EFFECTS OF OVARIECTOMY, SEASONAL CHANGES, AND ANATOMICAL
POSITION ON THE COMPACT BONE REMODELING AS SEEN IN THE ADULT
OVINE MODEL

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COMMITTEE MEMBERSHIP

TITLE: EFFECTS OF OVARIECTOMY, SEASONAL
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ADULT OVINE MODEL

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ABSTRACT

EFFECTS OF OVARIECTOMY, SEASONAL CHANGES, AND ANATOMICAL POSITION ON THE COMPACT BONE REMODELING AS SEEN IN THE ADULT OVINE MODEL

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The purpose of this study is to characterize the compact bone remodeling of the ovariectomized ewe and its effectiveness as an animal model for studies of postmenopausal osteoporosis. The use of animal models is a beneficial way to evaluate the effectiveness of medical devices and therapeutic agents for treating diseases. The ovine model is an attractive option because of its large size and similar bone remodeling to humans. However, this species, like most animals, does not undergo a natural menopause, making an ovariectomy necessary for studies of estrogen depletion. The OVX (ovariectomized) sheep has been widely accepted as a model for loss of BMD. However, even with its advantages seasonality has been shown to have an effect on the bone remodeling in the ovine model. Thus, this study focused on characterizing the seasonal and anatomical variation in the compact bone remodeling in the ovine model. In doing so, 28 skeletally mature Columbia-Rambouillet cross ewes underwent an ovariectomy. The animals were divided into groups of 7 based on each season: autumn, winter, spring, and summer. During its specified season each group of 7 underwent ovariectomy and then was sacrificed 12 months post-surgery. The radii and ulnae were harvested and then divided into 6 anatomical locations: craniolateral, cranial, craniomedial, craniolateral, caudal, and caudomedial. This allowed for analysis through the fabrication of microradiographs. Histomorphometric analysis involved measurements to quantify the bone volume to tissue volume ratio, the percent of tissue and material

remodeling, the mean secondary osteonal radius, and number of tissue and material cement line interfaces. Densitometry analysis was then performed to determine the density of each sector relative to an aluminum step wedge, serving as the key. Using a 2-way repeated measures ANOVA statistical analysis was performed to evaluate for seasonal and anatomical differences as well as a possible interaction between season and anatomical region. The OVX data showed significant seasonal and anatomical differences as well as seasonality within anatomical section in the remodeling parameters. When this data was combined with earlier data from a control, sham-cohort from the same experiment a 3-way ANOVA was also performed to evaluate the effects of season, anatomical sector, and treatment on the remodeling parameters. Again, there was significant seasonal, anatomical, and treatment differences, as well as interactions of all three. The results of this study showed that varying levels of remodeling occurs in the adult OVX ovine model and differences between the control and OVX model can be attributed to seasonal and anatomical variations. Thus, it will be an important consideration when developing new test protocols for research incorporating the sheep model for studying osteoporosis.

Keywords: Postmenopausal, Osteoporosis, OVX, Ovariectomy, Ewe, Bone Remodeling

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I. INTRODUCTION

1.1 Purpose

Osteoporosis is a major health threat for an estimated 44 million Americans or 55 percent of people 50 years of age or older [1]. In the U.S. today, 10 million individuals already have the disease with 8 million of them being women [1]. According to the World Health Organization, osteoporosis is defined on the basis of bone mineral density (BMD) assessment using specific criteria. A BMD that lies 2.5 standard deviations or more below the average value for a healthy individual is considered to be osteoporosis [2]. Osteoporosis involves a decrease in bone mass, and an alteration of microarchitecture leading to increased bone fragility. Increased fracture risk with low-energy trauma and age has proven to be the major complication of osteoporosis [3]. Osteoporosis frequently goes undetected until the bone fractures, with fractures commonly occurring in the spine, hip, wrist, humerus, and pelvis [4]. An understanding of the mechanism behind osteoporosis is necessary to improve therapeutic agents and the animal models required to test them. However, a discussion on the anatomy of bone must first be addressed in order to fully understand osteoporosis.

1.2 Skeletal Biomechanics

1.2.1 Bone Function and Structure

Bone provides a framework to support the body, protection of vital organs, an attachment site for muscles to allow for movement, a reservoir for calcium homeostasis, and blood cell formation [5]. As a highly dynamic tissue, bone is continually changing to its physiologic and mechanical environment. These changes in the environment impart energy to the bone [6]. Due to bone's flexibility, it has the ability to conform to the

absorbed energy. Bone is a unique material in that it is able to achieve stiffness while still remaining flexible, and strength while still maintaining lightness through its material composition and structural design [6].

Four cellular components are essential in bone's ability to support and maintain the body. Osteoclasts, multinucleated cells, are responsible for bone resorption [7]. Formed by the fusion of monocytes in the bone marrow, osteoclasts form a brush or ruffle border around the cell, allowing for resorption. Mononuclear, cuboidal cells called osteoblasts produce the new layers of bone and are derived from mesenchymal cells [7]. Osteocytes are former osteoblasts that have become buried in the bone and are in an active state within cavities called lacunae. Osteocytes have processes that allow them to form connections with the other bone cells [7]. The pores found in bone are interconnected through canaliculi, tying all the osteocytes together. This allows nutrients to reach the osteocytes by diffusing through the canaliculi [5]. Bone lining cells are also former osteoblasts. However, bone lining cells do not get buried in the bone matrix and instead line all bone surfaces. These cellular components play a key role in the remodeling of bone and will be discussed in a later section [7].

Bone surfaces are covered by two membranes: the periosteum and the endosteum. Aside from the joint surfaces, the external surface of the entire bone is composed of double-layered membrane called the periosteum. The outer layer is dense fibrous connective tissue while the inner layer consists primarily of osteoblasts and osteoclasts. The periosteum is richly supplied with nerve fibers, lymphatic vessels, and blood vessels, providing an insertion point for tendons and ligaments. Internal bone surfaces are covered

by the endosteal surface, a delicate connective tissue membrane that covers the trabeculae and lines the canals of cortical bone [5].

Cortical and trabecular bone are the two types of bone tissue. Cortical bone, also known as compact bone, is the dense bone that can be found in the shafts of long bones and on the outer layer of bone. The porosity of this type of bone ranges from 5 to 10 percent [7]. The main structural unit of cortical bone is the osteon, an elongated cylinder generally oriented parallel to the long axis of the bone that acts as load-bearing pillars as seen in Figure 1 [5]. Due to its stiffness, cortical bone is responsible for bearing most of the load from the body. Trabecular or cancellous bone is composed of thin plates, or trabeculae, in a loose mesh structure [8]. Trabecular bone is much more porous than cortical bone, with a porosity ranging from 75 to 90 percent. Trabecular bone can be found in the vertebrae, flat bones, and in the end of long bones [5].

Figure 1 Bone structure for cortical and trabecular bone [7]

The primary sources of porosity found in cortical bone are Haversian canals, Volkmann's canals, and resorption cavities. Haversian canals are found in the center of osteons and usually aligned to the long axis of bone. They contain capillaries and nerves that are necessary for providing nutrients to the bone. The size of Haversian canals are estimated to be 50 μm in diameter, approximately the diameter of a human hair [7]. Volkmann's canals, short and transverse canals, connect the Haversian canals to each other and the outside surfaces of the bone. Resorption cavities represent voids that have been created by the osteoclasts. These spaces are typically temporary, do not contain any bone formation, however bone formation sometimes can be seen, and are the initial stages of remodeling, a process that will be discussed in a later section [7].

Compact bone can be classified as either primary or secondary bone. Primary bone is the first bone formed on an existing bone surface during growth. Primary bone can be of two types: circumferential lamellar bone or plexiform bone. Lamellar bone forms slowly in a highly organized manner and is composed of parallel layers [7]. In circumferential lamellar bone, the lamellae are parallel to the bone surface. The formation of primary osteons occurs through blood vessels incorporating into the parallel layers until the vessel is completely surrounded by several lamellae. Plexiform bone formation occurs at a much quicker rate and involves continually constructing a trabecular network on the surface and filling in the gaps, resulting in a mixture of woven bone and lamellar bone [7]. Based on the shape of its vascular spaces, plexiform bone has a “brick wall” appearance. Plexiform is rarely seen in humans and is most typically seen in large, fast growing animals such as ungulates. Through remodeling, the resorption of preexisting bone and its replacement with new, lamellar bone results in secondary bone. In compact bone, secondary bone consists of secondary osteons that are about 200 μm in diameter and centered around a Haversian canal [7]. The boundary that surrounds the secondary osteon is known as the cement line. Like cortical bone, trabecular bone is remodeled in a similar manner. The main difference is the remodeling of trabecular bone rarely produces osteons because they usually do not fit inside the individual trabeculae. As a result, in the place of an osteonal canal a bone marrow space will form [7]. In adult humans, most of the cortical bone and trabecular bone is composed of secondary bone. This is due to the constant turnover of bone throughout one’s lifetime [7].

1.2.2 Material Composition of Bone

Bone is a two-phase compound with both organic and inorganic components. It is different than the other tissues in the body in that it is largely mineral or inorganic, 60 to 70 percent of its dry weight [8]. The minerals consist primarily of calcium and phosphate in the form of hydroxyapatite crystals, giving bone its solid consistency. The organic matrix is largely Type I collagen fibers and ground substance. Collagen plays a critical role in the structure and function of bone tissue; the fibers are tough and pliable, yet they resist stretching and have little extensibility [8, 9]. Other components of the organic matrix include proteoglycans and noncollagenous proteins. The relative amounts of each component of bone contribute to the overall strength of bone.

The structural organization of bone contributes to its mechanical properties. Cortical bone and trabecular bone have significantly different mechanical properties. Cortical bone is an anisotropic material, exhibiting a highly ordered arrangement of collagen fibers and mineral crystals. The longitudinal arrangement of collagen fibers and minerals make cortical bone stronger and stiffer in this direction [7]. It is much stronger in compression when loaded longitudinally as opposed to when loaded transversely. Also, osteonal remodeling tends to convert cortical bone from an orthotropic material to a transversely isotropic material [7].

Trabecular bone is substantially weaker and much more compliant than cortical bone. This is because trabecular bone is a porous material and its mechanical properties are governed by the magnitude of the porosity. In addition to porosity, the properties of trabecular bone are also determined by the anisotropy of the trabecular architecture, and

the material properties of the tissue within the individual trabeculae [7]. However, for the purpose of this experiment only cortical bone is of interest.

Osteons play a critical role in bone's response to a mechanical stimulus; however other factors have an effect. Porosity, the ratio of void volume to total volume, affects both cortical and trabecular bone. In cortical bone, Haversian canals and Volkmann's canals affect the mechanical properties. The bone marrow spaces between the trabeculae affect the properties of trabecular bone [7]. Several researchers have proposed relationships between the mechanical properties of bone and porosity. Figure 2 shows data compiled from several experiments exhibiting the relationship between porosity and strength. There is an initial linear relationship with small increases in porosity dramatically affecting bone strength. However, when the porosity is 0.1 the relationship changes and there is a more gradual decline in the ultimate stress in response to further changes in porosity [7].

Figure 2 The relationships between ultimate compress stress, porosity, and apparent density for fresh human bone (triangles), embalmed human bone (circles), and fresh bovine bone (squares) [7]

Variations in the mineral content of bone also play a role in its mechanical properties. The amount of mineral content is based on the degree and cause of mineralization. Bone formed at a higher rate consequently has reduced mineral content. This can be related to age, trauma, or metabolic disease. Reduced mineral content can also be caused by a delay in the complete formation of new osteons. Vose and Kubala conducted a study correlating strength and density, finding that a small difference in mineralization made large differences in breaking stress and found that the point of maximum toughness occurred when bone was 66 to 67 percent mineralized [7].

