

## Structural adaptation and intracortical bone turnover in an ovine model of osteoporosis.

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## Structural Adaptation and Intracortical Bone Turnover in an Ovine Model of Osteoporosis

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3 Title4  
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## 43 Running Title

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45 Compact bone and estrogen deficiency  
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## Abstract

Compact bone makes up approximately 80% of the human skeletal mass, however there is a relative paucity of studies examining the effects of estrogen deficiency in compact bone. This study examines the effect of estrogen deficiency on compact bone turnover and associated geometrical structural adaptation over a 31 month period.

Twenty –seven skeletally mature sheep were divided into control (n=16) and ovariectomy group (OVX, n=11). Animals were administered five different flouochrome dyes to label intracortical bone turnover and sacrificed at 31 months. Compact bone samples were analysed for cortical geometry, intracortical turnover at five time points, resorption cavities, porosity and compressive strength.

Intracortical bone turnover was significantly increased in OVX, which demonstrated seasonal variation. Cross sectional area in OVX was significantly with an associated increased resistance to bending. Intracortical porosity was significantly increased in OVX, however there was no significant difference in ultimate compressive strength between the groups.

Our results demonstrate sustained increased intracortical bone turnover, resorption spaces and porosity without adversely affecting compressive strength. Our results also support the hypothesis of geometrical adaptation of compact bone in response to estrogen deficiency. These results suggest an early structural compensatory response in compact bone despite increased intracortical turnover.

## Key Words

Compact bone, estrogen, ovine, bone turnover, bone geometry

## Introduction

Bone strength depends not only on material properties but also on structural properties.

The structural properties of bone depend on size and shape as well as its microarchitecture [1]. Adding mass away from the centre of the bone, periosteal apposition, can dramatically affect a bone's ability to resist bending and torsion. It has been estimated that an increase of 10% in periosteal diameter would lead to a 50% increase in axial compressive strength and a 70% increase in bending strength (second moment of inertia) [2]. These parameters are important considerations in the pathophysiology of skeletal diseases such as osteoporosis and fracture.

In this study, we used the OVX sheep model to study the effects of estrogen deficiency on compact bone over a 31 month period. The sheep has been used previously as an animal model for studying different aspects of osteoporosis [3-5]. Similarities between the hormone profiles of ewes and women, a comparable metabolic rate and commensurate bone remodelling cycles support the use of the Ovine model of osteoporosis [6-8]. We have previously demonstrated an increase compact bone turnover and porosity in an Ovine model of osteoporosis during the initial 12-months post ovariectomy without adversely affecting biomechanical properties [5]. Conversely, we have also demonstrated a significant reduction in the biomechanical properties of trabecular bone associated with increased turnover[9]. Here we examine the effect of estrogen deficiency on compact bone geometry, intracortical remodelling, porosity and biomechanical parameters in an ovine model of osteoporosis over a 31 month period..

## Materials and Methods

Twenty –seven skeletally mature mixed breed ewes were randomly divided into an ovariectomy group (OVX; n=11) and a control group (n=16). Animals in the OVX group had ovariectomy at month=0 under Irish Government animal license number B100/2443. Subsequent veterinary assessment of the animals showed no adverse effects of surgery.. Housing, feeding, and activity levels were the same for both groups at all times. Both animal groups received five different intravenous fluorochrome dyes to label sites of bone remodelling at intervals over the subsequent 24-month period (Table.1). Body mass was measured at each time point and prior to euthanasia at the end of month 31.

A cross sectional sample, 11mm in thickness, was removed from the mid-diaphysis of the left metatarsal of each animal using a slow speed diamond saw (Struers, Accutom 50, Ballerup, Denmark). Histological sections of 150 $\mu$ m thickness were taken from the proximal end of the bone specimen using the same instrument. Each slice was ground down to 100 $\mu$ m and mounted on a glass slide using standard preparation techniques for microscopy [10].

