Short-term Estrogen-depletion-related Changes in Anatomic Variation of Time-dependent Material Properties, and Evidence of Secondary Remodeling of Ovine Compact Bone

At California Polytechnic State University, San Luis Obispo

A Senior Project submitted in partial fulfillment of the requirements for the degree of Bachelor of Science in Biomedical Engineering

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Abstract

Recognized as the most common type of bone disease in humans, osteoporosis poses a major health threat to roughly 28 million Americans [1]. In women, the leading cause of osteoporosis is known to be a drop in estrogen during the time of menopause. Thus, it becomes essential to acquire a better understanding of postmenopausal osteoporosis, as many individuals desire a longer lifetime and an improved quality of life for the elderly. To further current knowledge of postmenopausal osteoporosis and help develop methods of overcoming it, it is important to find an appropriate animal model.

In this study, pretreated ovine compact bone is analyzed using histomorphometry to see if this type of animal model can be used to model osteoporotic bone in women experiencing menopause. Ready-made radiographs of ovine compact bone taken from the radius and ulna of 14 sham-ovariectomized sheep (control) and 14 ovariectomized sheep (OVX), sacrificed after 3 months in either the winter or fall seasons, were used to study the consequences of seasonal, anatomical, and hormonal variation on ovine bone remodeling. Three-way ANOVA analyses were performed to determine seasonal, anatomical, and hormonal interaction effects on the following variables (quantified via histomorphometry): bone volume to tissue volume ratio, average percent of tissue and material remodeling, average secondary osteonal radius, and number of cement line interfaces (CLI).

Anatomical and hormonal variations were found to have individual effects on average percent remodeled tissue and material, with 95% confidence. Tukey tests showed that estrogen depletion of OVX sheep caused significantly higher percent remodeled tissue and material averages than control sheep. In addition, there were seasonal and anatomical interaction effects on average CLI in both tissue and material, and a seasonal effect on average osteonal radius.

Introduction

Osteoporosis is a large and growing health problem in many countries [2]. Osteoporosis has been estimated to affect more than 75 million individuals in the United States, Europe, and Japan [3]. Its main consequence is increased risk of bone fracture, especially in the wrist, hip, and vertebral column. In fact, osteoporosis causes more than 8.9 million fractures annually worldwide, of which more than 4.5 million occur in the Americas and Europe [3]. Although bone fractures brought about by this deleterious disease often occur with little or no trauma, they are known to cause pain and injury during normal, everyday physical activity such as sitting, walking, and running, and commonly cause people to become bedridden. In Figure 1 below, the effect of osteoporosis is shown in a typical healthy, adult femur [4].

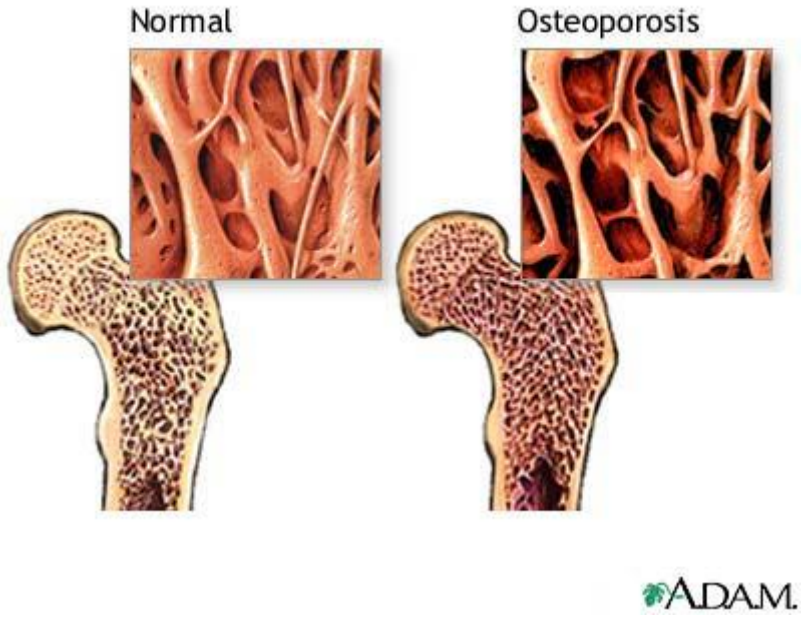

Figure 1: The effect of osteoporosis on spongy bone in the femur [5].

Osteoporosis, which literally means "porous bones," may be defined as a metabolic bone disorder resulting in a severe loss of bone density, causing increased bone fragility. Multinuclear cells called osteoclasts are responsible for bone resorption, and thus, the decrease in bone density. In bone resorption, osteoclasts break down and remove bone. In bone remodeling, however, a balance of bone resorption by osteoclasts and bone deposition by bone-forming cells called osteoblasts occurs to maintain bone density

and structure [6]. When bone resorption begins to outweigh bone deposition, osteoporosis becomes a major threat.

While the manifestations of osteoporosis may be apparent, the pathogeneses of the disease are currently incompletely understood [7]. However, certain pathogenic risk factors are known. These include having a diet low in certain micro-nutrients such as calcium and vitamin D, not exercising, smoking, long-term use of certain medications (e.g., corticosteroids), and low sex hormone (estrogen or testosterone) levels [8]. Low estrogen levels are a common cause of osteoporosis seen in postmenopausal women. At menopause, the secretion of estrogen by the ovaries is terminated. Since estrogen is used by the body to maintain bone density by inhibiting osteoclasts, the lack of estrogen enables osteoclast activity to outpace bone deposition by osteoblasts [4]. In essence, bone resorption occurs at a faster rate than bone formation, leading to a decrease in bone density. By age 70, the average white woman loses 30% of her bone tissue [4]. Thus, osteoporosis and increased fracture risk is more frequently seen in overage women than in men. As one of the leading causes of osteoporosis is a drop in estrogen during the time of menopause, it is important to determine a suitable animal model that will accurately mimic the pathogenesis of estrogen depletion in bone.

The human skeleton is mainly composed of two types of tissue: compact bone that forms the outer layer of bones, and spongy bone found inside bones. Bone is usually formed in layers or lamellae, which contain collagen fibers, hydroxyapatite, proteoglycans, and other substances [9]. In compact bone, lamellae form concentric cylinders called osteons that run parallel to the bone axis. In the center of the osteon is a passageway, called the Haversian canal, for nerves and blood vessels. An osteon is formed from bone remodeling, in which bone resorption proceeds radially outward from a blood vessel, followed by bone deposition proceeding radially inward and ending at the Haversian canal. When the osteon replaces primary bone, or the first bone tissue formed during youth, it is called a secondary osteon. Secondary osteons are weaker than primary bone because they do not have time to fully mineralize and harden during the relatively fast process of bone remodeling.

When analyzing bone at the tissue level, it is useful to distinguish the physical properties of the different regions in the bone. For example, the front side of a human tibia may be denser than the backside due to cyclical loading that is more distributed along the front side of the bone. For quadrupeds, axial sections of bone found in the limbs are categorized into the following regions: cranial (facing the front), caudal (facing the end), medial (facing the midline), and lateral (facing the sides of the body) [10].

Animal models are useful for studying similar tissues in the human body, including bone tissue. Rats are a common animal model used in studies due to their short life cycle, relatively lower cost, and small size. However, their lack of Haversian canal remodeling makes them poor candidates for modeling human bone remodeling [10]. Rabbits, on the other hand, undergo Haversian canal remodeling and have been used to model osteoporosis [10]. Their relatively small size and fast growth period result in less maintenance cost and less downtime, making them a more attractive model. However, their small size is inadequate for testing implant dynamics on a human scale [10]. Sheep are adequately large in size and may provide a more accurate model for osteoporosis in humans. More importantly, ovariectomized sheep have been shown in multiple studies as a feasible model for bone mineral density loss [10]. This suggests that they have similar hormonal effects to human females, and may be used to model postmenopausal osteoporosis.

In this study, ovine bone samples of ovariectomized sheep are analyzed to see if sheep bone undergoes postmenopausal osteoporosis similarly to humans. Ovariectomy has been performed on the sheep to study the effect of estrogen depletion in structural properties of ovine radius/ulna [11]. Sheep were sacrificed after 3 months post-operation. Sheep that received ovariectomies (OVX) and sheep that received control surgeries (sham) for winter and fall are used to compare seasonal effects of estrogen depletion. Cross-sectional sectors were cut out of the radius/ulna from six different locations: craniomedial, cranial, craniolateral, caudomedial, caudal, and caudolateral. These sectors were then used to make microradiographs capable of being imaged using a white light microscope. Dr. Les, who is leading this ongoing study, has provided the microradiographs of OVX and sham for 3-month fall and 3-month winter. Histomorphometry is performed on the microradiographs to analyze the secondary bone

remodeling present. In particular, bone remodeling physiognomies such as secondary osteonal density, cement line interface, fraction of remodeled bone, and the ratio of bone volume to tissue volume (BV/TV) are quantified. Below in Figure 2, an ovine bone microradiograph magnified at 100x is shown. The microradiograph shows much bone remodeling and large porosities due to bone resorption.

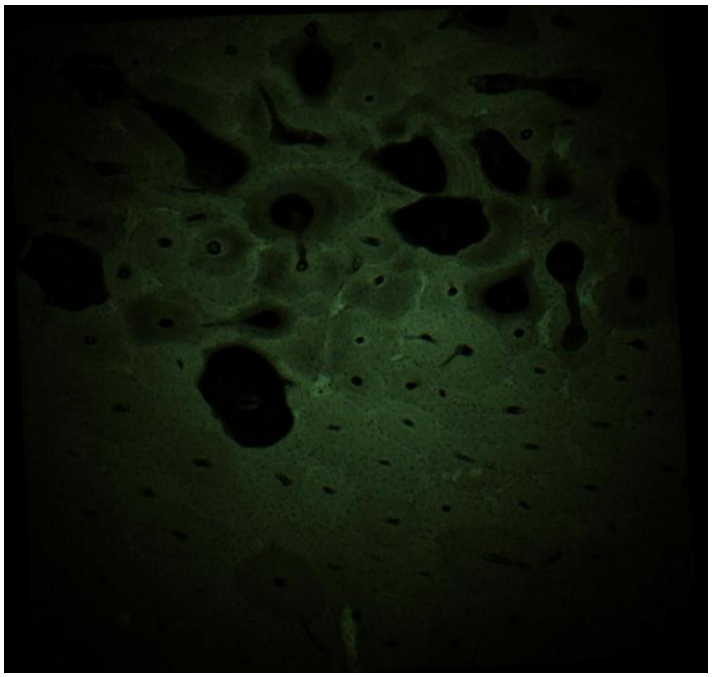

Figure 2: Ovine bone microradiograph at 100x magnification.