When considering the material properties of bone, density always has an effect. Density refers to the specific gravity of the solid matrix. While the apparent density represents the mass per unit volume of a region of bulk bone, including the bone voids

[7]. Depending on the area of interest, apparent density can be a much more useful property as it is a function of the porosity and mineralization.

1.2.3 Quantitative Analysis of Skeletal Biomechanics

The ability to quantitatively assess the skeletal system and more importantly prevent fractures from occurring is of utmost importance. Analyzing bone structure and bone mass can be performed both invasively and non-invasively. Micro computed tomographic scanning has been adopted as a method to measure bone microarchitecture. It has the ability to image small specimens of bone in three dimensions without destroying the tissue sample [10]. Other methods exist that allow for noninvasive measurement of bone mass. Most of the methods depend on the absorption of photons by the bone, and because minerals readily absorb photons the amount of bone mineral can be measured [7]. Dual energy x-ray absorptiometry (DEXA) was first used clinically in the 1990's and involves low-energy X-rays scanning back and forth along the bone to produce a two-dimensional image. These methods are accurate to within 1 to 2 percent of the patient's actual bone mineral content and exposes the patient to far less radiation than what would occur if a standard X-ray were used [7].

Stereology is the method for analyzing a three-dimensional space when only two-dimensional sections or their projections onto a surface are available. It is extremely useful in determining variables related to the structure and composition of bone [7]. Stereology involves taking histologic sections of the specimen and then analyzing them under a microscope. Several methods have been developed for measuring the porosity of the image. One method places a grid of points over the image and the number of points that falls on a void allows the user to find the percentage of porosity for that section [7].

Different types of grids have developed for this purpose. Grids that contain micrometers can be used to directly measure osteon diameters. A Merz grid is one example of a type of grid that can be used (Figure 3). It consists of 36 point markers and 6 sinusoidal lines. Typically, the Merz grid is superimposed onto an image through either an eyepiece reticule or by physically placing the grid onto an enlarged image.

Figure 3 Merz grid with point markers and sinusoidal lines

1.3 Bone Remodeling

The cellular mechanisms of modeling and remodeling are responsible for bone adaptations. In modeling and remodeling, the removal and addition of bone occurs through the same cellular components. However, the goals of the two processes are entirely different. The cellular components involved are the osteoclasts, cells that remove bone, and the osteoblasts, cells that add bone.

Modeling is vigorous during growth and produces a change in the size and shape of bone. This occurs when new bone is deposited by the osteoblasts without previous bone resorption [11]. Modeling involves independent actions of the osteoclasts and osteoblasts. Due to the fact that modeling occurs primarily during growth, the rate of modeling is greatly reduced after reaching skeletal maturity. At a particular site,

modeling is a continuous and prolonged process that is essential for adaptation of the body during growth and new loading occurs on the skeleton [7].

Remodeling occurs throughout one's life; however like modeling it is reduced after growth stops. It involves coupled actions by the osteoblasts and osteoclasts and does not typically affect the size and shape of bones. Each occurrence of remodeling is considered to have a definite beginning and ending. This process is essential for removing old and damaged bone and replacing it with new bone (Figure 4).

Figure 4 The Remodeling Cycle on a Trabeculum [11]

1.3.1 Remodeling: BMUs and ARF

Bone remodeling is an active process throughout the skeleton, essential for maintenance and renewal of the skeleton in adults. It is responsible for the removal of damaged bone and the subsequent formation phase restoring the structure of the bone [11]. Remodeling prevents accumulation of fatigue damage that could potentially lead to fatigue fracture [7]. Bone is remodeled through the coupled removal of bone and its replacement through the synthesis of a new bone matrix and its subsequent mineralization [12]. This process occurs through the teaming of osteoclasts and osteoblasts working

together in basic multicellular units, or BMUs. A BMU typically consists of about 10 osteoclasts and several hundred osteoblasts [7].

A BMU's lifetime consists of three principal stages: activation, resorption, and formation (ARF). During activation, a chemical or mechanical signal brings about the formation of osteoclasts through the fusion of monocytes. The signal initiation will be further discussed in a later section. The osteoclasts then remove a volume of bone in either the form of a ditch or a tunnel. The tunnel made by the osteoclasts will be about 200 μm in diameter [7]. During this tunneling, as seen in Figure 5, a resorption space is created to ultimately allow for the formation of a secondary osteon. The cutting cone creates the resorption area that occurs deep within the cortex of bone. To maintain nutritional access to the new bone, a new local vascular supply must be established. Thus, when the osteoblasts replace the resorbed bone, they do not completely fill in the tunnel. A Haversian canal is left in order to allow a constant blood supply to the newly formed osteon. The laying down of bone by the osteoblasts makes up the formation stage of the ARF cycle. The formation period is much slower than the resorption, with the total remodeling period taking 4 months [7].

Figure 5 Tunneling of osteoclasts to form a new osteon [13]

The ARF cycle can be further broken down into 6 sequential stages of bone remodeling: activation, resorption, reversal, formation, mineralization, and quiescence [7]. Activation does not include the initial recruitment of the osteoclasts and typically occurs over 3 days. After activation, the osteoclasts can begin resorbing bone. They resorb at a rate of $40\text{ }\mu\text{m/day}$, moving longitudinally creating the cutting cone shown in Figure 5 [7]. There is not an immediate switch from osteoclast to osteoblast activity, it can take several days. A transition phase known as reversal occurs, resulting in a cylindrical space between the resorption and refilling regions. The line surrounding the reversal area is known as the cement line or reversal line. The combined reversal and resorption period in humans takes about 30 days [7]. During formation, the osteoblasts lay down concentric lamellae at an average radial closure rate of $1\text{ to }2\text{ }\mu\text{m/day}$ [7]. To allow for vascularization, the osteoblasts do not completely refill the tunnel, but leave a Haversian canal of $40\text{ to }50\text{ }\mu\text{m}$ in diameter. In humans, the formation phase averages about 3 months [7]. Following deposition of the new bone, mineralization of the organic

matrix occurs within and between the collagen fibers. During the first few day of mineralization, 60 percent of the mineral content is laid down, referred as primary mineralization. Secondary mineralization occurs when the remaining 40 percent of the mineral content is laid down at a decreasing rate for 6 months [7]. The resorption of old, dense bone and its subsequent replacement with new, low-density bone brings about a change in the mechanical properties in the osteons and surrounding tissues. In the final phase of quiescence, the osteoclasts are no longer present and the osteoblasts either become osteocytes, bone lining cells in the Haversian canal, or disappear via apoptosis [7].

1.3.2 Signaling and Initiation of Bone Remodeling

The exact mechanism behind initiation of bone remodeling is not clearly understood. However, many researchers have published their theories on the initiation of bone modeling along with supporting scientific evidence. The idea of bone remodeling itself was popularized by Wolff [14]. Wolff wrote, “Thus the law of bone remodeling is the law according to which alterations of the internal architecture clearly observed and following mathematical rules, as well as secondary alterations of the external forms of the bones following the same mathematical rules, occur as a consequence of primary changes in the shape and stressing or in the stressing of the bones” [15]. His law focuses on the concept that the self-regulation of bone structures by cells is in response to a mechanical stimulus [7]. Investigators have developed theoretical models to quantify the relationship between mechanical loading and bone morphology. The backbone of the theories are founded in Harold Frost’s Mechanostat theory which states that if bone tissue experiences excess mechanical stimulation, additional bone will be deposited. However, if bone tissue

experiences insufficient mechanical stimulation, bone will be resorbed [16]. Other researchers claim that osteocytes are the key component in the remodeling cycle and act as the bone's mechanosensing cells. The osteocytes have the ability to sense strain on bone surfaces through stretch-activated ion channels, flow of interstitial fluid, and electrical potentials [14]. Additional research has been performed claiming that osteocytes that undergo apoptosis produce a signal initiating and guiding osteoclastic resorption of bone [17]. Osteocytes also have been suggested to sense fatigue damage and transmit signals activating remodeling in order to remove the damage [14]. Clearly, the theory behind bone remodeling is a controversial issue, with researchers each proposing their own mechanisms. Martin proposed a unifying theory for the control of bone remodeling based on five fundamental assumptions from other researchers as well as his own work [14]. His model first claims that bone lining cells are responsible for activating bone remodeling. Second, the network of osteocytes produces and transmits a signal proportional to the amount of mechanical loading. Third, osteocytes send an inhibitory signal to osteoblasts that reduces their rate of bone production. In summary, Martin argues that the signal produced by the osteocytes is in response to mechanical loading, and inhibits bone lining cells from activating remodeling [14]. The theories presented apply only to remodeling; however the signal generated by the osteocytes may also help control the modeling process.

1.3.3 Quantitative Analysis of Bone Remodeling

Analysis of histologic sections of bone provides a means for quantifying bone remodeling. Frost was the first to demonstrate this in the cortex of the human rib [7]. Today, the method has been adapted and applied to bone biopsies from the iliac crest.

The use of histomorphometric analysis requires the reduction of the mechanics of remodeling and bone formation to definitive steps for analysis. Additionally, it provides a useful method for identifying inconsistencies in theories and problems associated with diagnosing pathologic states and defining their etiology [7].

The methodology of Frost serves as the foundation for this field, but over time it has been updated with new technology for a much simpler approach. The method involves labeling bone with a vital fluorochrome stain for mineralizing bone such as tetracycline. The labels are given 7 to 14 days apart, with the second label ending at least 1 day before the bone specimen is taken [7]. The bone is cross sectioned to a thickness of 100 μm and then stained to distinguish osteoid from mineralized bone and allows the labels to be seen. This stain is typically tetrachrome or osteochrome. The sections are best observed using epifluorescent light [7]. Figure 6 diagrams the stained osteons as seen through the microscope. The BMUs labeled “R” are undergoing resorption, while the completed osteons are indicated with a “C”. BMUs that are still forming bone (F) have either a single label (F, S) or a double label (F, D) [7].

Figure 6 *Upper:* Schematic diagram of BMUs in a cross section of cortical bone. Black, irregularly shaped BMUs represent resorption spaces (R). BMUs with large canals are refilling; they have either a single label (F, S) or a double label (F, D). Completed osteons have small canals ©; some of these have a single label (C, S). *Lower:* A single BMU in the last stages of refilling is depicted. It has a double label; the distance between the labels is measured as shown (D_L) [7].

Measurements are taken from the histologic sections in order to calculate the remodeling characteristics of the bone. The number of resorption spaces are identified; they have scalloped surfaces and lack of osteoid or fluorochrome label [7]. The perimeter of each resorption space is measured, and the mean perimeter is calculated. Then the number of refilling BMUs is determined, identified by their osteoid seams. The mean perimeter and thickness of the osteoid seams is found. From the double labeled BMUs, the mean distance (μm) between the two labels can be determined. Finally, the number of completed osteons is recorded, indicated by the absence of osteoid seams [7]. The methodology of this study does not include staining. However, the number of secondary osteons as well as the number of cement line interfaces can be used in order to calculate the desired remodeling characteristics.

1.4 Osteoporosis

Bone remodeling plays an important role in the development of osteoporosis. Elderly patients, especially those at risk for osteoporosis, exhibit an imbalance between bone resorption and formation, particularly a negative balance. A negative balance occurs when the bone formation is less than the prior bone resorption, causing bone loss and compromising the strength of bone [11]. The micrographs shown in Figure 7 clearly depict the difference in microarchitecture between the 31 year old woman and 70 year old woman. The bone of the 70 year old is much more porous with an altered microarchitecture.