Each slide was examined using brightfield microscopy (Olympus IX51, Hamburg, Germany) at x1 magnification. The periosteal area and endosteal area were measured; cortical area (Ct.Ar) was calculated by subtracting endosteal area from periosteal area using a digital image analysis system (analySIS; Soft Imaging Systems, Munster, Germany). To calculate anterior, posterior, medial and lateral cortical thickness, a line was drawn through the widest diameter of the medullary cavity from medial to lateral. A line was then drawn from anterior to posterior, perpendicular to and bisecting the medial-lateral line. Cortical thickness measurements were taken at these four lines to represent

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3 anterior, posterior, medial and lateral cortices thickness. Measurements for area and  
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5 thickness were carried out four times and the average value used. Measurement were  
6  
7 scaled, by adjusting cross sectional area and thickness for the weight of the individual  
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9 sheep as compared with the mean weight for the flock to give the “effective area” and  
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11 “effective thickness” using the following equations [11].  
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17 Effective area = measured area x (mean weight / individual weight)<sup>0.75</sup>

18 Effective thickness = measured thickness x (mean thickness / individual thickness)<sup>0.375</sup>

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21 Second moment of Inertia (I) as a measurement of resistance to bending was calculated  
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23 using the following equation  
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$$I = \frac{\pi}{64} (\text{Periosteal Diameter}^4 - \text{Endosteal Diameter}^4)$$

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35 Each slide was then examined using a combination of ultra-violet (UV) ( $\lambda=365\text{nm}$ ), blue  
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37 ( $\lambda=470\text{nm}$ ) and green ( $\lambda=546\text{nm}$ ) epifluorescence microscopy at X10 magnification.

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39 The number of fluorochrome labelled osteons was measured and intracortical bone  
40  
41 turnover calculated for each time-point. The numbers of resorption spaces at time of  
42  
43 sacrifice were measured per unit area. Resorption spaces were identified by their  
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45 scalloped edges that lacked a cement line or any fluorochrome labels, and were  
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47 approximately the size of a completed osteon.  
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50 The central 4mm portion of the remaining metatarsal sample was then scanned using a  
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52 microCT scanner (Scanco,  $\mu\text{CT-40}$ , Bassersdorf, Switzerland). A measurement protocol  
53  
54 (control file) was created to ensure all scan parameters were the same. Source energy was  
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3 70 kV, scan time for each sample was less than 30 minutes and the voxel resolution was  
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5 15 $\mu$ m. A 3D reconstruction of the sample was created using Image Processing Language  
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7 (IPL, Scanco, Bassersdorf, Switzerland). Porosity was calculated as  $(1-BV/TV)$ , where  
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9 BV/TV is the relative bone volume as measured by voxel counting.  
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13 Cross-sectional areas were measured using the same image analysis system as used for  
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15 histomorphometry. Samples were subjected to unconfined compression testing along the  
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17 long axis of the bone. Testing was performed using servo-hydraulic materials testing  
18  
19 machine (Instron, 8501, Bucks, UK) with a cross-head displacement rate of 0.01mm/s.  
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21 Samples were loaded to failure and force-displacement curves were obtained from the  
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23 load cell and actuator output to determine ultimate compressive strength.  
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### 29 Statistical Analysis

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31 Data are presented as mean  $\pm$  standard error of the mean (SEM). For geometrical data  
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33 results are presented scaled for each individual animal relative to flock weight. For  
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35 statistical analyses, groups were assessed for normal distribution and then compared  
36  
37 using a t-test. For those variables failing the normality test, a nonparametric Mann-  
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39 Whitney rank sum test was used. SigmaStat 3.0 statistical package (SYSTAT Software  
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41 Inc, Chicago, IL 60606) was used for statistical analyses. A p value of  $<0.05$  was  
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43 considered to be significant.  
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## Results

At the time of sacrifice the mean weight of the flock was 83.3kg, with weight ranging from 67-101kg. There was no significant difference in weight between the study groups (control  $82.4 \pm 2.6$  Kg, OVX  $81.5 \pm 2.7$ ,  $p > 0.05$ )

Mean effective periosteal area was significantly greater in OVX compared with controls ( $188.9 \pm 3.7$  mm<sup>2</sup> and  $177.24 \pm 2.4$  mm<sup>2</sup> respectively,  $p = 0.008$ ). There was no significant difference in mean effective endosteal area of OVX and control ( $64.6 \pm 2.7$  mm<sup>2</sup> and  $61.3 \pm 2.6$  mm<sup>2</sup> respectively,  $p > 0.05$ ). Mean effective cross-sectional cortical area of OVX was significantly greater than control ( $129.3 \pm 2.6$  mm<sup>2</sup> and  $119.2 \pm 1.9$  mm<sup>2</sup> respectively,  $p = 0.005$ ).

