Changes of residual stress, diaphyseal size, and micro-nano structure in bovine femurs during growth and maturation

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Abstract
Bone tissue is subjected to multiple forms of mechanical stress. Even in the absence of external loads, however, residual stress is measured, although the underlying mechanisms remain unknown. This study measured the changes in residual stresses, diaphyseal size, and the micro- and nanostructures of bone during growth and maturation, periods associated with different in vivo mechanical loads due to increasing body weight. Mid-diaphyses from bovine femurs in the following three age groups were examined: 1) less than one month old, 2) two years old, and 3) 8−9 years old. Residual stresses along the bone axis at anterior, posterior, lateral, and medial positions on the diaphyseal surface were measured by X-ray diffraction and averaged. Diaphyseal size, porosity, mineral contents, and degree of hydroxyapatite crystal orientation of transverse cross-sections were investigated for relations with residual stress. Residual stress increased significantly from less than one month old (83.7 ± 53.3 MPa) to two years old (125.5 ± 61.9 MPa) in parallel with expanding diaphyseal width and cortical thickness. Residual stress plateaued until 8−9 years old (114.6 ± 42.2 MPa) and was correlated with local cortical thickness (p < 0.05). At the stage, diaphyseal width was only slightly greater than at 2 years and cortical thickness was not significantly different. For all measurements across groups, residual stress statistically correlated with porosity (p < 0.05), mineral contents (p < 0.01), and degree of crystal orientation (p < 0.01). These observations suggest that residual stresses are generated due to bone formation and reconstruction under changing in vivo mechanical loads with age. In conclusion, residual stresses in bone are generated during development and maintained in maturation, and are indirectly related to diaphyseal size and both bone micro- and nanostructure.

Keywords: Biomechanics, Bone, Residual stress, Hierarchical structure, X-ray diffraction

1. Introduction
The stress/strain state of bone is a critical determinant of bone strength and adaptability. Bone is subjected to static stresses from body weight and cyclic dynamic stresses due to daily movement. In addition, bone is under residual stresses, defined as the stresses that remain in a material in the absence of external forces and that satisfy the equilibrium of forces within the material. In general, residual stress is a significant factor determining a material’s strength. For instance, residual stress in blood vessel walls plays an important role in mechanical strength by reducing the in vivo stresses applied to the inner wall due to blood pressure (Fung, 1990). However, the residual stresses in bone have not been fully described or the sources identified.

Residual stress/strain is clearly present in bone tissue (Tadano and Yamada, 2016). When the material is cut to release residual stress, the material deforms depending on the direction and magnitude of the stress. Early studies noted changes in surface strain induced by cutting the whole bone in situ as measured by a strain gauge attached to the bone surface (Tanaka and Adachi, 1994; Adachi et al., 1998). In addition to strain gauges, X-ray diffraction (XRD) techniques have been used to investigate the presence of residual stress/strain in bone tissue (Todoh et al., 2000; Almer and Stock, 2005, 2007; Tadano and Okoshi, 2006; Giri et al., 2008; Yamada and Tadano, 2010, 2013; Yamada et al., 2011a, 2011b, 2014;
Hoo et al., 2011; Singhal et al., 2011; Stock et al., 2011; Tung et al., 2013). X-rays are advantages as they are nondestructive and noninvasive, and XRD has already shown promise as a tool to characterize bone nanocomposites (Tadano and Giri, 2011). The deformation of hydroxyapatite (HAp) crystals in bone tissue can be observed by XRD, and the HAp crystal strains can be calculated from the deformation by comparison to a reference state (Fujisaki et al., 2006; Tadano et al., 2008). When strip bone specimens are stretched, HAp crystal strain increases almost proportionally (Fujisaki and Tadano, 2007; Yamada et al., 2013). Based on this response, the residual stresses in bone tissue can be measured from the distribution of HAp crystal strains by the $\sin^2 \psi$ method of XRD (Yamada and Tadano, 2010, 2013; Yamada et al., 2011a, 2011b, 2014).

