Regional variation in oophorectomy induced trabecular bone osteopenia in the distal femur of the rat.

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Abstract

Postmenopausal osteoporosis is a condition resulting from altered bone turnover, producing excessive bone loss following the menopause. Characteristically atraumatic fractures are particularly evident in regions of trabecular bone. The oophorectomised rat has been established as an accepted model of this condition. The use of this model has revealed heterogeneity in response to ovarian hormone deficiency in trabecular bone.

This thesis examines regional variations in trabecular bone remodelling and bone loss following oophorectomy in the distal femur of the rat. The primary focus is the comparison of the weight bearing epiphyseal region to the less mechanically loaded diaphyseal and metaphyseal regions. This is performed using both static and dynamic histomorphometric techniques. Studies characterize the short term and long term response to oophorectomy in adult rats. The effects of oestradiol supplementation, dietary calcium restriction and prepubertal oophorectomy are also been investigated.

In the diaphysis and metaphysis, oophorectomy results in a rapid and permanent loss of trabecular bone, with no loss in the epiphysis, despite increased bone turnover. This epiphyseal immunity to osteopenia is consistent with the weight bearing activity within this region and the greater thickness of trabeculae.

The supplementation of oophorectomised animals with 17β-oestradiol (E_2) reduced bone turnover, thickening trabecuale. This increase in thickness is proposed as a factor in the antifracture efficacy of antiresorptive therapies, such as oestradiol.

The epiphysis was shown to be resistant to osteopenia resulting from calcium restriction. Dietary calcium restriction and oophorectomy produced extensive bone loss in the metaphysis and the epiphysis, highlighting the additive effects of calcium and ovarian hormone deficiency.

Prepubertal oophorectomy stopped development of trabecular bone in the diaphysis. In the metaphysis and epiphysis, the accretion of bone was not affected, with Tb.Th similar between operative groups. The production of trabecuale was viretually. Tb.Th was correlated with body weight in the metaphysis and epiphysis, suggesting that ovarian hormones are not obligatory for development of bone in weight bearing regions.

The studies of this thesis reveal a complex interaction between weight bearing and ovarian hormone deficiency, and show that physiological signals exist which can negate all adverse effects of postmenopausal osteoporosis.

Statement of Originality

This thesis contains no material which has been accepted for the award of any other degree or diploma in any other university of tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by any other person except where due reference has been made in the text.

I give consent to this copy of my thesis, when deposited in the University library, being available for photocopying.

Acknowledgement and Dedication

Many people have been involved in the completion of this thesis. The principal figure among these is my supervisor Dr Howard Morris, without whom this thesis may well never have been completed. It was born from his scientific insight and progressed due to his enthusiasm and commitment. I owe a debt of gratitude to him beyond my means to repay. He stuck with me when many would have waned. My future progress in science is founded on his good nature. It is to him alone that I dedicate this thesis.

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Chapter 1

Introduction

1.1 Function of bone

Bone is a highly specialised form of connective tissue that performs multiple functions within vertebrate organisms. Bony organs form the scaffolding from which muscle attachment enables movement and provides resistance to the force of terrestrial gravity. Flat bones such as the calvaria of the skull and the ribs form a protective barrier to the soft inner organs. Bone is also a reservoir for ion homeostasis. 99% of the body's calcium is crystallized in bone and teeth (Morris, 1994) and the regulation of this highly important mineral is an important aspect of bone physiology, as well as other ions including phosphorous and magnesium.

1.2 Composition of bone

Bone is composed of a collagenous matrix which gives structure and configuration to the skeletal organs, into this matrix hydroxy apatite ($[3Ca_3(Po_4)_2].(OH)_2$) crystals form (Bonar *et al.,* 1983), giving the skeleton its rigidity. The inorganic, mineral phase accounts for approximately two-thirds of the total dry weight of bone, with crystals located within and between the collagen fibrils. Both the matrix and the mineral phases of bone are impure, and contain many other constituents. While 90% of the protein content of bone is comprised of collagen, the matrix also contains 200 or more non-collagenous proteins (Delmas *et al.,* 1984). Among the most abundant are osteonectin, osteocalcin, osteopontin, matrix gla protein, and bone salioprotein (Robey and Boskey, 1995). The mineral phase also contains trace elements such as magnesium, aluminum, sodium, fluoride, carbonate and citrate (Bronner, 1995).

1.3 Anatomy and ultrastructure

The skeleton is derived from mesodermal origin, with the exception of some facial bones derived from neural crest tissue (Olsen, 1999). From an anatomical perspective, the skeleton is divided into two regions, the axial skeleton that includes the spinal column, skull, ribs and sternum and the appendicular skeleton, the limbs and pelvis. Morphologically bone is divided into two types cortical and trabecular.

Cortical bone is the solid or compact bone typically associated with the skeleton. It forms the external shell of all bony organs. Cortical bone is very dense, with up to 90% of its volume being calcified (Parfitt, 1988). The collagen matrix is highly organised in its orientation, giving cortical bone added strength and its distribution is consistent with mechanical function within the skeleton (Martin *et al.,* 1996). The external surface of the skeleton is covered with a layer of fibrous tissue and undifferentiated cells, the periosteum. The internal surface is continuous with the other type of bone, trabecular bone.

Trabecular bone is a spongue-like lattice of bone encased by the cortical shell (Whitehouse, 1977) and is less dense than cortical bone with the majority of its volume occupied by bone marrow, blood vessels, adipocytes and connective tissue. The lattice-like structure of trabecular bone results in a large surface area, accounting for 75% of the surface area of the skeleton and evidence of the metabolic role of this type of bone (Parfitt, 1988). The structure of the lattice in trabecular bone is consistent with a model of interconnecting rods, or plates with interconnecting rods (Fazzalari *et al.,* 1989, Mosekilde, 1989). The geometric and spatial organisation of the lattice is governed in part by local mechanical forces (Biewener *et al.,* 1996). While cortical bone is the main mechanical support within the skeleton, some regions such as the vertebrae and the ends of long bones have thin cortical shells. In these regions, such as

the distal femur, trabecular bone plays a greater mechanical role, with thicker trabeculae orientated along the predominant lines of force within the bone (Majumdar *et al.,* 1998, Millard *et al.,* 1998).

1.4 Cells of bone

1.4.1 The Osteoclast

1.4.1.1 Origin

Osteoclasts, the cell type responsible for resorption of skeletal tissue, are derived from the hemopoietic compartment and are members of the mononuclear phagocyte lineage (reviewed in Suda *et al.,* 1995). More specifically they differentiate from the granulocyte-macrophage colony-forming unit (GM-CFU) (Suda *et al.,* 1992).

1.4.1.2 Structure

The osteoclast is a large multinucleate giant cell containing abundant mitochondria, golgi apparatus and vacuoles (Baron *et al.,* 1986), giving the cytoplasm its characteristic "foamy" appearance. Mononuclear osteoclasts have also been shown to contribute to bone resorption (Kaye, 1984). At sites where they attach to the bone surface, active osteoclasts are highly polarized cells, and produce several characteristic membrane structures, the sealing zone and the ruffled border. The sealing zone is the attachment ring of the osteoclast. This region is rich in integrin receptors particularly of the $\alpha_v\beta_3$ (vitronectin) subtype (Nesbitt *et al.,* 1993), which attach to specific sequences in bone matrix proteins such as osteopontin and osteonectin. The sealing zone creates a tight attachment to the mineralised tissue and effectively isolates the subosteoclastic space in which the bone resorption process takes place. The ruffled

border, encircled by the sealing zone, is a highly convoluted structure through which the bone resorbing compounds are secreted.

1.4.1.3 Function

The subosteoclastic zone is acidified initially by fusion of acidic intracellular vesicles, which form the ruffled border (Baron *et al.,* 1988). Additional protons are provided by carbonic anhydrase II and are transported by vacuolar type proton pumps on the ruffled border (Bekker and Gay, 1990, Blair *et al.,* 1989). The decrease in pH resultant from the rise in proton concentration (Baron *et al.,* 1985) dissolves the mineral phase. Following this, proteolytic enzymes are secreted into the resorbing area to degrade the matrix. Lysosomal cysteine proteinases such as cathepsin K (Drake *et al.,* 1996) and the metalloproteinase MMP-9 (Tezuka *et al.,* 1994, Okada *et al.,* 1995) are involved in this process. Tartrate resistant acid phosphatase is also known to identify osteoclastic cells (Braidman *et al.,* 1990, Kalu *et al.,* 1993, Turner *et al.,* 1993, Bourrin *et al.,* 1995).

Osteoclasts are terminally differentiated cells with a limited life span, with the resorption cycle lasting 12 days in humans (Eriksen, 1986) and 1-2 days in rats (Baron *et al.,* 1984). It is generally accepted that following resorption osteoclasts undergo death by apoptosis or programmed cell death (Hughes, *et al.,* 1996, Parfitt *et al.,* 1996), although this view has been challenged (Solari *et al.,* 1995). A number of compounds have been associated with alteration in osteoclast life span. Oestrogen has been shown to stimulate osteoclast apoptosis, possibly mediated by transforming growth factor-beta (TGF-beta) (Hughes, *et al.,* 1996).

1.4.2 The Osteoblast

1.4.2.1 Origin

Osteoblasts, the cell type responsible for the formation of skeletal tissue, are derived from pluripotent mesenchymal stem cells, which also form adipocytes, fibroblasts and myoblasts (Aubin *et al.,* 1995). These precursors, given the right stimulation, undergo proliferation and differentiation to form committed, spindle shaped preosteoblast/stromal cells (Scott, 1967). The active osteoblast is a postproliferative cell, lining the bone surface at sites of bone matrix production (Lian *et al.,* 1999). The majority of osteoblasts involved in formation do not survive (65 %), with a proportion being incorporated into the bone matrix as osteocytes (30%), and the remaining cells differentiating to become bone lining cells (Weinstein and Manolagas, 2000).

1.4.2.2 Structure

The active osteoblast is characterised at the light microscope level as a mononuclear, cubiodal cell with a round nucleus at its base. Consistent with its protein secretory role, the osteoblast has well developed and prominent endoplasmic reticulum, circular golgi apparatus and secretory vesicles between the nucleus and the bone surface. The cytoplasm is strongly basophilic and the plasma membrane is strongly alkaline phosphatase positive (Nijweide *et al.,* 1986).

1.4.2.3 Function

The formation of bone involves the production of a protein matrix or osteoid, which is subsequently mineralised. The sequence of synthesis of the protein components of bone matrix has been elucidated (Aubin *et al.,* 1995, Stein and Lian, 1995). Matrix production, the initial phase of bone formation is characterised by production of type I

collagen, alkaline phosphatase and osteopontin. Matrix Gla protein and bone salioprotein are produced as the matrix matures. The onset of mineralisation is coincident with the production of osteocalcin and osteopontin production reappears. These components are secreted toward the bone formation front using the prominent vesicular apparatus.

Mineralisation follows slightly behind matrix formation. The initial precipitation of calcium salts occurs in the 'hole' zones between collagen fibrils (Glimcher, 1998). The low concentration of apatite in body fluids suggests this process must be facilitated by components of the matrix. Collagen alone does not suit the role of a nucleation promoter, but may act as a scaffold for such molecules (Anderson, 1989). Various matrix components have been described as initiators of mineral precipitation such as bone salioprotein (Hunter and Goldberg, 1993), osteopontin (Gerstenfeld, 1999), with others as inhibitors such as osteocalcin (Boskey *et al.,* 1998) and osteonectin (Anderson, 1989).

The location of the initial nucleation is uncertain, although vesicles have been shown to perform the function in calcifying cartilage and tendon (Robey and Boskey, 1996). Crystal growth is regulated by matrix macromolecules, which regulate crystal size and shape, with dietary cations such as magnesium and strontium (Johnson, 1973, Daculsi *et al.,* 1997) and fluoride (Grynpas, 1990) known to effect this process.

1.4.3 The Osteocyte

1.4.3.1 Origin

Osteocytes represent one mode of terminal differentiation in the osteoblast lineage (Aubin *et al.,* 1995) and are the most abundant cell in adult human bone (Parfitt, 1977). During bone formation some osteoblasts are incorporated into the newly

synthesizing matrix by a process believed to involve a reduction in matrix apposition rate (Marotti *et al.,* 1992) and become embedded as formation continues. These cells remain in the bone tissue indefinitely or until excavated by bone resorption. Reduction in osteocyte numbers with age however, suggests cell death by apoptosis prior to excavation (Mullender *et al.,* 1996, Tomkinson *et al.,* 1997).

1.4.3.2 Structure

The osteocyte has a characteristic stellate structure, enclosed within the lacuno-canalicular network of bone. This is a network of spaces (lacunae) containing the osteocyte cell bodies and small canals (canaliculi) containing osteocyte cell processes and is present throughout skeletal tissue (Palumbo *et al.,* 1990). The profuse processes of osteocytes connect to neighbouring osteocytes within an osteon and to the ostoblasts or lining cells at the bone surface. The connections at the tips of the processes (Doty, 1981) enable both metabolic and electric coupling through gap junction processes called connexins (Civitelli *et al.,* 1993). Thus the osteocyte network contains two interconnected systems within the bone, a cellular network from osteocyte to osteocyte via gap junctions and an extracellular network from lacunae to lacunae via canaliculi.

1.4.3.3 Function

The osteocytic network is involved in mechano-transduction within the skeleton (Turner and Forwood, 1995). Cellular deformation caused by interstitial fluid flow within the lacuno-canalicular network, responding to compression or tension is believed to be the signal by which mechanical load is sensed by the osteocytes (Klein-Nulend *et al.,* 1995, Ajubi *et al.,* 1996, Cowin, 1999). Osteocyte stimulation has been

shown to induce production of various bone active compounds such as prostaglandins (Klein-Nulend *et al.,* 1997) and nitric oxide (Zaman *et al.,* 1999). Osteocyte death has been linked to increased accumulation of bone microdamage (Mori *et al.,* 1997). Osteocytes may also play a role in plasma mineral homeostasis (el Haj *et al.,* 1990).

1.4.4 The Lining cell

1.4.4.1 Origin

Bone lining cells represent the other mode of terminal differentiation in the osteoblast, and are the cells left behind after a team of osteoblasts has finished forming new bone (Miller and Jee, 1987).

1.4.4.2 Structure

Lining cells are flattened, with few overt features of secretory activity. The cytoplasm is usually not visible on light microscopy. The nucleus is elongated and flattened. These cells form a continuous membrane over quiescent bone surfaces (Menton *et al.,* 1984), and cover a thin layer of non-osteoid connective tissue (Miller *et al.,* 1980).

1.4.4.3 Function

The function of lining cells is uncertain, although it has been suggested that they play a role in plasma calcium homeostais (Parfitt, 1989). Retraction in the sheet of lining cells is one of the initial stages in remodelling and thus they are thought to play a role in the localisation and initialization of bone remodelling (McSheehy and Chambers, 1989). This is consistent with their connection to the osteocyte network (Turner and Pavalko, 1998). Recently, lining cells have been shown to reactivate into secretory

cells following mechanical loading (Chow *et al.*, 1998) and intermittent PTH treatment (Dobnig and Turner, 1995).

1.5 Development and growth

The first bones to appear in the embryo arise from either mesenchyme or cartilage. Bones that arise from mesenchyme do so in a direct method of bone formation termed intramembranous ossification. This is restricted to the bones of the cranial vault, some facial bones, the mandible and the clavicle. Condensations of cells within the sheet-like mesenchyme transform into osteoblasts and secrete osteoid, which is then mineralised. The expanding bone engulfs blood vessels and eventually takes the form of an outer and inner plate of compact bone enclosing an inner space containing trabecular bone and marrow. The initially random arrangement of collagen fibrils produces woven bone, which is subsequently replaced by highly organised lamellar bone, with collagen fibrils laid down in layers of opposing orientation (Baron, 1999).

Endochondral ossification is the term for production of bone from a cartilage template and is responsible for the production of the majority of the skeleton, including the distal femur. This process is also initiated by condensations of mesenchymal cells, however in this case they differentiate into chondrocytes. These cells synthesize cartilage templates or anlagen, their shape reflecting both the position and form of the bone being produced. The primary ossification centre of a long bone develops in the middle of the immature shaft, with calcification of the cartilage and death of the chondrocytes (Recker, 1992). At the same time, cells at the outer collar of the bone differentiate and form the periosteum, depositing bone by intramembranous ossification. This is concurrent with vascular invasion of the central region of the developing bone, bringing osteoclasts. Resorption of the calcified cartilage is followed

by bone formation. This process continues toward the ends of the bone shaft. Around the time of birth, a secondary ossification centre begins in the end of the long bone, forming the epiphysis. This region develops by essentially the same process, however enlarges in a radial, not longitudinal direction (Olsen, 1999).

Increases in longitudinal growth in long bones occurs from a specialised region of remnant cartilage between the primary and secondary ossification centres, the epiphyseal growth plate. This region enables extension of the shaft of a long bone via the highly coordinated production of cartilage by columns of proliferating chondrocytes (Hunziker *et al.,* 1984). The matrix produced by these cells forms longitudinal septae. These cells undergo rapid hypertrophy (Eggli *et al.,* 1985), contracting the calcifying septa (Schenk *et al.,* 1967). The majority of chondrocytes undergo cell death, although differentiation into osteoblasts has been observed (Roach *et al.,* 1995).

The region surrounding the calcified septa is highly vascular, bringing osteoclasts, which resorb the majority of septa (Schenk *et al.,* 1968). The remaining septa form the scaffold upon which subsequent bone is formed. Osteoblasts produce a layer of woven bone upon the cartilage template, forming the primary spongiosa. This initial bone is characterised by a poorly organised collagen arrangement. The woven bone is subsequently resorbed and replaced with highly organised lamellar bone by the bone remodelling process, delineating the secondary spongiosa. By this process the proliferation of chondrocytes in the growth plate extends the shaft, leaving behind the trabecular bone network. The length of the trabecular network, thus formed, is determined by terminal destructive modelling, which removes trabeculae from the diaphysis as longitudinal growth continues (Turner *et al.,* 1994c). At the circumference of the growth plate condensation of the septa and subsequent bone

formation is such that no marrow cavity persists, producing the solid bone of the cortices.

1.6 Bone remodelling

Bone remodelling (Fig 1.1) is the coordinated process whereby small amounts of bone are eroded and replaced with new bone by an organized group of cells, the basic multicellular unit (BMU). Remodelling is a dynamic process occurring constantly throughout the mature skeleton, replacing around 11% of the skeleton per year (Parfitt, 1988). This process enables old or damaged bone to be replaced (Schaffler *et al.,* 1995, Parfitt, *et al.,* 1996), structural alterations to be made (Frost and Jee, 1994) and ion homeostasis to be maintained (Miller *et al.,* 1986). Remodelling is a four part process involving activation, resorption, reversal and formation. These processes are temporally distinct, occurring sequentially at any single site. They are also spatially distinct, occurring simultaneously at different locations.

1.6.1 Activation

Activation is the process that converts a resting bone surface into an active remodelling surface. A poorly understood process activates osteoclasts at specific, focal loci on the bone surface. This involves resorption of the thin layer of osteoid covering the bone surface by the resident lining cells (Chambers *et al.,* 1985) and their retraction to expose the mineralised surface to osteoclasts (Jones and Boyd, 1976), fusion of the mononuclear osteoclasts and the tight attachment of the osteoclast to the mineralised surface.