Figure 7 The structure of L3 vertebra in a 31 year old woman (top) and 70 year old woman (bottom) is shown using scanning electron micrographs. Note that many of the plate-like structures have become converted to thin rods [4]

The effects of aging appear to be an important determinant for the onset of osteoporosis. With increasing age, individuals are more susceptible to fragility fractures after a minor traumatic event. Overall, bone mineral density (BMD) significantly falls during adulthood as a result of both age-related and menopause-related bone loss [18]. The roles of estrogen deficiency and age related bone loss in women has been well documented [4]. Menopause is the most important risk factor for bone loss in woman in late adulthood and as a result osteoporosis affects 30% of postmenopausal women [19, 20]. Thus, women are more susceptible to osteoporosis than men. The peak bone mass of men is 20 to 30 percent higher than women [18]. Other key factors for developing osteoporosis include calcium deficiency, immobilization, alcoholism, smoking and corticosteroid use [18]. Another possible contribution to the development of osteoporosis is the decreased production of insulin like growth factor from the liver. Genetic factors are believed to also have an influence on the peak bone mass, attained during the third decade of life. Peak bone mass is an important determinant of bone mass later in life and is influenced by nutritional intake and physical activity [4].

1.4.2 Postmenopausal Osteoporosis

During menopause, a woman's ovarian function declines and the ovaries no longer function as endocrine organs. As a result, the woman produces less estrogen and progesterone [5]. The decreasing levels of estrogen make postmenopausal women more susceptible to fracture incidence and developing osteoporosis. Bone loss early in the postmenopausal period contributes to increased fracture incidence with estrogen levels playing a key role in increased bone loss [21].

The mechanism by which estrogen induces bone loss is either through an increase in osteoclastogenesis or an increase in osteoclast lifespan. Estrogen acts by altering the cytokine dependency of the body and its cellular pathway can be seen in Figure 8. Monocytes and macrophages are responsible for producing interleukin-1 (IL-1) and tumor necrosis factor (TNF). Under normal conditions, estrogen inhibits the activity of IL-1 and TNF. However, in an estrogen deficient individual, this does not occur and IL-1 and TNF continue to stimulate proliferation of a group of mediating cytokines. These cytokines, receptor activator of NFkB ligand (RANKL), macrophage colony stimulating factor (M-CSF), granulocyte/monocyte-colony stimulating factor (GM-CSF), interleukin-11 (IL-11), and interleukin-6 (IL-6), act directly on osteoclast progenitors to stimulate proliferation and inhibit apoptosis [22]. Additionally, IL-1 and TNF stimulate the synthesis of adhesion molecules, such as osteopontin, which contributes to increased cytokine production of IL-6. An important characteristic of the cytokines involved in regulation of osteoclastogenesis is their ability to stimulate their own as well as each other's synthesis (Figure 9) [22]. As previously stated, estrogen plays a role in bone loss by increasing osteoclast lifespan. Under normal conditions, all osteoclasts eventually die by apoptosis, a process stimulated through estrogen. Thus, in an individual who is estrogen deficient the osteoclasts do not undergo apoptosis as frequently, increasing the rate of remodeling and the volume of bone that is resorbed by prolonging the lifespan of osteoclasts [11, 22].

Figure 8 Cell signaling pathway occurring during estrogen deficiency

Figure 9 Regulation of IL-1, IL-6, and TNF production. Each arrow indicates a stimulatory effect of a cytokine on its own synthesis, or the synthesis of another cytokine. Demonstrated suppression of the stimulated synthesis of IL-6 and TNF by estrogen (“E”) is indicated by filled octagons, and the potential suppression of IL-6 induced IL-6 by estrogen is indicated by an open octagon [22]

1.4.3 Treatment Options for Osteoporosis

Current treatment options for preventing or delaying the onset of osteoporosis as well as the fragility fractures associated with osteoporosis involve either increasing an individual's peak bone mass or preventing/slowing the rate of bone loss in later life. Nutrition supplements such as calcium and vitamin D can be given as treatments for osteoporosis. Calcium slows the rate of bones loss, especially in elderly women and in those with low calcium intake. It is generally prescribed in conjunction with other drugs for osteoporosis [23]. Vitamin D is also used as a treatment. Studies have shown trends for a reduction in non-vertebral fractures in elderly men and women treated with annual injections of calcium and vitamin D. However, the efficacy of calcium and vitamin D supplementation in healthy elderly people has yet to be established [23].

Hormone replacement therapy (HRT) is a therapy option not sought after due to the associated adverse effects. The decision to use HRT involves balancing the unknown risks of breast cancer, menstrual bleeding, and other possible gynecologic symptoms [18]. However, HRT can reduce markers of bone resorption, the rate of bone loss, and the incidence of fractures [24]. When initiated at the time of menopause, HRT is able to prevent bone loss. If the estrogen is administered in established osteoporosis, the treatment is less effective. This is because estrogens can aid in the replacement of only a small fraction of the lost bone mass [23]. Data from observational studies show a 40 to 60 percent reduction in fracture risk in women receiving HRT [24]. It has also been hypothesized that the use of calcium supplements could enhance the effects of estrogen on BMD. Even with evidence for HRT as an appropriate therapy for osteoporosis, a large

portion of the population do not use it due to its contraindications or a perception of increased breast cancer [24].

Bisphosphonates are another type of treatment therapy for osteoporosis due to their high affinity for bone apatite. They are potent inhibitors of bone resorption, reducing the recruitment and activity of osteoclasts and increasing their apoptosis [23]. Long term treatment with bisphosphonates in human and nonhuman primates has shown increases in wall thickness, indicating an increase in osteoblast activity [25]. Bisphosphonates have also been studied in combination with other agents. Research has been done comparing the effects of bisphosphonates and HRT and suggests that both prevent bone loss relative to the controls [24]. Figure 10 diagrams the results from a 4-year study comparing etidronate, a bisphosphonate, with and without HRT. The spinal BMD increased by 6.8 percent with just HRT, by 6.8 percent with etidronate, and by 10.9 percent using both HRT and etidronate. [24]. These results indicate a possibility for a similar mechanism for treatment using etidronate and HRT, with both affecting the activity of the osteoclasts.

Figure 10 Lumbar spinal bone mineral density (BMD) increased similarly in estrogen and etidronate treated patients and by a greater amount using both. EHDP=etidronate; HRT=hormone replacement therapy [24]

1.5 Necessity for Animal Models

Before any medical device or pharmaceutical agent can be used in humans it must satisfy the safety and efficacy requirements set forth by the US Food and Drug Administration (FDA). This is shown through extensive bench and animal testing. Not only is animal testing used as a way to test potential therapies, but it also provides a means to better understand the progression of specific diseases. Animal testing typically begins with a smaller inexpensive model such as mice or rats and then progresses into larger diseased animals. The rationale for these procedures is to mitigate any risks and troubleshoot the treatment options in the smaller animals to minimize the time and costs. Thus, the more successful treatment option can then be placed in the larger and more physiologically relevant animal. Even with the more physiologically relevant animals, animal models cannot accurately simulate the differences amongst humans in nutrition, lifestyle, and physical activity, thus the use of human clinical trials is the next progression after the use of animals.

Animal models involved in osteoporosis must be able to simulate the osteoporotic conditions in order to test drugs [26]. Additionally, the conditions should be optimal for testing surgical treatment of osteoporotic fractures and the design of prosthetics devices with different coatings to promote osseointegration. The devices must be able to perform and not loosen during the decreased bone mass as seen in osteoporosis [26].

Rodents, specifically rats, are the most commonly used animal for osteoporotic studies, presenting numerous advantages. They are inexpensive, easy to house, and the general public is accustomed to the use of rodents in research. Their short life span facilitates studies on the effects of aging on bone. Due to the rat's extensive use in research, much is known about bone turnover and the effect of diet on bone aging [27]. Cortical thinning and increased fragility are also well documented in aging rat and mice models. Rodents do not experience natural menopause, thus ovariectomy is used to produce an artificial menopause. The ovariectomized rat model exhibits most of the characteristics of human postmenopausal osteoporosis and has been used to study the effects of bisphosphonates on estrogen deficiency [28, 29]. However, in a rat model the development of Haversian systems and impaired osteoblast function during the late stages of menopause requires 3 to 4 months, making it a poor model for studying the effects of ovariectomy on cortical bone [29].

Rabbit models are not as commonly used to study the effects of ovarian insufficiency on bone mass. They are more frequently used to study bone ingrowth into implants and the bone-implant interface. Rabbits undergo Haversian remodeling, have rapid bone turnover, and show skeletal maturity at 7 to 8 months. Despite these advantages, rabbit models are not fully characterized for osteoporosis. Studies conducted

using rabbits were only able to assess BMD and without an assessment of microarchitecture and bone mechanics a complete characterization of the rabbit model cannot be achieved [30].

Nonhuman primates demonstrate many advantages as they are physiologically relevant to humans. Their organ systems most closely resemble the human systems and include: the gastrointestinal tract, endocrine system, and bone metabolism. Baboon and macaques are the two most documented monkeys used. Female macaque monkeys cycle monthly and have hormonal patterns similar to humans. As a result, ovariectomy in these animals brings about significant reduction in vertebral cancellous volume. However, the female primate must be significantly aged to act as a model for postmenopausal women. Additionally, the use of primates is very costly, and they are difficult animals to handle to be used as a primary large animal model for osteoporosis [29].

Dogs have been used for studying the human skeleton because of their extensive BMU remodeling and larger size. Additionally, they are easy to work with and like humans they are monogastric. Unlike humans and the primates which are polyestrous, dogs are diestrous, with ovulation occurring twice a year. A clear disadvantage for dogs is removal of both ovaries and uteri doesn't appear to be sufficient to create significant bone loss [27]. Thus, the lack a sizeable response in histomorphometric, bone mass, and biochemical parameters limits the use of dogs in the study of postmenopausal osteoporosis [29].

1.5.1 Proposed Animal Model

A promising model for the study of osteoporosis is the skeletally mature sheep, more specifically ewes. Sheep present many advantages over other models. Its large size

allows for device implantation and subsequent analysis. They are easy to manage and are available in large numbers, allowing for large scale studies [31]. The metabolic rate of sheep, based on the oxygen consumption per gram of body weight, is 0.22, which is closer to that of humans (0.21) than to that of rats (0.87) or dogs (0.33) [32]. Another factor making the sheep an advantageous model is the hormone profile of ewes are temporally and quantitatively similar to those of women [32]. Young sheep have been shown to develop plexiform bone that is a combination of woven and lamellar bone. Additionally, 3 to 4 months after the development of plexiform bone, Haversian remodeling is visible in the cortical bone (Figure 11) [33].

Figure 11 (A) Plexiform bone from a three-year-old ewe (original magnification x 25).
(B) Haversian remodeling from the caudal femoral cortex of an eight-year-old ewe
(original magnification x 25)[33]

Ewes have similar reproductive cycles to that of women. However, they do not have a clear menopause at midlife. Thus, they are not able to develop postmenopausal osteoporosis on their own making an ovariectomy necessary. The OVX sheep has been used as a model for loss of BMD. However, unlike female humans, ewes have an annual anestrus period of 1 to 2 months [32]. Another clear disadvantage for the use of sheep is their different gastrointestinal systems. In order to administer drugs orally a surgical insertion is required to bypass the microflora of the rumen [29].