Taking several histomorphometric measurements for each specimen using the Merz grid, statistical analysis is then performed to study the effects of estrogen depletion on ovine radius/ulna. Using three-way ANOVAS, interaction effects between season, anatomical region, and estrogen variation, are tested for. Overall, more bone remodeling in OVX than in sham is expected. Through analysis of the ready-made ovine bone samples of OVX and sham, this study aims to help determine and quantify the potential of sheep being a valid animal model for postmenopausal osteoporosis in humans.

Methods

The following methods were performed at various places stated below and were not a part of this senior project at California Polytechnic University.

With approval from the Institutional Animal Care and Use Committee (IACAUC), twenty-six Rambouillet-cross ewes with fully matured skeletons were used for this study at Colorado State University, Ft. Collins, Colorado. Twelve of them were ovariectomized (OVX) meaning that their ovaries were removed through surgery. For the control group the remaining fourteen sheep underwent similar stresses from surgery but ovaries were kept intact so that they would continue estrogen production. The ewes were subjected to a sham abdominal surgery (sham) under general anesthesia, just as the ovariectomized ewes were. This all took place at the large animal surgery facilities at the College of Veterinary Medicine and Biomedical Sciences at Colorado State University.

The animals were kept in dry-lot conditions at an altitude of 1524m, 41 degrees North latitude, and fed a diet consisting of grass-hay and alfalfa. Groups of fourteen ewes were sacrificed at three months following the operation (seven OVX, and seven shams). One group of ewes was sacrificed in late August of the same year, while another was sacrificed in winter. At this time the left radius/ulna (Figure 3) was harvested and kept at -20°C in saline-soaked towels to await further processing.

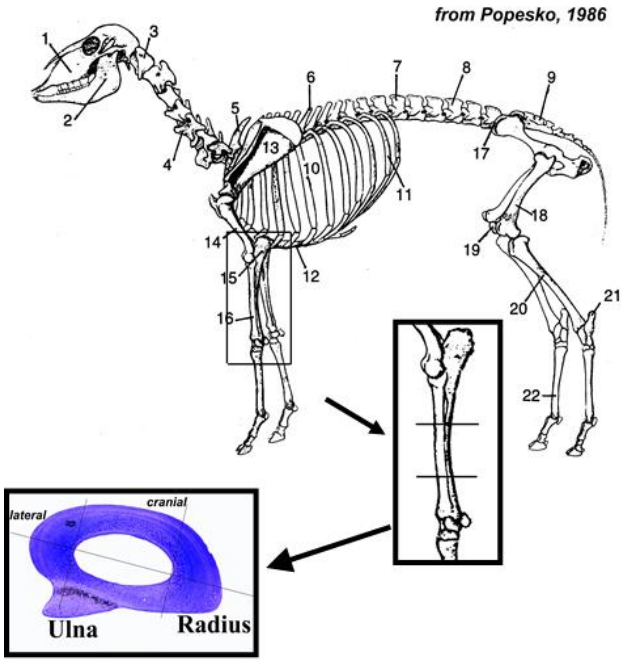

Figure 3: Radius and Ulna cross-sectional view taken from forelegs of a ewe [11].

Later, eighteen to twenty-five longitudinal cortical beams with 1.75x1.75x19mm dimensions were cut from the mid-radial diaphysis (Exakt Technologies Inc. USA, Oklahoma City). This took place at Henry Ford Medical Hospital and was not a part of the senior projects at Cal Poly. A single beam was randomly chosen from the craniomedial, cranial, craniolateral, caudomedial, caudal, and caudolateral. Specimens were tested non-destructively in 3-point bending via a DMA7e, Perkin Elmer controller. The force control ranged between 1-20 Hz with 0.2 Hz increments. There was 15 mm of outer support in normal saline at 37°C. A static load of 550 mN was applied, as well as a dynamic load of 500 mN. The Storage Modulus (analogous to the Elastic Modulus) E1, and the tangent of the phase angle between applied force and resulting strain, a measure of damping known as tangent delta, was calculated at each frequency. E1 data were fit to a curve represented by the equation $E=a\times freq^{b}$. In the equation, "a" represents E1 at a Frequency of 1 Hz representing the quasi-static material stiffness, and the exponent "b" represents a measure of stress-rate sensitivity [11]. After testing, a 100-micrometer section was taken from the center of the beam and sent to California Polytechnic University where they were microradiographed and analyzed.

The following methods were performed for this senior project at California Polytechnic University.

The microscope used was an Olympus BX-41 Microscope. To prepare the microscope for use the OptiScan II and the main switch on the microscope were turned on. Bright light to take wide field images was used. For this particular scope the filter settings were: "Filter Wheel 1" and "Filter Wheel 2" to #10 (because #10 does not have any filters on it), move the "Filter Wheel Cube" to the brightest field setting (BF 1), and open the "Iris Diaphragm Ring" all the way.

To analyze the bone under the microscope a Merz grid was used. The grid was placed in the left eyepiece in the micrometer-mounting frame and placed back on the microscope. The Merz grid (Figure 4) consists of six horizontal sinusoidal lines and thirty-six point markers that were used to measure if our porosities, secondary osteons, etc. are counted or not.

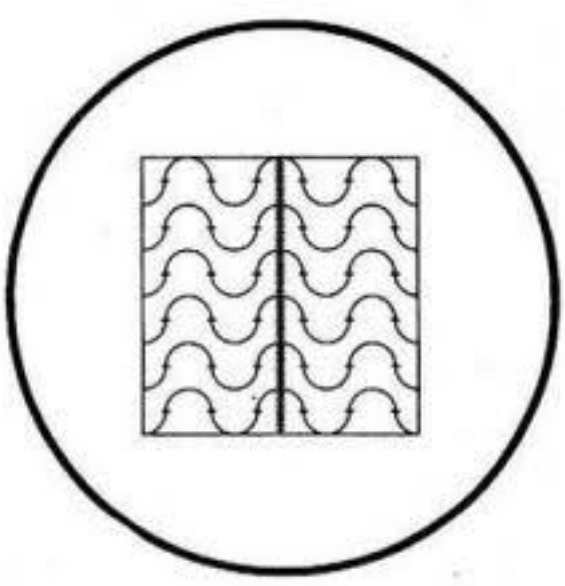

Figure 4: Merz grid example with 6 sinusoidal lines and 36 point markers [12].

Great care was taken in preparing the sample microradiograph because of how valuable they are. After locating the specific slide being analyzed, it was held only by the edges so as not to cause any scratches or fingerprints on the surface that would interfere with our data collection. A special sample holder was rapid prototyped to place the slide in so that it would fit the microscope dimensions. An example of a slide is shown in Figure 5 with a drafted rendition and a real picture.

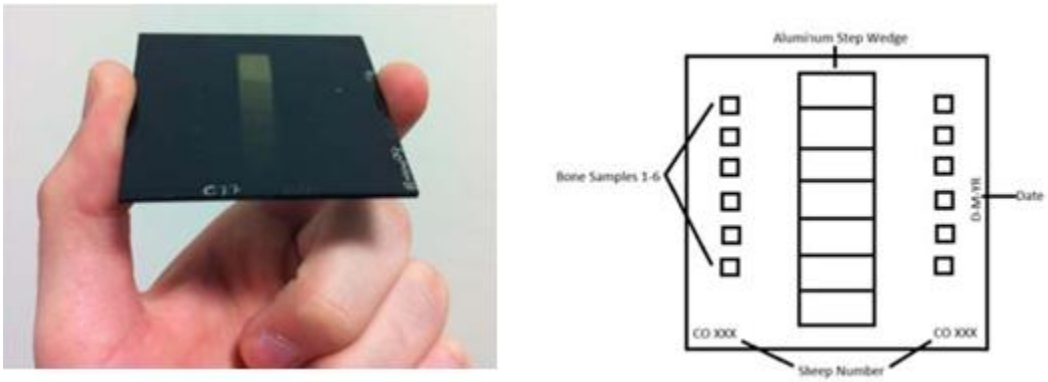

Figure 5: Microradiograph slide example picture and drafted rendition [12].

There are two sheep per Microradiograph identified by CO XXX on the bottom left and right hand corners. The bone sectors run vertically along the sides, starting with section 1 on the top, and going to section 6 on the bottom. Sectors 1, 2, 3, 4, 5, and 6 came from the craniomedial, cranial, craniolateral, caudomedial, caudal, and caudolateral regions of the radius/ulna, respectively. An aluminum step wedge is located in the middle as a reference for different shades. The imaging date and other general information are located vertically on the right hand side.

The sample slide should fit comfortably in the holder with the access holes on the top and bottom of the slide. The sample holder should be aligned against the bottom edge of the stage in the recess, with the spring loaded metal slide holder holding it firmly in place. Next the 40X objective lens should be removed to insure that there is no obstruction with the sample, and push the "Light Path Selector Knob" in completely so that all the light is transmitted to the eyepiece. We can then move and identify the bone on the slides by using the joystick, and bring it into focus with the coarse and fine focus adjustments. Once finding the slide being analyzed, rotate the eyepiece so that the Merz grid is aligned and move it to the top left hand corner of the section. On each section we will count the porosity, the number of secondary osteons, the number of cement line interfaces, and the number of points on remodeled bone.

Porosity

The porosity was measured using the 36 point markers on the Merz grid. Porosities were portrayed as any kind of black space on the slide and consisted of Haversian Canals, Volkmann's canals, or remodeling cavities. Any time a point laid on a porosity or dark spot, one count was made and placed into the Microsoft Excel spreadsheet. Figure 6 shows the point markers that are counted for the given porosity.

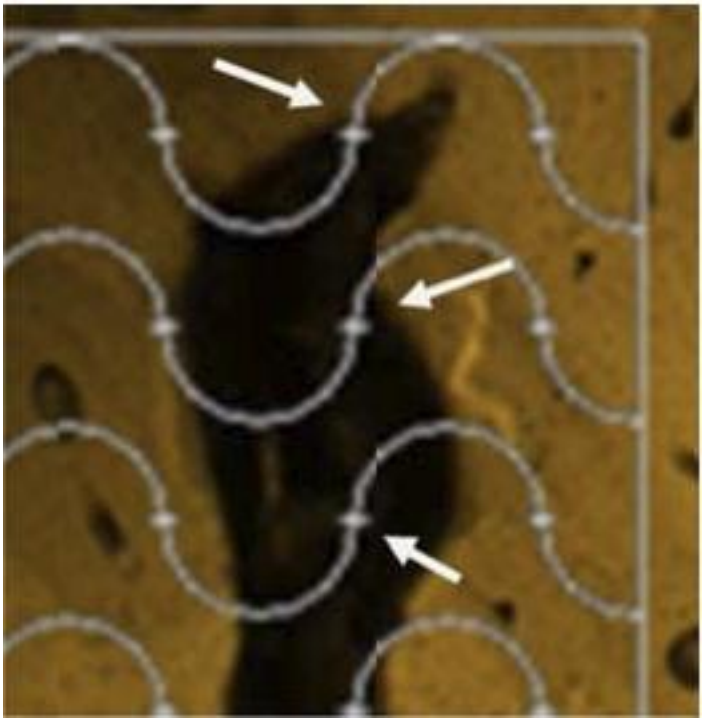

Figure 6: Point markers that were counted for the depicted porosity [12].