There was no significant difference in effective cortical thickness between the control and OVX, however the control group was consistently thinner than OVX (Table 2.). Second moment of inertia was significantly greater in OVX compared with controls ( $2526.28 \pm 85.6$  mm<sup>4</sup> and  $2210.17 \pm 53.8$  mm<sup>4</sup>,  $p = 0.002$ ).

There were a significantly greater number of resorption spaces per mm<sup>2</sup> at the time of sacrifice in OVX compared to control ( $0.252 \pm 0.056$  and  $0.111 \pm 0.026$  respectively,  $p = 0.022$ ). At 0 and 16 months post ovariectomy there was no significant difference in intracortical bone turnover as measured by fluorochrome labelled osteons between control and OVX. At 12/12, 20/12 and 24/12 there was a significantly greater number of labelled osteons in the OVX relative to control ( $p = 0.003$ ,  $p < 0.001$  and  $p = 0.044$  respectively) (Figure 1.).

The mean porosity for the OVX study group was significantly greater than control ( $2.51 \pm 0.096$  % and  $2.23 \pm 0.065$  % respectively,  $p = 0.015$ ). There was no significant difference in

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3 ultimate compressive stress between control and OVX ( $146.3 \pm 5.0$  MPa and  $148.1 \pm 5.7$   
4 MPa respectively  $p > 0.05$ ). There was no significant difference in modulus of elasticity  
5  
6 MPa respectively  $p > 0.05$ ). There was no significant difference in modulus of elasticity  
7  
8 between the control and OVX study groups ( $4269.5 \pm 354.3$  MPa and  $3998.9 \pm 233.9$  MPa  
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10 respectively,  $p > 0.05$ )  
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## 18 Discussion