When implementing the ewe as an animal model for postmenopausal osteoporosis, age and season of analysis are two factors that must be considered. An older ewe has been shown to better serve this purpose. Researchers have shown decreases in resorption surfaces, osteoblast surface, and bone formation rates when comparing biopsies of ewes 3 months apart. This supports the claim that changes in remodeling occur within 3 months of ovariectomy [34]. However, changes in bone mass has also been demonstrated in 3 to 4 year old ewes six months following ovariectomy (Figure 12) [34]. Variation does occur as other studies of osteoporosis did not see significant mechanical changes at the spine and the ilium until 12 to 24 months after ovariectomy [35].

Figure 12 (Left) Three-dimensional reconstruction of a sheep vertebral body biopsy from the control group (Right) Three-dimensional reconstruction of a sheep vertebral body biopsy after 6 months of osteoporosis induction with ovariectomy, steroid application and a calcium/vitamin D-restricted diet [36]

Humans have been shown to have alteration in BMD due to seasonal variation, with the lowered BMD occurring in winter. This type of variation also occurs in sheep. The nutrition of the sheep directly influences the bone metabolism of the sheep, in particular calcium and vitamin D levels. Vitamin D is produced in the skin of the animals by ultraviolet (UV) radiation. Thus, the time period that the animals are exposed to light, and the daytime length or season of the year are important parameters to consider [31].

Overall, the ovariectomized ewe provides an adequate large model for postmenopausal osteoporosis. It allows for testing of therapeutic agents and prosthetic devices. The effects of seasonality, however, need further characterization. Through this research, a quantifiable model for the seasonal and anatomical variations in the bone remodeling will be determined from an ovariectomized animal and then further characterized through comparison to the control animal.

1.6. Study Objectives

The purpose of this study is to quantify compact bone remodeling in adult ovine bone based on seasonal parameters. The amount of remodeling will be assessed using the following measurements: the ratio of bone volume to tissue volume, the percent of tissue remodeled, the percent of material remodeled, mean secondary osteonal radii, cement line interfaces as a function of tissue volume, cement line interfaces as a function of material volume, and bone mineral density. These measurements will be taken from the cortical bone of the radius and ulna of the ovine's left leg. Analysis will be made for variations across seasons, anatomical location, treatment, and a possible interaction among all three.

Research has been previously done showing that sheep do indeed undergo seasonal variations in bone properties [31]. However, little research has been done comparing the effect of anatomical location on remodeling parameters. It is expected that both seasonality and anatomical location will play a role in bone properties. In addition, a comparison will be made to sham operated sheep to see the effects of treatment. This model will determine the changes occurring based on season, anatomical location and treatment. The result of this study ensures future researchers are showing relevant information by using sheep as a model for postmenopausal osteoporosis.

2. MATERIALS AND METHODS

2.1 Animal Maintenance and Preparation

As part of a larger experiment, 112 skeletally mature Columbia-Rambouillet cross ewes, 5 years or older, were acquired and kept at Colorado State University, Fort Collins, Colorado. This was performed under approval from the Animal Care and Use Committee. For the duration of the experiment, the animals were kept in dry lots at 41° north and 1500 m altitude. Their diet consisted of a grass-alfalfa hay mixture. The 112 ewes were divided into four groups of 28 based on each season: autumn, winter, spring, and summer. These seasonal groups were then further divided into two groups of 14. At the large animal surgery facilities at the College of Veterinary Medicine and Biomedical Sciences at Colorado State University, the groups were anesthetized and underwent surgery. Two types of surgeries were performed. One group of 14 underwent an ovariectomy (OVX) in which the ovaries were identified and subsequently removed. The other group of 14 served as the control group and underwent a sham surgery in which the ovaries were identified, handled, and not removed. The groups of 14 were then again divided into two groups. One group of 7 was sacrificed 3 months postoperatively while the other 7 were sacrificed 12 months postoperatively. The seasonal group represents the month that either the sham or OVX surgery was performed. Summer sheep underwent surgery in August. The autumn, winter, and spring sheep underwent surgery in November, February, and May respectively. One of the 12 month autumn sham sheep died prematurely and thus was included in the 3 month data as part of another study. The 12 month OVX animals in combination with the 12 month control animals for all seasons were the experimental subjects for this study.

2.2 Specimen Preparation

After animal sacrifice, the sheep's right and left radius and ulna were removed. The bones were wrapped in a saline-soaked paper towel and stored in sealed plastic bags at a temperature of -20°C. The samples were then sent to Henry Ford Hospital. There, the samples were prepared for analysis. Using a band saw (Model 5212, Hobart Corporation, Troy, OH) the center 50 mm of the diaphysis was removed. Then the radius was divided into six anatomical sectors using the Exakt cutting-grinding system (Exakt Corporation, Oklahoma City, OK). The six sectors were cranial, caudal, craniolateral, craniomedial, caudomedial, and caudolateral (Figure 13).

Figure 13 Approximate anatomy of the radial-ulnar sectors divided into six sections as indicated by the gray lines. The top right is the cranial aspect and the top left is the lateral aspect [37].

From each of the anatomical sectors, 1.75x1.75x19mm longitudinal cortical beams were obtained. In a separate study, dynamic mechanical testing was performed on the cortical beams from the left radii and ulnae. A 150 μ m section was cut out from the center of the left radii and ulnae beams and the remaining distal end was used for drying and ashing. This provided a method to determine density. The proximal section was frozen for an analysis to be performed at a later time.

To create microradiographs of the specimens, the 150 μ m sections were ground down by hand using fine grit sand paper to a final thickness of 100 μ m. Using a

2506AGHD 2.5x2.5x0.06 High Definition Photo emulsion Plates and an HP Cabinet

Faxitron (HTA Enterprises, Microtome Technology Product, San Jose, CA)

microradiographs were taken of each sections. This was done at 25 kV for 20 minutes at 3mA. Each microradiograph contained sections from two sheep and an aluminum step wedge made with Reynolds Aluminum Foil in the middle (Figure 14). Aluminum Foil was chosen because it has an atomic number similar to the effective atomic number of hydroxyapatite [38]. By taping multiple layers of aluminum together in a stair step pattern, creating different levels of thickness, the aluminum step wedge was made. By taking the microradiographs of the aluminum and the specimens under the same conditions, we were able to standardize for exposure and development variation. The thickness of each step of aluminum is known and by comparing the specimen's image intensity to that of the aluminum step wedge a density measurement can be made relative to aluminum.

Figure 14 Schematic of the microradiograph layout

2.4 Microradiograph Analysis

The completed microradiographs were sent to California Polytechnic State University, San Luis Obispo from Henry Ford Hospital for analysis. The analysis included both histomorphometric and densitometry measurements. The histomorphometric measurements were performed to quantify the amount of remodeling occurring in the ovine bone. These measurements included: porosity, number of secondary osteons, number of cement line interfaces, and amount of remodeled bone. The densitometry measurements quantified the density of the bone specimens. For both

measurements, the microradiographs were observed under white light using an Olympus BX41 microscope (Olympus Optical Co., Ltd., Center Valley, PA) and the 10X objective.

2.5 Histomorphometry

For these measurements, every cortical beam from each anatomical sector (cranial, caudal, craniolateral, craniomedial, caudomedial, and caudolateral) was divided into four quadrants. To provide better precision, measurements were taken in each quadrant and then the values averaged. The measurements within each quadrant were taken with the assistance of a Merz grid at 100x magnification (Figure 3). To quantify porosity, the thirty six point markers on the Merz grid were used as reference points. Each time one of these point markers fell on a porosity it was counted as one (Figure 15). Porosity was considered a point that did not contain bone tissue and typically occurs from Haversian canals, Volkmann's canals, or remodeling cavities. The points were also used for finding the amount of remodeled bone. Each time one of the points landed on remodeled tissue it was counted as one (Figure 16). Remodeled tissue was identified by either whole or partial secondary osteons; primary osteons were not included in this count. The numbers for the porosity and the amount of bone cannot exceed 36, the number of points on the Merz grid [37].

Figure 15 Merz grid point markers located on a porosity

Figure 16 Merz grid points located on remodeled tissue

To quantify the number of secondary osteons, the borders of the Merz grid were used. Each time a secondary osteon was within the grid it was recorded as one (Figure 17). Prominent features of a secondary osteon were used to help identify them, particularly their circular nature, centering on a Haversian canal, circular pattern of osteocyte lacunae, and a cement line. In an effort to minimize the amount of repeating

counts made in the four quadrants, if a secondary osteon fell on the top or right border of the grid it was counted. However, if the osteon was on the left or bottom border it was not counted.

Figure 17 Secondary osteons located within the scope of the Merz grid

The six, linked semicircular lines on the Merz grid were guides for determining the number of cement line interfaces. Each time the line entered or left a secondary osteon, crossing the cement line, it was counted as one (Figure 18). After completion of all four measurements, the measurements were then repeated for the other three quadrants of that section. The collected raw data was then used to compute the amount of remodeling that occurred in the bone by finding the ratio of bone volume to tissue volume (BV/TV), percent of remodeled tissue and material, number of cement line interfaces as a function of tissue and material volume, and mean secondary osteonal radius.

Figure 18 Use of the Merz grid to count the number of cement line interfaces on secondary osteons

BV/TV indicates the amount of porosity. It is calculated by dividing the porosity by 36, the number of points on the Merz grid, and subtracting that from one (Equation 1).

$$\frac{BV}{TV} = 1 - \frac{porosity}{36} \quad (Eq. 1)$$

The amount of remodeling was then quantified in two ways: in the material and tissue.

The percent of tissue remodeled is the amount of remodeling that occurs in the entire section. While the percent of material remodeled is the amount of bone that is remodeled and excludes the porosity components. Percent tissue modeled was calculated by dividing the points on remodeled bone by 36. The percent of remodeled material was calculated by dividing the points on remodeled bone by 36 minus the points that lay on a porosity.

The calculations for cement line interface were calculated in a similar manner. The number of tissue cement line interfaces was the raw count of cement line interfaces. The number of material cement line interfaces was the raw count of cement line interfaces divided by the BV/TV. The final calculation was for mean secondary osteon radius. This

was calculated by multiplying the percent of tissue remodeled by 632025, a constant determined by Wessel [39], divided by the square root of π , times the number of secondary osteons (Equation 2).

$$\text{Mean Secondary Osteon Radius} = \frac{\text{Percent of tissue remodeled} \times 632025}{\sqrt{\pi \times \text{number of secondary osteons}}} \quad (\text{Eq. 2})$$

2.5 Densitometry

In order to quantify the bone density, a reference material of known composition and thickness was used. For this study, aluminum was the implemented material. Aluminum is commonly used in studies involving cortical bone due to its similar x-ray attenuation. The uniformity of aluminum allows for accurate measurements of its thickness [38, 40]. The measurements of density are described as equivalent thickness of aluminum (ETA) in units of millimeters. The slides were again observed under white light using a BX41 polarizing light microscope. Images were captured of the bone samples and each level of the step wedge, using a Retiga EXi color camera (QImaging, Surrey, BC, Canada) and the Q Capture Pro imaging program (QImaging). To begin, the light intensity was adjusted to allow the best visualization of the structures and a uniform light field within the sample. This light intensity remained the same for pictures of the aluminum step wedge and the sample. Each sample was again divided into four quadrants, with a picture taken of each quadrant at 100x magnification. Only one image was taken for each level of the aluminum step wedge at 40x magnification. In addition to a picture of each step, an image was also taken when the camera was off to control for electrical noise. Each sample was compared to its respective step wedge on the

microradiograph to determine the density of each sample. The exposure and light settings were reset for each microradiograph.