Secondary Osteons

The secondary osteons were measured using the sinusoidal lines as guides and each one inside the Merz grid was counted. A secondary osteon can be identified by its circular defined cement lines surrounding a Haversian canal. If an osteon lied on the top or right borders of the grid, they were counted. If an osteon laid on the left and bottom borders of the grid, they were NOT counted. In Figure 7 below, a microradiograph is shown at 40x magnification to show the osteons lying on the borders of the grid.

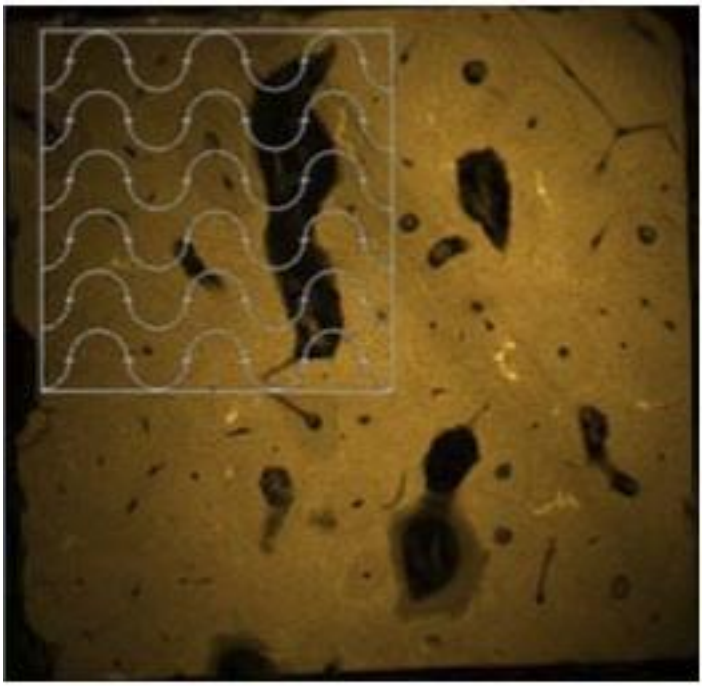

Figure 7: Microradiograph shown at 40x magnification to show the osteons lying on the borders of the grid [12].

Cement Line Interfaces

Cement line interfaces are the dark lines that surround an osteon and were counted using the six sinusoidal lines. Any time a line crossed a cement line, whether it was entering or leaving a secondary osteon, it counted as a cement line interface and was entered into Excel. This is depicted in Figure 8 below.

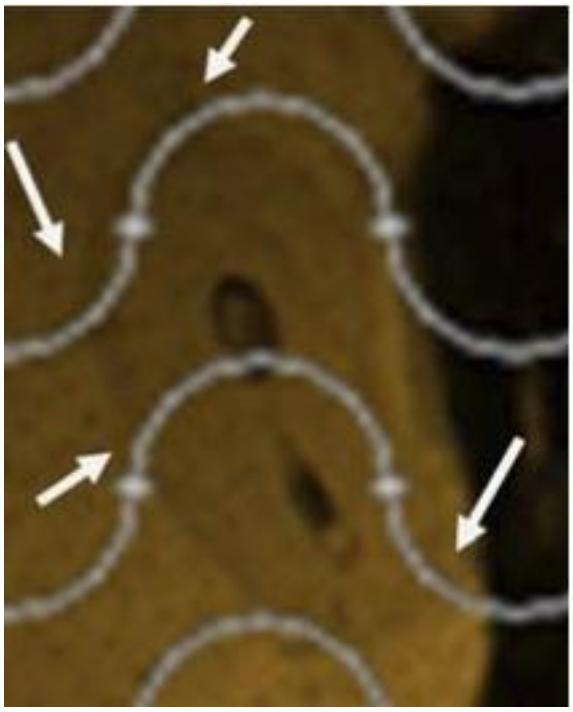

Figure 8: Microradiograph showing the cement line interface counts of an osteon using the Merz grid [12].

Points on Remodeled Bone

Using the 36 point markers, the total points on remodeled bone were counted. Any time a point marker lied on remodeled tissue (i.e. a secondary osteon) whole or partial, it was counted and put into Excel. This is depicted in Figure 9 below.

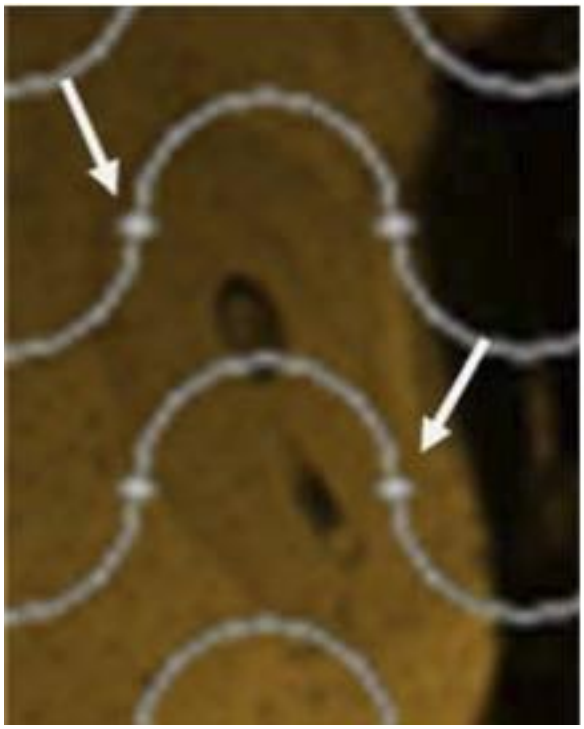

Figure 9: Microradiograph showing the points on remodeled bone using the Merz grid [12].

These measurements were repeated in each corner of the slide moving clockwise around the section. To minimize the overlap of measurements, the grid was placed in the corners of the sample. For the three-month trials that were analyzed, there were fourteen slides in total, seven from sheep that had their ovaries removed (OVX) and seven that did not (sham/control).

Data from each section above was then organized into an Excel spreadsheet where averages and standard deviations of each section were calculated. In Excel, formulas were used to compute and transpose the following information: the percent of remodeled tissue, the percent of remodeled material, the average osteon radius, the number of cement line interfaces in the tissue, the number of cement line

interfaces in the material, and the secondary bone volume to tissue volume ratio (BV/TV). These formulas may be found in the Appendix.

As stated earlier, ewes were sacrificed at three months following the operation and at twelve months following the operations. Ryan was responsible for gathering data from a three-month group sacrificed on an unspecified date in fall; and Kathleen was responsible for gathering data from a three-month group sacrificed on an unspecified date in winter. There was also a summer three-month section, a spring three-month section, a fall twelve-month section, a winter twelve-month section, a summer twelve-month section and a spring twelve-month section; but these samples were not included in this study.

In analyzing the data, 3-way ANOVAs were performed using a general linear model with a 95% confidence interval. The bone remodeling response variables were BV/TV, average % remodeled tissue, average % remodeled material, average secondary osteonal radius, average CLI (tissue), and average CLI (material). These variables were compared against a 3-way interaction between sheep type (sham or OVX), season, and sector. After determining which factors caused a significant difference in means, both Tukey and Fisher tests were done to determine which specific groups within each factor caused the significant difference.

Results

All data for both the three-month fall and the three-month winter was collected and organized in a Microsoft Excel spreadsheet and is shown in the Appendix. In Table 1 below, the resulting p-values for the 3-way ANOVA tests performed are shown. A 95% confidence interval was used for all tests. P-values less than 0.05 suggest the presence of an interaction between the response variable and factor(s), and are highlighted in green. P-values that are *not* significantly different but are *close* are highlighted in yellow. The reason that some of these are not significantly different *may* be due to the fact that the sample size was not large enough, however it is impossible to tell with the present data.

Table 1: P-Values for 3-Way ANOVA Tests on Sheep, Season, and Sector

3-way ANOVA p-values						
Response Variable	Sheep Sham/OVX	Season	Sector	Sheep & Season	Sheep & Sector	Season & Sector
BV/TV	P = 0.486	P = 0.184	P = 0.792	P = 0.647	P = 0.378	P = 0.467
% Remodeled Tissue	P = 0.031	P = 0.498	P = 0.000	P = 0.535	P = 0.768	P = 0.112
% Remodeled Material	P = 0.030	P = 0.502	P = 0.000	P = 0.503	P = 0.763	P = 0.124
Average Osteonal Radius	P = 0.655	P = 0.000	P = 0.343	P = 0.120	P = 0.162	P = 0.993
Average CLI (Tissue)	P = 0.107	P = 0.039	P = 0.000	P = 0.849	P = 0.841	P = 0.024
Average CLI (Material)	P = 0.102	P = 0.039	P = 0.000	P = 0.897	P = 0.835	P = 0.031

There was no significant difference between anatomical regions, sham and OVX, and seasons in BV/TV. Fall sheep showed to have a significantly smaller average osteonal radius than winter sheep. Seasonality and anatomical differences between sectors were seen to have significant effects on average cement line interface in both tissue and material. An interaction between season and sector was also seen in these two response variables.

Post-hoc analysis showed the difference in means amongst sham and OVX, revealing that OVX had a significantly higher average % remodeled tissue and average % remodeled material than sham. Figure 10 and Figure 11 show main effects plots of sheep, season, and sector for these response variables. Tukey tests showed that sector 5 was significantly different than sectors 1, 3, and 4, causing an interaction with average % remodeled tissue and material. Note that sectors 1, 2, 3, 4, 5, and 6 represent the craniomedial, cranial, craniolateral, caudomedial, caudal , and caudolateral sections of bone, respectively. In Figure 12, the main effects plot for BV/TV is shown for comparison.

