Secondary osteon structural heterogeneity between the cranial and caudal cortices of the proximal humerus in white-tailed deer

Jack T. Nguyen¹ and Meir M. Barak²,*

ABSTRACT
Cortical bone remodeling is an ongoing process triggered by microdamage, where osteoclasts resorb existing bone and osteoblasts deposit new bone in the form of secondary osteons (Haversian systems). Previous studies revealed regional variance in Haversian systems structure and possibly material, between opposite cortices of the same bone. As bone mechanical properties depend on tissue structure and material, it is predicted that bone mechanical properties will vary in accordance with structural and material regional heterogeneity. To test this hypothesis, we analysed the structure, mineral content and compressive stiffness of secondary bone from the cranial and caudal cortices of the white-tailed deer proximal humerus. We found significantly larger Haversian systems and canals in the cranial cortex but no significant difference in mineral content between the two cortices. Accordingly, we found no difference in compressive stiffness between the two cortices and thus our working hypothesis was rejected. As the deer humerus is curved and thus likely subjected to bending during habitual locomotion, we expect that similar to other curved long bones, the cranial cortex of the deer humerus is likely subjected primarily to tensile strains and the caudal cortex is subject primarily to compressive strains. Consequently, our results suggest that strain magnitude (larger in compression) and sign (compression versus tension) affect the osteoclasts and osteoblasts differently in the basic multicellular unit. Our results further suggest that osteoclasts are inhibited in regions of high compressive strains (creating smaller Haversian systems) while the osteoid deposition and mineralization by osteoblasts is not affected by strain magnitude and sign.

KEY WORDS: Cortical bone adaptation, Haversian system, Histomorphometry, Stiffness, Intracortical remodeling

INTRODUCTION
Cortical bone can be classified as primary or secondary bone tissue. Primary bone consists of new bone material that is laid in layers during appositional growth. There are several forms of primary bone; amongst them, plexiform bone (also called fibrolamellar bone) is characteristic of long bones’ cortices from large, fast-growing juvenile mammals such as cattle, horses, pigs and deer (Locke, 2004; Hillier and Bell, 2007; Barrera et al., 2016). This form of cortical tissue structure is built when bones need to grow faster than other cortical structures can be laid (Currey, 2002). Typically, in large mammals, plexiform bone is transitional and it is replaced as the animal matures by secondary bone tissue in a process called bone remodeling (Locke, 2004; Mori et al., 2005). Secondary bone refers to bone material that is deposited in concentric lamellae, called Haversian systems, where earlier existing primary bone tissue has been resorbed (Currey, 2002; Mori et al., 2005; Barak, 2019). Strong evidence indicates that the formation of Haversian systems may attenuate or repair fatigue microdamage (such as microcracks) as well as provide a mechanism for the skeleton to adapt to external loads in a manner known as targeted remodeling (Lipson and Katz, 1984; Heft et al., 1994; Reilly and Currey, 1999; Burr, 2002; Skedros et al., 2003).

It was previously shown that secondary cortical bone tissue tends to display regional variability between opposite cortices in Haversian system structure, bone material composition and mechanical properties; but see Skedros et al. (2013a) for a different view about the regional variability of Haversian system size, and Skedros and Doutré (2019) for an opposite trend in bat and pigeon wing bones. Different studies have demonstrated significant histomorphometric differences in Haversian systems’ size, shape and density (Skedros et al., 1994b, 1996, 1997, 2004; Mason et al., 1995; Pfeiffer et al., 2006; van Oers et al., 2008; Dominguez and Agnew, 2016; Keenan et al., 2017), collagen orientation in Haversian systems’ lamellae (Portigliatti Barbos et al., 1984; Boyde and Riggs, 1990; Skedros et al., 1996, 2004, 2006; Main, 2007), osteocyte density (Carter et al., 2014), porosity (Skedros et al., 1994b, 2001), mineral density (Mason et al., 1995; Skedros et al., 1996, 1997) and mechanical properties (Riggs et al., 1993b; Hiller et al., 2003; Gibson et al., 2006; Li et al., 2013; Mayya et al., 2016) between different sides of the cortex. Yet so far almost all studies investigated just one aspect of secondary bone regional variability (structure, composition or mechanical properties), and only a few studies have looked at both structure and mechanical property regional variability (Riggs et al., 1993b; Hiller et al., 2003; Gibson et al., 2006) or structure and composition regional variability (Skedros et al., 1996, 1997, 2003, 2004; Skedros et al., 2005) of their bone samples. Only one previous study, Skedros et al. (2006), examined the histomorphometric, composition and mechanical property variabilities in various locations along the cortex of the horse third metacarpal. Yet this study focused on collagen orientation and they only estimated average Haversian system size and did not measure Haversian canal size or Haversian system circularity. No previous study, to the best of our knowledge, had ever studied the regional variability of Haversian system size and shape, composition and mechanical properties between different sides of the cortex for the same bone samples. The goal of this study was to find if correlation exists between secondary bone structure and composition (i.e. mineral content), to the bone mechanical properties for the same bone samples. We chose the deer humerus as it is curved (Biewener, 1983b), and similar to other curved long bones, it is predicted to be loaded in bending (Lanyon et al., 1979; Riggs et al., 1993a; Goodwin and
Sharkey, 2002; Main and Biewener, 2004; Henderson et al., 2017). We decided to focus on the proximal humerus as it was demonstrated to be under bending (Pollock et al., 2008a,b) and at the same time to avoid the issue of torsional stress that was shown to increase from proximal to distal (Oh and Harris, 1978; Carter et al., 2014; Keenan et al., 2017). Cross-sections from the proximal humeri of young white-tailed deer were inspected using scanning electron microscopy (SEM) to determine areas of bone remodeling. These remodeled regions were analysed to quantify Haversian system size and shape. Next, we prepared bone cubes from the cranial and caudal remodeled cortices. Each bone cube was inspected again to verify its remodeled state and then loaded in compression to determine the stiffness along the three principal axes (axial, radial and transverse). Finally, all bone samples were ashed and their material composition was recorded (mineral, organic material and water content). Our null hypothesis was that the cranial and caudal cortices of the proximal humerus would demonstrate similar structural and material properties (Haversian system shape and size, and mineral content, respectively). We further postulated that these similar structural and material properties would correlate to a non-significant difference in compressive stiffness between the cranial and caudal cortices.

