Spring 5-2020

The Effect of Staphylococcus aureus Exposure on White-Tailed Deer Trabecular Bone Stiffness and Yield

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May, 2020

To the Dean of the Graduate School:

We are submitting a thesis written by Emily Long entitled The EFFECT OF *STAPHYLOCOCCUS AUREUS* EXPOSURE ON WHITE-TAILED DEER TRABECULAR BONE STIFFNESS AND YIELD. We recommend acceptance in partial fulfillment of the requirements for the degree of Master of Science.

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THE EFFECT OF *STAPHYLOCOCCUS AUREUS* EXPOSURE ON WHITE-TAILED
DEER TRABECULAR BONE STIFFNESS AND YIELD

A Thesis

Presented to the Faculty

Of the

College of Arts and Sciences

In Partial Fulfillment

Of the

Requirements for the Degree

Of

Master of Science

In Biology

Winthrop University

May, 2020

By

Emily Brooke Long

Abstract

Osteomyelitis is an infection of the bone or bone marrow caused by the infiltration of bacteria, resulting in destructive inflammation, bone necrosis and abnormal bone remodeling. With a growing number of osteomyelitis diagnoses, many of which are linked to *Staphylococcus aureus* (*S. aureus*), it is imperative to understand the pathology of *S. aureus* in relation to bone to provide better diagnostics and patient care. While the cellular mechanisms of *S. aureus* and osteomyelitis have been studied, little information exists on the biomechanical effects of such infections. We postulated that exposure to *S. aureus* for 72 hours would significantly decrease both the stiffness and yield of trabecular bone tissue. One hundred and three trabecular cubes (5 x 5 x 5 mm) from the proximal tibiae of *Odocoileus virginianus* (white-tailed deer) were used in this experiment. Bone cubes were sterilized and then swabbed to confirm sterilization before inoculation with *S. aureus*-ATCC-12600 (test group) or sterile nutrient broth (control group) for 72 hours. All cubes were then tested in compression until yield using an Instron 5942 Single-Column machine. Structural stiffness (N/mm) and yield (MPa) were calculated and compared between the two groups. Our results reveal that acute exposure to *S. aureus* does not significantly decrease trabecular bone stiffness or yield.

Acknowledgments

I would first like to thank my thesis advisor, Dr. Victoria Frost, for her unwavering support and encouragement over the past two years. My journey from undergraduate to postgraduate studies was not immediate or without its obstacles. Still, through this process, I have learned to conquer self-doubt, setbacks and to embrace challenges both personal and professional. Dr. Frost has been an integral part of this growth and I leave Winthrop a better scientist and teacher due in large part to our work together.

I would also like to thank my co-advisor, Dr. Meir Barak, who, despite relocating to pursue his career goals, unselfishly remained an essential part of this team. This project would not have been possible without Dr. Barak's guidance and expertise. Additionally, I would like to thank the other members of this committee, Dr. Matt Stern and Dr. Laura Glasscock, both of whom I had the pleasure of learning from as a part of my graduate course work. Also, thank you to Dr. Julian Smith and Dr. Scott Wertz for the use of their lab equipment to facilitate this project and to fellow graduate student Jack Nguyen for help in and out of the lab.

Furthermore, I would like to give a special thank you to the Winthrop University Research Council Grant Committee, whose financial support made this research possible.

I would also like to acknowledge my parents and fellow graduate students who have provided me with comic relief and helping hands along the way. Finally, I would like to recognize my husband, who encouraged me to seek out my goal of a graduate degree and continues to help me see the positivity in all life's challenges.

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Introduction

Osteomyelitis and *S. aureus*

Osteomyelitis is an infection caused by bacteria in the bone or bone marrow, resulting in inflammatory destruction and bone necrosis followed by new bone development (Tong et al. 2015). Osteomyelitis proves challenging to treat due to the difficulty of early diagnosis, persister cells within biofilms and evasion of host immune responses, ineffective antibiotic treatment and growing antibiotic resistance (Olson & Horswill 2013). In a population-based study, Kremers et al. (2015) showed that the rate of osteomyelitis cases (760 in total) in Olmsted County, Minnesota, increased significantly from 2000-2009 when compared with earlier data from 1969-1979. Incidence rates were steady amongst those less than fifty years old but nearly tripled in elderly patients; furthermore, of all infections, 44 percent involved the bacterium *Staphylococcus aureus* (*S. aureus*) (Kremers et al. 2015). With a growing number of osteomyelitis diagnoses, many of which are linked to *S. aureus*, it is essential to understand the short term pathology of *S. aureus* in relation to bone to provide better diagnostics and patient care, in addition to understanding the possible long term effects of osteomyelitis on the mechanical properties of bone.

Biofilm Formation and *S. aureus*

S. aureus is a gram-positive coccus shaped bacterium widely studied in connection with osteomyelitis and other pathogenic diseases (Berendt & Byren 2004). The capability of certain bacteria to produce biofilms has been associated with chronic bacterial infections, and *S. aureus* has been recognized as an active biofilm secretor on an array of living and non-living (i.e., prosthetic) surfaces (Guo et al. 2017). Biofilms are an adhesive protective matrix secreted by bacteria that enclose the microbes, allowing them

to grow on living tissues as well as foreign materials such as medical devices (Khatoon et al. 2018). *S. aureus* has recently been shown in murine models, using transmission electron microscopy (TEM), to invade the canaliculi of live cortical bone and carry out biofilm formation in osteocyte lacunae (Bentley et al. 2017). These studies serve not only to demonstrate the ability of *S. aureus* to invade deep within bone but also provide insight as to why osteomyelitis infections are so difficult to eradicate. Bacteria living within a biofilm are exceedingly impervious to antimicrobial products and residing within osteocyte lacunae offers protection against host immune responses. The capacity of certain bacteria to produce biofilms has been associated with chronic states of infection, including pneumonia, cystic fibrosis, chronic wounds, catheter-associated infections and orthopedic implant devices (Bjarnsholt 2013). In an article summarizing criteria for the classification of periprosthetic joint infections (PJI), Zimmerli (2014), describes delayed and late-onset of *S. aureus* infections, the latter of which can arise as many as 24 months after surgery.

Another method (closely linked with biofilms) employed by *S. aureus* to circumnavigate host immune responses and antimicrobials lies in their ability to form persister cells. Persister cells are those that can survive antimicrobial treatment (in a dormant like state) in which their formation is initiated by stress signaling within hostile environments (Brauner et al. 2016). These residual survivor cells have been found to be the main contributor to the antimicrobial tolerance of biofilms, and *S. aureus* is among the many species of bacteria capable of producing such a response (Keren et al. 2004). The development of medical interventions to attack these dormant cells in biofilms may aid in the prevention of recurring infections, particularly for those associated with using biofilm and persister cell strategies to avoid destruction (Arciola et al. 2018).