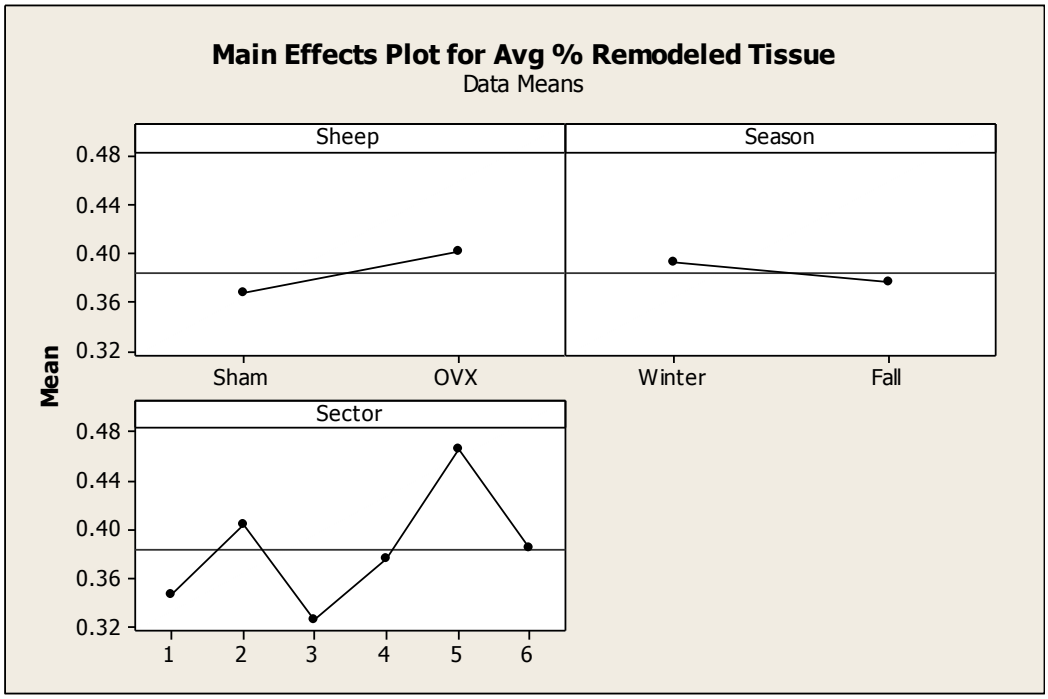

Figure 10: Main Effects Plot for Average % Remodeled Tissue.

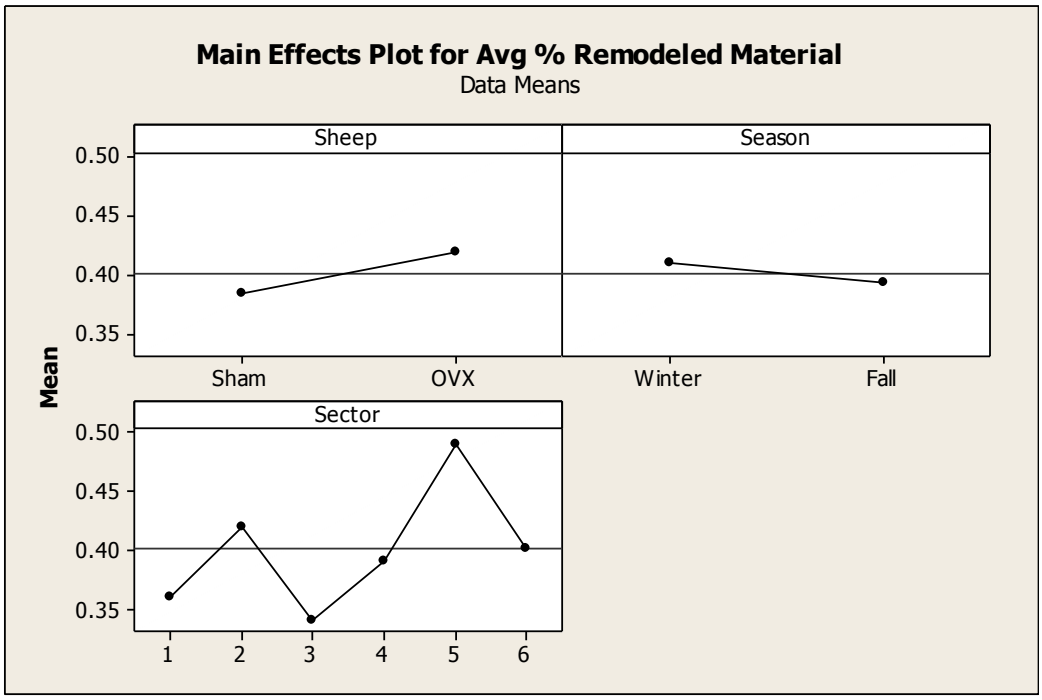

Figure 11: Main Effects Plot for Average % Remodeled Material.

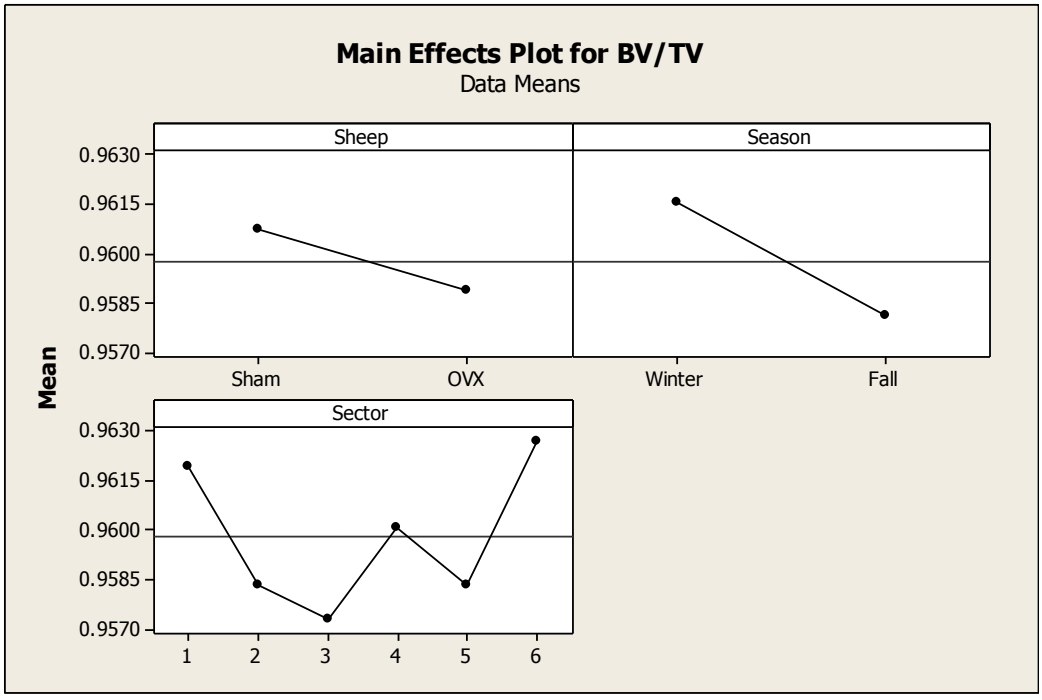

Figure 12: Main Effects Plot for BV/TV.

A summary of the average remodeling response variables and the standard deviations for each are given in Table 2 for both seasons and sheep type (sham/OVX). The averages for each sector may be found in the Appendix.

Table 2: Summary of Seasonal Effects on Bone Remodeling

Response Variable		Winter 3 Month		Fall 3 Month	
		Control	OVX	Control	OVX
Average BV/TV	AVG	0.9557	0.9560	0.9355	0.9578
	STDEV	0.0373	0.0239	0.1487	0.0176
Average % Remodeled Tissue	AVG	0.3722	0.4120	0.3545	0.3891
	STDEV	0.1501	0.1275	0.1159	0.1156
Average % Remodeled Material	AVG	0.3883	0.4307	0.3710	0.4061
	STDEV	0.1542	0.1336	0.1227	0.1198
Average Osteonal Radius (um)	AVG	67.502	66.067	59.018	62.964
	STDEV	9.183	7.415	12.566	7.950
Average CLI Tissue	AVG	40.11	42.76	35.21	39.25
	STDEV	15.37	14.55	11.96	11.57
Average CLI Material	AVG	41.854	44.773	36.866	41.004
	STDEV	15.73	15.402	12.648	12.096

Discussion

It is critical to understand the risk factors associated with developing osteoporosis so that we can diagnose and treat it before fractures occur. Some of the many factors that can increase the risk of fracture in older patients include: lack of exercise, smoking, alcohol intake, and age. However, a history of fractures before the age of 50 can increase the risk of fracture in elderly women [13]. It is critical to develop methods to diagnose and quantify fracture risk in these patients before fractures occur.

With sufficient bone loss analyses, potential models can be evaluated to determine their resemblance to human osteoporosis and osteopenia. By removing the ovaries (ovariectomizing) of animals or humans, osteoporosis can be induced. This is caused by a rapid increase in bone turnover resulting in increased osteoclast bone resorption and no increase in osteoblast bone deposition that has been shown in multiple animal models [14]. In this study, statistically significant changes were seen in several biomechanical properties of the ovariectomized ovine when compared to the sham. Specifically, a significant difference was seen in the percent remodeled tissue and in the percent remodeled material when comparing the OVX group to the control, with OVX having higher means than the control. This suggests that more bone remodeling occurred in OVX than in sham. This is what we expected to see, since estrogen depletion causes there to be more bone remodeling. As previously mentioned, estrogen inhibits osteoclasts to maintain a balance between bone resorption and formation; thus, a lack of estrogen causes osteoclast resorption to outpace osteoblast deposition. The caudal section (sector 5) had considerably higher percentages of average remodeled tissue and material than the craniomedial, craniolateral, and caudomedial sections of the radius/ulna cross-sections. Since higher levels of bone remodeling occur in areas of high compressive or tensile stress [10], the increased remodeling in the caudal section suggests that this region experiences relatively higher loads than the craniomedial and craniolateral sections.

Although there were no significant differences amongst sheep, season, and sector in BV/TV, the p-value for season ($p = 0.184$) seemed low enough to be taken into consideration. Fall showed a lower

average BV/TV than winter. Since the bone volume over the total volume is lower, it suggests that the fall sheep have more porosities than winter sheep. This result was not expected, since an earlier study has shown winter sheep to increase porosity when compared to fall sheep [10]. In the earlier study, however, 12-month sheep were used as opposed to the 3-month sheep used in this study. A shorter post-operation time span results in a shorter bone remodeling period. Therefore, variations between sheep samples from different post-operation time spans are likely.

In addition to having a lower average BV/TV, fall sheep showed to have a significantly smaller average osteonal radius than winter sheep. A smaller average osteonal radius and increased porosity reveal that fall sheep underwent less bone remodeling than winter sheep. This is consistent with the results shown in Figure 10 and Figure 11. Significant seasonality and anatomical differences were seen in average cement line interface (CLI) in both tissue and material, as well as an interaction effect between season and sector. Fall sheep had a significantly smaller average CLI in both tissue and material when compared to winter sheep. Similar to the results for average % remodeled tissue and material, the caudal section had a significantly higher average CLI in tissue and material than the craniomedial and craniolateral sections. A higher average CLI corresponds to a higher amount of remodeling, since the CLI is the interface between secondary osteons and interstitial primary bone.

While seasonality and anatomical differences were observed across the bone remodeling response variables, the important differences are attributed to those between OVX and sham. The higher average % remodeled tissue and material observed in OVX when compared to sham supports the hypothesis that sheep may be used to model postmenopausal osteoporosis. In this study, it is assumed that the OVX sheep lacked the necessary levels of estrogen needed to maintain a balance between bone resorption and bone deposition, which is similar to the case in women after experiencing menopause. More research is necessary to verify this hypothesis and the results of this study.