MATERIALS AND METHODS

Sample selection

Humeri from seven white-tailed deer, *Odocoileus virginianus* (Zimmermann 1780), were obtained from One Price Deer Processing Plant, York, SC, USA. All bones were intact with no signs of fracture or any other pathology. Age and sex were undetermined; however, all bones showed active growth plates at the proximal humerus, indicating that deer were juveniles between the age of 5 and 20 months (Purdue, 1983; Flinn et al., 2013). All soft tissue was removed, and humeri were stored in a −20°C freezer prior to cutting.

Cross-section preparation

Each humerus was measured from the proximal end of the humeral head to the distal end, and the proximal portion of each bone (approximately between 10 and 35% of bone length) was cut using a handsaw (Fig. 1A). Next, a thin 1 mm thickness cross-section was cut from the proximal and distal ends of each segment using a low-speed water-cooled diamond saw (TechCut 4, Allied Technologies). Each cross-section was then placed on an agitator and dehydrated using a series of 2 h washes with increasing ethanol concentration (35, 50, 75 and 95%), followed by a 2 h acetone wash. Once cross-sections were ready, each was embedded in EpoxySet (Allied Technologies) and allowed to harden for 24 h. Epoxy blocks were then polished (MiniMet, Buehler) and sputter-coated in gold for 30 s each (SPI Industries). Finally, each embedded cross-section was viewed using SEM (JSM-6010LA InTouchScope, JEOL USA Inc., Peabody, MA, USA) and areas of remodeling were recorded (Fig. 2). The SEM was calibrated using a resin block containing an aluminum stub and carbon rods. A gray value of 255 was set based on aluminium, and a gray value of 0 was set based on carbon. Images were taken of each region at 25× magnification.

Histomorphometric analysis

SEM images from the cranial and caudal cross-sectional areas were exported to ImageJ (version 1.50) for histomorphometric analysis.

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Fig. 1. Schematic diagram of the sample preparation process. Orientations were continually labeled throughout the cutting process to allow for correct identification of each principal axis: green, axial; blue, transverse; red, radial. (A) Initial cuts were made to separate the proximal region of each humerus. Subsequent radial cuts were made to isolate the cranial (Cr) and caudal (Ca) cortices. (B) Cranial and caudal cortices were first cut along the sagittal plane (two cuts, 2 mm apart) and then along the frontal plane (two cuts, 2 mm apart) to create a 2 mm×2 mm beam. (C) Each 2 mm×2 mm beam was cut proximally along the transverse plane to create a perpendicular surface and then every 2 mm (proximal to distal) as many times as possible to create 2 mm×2 mm×2 mm bone cubes. (D) Representative view of a 2 mm×2 mm×2 mm cube. Tr, transverse plane; Fr, frontal plane; Sa, sagittal plane.
Haversian system area, Haversian canal area and circularity were measured manually using the fit ellipse tool for more than 850 and 600 Haversian systems in the cranial and caudal cortices, respectively (Fig. 2, upper right inset). This number is significantly higher than previously recommended (25–100) (Pfeiffer et al., 2006; Skedros et al., 2009; Crescimanno and Stout, 2012; Dominguez and Crowder, 2012) and thus it accurately depicts Haversian system histomorphometric parameters in these locations.

