

## A comparative study of young and mature bovine cortical bone <sup>☆</sup>

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### ABSTRACT

The mechanical properties and microstructure of young and mature bovine femur bone were investigated by optical microscopy and compression testing in the longitudinal and transverse directions for untreated, deproteinized and demineralized cases. Optical microscopy revealed that mature bone has a more established and less porous microstructure compared to young bone. Mature bone was found to be stronger in both directions for the untreated and deproteinized cases. Mature untreated bone was also found to be stiffer and less tough compared to young bone in both directions. These results are related to the increase in mineralization of mature bone and significant microstructural differences. Young bone was found to be stronger in both directions for the demineralized case, which is attributed to alterations in the collagen network with age.

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### 1. Introduction

Bone is a composite material made out of 65 wt.% minerals (carbonated hydroxyapatite), 25 wt.% proteins (mostly collagen-I, with a small amount of non-collageneous proteins) and 10 wt.% water [1]. The main biological functions of bone include mineral storage, protection of internal organs, skeletal support for the body, sites for muscle attachments and shock absorption. Bone is not only a lightweight, tough, strong and stiff material, but also has the ability to sense mechanical stimuli, and can regenerate defected areas in order to maintain its structural and biological integrity [2,3]. These outstanding properties are due to its complex hierarchical structure. There are two main types of bone: cortical and trabecular. Cortical bone is denser and forms the outer sheath, while trabecular bone is present in the areas that need to absorb energy (skull, ribs, vertebra).

Cortical bone further consists of three subtypes, osteonal, interstitial and plexiform. The osteonal bone is made up of cylindrical structures (osteons) that span throughout the bone in the longitudinal direction. Osteons support nutritional needs and regeneration processes. The space in between the osteons is filled with interstitial bone, which consists of bone remnants after remodeling. The plexiform bone is made up of lamellar bone sheets that are perforated by a plexus of blood vessels. Plexiform bone is found in large, fast growing animals and is an indicator of non-human bone [4]. Katz and Yoon [5] showed that plexiform areas are

significantly stiffer than Haversian (osteonal) regions. Several studies that related the microstructure, strength and porosity of bone found that remodeled osteons are weaker and softer [6–9].

Several groups have investigated mineral contents of young and mature bovine and human bones [10–12]. Bovine and human bones reach their maturity level at different ages. According to Carter et al. [13], humans achieve full growth by the age of 16 years, while bovines are fully grown in 2 years. Therefore, the rate of bone growth is much higher for bovine bones than for human ones. This factor is extremely important in analyzing the structure and mechanical properties of bone. In bovines and humans, the bone mineral density increases significantly with age, resulting in corresponding changes in elastic properties, toughness and risk of fracture [14,15]. Furthermore, Currey and co-authors [12,16] demonstrated that bones from several species become more mineralized with increasing age, leading to greater stiffness and less toughness.

In addition, other factors, such as collagen deterioration with age, were found to influence age-related bone mechanical properties in humans [17,18]. Studies on bone of different species, including humans, have shown strong dependence of bone strength on collagen alignment and collagen content [19]. Research on osteogenesis imperfecta, a bone protein deficiency disease found in cattle and humans, also showed that a deficiency of proteins decreases bone strength and durability [20]. Several interesting results concerning the age related changes of bone microstructure and its influence on bone toughening mechanisms were reported by Nalla et al. [21] and Ritchie et al. [22]. They attributed the fracture sensitivity of aged human bones to an increasing density of Haversian systems and changes in collagen cross-linking at the nanolevel. A similar study by Zioupos and Currey [23] showed that, in human bones, an increase in stiffness with age leads to a de-

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crease in work of fracture and critical stress intensity factor, which is required to initiate a macrocrack.

Bone deproteinization and demineralization are powerful methods used to separate the two main constituents of bone and allow detailed investigation of properties of the mineral and protein phases separately. Compressive mechanical properties of bone and its main constituents were recently studied for mature bovine cortical bone [24] and mature bovine trabecular bone [25]. It was shown that both bone types are interpenetrating composite materials with mineral and protein constituents [1,4,24–26].

Mature cortical bone and its main constituents were found to have anisotropic mechanical properties [19,24,27–31]. The longitudinal direction was found to be the strongest for demineralized and deproteinized bone due to the preferential collagen fibers orientation in the former case and the mineral crystals preferential orientation in the latter case [24]. Skedros et al. [31] used acoustic microscopy to evaluate the elastic modulus of untreated, demineralized and deproteinized cortical bone of wild deer calcanei. They found that the anisotropy ratio (AR), calculated as the ratio between the longitudinal and transverse elastic coefficients, was significantly different from isotropy (where  $AR = 1$ ) not only for untreated bone, but also for demineralized and deproteinized bones. This demonstrated that both the mineral and collagen phases behave in an anisotropic manner, along with the whole bone.