In previous studies using the $\sin^2 \psi$ method of XRD, the diaphyses of adult bovine femurs exhibited evidence of tensile residual stresses around the surface region and compressive residual stresses inside the diaphysis (Yamada and Tadano, 2010, 2013; Yamada et al., 2011b, 2014). The surface residual stresses were lower in the circumferential axis than along the bone axis (Yamada and Tadano, 2010). Conversely, in the diaphyses of less than one-month-old bovine femurs, residual stresses were low and did not vary with depth from the surface (Yamada and Tadano, 2013). Furthermore, in rabbit extremities, the magnitude of residual stress at the surface along the bone axis correlated with osteon population density (Yamada et al., 2011a). It is expected that the residual stresses may be related to bone formation and/or reconstruction.

In general, residual stresses are generated inside a material by an indeterminate structure, which is assumed to be generated in bone by developmental changes in reference of deformation due to bone formation and reconstruction under body weight-dependent in vivo loading. If new tissue develops in a non-deformed state during in vivo loading, changes in loading during tissue development generate a difference in reference of deformation in the bone. Based on this hypothesis, the generation of residual stresses will differ during growth as new tissue develops under a rapid increase in body weight, and at maturation, during which new tissue develops and old tissue is absorbed or replaced without significant changes in body weight.

Therefore, the present study investigated changes in residual stresses in bone during growth and maturation and the associations with diaphyseal size, tissue microstructure, and tissue nanostructure as indicators of bone formation and reconstruction.

2. Materials and methods

2.1 Specimens

Five bovines were examined in three age groups, respectively: less than one month old (Group 1), two years old (Group 2), and 8–9 years old (Group 3). A femur was obtained from each individual. The specimens were frozen at –30°C until further preparation. The mid-diaphysis was cut from the femurs such that the measurement position was at both the center of the femur and the diaphyseal specimen (Fig. 1). The length of the diaphyseal specimen $l_s$ was chosen as 0.13 times the femoral length $l_f$. In Groups 2 and 3, $l_s$ was maintained uniform at 60 mm for convenience. The bone marrow and soft tissue around the surfaces were removed as much as possible. The specimens were kept in saline at 4°C until just before the residual stress measurements.

2.2 Residual stress measurements

Residual stresses along the bone axis were measured on the surface of the specimen center at four locations as shown in Fig. 1: 1) anterior, 2) posterior, 3) lateral, and 4) medial. The specimen was irradiated with characteristic Mo-Kα X-rays ($\lambda = 0.071$ nm) under no external forces in accord with a previous study (Yamada et al., 2014). The incident X-rays were collimated using a collimator with a diameter of 1.0 mm, and the resulting diffracted X-rays were detected by an imaging plate (IP; BAS-5R 127 × 127 mm², FUJIFILM, Japan) inside an X-ray diffractometer (RINT2200, Rigaku, Japan), as shown in Fig. 2. The sample–detector distance $L$ was 180 mm. The specimen was irradiated for 300 s; the tube voltage was 40 kV and the tube current was 40 mA. The specimens were surrounded by wet gauze except for the measurement location during irradiation. The measurements were conducted five times at each location, and the specimens were kept in saline between measurements. The residual stress along the bone axis was calculated from a synthetic Debye ring of the (211), (112), and (300) lattice planes of HAp crystals observed in the XRD pattern detected by the IP (Yamada et al., 2014).
The $x$–$y$–$z$ coordinate system is fixed at the specimen surface and the $x$-, $y$-, and $z$-axis correspond to the bone axial, circumferential, and radial directions, respectively. The HAp crystal strain $\varepsilon^H$ is defined by Eq. (1) as

$$
\varepsilon^H = \frac{d - d_0}{d_0},
$$

where $d_0$ is the interplanar spacing of the non-strained state. The present study assumed that the relationship between the tissue stress $\sigma^B$ and $\varepsilon^H$ in the cortical bone is as described in Eq. (2) (Yamada and Tadano, 2013; Yamada et al., 2014).