Only whole Haversian systems with a clear central canal were included in our measurements; any fragmented Haversian system due to recurring bone remolding was excluded. Circularity is a unitless parameter where values approaching 1 indicate a perfect circle, and values approaching 0 indicate an increasingly elongated ellipsoid. The differences between cranial and caudal histomorphometric parameters were analysed using a two-tailed t-test with equal variance; values smaller than 0.05 ($P<0.05$) were considered statistically significant.

**Cortical cubes preparation**

After the removal of the cross-sections from the proximal and distal ends of the proximal segment, each segment was further cut into four quadrants based on anatomical orientation (cranial, caudal, lateral and medial; Fig. 1A). Next, the cranial and caudal quadrants were cut into $2\text{ mm}\times2\text{ mm}\times2\text{ mm}$ cubes along the bone principal axes (axial, radial and transverse; see Fig. 1B–D) using a low-speed water-cooled diamond saw (TechCut 4, Allied Technologies). Thirty-six cubes were cut from the cranial cortex, and 39 cubes were cut from the caudal cortex.

Using a Nikon Eclipse E600 microscope, the transverse plane of each cortical bone cube was inspected to verify that the cube consisted of remodeled bone, and that no trabeculae were included (for cubes cut closer to the endosteum). Haversian systems (indicators of remodeling) would be visible on this plane as they run perpendicular to the long axis of the bone (Heft et al., 1994). Eight samples (six cranial and two caudal) were suspected to have...
some trabecular bone present and were omitted from the experiment. All remaining bone cubes (30 cranial and 37 caudal) were found to be fully remodeled (i.e. their entire transverse surface revealed Haversian systems with no evidence of primary plexiform bone) and they were stored in 1.5 ml Eppendorf tubes containing paper soaked in saline solution with 8% chloroform to prevent bacterial growth (Martos et al., 2013). Samples were kept frozen at −20°C until mechanical testing. Each sample was thawed at 4°C in the same saline solution 24 h prior to testing.

**Compression testing**

All cortical bone cubes were non-destructively tested in compression, within their elastic region, using an Instron 5942 universal testing machine (Instron Inc., Norwood, MA, USA). Each cube was tested three times, once in each of its principal orientations – axial, radial and transverse. Order of testing directions was alternated to prevent any possible effect of test order (i.e. the possibility that the orientation that is tested last is affected by the previous two tests). The tested cube was mounted in the orientation being tested on a stationary anvil with a thin layer of dental composite (Filtek Z250, 3M ESPE, St Paul, MN, USA). The Z250 3M composite material was used due to its stiffness value around 11 GPa – which is within the range of cortical bone stiffness values (Shahar et al., 2007) and because it does not irreversibly bond with the bone material and can be removed with no damage at the end of each experiment. The use of dental composite as a load-transfer layer was validated and used successfully in previous studies (Shahar et al., 2007; Barrera et al., 2016; Kunde et al., 2018). Another thin layer of composite was applied to the upper anvil, which was then manually lowered until contact was made with the surface of the sample. The addition of composite to both anvil faces helped to correct any surface incongruencies due to cutting and minimize stress concentration and shear stress, ensuring that the sample was loaded primarily in compression. Composite was then polymerized for 60 s using a hand-held light-cure device (Woodpecker 5W, GadgetWorkz). Prior to loading, 0.2 ml of saline solution containing 8% chloroform was added around the bone cube between the anvils to keep the bone moist during the experiment. As tests were short (about 35 s per cycle and about 6–7 min for an entire experiment), all cubes were kept moist throughout the testing process. After a small preload of 5 N was applied at the beginning of each testing cycle, load and deformation data were collected every 0.1 s (Bluehill 3 Software, Instron). Bone samples were loaded at a rate of 50 μm s−1 until a maximum load of 140 N, ensuring that bone samples remained within the elastic region and sustained no structural damage. A previous study (Kunde et al., 2018) tested in compression 2 mm×2 mm×2 mm bone cubes from white-tailed deer humeri and femora in all three orthogonal orientations (axial, radial and transverse) up to 460 N (close to the system and load cell maximum capacity of 500 N). Their results demonstrated that at 460 N, the cortical bone samples demonstrated no evidence of damage. In addition, comparable techniques with the same experimental set-up also demonstrated a lack of damage when bone samples were repeatedly loaded in a similar range of loads (Barak et al., 2008, 2009; Sharir et al., 2008; Barrera et al., 2016; Kunde et al., 2018). Accordingly, we have concluded that a load of 140 N was far from the bone’s yield point in all three orientations and thus within the elastic region. Each mounted sample was loaded ten cycles per test with the first seven cycles serving as preconditioning. Previous studies have shown improved reproducibility and precise stiffness results when using several conditioning cycles to achieve a viscoelastic steady state (Linde and Hvid, 1987; Ziopoulos and Currey, 1998). Load and deformation data were obtained from the final three load cycles in order to calculate the material stiffness (Young’s modulus) from the slope of the stress–strain curve. To test for differences within group, material stiffness values for the cranial and caudal cortices were compared between the different humeri in each of the three directions of loading using a non-parametric Kruskal–Wallis analysis of variance (R v.3.6.3; http://www.R-project.org/). All within-group differences were found to be statistically non-significant (P>0.05). The difference between cranial and caudal bone material stiffness was analysed using a two-tailed t-test with unequal variance; values smaller than 0.05 (P<0.05) were considered statistically significant.