Biomechanics of Bone

In the context of osteomyelitis, protecting bone structural integrity in the short and long terms becomes a significant concern for clinicians and patients. When considering the effect of microbes, such as *S. aureus*, on the biomechanics of bone, what we are interested in is how the bone or bone material responds to the presence of microbes, ultimately, considering the risk of failure or fracture within the bone. Key mechanical properties that can predict the risk of bone failure and fracture are structural and material stiffness, yield and strength. These mechanical properties can be calculated from the load-deformation relationship (or the normalized stress-strain relationship) of the bone when it is loaded in compression (Cullinane & Einhorn 2002; Turner and Burr 1993; Sharir et al. 2008). Structural stiffness is calculated from the slope of the load-deformation curve's linear portion. Material stiffness (Young's modulus) is derived from the slope of the stress-strain curve's linear region (a stress-strain curve is a normalized load-deformation curve) (Currey 2001). The steeper the slope, the stiffer the material. The yield point is defined as the stress at which a material first behaves plastically; in other words, it stops behaving elastically and the relationship between stress and strain is no longer linear. Thus, further deformation will be irreversible and after removal of the load, residual and permanent deformation will persist. The strength of bone refers to the ultimate load (stress) that bone can undergo and is identified as the highest stress point in the stress-strain curve. Strength, however, is more challenging to determine in trabecular bone due to its porous nature. Contrary to cortical bone, where there is a clear point of peak stress, strength measurement in a porous structure is less conclusive. Fyhrie and Schaffler (1994) and Hakamada et al. (2007) have studied the typical stress-strain behavior of trabecular bone. They found that when the first few trabeculae buckle and fail, there is a decrease in measured stress, yet as more and more trabeculae fail, the structure starts to collapse

and becomes compacted. At that stage, load (stress) may not change, or may even increase again, with the increase of deformation (denoted the “plateau region”) (Fyhrie and Schaffler 1994; Hakamada et al. 2007). Furthermore, it is important to note that all of these measurements (stiffness, yield and strength) are dependent on many additional factors including, the type of bone, mineral composition, hydration status, microstructure and the rate and mode of load, such as compression, tension and shear stress (Cullinane & Einhorn 2002).

Properties of Bone and *S. aureus*

Cortical (compact) and trabecular (cancellous or spongy) bone tissues are two structures made of the same bone material that differ in architecture and function (Turner et al. 1993). These bone types are found in variable percentages in different bones among vertebrates. Both cortical and trabecular macrostructure and microstructure contribute to overall bone stiffness and strength (Barak et al. 2008; 2010). In a recent comparative study of rat cortical and trabecular bone, Oftadeh et al. (2015) demonstrated that while cortical and trabecular bone differed in their architecture, their material composition was not significantly different. Both cortical and trabecular bone shared identical material properties such as bone tissue density, mineral content, tissue modulus (i.e., Young’s modulus), hardness, protein content and mineral content (Oftadeh et al. 2015). At the same time, cortical and trabecular bone tissue differed in their structural properties such as bone volume fraction, bone surface to volume ratio, stiffness and strength (Oftadeh et al. 2015). This hierarchical analysis further validates that the similarities and variances among trabecular and cortical bone reside primarily within the macro and microstructural levels, respectively and these properties are predictive of the mechanical behavior of these bone tissue types.

In general, bone can be considered in terms of its macrostructural and microstructural composition. When viewed at the macrostructural scale, trabecular bone is composed of a porous mesh-like structure, made up of beams (or struts) and plates. These beams and plates form an interconnected architecture that provides the scaffold for cellularized bone marrow and is designed to optimize load transfer, as seen in vertebral bodies and the epiphysis of long bones. Disruption of the connections between the structural elements of trabecular bone or deterioration of the beams themselves has been shown to reduce the strength of bone and increase susceptibility to fracture (Legrand et al. 2000; McCloskey et al. 2016; Samelson et al. 2019). At a microstructural scale, trabecular bone is a composite material primarily consisting of hydroxyapatite, collagen and water. As the building blocks of bone, collagen and mineral organization plus alignment are critical elements in determining bone tissue properties (Fritsch and Hellmich 2007).

Among the many factors that may affect critical structural features of bones are bacterial infections. Imaging analysis of murine models experiencing *S. aureus*-induced osteomyelitis have detected pathogen-induced bone remodeling and have validated the ability of *S. aureus* to induce profound changes in bone turnover (Cassat et al. 2013). As the remodeling of bone takes place throughout life, there exists a delicate balance between bone deposition and bone resorption. Osteoclasts and osteoblasts as part of BMUs (bone multicellular units) are the two main cell types directly involved in bone remodeling processes. Osteoclasts attach to the bone and form a sealing zone into which they secrete hydrogen ions through a portion of their plasma membrane called the ruffled border that breaks down the mineralized tissue and creates a cavity. In contrast, osteoblasts participate in the formation and deposition of unmineralized bone material called osteoid. In *ex vivo* organ cultures of neonatal mouse parietal bones and bone marrow and

periosteal cells, *S. aureus* cultured media significantly heightened the distribution of bone degradation fragments, the expression of osteoclastic genes (those which contribute to bone resorption) and osteoclastogenic transcription factors in a dose and time-dependent manner that was statistically significant at just 24 h (Kassem et al. 2016). Furthermore, there is evidence to support the degradation of bone, even in the absence of host cells. The ability of bacterial biofilms to degrade bone in the absence of active immune cells or osteoclasts was confirmed by Junka et al. (2017). This study demonstrated that the presence of *S. aureus* biofilm formation resulted in a significant reduction of total bone in both *in vivo* and *ex vivo* conditions.