There were several limitations to the study and aspects that could be improved upon. One major limitation to the study is that the measurements are somewhat open for interpretation. Although there are specific guidelines to follow there are some micrographs that are difficult to see, some points are unclear

as to whether they should be counted, and some micrographs that were missing altogether. This would be a difficult problem to fix; but if more detailed guidelines to counting points or even and automated program were created, the consistency of the measurement would improve. Another limitation to the study is that the microradiographs are essentially two dimensional pictures, and are representing a three dimensional structure. Essentially, the bone microstructure can change drastically in only a few millimeters and the bovine samples taken may not adequately represent the density of the rest of the bone. There were also some minor exposure issues during density measurements. More dense samples were washed out under the higher light intensities needed to visualize the less dense sectors. This can be fixed by capturing a new step wedge key for each section at the appropriate light intensity.

In previous studies, spongy bone loss was found in the proximal tibial metaphysic of ovariectomized rats during the first several months post-ovariectomy. Similar bone loss has been seen in human women in early stages of estrogen deficiency and ovariectomized monkeys [14]. Monkeys have very similar cortical bone remodeling compared to human cortical bone remodeling so this interaction is critical [15]. However, due to their difficult housing conditions and lengthy skeletal maturity time they provide an uneconomical model for large scale testing protocols. Regardless, further study of ovariectomized monkeys would greatly aid the knowledge of how human bone behaves in estrogen deficient women.

Smaller animals like rodents and rabbits are also a great first step for testing drug interactions and small scale replications of postmenopausal osteoporosis. However, these models are missing critical characteristics of human bone tissue, most obviously including size and shape, and do not provide a large enough model to test full scale device interactions. To develop a robust human model that is still economically reasonable is critical in the development of treatment protocols for osteoporosis. Since some significant changes were observed, future research needs to take into consideration that treatment times and follow up times must be kept consistent between the treatment groups in order to provide a valid comparison and that samples should be obtained from similar anatomic regions.

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Appendix

Histomorphometry Data

3 Month Winter Control

Average BV/TV

	Sector					
Sheep	1	2	3	4	5	6
C57	0.9722	0.9722	0.9444	0.9514	0.9722	0.9722
C63	0.9583	0.9722	0.9444	0.9861	0.9514	0.9792
C66	0.9792	0.9514	0.9583	0.9514	0.7778	0.9444
C72	0.9375	0.9583	0.9792	0.9583	0.8333	0.9444
C73	0.9653	0.9653	0.9514	0.9792	0.9375	0.9722
C79	0.9583	0.9792	0.9583	0.9722	0.9514	0.9861
C81	0.9722	0.9514	0.9514	0.9653	0.9792	0.9931
Standard Deviation	0.0137	0.0109	0.0119	0.0136	0.0775	0.0191
Average	0.9633	0.9643	0.9554	0.9663	0.9147	0.9702

Average Percent Remodeled (Tissue)

	Sector					
Sheep	1	2	3	4	5	6
C57	0.4028	0.4653	0.4583	0.4167	0.4722	0.3889
C63	0.3819	0.4028	0.2847	0.4653	0.5000	0.2917
C66	0.1181	0.5486	0.1736	0.1806	0.2222	0.1944
C72	0.2431	0.4583	0.2639	0.3264	0.2222	0.3611
C73	0.4097	0.3681	0.1458	0.3611	0.5417	0.4931
C79	0.1250	0.4444	0.2292	0.4931	0.4931	0.2153
C81	0.1597	0.4167	0.5764	0.7708	0.6111	0.5347
Standard Deviation	0.1331	0.0575	0.1568	0.1822	0.1538	0.1303
Average	0.2629	0.4435	0.3046	0.4306	0.4375	0.3542

Average Percent Remodeled (Material)

	Sector					
Sheep	1	2	3	4	5	6
C57	0.4150	0.4788	0.4864	0.4404	0.4894	0.3998
C63	0.3979	0.4137	0.2966	0.4722	0.5243	0.2980
C66	0.1209	0.5765	0.1807	0.1908	0.2846	0.2079
C72	0.2567	0.4813	0.2689	0.3399	0.2606	0.3824
C73	0.4245	0.3798	0.1531	0.3698	0.5802	0.5069
C79	0.1307	0.4519	0.2380	0.5063	0.5176	0.2171
C81	0.1639	0.4377	0.6072	0.7993	0.6245	0.5381
Standard Deviation	0.1380	0.0626	0.1670	0.1877	0.1413	0.1308
Average	0.2728	0.4600	0.3187	0.4455	0.4687	0.3643

3 Month Winter Control (cont.)

Average Osteonal Radius (um)

	Sector					
Sheep	1	2	3	4	5	6
C57	57.854	56.817	61.019	68.144	49.944	57.163
C63	66.057	73.206	65.180	76.254	66.707	77.127
C66	63.845	75.235	78.275	67.784	75.265	66.459
C72	63.867	73.141	65.842	70.011	72.055	74.328
C73	70.804	72.784	64.572	67.165	70.294	77.923
C79	55.449	56.270	63.157	52.474	55.749	48.773
C81	58.647	80.237	73.680	87.666	85.823	72.037
Standard Deviation	5.343	9.324	6.215	10.604	12.041	11.007
Average	62.360	69.670	67.389	69.928	67.977	67.687

Average CLI (Tissue)

	Sector					
Sheep	1	2	3	4	5	6
C57	46.25	51.25	47.00	49.75	72.50	47.75
C63	42.50	45.00	30.75	45.00	59.25	24.00
C66	14.75	57.00	13.00	20.50	21.25	23.00
C72	26.25	45.75	29.25	42.75	24.75	39.50
C73	40.00	42.50	16.00	42.50	54.50	44.00
C79	16.25	53.50	26.00	70.75	63.00	32.75
C81	19.25	40.50	55.25	52.25	47.25	49.50
Standard Deviation	13.34	6.09	15.39	14.94	19.34	10.88
Average	29.32	47.93	31.04	46.21	48.93	37.21

Average CLI (Material)

	Sector					
Sheep	1	2	3	4	5	6
C57	47.692	52.574	49.905	52.647	74.735	49.098
C63	44.342	46.357	32.060	45.664	62.221	24.529
C66	15.034	59.887	13.553	21.645	27.539	24.666
C72	27.920	47.535	29.863	44.645	29.380	41.824
C73	41.463	43.897	16.882	43.487	58.479	45.140
C79	16.896	54.359	27.133	72.750	66.207	33.150
C81	19.800	42.642	58.194	54.082	48.167	49.843
Standard Deviation	13.861	6.232	16.340	15.290	18.206	10.886
Average	30.450	49.607	32.513	47.846	52.390	38.321

3 Month Winter OVX

Average BV/TV

	Sector					
Sheep	1	2	3	4	5	6
C60	0.9306	0.9792	0.9236	0.9722	0.9722	0.9444
C61	0.9653	0.9653	0.9792	0.9722	0.9792	0.9722
C65	0.9653	0.9375	0.9653	0.9236	0.8889	0.9583
C69	0.9792	0.9514	0.9306	0.9722	0.9653	0.9514
C74	0.9514	0.9514	0.9653	0.9375	0.9583	0.8681
C76	0.9792	0.9861	0.9514	0.9514	0.9583	0.9792
C78	0.9653	0.9722	0.9514	0.9792	0.9514	0.9514
Standard Deviation	0.0169	0.0173	0.0198	0.0212	0.0299	0.0367
Average	0.9623	0.9633	0.9524	0.9583	0.9534	0.9464

Average Percent Remodeled (Tissue)

	Sector					
Sheep	1	2	3	4	5	6
C60	0.2292	0.5139	0.2153	0.3542	0.4375	0.3958
C61	0.4583	0.3611	0.2361	0.4375	0.3958	0.2500
C65	0.5000	0.4722	0.3750	0.4097	0.4583	0.2708
C69	0.3611	0.4236	0.2153	0.3056	0.2153	0.2847
C74	0.5069	0.5833	0.3889	0.5069	0.6319	0.4514
C76	0.6389	0.5903	0.3264	0.3889	0.6806	0.4236
C78	0.4167	0.3542	0.4722	0.2778	0.7083	0.3819
Standard Deviation	0.1285	0.0972	0.0999	0.0785	0.1784	0.0810
Average	0.4444	0.4712	0.3185	0.3829	0.5040	0.3512

Average Percent Remodeled (Material)

	Sector					
Sheep	1	2	3	4	5	6
C60	0.2471	0.5254	0.2327	0.3628	0.4500	0.4191
C61	0.4746	0.3729	0.2399	0.4508	0.4019	0.2578
C65	0.5181	0.5038	0.3846	0.4415	0.5072	0.2824
C69	0.3708	0.4489	0.2325	0.3137	0.2224	0.2984
C74	0.5341	0.6120	0.4034	0.5408	0.6597	0.5183
C76	0.6519	0.6001	0.3428	0.4086	0.7105	0.4327
C78	0.4297	0.3623	0.4962	0.2824	0.7450	0.4006
Standard Deviation	0.1291	0.1001	0.1026	0.0884	0.1886	0.0954
Average	0.4609	0.4893	0.3332	0.4001	0.5281	0.3727

3 Month Winter OVX (cont.)

Average Osteonal Radius (um)

	Sector					
Sheep	1	2	3	4	5	6
C60	69.971	62.155	59.413	60.575	57.539	60.286
C61	72.995	67.859	68.486	78.502	68.904	61.321
C65	74.295	75.596	78.681	58.039	63.030	74.740
C69	85.716	66.222	69.357	69.677	51.551	69.863
C74	62.857	68.235	62.296	64.231	62.784	65.690
C76	73.894	65.822	61.053	55.907	61.921	52.385
C78	69.336	69.925	60.024	75.567	61.354	56.743
Standard Deviation	6.942	4.154	7.006	8.748	5.354	7.683
Average	72.723	67.974	65.616	66.071	61.012	63.004

Average CLI (Tissue)

	Sector					
Sheep	1	2	3	4	5	6
C60	20.50	37.25	22.50	39.00	51.50	43.00
C61	38.00	34.75	20.00	41.50	42.25	25.25
C65	45.00	45.00	35.00	43.75	50.25	19.50
C69	29.75	47.00	19.00	26.50	25.75	29.25
C74	62.25	68.00	48.50	60.75	66.25	47.75
C76	58.00	56.25	38.75	53.75	68.25	50.00
C78	40.25	38.75	54.25	26.75	71.75	54.25
Standard Deviation	14.76	11.85	14.13	12.74	16.45	13.59
Average	41.96	46.71	34.00	41.71	53.71	38.43

Average CLI (Material)