**Bone ashing and mineral content analysis**

The bone cubes from each proximal humerus were crushed into a fine powder using a pestle and mortar in order to analyse the ratio of mineral, organic material and water. The powder from each humerus was then divided into 70–100 mg samples and placed into 1.5 ml Eppendorf vials.

Two humeri (no. 5 and no. 3) lacked enough material from both cortices to test for mineral content (30–40 mg). Additionally, there was also an insufficient amount of material from the cranial cortex of humerus no. 7 to be properly evaluated. The bone powder from each vial was weighed to determine the original weight and then placed into a ceramic boat. Next, the samples were heated to 100°C for 3 h inside an Isotemp programmable forced-draft furnace (Thermo Fisher Scientific, Waltham, MA, USA) and their dry weight was measured. The difference between initial weight and dry weight was calculated to determine the amount of water present in each sample. All samples were then placed back into the furnace at 500°C for a period of 15 h after which they were re-weighed one last time. The amount of organic material present in each sample was calculated by subtracting the final mineral weight from the previously recorded dry weight. A two-tailed t-test with unequal variance was used to compare the water, organic material and mineral weights of samples from cranial and caudal cortices. Values smaller than 0.05 (P<0.05) were considered statistically significant.

**RESULTS**

**Structural variability: bone histomorphometric analysis**

Average Haversian system area in the cranial cortex of the proximal humerus (2833±1075 µm²) was significantly larger than the average Haversian system area in the caudal cortex (354±875 µm²) (P<0.01; Fig. 3, left panel). Similarly, average Haversian canal area in the cranial cortex (454±875 µm²) was significantly larger than the average Haversian canal area in the caudal cortex (354±257 µm²) (P<0.01; Fig. 3, right panel). However, relative Haversian canal area (ratio between Haversian canal area to Haversian system area) was significantly larger in the cranial cortex (4.7±3.0%) compared with the cranial cortex (3.7±2.7%) (P<0.01). No significant difference between the cranial and caudal cortices was found in Haversian system circularity (0.950±0.004 and 0.958±0.037 for the cranial and caudal cortices, respectively) and Haversian canal circularity (0.950±0.081 and 0.955±0.065 for the cranial and caudal cortices, respectively).

**Material variability: bone material composition**

Percentage of mineral, organic material and water between the cranial and caudal cortices of the proximal humerus were all found to be non-significantly different (Fig. 4). Mineral content was found to be 62.9±2.0 and 63.7±2.1% in the cranial and caudal cortices, respectively. Organic material content (mostly collagen) was found
to be 25.8±1.2 and 24.8±1.6% in the cranial and caudal cortices, respectively. Water content was found to be 11.3±1.3 and 11.4±1.8% in the cranial and caudal cortices, respectively (Table 1).

Mechanical properties variability: Young’s modulus
Average axial stiffness in the cranial cortex of the proximal humerus (17.1±4.0 GPa) was not significantly different from average axial stiffness in the caudal cortex (18.9±3.1 GPa) (P≥0.05; Fig. 5A). Similarly, average radial stiffness in the cranial cortex (10.9±2.5 GPa) was not significantly different from average radial stiffness in the caudal cortex (10.2±2.2 GPa) (P≥0.05; Fig. 5B). Finally, average transverse stiffness in the cranial cortex (11.3±2.5 GPa) was not significantly different from average transverse stiffness in the caudal cortex (11.0±1.8 GPa) (P≥0.05; Fig. 5C). Both cranial and caudal samples demonstrated transverse isotropy (Fig. 5D,E; Table 2). Young’s moduli for the radial and transverse orientations in the cranial and caudal cortices were not significantly different from each other, but were significantly lower than for the axial orientation (Fig. 5F).