Our study examined the effect that *S. aureus* exposure has on the mechanical properties of trabecular bone from white-tailed deer (cervine) tibiae. Deer tibiae were found to be a better human bone model than pigs (porcine), goats (caprine) and sheep (ovine) for assessing overall morphology and mechanical properties. In an analysis of tibiae modeling, Throop et al. (2015) tested a variety of measurements on cervine tibiae. They found that of the 13 dimensions measured, none of the cervine measurements exceeded a 5% difference from the human tibia. Additionally, the stiffness of the cervine tibia is within the range of values reported for human tibiae

Recently Kunde et al. (2018) conducted a study on the mechanical properties of cortical bone tissue stiffness, post-exposure (48 h) to *S. aureus*. While they demonstrated that *S. aureus* was able to penetrate the bone tissue, no significant difference in bone stiffness was found between the test group, exposed to *S. aureus* and control group immersed in distilled water (dH₂O). Trabecular bone porosity ranges between 50-90% (compared to 5-10% for cortical bone) and it is a prominent architectural characteristic that significantly increases bone surface area to bone volume (BS/BV) (Burr 2011; Clarke 2008; Sikavitsas et al. 2001). Due to the porous nature and increased surface area of

trabecular bone tissue, the potential of *S. aureus* to penetrate and negatively affect trabecular bone material is markedly increased. Thus, we have extended the work of Kunde et al. (2018) to trabecular bone. We hypothesized that exposure to *S. aureus* would significantly decrease the stiffness of trabecular bone tissue. In addition, we sought to assess trabecular bone yield and strength (a component missing from Kunde et al.) identifying possible correlations between *S. aureus*, bone stiffness and yield. Again, due to the lattice-like structure and ease of bacterial entry into trabecular bone tissue, we hypothesized that exposure to *S. aureus* would significantly decrease both yield and strength.

To test our hypotheses, we subjected 5 mm³ trabecular bone cubes cut distally to the proximal physis of the tibia of white-tailed deer to compressional stress after inoculation with *S. aureus* for 72 h and measured their tissue stiffness, yield and strength. The second set of cubes (control group) was subjected to the same experimental protocol and timeframe except for *S. aureus* inoculation. Ultimately, we were unable to obtain strength data for all cubes, as many exhibited strength values above the capability of our machine (500 N). Thus, only the structural stiffness and yield (a proxy for strength) of the bone cubes between each group (control and test) were compared to determine if they were negatively impacted after acute exposure to *S. aureus*.

Our study contributes to what we see as an area of need. The cellular and molecular responses of bone to bacterial infections such as *S. aureus* have expanded our view of bone influencers, but less is known about the biomechanical effects these microbes have on bone. Additionally, when considering studies that have been conducted on the mechanical properties of bone, trabecular bone studies are underrepresented in comparison to cortical bone. Our team is uniquely qualified to investigate this hypothesis based on the recently published research by the senior members of our team on the

mechanical properties of cortical bone after acute exposure to *S. aureus* (Kunde et al. 2018). We sought to build upon the previous research of Kunde et al. (2018) by extending the exposure time of *S. aureus* from 48 h to 72 h in addition to considering the mechanical effect of this microbe on the other primary type of mature mammalian bone structure, trabecular bone.

Materials and Methods

Preparation of Bone Samples

Nine white tailed-deer tibiae were obtained from One Price Deer Processing in York, South Carolina. Deer were killed during hunting season and tibiae were harvested freshly from the carcasses. Each tibia was identified (right or left) and then inspected to rule out any evidence of damage or abnormalities. Explicit sex and precise age were unknown, but all tibiae were from juveniles, between the ages of 5 and 20 months, as they exhibited active growth plates (Flinn et al. 2013; Purdue 1983). Bones were cut using a hand saw to isolate the proximal tibia and then cleaned of soft tissue and stored at -20 °C until further preparation.

Two 5 mm slices (exception tibia 1, one slice only) were cut from the proximal end of each tibia distal to the physis. These slices were used to create 5 mm³ trabecular bone cubes. In total, 200 cubes were cut from the proximal end of the tibiae parallel to the three principal axes of the bone (Fig. 1). Sixty-three cubes were prepared for the preliminary tests (chlorhexidine exposure and time exposure, see sections 3.2 and 3.3) and 137 were initially assigned to the main experiment (test and control groups, Fig. 2). Three of the 63 preliminary cubes were used to verify the mechanical testing protocol prior to the beginning of the actual experiment. For the preliminary chlorhexidine (CHG) experiment, two cubes were removed. For the preliminary exposure timeline test, three 72 h cubes

(see sections 3.2 and 3.3) were removed for abnormal trabecular bone behavior, in which linear regions could not be measured. Thus, in total, 38 cubes were used in the preliminary CHG testing experiment (see section 3.2), and 36 cubes were used in the exposure timeline testing experiment (see section 3.3). Six control cubes and 11 test cubes were removed due to the identification of cartilage by SEM imaging (see section 3.11). In addition, 10 cubes from the second test group batch were removed due to bacterial contamination. Finally, 1 cube per test group batch (7 in total) were crushed prior to the compression test to verify the decontamination of the bone tissue (part of the experimental protocol, see section 3.8). Thus, in all 52 control cubes and 51 test cubes were used for stiffness data analysis in this experiment (a total of 103 cubes, Fig. 2). Test cube T9R-B1-C05 was excluded from the yield analysis as the machine experienced an early stop before the yield point, resulting in 52 control and 50 test cubes for yield data analysis.

Bone cubes were cut using a water-cooled diamond saw (TechCut4™ Precision Low-Speed Saw). Cube edges were color-coded red (anterior/posterior), blue (proximal/distal) and black (medial/lateral) (Fig. 3). Bone slices were drawn (cross-section) to scale and the location of each cube was recorded (see appendix A). To randomize our groups and prevent any effect due to possible inter and intra bone variance, we included both control and test groups samples from all tibia ($n=9$), from both slices per bone, and from all anatomical locations per slice (anterior/posterior and medial/lateral). Prior to storage, the volume (mm^3) was measured for all cubes along with the mass (mg) and these values were used to calculate the apparent density of each cube (mg/mm^3). A two-tailed independent t-test revealed a significant difference ($P < 0.05$) between the apparent density of the control group and that of the test group. Density was therefore controlled as a covariate for our ANCOVA results of stiffness and yield (see results 3.1). Labeled

samples were placed in a 1.5 mL microcentrifuge tube (Fisherbrand®) stuffed with paper soaked in 0.2 mL saline + 8% chloroform and stored frozen at -20 °C (Martos et al. 2013).

Fig. 1. A schematic illustration of the white-tailed deer tibia and the location from which the bone cubes were cut. The cubes (measured 5x5x5 mm) were prepared from slices cut distal to the proximal physis, parallel to the tibial plateau.

Fig. 2. A schematic illustration of cube group allocation per batch and removal for both preliminary and experimental testing.

Fig. 3. A single 5 mm³ trabecular bone cube color-coded before experimentation to mark anatomical orientation. Red: anterior/posterior; blue: proximal/distal; and black: medial/lateral.