![Fig. 1 Specimen preparation and positions of the residual stress measurements.](image1)

![Fig. 2 Scheme for the residual stress measurements.](image2)
The cross-sectional area, cortical thickness at the four locations, and diaphyseal width in anterior–posterior and lateral–medial directions were measured.

After CT observations, the specimens were cut out at the specimen center using a slow-speed diamond wheel saw (model 650, South Bay Technology, USA). The cross-sections were ground with emery paper and then buffed by a buffing machine (model 900, South Bay Technology, USA). The cortical surface regions at the four locations on the section were

\[
\begin{bmatrix}
\varepsilon_x^H \\
\varepsilon_y^H \\
\varepsilon_z^H
\end{bmatrix} =
\begin{bmatrix}
1 & \frac{\nu'}{E'} & -\frac{\nu'}{E'} \\
-\frac{\nu'}{E'} & 1 & -\frac{\nu'}{E'} \\
\frac{\nu'}{E'} & \frac{\nu'}{E'} & \frac{1}{E'}
\end{bmatrix}
\begin{bmatrix}
\sigma_x^H \\
\sigma_y^H \\
\sigma_z^H
\end{bmatrix}
\] (2)

In Eq. (2), \( E' \) and \( \nu' \) are the X-ray elastic constant and X-ray Poisson’s ratio, respectively, which describe the elastic properties between the bone tissue stress and the HAp crystal strain. The HAp crystal strain in the direction corresponding to the \( \beta \)-direction on IP, \( \epsilon_x^H' \), was approximated as in Eq. (3) (Yamada et al., 2014).

\[
\epsilon_x^H = \epsilon_x^H' \sin^2 \psi + \epsilon_y^H' (1 - \sin^2 \psi) = \frac{1 + \nu'}{E'} \epsilon_x^H' \sin^2 \psi - \frac{\nu'}{E'} (\epsilon_x^H + \epsilon_y^H)
\] (3)

In Eq. (3), \( \psi \) is the angle between the direction of \( \epsilon_x^H \) and the z-axis, and is described by \( \beta \) in Eq. (4).

\[
\psi = \cos^{-1}(\cos \theta_x \cos \theta_y \cos \beta + \sin \theta_x \sin \beta)
\] (4)

The term \( 2\theta_x \) was defined as the synthetic diffraction angle of the lattice planes in the \( \beta \)-direction and was calculated from the radius of the Debye ring, \( r_{\beta} \), and \( L \). The angle \( \theta_x \) was set to 7.25°, which is the Bragg angle of the (211) lattice planes in the HAp crystals in a non-strained state using characteristic Mo-K\( \alpha \) X-rays from the International Center for Diffraction Data - Powder Diffraction Files No. 9-432. From the partial differentiation of Eq. (3) with respect to \( \sin^2 \psi \), \( \sigma_x^H \) is described by \( \psi \) and \( 2\theta_x \) in degrees as in Eq. (5) (Yamada et al., 2014).

\[
\sigma_x^H = \left( -\frac{E'}{2(1 + \nu')} \frac{\pi}{180} \cot \theta_x \right) \frac{\partial (2\theta_x)}{\partial (\sin^2 \psi)} = K_s \frac{\partial (2\theta_x)}{\partial (\sin^2 \psi)}
\] (5)

In Eq. (5), \( K_s \) is a stress constant with a value of \(-660 \text{ MPa/degree} \) (Yamada and Tadano, 2010, 2013). Therefore, the macroscopic residual stress in the bone axial direction can be calculated via the portion of the Debye ring. This stress is the deviatoric stress because the hydrostatic deformation resulting from hydrostatic stress cannot be detected.

