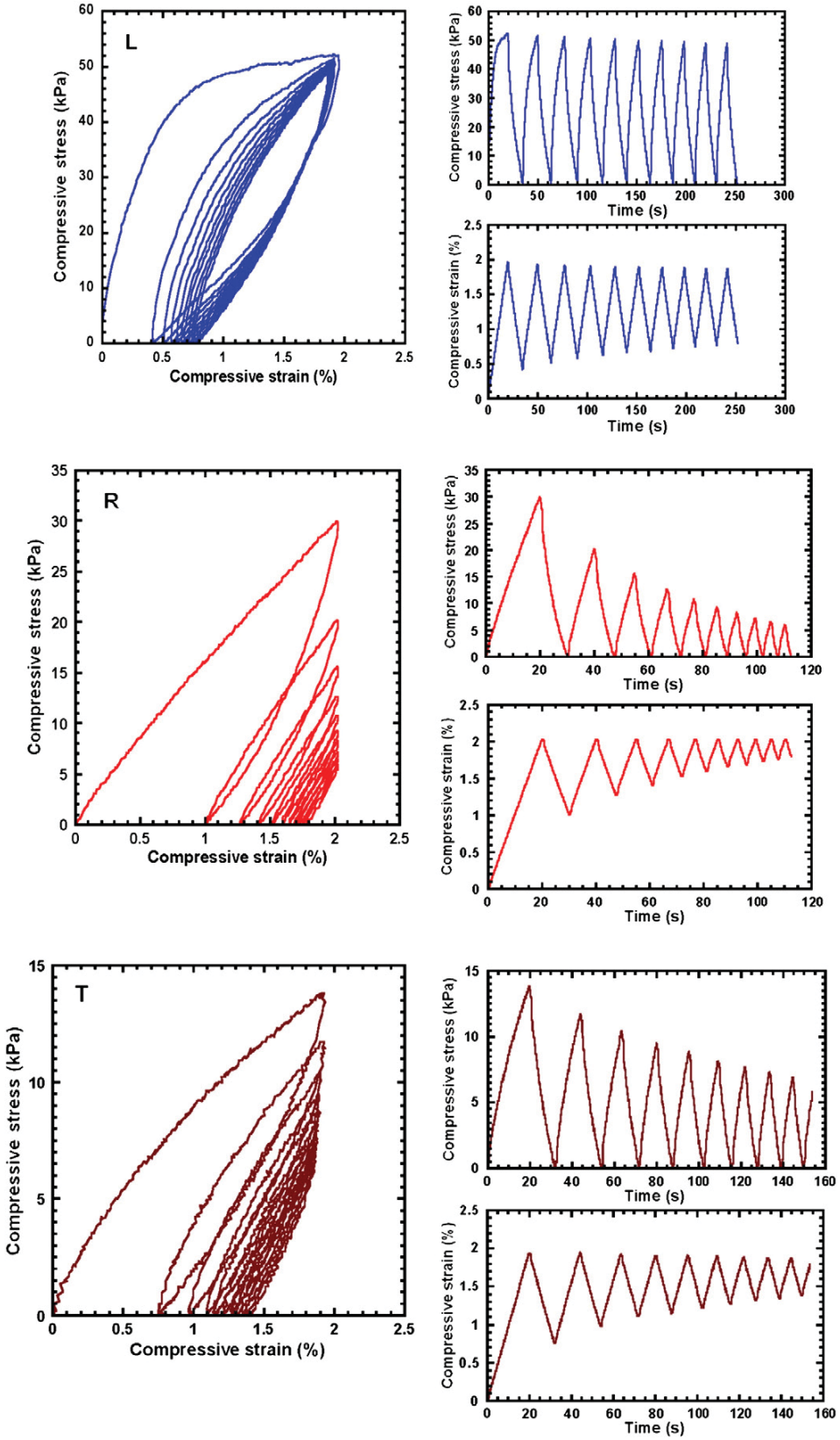

Fig. 4. Representative stress–strain, stress–time, and strain–time curves for cyclic loading–unloading compression (10 consecutive cycles) for demineralized cortical bone in three anatomical directions. L = longitudinal, R = radial, T = transverse.

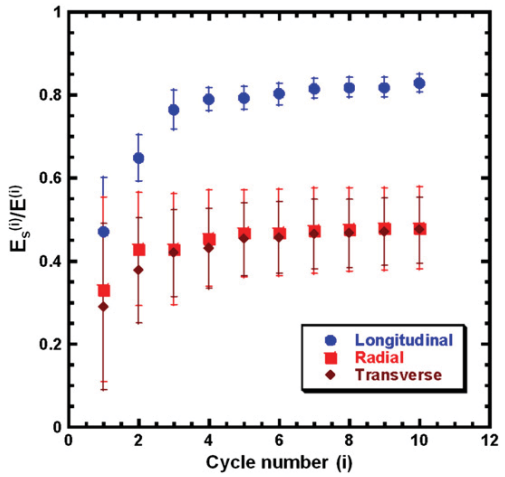

Fig. 5. The variation of the secant/tangent moduli ratio $E_s^{(i)}/E^{(i)}$ with the cycle number during first ten loading–unloading compression cycles for longitudinal, radial, and transverse directions.

Fig. 5 show that there was no significant difference in $E_s^{(i)}/E^{(i)}$ between the radial and transverse directions. Additionally, these results indicate that this ratio reached an equilibrium value for all three anatomical directions by the fourth cycle. The equilibrium value for the radial and transverse directions was close to 0.5, while for the longitudinal direction it was ~0.8, which means that the stiffness in the longitudinal direction was least affected by cyclic loading–unloading compression. The gradual increase of the secant modulus with the number of cycles can be attributed to gradual collagen compaction and rearrangement of its microstructure during repeated compressive loading.

4. Conclusions

The mechanical properties under cyclic loading–unloading compression for strains up to 2% were investigated for demineralized (100% protein) bovine femur cortical bone in three anatomical directions. The main findings are the following:

- Demineralized bone shows anisotropic mechanical behavior in the three anatomical directions. It is significantly stiffer in the longitudinal than in the radial and transverse directions due to preferential collagen fibers orientation.
- The radial direction is the most energy dissipative due to the periosteal sheath.
- There is no significant difference in the cyclic hysteresis area between the longitudinal and transverse directions due to uniform internal microstructure.
- The initial tangent modulus at the beginning of each loading cycle was little affected during first ten cycles of compressive loading within the physiological strain range.
- Stiffness in longitudinal direction was least affected by cyclic loading–unloading compression.

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