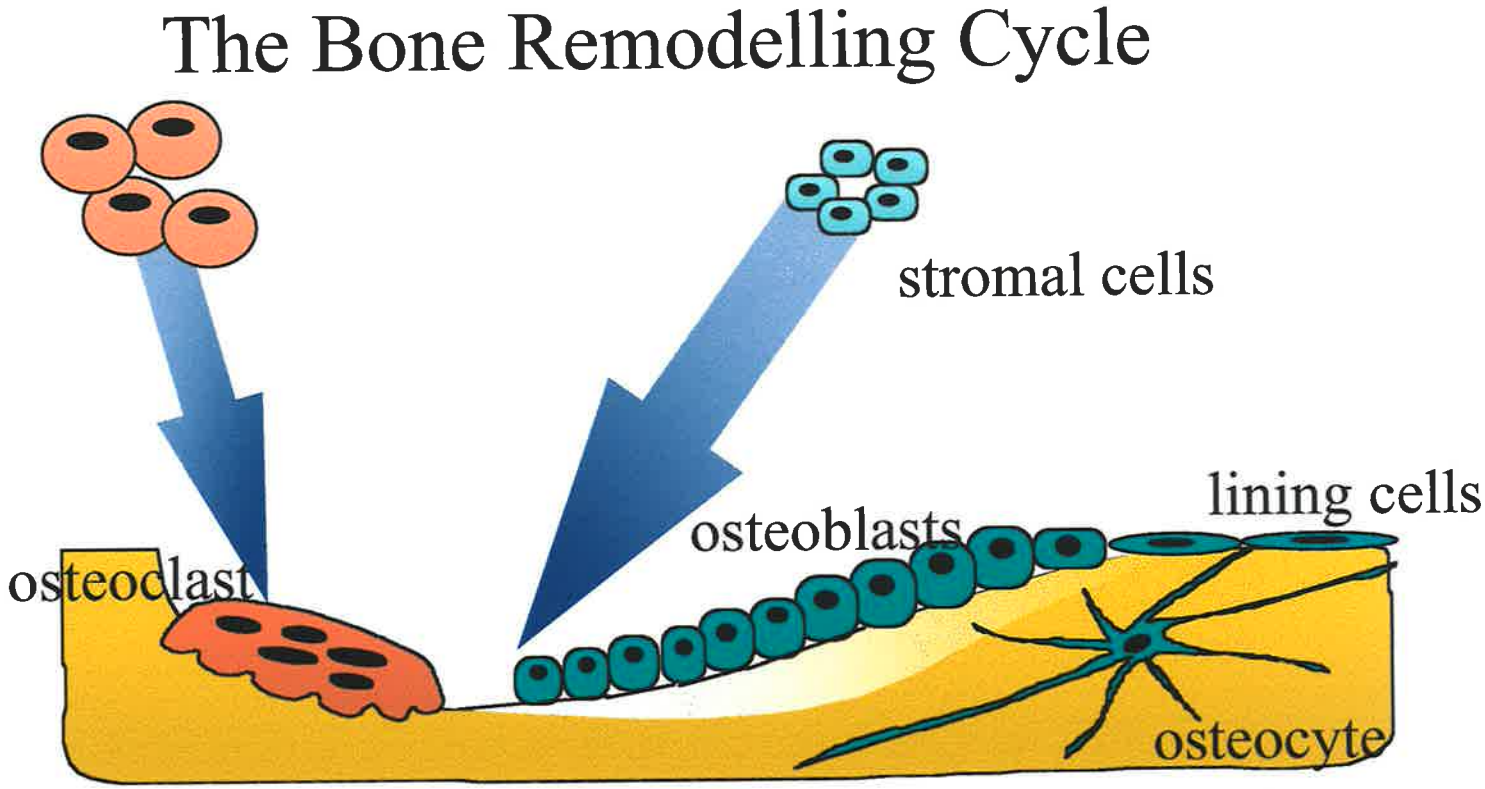


Figure 1.1 The bone remodelling cycle in trabecular bone. Mature, bone resorbing osteoclasts are produced by fusion of mononuclear precursor cells. Osteoblasts, attracted to the site of resorption following their own proliferation and differentiation from immature precursors, replace the resorbed bone with new matrix, which is subsequently mineralised. Surviving osteoblasts terminally differentiate into lining cells or are embedded as osteocytes.

1.6.2 Resorption

Resorption is a dynamic process, with osteoclasts working in teams producing a moving resorption front. In cortical bone this results in a cutting cone burrowing through the bony tissue (Moseklide 1990). In trabecular bone this results in a shallow, scalloped bay in the surface of the bone termed a Howsip's lacunae. Resorption is mediated by many factors, including parathyroid hormone (PTH) and 1,25 dihydroxyvitamin D_3 (1,25 $(OH)_2$ D_3) (Teti *et al.,* 1988, Suda *et al.,* 1997, Bushinsky and Monk, 1998). The enhancement of osteoclast differentiation is largely mediated via immature cells of the osteoblast lineage (Martin and Ng, 1994).

The regulatory factors involved in this interaction have been identified. Stromal and osteoblast precursor cells express a member of the TNF receptor ligand family, receptor activator of NFκB ligand (RANKL), also identified as ODF, OPF-L and TRANCE (Anderson *et al.,* 1997, Wong *et al.,* 1997, Yasuda *et al.,* 1998). This cell surface ligand stimulates osteoclastogenesis and osteoclast activity by binding to its cognate receptor, RANK, also termed ODF receptor (ODFR), on the surface of osteoclast precursors (Hsu *et al.,* 1999). A further level of regulation is provided by a RANKL decoy receptor, termed osteoprotogerin (OPG) or osteoclastogenesis inhibitory factor (OCIF), produced by osteoblasts and other cell types (Simonet *et al.,* 1997, Yasuda *et al.,* 1998b). OPG binds to RANKL, blocking its interaction with RANK on osteoclast precursors and thus inhibits osteoclast activity (Lacey *et al.,* 1998, Yasuda *et al.,* 1998, Hsu *et al.,* 1999). Based on these and other studies, it has been suggested that the ratio between RANKL and OPG expression levels in osteoblastic cells is a key factor in osteoclast regulation (Horwood *et al.,* 1998, Nagai and Sato, 1999).

1.6.3 Reversal

The reversal phase is characterised by the disappearance of multinucleated osteoclasts and the appearance of mononuclear cells. The majority of resorption results from the action of multinuclear osteoclasts. It has been suggested however, that mononuclear cells, found in resorption pits after multinuclear cells are responsible for up to one third of resorption (Eriksen, 1986). During this period the resorbed surface is smoothed and a non-collagenous, cement layer is laid down (Vignery and Baron, 1980).

1.6.4 Formation

The initiation of bone formation involves the chemotactic attraction of osteoblast precursors to the site of previous resorption. This process is likely mediated by local factors produced during bone resorption, with evidence from in vitro studies of the chemotactic effect of collagen 1 and osteocalcin (Mundy and Poser, 1982, Mundy *et al.,* 1983), TGF-beta (Pfeilschifter *et al.,* 1990) and BMP-2 (Lind *et al.,* 1996). Once attracted to the site, precursors proliferate and differentiate into mature osteoblasts, influenced by local factors such as IGF-I and II (Hock *et al.,* 1988, Wergedal *et al.,* 1990) and TGF-beta (Rickard *et al.,* 1993).

The assembled team of osteoblasts begins production of new matrix, which is subsequently mineralised. As the resorption front proceeds, so does the formation front, with newly exposed portions of the Howsip's lacunae occupied by newly differentiated osteoblasts. Formation occurs at a much slower rate than resorption, resulting in osteoblasts rather than osteoclasts representing a larger proportion of the cells active on bone at any time. As formation continues, osteoblasts are incorporated into the matrix and differentiate into osteocytes, connecting with other osteocytes in

the newly synthesised section of bone. The total volume of bone made at one remodelling site is termed an osteon.

1.7 Consequences of altered remodelling on trabecular structure

The basic framework around which remodelling is discussed has been termed the quantum concept of bone remodelling (Parfitt, 1979, Parfitt, 1984b). In a steady state, bone remodelling will continue without a change in the amount of bone in the skeleton. Alteration to the remodelling cycle however, can produce rapid and permanent changes in skeletal structure and strength. Particular alterations in remodelling will have characteristic effects on bone structure, particularly evident in trabecular bone.

1.7.1 Activation frequency

Changes in the activation frequency of remodelling, the generation of new BMUs, can alter bone balance without a change in the activity of individual BMUs. In trabecular bone, these changes result from an alteration in the number of Howsip's lacunae and therefore the volume of bone absent from the skeleton at any time, termed the remodelling space. An increase in activation frequency will result in a remodelling transient that will reduce the volume of bone present due to the relative increase in resorption sites (Heaney *et al.,* 1997). In trabecular bone this increase will result in a decrease in trabecular thickness, which is generally reversible. The increased frequency of resorption however, will increase the probability of trabecular perforation (Eriksen *et al.,* 1990), a process known to trigger the permanent removal of the affected strut (Mosekilde, 1990). Elevated bone turnover is recognised as an independent risk factor for fracture (Garnero *et al.,* 1996, Delmas, 1997, Melton III *et*

al., 1997) and is associated with lower BMD in postmenopausal women (Ravn *et al.*, 1996). The decrease in trabecular number is permanent and will not be restored by a return to previous levels of bone turnover.

Conversely, a reduction in activation frequency will transiently over represent formation, decreasing the remodelling space and increasing bone volume. In trabecular bone this will increase the thickness of trabeculae. This is reflected clinically, with rapid increases in BMD following short term antiresorptive therapy (Storm *et al.*, 1993, Liberman *et al.*, 1995, Heaney *et al.*, 1997). The increased trabecular thickness will persist as long as the activation frequency remains reduced.

1.7.2 Resorption

The short time course of the resorption process results in rapid manifestation of changes in bone structure brought about by increased resorption. Resorption may be increased by a number of alterations. These include an increase in the number of osteoclasts comprising the resorption team (Cohen-Solal *et al.*, 1991) or the number of mononuclear osteoclasts fused to form the mature cells (Piper *et al.*, 1992). The osteolytic capacity of the individual osteoclast or team may be increased (Wassermann *et al.*, 1996) or the lifespan of the team (Jimi *et al.*, 1995). These alterations will either increase the extent of Howsips lacunae, their depth or both.

The exact nature of the changes in osteoclast function will determine the effect on bone structure. An increase in the surface extent of resorption in trabecular bone will tend to decrease the thickness of trabeculae. This in itself is not an irreversible change, but will increase the probability of perforation (Eriksen, 1986). An increase in the depth of resorption will increase the probability of perforation to a greater extent, leading to irreversible bone loss (Mosekilde, 1990). Clearly an increase in both extent

and depth will have compounding effects. Perforation of trabeculae has a secondary effect on remodelling balance. The removal of a trabecular strut leaves no template upon which subsequent formation can occur, producing a physical limitation to coupling, and amplifying bone loss (Eriksen, 1986).

Conversely, a reduction in the extent of resorption will tend to thicken trabeculae slowly as formation continues unchanged. A decrease in the depth of resorption will also produce a slow thickening of trabeculae, which would be associated with a reduction in perforation, lessening bone loss with time.

1.7.3 Formation

Alterations in bone structure brought about by changes in formation are mainfested more slowly than those due to altered resorption. Formation may be increased by an increase in the proliferation of preosteoblasts, increasing the density of cells at the formation front (Erben *et al.,* 1997). The anabolic capacity of the team may be increased (Sakai *et al.,* 1999) or life span may be increased (Jilka *et al.,* 1999.

These alterations will increase the depth of bone formed following resorption, measured from the cement line to a resting surface, termed wall thickness (Lips *et al.,* 1978). In trabecular bone increased wall thickness will increase the thickness of trabeculae (Ejersted *et al.,* 1995). This will increase the volume of bone, as well as reducing the probability of perforation, in a similar manner to a reduction in resorption depth.

Conversely, a reduction in wall thickness will reduce the thickness of trabeculae and increase in the probability of perforation, similar to an increase in resorption. A reduction in wall thickness has been associated with thinning of trabeculae in age related bone loss (Parfitt, 1984). A decrease in osteoblast proliferation and promotion

of apoptosis, as evident in glucocorticoid excess, will result in a reduction in bone via a decrease in trabecular thickness (Weinstein *et al.,* 1998). Thus similar structural alterations can result from differing alterations to bone remodelling. The bone biopsy offers a method for investigating the mechanism of these structural changes, and assigning an appropriate treatment regime.

1.8 Methods for studying bone remodelling

The study of bone remodelling in metabolic bone disorders in vivo requires the examination of a sample of bone removed from the patient. This is most frequently achieved, with a minimum of trauma, using the transilial bone biospy technique (Duncan *et al.,* 1980). This involves a small incision through which a biopsy needle is pushed. A trephine of around 7mm in diameter is removed from the iliac crest containing two corticies and the intervening trabecular bone (Recker *et al.,* 1988).

1.8.1 Parameters measured

The primary indices for estimating bone remodelling and their nomenclature has been standardized (Parfitt *et al.,* 1987). The primary measurements for trabecular bone are area and perimeter. These are often reported in three dimensional nomenclature, as volume and surface. Thus the proportion of the sample area which contains bone will be expressed as trabecular bone volume (BV/TV, %). Following the rod and strut model (Parfitt *et al.,* 1983) this volume is further described by the number (Tb.N, /mm) and thickness (Tb.Th, um) of the trabeculae present, using standardized equations (2.14).

The primary measurements for bone turnover represent the perimeter of the bone surface occupied by resorption or formation. Resorption is estimated by the

identification of osteoclasts on the surface of the bone. These cells can be visualized in a number of ways (2.12.1). This perimeter data will often be expressed as the percentage of bone perimeter covered by osteoclasts, termed osteoclast surface (Oc.S, %). This enables resorption activity in biopsies of different structure to be compared. In human biopsies, the low speed of turnover enables the estimation of erosion depth (Eriksen *et al.,* 1984), thus providing estimations of both the extent and vigor of osteoclast activity.

The estimation of bone formation is greatly aided by the use of intravital labelling with tetracycline derivatives prior to biopsy (Frost, 1969). These compounds are incorporated into the mineralisation front at active formation sites, upon excitation with UV light they can be visualised as fluorescent deposits within the bone tissue (2.11.3). At sites of lamellar bone formation these deposits appear as bands displaying the extent of bone mineralisation. Two doses of label are administered with a gap of generally 10 days between doses and a 3 day period before biopsy (Compston, 1997).

As with Oc.S, formation is estimated by the percentage of bone perimeter covered by fluorescent label. In order to assure that measurement represents a site of continuous bone formation, only surfaces covered by both labels are incorporated into the estimate of perimeter, termed double labelled surface or mineralising surface (dLS, %). In order to account for the resorption of previously incorporated label and the staggered initiation of formation during the labelling period (Schwartz and Recker, 1982), the perimeter may also include one half of the single labelled surface. The mineral apposition rate (MAR, um/d) can be estimated by the distance between the inner edges of the labels divided by the time period between their administration (Tam and Anderson, 1980). This gives a dynamic estimation of the activity of osteoblastic

cells at the time of biopsy. Bone formation rate (BFR, $um^3/um^2/d$) can then be calculated from the product of dLS and MAR.

1.8.2 Advantages

Information gained from the bone biopsy enhances both biochemical, radiological and densitometric data. Biopsy data enables envelope specific investigations to be made without the additional influence of other physiological pathways both of which may confound biochemical studies. The assessment of actual bone cell activity and dynamic rates of activity add enormously to the understanding of calcified tissue pathology produced by radiology. The microarchitectural information can relate strongly to bone strength adding to densitometric analysis (Cummings *et al.* 1996).

1.8.3 Limitations

The major limitation of the bone biopsy is that it samples only a single skeletal site. Some metabolic disorders, such as osteomalacia and renal osteodystrophy appear to affect the entire skeleton, making the biopsy a true representation of the disease process. In osteoporosis however, there is evidence of disease heterogeneity both in bone loss (Davis *et al.,* 1994) and bone cell activity (Podenphant and Engel, 1987, Byers *et al.,* 1997), reducing the diagnostic power of the biopsy. Bone turnover may also differ at various skeletal sites (Parfitt *et al.,* 1985, Eventov *et al.,* 1991).

The possibility of serial biopsies is severely limited, with a maximum of two recommended, one on either side of the pelvis (Weinstein, 1992). Thus the tracking of a patient's response to treatment using this technique is limited. This is compounded by the necessity to sample a new section of bone, with the inevitable increase in variation. A number of studies have reported variance in the measurement of

histomorphometric indices, arising from intra and inter observer variation (Wright *et al.,* 1992), methodological variation (Chavassieux *et al.,* 1985) and sampling variation (de Vernejoul *et al.,* 1981, Chavassieux *et al.,* 1985b).

The procedure for biopsy is by necessity invasive, although it is performed as an outpatient procedure (Compston, 1997). Nevertheless patients may be reluctant to agree to biopsy. Complications of the procedure are minimal with a total incidence of complication of 0.7% (Duncan *et al.,* 1980).

1.9 Models for studying bone remodelling

The difficulties associated with bone biopsies from human subjects increased the need for an animal model of human skeletal physiology. The rat has become an accepted model for investigation of human bone disorders. This acceptance is such that FDA guidelines recommend the use of the oophorectomised rat model for preclinical evaluation of agents for the treatment of osteoporsis (Thompson *et al.,* 1995). Confidence in this model is based on many years of research, which has established the similarity of skeletal physiology between humans and rats.

Trabecular bone production occurs in humans during longitudinal growth from growth plate cartilage (1.5). The process has been shown to be similar in rats (Kimmel and Jee, 1980). Although the rat growth plate does not close as consistently or as early in life as in humans, longitudinal growth rates slow in rats at around 6 months of age and eventually ceases in long bones (Berg and Harmison, 1957, Kalu *et al.,* 1989, Sontag, 1992) and vertebrae (Mori *et al.,* 1990). Rats undergo age related loss of trabecular bone (Wronki *et al.,* 1989, Sontag, 1992), as evident in humans (Parfitt, 1984, Sherman *et al.,* 1995). This loss occurs as a result of a decrease in trabecular

number in rats (Wronki *et al.,* 1989, Li *et al.,* 1990) and humans (Atkinson, 1967, Parfitt, 1984).

BMU based bone remodelling has been demonstrated in the rat (Vignery and Baron, 1980), and shown to follow a similar, but more rapid pattern (Tran Van *et al.,* 1982, Baron *et al.,* 1984). In 12 week old rats the entire remodelling cycle was completed in approximately 38 days, with resorption lasting 1-2 days, formation 33 days and reversal 4 days (Baron *et al.,* 1984), compared to 27 days of resorption, 89 days of formation in young humans (Agerbaek *et al.,* 1991).