In the present study, the same value of \( K_s \) was used to calculate the residual stresses for all age groups according to previous results. First, a four-point bending test of strip bone specimens under X-ray irradiation indicated that \( K_s \) did not differ between Groups 1 and 2 (Yamada and Tadano, 2013). Furthermore, the \( K_s \) value of Group 3 did not differ from those of Groups 1 and 2 according to measurement of a strip specimen (\( 3 \times 28 \times 1 \text{ mm} \)) taken from a diaphyseal specimen of Group 3 using a previously described procedure (Yamada et al., 2011a).

2.3 Morphological measurements

The diaphyseal specimens were air-dried after X-ray measurements and the shape of the transverse cross-section at the specimen center was observed using a microfocus X-ray CT instrument (inspeXio SMX-90CT, Shimadzu, Japan). The cross-sectional area, cortical thickness at the four locations, and diaphyseal width in anterior–posterior and lateral–medial directions were measured.

After CT observations, the specimens were cut out at the specimen center using a slow-speed diamond wheel saw (model 650, South Bay Technology, USA). The cross-sections were ground with emery paper and then buffed by a buffing machine (model 900, South Bay Technology, USA). The cortical surface regions at the four locations on the section were...
observed by a color 3D laser microscope (VK-9700, KEYENCE, Japan). Figure 3 shows a typical microscopic image of the measurement region for residual stress. The observation area reached 100 µm below the outer cortical surface (the approximated X-ray penetration depth, dashed line in Fig. 3) and 1.4 mm across centered on the z-axis of the section. The total area of canals in the region was measured and the porosity was calculated.

To evaluate the mineral contents of the tissue, X-ray fluorescence analysis (XRF) was performed at the four locations of the cross-section using an energy-dispersive X-ray fluorescence spectrometer (JSX-3100R1I, JEOL, Japan). The irradiated area was an elliptical region (1 mm deep and 1.4 mm wide) around the cortical surface, and the intensity of XRF from calcium, which is derived from HAp, was measured using an artificial HAp sample as a reference. The intensity was normalized to the average intensity in Group 1.

An XRD profile from the cortical surface region at the four locations in the transverse cross-section of the specimen was measured using a scintillation counter in the X-ray diffractometer. The incident X-rays were also collimated to 1 mm diameter. Figure 4 shows a typical XRD profile. To evaluate the degree of HAp crystal orientation for the bone axis, the ratio of intensity from the (002) plane \( I_{(002)} \) to the intensity from the (211) plane \( I_{(211)} \) was calculated, and the ratio was normalized to the average ratio in Group 1.

### 2.4 Elastic modulus measurements

A strip specimen (3 mm × 28 mm × 1 mm) was taken from each diaphysis along the bone axis using the diamond wheel saw and a strain gauge (KFG-1N-120-C1-11L3M3R, KYOWA, Japan) was bonded to the surface. The strip specimen was deformed by four-point bending (outer span length = 24 mm, inner span length = 12 mm) using a material testing machine (Model 4411, Instron, USA), and the elastic modulus was calculated from the force–strain relationship.
3. Results

Table 1 presents the sizes and shapes of the specimens from the three age groups. The femurs grew at a good pace, with increases in femoral length, diaphyseal width, and cortical thickness until two years of age, after which the femoral length and width increased only slightly.

Figure 5 shows the average residual stress in the specimens from each group. The average residual stress at four locations (anterior, posterior, lateral, and medial) in five specimens from each group were (mean ± standard deviation) 83.7 ± 53.3 MPa in Group 1, 125.5 ± 61.9 MPa in Group 2, and 114.6 ± 42.2 MPa in Group 3. There was a significant difference between Group 1 and Group 2 (p < 0.05) but no statistically significant difference between Group 2 and Group 3. In both Groups 1 and 3, there was no statistically significant difference in the locations. In Group 2, there were statistical differences of residual stresses between anterior and posterior (p < 0.01), anterior and medial (p < 0.05), and lateral and posterior (p < 0.01). Anterior and lateral locations had higher residual stresses than posterior, corresponding to the trends observed in previous studies (Yamada and Tadano, 2010; Yamada et al., 2014).