	Sector					
Sheep	1	2	3	4	5	6
C60	22.043	38.096	24.185	40.087	53.009	45.529
C61	39.381	35.897	20.371	42.805	43.110	26.074
C65	46.729	48.227	35.822	47.280	56.194	20.390
C69	30.528	49.637	20.548	27.074	26.549	30.813
C74	65.580	71.308	50.218	64.858	69.134	54.943
C76	59.289	57.059	40.906	56.496	71.266	51.114
C78	41.610	39.708	56.987	27.143	75.456	57.020
Standard Deviation	15.256	12.511	14.652	14.075	17.443	14.861
Average	43.594	48.562	35.577	43.677	56.388	40.840

3 Month Fall Control

Average BV/TV

	Sector					
Sheep	1	2	3	4	5	6
C43	0.9653	0.9444	0.9653	0.9861	0.9444	0.9653
C45	0.9653	0.9306	0.9722	0.9583	0.9583	0.9583
C49	0.9722	0.9514	0.9722	0.9514	0.9444	0.9722
C51	0.9653	0.9653	0.9514	0.9514	0.9375	0.9653
C52	0.9583	0.9444	0.9722	0.9653	0.9375	0.9653
C53	0.0000	0.9375	0.9653	0.9444	0.9931	0.9236
C56	0.9722	0.9514	0.9931	0.9444	0.9583	0.9514
Standard Deviation	0.3653	0.0111	0.0125	0.0147	0.0195	0.0163
Average	0.8284	0.9464	0.9702	0.9573	0.9534	0.9573

Average Percent Remodeled (Tissue)

	Sector					
Sheep	1	2	3	4	5	6
C43	0.3681	0.3611	0.2222	0.2083	0.3958	0.4097
C45	0.2847	0.2153	0.3333	0.1944	0.3333	0.5208
C49	0.3611	0.3958	0.3542	0.2917	0.4514	0.3264
C51	0.3681	0.4097	0.2986	0.4306	0.5208	0.5208
C52	0.2917	0.3125	0.4097	0.2847	0.4861	0.2153
C53	0.0000	0.3403	0.2361	0.6667	0.5000	0.4722
C56	0.3611	0.3958	0.2708	0.2917	0.4514	0.3264
Standard Deviation	0.1332	0.0677	0.0671	0.1639	0.0650	0.1151
Average	0.2907	0.3472	0.3036	0.3383	0.4484	0.3988

Average Percent Remodeled (Material)

	Sector					
Sheep	1	2	3	4	5	6
C43	0.3824	0.3823	0.2332	0.2104	0.4190	0.4258
C45	0.3004	0.2289	0.3436	0.2019	0.3521	0.5439
C49	0.3725	0.4174	0.3694	0.3078	0.4766	0.3369
C51	0.3808	0.4237	0.3141	0.4496	0.5553	0.5391
C52	0.3059	0.3274	0.4264	0.2943	0.5163	0.2246
C53	0.0000	0.3605	0.2430	0.7059	0.5036	0.5183
C56	0.3725	0.4174	0.2728	0.3094	0.4697	0.3456
Standard Deviation	0.1377	0.0697	0.0705	0.1753	0.0672	0.1224
Average	0.3021	0.3654	0.3146	0.3542	0.4704	0.4192

3 Month Fall Control (cont.)

Average Osteonal Radius (um)

	Sector					
Sheep	1	2	3	4	5	6
C43	70.360	58.670	50.995	57.807	56.924	51.618
C45	49.905	47.650	62.027	50.269	58.957	63.024
C49	57.441	57.005	56.557	56.716	60.022	53.554
C51	59.738	60.522	66.034	59.594	56.451	59.152
C52	64.659	70.181	80.975	75.525	71.871	72.008
C53	0.000	81.041	49.696	79.388	57.615	56.974
C56	57.441	57.005	53.093	56.716	60.022	53.554
Standard Deviation	23.530	10.786	11.009	10.813	5.311	7.094
Average	51.363	61.725	59.911	62.288	60.266	58.555

Average CLI (Tissue)

	Sector					
Sheep	1	2	3	4	5	6
C43	27.00	39.25	26.00	19.75	46.75	51.75
C45	32.25	18.50	31.00	20.25	45.50	48.00
C49	35.50	40.50	32.75	35.50	49.50	40.50
C51	37.25	36.50	29.00	43.25	44.00	51.50
C52	25.50	23.75	25.25	19.50	43.75	19.00
C53	0.00	27.25	26.00	46.00	52.25	58.25
C56	35.50	40.50	29.00	35.50	49.50	40.50
Standard Deviation	12.95	9.03	2.82	11.47	3.19	12.81
Average	27.57	32.32	28.43	31.39	47.32	44.21

Average CLI (Material)

	Sector					
Sheep	1	2	3	4	5	6
C43	28.044	41.602	27.187	19.985	49.678	53.792
C45	34.011	19.502	31.892	20.934	47.824	50.150
C49	36.453	42.686	34.001	37.451	52.270	41.783
C51	38.575	37.900	30.585	45.624	46.941	53.382
C52	26.724	24.811	26.203	21.055	46.633	20.012
C53	0.000	28.972	26.732	48.706	52.621	63.320
C56	36.453	42.686	29.229	37.680	51.520	42.772
Standard Deviation	13.379	9.520	2.917	12.284	2.549	13.751
Average	28.609	34.023	29.404	33.062	49.641	46.459

3 Month Fall OVX

Average BV/TV

	Sector					
Sheep	1	2	3	4	5	6
C31	0.9583	0.9583	0.9583	0.9653	1.0000	0.9653
C32	0.9653	0.9444	0.9861	0.9653	0.9375	0.9653
C34	0.9375	0.9792	0.9444	0.9653	0.9167	0.9653
C35	0.9236	0.9514	0.9375	0.9653	0.9583	0.9722
C36	0.9861	0.9514	0.9514	0.9722	0.9583	0.9514
C37	0.9722	0.9514	0.9444	0.9167	0.9653	0.9653
C38	0.9514	0.9792	0.9375	0.9583	0.9722	0.9583
Standard Deviation	0.0211	0.0141	0.0170	0.0188	0.0263	0.0066
Average	0.9563	0.9593	0.9514	0.9583	0.9583	0.9633

Average Percent Remodeled (Tissue)

	Sector					
Sheep	1	2	3	4	5	6
C31	0.3819	0.4236	0.4861	0.5625	0.4931	0.5208
C32	0.1667	0.3125	0.3819	0.2847	0.4861	0.2431
C34	0.3958	0.2014	0.2431	0.4167	0.3819	0.4861
C35	0.4444	0.4375	0.3403	0.4236	0.6597	0.4306
C36	0.3333	0.3333	0.4653	0.2431	0.4306	0.5764
C37	0.2569	0.2292	0.3542	0.2639	0.4792	0.2153
C38	0.4028	0.5347	0.3750	0.2847	0.3958	0.5625
Standard Deviation	0.0972	0.1194	0.0812	0.1171	0.0926	0.1480
Average	0.3403	0.3532	0.3780	0.3542	0.4752	0.4335

Average Percent Remodeled (Material)

	Sector					
Sheep	1	2	3	4	5	6
C31	0.3989	0.4423	0.5087	0.5793	0.4931	0.5393
C32	0.1731	0.3274	0.3883	0.2943	0.5163	0.2523
C34	0.4201	0.2071	0.2530	0.4307	0.4173	0.5027
C35	0.4811	0.4572	0.3693	0.4410	0.6881	0.4408
C36	0.3383	0.3514	0.4889	0.2503	0.4501	0.6022
C37	0.2648	0.2421	0.3762	0.2879	0.4986	0.2231
C38	0.4237	0.5460	0.4008	0.2968	0.4069	0.5871
Standard Deviation	0.1066	0.1217	0.0846	0.1188	0.0945	0.1546
Average	0.3572	0.3677	0.3979	0.3686	0.4957	0.4496

3 Month Fall OVX (cont.)

Average Osteonal Radius (um)

	Sector					
Sheep	1	2	3	4	5	6
C31	64.761	65.441	65.603	64.602	62.917	60.118
C32	59.874	70.181	78.739	75.525	71.871	54.333
C34	63.588	62.009	62.620	69.596	56.632	65.377
C35	65.262	59.990	54.555	64.821	55.319	52.608
C36	85.402	71.006	67.991	69.164	53.377	66.710
C37	48.053	50.867	61.030	48.185	51.835	57.192
C38	63.160	65.570	69.851	64.852	56.884	67.018
Standard Deviation	11.044	6.869	7.615	8.483	6.894	6.003
Average	64.300	63.581	65.770	65.249	58.405	60.479

Average CLI (Tissue)

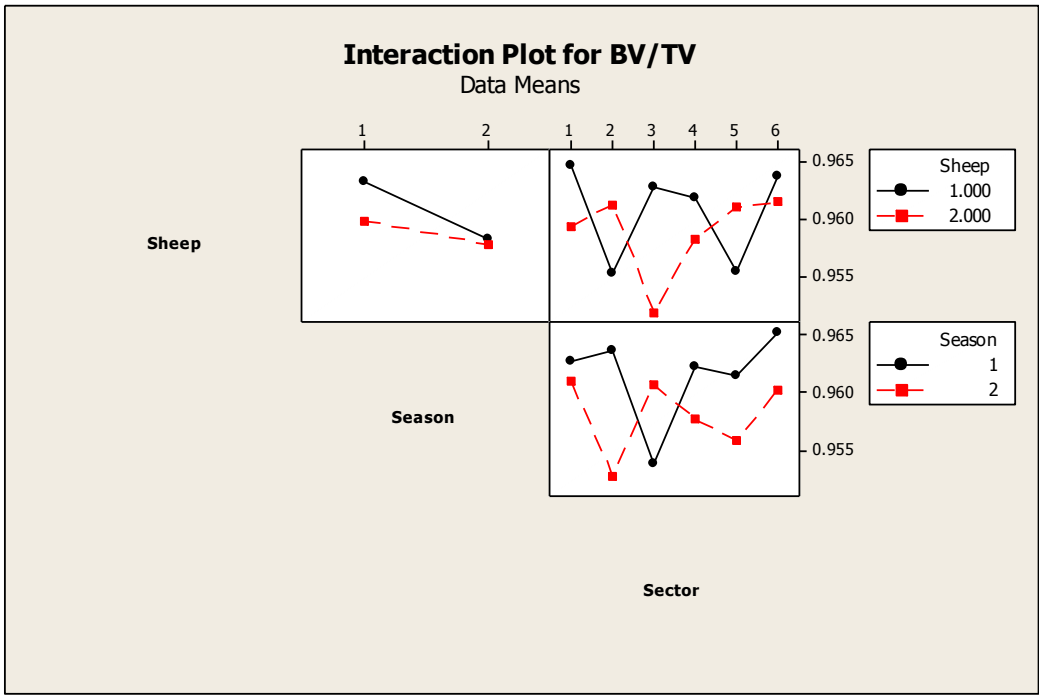
	Sector					
Sheep	1	2	3	4	5	6
C31	34.75	33.25	44.25	54.75	45.25	53.25
C32	24.75	27.25	26.75	24.25	43.75	38.75
C34	32.25	22.75	26.50	33.00	41.25	46.50
C35	48.75	46.50	32.00	47.75	70.25	50.50
C36	31.75	30.50	45.75	20.00	46.25	57.75
C37	21.25	28.00	39.00	38.25	54.75	22.50
C38	42.75	53.50	44.00	38.25	36.50	48.75
Standard Deviation	9.58	11.22	8.39	12.25	11.15	11.70
Average	33.75	34.54	36.89	36.61	48.29	45.43