Preliminary CHG Testing

As trabecular and cortical bone tissues have different macrostructures, the decontamination technique previously used by Kunde et al. (2018) for cortical bone had to be verified for trabecular bone. To this end, 38 trabecular bone cubes were thawed for 24 h and equally divided into two groups. Nineteen cubes were suspended in 1 mL of sterile dH₂O (this group also served as the control group for the exposure time testing, see section 3.3). The other 19 cubes were suspended in 1 mL of 4% w/v chlorhexidine gluconate (Hibiclens™ Liquid Antiseptic Skin Cleanser, Fisher Scientific™). These initial washes were under constant agitation (200 rpm) in a temperature-controlled shaker (Eppendorf™ Thermomixer™ R) at 21 °C for 15 min. Next, all samples underwent rinsing and rehydration by shaking (200 rpm) with 1 mL of sterile dH₂O at 21 °C for 5 min two consecutive times. After all 38 bone cubes were rinsed and rehydrated, they were tested in compression and structural stiffness and yield were recorded (see section 3.9 for mechanical testing protocol). A two-tailed independent t-test revealed a significant difference ($P < 0.05$) between the apparent density of the water group and that of the CHG group. Density was therefore controlled as a covariate for our ANCOVA results of stiffness and yield for this preliminary experiment. The mean stiffness of the dH₂O samples was 8899.9 ± 7442.6 (N/mm) and the stiffness of the CHG samples was 7019.2 ± 4908.7

(N/mm). We found no significant difference in stiffness between cubes washed with CHG and cubes washed with sterile dH₂O only ($P > 0.05$). The mean yield of the dH₂O group was 16.13 ± 3.93 (MPa) and the yield of the CHG samples was 15.21 ± 3.71 (MPa). We did find a significant difference between the yield of the water group and that of the CHG group ($P = 0.036$).

Preliminary Exposure Timeline Testing

Bone tissue slowly degrades at temperatures above freezing and therefore, experimental time itself may influence bone stiffness. To test the mechanical effect of this deterioration, Barrera et al. (2016) showed a nonsignificant decrease in stiffness of white-tailed deer cortical bone cubes from the femur after seven days of thawing. Similarly, Kunde et al. (2018) showed a nonsignificant decrease in stiffness of white-tailed deer cortical bone cubes from the humerus after 48 h of thawing. To account for the difference in surface area between cortical and trabecular bone (the larger surface area of trabecular bone may lead to faster deterioration) and the presence of bone marrow (which can supply additional nutrients for bacterial growth), a test was needed to verify that no significant difference in stiffness and yield occurs during that time or if so to quantify the effect. Nineteen trabecular bone cubes were loaded in compression after thawing for 24 h; these cubes are the same group as the water suspended group in the CHG test (see section 3.2) and 17 trabecular bone cubes were loaded in compression after thawing for 72 h. A two-tailed independent t-test revealed no significant difference ($P > 0.05$) between the apparent density of the 24 h and that of the 72 h group. The results were analyzed with an independent one-tailed t-test to determine the effect of thawing time on trabecular bone tissue stiffness and yield. The mean stiffness of the 24 h samples was 8899.9 ± 7442.6 (N/mm) and the stiffness of the 72 h samples was 6386.6 ± 4260.6 (N/mm). We found no significant difference in stiffness between cubes thawed for 24 h and those thawed for 72

h ($P > 0.05$). The mean yield of the 24 h group was 16.13 ± 3.93 (MPa) and the mean of the 72 h was 14.71 ± 4.49 (MPa). We found no significant difference in yield between cubes thawed for 24 h and those thawed for 72 h ($P > 0.05$).

Experimental Timeline

Each experimental trial consisted of 10-12 bone cubes and lasted eight days from the defrosting of the *S. aureus* stock culture (Fig. 4). On day one, 1 mL of *S. aureus* stock culture was defrosted and moved to new sterile broth to grow for 48 h. On day three, these samples were transferred to individual 5 mL centrifuge tubes to grow for an additional 48h. On day four, bone cubes were transferred to a refrigerator to thaw at 4 °C for 24 h. On day five, bone cubes were disinfected using CHG (see section 3.6) and swabbed to confirm initial bacterial removal followed by inoculation of *S. aureus* (test group) or sterile nutrient broth (control group) for 72 h. All cubes were removed from 72 h suspension on day eight, disinfected with 95% ethyl alcohol (Ward's Science®), rehydrated (dH₂O washes x 4) and swabbed to confirm decontamination (except for one test cube from each batch that was crushed prior to the compression test to verify the decontamination of the internal bone surfaces). Lastly, all bone cubes were tested in compression immediately following decontamination on day eight. Overall, 52 cubes were included in the control group and 51 cubes in the test group for stiffness analysis. Fifty-two control cubes and 50 test cubes were used in the yield analysis.

S. aureus Test Suspension Protocol

S. aureus (ATCC-12600) was purchased from the American Type Culture Collection (Manassas, VA, USA) and directly inoculated in nutrient broth (Difco™ Nutrient Broth, BD). This initially inoculated broth was incubated at 37 °C for 48 h. The inoculum was then frozen at -80 °C as a 1 mL-40% glycerol stock culture. In preparation for each

experiment, an initial passage one from frozen (P1FF) test suspension was created by transferring 10 μ L of the frozen bacterial stock to 10 mL nutrient broth and incubated at 37 °C for 48 h. Before incubation with the bone sample, 10 μ L of stock culture and P1FF solutions were taken and streaked separately on nutrient agar (Difco™ Nutrient Agar, BD) and incubated at 37 °C for 48 h to verify purity and growth (Fig. 5A). To make the P2FF (second passage from frozen) solution, P1FF cultures were vortexed and 10 μ L was transferred into individual sterile 5 mL centrifuge tubes (VWR™) containing 2 mL of nutrient broth. Again, 10 μ L of all P2FF samples were taken and streaked individually on nutrient agar at 37 °C for 48 h to verify the purity and growth of P2FF (Fig. 5B). These P2FF 5 mL tubes were under continuous agitation (200 rpm) in a temperature-controlled shaker at 37 °C for 48 h and were used as the test solution for *S. aureus* inoculated groups. This bacterial suspension was tested against the Remel McFarland Equivalence Turbidity Standard (Thermo Scientific™), which uses visual inspection to identify the distortion of black lines that are equal to bacterial counts within an expected range. Our density was 1.5×10^8 bacteria/mL at the beginning of the inoculation. *S. aureus*, along with other Staphylococcal strains, have been identified as highly effective biofilm secretors on a variety of biotic and abiotic surfaces (Guo et al. 2017). We used the tube method (TM) as described by Christensen et al. (1985) to verify the ability of our strain of *S. aureus* to produce a biofilm (Fig. 6).