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20 In advancing age bone quality and quantity deteriorates, a process accelerated by  
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22 estrogen deficiency. Understanding the pathogenesis of bone fragility is crucial to the  
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24 prevention and treatment of osteoporotic fractures. We have previously examined the  
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26 effects of estrogen deficiency over a 12 month period on ovine compact bone, here we  
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28 assess the changes in bone turnover, geometrical adaptation, porosity and biomechanical  
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30 strength over a 31 month period [5].  
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34 The size and shape of bone are known to alter its biomechanical properties. Bone loss  
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36 from the inner endocortical surface contributes to bone fragility, whereas deposition on  
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38 the outer periosteal surface is believed to be an adaptive response to maintain resistance  
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40 to bending. Compared to endosteal osteoblasts, periosteal osteoblasts have been shown to  
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42 have more estrogen receptors [12]. Studies in animals have shown that estrogen inhibits  
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44 periosteal expansion whereas estrogen deficiency stimulates periosteal expansion [13,  
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46 14]. In a prospective study of postmenopausal women, the endosteal diameter was shown  
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48 to expand along with an increase in the periosteal diameter, with periosteal diameter was  
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50 inversely associated with postmenopausal estradiol levels [15]. In our study, the effective  
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52 periosteal area of OVX and cross sectional area were significantly greater than control  
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3 These results support the hypothesis that bone adapts structurally in response to estrogen  
4 deficiency as early as 31 months post estrogen withdrawal, which resulted in a significant  
5 increase in second moment of inertia in the OVX group.  
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10 The fluorochrome labelling sequence used in this study allowed us to measure and  
11 compare intracortical bone at these five different time-points. The estrous cycle in sheep  
12 is subject to seasonal variation, with periods of anestrus during summer months or  
13 specifically months with long day light hours[16]. This phenomenon varies with latitude,  
14 with an increase in estrous cycles with increasing latitude. Our results demonstrate a  
15 distinct seasonal influence, with the highest rate of bone turnover in control and OVX  
16 during the summer and autumn months, which corresponds to the fluorochrome dye  
17 administration at 12, 20 and 24 months. At 12, 20 and 24 months there was a significant  
18 increase in bone turnover in the control group compared to OVX. We would expect no  
19 significant difference between the groups at the commencement of the study (0 months),  
20 at 16 months the lack of significance may be explained by the relatively low bone  
21 turnover in both groups for this period. When we compare our findings with the results of  
22 a study over a one year period we can see clearly the seasonal variation in bone turnover  
23 and of note bone turnover in OVX does not significantly increase from the Summer of  
24 our previous study to Summer of the year two study group (24 month time point),  
25 suggesting that the increased rate of bone turnover, depending on seasonal influence, is  
26 relatively constant in the initial two year period and does exhibit an additive or  
27 exponential increase[5].  
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52 Resorption spaces, which represent an active bone multicellular unit or an area excavated  
53 by an osteoclast, were significantly greater in number in OVX compared to controls.  
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3 Overall the number of resorption spaces was increased relative to the data from year one;  
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5 however this reflects the time of sacrifice [5]. Year one was sacrificed during winter,  
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7 which is a period of low turnover and hence decreased activity of the bone multicellular  
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9 unit, whereas year two were sacrificed during summer, a period of increased bone turnover  
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11 as evidenced by the labelling of osteons.  
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15 MicroCT measurement of intracortical porosity of OVX was significantly greater than  
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17 the control group. Burr et al found similar results in a study that used sham and OVX  
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19 cynomolgus monkeys to assess the effect of human parathyroid hormone on bone  
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21 turnover and strength at 18 months [17]. However there was however no significant  
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23 correlation between the number of resorption spaces with porosity the ( $r^2 = 0.18$ ). If the  
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25 number of resorption spaces were to correlate with porosity, the resorption spaces should  
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27 be uniform in size. A study by Thomas et al suggests that size of resorption space and not  
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29 density is the main determinant of porosity in cortical bone [18]. They found a significant  
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31 difference between mean resorption space size between young, middle and elderly males  
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33 and females and found that porosity mean pore area could almost wholly be explained by  
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35 mean pore area. Inferring from this study, our results suggest that whilst the number of  
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37 resorption spaces is increased in OVX, the actual size of the space is not concomitantly  
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39 increased.  
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46 The primary factor that affects strength as a material is bone density or mineralisation,  
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48 which accounts for 60-90% of its strength [19]. We also know that small changes in the  
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50 geometry can affect strength [2]. In our study we see found no significant changes in  
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52 biomechanical strength of the study groups, which is consistent with our previous study  
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54 [5]. This suggests that the OVX, despite an increase rate of bone turnover and porosity,  
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3 do not exhibit gross differences in strength at 31 months. An explanation why these  
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5 changes did not affect the mechanical strength may be that the increases in intracortical  
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7 bone turnover and porosity take place at the microscopic level and thus require longer to  
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9 exert a detectable effect at the macroscale level. This finding may be useful in clinical  
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11 settings, where the onset of osteoporosis may be detected by increased bone turnover and  
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13 intracortical porosity before it affects mechanical strength, and preventative measures  
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15 instituted.  
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19 In conclusion our data support the hypothesis that estrogen deficiency increases bone  
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21 turnover, albeit in a seasonal manner, in ovariectomised sheep. There is compensatory  
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23 geometrical adaptation of the distribution of bone mass that increases resistance to  
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25 bending and also ameliorates a potential reduction in compressive strength and that pore  
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27 density does not correlate with porosity as measured with MicroCT.  
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31 Therefore evidence of early changes in bone geometry and increased bone turnover could  
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33 be used to detect osteoporosis before estrogen deficiency has adversely affected bone  
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35 strength.  
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For Peer Review