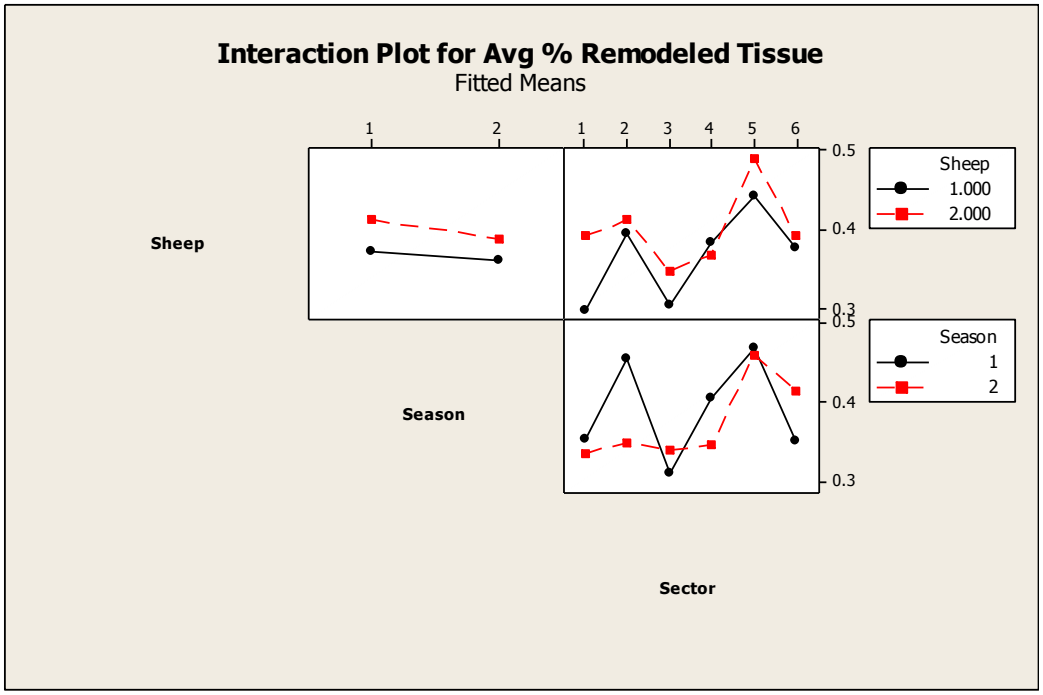
Average CLI (Material)

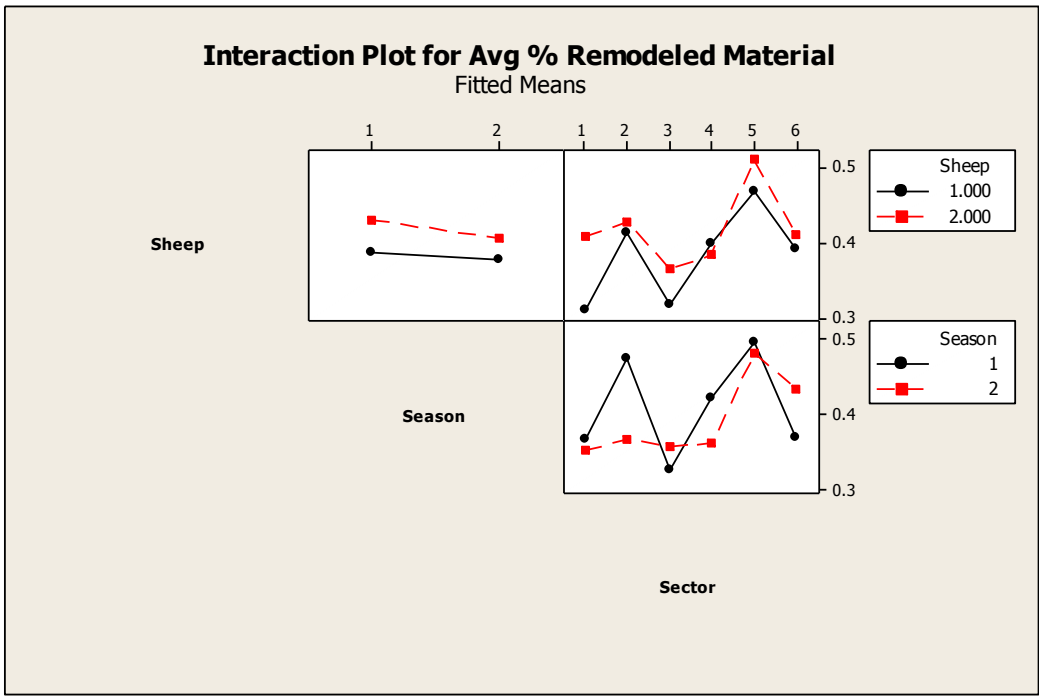
	Sector					
Sheep	1	2	3	4	5	6
C31	36.272	35.176	46.356	56.678	45.250	55.098
C32	25.613	28.749	27.157	25.192	46.633	40.031
C34	34.399	23.320	27.671	34.176	45.142	48.396
C35	52.723	48.838	34.662	49.673	73.332	51.838
C36	32.243	32.159	48.082	20.645	48.273	60.822
C37	21.867	29.522	41.451	42.099	57.164	23.339
C38	44.939	54.657	44.168	39.919	37.541	50.890
Standard Deviation	10.660	11.428	8.708	12.810	11.618	12.283
Average	35.437	36.060	38.507	38.340	50.476	47.202

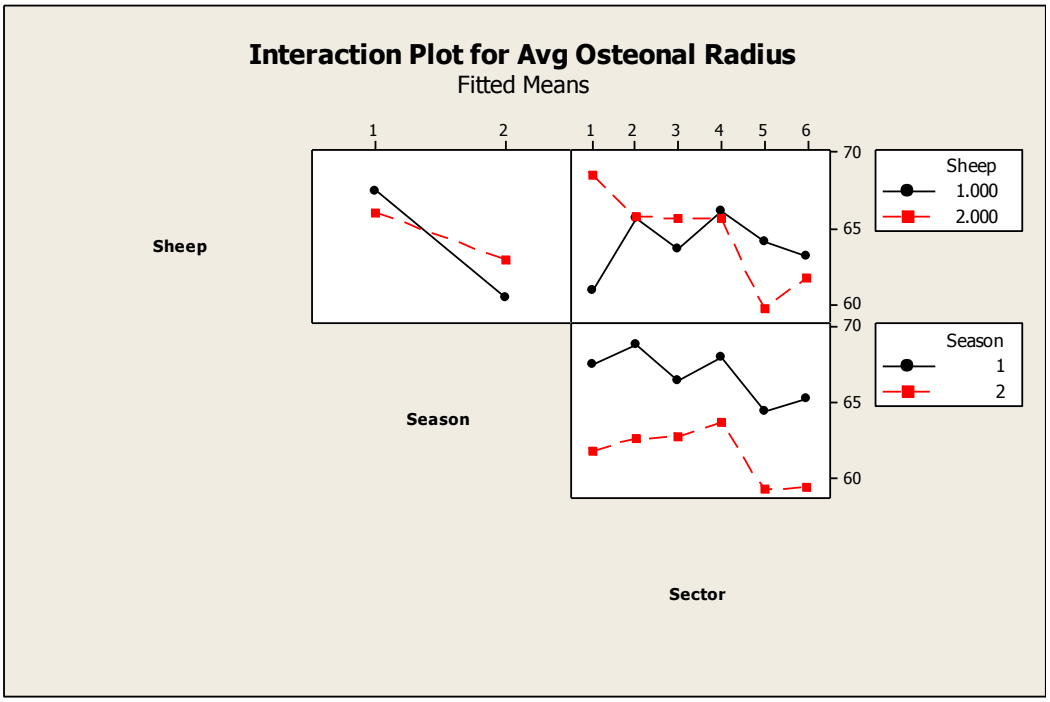
Interaction Plots and Main Effects Plots Not Included in Results

Key:
Sheep 1.000 = Sham Sheep 2.000 = OVX
Season 1 = Winter Season 2 = Fall






Interaction Plot for Avg % Remodeled Material
Fitted Means
Sheep
Season
Sector
1
2
1
2
3
4
5
6
0.5
0.4
0.3
Sheep
1.000
2.000
Season
1
2


Interaction Plot for Avg Osteonal Radius
Fitted Means
Sheep
Season
Sector
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2
1
2
3
4
5
6
70
65
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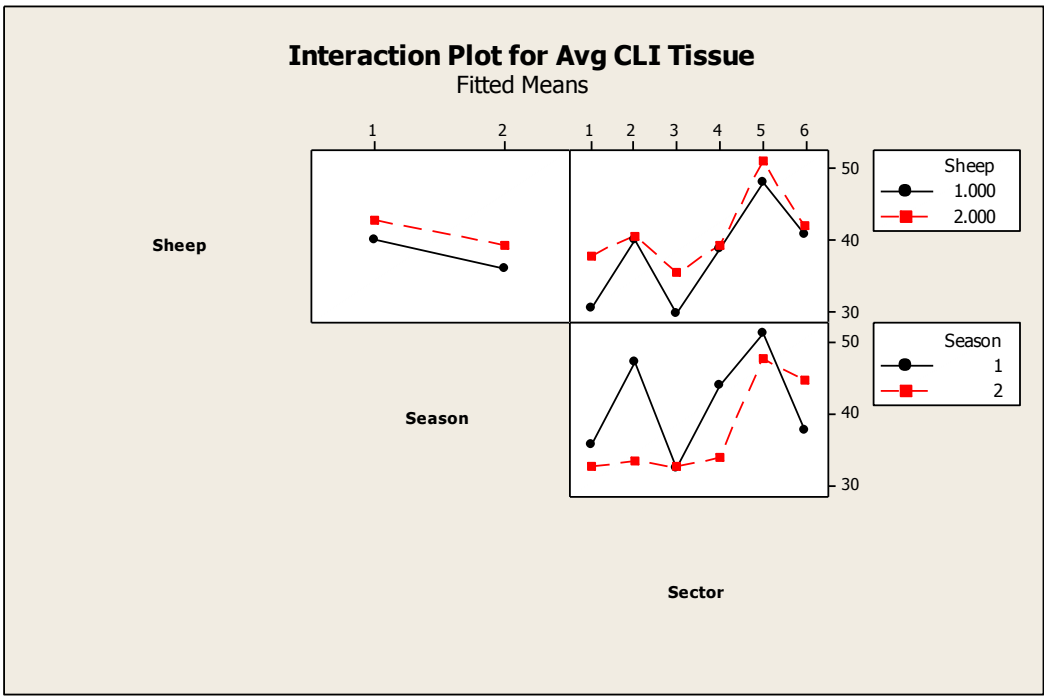
Sheep
1.000
2.000
Season
1
2