**DISCUSSION**
Previous studies demonstrated regional variability in cortical bone Haversian system size and morphology (Martin et al., 1996; Skedros et al., 1997, 2004; Dominguez and Agnew, 2016), material composition (Mason et al., 1995; Skedros et al., 1996, 1997) and mechanical properties (Riggs et al., 1993b; Hiller et al., 2003; Gibson et al., 2006; Mayya et al., 2016), yet no study had linked all three regional heterogeneities for the same bone. The goal of our study was to investigate if correlation exists between secondary cortical bone structure (Haversian system size and morphology) and composition (i.e. mineral content), and the bone mechanical properties (i.e. stiffness) for the same cortical bone samples. To this end, we used cortical bone samples from the cranial and caudal cortices of the proximal humeri from juvenile white-tailed deer. Consistent with previous findings, we expected to find differences in Haversian system size, shape and mineralization between the cranial and caudal cortices of the proximal humerus from juvenile white-tailed deer. Consistent with previous findings, we expected to find differences in Haversian system size, shape and mineralization between the cranial and caudal cortices of the proximal humerus, and that these differences would correlate to a significant difference in compressive stiffness between the two cortices.

In line with our predictions, and in agreement with previous studies (Burr et al., 1990; Skedros et al., 1994a,b, 1997, 2004; Martin et al., 1996; van Oers et al., 2008; Dominguez and Agnew, 2016), we found regional variability in Haversian system size and Haversian canal size. Both cranial and caudal samples demonstrated transverse isotropy (Fig. 5D,E; Table 2). Young’s moduli for the radial and transverse orientations in the cranial and caudal cortices were not significantly different from each other, but were significantly lower than for the axial orientation (Fig. 5F).
was larger in the smaller Haversian systems on the caudal side. This may imply that Haversian canal size is dictated by the size of the blood vessel it carries, which has a minimum diameter boundary and thus smaller Haversian systems need relatively larger canals. Skedros et al. (2013b) came to a similar conclusion after comparing Haversian system size and Haversian canal size between human ribs and lower limb bones. They have found that Haversian system diameter varies much more than Haversian canal diameter, and thus they argued that as all osteocytes within an osteon must receive their nutrients from the capillary in the central canal (no canaliculi cross the cement line), a minimum capillary diameter may exist (Skedros et al., 2013b). Similarly, Metz et al. (2003) found support for their hypothesis that osteocytes inhibit refilling of forming Haversian systems so that the Haversian canal is large enough, to allow adequate delivery of nutrients to the osteocytes (Metz et al., 2003).

Contrary to our expectations, Haversian system shape (circularity) did not differ significantly between the cranial and caudal cortices of the bone. This finding is in line with a previous study by Skedros et al. (2019) that also did not find differences in Haversian system circularity between the opposing cortices of various bones. In addition, bone material composition, and specifically mineral content, did not differ significantly between the cranial and caudal cortices of the bone. Consequently, we found no significant difference in compressive stiffness between bone samples from the cranial and caudal cortices in all three loading orientations (axial, radial and transverse). As mineral content is a key contributor to bone stiffness (Currey, 1988; Martin and Boardman, 1993; Wu et al., 2006; Barak et al., 2009), the lack of difference in compressive stiffness in light of the similar mineral content (and despite the difference in Haversian system size) is not surprising. Therefore, our working hypothesis that structural differences between the cranial and caudal cortices will correlate with differences in compressive stiffness was not supported. We did find, however, that as our bone samples consisted of remodeled bone (Haversian systems) they have demonstrated transverse isotropic behavior (Lipson and Katz, 1984; Shahar et al., 2007), where the radial and transverse orientations had non-significant differences in stiffness but both were significantly less stiff than the axial orientation. This transverse isotropy mechanical behavior is interesting, especially in light of previous studies by our group that demonstrated orthotropic behavior in the proximal femur (Barrera et al., 2016) and the humerus mid-diaphysis (Kunde et al., 2018) of similarly juvenile white-tailed deer. These results imply that cortical bone remodeling in white-tailed deer happens at a much earlier age at the proximal humerus compared with the proximal femur and mid-diaphysis of the humerus, and support the use of the proximal humerus for the current study. These data are also in line with the results of Purdue (1983), who found the white-tailed deer proximal humerus to be the most active site of growth and the last site to fuse its growth plate.