Image analysis for all the pictures was performed using Image J (Wayne Rashband (NIH)). The appearances of the image are affected by the density of the material. The thicker and denser materials appear lighter and brighter. As seen in the step wedge, as the thickness of aluminum increases the image brightness also increases (Figure 19)

Figure 19 Images of each level of an aluminum step wedge, increasing thickness from left to right, top to bottom (0mm, 0.02mm, 0.04mm, 0.06mm, 0.08 mm, 0.1mm, 0.12mm, and 0.14mm respectively)

Each level in the step wedge is correlated with an increase in thickness of aluminum with each step equaling 0.02 mm. Using the histogram option in Image J, the number of pixels within the image was measured at every pixel intensity from 0 to 255. From there, mean pixel intensity could be determined for each level in the step wedge and this served as the key. The aluminum thickness was graphed against corresponding mean pixel intensity and then fit to a 4-parameter sigmoid curve (Equation 3) in

SigmaPlot 11.0 (Systat Software, Inc.). The sigmoidal fit provided the calibration parameters necessary for determining the ETA of each sample.

$$y = y_0 + \frac{a}{1 + e^{-\left(\frac{x-x_0}{b}\right)}} \quad (Eq. 3)$$

After the key had been determined, analysis was performed on the samples. Again using Image J, the brightness was measured at each pixel intensity. This was done for all four quadrants of the sample and then using the calibration curve from the key, the pixel intensity values were converted to ETA. This measurement accounted for porosity, by counting all the non-bone points on the four images and dividing it by 144, the total possible points. This number represented the proportion of pixels that represent non-bone, Q. Then starting with the lowest ETA pixels, data was deleted until the remaining data was 1 minus Q. These measurements were performed for all the samples and its corresponding step wedge.

2.6 Statistical Analysis

To analyze the histomorphometric and densitometry measurements, the data were first tested using a 2-way Repeated-Measures ANOVA in SigmaPlot 11.0 (Systat Software, Inc.) to measure the differences in BV/TV, percent of remodeled material, percent of remodeled tissue, CLI (bone), CLI (tissue), and mean secondary osteonal radius. The ANOVA model was performed to compare the effects of season of sacrifice and anatomical sector of the bone on the remodeling parameters. In addition to the main effects, the interaction between season and sector were also evaluated. Comparisons in the analysis were made using *post-hoc* Fisher Least Significant Difference (LSD) tests.

The histomorphometric measurements for the control sheep were done as part of a previous thesis and was incorporated into this study to allow for comparison between the control and OVX groups [37]. Thus, a second analysis was performed using a 3 -way ANOVA in SPSS 19.0 to measure the differences in BV/TV, percent of remodeled bone, percent of remodeled tissue, CLI (bone), CLI (tissue), and mean secondary osteonal radius. This model tested the differences in the interaction of season, sector, and treatment across all subjects in addition to the main effects. The comparisons for main effects were again made using a post-hoc Fisher LSD test. The interactive effects were performed using t-tests. In all of the statistical analysis, p-values less than 0.05 were considered significant.

3. RESULTS

3.1 Histomorphometry

Measurements were taken for autumn, winter, and spring. The histomorphometric measurements for summer were previously made as part of a larger experiment [41]. For each sector, the average value of the four quadrants was found for BV/TV, the percent of remodeled tissue, the percent of remodeled material, mean secondary osteon radius, cement line interfaces of the tissue, and cement line interfaces of the material. For each season and anatomical sectors, the averages of the remodeling factors and their standard error are found in Tables I, II, III, IV, V, and VI.

Table I Bone volume to tissue volume for adult 12 month OVX ovine compact bone

BV/TV		
Season		
Group	Mean	Standard Error
Autumn	0.927	0.00567
Winter	0.919	0.00567
Spring	0.930	0.00525
Summer	0.954	0.0021
Anatomical Sector		
Group	Mean	Standard Error
Craniomedial	0.932	0.00614
Cranial	0.939	0.00555
Craniolateral	0.936	0.00581
Caudomedial	0.929	0.00669
Caudal	0.929	0.00667
Caudolateral	0.934	0.00704

Table II Percent remodeled tissue for adult 12 month OVX ovine compact bone

Percent Remodeled Tissue (Remodeled Tissue/Total Area)		
Season		
Group	Mean	Standard Error
Autumn	33.7	0.0276
Winter	27.1	0.0276
Spring	20.7	0.0255
Summer	32.2	0.0302

Anatomical Sector		
Group	Mean	Standard Error
Craniomedial	22.6	0.0273
Cranial	30.4	0.0242
Craniolateral	22.9	0.0255
Caudomedial	35.3	0.0281
Caudal	35.3	0.0203
Caudolateral	29.5	0.0301

Table III Percent remodeled material for adult 12 month OVX ovine compact bone

Percent Remodeled Material (Remodeled Tissue/Bone Area)		
Season		
Group	Mean	Standard Error
Autumn	36.5	0.0287
Winter	29.6	0.0287
Spring	22.4	0.0266
Summer	33.8	0.0315
Anatomical Sector		
Group	Mean	Standard Error
Craniomedial	24.8	0.0274
Cranial	32.3	0.0253
Craniolateral	24.3	0.0273
Caudomedial	32.3	0.0307
Caudal	38.0	0.0204
Caudolateral	31.8	0.0335

Table IV Mean secondary osteon radius for adult 12 month OVX ovine compact bone

Mean Secondary Osteon Radius (μm)		
Season		
Group	Mean	Standard Error
Autumn	58.535	3.536
Winter	57.295	3.536
Spring	56.306	3.555
Summer	64.455	3.874
Anatomical Sector		
Group	Mean	Standard Error
Craniomedial	55.137	2.821
Cranial	61.650	2.744
Craniolateral	58.527	2.741
Caudomedial	59.559	2.741

Caudal	61.665	2.685
Caudolateral	58.348	2.685

Table V Cement line interfaces (tissue) for adult 12 month OVX ovine compact bone

Cement Line Interfaces (Tissue) (mm/mm²)		
Season		
Group	Mean	Standard Error
Autumn	23.826	2.504
Winter	23.819	2.504
Spring	21.405	2.318
Summer	32.884	2.743
Anatomical Sector		
Group	Mean	Standard Error
Craniomedial	19.654	2.35984
Cranial	28.717	2.37181
Craniolateral	21.542	2.10055
Caudomedial	24.796	2.14402
Caudal	32.046	2.00219
Caudolateral	26.147	2.79263

Table VI Cement line interfaces (material) for adult 12 month OVX ovine compact bone

Cement Line Interfaces (Material) (mm/mm²)		
Season		
Group	Mean	Standard Error
Autumn	25.717	2.626
Winter	25.954	2.626
Spring	22.349	2.431
Summer	34.554	2.877
Anatomical Sector		
Group	Mean	Standard Error
Craniomedial	20.169	2.43555
Cranial	30.035	2.51807
Craniolateral	23.446	2.17466
Caudomedial	26.480	2.30447
Caudal	34.604	2.02224
Caudolateral	28.128	2.90092

3.1.2 Two Way Repeated Measures ANOVA

A 2-way repeated measures ANOVA was performed to obtain the means and standard errors for the OVX sheep. Additionally, the statistical test was used to determine seasonal and anatomical differences as well as possible seasonality within the anatomical sector. A p-value less than 0.05 was considered significant. Table VII summarizes the p-values obtained from the 2-way repeated measures ANOVA for each bone remodeling parameter.

Table VII P-values for 2-way repeated measures ANOVA of remodeling parameters

2-way Repeated Measures ANOVA: p-values			
Parameter	Season	Sector	Interaction
BV/TV	0.004	0.691	0.280
Percent Remodeled (Tissue)	0.011	0.000	0.072
Percent Remodeled (Material)	0.010	0.000	0.049
Mean Secondary Osteon Radius	0.443	0.564	0.711
Cement Line Interface (Tissue)	0.029	0.000	0.004
Cement Line Interface (Material)	0.031	0.000	0.004

A significant difference was seen in the BV/TV between seasons (Figure 20). Animals sacrificed in summer had significantly larger BV/TV values than all other season. No significant differences were shown among autumn, winter, and spring. There was no significant anatomical variation or seasonality within sector in the BV/TV values. Significant seasonal and anatomical variation was seen in the percent of tissue remodeled as shown in Figure 21 and Figure 22. Higher levels of remodeling were seen on the cranial and caudal regions of the bone. The cranial and caudal regions correspond to areas of high tension and compression respectively [42]. The regions surrounding the neutral axis exhibited lower amounts of remodeled tissue. The p-value for the interaction of season and sector was not significant; however it was near the 0.05 cut off and could potentially provide useful information. There was significant seasonality within region in

the percent of remodeled material as shown in Figure 23. The caudomedial and craniomedial regions exhibited less variation among the seasons. However, the other sectors showed more variability between seasons and anatomical sector.

Figure 20 Mean BV/TV values and standard deviations for each season of the OVX sheep

Figure 21 Mean percent of remodeled tissue values and standard deviations for each season of the OVX sheep

Figure 22 Anatomic variability of the OVX sheep in the percentage of tissue remodeled. The polar graph orients the values for each sector in their approximate anatomical location. The radial distance indicates the value of the remodeling parameter.

Figure 23 Anatomic variability of OVX sheep within season for the percentage of material remodeled. The polar graph orients the values for each sector in their approximate anatomical location. The radial distance indicates the value of the remodeling parameter.

Analysis of secondary mean osteon radius showed no significant effects of seasonality or anatomical region. Cement line interfaces for both the tissue and material demonstrated significant seasonality within anatomical sectors as seen in Figure 24 and Figure 25. The craniomedial region showed no seasonal variability in the tissue cement line interfaces, as opposed to the other regions which saw the effects of season and anatomical region. Analysis of the material cement line interfaces indicated the craniomedial and craniolateral regions had minimal seasonal effects. However, seasonal and anatomical variation were seen in the other sectors.

Figure 24 Anatomic variability of OVX sheep within season for the number of tissue cement line interfaces. The polar graph orients the values for each sector in their approximate anatomical location. The radial distance indicates the value of the remodeling parameter.

Figure 25 Anatomic variability of OVX sheep within season for the number of material cement line interfaces. The polar graph orients the values for each sector in their approximate anatomical location. The radial distance indicates the value of the remodeling parameter.

3.1.2 Three Way ANOVA

The histomorphometric data for the 12 month control sheep was collected as part of a previous thesis [37]. A 3-way ANOVA was performed to obtain the means and standard errors for the control and OVX data. Additionally, the statistical test was used to assess seasonal, anatomical, and treatment differences as well as possible interactions between all three factors. A p-value less than 0.05 was considered significant. Table VIII summarizes the p-values obtained from the 3-way ANOVA for each parameter.

Table VIII P-values for 3-way ANOVA for comparison of OVX and control remodeling parameters

3-way ANOVA: p-values							
Parameter	Season	Sector	Treatment	Season & Sector	Season & Treatment	Sector & Treatment	Season, Sector, & Treatment
BV/TV	0.000	0.041	0.060	0.052	0.093	0.121	0.018
Percent Remodeled (Tissue)	0.003	0.000	0.455	0.872	0.219	0.430	0.012
Percent Remodeled (Material)	0.004	0.000	0.796	0.803	0.171	0.428	0.008
Mean Secondary Osteon Radius	0.000	0.602	0.084	0.544	0.844	0.816	0.914
Cement Line Interface (Tissue)	0.010	0.000	0.003	0.358	0.116	0.089	0.000
Cement Line Interface (Material)	0.013	0.000	0.007	0.256	0.146	0.095	0.000

Seasonality and anatomical sector within treatment had a significant effect on the BV/TV as seen in Figure 26. The control sheep have higher ratios of BV/TV when compared to the OVX sheep across all seasons. However, the differences are only statistically different during the autumn, winter, and spring seasons. The percent of remodeled tissue and bone showed significant differences due to season (Figure 27 and Figure 29) and anatomical variation (Figure 28 and Figure 30). In the percent of remodeled tissue, there was a significant difference between the control and OVX sheep during spring. In addition, there was also a difference related to anatomical region. In the cranial and caudolateral regions, the level of tissue remodeling of was significantly different than the control with remodeling decreasing with OVX. The control sheep also

exhibited significantly larger amounts of material modeling during spring when compared to the OVX sheep. An overall effect of sector was seen in the data for percent of remodeled material, however there were no significant differences between the control and OVX sheep. There was a significant difference in the interaction of season, treatment, and sector in the percent of material and tissue remodeled.