However, the rat has been shown to lack some characteristics of human bone physiology. Human cortical bone is remodelled by intracortical cutting cones, creating a Haversian system within the cortices (Recker, 1992). Such intracortical remodelling is rare in rats, with remodelling generally confined to the bone surface. However, it has been observed in association with castration (Wink and Felts, 1980) and prostaglandin administration (Jee *et al.,* 1990). It has been suggested that rats do not show an age related decrease in osteoblast function (Wronski *et al.,* 1989b) as seen in very old humans (Parfitt 1984). However, the same group has recently shown a decrease in wall width with age, 12 months after oophorectomy at 3 months of age (Wronski *et al.,* 1999), suggesting that osteoblast activity is reduced. Most importantly, this rodent model does not develop spontaneous fracture following ovarian hormone deficiency. Thus studies in this model investigate osteopenia not osteoporosis. This also limits the usefulness of this model more to alterations in bone cell activity and changes in trabecular bone structure.

1.10 Factors influencing remodelling

1.10.1 Ovarian hormone deficiency

Cessation of the normal supply of ovarian hormones, as occurs naturally at the menopause, or following surgical intervention such as oophorectomy, has a profound and characteristic effect on bone turnover. The initial change is an increase in bone resorption, detected biochemically as an increase in breakdown products of bone in urine (Gertz *et al.,* 1994, Lewis *et al.,* 2000). The coupled nature of bone remodelling results in a compensatory increase in bone formation, detected biochemically as an increase in bone formation metabolites in the blood (de Leo *et al.,* 2000). These changes are rapid and may manifest 6 months after the cessation of ovarian hormone production (Stephan *et al.,* 1987).

The postmenopausal increase in bone turnover however, is not balanced. Trans-ilial biopsy data suggest the increase in resorption is greater than the formative compensation, and as a result bone is lost from the skeleton (Eriksen *et al.,* 1985). This bone loss is greater from trabecular sites than cortical (Riggs *et al.,* 1981, Stephan *et al.,* 1987), involving loss of entire trabeculae struts not generalised thinning (Arlot *et al.,* 1990, Parisien *et al.,* 1995). The bone loss is initially rapid, reducing BMD by around 12% in the first five years (Nordin *et al.,* 1990), followed by a period of slower bone loss (Stephan *et al.,* 1987, Eastell *et al.,* 1988), increasingly the result of age related processes.

The changes in bone cell activity are reflective of the skeletal losses. The initial changes are proresorptive. Activation frequency increases by around 45% (Eriksen *et al.,* 1990). Consistent with an increase in turnover after ovarian hormone deficiency, biochemical indices of resorption reach a peak prior to those of formation after oophorectomy in women, with both reducing during subsequent years (Stephan *et al.,*

1987). While resorption depth is not increased with age (Eriksen *et al.,* 1985b) it has been suggested that individual osteoclast activity is increased in the perimenopause (Eriksen *et al.,* 1985) and in osteoporotics (Gruber *et al.,* 1986, Eriksen *et al.,* 1990). The latter finding however, has been challenged (Cohen-Solal *et al.,* 1991b) and may be subject to variation with technique (Compston, 1997). The histologic features of osteoporosis have been shown to be heterogeneous, independent of difficult techniques such as resorption depth assessment. Osteoporotic patients have been shown to display a spectrum of skeletal kinetics varying from high to low turnover disease (Whyte *et al.,* 1982, Kimmel *et al.,* 1990). This may relate to the variation in the kinetics of bone loss with time after menopause, as mentioned above. The initial phase of rapid bone loss is dominated by osteoclast activity, with osteoblast activity increasingly important with time.

The increase in resorption following ovarian hormone deficiency may be temporary, however there is evidence for a sustained reduction in individual osteoblast activity (Arlot *et al.,* 1984, Cohen-Solal *et al.,* 1991b). This reduction in formation is directly responsible for the long term imbalance in remodelling, which results in a reduction in wall thickness and therefore trabecular thickness with increasing age (Lips *et al.,* 1978). This reduction is enhanced in osteoporotics, with reductions of over 20% reported 10 years after the menopause (Cohen-Solal *et al.,* 1991).

The osteopenia resulting from these alterations in skeletal physiology is often of such a magnitude as to result in characteristic atraumatic or postmenopausal fractures, most often in the spine, hip and wrist (Wasnich, 1999). This pathological weakening of the skeleton is so common that 40% of women and 13% of men over the age of 50 can expect such a fracture in their lifetime (Melton III *et al.,* 1992). In addition, these fractures are associated with significant morbidity and mortality, with 18% of fracture

patients requiring long term care following a hip fracture, with 24% not surviving the subsequent 12 months (Schurch *et al.,* 1996). Clearly osteoporosis is a significant medical and social issue, continually compounded by the advance in world population life expectancy (Sowers, 2000).

1.10.1.1 Studies in the Rat

The ability of rat skeletal physiology to model human conditions has enabled the changes following surgical oophorectomy to be further characterised. This model has been shown to reproduce menopausal skeletal and bone cell changes, via surgically induced ovarian hormone deficiency. Foundation studies by Wronski *et al.* demonstrated significant increases in resorption and formation (Wronski *et al.,* 1988), which were associated with progressive and profound trabecular bone loss in the proximal tibial metaphysis (Wronski *et al.,* 1989).

The rat has consistently shown increased bone turnover following oophorectomy, which develops rapidly after operation. Increased resorption has been recorded within one week of operation (Dempster *et al.,* 1995, Sims *et al.,* 1996), with bone changes as early as 5 days (Lane *et al.,* 1998, Tanizawa *et al.,* 2000) and well developed by 10 days (Dempster *et al.,* 1995). Bone formation increases follow the rise in resorption (Wronski *et al.,* 1988, Sims *et al.,* 1996). Bone loss is progressive, and similar to human postmenopausal bone loss, continues rapidly for an initial period followed by a slowing of bone loss (Wronski *et al.,* 1989(b), Dempster *et al.,* 1995). This initial bone loss is severe, with losses of over 50% not uncommon in the metaphyses of long bones (Shen *et al.,* 1995, Qi *et al.,* 1995, Sims *et al.,* 1996).

Osteopenia is a characteristic finding following oophorectomy in the rat (reviewed in Kalu, 1991, Frost and Jee, 1992, Miller *et al.,* 1995). Cortical bone loss is

not a feature of ovarian hormone deficiency in long bones (Kimmel and Wronski, 1990, Shen *et al.,* 1995) or the vertebrae (Chachra *et al.,* 2000). Indeed, at the tibio-fibular junction, cortical area and periosteal bone formation rate were increased 16% and 900% 6 weeks after operation and 31% and 260% 12 weeks after operation, respectively (Bagi *et al.,* 1993). Oophorectomy-induced osteopenia has been characterised as occurring due to trabecular perforation and not trabecular thinning (Dempster *et al.,* 1995, Sims *et al.,* 1996). Thinning of trabeculae however, has been recorded in regions of greater initial trabecular thickness, such as the femoral neck (Bagi *et al.,* 1997, Li *et al.,* 1997). This perforation of trabeculae results in an increase in disconnectivity within the trabecular lattice as measured by node-strut analysis (Dempster *et al.,* 1995), direct sterology (Boyce *et al,* 1995) or X-ray tomography (Lane *et al.,* 1998), decreasing its strength (Jiang *et al.,* 1997). However, this decrease is revealed only upon mechanical testing, and not as the result of spontaneous fracture. The trabecular bone of the oophorectomised rat is therefore an excellent model for certain aspects of postmenopausal osteoporosis. Similar bone cell and structural changes in trabecular bone facilitate the study of pathogenesis. The osteopenia is large, rapid and widespread allowing definite reactions to be demonstrated and multiple remodelling cycles to include in a relatively short experimental period. However, it is unable to directly relate to the most important outcome for the osteoporotic patient, bone fracture.

Oophorectomy induced bone loss has been reported in many regions of trabecular bone such as the distal femur (Westerlind *et al.,* 1994, Sims *et al.,* 1996), proximal femur (Li *et al.,* 1997, Shen *et al.,* 1997), vertebrae (Wronski *et al.,* 1989, Miyakoshi *et al.,* 1999), ilium (Kalu *et al.,* 1989) and mandible (Tanaka *et al.,* 1999). The variation between these sites being quantitative rather than qualitative. Bone loss is

equally rapid in the metaphyses of long bones, but is reduced in speed and magnitude in vertebrae. Bone loss is absent in the first lumbar vertebrae at 18 days post oophorectomy (Sims *et al.,* 1996), but is developed by 30 days, although at a reduced level of bone loss compared to the long bone metaphysis (Wronski *et al.,* 1989b). This vertebral response has been shown to involve reduced increases in bone turnover compared to the proximal tibial metaphysis (Qi *et al.,* 1995).

Interestingly, some regions of trabecular bone have been shown resistant to oophorectomy induced bone loss (Yamazaki and Yamaguchi, 1989, Durbridge *et al.,* 1990, Turner *et al.,* 1994b, Westerlind *et al.,* 1997, Tanaka *et al.,* 1999, Turner *et al.,* 1999). The trabecular bone of the femoral epiphysis did not loss bone 11.5 months after operation (Westerlind *et al.,* 1997), similar to the cortices of long bones as mentioned above. Osteopenia was absent in this region despite elevated bone turnover at this time. This epiphyseal resistance to osteopenia is known to be reversed by functional unloading and metaphyseal bone loss is reduced by treadmill exercise (Turner *et al.,* 1999). Thus factors intrinsic to some regions of bone, such as mechanical strain gradients, may have a strong modulatory effect on ovarian hormone deficient bone loss in the rat. This is consistent with the recovery of initial bone loss in the heavily loaded subchondral region of the rat mandibuar condyle (Tanaka *et al.,* 1999), while bone loss was permanent from the less loaded central region. Modulation of bone loss by loading is also consistent with the reduction in the extent of osteopenia following oophorectomy, due to increased body weight (Wronski *et al.,* 1987).

1.10.2 Increased ovarian hormone levels

The increased turnover and bone loss associated with ovarian hormone deficiency is stopped by exogenous oestrogen treatment in humans (Raisz *et al.,* 1996, Heikkinen *et*

al., 1997). Hormone replacement therapy (HRT) has been the cornerstone of osteoporosis treatment. The rationale that exogenous ovarian hormone replacement will prevent the osteopenia associated with postmenopausal osteoporosis has been supported by a variety of study procedures. Biochemical markers of bone turnover are reduced by HRT in the short (Brown *et al.*, 1984, Raisz *et al.*, 1996) and long term (Wimalawansa, 1995, Heikkinen *et al.*, 1997). Further, these changes reflect the changes in BMD in short (Steiniche *et al.*, 1989, Akesson *et al.*, 1995, Cosman *et al.*, 1996) and long term trials (Storm *et al.*, 1990, Liberman *et al.*, 1995, Eiken *et al.*, 1996).

1.10.2.1 Studies in the rat

The response to exogenous oestrogen supplementation has been shown to be similar in the oophorectomised rat (reviewed in Kalu, 1991, reviewed in Turner *et al.*, 1994b). The effects of excess oestrogen have also been studied in the rat. Increased doses of 17β oestradiol have been shown to continue to reduce bone turnover, with subsequent increases in bone volume due to contraction of the remodelling space (Takano-Yamamoto and Rodan, 1990, Kalu *et al.*, 1991). Direct infusion of high doses of 17β oestradiol into the medullary cavity of oophorectomised rats resulted in depression of resorption and increased osteoblast proliferation, suggesting a direct effect of this compound on osteoblastic cells (Samuels *et al.*, 1999), as seen in vitro (Gray *et al.*, 1987, Ernst *et al.*, 1988).

The exact nature of oestrogen's effect on bone cells *in vivo* has attracted considerable investigation. It has been reported that oestrogen may exert an anabolic effect on osteoblasts (Abe *et al.*, 1992, Chow *et al.*, 1992). This has not been a common finding however, with oestrogen's effect on osteoblasts generally confined to

decreasing bone formation as part of a general reduction in bone turnover, secondary to its antiresorptive effects (reviewed in Turner *et al.,* 1994b). The inconsistencies may relate to the use of oophorectomised animals in studies of oestrogen replacement, as the anabolic effect has only be observed in intact animals (Abe *et al.,* 1992). This may relate to the increase in bone resorption, which results in a simultaneous reduction in preexisting oestrogen dependent bone formation (Chow *et al.,* 1992).

1.10.3 Mechanical forces

The role of mechanical signals in modulating bone structure has long been acknowledged. Weight bearing has been one of the essential functions of the skeleton since terrestrial habitation. In order for the skeleton to adapt to altering mechanical needs, bone remodelling must be controlled to some extent by the external forces experienced by the skeletal tissue. Modern examples of this interaction are seen in the osteopenia that develops during space flight (Stupakov *et al.,* 1984) and the increased bone density of elite athletes (Gleeson *et al.,* 1990). It can even be measured within an individual, with greater cortical thickness in the humerus of the dominant arm of professional tennis players (Jones *et al.,* 1977).

A number of theories have been proposed to explain the relationship between loading and bone adaptation (Hart *et al.,* 1984, Fyhrie and Carter 1986, Frost 1987). However the emerging paradigm appears most closely aligned with the latter theory. The Frost "mechanostat" model states that bone adapts in predictable ways dependent on the dynamic strain environment experienced by the bone tissue. Basically stated, low strain results in loss of bone and high strain results in gain of bone. It further states that these transitions occur within well defined mechanical usage windows, by well defined cellular processes, with modelling at the extremes and remodelling at

moderate levels. More recently however, the increased use of computer models (Garrahan *et al.,* 1990, Jensen *et al.,* 1990, Van Rietbergen *et al.,* 1999, Huiskes *et al.,* 2000), animal models (Mosley *et al.,* 1997, Hsieh *et al.,* 1999, Zaman *et al.,* 1999) and critical interpretation (Lanyon, 1992, Lanyon, 1996, Skerry *et al.,* 1997, Turner, 1998, Turner, 1999, Weiner *et al.,* 1999) has enabled Frost's hypothesis to be modified to more closely match clinical observations.

1.10.3.1 Decreased mechanical usage

This situation is seen most commonly in patients after limb immobilsation (Kannus *et al.,* 1994), chronic bed rest (Le Blanc *et al.,* 1990) and as the result of space flight (Stupakov *et al.,* 1984). Bone loss is greatest from those bones most stressed by gravity such as the calcaneus. Calcaneal BMD decreased by up to 19% following a flight over 4 months in duration (Stupakov *et al.,* 1984) and 10% following bed rest of a similar period (LeBlanc *et al.,* 1990). The response of bone cells to decreased mechanical usage typically involves a decrease in bone formation. This has been recorded following both space flight (Morey and Baylink, 1978) and extended bed rest (Vico *et al.,* 1987, Zerwekh *et al.,* 1998). Observations of the osteoclastic response have not been so consistent. An increase in both resorption depth and osteoclast surface has been reported in iliac crest trabecular bone following 12 weeks of bed rest (Zerwekh *et al.,* 1998). This finding has been supported by short term (Nishimura *et al.,* 1994) other bed rest studies (Vico *et al.,* 1987) and following paraplegia (Miniare *et al.,* 1974). However, studies of longer duration have failed to record an increase in resorption, leading to the suggestion that the increase in resorption is transient (Palle *et al.,* 1992).

1.10.3.2 Studies in the Rat

Microgravity, such as experienced during space flight, can be reproduced in rats using hind limb immobilisation via bandaging (Chen *et al.,* 1992, Bagi and Miller, 1994, Iriji *et al.,* 1995, Ma *et al.,* 1995) or denervation (Yoshida *et al.,* 1991, Shen *et al.,* 1997, Yonezu *et al.,* 1999), hind limb elevation models (Globus *et al.,* 1984, Abram *et al.,* 1988, Dehority *et al.,* 1999) or short term space flight (Wronski and Morey, 1983, Vico *et al.,* 1991, Vailas *et al.,* 1992, Westerlind *et al.,* 1997). The bone cell changes in cortical bone following unloading, in a similar manner to human studies, display a consistent reduction in periosteal mineral apposition rate (Weinreb *et al.,* 1989, Bagi *et al.,* 1993, Kodama *et al.,* 1997). Endocortical resorption is also elevated (Bagi *et al.,* 1993, Zeng *et al.,* 1996, Shen *et al.,* 1997). The resulting bone loss has been reported by densitometry (Kodama *et al.,* 1997), peripheral quantitative computed tomography (Yonezu *et al.,* 1999) and histology (Zeng *et al.,* 1996). These methods report a decrease in periosteal perimeter and increase in endosteal perimeter, with resultant decreases in strength (Bagi *et al.,* 1993).

In trabecular bone, formation is also consistently decreased with reduced formation surface (Shen *et al.,* 1997), or both surface and mineral apposition rate (Thompson *et al.,* 1990, Akamine *et al.,* 1992, Chen *et al.,* 1992) contributing to the reduction in bone formation rate. The osteoclastic response to skeletal unloading is very rapid in trabecular bone, with increased resorption recorded as soon as 24 hours after sciatic neurectomy (Thompson *et al.,* 1990). This elevation has been shown to return to control levels 14 weeks (Chen *et al.,* 1992) to 16 weeks (Iriji *et al.,* 1995) after bandaging. Similar increases have been demonstrated after neurectomy (Zeng *et al.,* 1996). Increases in resorption following space flight have not been as consistent, with no increase in trabecular bone resorption following a 7 day flight (Vico *et al.,*

1991), suggesting fluid shifts within the bone due to usage alterations at normal gravity may be important to the osteoclastic response.

The structural changes involve reduced trabecular number and thickness (Chen *et al.,* 1992, Bagi and Miller, 1994, Iriji *et al.,* 1995, Zeng *et al.,* 1996, Kodama *et al.,* 1997), with corresponding declines in both connectivity (Chen *et al.,* 1992, Zeng *et al.,* 1996 and strength (Kodama *et al.,* 1997). These trabecular bone reductions do not display the regional variation evident after oophorectomy, with significant bone loss after 2 and 6 weeks (Westerlind *et al.,* 1997) and 18 weeks (Iriji *et al.,* 1995) after immobilisation in the femoral epiphysis.

1.10.3.3 Increased mechanical usage

This situation is most commonly seen in elite athletes such as weight lifters (reviewed in Karlsson *et al.,* 1995) and in response to high impact exercise (Heinonen *et al.,* 1996, Welsh and Rutherford, 1996). The changes in bone mass in response to controlled exercise regimes have been generally modest. Changes of 1-2%/year have been reported following aerobics and weight training (Friedlander *et al.,* 1995) and resistance training (Lohman *et al.,* 1995), although gains up to 5% have been reported after just 5 months of training (Simkin *et al.,* 1987). The nature of the training regimes however, may not have promoted the most appropriate stimulus for osteogenesis. Bone mass is higher in athletes undergoing high impact activities, such as gymnastics, when compared to compared to running (Robinson *et al.,* 1995) and swimming (Taaffe *et al.,* 1995). It must be noted however, that even low impact training in the elderly can reduce fracture rates, without increase in bone mass, by reduction in falls (Cummings *et al.,* 1995) due to increased balance and postural stability (Lord *et al.,* 1996).