Cortical bone adaptation to external loads via the process of remodeling is mediated by mechanical strain (Lanyon et al., 1979; Frost, 1990). Bone tissue deformation (strain) due to loading causes fluid flow in the bone canaliculi, which in turn induces shear stresses in the osteocyte cell processes running in these canaliculi. In vivo measurements of mechanical strain in long bones loaded in bending from various mammals pointed to strain magnitude and sign (tension or compression) as factors influencing cortical bone remodeling (Skedros et al., 1994b; 1996; Boyce et al., 1998; Reilly and Currey, 1999). It was further shown that peak compressive strains tend to be significantly higher than peak tensile strain (Lanyon, 1974; Turner et al., 1975; Lanyon et al., 1979; Carter et al., 1981; Biewener et al., 1983; Rubin, 1984;
Goodwin and Sharkey, 2002; Pollock et al., 2008a). This is due to the cumulative effect of axial compressive strains transmitted via the more proximal joint surface overlaid on bending compressive strains generated from the curvature of the bone (Lanyon et al., 1982; Lieberman et al., 2004). Therefore, it is predicted that bone remodeling may hold valuable information on bone loading history (Her et al., 1994; Mason et al., 1995; Skedros et al., 2004, 2009), particularly in curved long bones that are loaded in bending and thus experience various strain magnitudes and sign at opposite cortices (Riggs et al., 1993a,b; Skedros et al., 1996; Reilly and Currey, 1999). Previous studies demonstrated that the humerus of many terrestrial quadrupedal mammals, among them the deer and other members of the family Cervidae, is curved (Biewener, 1983a,b; Rubin, 1984; Bertram and Biewener, 1988; Bertram and Biewener, 1992), and thus is subjected like many other curved long bones to bending (Lanyon et al., 1979; Riggs et al., 1993a; Main and Biewener, 2004; Goodwin and Sharkey, 2002; Henderson et al., 2017). While we did not measure the in vivo strains of the deer humerus, based on a previous study that measured stresses in humeri of rodents (Biewener, 1983a) and studies that measured in vivo strains in various curved long bones (Riggs et al., 1993a,b; Skedros et al., 1996; Reilly and Currey, 1999), we expect that during normal quadrupedal locomotion, the cranial cortex in the deer humerus is

![Stress-strain curves and stiffness boxplots](image-url)

**Fig. 5.** Stress–strain curves and stiffness boxplots with individual data points overlaid from the cranial and caudal cortices of the proximal humerus when loaded in the axial, radial and transverse directions. Cranial cortex, *N*=30; caudal cortex, *N*=37. (A–E) Stress–strain curves are linear regressions with 95% confidence intervals. Note that due to the very high *R*² values (values in parentheses), the 95% confidence intervals are very narrow. Bone cubes tested in the axial (A), radial (B) and transverse (C) directions showed no significant difference of stiffness when comparing the cranial versus caudal cortices of the bone (two-tailed *t*-test with unequal variance; *P*<0.05). Bone cubes tested in the axial direction were significantly stiffer than the radial and transverse directions for both the cranial (D) and caudal (E) cortices. Transverse and radial directions showed non-significant differences in their stiffness for both the cranial and caudal cortices (D,E), highlighting the transverse isotropic behavior of all cubes tested. (F) Boxplots give stiffness in GPa. The horizontal line inside the boxes is the median. Box hinges represent the 25th and 75th percentiles. The box notch represents the 95% confidence interval of the median. If the notches of two plots overlap, this indicates that the two medians do not differ. Whiskers represent minimum and maximum measured values, not including outliers. The individual data points of the different humeri are overlaid on the boxplots (color coded). All within-group differences (comparing data points between humeri for the same direction and cortex) were found to be statistically non-significant (non-parametric Kruskal–Wallis analysis of variance; *P*≥0.05).

<table>
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<th>Cranial cortex (<em>N</em>=30) (GPa)</th>
<th>Caudal cortex (<em>N</em>=37) (GPa)</th>
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<tr>
<td>Axial</td>
<td>17.1±4.0</td>
<td>18.9±3.1</td>
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<td>Radial</td>
<td>10.9±2.5</td>
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<tr>
<td>Transverse</td>
<td>11.3±2.5</td>
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Data are given as means±s.d.
most likely experiencing predominantly tensile strains and the caudal cortex is experiencing predominantly compressive strains. These predicted differences of strain magnitude and signs may explain the significant difference in Haversian system size we found between the cranial and caudal cortices.