Figure 26 Mean BV/TV and standard deviations for control and OVX sheep for each season.

Figure 27 Mean percentage of tissue remodeled and standard deviations for control and OVX sheep for each season.

Figure 28 Anatomic variability in the percentage of tissue remodeled for control and OVX sheep. The polar graph orients the values for each sector in their approximate anatomical location. The radial distance indicates the value of the remodeling parameter.

Figure 29 Mean percentage of remodeled material and standard deviations for control and OVX sheep for each season.

Figure 30 Anatomic variability in the percentage of material remodeled for control and OVX sheep. The polar graph orients the values for each sector in their approximate anatomical location. The radial distance indicates the value of the remodeling parameter.

Statistical analysis of mean secondary osteon values indicates a significant difference due to season (Figure 31). Across the OVX and control sheep, animals sacrificed in summer have larger osteons than all other seasons. However, there was only a significant difference in osteon size in the winter seasons, with the OVX sheep having larger osteons. Additionally, no significant anatomical or treatment differences were seen in the values for mean secondary osteon radius. Cement line interfaces for tissue and bone material showed significant effects from a combination of season, anatomical region, and treatment. For both cement line interface measurements, significant differences between the control and OVX were seen in the autumn and spring sheep

(Figure 32, Figure 33). However, no significant differences were seen in the winter and summer sheep.

Figure 31 Mean secondary osteon radius (um) and standard deviations for control and OVX sheep for each season.

Figure 32 Mean number of tissue cement line interfaces and standard deviations for control and OVX sheep for each season.

Figure 33 Mean number of material cement line interfaces and standard deviations for control and OVX sheep for each season.

3.2 Densitometry

Images were captured of the bone specimens and its corresponding aluminum step wedge. The mean pixel intensity values of the step wedge were determined and in conjunction with its equivalent thickness of aluminum (ETA) were graphed and then fit to a 4-parameter sigmoid curve to establish the calibration curve. The mean pixel intensities were determined for each quadrant of the specimen and using the key the ETA for the specimens could be determined. Measurements were taken for autumn, winter, spring, and summer. The mean ETA values and the standard error for each season and anatomical sector are shown in Table IX.

Table IX Densitometry values (ETA) for OVX sheep

Densitometry (ETA) (mm)		
Season		
Group	Mean	Standard Error
Autumn	0.0663	0.00510
Winter	0.0608	0.00491
Spring	0.0654	0.00446
Summer	0.0561	0.00531
Anatomical Sector		
Group	Mean	Standard Error
Craniomedial	0.0650	0.00159
Cranial	0.0597	0.00159
Craniolateral	0.0643	0.0174
Caudomedial	0.0627	0.0164
Caudal	0.0577	0.0171
Caudolateral	0.0633	0.00164

Statistical analysis indicates a significant anatomical difference in the density of the bone (Figure 34). The densest part of the bone was observed in the craniomedial region. Higher levels of density were also seen in the regions surrounding the neutral axis, the craniomedial, craniolateral, caudomedial, and caudolateral regions. The lowest density was seen in the caudal region of the radius/ulna. There was no significant seasonality or seasonality within the anatomical region.

Figure 34 Anatomic variability in density measured in equivalent thickness of aluminum (ETA) for OVX sheep. The polar graph orients the values for each sector in their approximate anatomical location. The radial distance indicates the value of the remodeling parameter.

4. DISCUSSION

Bone disease, specifically osteoporosis, is a health crisis affecting millions of men and women [43]. Osteoporosis is characterized by a decrease in bone mass, making bone more susceptible to fracture. Fragility fractures are the first clinical manifestation of osteoporosis and can occur after minor trauma. Osteoporotic fractures are considered to have the most severe impact on patient's lives, indicated by a decrease in their quality of life and increased dependence on walking aids or help from others [44]. Thus, there is an ever growing need for detection methods for osteoporosis, especially for patients over 50 who suffer fractures. Current detection methods involve dual X-ray absorptiometry to assess patient's bone mineral density. Many patients aren't diagnosed with osteoporosis until after their first fracture and as a result the main challenge in detection methods is identifying patients with fractures who require screening for osteoporosis [45]. Therefore, it is important to understand the risk factors associated with osteoporosis. Increased risk for osteoporosis can be caused by smoking, excessive alcohol and caffeine consumption, increased age and a sedentary lifestyle [43]. Smoking, excessive alcohol and caffeine consumption, and increased age also have shown to increase the risk of osteoporosis related fractures.

There is a great importance in detection methods of osteoporosis, and in addition to dual X-ray absorptiometry other methods have been used for the assessment of bone microarchitecture. Dual X-ray absorptiometry is a well-established method for determining bone mineral density, however that is not the only indicator of bone strength. The fragility of bone is also influenced by mineralization, bone turnover rate, and trabecular and cortical microarchitecture [46]. Computed tomography and magnetic

resonance are attractive options for assessment of bone microarchitecture. They are feasible primarily because they can be used *in vivo*. High-resolution peripheral quantitative computed tomography is another promising method to assess bone density and microarchitecture. As seen in Figure 35 it is a method that would allow physicians to determine a patient's osteoporotic risk.

Ovariectomy surgery involves the removal of the ovaries, and as a result can simulate osteopenia in animals. This has been shown to be an effective treatment as many animal models have shown to exhibit osteoporotic characteristics such as increased bone turnover that can eventually lead to bone loss [27]. In addition to providing a model for osteoporosis, ovariectomy provides a means to induce an animal model for postmenopausal osteoporosis. With ovariectomy, the rat model can produce alterations in cancellous bone that is identical to the bone loss seen in the human skeleton during aging and menopause [47]. In addition to rats, monkeys undergo a reduction in cancellous bone in response to ovariectomy. However, due to their high cost they are primarily used after rats and larger animals have been used.

Figure 35 Images of the tibia (left) and radius (right). A. View demonstrating the reference line (solid line) and the measurement site (between dotted lines); B-E, images from a premenopausal (B), postmenopausal osteopenic (C), postmenopausal osteoporotic (D), and postmenopausal severe osteoporotic (E) subject. [48]

Generally, smaller animals such as rats and mice are the initial animal model for testing the pathophysiology of bone loss and subsequent therapy options. These animals are advantageous due to their lower housing costs; however they are not able to mimic

human osteoporosis as well due to differences in their reproductive physiology, hormone profiles, and biomechanical bone characteristics. Rats and mice also do not provide a large enough model to test full scale device interactions with bone tissue [49]. Primates demonstrate many advantages over other models for osteoporosis due to their resemblance to the human systems. However, as previously stated, primates are extremely costly and difficult to handle and therefore are not the primary model for osteoporosis [27].

There is a need for developing a physiologically relevant and economical large animal model for testing of therapeutic agents to treat osteoporosis. Adult sheep are well suited for this role for many reasons. Sheep present bone loss with estrogen deficiency which is beneficial for analysis of postmenopausal osteoporosis. They exhibit similar hormone profiles to women and Haversian remodeling. Some breeds are seasonally polyestrous and during anestrus periods the animal undergoes a period of estrogen depletion [29]. This contributes to the seasonal effect on the bone remodeling of the animal. As a result, this is an important component that must be considered when implementing sheep as a model for osteoporosis. This is specifically important when comparing different treatments and follow up times. The differences in daylight observed across the seasons plays a crucial part in the activity of the sheep, in turn affecting the animal's loading pattern. The change in loading patterns could potentially affect the rate of bone turnover seen in the varying seasons [33].

The cyclic reproductive pattern seen in sheep is believed to be regulated by melatonin secretion. Research has shown that sheep experience regular 16 to 18 day estrus cycles, period where estrogen has its greatest influence, from mid-November to

late January. Then from May to September no cycling occurs and sheep go through anestrus cycles, period where the sexual cycle rests [50, 51]. However, there is disagreement on the exact time periods for the beginning of the anestrus cycle. Other research from Dr. Dana Ruehlman indicates sheep undergo estrous during early summer to late fall followed by the anestrus period in the late fall through the winter [52]. The cortical bone remodeling of the control sheep are directly affected by the hormonal cycling. However, the OVX sheep are in a constant period of anestrus due to the removal of their ovaries and the different treatments will affect the cortical bone remodeling.

Seasonality had a significant effect on the data presented in this study. Analysis of the OVX data showed seasonality in the BV/TV and percent of remodeled tissue. The most porous bone was seen in the winter months, possibly indicative of the inactivity of sheep during the winter months. The inactivity results in lower amounts of loading on the bone which potentially decreases the initiation of the bone remodeling cycle. Spring had a minor recovery period where the porosity decreased. It was not until summer that a full recovery occurred and the BV/TV levels reached a maximum. The higher percentages of remodeled tissue were observed in summer and autumn. This can be attributed to the recovery period where bone remodeling is occurring more frequently to circumvent the more porous bone seen in winter. In addition to seasonal variation, the data for the percent of remodeled tissue had significant anatomical variation. Higher levels of remodeling were seen on the cranial and caudal regions of bone, regions of increased compressive and tensile stress.

Significant seasonality, anatomical variation, and treatment effects were seen during the comparison of the control and OVX sheep. The cement line interface

measurements for both the tissue and the bone showed significant effects of variation due to season, anatomical region, and treatment. Comparisons of the autumn caudal region and the autumn cranial region indicated no difference in the OVX and the control sheep. However, the autumn caudal and cranial sectors for the OVX sheep were significantly different than the autumn caudal and cranial sectors for the control sheep. This can be explained by the estrous cycle of the ewe that as previously stated begins in November. During the autumn months, the control sheep are able to produce estrogen as opposed to the OVX sheep which cannot. As a result, the OVX bone is much more porous due to the lack of estrogen. On the other hand, no differences were seen in the summer caudal region and the summer cranial region across the control and OVX sheep, indicating treatment did not have an effect. The summer sheep should be approximately four months into their anestrus cycle, at which point estrogen production ceases. This presents an animal that is similar to the OVX sheep and thus supports the study results indicating no differences between the summer months. In addition to supporting the belief of an estrous cycle during autumn and winter, the results also supported Dr. Ruehlman's claims of an estrous cycle during summer and autumn. Looking again at both cement line measurement the differences during the autumn seasons are indicative of the control sheep undergoing estrogen production during those seasons compared to the OVX sheep which would not. However, no differences were seen in any parameter during the summer months.

One limitation of this study is the purchase cohort effect. Upon arrival to Colorado State University, the sheep were already skeletally mature and as a result the previous remodeling cycles must be taken into consideration. To minimize the cohort

effect, the sheep underwent a post-operative time of 12 months. This was done to allow for the previous remodeling cycles to be remodeled away. Additionally, to maintain uniformity the sheep were all housed in similar conditions using the same feed and exercise conditions.