1.10.3.4 Studies in the rat

The ability to precisely control externally applied loads to rats has resulted in considerable advances in the knowledge concerning the effects of increased mechanical usage on bone physiology. The majority of studies have employed bending forces to the cortical regions of long bones *in vivo* (Turner *et al.,* 1991, reviewed in Forwood and Turner, 1995) although isolated bones (reviewed in Lanyon, 1996) and the application of external weight during exercise (van der Wiel *et al.,* 1995) have also been shown successful. The increases following external loading can be very large, with woven bone formation possible on the periosteal surface above a defined load threshold (Forwood *et al.,* 1998). However reduction in loading conditions produces lamellar bone on both periosteal and endosteal surfaces (Turner *et al.,* 1994e). This technique has shown the importance of dynamic loads compared to static loads (Lanyon and Rubin, 1984), strain rate (Turner *et al.,* 1994b) and magnitude (Forwood *et al.,* 1998). These studies have enabled substantial modification of Frost's 'mechanostat' theory (Turner, 1998).

The effect of increased mechanical usage in trabecular bone can also be studied in contralateral limb in concert with unloading (Tuukkanen *et al.,* 1991) or following reversal of bandaging induced immobilisation (Tuukkanen *et al.,* 1991, Maeda *et al.,* 1993, Ijiri *et al.,* 1995, Kannus *et al.,* 1996). These studies have shown increases in bone volume associated with restoration of function, albeit incomplete. This is associated with an increase in trabecular thickness, with no recovery of lost trabecular struts (Maeda *et al.,* 1993, Ijiri *et al.,* 1995). This permanent loss of trabeculae accounts for the deficit in bone mass recorded after recovery. This is consistent with the response in the distal femur (Kannus *et al.,* 1996), proximal femur (Sogaard *et al.,*

1994), and proximal tibial metaphysis following treadmill training (Bourrin *et al.*, 1995). Reduction in osteoclast surface and number, and an increase in mineralising surface accompanied the latter post training increases in trabecular thickness.

1.10.4 Dietary calcium intake

1.10.4.1 Increased

The physiology of calcium homeostasis involves complex regulation, enabling rigid control of serum ionized calcium levels whilst also permitting the myriad of calcium dependent functions to continue. Bone and teeth contain 99% of the bodies calcium (Morris, 1994), and is thus is a vital region of regulation within this system. The other major organs involved in calcium homeostasis are the kidney and the gut (Broadus, 1999), via the precise monitoring of the calcium sensing receptor (Brown *et al.*, 1993) and the actions of PTH and 1,25 $(OH)_2 D_3$ (Bushinsky and Monk, 1998).

The recommended human calcium intake varies with age, high during growth leveling off, then increasing again at the menopause, with a recommended daily intake of between 1200mg (Nordin, 1997) and 1500 mg (NIH, 1994) during this latter period. However, if this requirement is not met through dietary intake, bone will be mobilised to supply the persistent homeostatic requirements (Bushinsky and Lechleider, 1987). The magnitude of the store of calcium in bone relative to daily metabolic requirements is such that a significant lag time exists between calcium deficiency and pathological manifestation such as increased fragility (Power *at al.*, 1999). However, as self chosen calcium intakes remain generally below recommended levels (Musgrave *et al.*, 1989), chronic low dietary calcium intake remains a significant factor in osteoporosis (Heaney, 1993).

The primary effect of calcium deficiency on bone remodelling is an increase in resorption (Wright and McMillan, 1994). This is PTH mediated (Jowsey and Raisz, 1968), with extracellular calcium concentration regulating the secretory rate of the parathyroid gland (Orloff *et al.,* 1989). Studies investigating the effect of calcium supplementation on skeletal health may be associated with some difficulties (Dawson-Hughes, 1991), however there is clear evidence for a positive relationship of calcium intake and bone mass (reviewed in Dawson-Hughes, 1991, reviewed in Nordin, 1997). A study involving 1800 very old women, elevated intake from 500 mg/day to 1700 mg/day for 18 months, with a resulting reduction in fracture rate at the hip and other extremities of 20-40% (Chapuy *et al.,* 1992). Consistent with this reduction in fracture, the supplemented group gained 2.7% BMD, while the controls lost 4.6%. More modest increases in BMD (+1.5%) were reported following 4 years supplementation at 1200 mg/day, with a significant reduction in fracture rate (Recker *et al.,* 1996).

1.10.4.2 Studies in the rat

The ability to easily control the dietary intake of laboratory animals has enabled precise evaluation of the effects of diminished and elevated dietary calcium intake on bone cell activity and trabecular structure. Consistent with human studies, the rise in resorption following dietary calcium reduction has been shown to be PTH mediated, with no increase in resorption after thyroparathyroidectomy (Antic *et al.,* 1996). In cortical bone, reduction in BMD is a consistent finding following prolonged calcium deficiency (Kunkel *et al.,* 1990, Shen *et al.,* 1995, Moriya *et al.,* 1998), with rapid recruitment of osteoclasts to the endosteal surface (Wright and McMillan, 1994), and reduced cortical thickness (Weinreb *et al.,* 1991).

In trabecular bone this loss is manifest rapidly, with osteopenia reported 24 hours after dietary restriction. Bone turnover is elevated, with resorption increased within 24 hours and formation within 72 hours (Masarachia *et al.,* 1998, Seto *et al.,* 1999). Increases in both osteoclast and osteoblast cell numbers occur within this time. The initial structural change is a reduction in trabecular number (Seto *et al.,* 1999). Trabecular thickness may be subsequently reduced during this early period, with reduced thickness reported after 72 hours (Masarachia *et al.,* 1998, Seto *et al.,* 1999). One month of low calcium feeding at 0.1% dietary calcium revealed decreased bone volume, trabecular number and strength with no elevation in osteoclast surface in the ends of long bones (Shen *et al.,* 1995), indicating that the increase in resorption and reduction in trabecular thickness are transient. Studies of longer duration using a similar diet have reported similar findings after 12 and 28 weeks (Yoshitake *et al.,* 1999). Longer studies also detect a reduction in cortical bone mass and strength. Calcium deficient bone loss has also been reported in the mandible (Rosenquist and Lundgren, 1992) and the membranous bones of the calvaria (Liu *et al.,* 1989). The extent of bone loss can be extensive with a 32% reduction in tibial cortical area after 18 days of complete calcium deprivation (Liu *et al.,* 1989). Thus calcium deficient osteopenia does not show the regional variation evident after oophorectomy, but rather, effects both cortical and trabecular surfaces.

Calcium replenishment in deficient rats reduces osteoclastic resorption within 3 hours (McMillan *et al.,* 1989), with complete osteoclast recession and replacement by osteoblasts after 1-3 days (Liu *et al.,* 1982). The dietary calcium requirement for immature rats to achieve normal skeletal status has been estimated at 0.4% (Bell *et al.,* 1941). Thus most laboratory animals receive excess dietary calcium. Consistent with changes induced by reduced calcium intake, the high calcium diets of normal

laboratory chow, would reduce calcium homeostatic resorption of bone. This would produce a thickening of trabeculae and a decreased probability of trabecular perforation (Eriksen, 1986).

1.11 Summary and hypotheses

It is clear that the regulation of skeletal tissue is complex, with simultaneous impact of multiple regulatory stimuli being modulated to optimise both mechanical and homeostatic functions. These functions however, are not uniformly distributed across the skeleton. Mechanical functions are preferentially performed by cortical bone, while trabecular bone is preferentially responsive to ovarian hormone deficiency. This heterogeneity suggests an element of local control in bone response to systemic modulators of bone turnover.

There is substantial evidence of Local modulation of bone turnover in postmenopausal osteoporosis. The disease process is known to display heterogeneity in bone loss and bone cell activity (1.8.3), with fracture typically affecting some bones and not others. Local control is also evident in the pathology of focally active skeletal disorders such as pagets disease (Davie *et al.,* 1999) and regional osteoporosis (McCarthy *et al.,* 1998).

Mechanical load is not uniformly distributed throughout the skeleton, and has been shown to affect local bone turnover after disuse (1.10.3.1) and oophorectomy (1.10.1.1). Calcium deficiency in contrast, appears to manifest a more generalised osteopenia (1.10.4.1), suggesting lessened or alternate levels of local control of bone turnover.

The rat model provides the level of control necessary to investigate the interaction of these systemic factors, such as calcium and ovarian hormone deficiency, with local factors. Ovarian hormone levels and calcium intake, both difficult to control in

humans, are easily maintained in rats. Further, the interaction of these two systemic regulators and local factors such as mechanical load, can be effectively monitored in the distal femur. The trabecular bone of the epiphysis experiences high load, while in the metaphysis load decreases with distance from the growth plate (Westerlind *et al.*, 1997). This region therefore represents an *in vivo* system for examination of loading interactions in trabecular bone at physiological strains without the necessity of external force transducers. The distal femur also provides the opportunity to examine the effects of altered turnover in regions of varied architecture, particularly trabecular thickness, which varies widely from the epiphysis to the distal regions of the metaphysis.

This thesis will examine the following hypotheses:

Hypothesis 1

The estimation of osteoclast surface in rat histological sections following oophorectomy is effected by the method used and the area sampled.

Hypothesis 2

The short term temporal relationship between oestrogen deficiency and altered bone cell activity and osteopenia in the distal femur is effected by local mechanical strain.

Hypothesis 3

The long term temporal relationship between oestrogen deficiency and altered bone cell activity and osteopenia in the distal femur is effected by local mechanical strain.

Hypothesis 4

Exogenous oestrogen supplementation will inhibit the ability of mechanical strain to alter the local response to oestrogen deficiency.

Hypothesis 5

The effects of dietary calcium restriction will be independent of those of mechanical strain following oophorectomy.

Hypothesis 6

Oestrogen deficiency has specific effects on skeletal development during growth and these are effected by local mechanical strain.

Chapter 2
Materials and Methods

2.1 Animals

Virgin female, outbred Sprague-Dawley rats were used in all studies and were obtained from the Gilles Plains Animal Resource Centre (Gilles Plains, South Australia) and kept at 22°C on a 12 h light:dark cycle in cages of six. Animals oophorectomised prior to puberty were supplied at three weeks of age and underwent operation immediately upon arrival. All other animals were supplied at 5 months of age, and allowed to acclimatise to conditions at the IMVS animal care facility prior to use.

2.2 Diet

Rats were fed commercial rat chow containing 0.76% calcium, 0.46% available phosphorus and 2000 IU/kg vitamin D_3 (Milling Industries, Murray Bridge, South Australia) and tap water *ad libitum* unless otherwise stated.

In studies restricting dietary calcium intake (Chapters 5 and 6), a semi-synthetic diet (AIN-76A) was produced (American Institute of Nutrition, 1977) in which the calcium content was either to 0.2 or 0.04 g/100g diet. The ingredients of this diet are shown in tables 2.1 and 2.2.

AIN-76A-starch semi-synthetic diet

Ingredient	g/100g
casein	20
corn starch	65
cellulose	5
corn oil	5
DL-methionine	0.3
choline bitartate	0.2
calcium carbonate (0.04-0.2%)	1 - 5
mineral mix (calcium deplete)[1]	3.5
vitamin mix[2]	1

Table 2.1 Ingredients for the AIN-76A semi-synthetic rodent diet.

AIN-76A-mineral mixture

Ingredient	mg/kg diet
sodium di-hydrogen phosphate	6895
potassium di-hydrogen phosphate	9625
potassium sulphate	1820
magnesium oxide	840
manganous carbonate	123
ferric citrate	210
zinc carbonate	56
chromium potassium sulphate	19
cupric carbonate	11
potassium iodate	0.4
sodium selenite	0.4
sucrose	15400

Table 2.2 Ingredients for the mineral mix of the AIN-76A semi-synthetic rodent diet.

Semi-synthetic diet preparation protocol was derived from previous studies (O'Loughlin and Morris, 1998). Cornstarch, casein and cellulose were mixed for 30 minutes in a dough mixer (OEM, Modena, Italy). The remaining dry ingredients were added slowly and the mixture combined for a further 30 minutes. The ingredients of the mineral mix were weighed, crushed in a mortar and pestle and mixed manually for 5 minutes before addition with the remaining dry ingredients. Following this procedure, the corn oil was drizzled into the mixture and combined for a further 30 minutes. Finally distilled water was added to form a thick paste, which was poured

into trays and allowed to partially solidify before being cut into blocks and frozen at -20°C. Animals were fed 120 g of diet per cage of 6 rats at 5 pm every day. Diet was not kept for periods exceeding 6 weeks.

2.3 Fluorochrome labelling

Demeclocycline and calcein were used as the two fluorochrome labels throughout all studies. All solutions were prepared on the day of administration. Demeclocyline (Lederle, Sydney, Australia) was prepared by adding 0.5g of declomycin, containing 0.3g of demeclocycline, to 10 mL of normal saline and stirring for 2 hours. The supernatant was decanted and 0.1 mL/100g body weight was injected i.p. 6 days prior to killing, for the desired dose of 30 mg/kg. Calcein (Sigma Chemical Co., St Louis, USA) was prepared by adding 0.2g of calcein and 0.4g of sodium bicarbonate to 10 mL of normal saline and stirring for 30 minutes. 0.1 mL/100g body weight of this solution was injected i.p. 2 days prior to killing, for the desired dose of 20 mg/kg.

2.4 Surgery

2.4.1 Anaesthesia

Anaesthesia was provided by inhalation of a gas mixture containing 3.5% isofluothane (Zeneca Ltd., Macclesfield, UK) in a 2:5 NO_2/O_2 gas mixture (BOC Gases, Sydney, Australia). Immediately prior to surgery the animal was placed in a clear perspex box connected to the anaesthetic supply, enabling observation during initial anaesthesia. Once immobile the animal was weighed and a plastic cap fitted over the head which enabled continuous anaesthesia. Completeness of anaesthesia was assessed prior to any operative procedure by applying acute pressure with non-toothed forceps to the

skin between the toes of the left hind foot. Any retraction of the limb was evidence of incomplete anaesthesia. No deaths were recorded as a result of anaesthesia.

2.4.2 Prepubertal oophorectomy: Ventral approach

Prepubertal surgical procedures were performed using a ventral approach, providing greater exposure of the abdominal organs, in order to locate the immature sex organs. Following anaesthesia, animals were secured ventral side up with their hind limbs extended. The abdomen was shaved of hair from the distal margin of the sternum to the proximal margin of the genitals and swabbed with iodine antiseptic (Fauldings, Adelaide, Australia). A sterile shroud with a 5 cm circular aperture was placed over the animal.

A 1.5 cm opening was made in the abdominal skin perpendicular to the long axis of the spine. The skin was torn rather than cut in order to facilitate postoperative healing, by extension of scissor blades. The abdominal cavity was entered through a peritoneal incision of the same size made in the same manner. The uterine horn was traced from its junction behind the bladder to its end point at the fallopian tube and ovary. The ovary was externalised, and in the case of oophorectomy (Oophx), the uterine horn was clamped below the fallopian tube then tied with '000' silk (Johnson and Johnson, Sydney, Australia) below the clamp. The ovary and the fallopian tube were then dissected from the uterine horn above the clamp, which was slowly released to check for excessive bleeding and the uterine horn replaced. The process was repeated for the other ovary. The procedure took approximately 20 minutes. In the case of sham operation (Sham), the ovary was externalised for the same period of time taken to complete oophorectomy. The wound was closed in layers using dissolving

polyglactin suture (Johnson and Johnson, Sydney, Australia) and '00' silk (Johnson and Johnson, Sydney, Australia).

2.4.3 Mature oophorectomy: Dorsal approach

The trabecular bone of the distal femur changes constantly with age. Chapter 7 reports the extent to which this region is modified during the first 18 months of life. Peak BV/TV is achieved at around 5 months of age in all regions (7.3.4), as femoral lengthening ceases (7.3.2). As age increased BV/TV fell 6% in the metaphysis due to decreasing trabecular number but increased 8% in the epiphysis due to increasing trabecular thickness. As a result, an operative age of 7 months was chosen for studies of the mature skeleton, to optimise the volume of bone remaining within each region and the stability of the remnant volume.

A dorsal approach was used in mature animals in order to minimise the physiological disruption due to the operative procedure. Dorsal incisions avoided weight bearing on the wound and the possibility of infection by contact with the cage floor. Entry through the thinner musculature of the lateral peritoneum reduced the amount of tissue damage and prevented the animal interfering with the wound.

Following anaesthesia, animals were secured, abdomen down, the lower half of the dorsal aspect was shaved of hair, swabbed with iodine antiseptic (Fauldings, Adelaide, Australia), and covered with a sterile shroud. A 1.5 cm incision was made in the skin perpendicular to the long axis of the spine, approximately 4 cm above the base of the tail. Using round tipped scissors the skin was blunt dissected from the underlying layers, parallel to the line of the incision, allowing lateral access to the abdominal cavity through a 1 cm incision. The ovary was located in a fat pad distal to the kidney and was externalised. Unlike the prepubertal animals, the mature ovary was

easily located due to its rich red colour within the fat and the prominent blood vessels of the uterine horns. Oophorectomy and sham procedures were identical to the ventral approach (2.3.1). The lateral entry and small incision made the closure of the musculature unnecessary. The incision in the skin was closed using wound clips (Becton Dickson, Sparks, MD), which were shed onto the cage flooring following healing.

2.4.4 Recovery

Rats recovered from the surgery in individual cages, with overhead heating lamps to aid thermoregulation until conscious and mobile. Checks were performed every 20 minutes during the immediate postoperative period. Upon initial recovery, when conscious and mobile, animals were returned to communal cages. A further check was carried out the following morning in which the sutures and the general behaviour of the animal were inspected. If an animal recovered poorly it was isolated for observation. Indications of poor recovery included slow or stilled ambulation, a contracted, balled stance, raised fur and prominent chromodacryorrhea. Following full recovery, animals were returned to communal cages. Weekly health checks were carried out on all animals to assess general condition and behaviour, ambulation and teeth growth. Overgrown or misaligned teeth were trimmed manually.

2.5 17β oestradiol supplementation

Using sterile technique, under UV light in a laminar flow hood, 2 mm diameter silastic tube was cut into 15 mm lengths and tied closed at one end with '00' silk (Johnson and Johnson, Sydney, Australia). Powdered 17β oestradiol (Sigma Chemical Co., St Louis, USA) was lightly packed inside the tube to a length of 10 mm and the open end tied. These were sterilised by irradiation and maintained sealed and refrigerated until the

time of operation. One implant was placed in the distal abdominal fat pad during location of the ovary (2.4.2).

2.6 Bone harvesting and fixation

Rats were anaesthetised (2.4.1), weighed, and exsanguinated by cardiac puncture. Blood was stored on ice , centrifuged at 3000 rpm for 10 minutes and the serum stored at -70°C. Exsanguination was considered complete in mature animals when 10 mL of blood had been collected. Animals then underwent cervical dislocation in accordance with IMVS Animal Ethics Committee. Completeness of oophorectomy was confirmed at death in all Oophx rats by the absence of ovarian tissue and atrophied uterine horns. Both femora and the right tibia were harvested, being separated out and defleshed before being placed in neutral buffered formalin at 4^0C for 4h when processing to methyl methacrylate (MMA) or overnight when processing to glycol-methyl methacrylate (GMMA) (Chapter 7 only). The animal was discarded in a waste bin and disposed of by the IMVS Animal House.

2.7 Bone sample cutting

Following fixation, femora were washed and stored in 70% ethanol. The right femur was selected for processing, with the left kept at 4^0C until successful processing of the initial sample had been completed. In the prepubertal study, the length of the right femur was determined using a digital micrometer (Digimatic, Mitutoyo, Japan) and the bone was cut transversely one third of total length from the distal end using a slow speed saw (Beuhler Ltd, Lake Bluff, USA) equipped with a diamond tipped blade (van Moppes, Gloucester, UK).