**Table 1.** Details of fluorochrome administration during experimental period

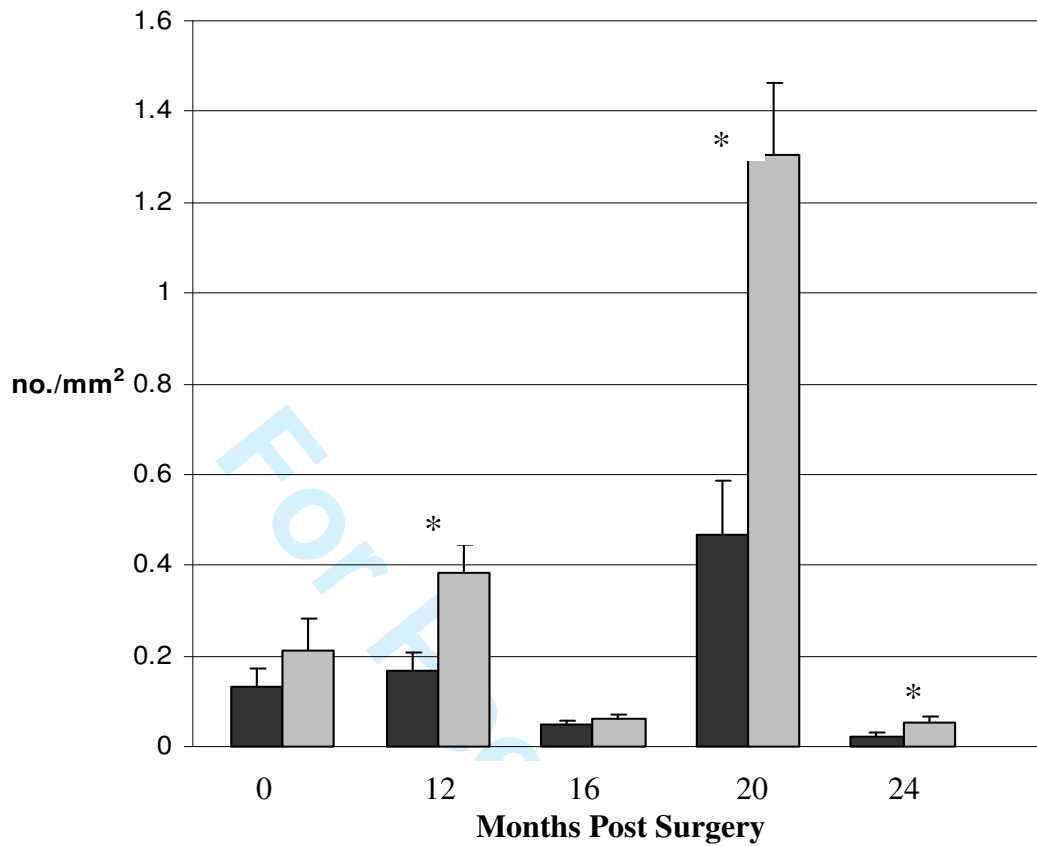
Time	Season	Fluorochrome	Supplier	Dose (mg/kg)
0/12	Winter	Oxytetracycline	Pfizer	50*
12/12	Autumn	Alizarin Complexone	Sigma-Aldrich	25*
16/12	Spring	Calcein	Sigma-Aldrich	10¶*
20/12	Summer	Xylenol Orange	Sigma- Aldrich	90¶*
24/12	Autumn	Calcein Blue	Sigma-Aldrich	30¶*
31/12	Sacrifice			

\* [20], ¶ [21]

(1) Pfizer Animal Health, Dublin,Ireland (2) Sigma Aldrich, Dublin

**Table 2.** Effective cortical thickness, Mean ( $\pm$  sem)

	Medial (mm)	Lateral	Anterior	Posterior
Con	3.50 ( $\pm$ .07)	3.21 ( $\pm$ .08)	2.71 ( $\pm$ .07)	2.32 ( $\pm$ .07)
Ovx	3.64 ( $\pm$ .09)	3.41 ( $\pm$ .08)	2.81 ( $\pm$ .08)	2.43 ( $\pm$ .06)



**Figure 1.** Mean ( $\pm$  sem) number of labeled osteons at time point.  
Control grey bar. OVX black bar. \*  $p < 0.05$