Another constraint of this study was the projection of a 3D structure onto a 2D plane as seen in the microradiographs. The thickness of the specimen can affect the dimensions of the features being measured. This can present a problem when analyzing the specimen for the histomorphometric measurement as certain features may not be visible within the 2D plane.

Microradiograph exposure had an effect on the ability to make the histomorphometric measurements. Some sectors were removed because the features of the specimen were not visible. Problems with the exposure also played a role during the densitometry measurement. Issues with sectors being either washed out or too dark were resolved by changing the lighting to optimize visualization and imaging a new step wedge key for that sector. However, even after changing the lighting the key for some sectors did not exhibit a sigmoidal curve and were therefore omitted.

Based on the results from this study, there are potentially many future studies to be considered. One area of interest involves comparison of densitometry data between the control and OVX. This allows for a more complete analysis to understand the effect of ovariectomy on bone density. Another consideration is the use of the OVX sheep for analysis of the bone remodeling cycle in response to estrogen therapy or another therapeutic agent. When compared to the control sheep the hope is that sheep that

underwent osteoporotic therapy have a similar BMD, thus indicating the effectiveness of the treatment.

Within the OVX data alone, there was significant seasonal and anatomical variation, as well as seasonality within anatomical region. Then when compared to the control sheep there was significant seasonal, anatomical, and treatment differences. In addition, the comparison saw variation due to seasonality within anatomical region, seasonality within treatment, and then an interaction between season, sector and treatment. These results indicate the need for a robust model for compact bone remodeling. The effects of seasonality and region must be considered in the analysis of bone remodeling parameters. As indicated by the results, it cannot be assumed that each region of bone remodels in a similar manner. For example, during comparison of a caudal region and cranial medial region, it would be incorrect to assume that the remodeling in both regions is equivalent. The same can be said for different time points of sheep sacrifice and treatment; the summer OVX and the autumn control sheep did not have comparable amounts of remodeling. As a result, when developing protocols for studies involving bone remodeling and osteoporosis it is important to consider the changes that occur in remodeling due to season, treatment and anatomical region. This research showed that there are varying levels of remodeling in the adult OVX ovine model, provided a model for post-menopausal osteoporosis using 12 month OVX sheep, and indicates that ovariectomy does indeed have an effect on bone remodeling in the ovine model.

5. CONCLUSION

The ovariectomized adult ewe provides an economical large animal model for the study of postmenopausal osteoporosis and compact bone remodeling. Sheep are advantageous because their large size allows for device implantation and studying treatment options for osteoporosis. In addition, sheep have been shown to exhibit Haversian remodeling in the cortical bone 3 to 4 months after the development of plexiform bone. The ovariectomized model involves removal of the sheep's ovaries simulating menopause, making it an ideal model for studies of postmenopausal osteoporosis.

This research involved the use of ovariectomized sheep divided into four seasonal groups based on time of animal sacrifice, performed at Colorado State University. The animal's right and left radius and ulna were removed and then sent to Henry Ford Hospital. There, the radius was divided into six anatomical sectors and then ground down to a final thickness of 100 μm for microradiograph imaging. Finally, an aluminum step wedge was created to provide a key for densitometry analysis. After completion, the microradiographs were sent to California Polytechnic State University, San Luis Obispo for histomorphometric and densitometry analysis.

Histomorphometry involved observation of the microradiographs under white light using a Merz grid to quantify the bone remodeling parameters. The cortical beams for each anatomical sector were divided into four quadrants; measurements were taken in each quadrant and then the values averaged. To quantify remodeling the following measurements were taken: porosity, number of secondary osteons, number of cement line interfaces, and amount of remodeled bone. These measurements were then input into a

Microsoft Excel spreadsheet to compute the amount of remodeling that occurred in the bone by finding the ratio of bone volume to tissue volume (BV/TV), percent of remodeled tissue and bone, cement line interface of tissue and bone (CLI), and mean secondary osteonal radius.

Densitometry required taking images of each specimens and its corresponding step wedge key. This light intensity and exposure times remained the same for pictures of the aluminum step wedge and the sample. Image analysis for all the pictures was performed to measure the number of pixels in the image at every pixel intensity from 0 to 255. From there mean pixel intensity was found for each level in the step wedge and a key could be determined from this calibration. Mean pixel intensities were also found for each specimen. Then based on the calibration curve and the porosity count, an equivalent thickness of Aluminum (ETA) could be determined for the specimen.

After data collection, statistical analysis was then performed. To analyze the histomorphometric and densitometry measurements for the OVX data, the data was tested using a 2-way Repeated-Measures ANOVA. The ANOVA model was performed to compare the effects of season of sacrifice and anatomical sector on the remodeling parameters. The final statistical test was a 3-way AOVA comparing the OVX sheep with the controls. This model compared the effects of season, treatment, anatomical location, and interactions between all three.

The presented data showed significant seasonal and anatomical variations in compact bone remodeling. The OVX model alone showed seasonal variation in the ratio of bone volume to tissue volume and the percent of remodeled tissue. Anatomical variation was only seen in the percent of remodeled tissue. No demonstrable differences

due to season or sector were seen in the mean secondary osteon radius. Significant seasonality within anatomical location was exhibited in the percent of remodeled material, and in both cement line interface measurements. Comparison of the OVX and control sheep also resulted in significant seasonal, anatomical, and treatment variations. A significant interaction of season, sector, and treatment were seen in BV/TV, both percent remodeling measurement, and both cement line interface measurements. Seasonal variation was seen in the mean secondary osteon radius. Pairwise comparisons showed similarities and differences between the control and OVX sheep that were indicative of the hormonal changes that occur in the control sheep. The effects of estrogen were seen in the control sheep during their estrous cycles, period of greatest estrogen influence, and comparison of these sheep to the OVX presented significant results.

The large amount of significance indicates there is much variation in the cortical bone remodeling of sheep. It is important that the conclusions of this study be taken into consideration when developing new test protocols for research incorporating the sheep model for studying osteoporosis. The follow up times, treatments, and anatomical region of interest must all be considered because each will have a different effect on the remodeling of the animal. The treatment in relationship to the cyclic hormonal patterns of the sheep also must be clearly understood for better result interpretation. The results of this study showed that varying levels of remodeling occurs in the adult OVX ovine model and differences between the control and OVX model can be attributed to seasonal and anatomical variations, with the polyestrous cycle of the sheep also playing a key role.

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Appendix A: 12 Month OVX Histomorphometry Data

12 Month Autumn OVX						
Average BV/TV						
	Sector					
Sheep	Craniomedial	Cranial	Craniolateral	Caudomedial	Caudal	Caudolateral
C29	0.875	0.8958	0.9236	0.8889	0.9236	0.9514
C30	0.9236	0.9306	0.9097	0.9167	0.9514	0.9583
C33	0.8889	0.9444	0.9306	0.9167	0.9444	0.9444
C39	0.9306	0.9167	0.9236	0.875	0.9444	0.9167
C40	0.9306	0.9352	0.9236	0.9375	0.9236	0.9306
C42	0.9444	0.9653	0.9306	0.9236	0.9583	0.9583
Average Percent Remodeled (Tissue) (Remodeled Tissue/Total Area)						
	Sector					
Sheep	Craniomedial	Cranial	Craniolateral	Caudomedial	Caudal	Caudolateral
C29	0.014	0.104	0.222	0.208	0.424	0.188
C30	0.431	0.479	0.194	0.514	0.417	0.333
C33	0.257	0.396	0.451	0.354	0.313	0.333
C39	0.472	0.285	0.361	0.521	0.396	0.549
C40	0.299	0.324	0.403	0.340	0.375	0.319
C42	0.458	0.201	0.236	0.313	0.389	0.264
Average Percent Remodeled (Material) (Remodeled Tissue/Bone Area)						
	Sector					
Sheep	Craniomedial	Cranial	Craniolateral	Caudomedial	Caudal	Caudolateral
C29	0.147	0.116	0.241	0.239	0.458	0.197
C30	0.465	0.516	0.212	0.565	0.438	0.350
C33	0.290	0.417	0.486	0.385	0.331	0.354
C39	0.506	0.313	0.292	0.594	0.419	0.601
C40	0.321	0.347	0.437	0.365	0.406	0.343
C42	0.490	0.208	0.254	0.339	0.408	0.276
Average Osteon Radius (µm)						
	Sector					
Sheep	Craniomedial	Cranial	Craniolateral	Caudomedial	Caudal	Caudolateral
C29	18.689	51.235	55.840	60.763	67.174	59.269
C30	58.741	63.080	49.277	56.816	56.576	64.230
C33	50.852	63.108	60.450	53.229	68.235	63.517
C39	83.165	56.682	66.343	78.244	73.216	72.045
C40	47.703	56.160	65.152	52.142	60.981	56.311

C42	52.952	56.029	43.563	49.211	53.384	62.889
Average CLI (Tissue) (mm/mm²)						
	Sector					
Sheep	Craniomedial	Cranial	Craniolateral	Caudomedial	Caudal	Caudolateral
C29	0.750	11.250	26.750	22.250	33.000	15.750
C30	14.000	19.500	23.250	19.000	23.500	17.250
C33	30.750	43.750	47.750	41.250	27.250	35.500
C39	23.250	26.250	26.250	31.250	31.000	27.750
C40	8.250	13.000	21.000	19.500	30.250	30.750
C42	25.000	18.500	19.750	12.750	29.500	11.250
Average CLI (Material) (mm/mm²)						
	Sector					
Sheep	Craniomedial	Cranial	Craniolateral	Caudomedial	Caudal	Caudolateral
C29	0.865	12.453	29.027	25.458	35.868	16.571
C30	15.098	20.912	25.353	20.890	24.724	17.912
C33	34.728	46.100	51.313	44.778	29.014	37.544
C39	24.866	28.817	28.382	36.050	32.878	30.053
C40	8.847	14.909	22.734	20.790	32.679	33.142
C42	26.507	19.141	21.172	13.754	30.736	11.736

12 Month Winter OVX						
Average BV/TV						
	Sector					
Sheep	Craniomedial	Cranial	Craniolateral	Caudomedial	Caudal	Caudolateral
C58	0.9097	0.9375	0.9167	0.9236	0.8472	0.9444
C62	0.8819	0.9236	0.9514	0.9028	0.9306	0.8958
C67	0.9306	0.9167	0.9792	0.9236	0.9306	0.9236
C68	0.8889	0.9306	0.9306	0.9653	0.9375	0.9236
C70	0.9306	0.9514	0.9375	0.9583	0.875	0.9236
C71	0.9236	0.8542	0.8542	0.8681	0.9236	0.9514
Average Percent Remodeled (Tissue) (Remodeled Tissue/Total Area)						
	Sector					
Sheep	Craniomedial	Cranial	Craniolateral	Caudomedial	Caudal	Caudolateral
C58	0.194	0.139	0.174	0.396	0.174	0.333
C62	0.097	0.229	0.208	0.229	0.257	0.111
C67	0.194	0.188	0.208	0.306	0.306	0.104
C68	0.201	0.354	0.188	0.271	0.424	0.174
C70	0.292	0.438	0.410	0.347	0.319	0.243