As femoral lengthening was virtually ceased in the rat after 7 months of age (7.3.3), it was not assessed in rats oophorectomised at this age. In these animals the

femora were cut transversely 20 mm from the distal end. The distal sample was cut in the sagittal plane between the greater condyles, retaining two thirds of the cortical bone at the diaphyseal margin within the sample. The outer cortical casing was removed from the central region of the epiphysis and metaphysis to ensure adequate transfer of fluids within the trabecular network, using the slow speed saw.

Similarly, the tibiae were cut transversely 20 mm from the proximal end and the cortical casing was removed from the central region of the epiphysis and metaphysis. The bone sample was bisected in the coronal plane for processing, retaining two thirds of the cortical bone at the diaphyseal margin within the sample.

2.8 Scanning electron microscopy

Femora selected for electron microscopy were cut as per the histological samples (2.7) and marrow was removed manually using a fine brush under running water. The samples were then immersed in 4.5% sodium hypochlorite solution for 40 minutes and rinsed thoroughly under running tap water. Completeness of the marrow removal was confirmed using a dissecting microscope (Olympus Ltd., Tokyo, Japan). The specimens were then dehydrated in ethanol (70%, 2x90%, 2x100%) for one hour each and air dried. They were then attached to aluminium stubs with silver dag and sputter coated with gold for examination in a JOEL JSM 5300 scanning electron microscope at 15 kV (JOEL, Tokyo, Japan). The scans were interfaced directly with a graphics package (Adobe Photoshop, San Jose, USA), allowing compilation of the micrographs without the need for film processing.

2.9 Histology

2.9.1 Resin processing

2.9.1.1 Glycol-methyl methacrylate resin processing for bone samples

All embedding protocols were derived from standard laboratory techniques (Moore, 1996). Following 24h fixation (2.6), samples were dehydrated in graded ethanol (70%, 95%, 2x 100%) for 1 hour each, then transferred into two changes of a 50:50 mixture of glycol (hydroxyethyl) methacrylate monomer (GMA) (Kasei, Tokyo, Japan) and methyl methacrylate monomer (MMA) (BDH, Poole, England), for 48 hours. The final embedding mixture, containing 50:50 GMA/MMA (GMMA), 0.1% w/v benzyl peroxide (Sigma Chemical Co., St Louis, USA) and 0.05% v/v N,N-dimethylanaline (BDH, Poole, England), was poured into moulds (Bio-Rad, North Ryde, Australia). The bone samples were positioned cut side down within the moulds, an aluminium block holder (Bio-Rad, Sydney, Australia) placed on top and the embedding fluid sealed air tight with paraffin. Polymerisation was completed overnight at room temperature. All work was performed in a fume hood and contact with skin was avoided.

2.9.1.2 Resin processing MMA

MMA was considered superior to GMMA due to its hydrophobic nature which resisted swelling during sectioning, the ability to polymerise in the presence of limited amounts of air, adhere sections to slides and depolymerise processed samples (Chappard *et al.,* 1983) MMA was used in all studies in this thesis, with the exception of Chapter 7. Following 4h fixation (2.6), samples were dehydrated in graded acetone (70%, 95%, 2x 100%) for 1 hour each, then transferred into two changes of MMA and 8% w/v K-Plast plasticiser (Medim, Giessen, Germany) each for 24 hours. 4 mL of the

final embedding mixture containing MMA, 8% w/v plasticiser and 0.9% (w/v) K-Plast initiator (Medim, Giessen, Germany) was poured into 25 mL polypropylene tubes and the bone sample immersed, cut surface down. The tubes were tightly capped and transferred to a 37^{0}C waterbath for overnight polymerisation. All work was performed in a fume hood and contact with skin was avoided, with samples maintained at 4^{0}C prior to polymerisation. The embedded samples were cut from the tubes using a band saw and fixed to aluminium block holders (Bio-Rad, Sydney, Australia) with epoxy glue (Selleys, Sydney, Australia).

2.9.2 Section production

The complex geometry of the distal femur required strict adherence to protocol in order to ensure sampling of a standardised region throughout the studies. Following embedding the samples were trimmed to expose the sample area by removing and discarding 10 um sections with a Jüng K motorised microtome (Reichert, Heidelberg, Germany). The sample area was defined as the level at which the greater condyle of the epiphysis was bisected, reaching its maximal area. Excess cortical bone in the diaphysis was removed during trimming, bringing the sample area in this region back to the midline. The correct orientation of the bone sample for sectioning was maintained by ensuring that the cut surface of the bone sample was parallel to the block holder and hence the plane of trimming. Similarly, the tibial region used in osteoclast estimate studies was defined as the level where the epiphyseal region showed maximal dimension, and the diaphysis was midline within the shaft.

Once the sample area was exposed, the block surface was moistened with tap water for GMMA sections or 50% ethanol for MMA sections and three consecutive 5 um sections were cut. Each section was checked under a microscope prior to storage to

eliminate those damaged by the cutting process. GMMA sections were stored and stained free floating and attached to slides with glycergel (Dako, Carpinteria, USA), an aqueous based mountant. MMA sections were flattened onto slides following immersion in a 30:70 mixture of ethlyene glycol mono-ethyl ether (Merck, Kilsyth, Australia) and 70% ethanol heated to 65-70°C. The section was then covered with polyethylene plastic and cartridge paper, clamped and annealed to the slide overnight in a 37°C oven. Prior to staining, MMA was removed by 2x5 minute immersions in 100% acetone. Following staining, sections were dehydrated in ethanol (2x 100%), cleared in xylene (2x 100%) and mounted in xylene based mountant Eukitt (Kinder GmbH and Co., Freiburg, Germany) with the exception of acid phosphatase stained sections which were mounted in glycergel (Dako, Carpinteria, USA). Sections were identified by comparison with the blocks from which they were cut.

2.9.3 Staining methods

2.9.3.1 Modified von Kossa method for identification of calcified tissue

Procedure for modified von Kossa staining was derived from previously published protocol (Page, 1977). Sections previously stored in tap water were washed 2x with distilled water and transferred to an aqueous solution of silver nitrate 0.5% for GMMA sections, 0.1% for MMA sections and exposed to UV light for 45 minutes. Sections were then washed in distilled water and transferred to an aqueous solution of sodium thiosulphate 5% (GMMA), 2.5% (MMA) for 5 minutes. Over exposure obscured trabecular surfaces, therefore staining was conservative, the appropriate end point was dark brown stained mineralised tissue with well defined surfaces (Fig 2.1). Sections were rinsed in distilled water before mounting.

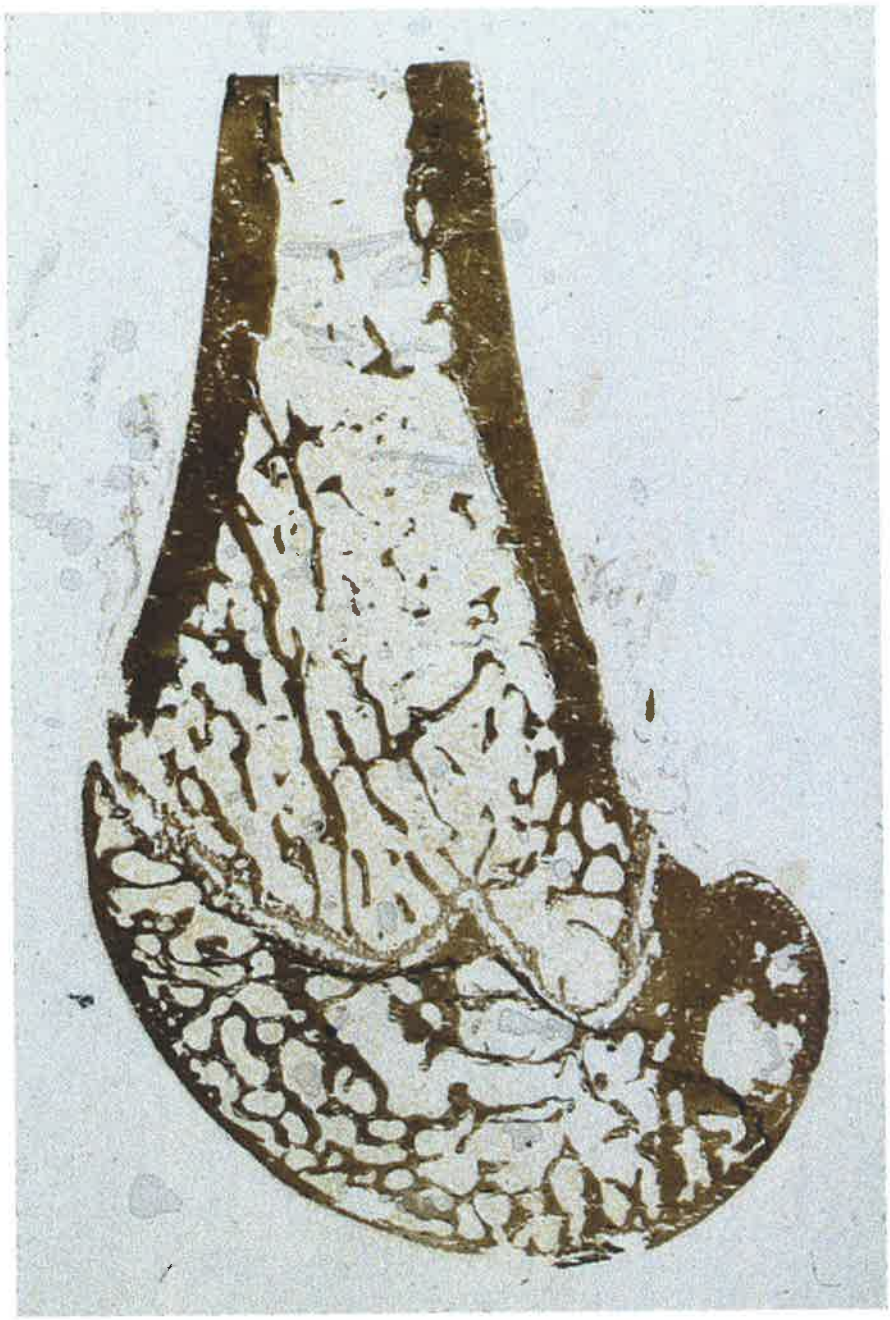


Figure 2.1 Modified von Kossa stained sagittal section of the distal femur of the rat. Calcified tissue is stained dark brown while noncalcified tissue remains unstained. (original magnification x2.5)

2.9.3.2 Von Kossa method for sites of calcium deposition with haematoxylin and eosin counterstain

Procedure for haematoxylin and eosin counterstaining was derived from previously published protocol (Page, 1977). Following the final distilled water wash of the von Kossa staining protocol (2.9.3.1) sections were stained in Lillie Mayer's alum haematoxylin (Page, 1977) for 10 minutes and washed in tap water for 1 minute. The next step involved a combination of differentiation in an acid alcohol solution and blueing in a saturated aqueous solution of lithium carbonate (Sigma Chemical Co., St Louis, USA). The appropriate end-point for this component was blue nuclei and basophilic cytoplasm over a colourless background, overstaining was reduced by further exposure to acid alcohol. Sections were then rinsed in running tap water for 30 seconds and stained with a 1% aqueous eosin solution (Sigma Chemical Co., St Louis, USA) for 3 minutes. The appropriate end point of this component was pink osteoid, and strong staining of acidophilic cytoplasm, such as osteoclasts, while still retaining cytoplasmic texture (Fig 2.2).

2.9.3.3 Enzyme localisation of acid phosphatase

The procedure for enzyme localisation of acid phosphatase was derived from previously published protocol (McNeil *et al.,* 1997). Sections were brought to distilled water and the enzyme reactivated overnight in Tris buffer (pH 9). Following equilibration in acetate buffer (pH 5) for 1 hour, sections were incubated in staining solution. The staining solution contained the enzyme substrate, 40 mg of napthol AS-BI phosphate (Sigma, St Louis, USA) dissolved in 2 mL of dimethyl formamide (BDH, Poole, UK) and a chromophore, 0.1 mL of acidified basic fuschin (Gurr, Poole, UK) in 0.1 mL of 4% aqueous sodium nitrite solution (BDH, Poole, UK). The sodium

nitrite was prepared just prior to use. The fuschin and sodium nitrite solutions were mixed and left for 60 seconds. The final staining solution was freshly prepared by adding 35 mL of the acetate buffer to the substrate solution followed by the acidified basic fuschin, sodium nitrite mixture, the solution was then used immediately. The section were incubated immersed in the final staining solution in a 37°C waterbath for 20 minutes then rinsed in distilled water for 15 minutes (Parkinson *et al.,* 1991). The appropriate endpoint was a strong red stain in the cytoplasm of some cells with a colourless background. The cell nuclei were counterstained with Harris's haematoxylin (Page, 1977) for 2 minutes, rinsed in tap water for 1 minute, then mounted in glycergel (Dako, Carpinteria, USA) (Fig 2.3).

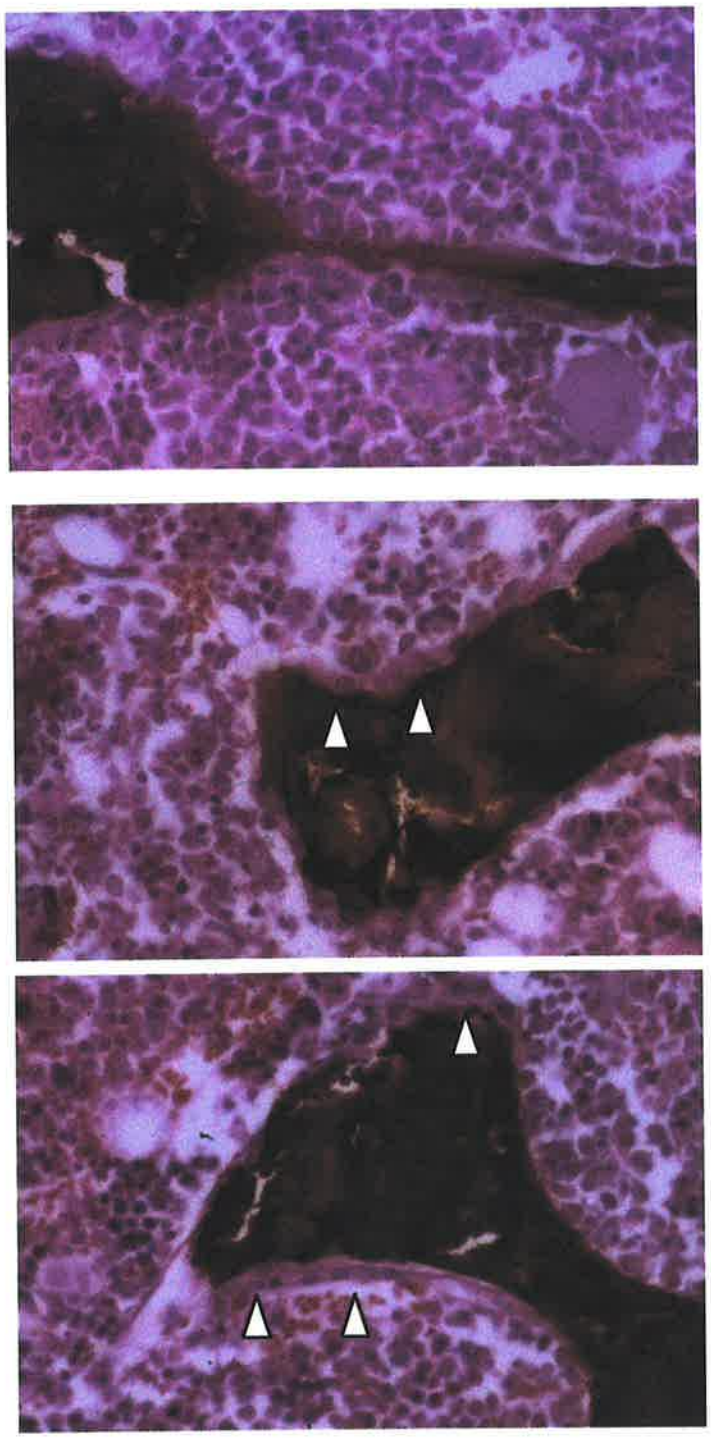


Figure 2.2 Osteoclast identification using modified von Kossa stained sections with a haemotoxylin and eosin counterstain. Osteoclasts were identified as eosinophilic, multinucleated cells adjacent to the bone surface (white arrow heads). Howsips lacunae are evident as crenations in the bone surface (dark brown). (original magnification x400)

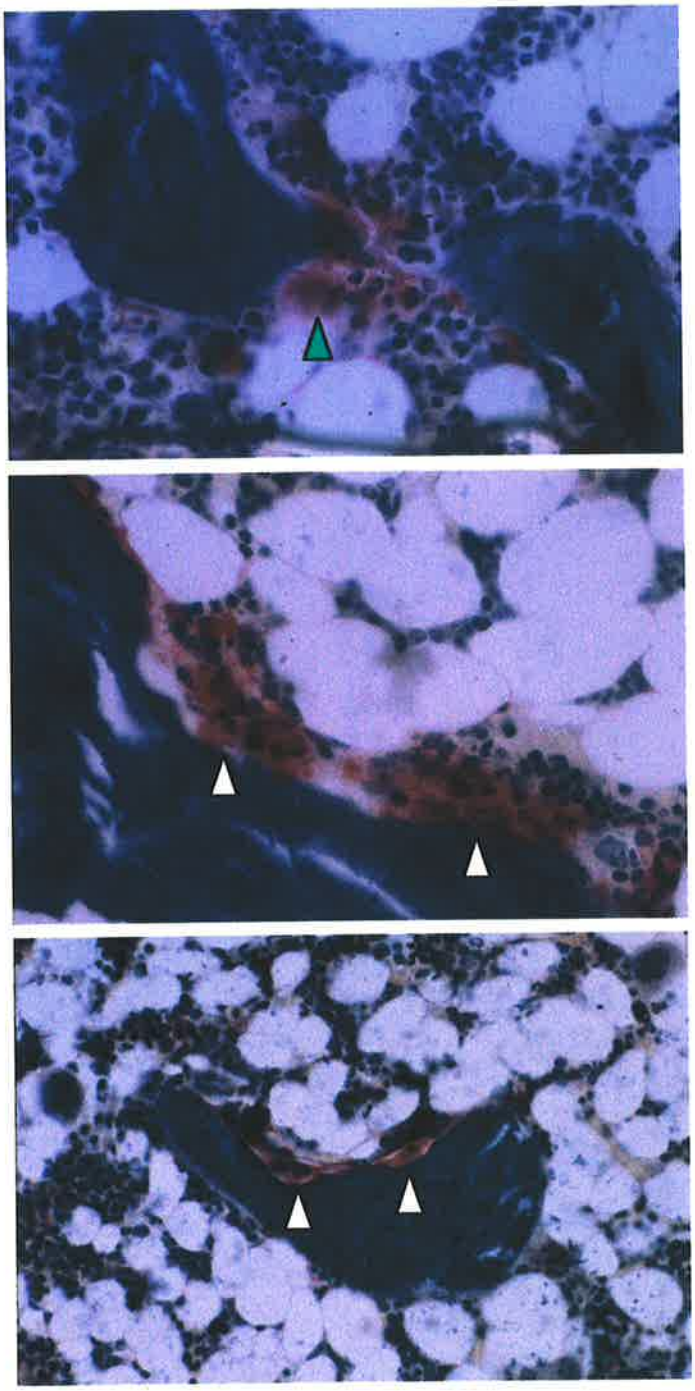


Figure 2.3 Osteoclast identification using enzyme localisation of acid phosphatase. Osteoclasts (white arrow heads) were identified as having red stained cytoplasm, lying adjacent to bone surfaces devoid of osteoid. Haemotoxylin counterstain enables visualisation of cell nucleii and calcified tissue (dark blue). The green arrow head shows an anuclear profile. (original magnification x400)

2.10 Histomorphometry

2.10.1 Sample areas for trabecular bone morphometry

Sections were partitioned into three regions unless otherwise stated. The epiphyseal region (Epi) below the growth plate, the metaphyseal region (Meta) extending from 3 mm above the most distal point of the growth plate, excluding primary spongiosa from analysis, to 7 mm above the growth plate and the diaphyseal region (Dia) from 7 mm to 12 mm above the growth plate. Estimation of trabecular morphological indices was restricted to the largest rectangle that could be positioned within the region, excluding all cortical bone. In the epiphysis the sampling rectangle was positioned with the proximal margin at a tangent to both arcs of the growth plate (Fig 2.4). For estimation of cellular indices, all trabecular bone within the epiphysis was counted. In the metaphysis and diaphysis all trabecular bone bordered by the proximal and distal margins of the sample areas was counted.