Several possible explanations were previously suggested for the regional variance we and other studies found in Haversian system size. One possibility is that during the process of remodeling, the BMU that is activated to resorb old bone (cutting cone) and deposit new osteoid is responding differently to tensile versus compressive strain (Carter et al., 1981; Biwener et al., 1986). Carter et al. (1981) suggested that the different cellular response to strain-mediated stimuli is based on stress-generated electrical potential in bone, where bone tissue under compressive stress has negative electrical potential and bone tissue under tensile stress has more positively charged regions. Although this idea explains the mechanism that leads to the difference, it does not clarify why larger cutting cones are generated in response to tensile strains. Another possibility is that larger Haversian systems are better suited to resist tensile forces (Pope and Murphy, 1974; Hiller et al., 2003; Gibson et al., 2006). Hiller et al. (2003) found significant differences in Haversian system pullout between the compressive and tensile cortices of the horse third metacarpal bone. Haversian system pullout develops under tensile strains when the tensile strength of the Haversian system surpasses the shear strength of its cement line. In such a case, the Haversian system will separate and pull out of the crack surface like a telescopic pole. This phenomenon is beneficial to the bone as the pullout dissipates some of the energy and thus decreases crack propagation. Furthermore, it allows the pullout Haversian system to bridge the forming crack and maintain the integrity of structure for a longer duration. As Haversian systems increase in diameter, their volume increases faster than their surface area (i.e. cement line) and thus their tensile strength will increase relative to their shear strength. Hence, larger Haversian systems are advantageous in bone regions subjected to tensile stress. This proposition is interesting, especially as it implies that the size difference will affect bone stiffness and strength under tensile loading and therefore may be the reason why we did not find differences in compressive stiffness between the cranial and caudal cortices. However, Skedros et al. (2013a,b) have postulated based on their results that collagen fiber orientation (CFO) within the lamellae may be a stronger indicator for tensile versus compression loading compared with Haversian system size (Skedros et al., 2013a). Furthermore, it is possible that the simplified interpretation of ‘one cortex is primarily subjected to compressive strains while the opposite cortex is primarily subjected to tensile strains’ is ignoring a third important component, namely shear strains (Keenan et al., 2017; Skedros et al., 2013a, 2019). Skedros et al. (2019) found that the plantar cortex of the deer calcaneus, which was previously believed to be loaded primarily in tension, is also actually experiencing significant shear strains. Accordingly, they have changed their description from tension/ compression to tension-shear/compression (Skedros et al., 2019). Yet another possibility is that higher strains (which are expected in the compressive cortex) will inhibit osteoclast activity and thus will generate smaller diameter cutting cones, and consequently smaller Haversian systems during the remodeling process (van Oers et al., 2008). This again is an interesting idea that fits our experimental findings, as it explains the difference in Haversian system size between the cranial and caudal cortices (differences in osteoclast activity), with no effect on bone mineral content and compressive stiffness (no difference in osteoblast activity). This explanation is supported by Schulte et al. (2013), who demonstrated in their study of mice caudate vertebrae loaded in compression that bone resorption (via osteoclasts) is more strictly controlled than bone formation (via osteoblasts) (Schulte et al., 2013). Their interpretation of this phenomenon is that it is mechanically more risky when bone is resorbed improperly (e.g. at the wrong place or to a larger extent than the bone can safely sustain), than when bone is formed unnecessarily. The final possibility is that Haversian system size is dependent on the form of microdamage, which differs between the tensile and compressive cortices (Boyce et al., 1998; Reilly and Currey, 1999, 2000; Ebacher et al., 2007; Burr, 2011; Skedros et al., 2011). It was observed that compressive microcracks are relatively straight and long (tens to a hundred micrometers in length), while tensile microdamage is of a diffuse nature, consisting of numerous smaller microcracks (up to 10 μm in length) forming a flame-like array that covers a much larger area of bone. Hence, it is possible that a linear microcrack in the compressive cortex will initiate smaller BMUs, as one or two smaller cutting cones (and thus smaller Haversian systems) will be sufficient to remove the damaged tissue. In contrast, the larger area covered by the diffuse array of numerous shorter microcracks in the tensile cortex may initiate larger BMUs that will result in larger Haversian systems.

The fact that we did not find a difference in mineral content between the cranial and caudal cortices was not very surprising, as previous studies found conflicting evidence to which cortex, if any, is more mineralized. Although some studies found higher mineral content in the compression cortex of calcanei from deer, horse, elk and sheep (Skedros et al., 1994a, 1997) and the sheep radius (Lanyon et al., 1979), Skedros et al. (1996) found an opposite trend where the tension cortex of the horse third metacarpal bone demonstrated higher mineral content. Yet other studies found no significant difference in mineral content between the compressive and tensile cortices of horse radii (Riggs et al., 1993b; Mason et al., 1995), sheep tibia (Lanyon and Bourn, 1979) and mule deer forelimb bones and ribs (Skedros et al., 2003). A possible explanation for the similar mineral content in the cranial and caudal cortices in our study is the young age of our deer. Skedros et al. (2004) found that mineral content differences between the compressive and tensile cortices of mule deer calcanei start to appear only after a certain age (sub-adults), and that young fawn calcanei mineral content was lower compared with older age groups (62–64 and 66–71% in young fawns and older deer, respectively). We too found lower values of mineral content in the proximal humerus, around 62–63%.