C71	0.201	0.285	0.375	0.438	0.493	0.458
Average Percent Remodeled (Material) (Remodeled Tissue/Bone Area)						
	Sector					
Sheep	Craniomedial	Cranial	Craniolateral	Caudomedial	Caudal	Caudolateral
C58	0.215	0.150	0.189	0.430	0.212	0.361
C62	0.108	0.251	0.218	0.256	0.273	0.123
C67	0.207	0.201	0.214	0.334	0.328	0.113
C68	0.234	0.380	0.205	0.280	0.457	0.193
C70	0.315	0.461	0.437	0.364	0.365	0.264
C71	0.216	0.344	0.442	0.507	0.534	0.481
Average Osteon Radius (μm)						
	Sector					
Sheep	Craniomedial	Cranial	Craniolateral	Caudomedial	Caudal	Caudolateral
C58	57.386	43.298	56.208	74.487	64.944	65.268
C62	54.969	45.995	52.048	51.418	50.025	47.594
C67	47.907	47.506	52.994	53.833	61.040	58.271
C68	49.772	71.154	49.411	55.087	67.030	46.696
C70	53.920	62.140	67.358	56.877	68.641	54.287
C71	43.897	65.277	62.983	65.213	66.991	70.713
Average CLI (Tissue) (mm/mm^2)						
	Sector					
Sheep	Craniomedial	Cranial	Craniolateral	Caudomedial	Caudal	Caudolateral
C58	15.000	20.000	15.000	27.000	16.500	25.000
C62	7.000	19.500	19.250	25.750	23.500	11.250
C67	21.000	21.500	21.500	25.750	25.000	8.750
C68	21.500	24.250	21.250	21.250	32.500	18.000
C70	36.000	37.750	28.250	35.000	28.000	24.250
C71	23.750	27.250	28.000	37.000	36.000	29.250
Average CLI (Material) (mm/mm^2)						
	Sector					
Sheep	Craniomedial	Cranial	Craniolateral	Caudomedial	Caudal	Caudolateral
C58	16.457	21.299	16.261	29.007	19.926	27.143
C62	7.955	21.187	20.238	28.737	25.329	12.446
C67	22.467	23.140	21.900	27.596	26.727	9.406
C68	24.835	25.922	23.227	21.987	34.889	19.700
C70	38.621	39.457	30.157	36.616	31.372	26.254
C71	25.438	33.748	32.643	42.332	39.122	30.812

12 Month Spring OVX						
Average BV/TV						
	Sector					
Sheep	Craniomedial	Cranial	Craniolateral	Caudomedial	Caudal	Caudolateral
C101	0.9167	0.9792	0.9167	0.9444	0.8958	0.9514
C102	0.9236	0.9306	0.9514	0.875	0.9375	0.9028
C104	0.9653	0.9167	0.9375	0.9306	0.9236	0.875
C111	0.9583	0.9444	0.9028	0.8958	0.8681	0.9375
C112	0.9236	0.9352	0.8958	0.8958	0.9236	0.9444
C89	0.9861	0.9444	0.9306	0.9306	0.9653	0.9167
C98	0.9444	0.9514	0.9514	0.9514	0.9444	0.9514
Average Percent Remodeled (Tissue) (Remodeled Tissue/Total Area)						
	Sector					
Sheep	Craniomedial	Cranial	Craniolateral	Caudomedial	Caudal	Caudolateral
C101	0.243	0.201	0.285	0.174	0.243	0.382
C102	0.076	0.194	0.083	0.021	0.389	0.069
C104	0.201	0.264	0.049	0.125	0.229	0.139
C111	0.146	0.292	0.340	0.118	0.278	0.389
C112	0.181	0.361	0.229	0.264	0.208	0.069
C89	0.326	0.104	0.035	0.076	0.236	0.264
C98	0.306	0.174	0.056	0.285	0.313	0.292
Average Percent Remodeled (Material) (Remodeled Tissue/Bone Area)						
	Sector					
Sheep	Craniomedial	Cranial	Craniolateral	Caudomedial	Caudal	Caudolateral
C101	0.275	0.207	0.308	0.183	0.273	0.400
C102	0.086	0.207	0.087	0.025	0.415	0.080
C104	0.209	0.289	0.053	0.145	0.251	0.161
C111	0.160	0.308	0.379	0.133	0.323	0.413
C112	0.197	0.388	0.258	0.298	0.224	0.075
C89	0.329	0.111	0.037	0.081	0.245	0.292
C98	0.323	0.187	0.059	0.299	0.331	0.307
Average Osteon Radius (µm)						
	Sector					
Sheep	Craniomedial	Cranial	Craniolateral	Caudomedial	Caudal	Caudolateral
C101	0.000	0.000	61.334	61.553	57.048	62.791
C102	43.404	54.632	39.942	26.319	62.853	47.628
C104	53.981	60.066	43.153	0.000	54.259	56.263
C111	0.000	79.082	78.083	54.093	65.259	70.099
C112	54.675	61.875	50.512	59.552	51.382	40.742

C89	62.125	44.694	31.154	35.042	61.359	47.537
C98	68.650	55.271	0.000	70.698	65.243	59.029
Average CLI (Tissue) (mm/mm²)						
	Sector					
Sheep	Craniomedial	Cranial	Craniolateral	Caudomedial	Caudal	Caudolateral
C101	27.333	30.333	26.750	15.000	28.750	38.500
C102	11.500	17.750	12.250	5.750	31.250	10.000
C104	25.000	27.250	6.000	18.333	29.250	13.750
C111	18.500	26.000	25.750	10.000	25.750	29.750
C112	15.500	30.750	27.500	25.500	25.000	6.000
C89	31.000	16.500	6.250	12.750	25.000	30.500
C98	26.500	18.250	9.000	22.750	27.750	32.000
Average CLI (Material) (mm/mm²)						
	Sector					
Sheep	Craniomedial	Cranial	Craniolateral	Caudomedial	Caudal	Caudolateral
C101	23.069	23.493	29.123	15.912	32.086	40.551
C102	12.749	19.086	12.865	6.565	33.361	11.271
C104	26.069	29.824	6.663	16.258	31.855	15.655
C111	10.042	27.442	28.548	11.141	29.832	31.617
C112	16.838	30.849	30.912	27.849	22.423	14.818
C89	28.138	12.828	11.182	13.555	31.760	28.102
C98	24.015	17.880	13.423	23.349	32.081	33.600

12 Month Summer OVX						
Average BV/TV						
	Sector					
Sheep	Craniomedial	Cranial	Craniolateral	Caudomedial	Caudal	Caudolateral
C04	0.917	0.965	0.951	0.965	0.868	0.965
C05	0.965	0.958	0.986	0.958	0.958	0.969
C08	0.944	0.965	0.972	0.944	0.972	0.979
C13	0.972	0.938	0.958	0.979	0.935	0.824
C18	0.979	0.979	0.951	0.979	0.944	0.972
Average Percent Remodeled (Tissue) (Remodeled Tissue/Total Area)						
	Sector					
Sheep	Craniomedial	Cranial	Craniolateral	Caudomedial	Caudal	Caudolateral
C04	0.181	0.375	0.153	0.118	0.278	0.125
C05	0.076	0.424	0.097	0.458	0.556	0.424
C08	0.028	0.465	0.398	0.278	0.481	0.438
C13	0.120	0.496	0.139	0.410	0.361	0.602

C18	0.465	0.326	0.174	0.417	0.458	0.333
Average Percent Remodeled (Material) (Remodeled Tissue/Bone Area)						
	Sector					
Sheep	Craniomedial	Cranial	Craniolateral	Caudomedial	Caudal	Caudolateral
C04	0.196	0.389	0.161	0.122	0.322	0.130
C05	0.080	0.444	0.099	0.478	0.579	0.432
C08	0.029	0.481	0.408	0.292	0.494	0.449
C13	0.124	0.519	0.145	0.422	0.386	0.731
C18	0.474	0.333	0.183	0.425	0.485	0.342
Average Osteon Radius (μm)						
	Sector					
Sheep	Craniomedial	Cranial	Craniolateral	Caudomedial	Caudal	Caudolateral
C04	94.020	52.460	67.630	41.440	39.040	29.710
C05	32.120	57.670	45.210	94.540	81.020	56.270
C08	44.140	136.050	116.100	82.380	63.870	84.640
C13	47.320	60.680	52.140	63.990	60.210	71.240
C18	65.320	56.340	62.490	66.610	59.150	49.840
Average CLI (Tissue) (mm/mm^2)						
	Sector					
Sheep	Craniomedial	Cranial	Craniolateral	Caudomedial	Caudal	Caudolateral
C04	19.000	42.000	14.250	11.250	26.500	8.750
C05	5.750	49.250	8.000	38.250	62.750	55.250
C08	4.000	51.500	43.440	22.500	47.000	45.250
C13	12.330	46.500	15.000	44.250	37.000	48.000
C18	50.000	30.500	20.750	36.250	50.000	38.750
Average CLI (Material) (mm/mm^2)						
	Sector					
Sheep	Craniomedial	Cranial	Craniolateral	Caudomedial	Caudal	Caudolateral
C04	20.570	43.480	15.120	11.650	30.520	9.040
C05	6.000	51.760	8.150	39.820	65.490	56.400
C08	4.230	53.430	44.690	23.810	48.340	46.230
C13	12.780	49.670	15.740	45.390	39.610	58.320
C18	53.570	31.160	21.760	37.050	52.970	39.860

Appendix B: 12 month OVX Densitometry Data

12 Month Autumn OVX						
ETA (mm)						
	Sector					
Sheep	Craniomedial	Cranial	Craniolateral	Caudomedial	Caudal	Caudolateral
C29	0.0938	0.0862	0.6807	0.0828	0.0720	0.0889
C30	0.0584	0.0543	0.8727	0.0653	0.0598	0.4889
C33	0.0712	0.0681	0.0592	0.0774	0.0637	0.0689
C39	0.0585	0.0570	0.0526	0.0427	0.0490	0.0560
C40	0.0721	0.0728	0.0773	0.0570	0.0765	0.0788
C42	0.0372	0.0637	0.0685	0.0597	0.0550	0.0666

12 Month Winter OVX						
ETA (mm)						
	Sector					
Sheep	Craniomedial	Cranial	Craniolateral	Caudomedial	Caudal	Caudolateral
C58	0.0662	0.0727	0.0736	0.0724	0.0000	0.0751
C62	0.0650	0.0649	0.0754	0.0703	0.0646	0.0692
C67	0.0768	0.0644	0.0677	0.0814	0.0709	0.0741
C68	0.0745	0.0663	0.0742	0.0584	0.0564	0.0725
C70	0.0486	0.0324	0.0389	0.0246	0.0315	0.0448
C71	0.0521	0.0436	0.0475	0.0503	0.0430	0.0414

12 Month Spring OVX						
ETA (mm)						
	Sector					
Sheep	Craniomedial	Cranial	Craniolateral	Caudomedial	Caudal	Caudolateral
C101	0.0682	0.0639	0.0644	0.0781	0.0577	0.0582
C102	0.0585	0.0600	0.0798	0.0677	0.0663	0.0725
C104	0.0640	0.0665	0.0765	0.0865	0.0679	0.0738
C111	0.0752	0.0504	0.0547	0.0762	0.0623	0.0587
C112	0.0808	0.0750	0.0777	0.0775	0.0776	0.0846
C89	0.0652	0.0499	0.0637	0.0574	0.0712	0.0505
C98	0.0656	0.0407	0.0307	0.0555	0.0415	0.0511

12 Month Summer OVX						
ETA (mm)						
	Sector					
Sheep	Craniomedial	Cranial	Craniolateral	Caudomedial	Caudal	Caudolateral
C04	0.0656	0.0431	0.0445	0.0376	0.0461	0.0648
C05	0.0793	0.0633	0.0817	0.0586	0.0613	0.0546
C08	0.0440	0.0407	0.0258	0.0390	0.0266	0.0346
C13	0.0481	0.0646	0.0765	0.0632	0.1213	0.0494
C18	0.0776	0.0710	0.0684	0.0665	0.0608	0.0740