Fig. 4. Timeline showing experimental workflow by day. NB: nutrient broth.

Fig. 5. A picture showing bacterial growth of test group #3. A) 10 μ L loop transfer from *S. aureus* glycerol stock (upper half), P1FF tube 1 (lower right) and P1FF tube 2 (lower left) onto nutrient agar. This picture shows axenic *S. aureus* growth with no contamination. B) 10 μ L loop into P2FF (cubes 1-5 shown) onto nutrient agar; axenic *S. aureus* growth, no contamination.

Fig. 6. A picture of the tube method (TM) results. Left: *S. aureus* biofilm formation (purple). Right: negative control.

Disinfection

All samples were placed in sterile 5 mL centrifuge tubes containing 1 mL of 4% w/v CHG under continuous agitation (200 rpm) at 21 °C for 15 min. Next, samples underwent four cycles of sterile dH₂O washing for 5, 10, 5 and 5 min. Cubes were transferred to a new sterile 5 mL centrifuge tubes with 2 mL of sterile dH₂O between washes. All sterile dH₂O washes were under continuous agitation (200 rpm) and kept at 21 °C. Each cube was then removed using sterile forceps, swabbed with a sterile cotton tip and streaked on nutrient agar. These plates were incubated for 48 h at 37 °C to confirm disinfection. All control cubes underwent the same disinfection procedure as outlined above.

Introduction of *S. aureus*

Test bone cubes were contaminated via submersion in 2 mL of *S. aureus* P2FF test suspension under constant agitation (200 rpm) in a temperature-controlled shaker at 37 °C for 72 h. Control samples were suspended in 2 mL of sterile nutrient broth on a shaker under the same conditions.

Decontamination

For safety purposes and to maintain exact exposure time, all samples were decontaminated after 72 h. All bone cubes were removed from *S. aureus* test suspension (test group) and sterile broth suspension (control group) and placed in sterile 5 mL centrifuge tubes containing 1 mL of 95% ethyl alcohol (ETOH) under agitation (200 rpm) on a shaker at 21 °C for 30 min. Previous researchers have found that brief periods of ethanol washing do not significantly affect the mechanical properties of the bone (i.e., stiffness) when the bone is rehydrated with water washing prior to testing (Beaupied et al. 2006; Linde and Sørensen 1993; Turner and Burr 1993). Each cube was then washed four times (5, 10, 5 and 5 min) in 2 mL of sterile dH₂O under constant agitation (200 rpm) on a shaker at 21 °C to remove any ETOH residue and to rehydrate tissue. Between each wash, samples were moved to new sterile 5 mL centrifuge tubes. After washing, the surfaces of each sample were swabbed, streaked on onto nutrient agar and incubated (37 °C, 48 h) to verify the lysing of all bacteria. One cube from each test batch was sacrificed to verify no bacterial growth persisted on internal surfaces; these sacrificed cubes were aseptically crushed, swabbed, streaked on nutrient agar and incubated at 37 °C for 48 h.

Mechanical Testing

An Instron 5942 Single-Column Table Frame in compression mode was used to determine the stiffness and yield of all bone samples. All trabecular bone cubes were loaded at room temperature (18-22 °C) in the axial orientation until failure, determined as a sudden visible decrease in load on the load-deformation curve, or up to 480 N (system and load cell maximum are 500 N) if failure was not reached. By 480 N, stiffness and yield (but not strength) for all trabecular bone samples were observed.

All bone cubes were kept in sealed centrifuge tubes with 2 mL of dH₂O until time of compression. At the start of each loading cycle, one sample was centered on a lower stationary anvil and the upper mobile anvil was lowered until contact (Fig. 7). To mimic physiological conditions, a small amount of saline (approx. 0.2 mL) was added around the sample to keep the bone hydrated during compression (around 5 min per test). A small preload (1-5 N) was applied at the start of each experiment. Beginning at 5 N, load and displacement data was collected every 0.1 s (BlueHill 3 Software, Instron, USA). The upper anvil of the Instron loaded the bone cubes against the lower stationary anvil at a rate of 0.05 mm/min up to 480 N. At the end of the loading cycle, compressed bone cubes were removed and placed back into labeled centrifuge tubes and stored frozen at -20 °C. To determine the structural stiffness of each cube, we calculated the slope (N/mm) from the linear portion of each load-displacement curve. To determine the compressive yield, each load-displacement curve was transformed into a stress-strain curve by dividing the measured load by the cube loaded area and the displacement by the cube height. Next, we determined the point where the stress-strain curve stopped behaving linearly (usually around 0.02%) as yield (Moore and Gibson 2002).

Fig. 7. A picture showing a color-coded trabecular bone cube (loaded in the axial orientation: blue) as it was centered on the lower stationary anvil prior to compression.

SEM Imaging

Trabecular bone cubes that demonstrated yield strains above 0.025% were inspected under a multitouch panel scanning electron microscope (JSM-6010PLUS/LA InTouchScope™) to verify the sample was normal (e.g., did not contain cartilage). These cubes were prepped for imaging by soaking in acetone for 24 h to remove all orientation markings and then dried for three days in increasing percentages of ethyl alcohol (24 h in each of the following concentrations: 70%, 80% and 90%). On day five, cubes were dried in an oven overnight (Precision Scientific Model 19 Thelco Vacuum Lab Oven®) at 70 °C. The following day, cubes were attached to mounts and imaged to visualize multiple planes; all images were inspected for the presence of cartilage. In all, of the 56 trabecular bone cubes that demonstrated yield strains above 0.025%, 11 test cubes and 6 control cubes were removed due to evidence of cartilage.

Bone Ashing Procedure

To confirm that there was no significant difference in percent (%) of mineral, organic material and water among tibia tested, samples from all bones were ashed. Between 100-300 mg from all nine tibiae were crushed and ground using a mortar and pestle. Ground samples were washed in production-grade acetone (VWR™) to remove all lipids and then placed for 12 h under a heat lamp to evaporate the acetone. Initial weight measurements (± 0.5 mg) were taken to determine lipid-free wet mass. Next, samples were placed in a ceramic cup and heated in an isotherm programmable forced-draft furnace (ThermoFisher Scientific ©) at 100 °C for three h to remove all unbound water. After, 3 h samples were weighed to determine the dry mass (organic and mineral content). To remove all organic material, samples were returned to their ceramic cups and heated to 500 °C for 16 h. Final weight measurements were used to determine the dry bone ash content. Water content (%) was calculated by subtracting the wet mass from the dry mass; organic material (%) was calculated by subtracting the dry mass from the ash mass and ash content (%) was calculated by dividing by the wet mass.