Figure 6a shows the changes in the elastic modulus of the strip specimens. The elastic modulus of Group 1 was almost half that of Groups 2 and 3. After growth, the elastic modulus did not change (2 years to 8–9 years). Figure 6b shows the changes in surface porosity of the transverse cross-sections from diaphyseal specimens. In Group 1, many holes related to bone formation were observed in the surface region; on the other hand, few canals were observed in Group 3. The microstructure at the surface region changed markedly with age. Figure 6c shows the changes in mineral contents within the tissue. The bone tissue in Group 1 had lower mineral contents than Groups 2 and 3 (p < 0.01). Furthermore, as shown in Fig. 6d, the degree of HAp crystal orientation along the bone axis was largest in Group 2. There were significant differences between Groups 1 and 2 (p < 0.01) and between Groups 2 and 3 (p < 0.05). During growth, both bone mineral density and the degree of crystal orientation increased. After the period of rapid growth (2 years to 8–9 years), the degree of orientation decreased while the bone mineral density stabilized.

As shown in Fig. 7, there were statistical correlations of residual stress with cortical thickness (r = 0.33, p < 0.05), porosity (r = −0.28, p < 0.05), mineral contents (r = 0.48, p < 0.01), and crystal orientation (r = 0.52, p < 0.01) for all 54 measurements (excluding the locations where the residual stresses could not be measured). In contrast, there was no statistical correlation of residual stress with elastic modulus (r = 0.51, p > 0.05) or cross-sectional area (r = 0.45, p > 0.05).

Table 1  Specimen size and shape in the three age groups (n = 5 femur specimens for each group): 1) less than one month old (Group 1); 2) two years old (Group 2); and 3) 8–9 years old (Group 3). The length of diaphyseal specimens l_s was 0.13 times the femoral length l_f. In Groups 2 and 3, l_s was uniform as 60 mm.

| Specimen size | Group 1 mean | s.d. | Group 2 mean | s.d. | Group 3 mean | s.d. |
|---------------|--------------|------|--------------|------|--------------|------|
| Femoral length l_f (mm) | 218 ± 20 | 460 ± 4 | 477 ± 10 |
| Specimen length l_s (mm) | 28.4 ± 2.6 | 60.0 ± 0.0 | 60.0 ± 0.0 |
| Cross-sectional area (mm²) | 165 ± 46 | 1046 ± 46 | 1194 ± 57 |

| Diaphyseal width | Group 1 mean | s.d. | Group 2 mean | s.d. | Group 3 mean | s.d. |
|------------------|--------------|------|--------------|------|--------------|------|
| Anterior-Posterior (mm) | 21.1 ± 1.9 | 47.7 ± 1.6 | 55.4 ± 2.7 |
| Lateral-Medial (mm) | 21.5 ± 2.6 | 42.2 ± 1.0 | 45.7 ± 2.3 |

| Cortical thickness | Group 1 mean | s.d. | Group 2 mean | s.d. | Group 3 mean | s.d. |
|-------------------|--------------|------|--------------|------|--------------|------|
| Anterior (mm) | 2.6 ± 0.4 | 12.9 ± 1.0 | 12.4 ± 1.1 |
| Posterior (mm) | 3.3 ± 0.8 | 7.8 ± 0.5 | 8.1 ± 1.0 |
| Lateral (mm) | 2.5 ± 0.5 | 8.2 ± 0.7 | 7.9 ± 0.6 |
| Medial (mm) | 3.4 ± 0.9 | 9.9 ± 1.2 | 9.4 ± 0.8 |
| Average (mm) | 2.9 ± 0.8 | 9.7 ± 2.2 | 9.4 ± 2.0 |
Fig. 5  Mean residual stress in femurs of the three age groups. Solid bars indicate the average of four measurement locations (anterior, posterior, lateral, and medial) at the cortical surface from five specimens for each age group. The error bars indicate the standard deviations of 20, 19, and 15 measurements in Groups 1, 2, and 3, respectively. In a few cases, the soft tissues on the posterior location could not be removed so these locations were not examined.