In the rats operated upon prior to puberty (Chapter 7) the growth of the femora precluded the use of sample areas of constant dimension. To account for this, the length of the section was maintained at 1/3 of total femoral length and the proximal region from the apex of the most distal aspect of the growth plate to the proximal margin of the section was trisected. The region adjacent to the growth plate was excluded, the next two regions corresponded to the metaphysis and diaphysis, respectively. The epiphyseal region was similar to that used for mature rats. In the study of resorption estimates (2.12) the Meta and Dia regions were pooled to simplify analysis and increase the consistency of the region with the tibial estimates.

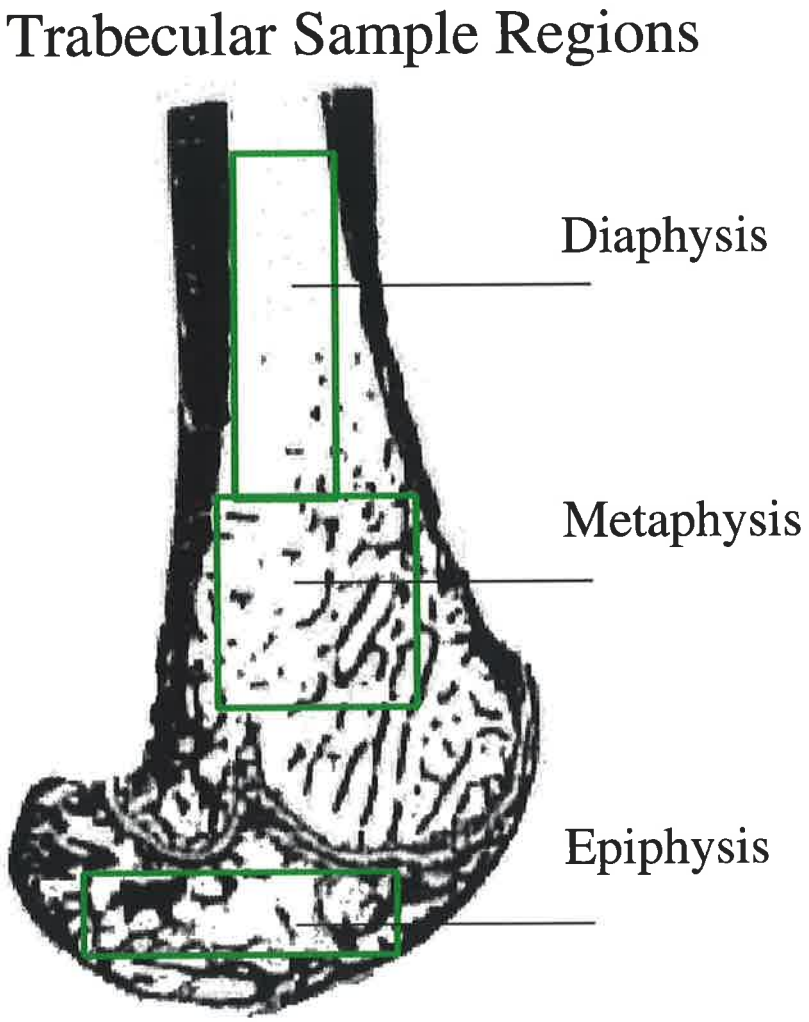


Figure 2.4 Sample regions for histomorphometric analysis. The epiphyseal region was positioned with the proximal margin at a tangent to both arcs of the growth plate. Th metaphyseal region was positioned 3mm proximal to the most distal aspect of the growth plate, extending 4mm proximally. Th diaphyseal region was positioned contiguous to the metaphysis, extending to a maximum of 12mm proximally. Sample regions excluded cortical bone. Sampling of cellular indices included all trabecular bone surfaces within the epiphysis, and all trabecular bone surfaces bordered by the proximal and distal margins of the metaphysis and diaphysis was included.

2.10.2 Trabecular bone indices: Estimation using the semi-automated image analysis system

Trabecular bone indices were estimated using a high resolution camera (Sony, Tokyo, Japan) connected to an Olympus BH-2 microscope (Olympus, Tokyo, Japan) and interfaced with a Quantimet 520 Image Analysis System (Cambridge Instruments, Cambridge, UK). The final image at x100 magnification was adjusted to an appropriate threshold for staining intensity and manually edited for artifacts of sectioning or staining. A binary image was created representative of the darkly stained calcified tissue within the region. From this image the total sample area (TA), bone area (BA) and bone perimeter (Peri) are recorded. From these data trabecular bone volume (BV/TV, %), trabecular thickness (Tb.Th, um) and trabecular number (Tb.N, /mm) were calculated (2.14) using the plate model (Parfitt *et al.,* 1983). These calculations were performed without correction for anisotropy.

2.10.3 Formation indices: Estimation using fluorochrome labelled sections

Sections were brought from tap water to distilled water, dehydrated in ethanol (2x 100%) cleared in xylene (2x100%) and mounted unstained (2.9.2). Tetracycline derivatives such as demeclocycline and calcein chelate to calcium as it is laid down during the mineralisation process and are thus embedded in the bone at all sites of mineralisation after their administration. When viewed under UV light with a 420 nm filter (Zeiss, Heidelberg, Germany) with a fluorescence microscope (Zeiss, Heidelberg, Germany) the tetracycline labels (2.3) labels were seen as distinct fluorescent bands near the surface of the trabeculae, demeclocycline a yellow/orange band and calcein a green band (Fig 2.5).

Double fluorochrome labelled surface as a percentage of total trabecular surface (dLS) was determined manually using an ocular mounted Weibel II graticule (Graticules Ltd., Tonbridge, UK) at 250x magnification. All intersections of the trabecular surface with the grid lines of the graticule were categorised as either inactive, single or double labelled, in a line perpendicular to the surface from the point of intersection. Interlabel distance was measured at multiple sites along the labelled surface using an ocular mounted micrometer (Selbys Scientific Ltd, Kent, UK) at 400x magnification. The distance was measured from the internal origin of each fluorescent band (Fig 2.5). Three or four evenly spaced estimations were made at each site with a maximum of 10 sites per region.

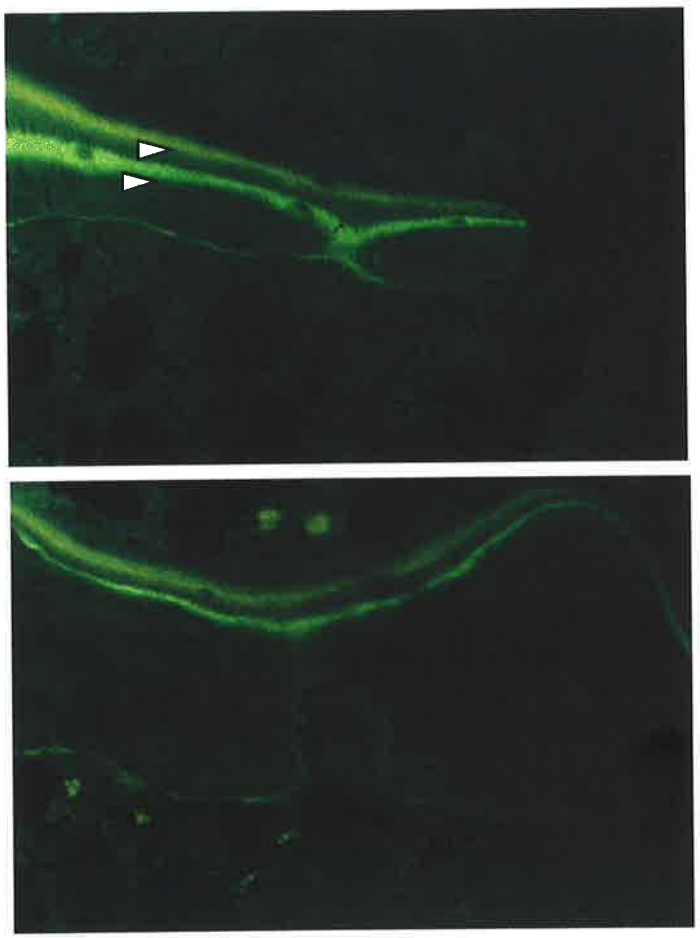


Figure 2.5 Fluorochrome labelled sites of active bone mineralisation. UV excitation reveals demeclocycline as a green band and calcein as an orange band. The percentage and linear extent of the trabecular bone surface covered by double labels was used to estimate bone formation activity. The distance between the inner margin of each band perpendicular to the bone surface (white arrow heads) represents 4 days of continuous bone formation and was used to estimate mineral apposition rate. (original magnification x200)

2.11 Resorption indices: Comparison of three methods for estimation of bone resorption following oophorectomy

2.11.1 Introduction

Osteoclast activity can be estimated using a variety of techniques resulting in a wide range of reference values. Histomorphometric techniques for assessing the extent of bone resorption often report the percentage of the total trabecular surface covered by multinuclear cells with the morphological appearance of giant cells or macrophages. These methods show significant variation when performed in human biopsy (Parfitt, 1993). When performed in small animal models such as the rat, the potential for error is magnified by the increased rate of turnover, the small sample space and by the diminished surface extent relative to formation.

Variation in estimation of resorption parameters for the rat may arise from a number of sources, including the method of osteoclast identification. These vary from purely morphological techniques (Wronski *et al.,* 1985, Shen *et al.,* 1995, Dempster *et al.,* 1995, Wronski *et al.,* 1988, Takano-Yamamoto and Rodan 1990, Westerlind *et al.,* 1994, Bagi and Miller 1994, Tobias *et al.,* 1993, Gallagher *et al.,* 1993), or those based upon bone surface characteristics (Akamine *et al.,* 1993, Chen *et al.,* 1992). Further variation may arise from the use of different bones in the appendicular skeleton, most commonly the proximal tibia (Wronski *et al.,* 1985, Wronski *et al.,* 1988, Hagino *et al.,* 1993, Gallagher *et al.,* 1993, Bagi and Miller 1994, Shen *et al.,* 1995, Dempster *et al.,* 1995) and the distal femur (Takano-Yamamoto and Rodan 1990, Martin and Zissimos 1991, Akamine *et al.,* 1992, Chen *et al.,* 1992, Westerlind *et al.,* 1994, Sims *et al.,* 1996) and analysis of different regions within these bones such as the metaphysis (Wronski *et al.,* 1988, Takano-Yamamoto and Rodan 1990, Hagino *et al.,* 1993, Tobias *et al.,* 1993, Bagi and Miller 1994, Westerlind *et al.,* 1994, Dempster *et*

al., 1995) or the epiphysis (Martin and Zissimos 1991, Westerlind *et al.,* 1994, Iriji *et al.,* 1995). More recently, acid phosphatase (AcP) has been used to identify osteoclasts (Braidman *et al.,* 1990, Kalu *et al.,* 1993, Turner *et al.,* 1993, Bourrin *et al.,* 1995, Sims *et al.,* 1996). This technique may also detect cells that appear in thin tissue sections to be mononuclear or even to have no nucleus at all (Kaye, 1984) and progenitor cells of the osteoclast lineage (Baron *et al.,* 1986).

A wide spread of values for bone resorption estimates have been reported. In the proximal tibial metaphysis values of the extent of osteoclasts in 21 week old ovary-intact rats range from 0.15% using toluidine blue stain (Tobias *et al.,* 1993) to 11% using Masson-Goldner stain (Wronski *et al.,* 1986). Estimates for oophorectomised animals range from 0.5% at 21 days after operation on 230g rats (Gallagher *et al.,* 1993) to 27% at 6 weeks after operation at 90 days of age (Bagi and Miller 1994), both using toluidine blue stain.

Thus clearly the criterion for osteoclast and erosion surface identification vary significantly. In order to identify factors contributing to this variation, three techniques for assessing osteoclast surface were compared, at four anatomical sites of the adult rat. The three techniques for osteoclast identification were, enzyme localisation of acid phosphatase (AcP) (2.9.3.3) cell morphology following von Kossa with haematoxylin and eosin counterstain (VK/HE) (2.9.3.2) and an estimate based on (VK/HE) morphology with the additional criterion of cells adjacent to Howship's lacunae. The four regions in which these estimates were made were the metaphysis and epiphysis of the distal femora and proximal tibiae. The study was performed on skeletally mature oophorectomised and sham-operated rats killed at 0, 9 or 18 days following operation.

2.11.2 Materials and Methods

30 female Sprague-Dawley rats at 7 months of age were allocated to either oophorectomy or sham operations with 6 animals killed at operation to form a baseline group. At 9 and 18 days post operation 6 Oophx and 6 Sham rats were killed and the right femora and tibiae excised. The distal 20 mm of the femora and the proximal 20 mm of the tibiae were bisected polymerised in MMA and 5 um thick longitudinal sections were cut as per preceding protocols. One section from each bone was stained for AcP and a second section was stained with VK/HE.

In the AcP stained sections osteoclast-like cells were identified as those having a red staining cytoplasm lying adjacent to the bone surface (AcP-Oc). In the VK/HE sections identification of osteoclasts was based upon their multinucleate morphology and foamy eosinophilic cytoplasm and location adjacent to trabecular surfaces devoid of osteoid. Two separate counting procedures were used on the VK/HE sections. The first included all trabecular surfaces (VK-Oc), while the second included only those cells associated with Howship's lacunae of a depth 5um or greater (Pit-Oc). The percentage of trabecular surface covered by these cells was estimated manually using an ocular-mounted Weibel II graticule (Graticules Ltd., Tonbridge, UK) for AcP and VK/HE stained sections at 100x and 200x magnification respectively.

Sections were partitioned into epiphyseal and metaphyseal regions (2.11.1). To exclude primary spongiosa from analysis in the metaphysis, counting was begun 1 mm below the most distal point of the growth plate in the tibial specimens and 3 mm above the most distal point of the growth plate in the femora. The epiphysis consisted of the region bordered by the growth plate and the cortical casing of the respective condyles. Sampling was via contiguous fields and included all trabecular bone in each region.

To compare the methods for estimating osteoclast surface, mean values for each group were scaled by dividing their mean by the mean of all the ovary-intact groups for each respective bone region and analytical method. The three analytical methods were compared using a stylized model of osteoclast response to oophorectomy, predicting an increase in the oophorectomised groups at both 9 and 18 days post operation as reported previously in the metaphyses of both distal femur (Sims *et al.*, 1996) and proximal tibia (Dempster *et al.*, 1995). The methods were compared for their ability to discriminate between oophorectomised and sham operated sections (operative discrimination) and for the minimum variance in ovary-intact groups over all sampling times (baseline stability). A repeated measures analysis of variance was used to assess the ability of each method to describe the stylised model of response to oophorectomy, alone and against the other methods (2.15).

The variance unaccounted for by either operation or time after oophorectomy for each method was also compared without scaling. The accuracy of each method was assessed by comparing this unexplained variance using Maxwell's variance ratios test (2.15). Finally, the mean values from the two anatomical regions within the two separate bones were compared by repeated measures analysis of variance with contrast, to examine the uniformity of osteoclast surface parameters between the two bones (2.15).

2.11.3 Results

The femoral estimates (Fig 2.6) display the pattern of a generalised increase in the percentage of osteoclast surface following oophorectomy in both the metaphysis and epiphysis. The AcP-Oc method produced the highest absolute values, with Pit-Oc an order of magnitude lower than the other two methods. The graphs for the tibial estimates (Fig 2.7) also display the increase in osteoclast surface after oophorectomy. The AcP-Oc method failed to detect an increase at 18 days post-oophorectomy in the metaphysis and at both times in the epiphysis. Comparison between the two bones suggests a larger initial increase in osteoclast surface in the distal femur compared to the proximal tibia.