Results

Mineral, Organic Material and Water Content

To confirm that there was no significant difference in bone material composition between the tibiae, namely mineral, organic material and water content, samples from all nine tibiae were ashed. Our results revealed highly similar mineral, organic material and water content (measured as % of total weight) between all tibiae with low standard deviations (S.D.) (Table 1). A ternary diagram shows our deer tibia data (n=9) plotted alongside data derived from Zioupos et al. (2000) regarding the relative amount of water, mineral and organic material (%) in bones from other vertebrates (Fig. 8).

Table 1 The weight (g) fractions of water, mineral and organic content per dry bone matrix weight of the tibia used in this experiment (mean $\pm$ S.D., n = 9)

Tibia	Mineral (%)	Organic (%)	Water (%)	Total (%)
1	63.3	27.8	8.9	100.00
2	61.8	26.9	11.3	100.00
3	63.0	27.9	9.1	100.00
4	63.2	26.0	10.8	100.00
5	62.3	27.6	10.1	100.00
6	62.3	28.6	9.1	100.00
7	62.5	28.2	9.3	100.00
8	60.5	29.8	9.7	100.00
9	62.8	27.9	9.3	100.00
Mean $\pm$ SD	62.4 $\pm$ 0.8	27.9 $\pm$ 1.0	9.7 $\pm$ 0.8

Fig. 8. Ternary diagram of mineral, organic material and water content (% weight) in various bones from different animals. The nine deer tibiae from the current study are marked in black. All other data points are derived from Zioupos et al. (2000).

Bone Density and its Correlation to Bone Stiffness and Yield

The mean density of the control group (n=52) was $1.213 \pm 0.124 \text{ mg/mm}^3$, and the mean density of the test group (n=51) was 1.276 ± 0.127 (Fig. 9). While the difference is minimal and probably has no biological significance, a two-tailed t-test revealed that there was a significant difference in the density between the control and test group ($P > 0.05$). Therefore, an ANCOVA with density controlled for as a covariate was used in the comparison of stiffness and yield values between the control and test group (see results 4.3). A Spearman's correlation was run to determine the relationships between density and stiffness, density and yield and stiffness and yield. Results of the Spearman correlations indicated that there was a significant positive association between density and stiffness, ($r_s = 0.65$, $n=103$, $P < 0.001$), density and yield ($r_s = 0.67$, $n=102$, $P < 0.001$) and stiffness and yield ($r_s = 0.72$, $n=102$, $P < 0.001$). Three separate scatterplots with trend lines were created to show the correlation between the comparisons detailed above (Fig. 10 A-C).

Fig. 9. Boxplot diagram showing the density comparison between the control group and the test group. The horizontal line inside the boxes is the median. Box hinges represent 25th and 75th percentiles. Whiskers represent the minimum and maximum measured values, not including outliers. An outlier (denoted by a circle) is defined as a data point that is located 1.5 times interquartile range above the upper quartile and below the lower quartile. These outliers are only shown in the figure; no values were excluded from statistical calculations. There was a significant difference between the density of the control group compared to the density of the test group ($P < 0.05$).

A

B

C

Fig. 10. Scatterplots showing positive correlations and trend lines. Each data point is color coded by group: blue: control; red: test. A) Density and stiffness, $r_s = 0.65$, $n=103$, $P < 0.001$. B) Density and yield, $r_s = 0.67$, $n=102$, $P < 0.001$. C) Stiffness and yield, $r_s = 0.72$, $n=102$, $P < 0.001$.

Structural Stiffness and Yield

Fig. 11A and 11B show a representative load-displacement curve (used to calculate structural stiffness) and a stress-strain curve (used to calculate yield) for one of the trabecular bone samples loaded in the axial orientation. The mean stiffness of the control group and test groups were 5561.2 ± 4027.3 and 5798.4 ± 4181.5 N/mm, respectively (Fig. 12). An ANCOVA (with density as the covariate) revealed that after inoculation with sterile nutrient broth or *S. aureus* for 72 h, the structural stiffness (N/mm) of the control group ($n=52$) was not significantly different from the test group ($n=51$, $P > 0.05$). The mean yield point of the control group and test groups were 10.95 ± 5.11 and 12.86 ± 4.87 MPa, respectively (Fig. 13). An ANCOVA (with density as the covariate)

revealed that after inoculation with sterile nutrient broth or *S. aureus*, the mean yield point (MPa) of the control group (n=52) was not significantly different from the test group (n=50, $P > 0.05$). A review table with p values summarizes the mean apparent density (mg/mm^3) $\pm$ S.D, stiffness (N/mm) $\pm$ S.D. and yield (MPa) $\pm$ S.D. by group (Table 2).

A **B**

Fig. 11. A typical load-displacement and stress-strain curve for a trabecular bone cube loaded in the axial direction undergoing compressive strain testing. A) The structural stiffness of each cube was calculated using the slope (N/mm) from the linear portion (marked with a dashed line) of each load-displacement curve. B) The compressive yield was derived from the transformation of each load-displacement curve into a stress-strain curve. The point where the stress-strain curve stopped behaving linearly (marked with an open circle) was identified as the yield.

Fig. 12. Boxplot diagram showing the stiffness comparison between the control group and the test group. The horizontal line inside the boxes is the median. Box hinges represent 25th and 75th percentiles. Whiskers represent the minimum and maximum measured values, not including outliers. An outlier (denoted by a circle) is defined as a data point that is located 1.5 times interquartile range above the upper quartile and below the lower quartile. These outliers are only shown in the figure; no values were excluded from statistical calculations. There is no significant difference between the stiffness of the control group compared to the test group ($P > 0.05$).

Fig. 13. Boxplot diagram showing the yield comparison between the control group and the test group. The horizontal line inside the boxes is the median. Box hinges represent 25th and 75th percentiles. Whiskers represent the minimum and maximum measured values, not including outliers. No values were excluded from statistical calculations. There was no significant difference between the yield of the control group compared to the test group ($P > 0.05$).

Table 2 Trabecular bone mechanical properties in compression (mean $\pm$ S.D.) by experimental group. NS indicates no significant difference ($p > 0.05$).