Interaction Plot for Avg CLI Tissue
Fitted Means
Sheep
Season
Sector
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2
1
2
3
4
5
6
50
40
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Sheep
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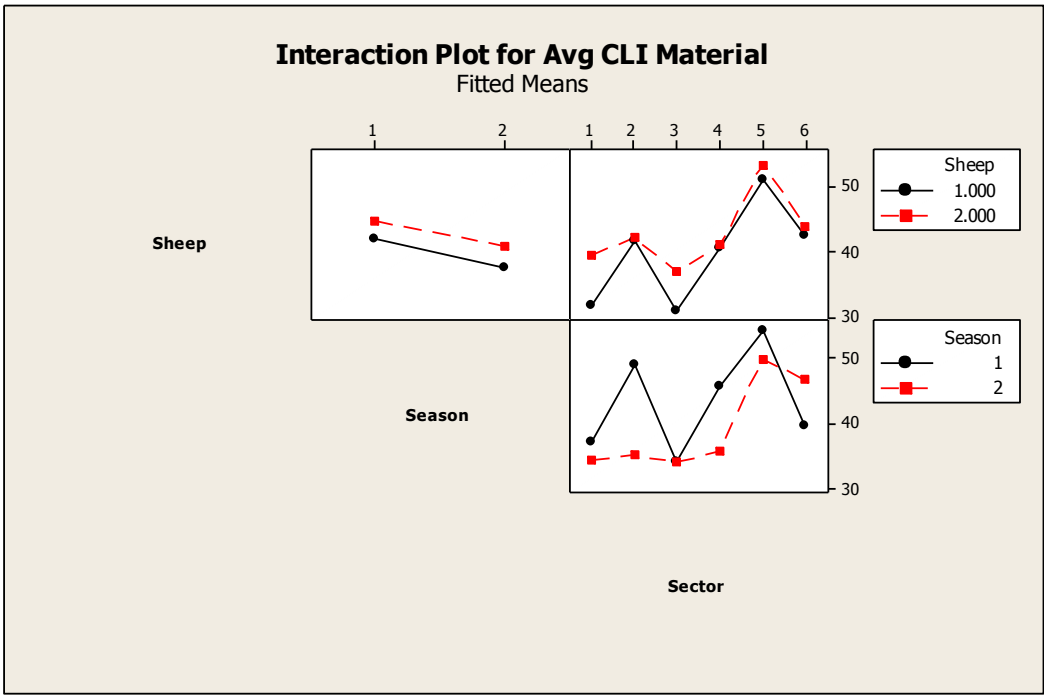
2.000
Season
1
2


Interaction Plot for Avg CLI Material
Fitted Means
Sheep
Season
Sector
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1
2
3
4
5
6
50
40
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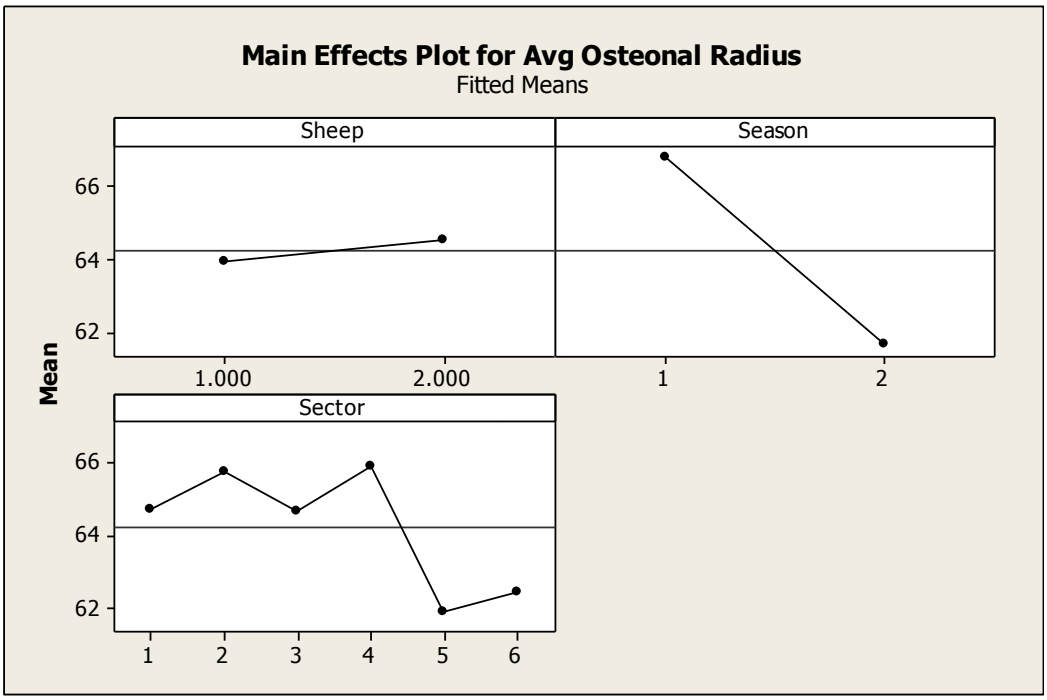
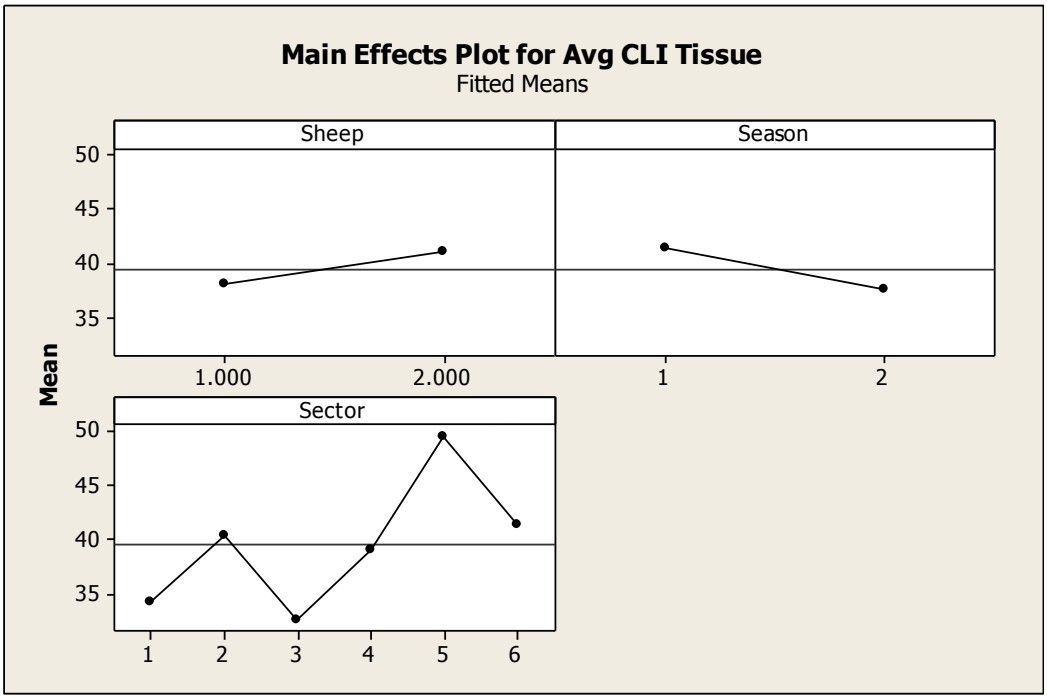
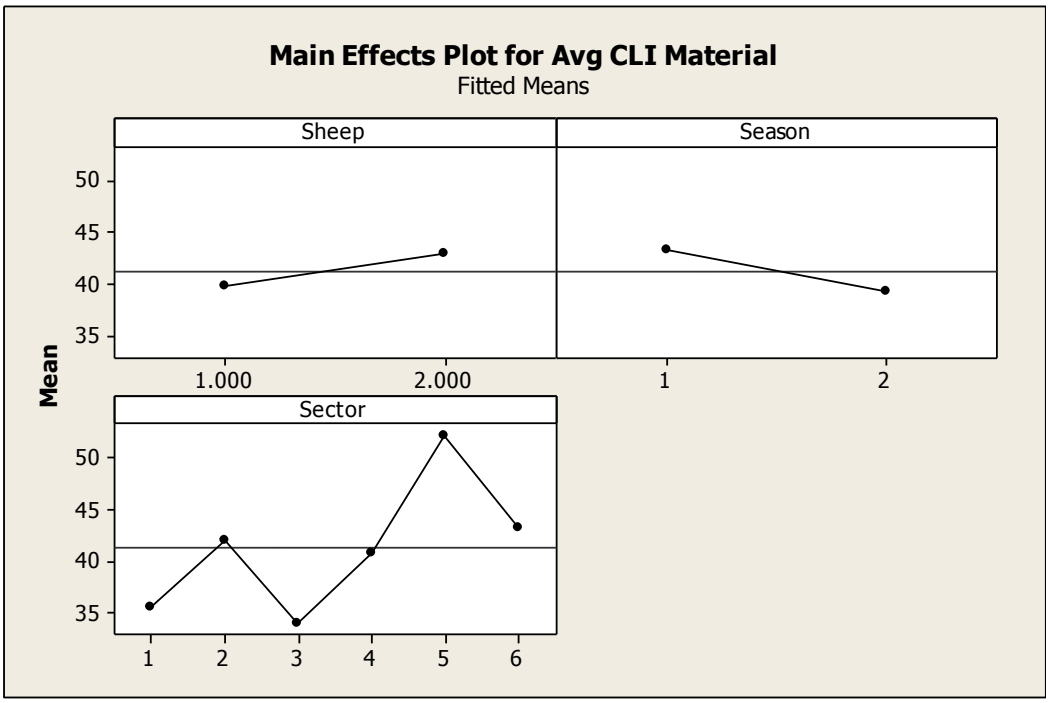
2.000
Season
1
2


Main Effects Plot for Avg Osteonal Radius
Fitted Means
Sheep
Season
Sector
Mean
66
64
62
1.000
2.000
1
2
1
2
3
4
5
6


Main Effects Plot for Avg CLI Tissue
Fitted Means
Sheep
Season
Sector
Mean
50
45
40
35
1.000
2.000
1
2
1
2
3
4
5
6


Main Effects Plot for Avg CLI Material
Fitted Means
Sheep
Season
Sector
Mean
50
45
40
35
1.000
2.000
1
2
1
2
3
4
5
6