Fig. 6  (a) Elastic modulus of the strip bone specimens measured by four-point bending tests ($n = 5$). (b) Porosity of the cortical surface region. (c) Normalized mineral contents relative to Group 1 as analyzed by X-ray fluorescence. (d) Normalized degree of hydroxyapatite crystal orientation in the bone axis relative to Group 1 as analyzed by X-ray diffraction. In (b)–(d), solid bars and error bars indicate the average and standard deviation of four locations in five specimens per age group.
Fig. 7  Relationship between residual stress and (a) cortical thickness \( (r = 0.33, p < 0.05) \), (b) porosity \( (r = -0.28, p < 0.05) \), (c) mineral contents \( (r = 0.48, p < 0.01) \), and (d) crystal orientation \( (r = 0.52, p < 0.01) \) from 54 measurements (excluding locations where the residual stress could not be measured). Relationship between averaged residual stress in each diaphyseal specimen and (e) elastic modulus \( (r = 0.51, p > 0.05) \) and (f) cross-sectional area \( (r = 0.45, p > 0.05) \) of the specimens.

4. Discussion

The use of air-dried specimens is a limitation of previous studies on bone residual stress (Yamada and Tadano, 2010, 2013; Yamada et al., 2011b, 2014). Therefore, residual stress was measured under wet conditions in the current study. The trends in residual stress as measured in 2-year-old bone (Group 2) are consistent with previous results from air-dried specimens. However, when a specimen from Group 2 was air-dried and residual stress re-measured after the initial measurements under wet conditions, the relationship between \( 2\theta \beta \) and \( \sin^2\psi \) was shifted vertically with no change in slope (Fig. 8). This suggests that air-drying affects the hydrostatic deformation of the tissue, while the measured deviatoric residual stress is not affected.

The present study demonstrates that the residual stress in bovine femur changes substantially during growth and then more slowly during maturation. Residual stresses at the outer surface of the diaphysis increased significantly during growth, in accord with our previous results on air-dried specimens from less-than-one-month-old and 2-year-old bovine
femurs using a high-energy synchrotron white X-ray beam (Yamada and Tadano, 2013). On the other hand, the average residual stress changed little during maturation (from 2 years to 8–9 years). Bone porosity at the outer surface is indicative of bone formation since growth of new bone tissue is accompanied by the formation of pores around the outer surface of the diaphysis that are still not filled with lamellae, especially in large mammals (Currey, 2002). According to Table 1 and Fig. 6b, during growth, new tissue is actively constructed at the outer surface, and diaphyseal width increases. On the other hand, during maturation, new tissue is slowly constructed at the outer surface and the tissue around the inner surface is absorbed in some locations. Furthermore, there was a statistical correlation of residual stress with cortical thickness. Therefore, residual stresses may be substantial during growth due to construction of new tissue at the outer surface and increasing diaphyseal size. After growth, residual stresses may be maintained on average under slowly construction of new tissue at the outer surface and absorption at the inner surface. A previous study noted that the 2-year-old bovine femurs showed a trend toward tensile residual stresses at the outer surface region and compressive residual stresses in the deeper region, suggesting the equilibrium of forces between the surface and the deeper regions of the diaphysis (Yamada and Tadano, 2013). These suggest that the balance between the new tissue and deeper initial tissue is essential and the bone formation and reconstruction during growth and maturation relate to the mechanisms.