Parameter		Control	Test	p-value
Apparent Density	(mg/mm ³)	1.213 $\pm$ 0.124 ^a	1.276 $\pm$ 0.127 ^b	< 0.05
Stiffness	(N/mm)	5561.2 $\pm$ 4027.3 ^a	5798.4 $\pm$ 4181.5 ^b	NS
Yield	(MPa)	10.95 $\pm$ 5.11 ^a	12.86 $\pm$ 4.87 ^c	NS

^a n = 52, ^b n = 51, ^c n = 50

Discussion

The purpose of this study was to determine if trabecular bone stiffness and yield deteriorated after acute exposure to *S. aureus*. We were interested in this question for its clinical relevance in risk factor assessments of *S. aureus*-based bone infections, as the mechanical properties of trabecular bone have been associated with the strength of whole bones and an indicator of fracture risk (Ciarelli et al. 1991; Hodgkinson and Currey 1993). One of the main advantages of this study is that we were able to reference recently published research investigating a similar question related to cortical bone, carried out by the senior members of this team (Kunde et al. 2018). As a result, we were able to refine our methods and address some previous limitations (i.e., extended bacterial exposure time, the inclusion of more tibia samples and collection of yield data) as well as address the question for trabecular bone. In addition, our extrinsic or *ex vivo* loading model allowed for greater control over mechanical parameters and better reproducibility in comparison with *in vivo* modeling. For example, we were able to design an experimental procedure and exact compression protocol that was consistent between experimental groups and across time.

In agreement with the conclusions of Kunde et al. (2018) on cortical bone, our results showed that despite the larger surface area of trabecular bone, there was no significant decrease in the compressive stiffness of our trabecular bone samples after inoculation with *S. aureus* (test group) in the axial direction compared to immersion in sterile nutrient broth only (control group). In addition, we found no significant decrease in the yield of our test group when compared with the control group in the same compressive direction. The axial direction corresponds to the physiological direction loading of the proximal tibia that would occur as a result of normal animal activities (walking, running, jumping, etc.). These results thereby indicate that acute exposure to *S. aureus*, within the

context of our model, does not adversely affect trabecular bone stiffness or yield during daily compressive loading activities. There was a small but significant difference between the apparent densities of the control and test groups. This difference is due to our prioritizing of equal representation of all nine tibia and all anatomical locations within each tibial slice in both groups over equal densities. While this small apparent density difference (the average difference was 0.063 mg/mm^3) is statistically significant, it is unlikely to contribute to any meaningful mechanical difference. Nevertheless, we accounted for this difference in our statistical analysis (see section 4.3) by controlling for density as a covariate.

It has long been noted that the compressive modulus of trabecular bone can vary as much as 100-fold from one location to another within a singular area of bone, such as was found in the proximal tibia of humans (Goldstein et al. 1983). Moreover, large SD for both structural (N/mm) and material stiffness (MPa) have been reported for trabecular bone, including canine, horse and human tibiae (Garrison et al. 2009; Hodgskinson and Currey 1993; Kang et al. 1998; Morgan and Keaveny 2001). This natural variation in compressive properties, therefore, helps to explain the rather large SD seen in our structural stiffness results. The large variation of trabecular bone mechanical properties has been linked to the heterogeneous and anisotropic nature of trabecular architecture. For instance, Nazarian et al. (2007) found that trabecular bone microarchitecture greatly determined the mechanical properties of individual regions within the human proximal femur. As trabecular microarchitecture influences mechanical properties, a sample large enough to represent the trabecular tissue accurately is required. Harrigan et al. (1988) report a span of 3-5 trabeculae as necessary to estimate trabecular structural parameters correctly. As trabecular bone typically has 5 trabecula per mm, this brings us to our cubical sample size of 5 mm.

To the best of our knowledge, no previous studies have examined the biomechanical properties of trabecular bone post *S. aureus* inoculation. However, previous studies have examined trabecular bone density and ash content, together with some of the biomechanical measurements assessed in this study. For instance, it has been established that bone density and bone quality (including mineral, organic and water content) are significant contributors to overall bone strength (Currey 1969). As the main constituent of mature bone, mineral content primarily contributes to the stiffness and strength of bones in such a way that these mechanical indices increase as the ash mineral fraction increases (Follet et al. 2004). The relationship between bone mineralization and mechanical properties has been demonstrated even when adjusting for other factors such as bone tissue volume (BV/TV) and microarchitecture (Loveridge et al. 2004). The results from the ashing of our nine tibiae reveal remarkably similar mineral, organic and water content (%) with low SD of less than 1.01 % (Table 1). Additionally, our mineral, organic and water content are similar to those reported for comparable bones across several species (Zioupou et al., 2000). This uniformity in ash content helps to ensure that any difference in mechanical properties is unlikely the result of individual bone mineral differences.

Regarding density, our average apparent density for all cubes (n=103) of $1.244 \pm 0.128 \text{ mg/mm}^3$ is higher than what was reported by some previous studies (Kang et al. 1998; Kuhn et al. 1989; Rho et al. 1997). For example, Kang et al. (1998) reported an average apparent density for canine tibia at $0.83 \pm 0.20 \text{ mg/mm}^3$ and Rho et al. (1997) reported an average apparent density of bovine tibia at $0.41 \pm 0.16 \text{ g/cm}^3$. This difference in density is likely a result of variations in sample preparation and the complete vs. partial removal of bone marrow by different washing techniques (we sought to retain bone marrow as a nutrient to assist bacterial growth). Increases in bone density (albeit to a

point) are associated with increased mechanical properties, such as stiffness and yield (Fonseca et al. 2014). In agreement with previous research, we found a significant positive association of density between both stiffness and yield (Fig. 10A & 10B) (Hodgskinson and Currey 1993; Kang et al. 1998; Keaveny et al. 1994; Morgan and Keaveny 2001; Morgan et al. 2003). Therefore, it is not surprising that our overall denser population showed on average higher stiffness and yield values in comparison with other studies using less dense samples. To illustrate this point, Kang et al. (1998), reports an average material stiffness of 1394 ± 649 (N/mm) for canine tibia samples in comparison to our combined (test and control) average stiffness of 5680 ± 4104 (N/mm); yet they do not report mineral content. Our higher average and larger SD may be explained by a denser population as well as varied sample collection within the tibia itself. Consistent with our finding's stiffness and yield (used here as a proxy for strength) have also been positively correlated in previous research (Fyhrie and Vashishth 2000; Mittra et al. 2005). For instance, Fyhrie and Vashishth (2000) found that for human vertebral trabecular bone, the coefficient of determination (r^2) for regression between yield strength and stiffness was 0.94. Again, we see this same trend between stiffness and yield within and across our experimental groups (Fig. 10C).