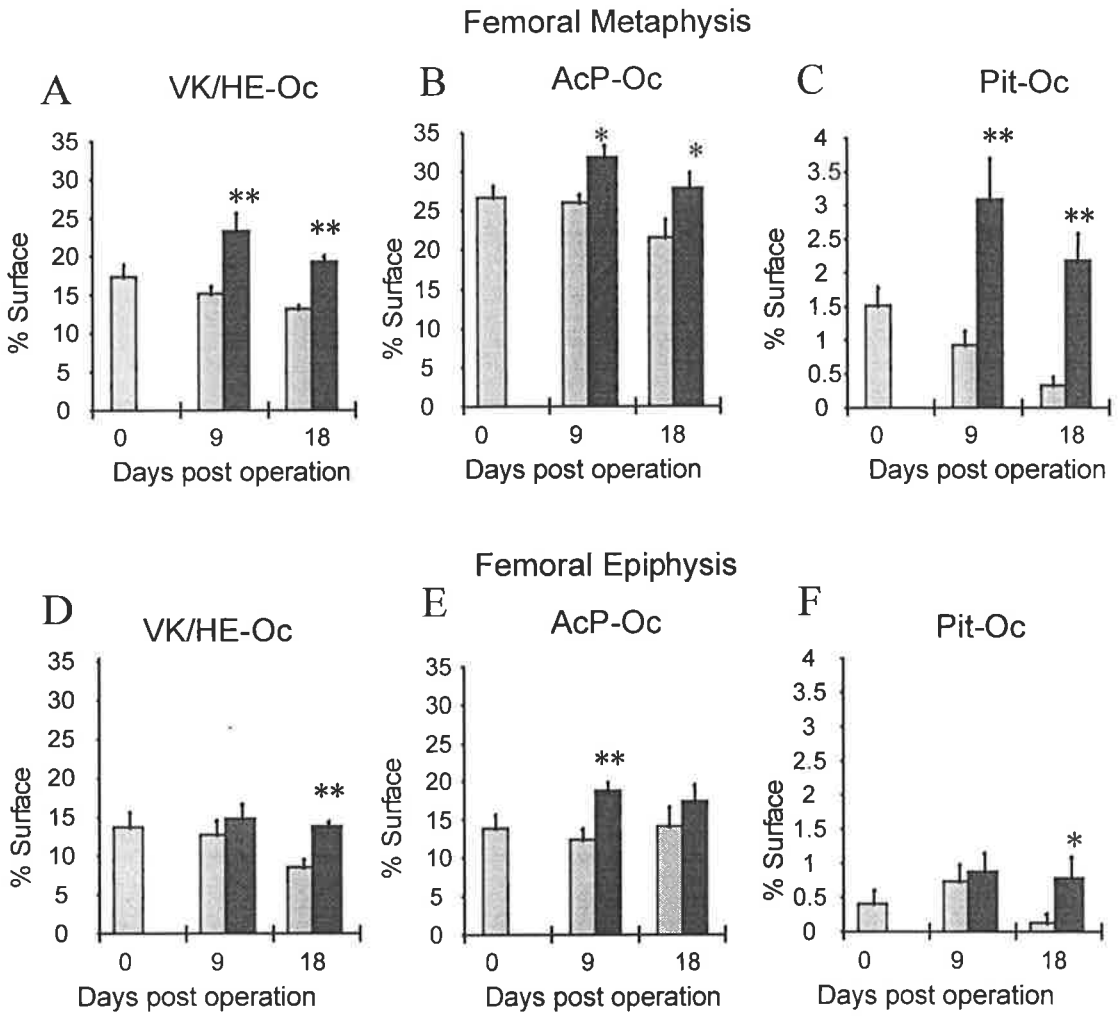


Figure 2.6 Unscaled resorption estimates (mean+SE) from the distal femoral metaphysis (a-c) and epiphysis (d-f), using three methods; based on osteoclast morphology (VK-Oc), acid phosphatase expression (AcP-Oc) or bone surface morphololgy (Pit-Oc), displaying the effect of oophorectomy (Oophx black bars, Sham grey bars) and the stability of ovary intact values at 0, 9 and 18 days post operation. * $P<0.05$, ** $P<0.01$.

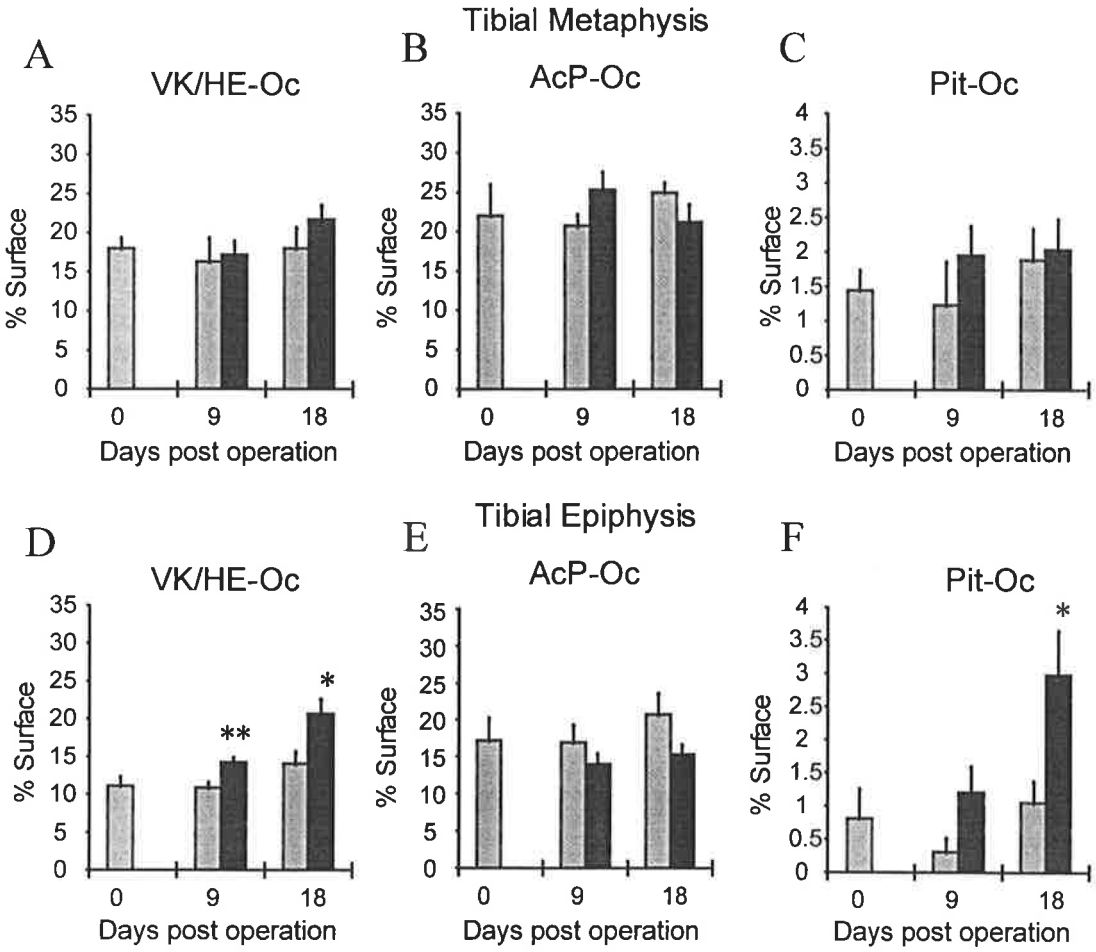


Figure 2.7 Unscaled resorption estimates (mean+SE) from the proximal tibial metaphysis (A-C) and epiphysis (D-F), using three methods; based on osteoclast morphology (VK-Oc), acid phosphatase expression (AcP-Oc) or bone surface morphololgy (Pit-Oc), displaying the effect of oophorectomy (Oophx black bars, Sham grey bars) and the stability of ovary intact values at at 0, 9 and 18 days post operation. * $P<0.05$, ** $P<0.01$.

The validity of the scaling process was confirmed by examining the results from analysis conducted within each method for both non-scaled and scaled data. There was no significant difference between the two data sets (data not shown), indicating that the scaling procedure did not effect the pattern of the data. Comparison of the scaled data for osteoclast surface estimates from the femora and tibiae between ovary-intact and oophorectomised rats indicated that the reporting of the effect of oophorectomy varied significantly between the methods (Table 2.3). The Pit-Oc method demonstrated the largest effect of oophorectomy with a 250 to 300% increase in the femoral metaphysis and 200% increase in the femoral epiphysis. The VK-Oc and AcP-Oc methods demonstrated a 50% increase with oophorectomy in both regions of the distal femur, however with greatly reduced variance. Effects of oophorectomy in the tibiae were generally less than in the femora but the same relationship between the methods was maintained.

	Femur			Tibia		
	AcP-Oc	VK-Oc	Pit-Oc	AcP-Oc	VK-Oc	Pit-Oc
Metaphysis						
Sham 0 days	1.09(0.13)	1.14(0.23)	1.64(0.70)	1.11(0.16)	0.98(0.28)	0.95(0.46)
Sham 9 days	1.06(0.09)	1.00(0.13)	1.00(0.53)	0.86(0.13)	0.96(0.40)	0.81(0.98)
Oophx 9 days	1.26(0.13)	1.50(0.40)	3.16(1.71)	1.06(0.25)	1.01(0.21)	1.54(1.03)
Sham 18 days	0.85(0.19)	0.86(0.06)	0.36(0.28)	1.03(0.12)	1.06(0.36)	1.24(0.71)
Oophx 18 days	1.13(0.20)	1.26(0.12)	2.50(1.10)	0.91(0.20)	1.26(0.28)	1.43(0.72)
Epiphysis						
Sham 0 days	1.06(0.23)	1.18(0.37)	0.98(1.08)	0.97(0.41)	0.93(0.20)	1.13(1.45)
Sham 9 days	0.91(0.23)	1.10(0.34)	1.73(1.43)	0.96(0.30)	0.90(0.11)	0.42(0.66)
Oophx 9 days	1.41(0.17)	1.26(0.44)	2.02(1.66)	0.81(0.20)	1.18(0.10)	1.66(1.15)
Sham 18 days	1.04(0.44)	0.72(0.20)	0.29(0.72)	1.07(0.21)	1.17(0.31)	1.45(1.03)
Oophx 18 days	1.27(0.44)	1.17(0.10)	1.91(1.89)	0.86(0.15)	1.58(0.17)	3.34(1.86)

Table 2.3 Mean (standard deviation) of osteoclast surface estimates from the femora and tibiae of sham-operated or oophorectomised rats. The data were scaled to enable comparison, by dividing each mean value by the mean of all sham-operated animals for that particular method and region.

Applying the stylised model of osteoclast response to oophorectomy, all three methods were able to detect a statistically significant response to oophorectomy (Table 2.4), although no rise in osteoclast surface was detectable in the tibial metaphysis, despite a consistent trend for greater values in the oophorectomised groups for VK-Oc and Pit-Oc. The VK-Oc method was the most consistent method overall, producing significant discrimination between the two operative groups in three of the four anatomical regions examined. The significance values produced by the VK-Oc method were greater than or equal to the other methods in all responsive regions. The AcP-Oc and Pit-Oc methods were only able to detect a significant rise in osteoclast surface in two regions, the femoral metaphysis and epiphysis for AcP-Oc and femoral metaphysis and tibial epiphysis for Pit-Oc. There were no significant differences between any of the analytical methods for demonstrating baseline stability. A reduction in the osteoclast surface estimates in the femoral metaphysis between the group of rats killed on Day 18 from the Day 0 group, detected by all methods, was the only consistent variation in the Sham rats.

Comparison of the T values calculated from the scaled means indicated differences between methods in the level of operative discrimination produced in both the femoral metaphysis and tibial epiphysis (Table 2.4). In both anatomical sites the Pit-Oc method produced significantly greater operative discrimination than the other two methods. While the VK-Oc method detected significantly greater operative discrimination than the AcP-Oc method in these two regions.

	Femur			Tibia		
	AcP-Oc	**VK-Oc**	**Pit-Oc**	**AcP-Oc**	**VK-Oc**	**Pit-Oc**
Metaphysis						
Oophx vs Sham	3.64(0.005)	4.92(0.0001) [af]	5.27(0.0001) [ad, be]	ns	ns	ns
Sham 0 vs Sham 9	ns	ns	ns	2.49(0.05)	ns	ns
Sham 0 vs Sham 18	2.69(0.05)	2.24(0.05)	2.34(0.05)	ns	ns	ns
Epiphysis						
Oophx vs Sham	2.68(0.05)	2.24(0.05)	ns	ns	4.14(0.001) [ad]	2.28(0.01) [ae, bc]
Sham 0 vs Sham 9	ns	ns	ns	ns	ns	ns
Sham 0 vs Sham 18	ns	2.51(0.05)	ns	ns	ns	ns

Table 2.4 Discrimination between operation groups and the stability of estimates of osteoclast surface. T values (P) for the effect of discrimination between operation groups and the stability of estimates of osteoclast surface within the Sham group for each method. [a] Significant difference from AcP-Oc, [b] Significant difference from VK-Oc: [c] $P< 0.05$, [d] $P< 0.005$, [e] $P<0.001$, [f] $P< 0.0001$.

The imprecision of each method was examined by comparing the residual variance i.e. that variance unaccounted for by either operation or time after oophorectomy (Table 2.5). The Pit-Oc method produced a greater relative error than either AcP-Oc or VK-Oc in all regions.

Comparison between femora and tibiae (Table 2.6) demonstrated consistently higher estimates of osteoclast surface following oophorectomy in the femoral metaphysis compared to the tibial metaphysis at day 9 for all methods. The VK-Oc method produced significantly greater estimates of osteoclast surface in the tibial epiphysis for both operative groups at 18 days. The AcP-Oc method demonstrated higher values for femoral estimates in all Oophx groups consistent with an inability to demonstrate a difference between operation groups in the tibia. There were no significant differences at 0 days.

	Femur			Tibia		
	AcP-Oc	**VK-Oc**	**Pit-Oc**	**AcP-Oc**	**VK-Oc**	**Pit-Oc**
Metaphysis	0.02	0.05	0.91[a]	0.03	0.10[b]	0.64[a]
Epiphysis	0.10	0.10	1.91[a]	0.08	0.04	1.62[a]

Table 2.5 Residual variance not accounted for by time post-operation or operation, of the three methods of osteoclast surface estimation, calculated on unscaled data. [a] Significant difference from AcP-Oc and VK-Oc ($P<0.001$), [b] Significant difference from AcP-Oc ($P<0.01$)

	Metaphysis			Epiphysis		
	AcP-Oc	**VK-Oc**	**Pit-Oc**	**AcP-Oc**	**VK-Oc**	**Pit-Oc**
Sham 0 days	ns	ns	ns	ns	ns	ns
Sham 9 days	0.05[a]	ns	ns	ns	ns	ns
Sham 18 days	0.05	ns	ns	ns	0.01	ns
Oophx 9 days	0.05[a]	0.01[a]	0.01[a]	0.01[a]	ns	ns
Oophx 18 days	0.05[a]	ns	ns	0.05[a]	0.05	ns

Table 2.6 P values for the comparison of estimates of osteoclast surface by method between the femora and tibiae in the metaphysis and epiphysis at each time point. [a] femora>tibiae

2.11.4 Discussion

This study demonstrated significant differences between the three methods used to estimate osteoclast surface and their response to oophorectomy at four sites of the rat appendicular skeleton. The VK-Oc method proved the most powerful and consistent, demonstrating an increase in osteoclast surface following oophorectomy in three of the four regions studied, while the other methods were only able to demonstrate a difference in two regions. The significance of the response detected by VK-Oc was equivalent or better than the other two methods in every region that showed a response. As a result this method was adopted for all studies in this thesis.

The Pit-Oc method was found to indicate a larger absolute increase in osteoclast surface following oophorectomy compared to both VK-Oc and AcP-Oc methods. The variance associated with the Pit-Oc method however, was larger than for the other methods. The presence of lacunae as a requirement for counting produced a marked reduction in the number of positive counts relative to the other methods, reducing the sample size of trabecular surface available for estimation of resorption surface. The sample size could be increased by increasing the number of sections counted which may reduce the variance of this method, although such a modification may not always be practical. The AcP-Oc method, whilst producing the least variance, lacked the sensitivity of the other methods to detect the increase in osteoclast surface following oophorectomy, suggesting the likelihood of a sampling inaccuracy with this method. The inconsistency of the AcP-Oc method was most likely the result of counting all AcP positive cell profiles including mononuclear and anuclear cells. The inclusion of these cells appears to raise the Sham values, suggesting that multinuclearity is an important discriminator of the post oophorectomy resorptive response.

Comparison of the two bones reveals a difference between the magnitude of the response immediately following oophorectomy. The extent of osteoclast surface in the femoral metaphyses of the oophorectomised groups was greater at day 9 in the VK-Oc and Pit-Oc groups. There was also a difference in the response to oophorectomy between the epiphysis and metaphysis for each bone, with a larger response in the metaphysis of the distal femur compared with the epiphysis whereas in the tibia the opposite pattern was evident. Increased resorption has been reported at 6 days (Sims *et al.,* 1996) and 5 days (Dempster *et al.,* 1995) post oophorectomy in the femoral and tibial metaphyses, respectively. These findings are consistent with a larger initial response to oestrogen deficiency in the distal femur. However, the lack of an increase in the proximal tibiae may have related to researcher inexperience with this anatomical region.

Whilst each method demonstrated consistent values of osteoclast surface in ovary-intact rats, the Pit-Oc method was associated with greater variance than the other methods. In addition, the VK-Oc and Pit-Oc methods were more sensitive than the AcP-Oc method for detecting a response to oophorectomy. Thus the VK-Oc method combines both accuracy and precision to provide the most reliable estimate. The AcP-Oc method was the least statistically powerful, perhaps due to its detection of a wider population of cell morphologies. The immediate increase in osteoclast surface as a result of oophorectomy was larger in the distal femur than in the proximal tibia. The significance of the response detected by VK-Oc was equivalent or better than the other two methods in every region that showed a response. As a result this method was adopted for all studies in this thesis.

2.12 Growth plate thickness

Growth plate thickness was estimated from VK/HE sections manually using an ocular mounted micrometer (Graticules Ltd., Tonbridge, UK). Measurement was taken from the proximal margin of the calcified tissue in the epiphysis to the proximal margin of the mineralised metaphyseal primary spongiosa, parallel to the condrocyte columns. 10 evenly spaced measurements were taken per section.

2.13 Serum oestradiol estimation

Circulating oestradiol levels were estimated from serum samples collected at death (2.6) by radioimmunoassay (Spectria Orion Diagnostica, Finland) following extraction with 60% cyclohexane/40% ethyl acetate and reconstruction with charcoal stripped human serum.

2.14 Calculations

2.14.1 Static trabecular indices

The surface and area data produced by the image analysis system (2.11.2) was used to estimate trabecular morphology using the following equations.

Trabecular bone volume (%)	BV/TV = (BA/TA)*100
Trabecular thickness (um)	Tb.Th = (BA/TA)/((Peri/TA)/2)
Trabecular number (/mm)	Tb.N = (BA/TA)/Tb.Th

[total sample area (TA), bone area (BA) and bone perimeter (Peri)]

2.14.2 Cellular indices

Resorption extent (um)	Oc.E = Oc.S x Peri

Formation extent (um)	dLE = dLS x Peri
Mineral apposition rate (um/d)	MAR
	= interlabel distance/interlabel period

Bone formation rate (tissue level, surface referent, $um^3/um^2/d$)

(BFR) = dLS x MAR

[osteoclast surface (Oc.S) and double fluorochrome labelled surface (dLS)]

Turnover ratio[1] (ToR) = Oc.E/(dLE x interlabel distance)

[1] assumption: rate of bone loss ≡ $\frac{\text{volume resorbed (Oc.E x resorption depth)}}{\text{volume formed (dLE x interlabel distance)}}$

2.15 Statistics

Within region analysis repeated measures analysis of variance with contrast was used to compare the mean values for each variable for the two operative groups at each time point. Change through time was estimated by regression analysis assessed for each group and compared to the corresponding operative group in that region. Inter-region analysis was conducted by analysis of variance with contrast on the slopes of the regression equations of time on the difference between the individual Oophx values and the mean of the Sham value for each time point. Due to the difference in baseline values between regions, structural trabecular bone indices were normalised by expressing the resultant individual Oophx values as a percentage of the mean Sham value for that time point and region. The design of the statistical analysis included a correction for multiple analyses Statistics were performed using PC-SAS (SAS Institute, Cary, NC) and $P < 0.05$ was considered significant.

Chapter 3

Variation in the short-term changes in bone cell activity following oophorectomy in three regions of the distal femur.