Cortical bone stiffness was found to not differ significantly between the cranial and caudal cortices. As we have not found any significant difference in mineral content between the two cortices, these results were expected. Although we did find larger Haversian systems in the cranial cortex, this morphological difference seems to have little or no influence on the compressive stiffness of cortical bone. We have listed above several possible explanations for this difference in size, and it may be that the effect of a larger Haversian system is more important in tensile loading or in the fatigue life of bone (attenuating and arresting the propagation of microcracks). Another interesting finding was that our current compressive stiffness values are lower compared with similar samples from the proximal femur and the humerus mid-diaphysis of juvenile white-tailed deer (Barrera et al., 2016; Kunde et al., 2018). Our axial, radial and transverse stiffness values were around 18, 10 and 11 GPa, respectively; however, Barrera et al. (2016) found compressive stiffness in the proximal femur of white-tailed deer to be around 21, 18 and 15 GPa for the axial, radial and transverse directions, respectively. Similarly, Kunde et al. (2018) found...
compressive stiffness in the mid-diaphysis humerus of white-tailed deer to be around 25, 18 and 15 GPa for the axial, radial and transverse directions, respectively. These differences in stiffness values (lower for secondary Haversian bone compared with primary plexiform bone) and mechanical behavior (transverse isotropic versus orthotropic) are indicative of the differences in bone structure.

There are a few potential limitations to our study. The main limitation is that our analysis is based on an over-simplified model of the curved deer humerus, which assumes that normal loading occurs mainly in tension (cranial cortex) and compression (caudal cortex). Therefore we did not address shear stresses and strains that may be significant especially in the tensile cortex (Skedros et al., 2019). Nevertheless, there is ample evidence that most curved long bones of quadrupedal animals, including the humerus, are loaded in bending and that compressive and tensile strains are experienced on opposite cortices (Biewener, 1983a,b; Rubin, 1984; Pollock et al., 2008a,b; Henderson et al., 2017). In addition, we decided to focus our study on the proximal humerus to avoid torsional stresses that were revealed to increase as we move distally along the bone shaft (Oh and Harris, 1978; Skedros and Baucom, 2007; Carter et al., 2014). Another related limitation is our inability to investigate the relationship between CFO and Haversian system size. A previous study (Skedros et al., 2013a) demonstrated that Haversian system morphotype, which is determined by the predominant CFO, is much more consistent with the distribution of tension and compression strains of habitual bending. Thus, a more complete analysis should also investigate the Haversian system CFO and bone toughness (i.e. microcrack propagation) between the two opposite cortices. Yet another possible limitation is that as we acquired the bones from a processing plant, we could not assess the potential effect of sex, age and body size in our samples. Data collected for deer populations in the USA (Hillman et al., 1973; Purdue, 1983; Flinn et al., 2013) demonstrated that age, sex and especially nutrition are all parameters that may affect the timing of epiphyseal growth plate closure. Yet Purdue (1983) noted that deer from South Carolina (the location where we collected our bones) were from an environment with a long frost-free period, and thus they were expected to feed well. Furthermore, we collected our bones during the hunting season (autumn), which is the time of the year when deer are best fed and bone remodeling is at its peak (Hillman et al., 1973). Finally, we were unable to mechanically test our samples until failure as the strength of our bone samples exceeded the limit of our testing machine and load cell (500 N). Thus we were unable to detect any strength and toughness (crack propagation) differences between the cranial (tensile) and caudal (compressive) cortices. Similarly, our samples were only tested in compression and not in tension, and it is possible that larger Haversian systems are beneficial specifically under tensile stress. Skedros et al. (2006) were able to test samples from the same bone in compression and tension, yet they studied the horse third metacarpal, which is not a simple bending model but experiences complex loading during locomotion (Keenan et al., 2017) and thus is less suitable for comparison of regional variability between opposite cortices of a bone subjected to bending.

In conclusion, the aim of this study was to find a correlation between secondary cortical bone structure and tissue composition, and the mechanical properties of the cranial and caudal cortices of the proximal humerus from white-tailed deer. Similar to previous studies, we found larger Haversian systems in the cranial cortex compared with the caudal cortex, yet no difference in mineralization was detected. Predicating differences in strain magnitude and sign between the cranial and caudal cortices, these results may imply that strain magnitude and sign affect osteoclasts in the BMU during the resorption phase of bone remodeling but not osteoblasts in the BMU during the bone deposition phase of bone remodeling. Consequently, we found no difference in compressive stiffness between the two cortices and thus our working hypothesis of correlation between bone structure and function was not supported.

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Author contributions

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