To the best of the authors' knowledge, there is no side-by-side investigation of the anisotropic properties of mature and young bones and their main constituents. Such an investigation is the main goal of this study.

## 2. Materials and methods

### 2.1. Sample preparation

Mature and young bovine femur bones from the mid-diaphysis region were purchased from a local butcher's shop (La Jolla, CA). The slaughter age was about 18 months for the mature bone samples and about 6 months for the young ones. All the bones were either kept frozen or refrigerated (4 °C) in Hank's balanced saline solution. Cross-sectional samples were first roughly cut with a band saw, then precisely shaped with a diamond blade under constant water irrigation into rhomboid parallelepipeds with dimensions  $5 \times 5 \times 7.5 \text{ mm}^3$  for compression testing [32]. The samples were cut in two anatomical directions. The longitudinal direction coincided with the direction of bone growth, and the transverse direction to be perpendicular to the longitudinal one (Fig. 1). The samples came from one mature and one young bovine femur bone. Sixty bone samples were prepared (30 for mature and 30 for young bone). Five mature and five young samples from each group (longitudinal and transverse) were demineralized, and five mature and five young samples from each group (longitudinal and transverse) were deproteinized.

Additionally, samples for optical imaging, which consisted of the entire mid-diaphysis cross-section (1 cm thick), were prepared using four separate grinding papers and two additional polishing papers. A total of four cross-sectional samples (two for mature and two for young bones) were prepared. The compression testing and optical analysis were performed on different bone samples (from the same mature and young bovine animals) due to the difficulties of obtaining mechanical properties and optical analysis data from the same bone samples.

### 2.2. Mineral content

The ash content of young and mature bovine samples was determined by heating the samples in an oven for 4 h at 105 °C first

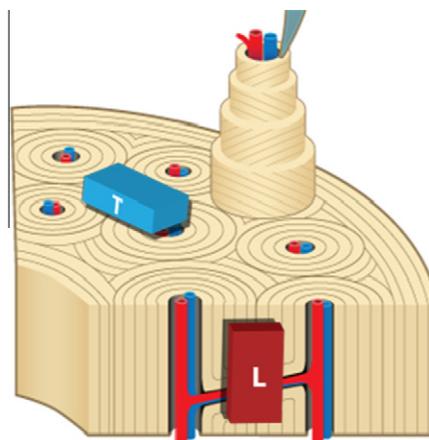


Fig. 1. Bone sample orientations. L = longitudinal, T = transverse. Samples are not shown to scale.

to evaporate the water, then for 24 h at 550 °C to eliminate the collagen content. The weights of the individual samples were measured before and after the heating processes. The weight percent of mineral was calculated by dividing the weight after heating by the weight before heating. The mineral volume percent was calculated according to equation:

$$\text{vol.}\% = \text{wt.}\% \rho_{\text{app}} / \rho_{\text{HA}}$$

where  $\rho_{\text{app}}$  is the apparent density of mature or young bone samples and  $\rho_{\text{HA}}$  is the density of hydroxyapatite ( $3.15 \text{ g cm}^{-3}$ ) [37].

### 2.3. Deproteinization and demineralization processes

Deproteinization was performed by aging the samples in 5.25 wt.% NaOCl (bleach) at 37 °C [33]. The bleach solution was replaced daily for 2 weeks. Previous reports on deproteinized bone showed that the amount of protein left in the solution after subsequent demineralization of previously deproteinized samples was less than 0.001 wt.% [24,25,34]. At the end of the deproteinization process, samples were left overnight under running water to wash away the bleach solution to avoid any undesirable chemical side effects. Demineralization was performed by aging the samples in 0.6 N HCl solution at room temperature [25]. The acid was replaced daily for 10 days. Complete demineralization was verified by the absence of mineral in the solution, according to the procedure outlined in Castro-Ceseña et al. [35]. At the end of the demineralization process, samples were left overnight under running water to wash away the acid solution to avoid any undesirable chemical side effects. The sample sizes used in the present research were similar to those used in previous studies [24,25,34,35].

### 2.4. Structural characterization

Mature and young bone samples from all three groups (untreated (UT), demineralized (DM) and deproteinized (DP)) were analyzed by optical microscopy using a Zeiss Axio imager equipped with a CCD camera (Zeiss Microimaging Inc., Thornwood, NY). Entire cross-sections were analyzed along axes of major angles (0°, 45°, 90°, 135°, 180°, 225°, 270°, 315°), with 0° corresponding to lateral (outer) side of the femur (Fig. 2). Five photos were taken across each angle of the mature cross-sectional sample and approximately three images across each angle of the young cross-sectional sample. Fracture surfaces of the specimens were examined by scanning electron microscopy (SEM) using a microscope equipped for energy-dispersive spectroscopy (EDS) (FEI-XL30, FEI Company, Hillsboro, OR). DM samples were subjected to critical point drying before SEM imaging to avoid excessive shrinkage. All samples were

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