Bovine body weight increases rapidly from birth to age two years, and more slowly thereafter. In one study, bovine weight increased to approximately 450 kg at two years old, and then to approximately 550 kg over the next three years before reaching a plateau (Brown et al., 1976). It appears that body weight-dependent in vivo mechanical loadings applied to the bone axis during development change markedly during growth and development but stabilize during maturation. Hence, the outer surface region of bone may have been constructed under higher in vivo loadings than the deeper initial tissue. Indeterminate structures between new bone growth and older, deeper bone tissue should then be generated. This creates tensile residual stresses in the bone axis at the outer surface and compressive residual stresses in deeper region. At maturity, however, body weight-dependent in vivo loadings may stabilize during bone formation and/or reconstruction. With continued development of the outer surface region, the differences may stabilize, resulting in stable residual stresses. Furthermore, the residual stresses at the outer surface may decrease at locations where the inner surface is absorbed according to Fig. 7a. This may explain the differences in residual stresses among ages. However, the observed correlation between residual stress and cortical thickness was not strong.

Changes in micro- and nanostructure with age were observed and there were also significant correlations between residual stresses and both mineral contents and crystal orientation. Local heterogeneity in hierarchical structure may occur depending on body weight-dependent in vivo loadings during bone formation and/or reconstruction. These differences in micro- or nanostructure may also affect the generation of residual stresses. According to Figs. 6c and 6d, during growth, the mineral contents and degree of crystal orientation at the new tissue gradually become higher than those in young age. It may generate the differences in the nanostructure between the new tissue and deeper initial tissue as well as the differences in reference of deformation, resulting in the indeterminate structures. Thereafter, the new tissue with still higher mineral contents and degree of crystal orientation than the young age is slowly constructed at the outer surface under small increasing body weight, although the degree of crystal orientation slightly decreases after growth. It may have little impact on further generation of the indeterminate structures. These satisfy the correlations with mineral contents and degree of crystal orientation as in Figs. 7c and 7d, reflecting the significant changes during growth mainly. Furthermore, the variation of residual stresses in the locations may depend on the differences in bone micro- and nanostructure as well as the diaphyseal size, relating to the spatial variation of in vivo mechanical environment. Further studies on the in vivo mechanical environment and the distributions of micro- and nanostructures within the bone are essential for a complete understanding of the mechanisms underlying residual stresses in bone. A long-term study is needed to better understand the generation mechanisms of residual stress in bone tissue throughout the hierarchical structure resulting from functional adaptation.

The present study has several limitations. First, the study used specimens cut from whole bones. However, this cutting process had little impact on the measured residual stresses according to strain gauge measurements during the same cutting process (Yamada and Tadano, 2013). Also, the difference of external stress state between in vivo and in vitro, for instance effects of ligament tension and muscle forces around the bone, might be one of the factors. Second, the measurement regions for elastic modulus, mineral contents, and crystal orientation did not exactly correspond to the region for residual stress measurements due to technical issues, although the regions for mineral contents and crystal orientation measurements were close to those for residual stress measurements. Hence, it was difficult to describe the precise effects of structure on residual stresses. Furthermore, the current results did not exactly correspond to the general
trend between porosity and elastic modulus. Third, the direct influences of \textit{in vivo} loadings on residual stress generation could not be described because quadrupedal limb bones are subjected to complex loadings, such as compression, bending, and torsion. Fourth, the present study investigated only the residual stresses around the surface region. The radial distribution of micro- and nanostructures should be investigated as well as the balance of residual stresses. Fifth, the sex of the bovines was not specified in the experiments and the effects may be included in the results. Sixth, only three age groups were examined. The influences of aging, osteoporosis, and adapted state of bone on residual stresses are outstanding issues for future study.

![Fig. 8](image)

Fig. 8 Relationships between $2\theta_{b}$ and $\sin^2\psi$ under wet and air-dried conditions in a specimen from Group 2. The marks indicate the average values of five measurements in a location and the errors indicate the standard deviations.

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