While several classifications systems of osteomyelitis have been proposed, there is no clear consensus as to which one is the most suitable. However, in general, osteomyelitis can be classified as acute or chronic. For example, acute osteomyelitis is characterized by inflammatory bone alterations which usually present within two weeks, while chronic infections are those that involve bone necrosis and sequestra (dead bone pieces that have separated from surrounding healthy bone) at six or more weeks (Hatzenbuehler and Pulling 2011; Sia and Berbari 2006). Recently, de Mesy Bentley et al. (2017) demonstrated the *in vivo* ability of *S. aureus* to penetrate through a porous 0.5 μm

membrane in as little as 6.5 h. Moreover, Kunde et al. (2018) confirmed the ability of *S. aureus* to migrate through deer cortical bone (approx. thickness of 2 mm) within their experimental timeframe of 48 h. However, when left for prolonged periods at or above room temperature, bone stiffness naturally deteriorates (Barrera et al., 2016). Our preliminary timeline testing results (see section 3.3) revealed no significant difference in stiffness or yield of cubes thawed for 24 hours and those thawed for 72 hours. Thus, we sought to extend the *S. aureus* exposure time of Kunde et al. (2018) in cortical bone from 48 h to 72 h to replicate acute *in vivo* exposure length more accurately. Despite the additional 24 h of *S. aureus* exposure, we found no significant difference in the stiffness or yield of our test cubes (see section 4.3). We acknowledge that while accounting for the natural deterioration of bone at or above room temperature as a necessary time restraint in this study, our exposure time of 72 h may still fall short of the time required for *S. aureus* to induce detectable mechanical changes.

Our study has several limitations. One limitation was that our *S. aureus* inoculation was carried out *in vitro* and, therefore, the naturally occurring cellular and chemical responses of the immune system to such infections were not present. The pathology of *S. aureus* based diseases, such as osteomyelitis, are driven by numerous bacterial-host interactions and immune interference, a component missing from this study (Bentley et al. 2017; Ciampolini 2000; Olson and Horswill 2013; Tong et al. 2015). Despite this, it is worth mentioning again that deterioration of bone has been previously studied successfully under *in vitro* conditions (Junka et al. 2017). Another limitation is that we did not include microstructural measurements, such as trabecular thickness (Tb.Th), trabecular spacing (Tb.Sp) and trabecular number (Tb.N). As described in the introduction (see section 1.4), trabecular bone is a complex 3D web of branching and interconnecting beams and plates, resulting in a unique microarchitecture throughout and between bones. Of note, while

investigating microstructural parameters and the mechanical properties of porcine trabecular bone, Teo et al. (2006) found that trabecular microarchitecture parameters including; Tb.Th, Tb.Sp and Tb.N, varied both within and between samples. Results from additional studies support the hypothesis that trabecular bone microarchitecture is an essential determining factor of bone mechanical properties (Chesnut et al. 2005; Kreipke et al. 2014; Legrand et al. 2000; Mittra et al. 2005). Recent technological developments now allow for trabecular bone microarchitecture indices to be quantified *in vivo* with advanced imaging techniques such as HR-pQCT (high resolution peripheral quantitative computed tomography), which currently has a maximum resolution of 60 micrometers and it is approved to use in live humans, as well as high-resolution MRI (HR-MRI). Looking forward, the imaging of trabecular bone samples before experimentation could aid in the creation of a system in which the differences in trabecular microarchitecture could be better controlled and accounted for in the grouping of samples (control vs. test) and the final analysis. The availability of and expertise in the use of such equipment, as well as a significant increase in sample size to accommodate such detailed groupings, will be a future hindrance in need of consideration. Yet another limitation of our study relates to the nature of trabecular bone and the development of microcracks during mechanical testing. Results from Nagaraja et al. (2005) showed that microdamage initiation in bovine tibial trabecular bone preceded apparent compressive yield (the endpoint of our mechanical testing). This behavior prohibited our trabecular bone samples from undergoing mechanical stress (i.e., compression) more than once, as even small forces introduce microcracks that will negatively affect bone stiffness and strength in a subsequent loading cycle. Therefore, contrary to Kunde et al. (2018), a cube in our experiment could not serve as its own control and instead, this research required an independent group design. It has been demonstrated that trabecular architecture varies and is unique (Chesnut et al. 2005; Kreipke et al. 2014; Legrand et al. 2000; Mittra et al. 2005). Thus, we have relatively large

SDs; however, as each cube could only be compressed once this was an unavoidable compromise and certainly not an issue unique to this study. This independent group design also limited us to testing a single orientation (contrary to cortical samples, where multiple directions can be compared). However, our direction of loading (axial) represents the main physiological direction of compressive stress and is, therefore, the most biomechanically relevant when considering the probability of bone fracture or failure. A final limitation that we did not expect was the relatively large size of our cubes, which was required for the correct representation of the tissue structure (5 mm³). This sample size prohibited us from reaching maximum load (strength) due to a load limitation of our mechanical testing machine; therefore, we had to use the yield point as a proxy. In the future, it would be of interest to consider an *in vivo* murine model in combination with more comprehensive imaging techniques to better replicate real-world conditions while also accounting for the microstructural variability of bone.

In conclusion, our results show no significant decrease in tibiae trabecular stiffness or yield in the axial direction after 72 h exposure to *S. aureus*. Thus, our working hypothesis was not supported and our current findings for trabecular bone agree with those of cortical bone by Kunde et al. (2018). Nevertheless, a growing number of *S. aureus* positive osteomyelitis cases in combination with the known ability to circumvent traditional medical treatments warrants further *in vivo* studies that investigate the biomechanical effects of such infections. While the variable nature of trabecular bone presents a unique set of challenges, we believe that with further technological advances and a continuing collection of data, the ability to assess the effects of *S. aureus* related osteomyelitis on the mechanical properties of bone remains a promising clinical application in regards to skeletal integrity and fracture risk.

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Appendix

Tibia Maps

A

B

* T2R B2 & B4 one cube only!
large amount of marrow

C

T3L 11-29-18 collected 11-19-18

Slice 1 proximal

Slice 2: distal

D

T4L

Slice 1 proximal

Relabel after B1 is complete!

Slice 2 distal

E

F

G

Tibia 7 Right

Slice 1 - Proximal

Slice 2 - Distal

H

Tibia 8 Left

Slice 1 - Proximal

Slice 2 - Distal

Fig. 14. Hand drawn maps of all tibia slices, with beam (B) and cube (#) locations. Unused or unusable areas noted.