3.1 Introduction

While the loss of ovarian hormones following oophorectomy is systemic, the development of osteopenia at different skeletal sites varies with regard to both rate and magnitude (Wronski *et al.,* 1989, Durbridge *et al.,* 1990, Sims *et al.,* 1996). Osteopenia induced by ovarian hormone deficiency has been consistently reported in the trabecular bone of the femoral metaphysis, associated with increased bone cell activity (Takano-Yamamoto and Rodan 1990, Martin and Zissimos 1991, Akamine *et al.,* 1992, Chen *et al.,* 1992, Sims *et al.,* 1996). However, the neighbouring epiphysis has been reported to be resistant to such loss (Westerlind *et al.,* 1997). Similar heterogeneity in the response of femoral trabecular bone to oestrogen deficiency has also been alluded to by other researchers (Durbridge *et al.,* 1990, Turner *et al.,* 1994b). Only one study of turnover in the trabecular bone of the epiphysis shortly after oophorectomy has been conducted (Martin and Zissimos, 1991). An increase in formation 1 month after oophorectomy was reported with no increase in resorption or osteopenia however a reversal of this pattern was reported at 3 months, associated with osteopenia. Thus both the proliferative and osteopenic response of bone cells to ovarian hormone deficiency in this region are uncertain, but suggest the existence of large variations in response to ovarian hormone deficiency.

No study has measured both epiphyseal morphometry and bone cell activity during this period of accelerated turnover and bone loss in the metaphysis. Thus it is not known whether bone cell activity rises in a similar fashion in these different regions within the femur, and secondly, whether the architectural differences between the regions, particularly differences in Tb.Th, are involved in the resistance to ovarian hormone induced osteopenia.

Increased bone remodelling has been associated with osteopenia, however when examining the short term effects of ovarian hormone deficiency the impact of the remodelling transient must be considered. The increase in activation frequency triggered by oophorectomy has the ability to produce permanent bone loss independent of the steady state changes to bone remodelling (Heaney, 1994). By estimating bone cell activity at regular intervals, the relative contribution of the remodelling transient to the overall loss of bone following oophorectomy can be investigated. Thereby isolating the contribution of this initial change in activation frequency from long term bone loss induced by steady state increased turnover.

In response to these questions, this study sampled three separate regions in the distal femur of the rat for 30 days following oophorectomy. Static and dynamic histomorphometric techniques were used to examine the effect of oestrogen deficiency on the morphology of the trabecular bone as well as resorption and formation parameters. This enabled the characterisation of the changes in bone turnover immediately following oophorectomy and their effect on bone structure in the three regions of the distal femur.

3.2 Materials and Methods

3.2.1 Animals

90 Female Sprague-Dawley rats 6 months of age were maintained on commercial rat chow (2.2) and tap water *ad libitum*. All procedures involving the animals were approved by the IMVS Animal Ethics Committee.

3.2.2 Surgery

At 7 months of age 6 animals were killed as a baseline group and the remainder allocated to either oophorectomy or sham operation, via the dorsal approach (2.4.3). All rats received demeclocycline and calcein (2.2), 6 and 2 days respectively, prior to killing. At 9, 15, 18, 21, 24, 28 and 30 days post operation, 6 Oophx and 6 Sham rats were killed and the femora were excised.

3.2.3 Histomorphometry

The distal 20 mm of the right femora were bisected in the sagittal plane processed into MMA resin (2.9.1.2). One half of the left femora of a randomly chosen sample from each group killed at 150 days were used for scanning electron microscopy (2.8).

Sections were partitioned into three regions, the epiphysis, metaphysis and diaphysis (2.11.1).

BV/TV, Tb.N, Tb.Th and Peri were calculated (2.14) from von Kossa stained sections (2.9.3.1).

Interlabel distance and dLS were estimated from unstained sections (2.11.3) and Oc.S was estimated (2.14) from VK/HE stained sections (2.11.4.2).

3.2.4 Calculations and statistics

MAR, BFR, Oc.E, dLE and ToR were calculated as described previously (2.14). The design of the statistical analysis included a correction for multiple analyses, $P<0.05$ was considered significant for all analyses (2.18.4).

3.3 Results

3.3.1 Trabecular bone structure

3.3.1.1 Diaphysis

In the diaphysis BV/TV (Fig 3.1A) fell in both groups (Sham $P<0.005$, Oophx $P<0.0001$). The loss was greater in the Oophx group ($P<0.05$), which fell to 29% of Sham levels at day 30. Osteopenia resulted from decreases in both Tb.N (Fig 3.1B) (Sham $P<0.01$, Oophx $P<0.0001$) and Tb.Th (Fig 3.1C) (Sham $P<0.0001$, Oophx $P<0.005$). Tb.N was decreased in the Oophx group compared to Sham at various times post operation, beginning at day 9 and Tb.Th from day 28 ($P<0.005$).

Peri fell in both groups (Sham $P<0.05$, Oophx $P<0.0005$) (Table 1). There was a trend for this reduction to be significantly greater in the Oophx group over time ($P<0.07$), and was significantly different at various time points post operation.

Figure 3.1A Temporal changes in trabecular bone volume (BV/TV, %) following oophorectomy (Oophx, ■ , solid line) or sham-operation (Sham, □ , broken line) in the diaphysis of the distal femur in the rat, expressed as mean ± SEM. Φ Significant change with time (P<0.005). # Significantly different to Sham with time (P<0.0001). * Significantly different to Sham (P<0.05).

Figure 3.1B Temporal changes in trabecular number (Tb.N, /mm) following oophorectomy (Oophx, ■ , solid line) or sham-operation (Sham, □ , broken line) in the diaphysis of the distal femur in the rat, expressed as mean ± SEM. Φ Significant change with time (P<0.05). # Significantly different to Sham with time (P<0.01). * Significantly different to Sham (P<0.05).

Figure 3.1C Temporal changes in trabecular thickness (Tb.Th, um) following oophorectomy (Oophx, ■ , solid line) or sham-operation (Sham, □ , broken line) in the diaphysis of the distal femur in the rat, expressed as mean ± SEM. Φ Significant change with time (P<0.05). # Significantly different to Sham with time (P<0.01). * Significantly different to Sham (P<0.05).

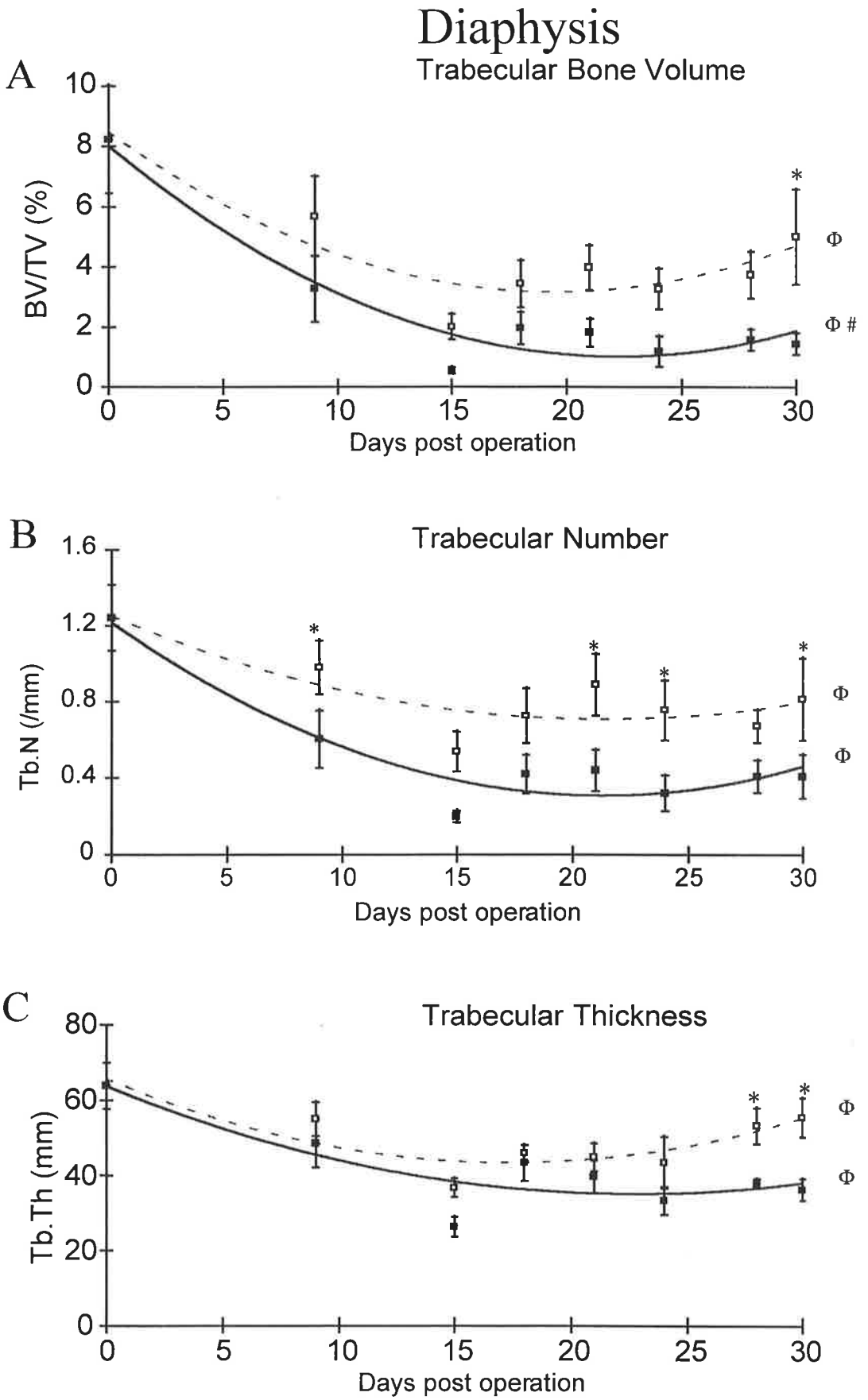

Diaphysis
A
Trabecular Bone Volume
BV/TV (%)
10
8
6
4
2
0
0
5
10
15
20
25
30
Days post operation
Φ
Φ #
*
B
Trabecular Number
Tb.N (/mm)
1.6
1.2
0.8
0.4
0
Days post operation
Φ
Φ
*
C
Trabecular Thickness
Tb.Th (mm)
80
60
40
20
0
Days post operation
Φ
Φ
*

Table 3.1 Results of diaphyseal morphometric analysis, by days post oophorectomy

Days post operation		0	9	15	18	21	24	28	30
Peri (um)									
	ShamΦ	20559(1909)	17940(2867)	11126(2497)	13687(3221)	18304(2444)	13882(2947)	12515(2139)	13265(3398)
	OophxΦ		13360(3384)	3946(1030)*	8259(2477)	8778(2893)*	5935(1991)*	9915(2544)	7880(1869)*
Oc.E (um)									
	ShamΦ	4548(454)	3972(1233)	3400(888)	2774(578)	3818(1465)	2837(469)	2100(462)	2135(613)
	OophxΦ		3129(844)	1641(335)	2645(764)	2814(988)	1237(344)	2818(711)	2810(919)
dLE (um)									
	Sham	1745(378)	1841(392)	1236(549)	2499(945)	2849(785)	2724(881)	1944(349)	764(283)
	Oophx		3980(1140)*	1106(242)	2271(647)	3986(1917)	2081(806)	2606(675)	3316(774)*
ToR									
	ShamΦ	0.64(0.15)	0.22(0.04)	0.33(0.03)	0.49(0.04)	0.37(0.18)	0.14(0.02)	0.22(0.07)	0.31(0.13)
	OophxΦ		0.11(0.01)	0.29(0.03)	0.26(0.06)	0.19(0.06)	0.12(0.02)	0.17(0.09)	0.17(0.05)

Values are mean (SEM) for trabecular perimeter (Peri), osteoclast extent (Oc.E) double labelled extent (dLE) and turnover ratio (ToR) measured in the diaphyseal region of the distal femur in non-operated rats (0 days) and sham operated (Sham) and oophorectomised rats (Oophx) at 9, 15, 18, 21, 24, 28, and 30 days post operation at 7 months of age. Φ Significant change with time. # Significantly different to Sham with time. * Significantly different to Sham.

3.3.1.2 Metaphysis

In the metaphysis BV/TV (Fig 3.2A) fell in both groups (Sham $P<0.01$, Oophx $P<0.0001$). The loss was greater in the Oophx group ($P<0.0001$) from day 15, and had fallen to 65% of Sham values at day 30. Osteopenia resulted from a decrease in both Tb.N (Fig 3.2B) ($P<0.0001$) and Tb.Th (Fig 3.2C) ($P<0.0001$) in the Oophx group, while in the Sham group only Tb.Th was decreased ($P<0.005$). Tb.N was lower in the Oophx group compared to Sham from day 15 ($P<0.0001$), and Tb.Th only at day 28 ($P<0.05$).

Peri decreased in both groups (Sham $P<0.05$, Oophx $P<0.0005$), the decrease was greater in Oophx ($P<0.05$), from day 9 (Table 2).

Figure 3.2A Temporal changes in trabecular bone volume (BV/TV, %) following oophorectomy (Oophx, ■ , solid line) or sham-operation (Sham, □ , broken line) in the metaphysis of the distal femur in the rat, expressed as mean ± SEM. Φ Significant change with time (P<0.05). # Significantly different to Sham with time (P<0.0001). * Significantly different to Sham (P<0.05).

Figure 3.2B Temporal changes in trabecular number (Tb.N, /mm) following oophorectomy (Oophx, ■ , solid line) or sham-operation (Sham, □ , broken line) in the metaphysis of the distal femur in the rat, expressed as mean ± SEM. Φ Significant change with time (P<0.05). # Significantly different to Sham with time (P<0.01). * Significantly different to Sham (P<0.05).

Figure 3.2C Temporal changes in trabecular thickness (Tb.Th, um) following oophorectomy (Oophx, ■ , solid line) or sham-operation (Sham, □ , broken line) in the metaphysis of the distal femur in the rat, expressed as mean ± SEM. Φ Significant change with time (P<0.05). # Significantly different to Sham with time (P<0.01). * Significantly different to Sham (P<0.05).

Metaphysis

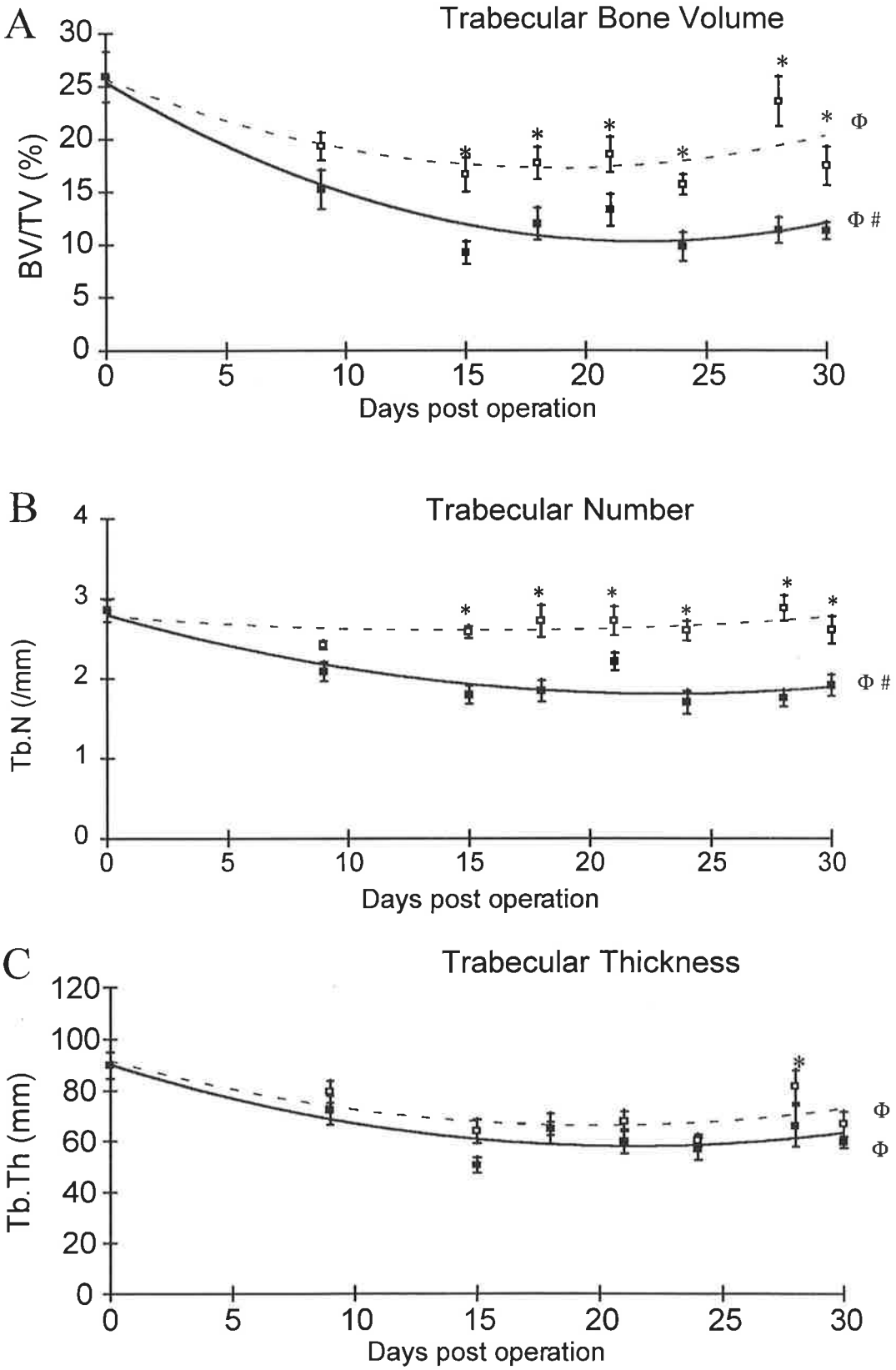

A
Trabecular Bone Volume
BV/TV (%)
Days post operation
Φ
Φ #
B
Trabecular Number
Tb.N (/mm)
Days post operation
Φ #
C
Trabecular Thickness
Tb.Th (mm)
Days post operation
Φ
Φ

Table 3.2 Results of metaphyseal morphometric analysis, by days post oophorectomy

Days post operation		0	9	15	18	21	24	28	30
Peri (um)									
	ShamΦ	74774(2989)	62193(3253)	78553(4715)	68988(5770)	84245(9370)	71846(7717)	91341(10945)	73022(6259)
	OophxΦ#		45789(2511)*	51845(4784)*	50605(5002)*	61045(3447)*	54834(4553)*	56688(4246)*	64799(4906)
Oc.E (um)									
	ShamΦ	12389(1041)	9070(848)	13356(789)	8337(690)	8037(702)	6244(1500)	11374(1728)	8636(1299)
	OophxΦ#		10625(1289)	13490(1885)	8987(931)	11558(1330)	12628(1880)	14537(1735)	18584(2311)
dLE (um)									
	ShamΦ	8262(894)	5154(1140)	10000(2166)	10648(1175)	10174(2674)	15755(1532)	18808(3620)	9328(839)
	OophxΦ#		5538(967)	8791(1619)	11263(1224)	20674(1555)	20182(2111)	23020(1667)	23642(2037)
ToR									
	Sham	0.28(0.04)	0.37(0.04)	0.42(0.12)	0.11(0.01)	0.15(0.04)	0.06(0.01)	0.12(0.03)	0.12(0.03)
	OophxΦ#		0.48(0.09)	0.38(0.04)	0.18(0.03)	0.10(0.02)	0.09(0.01)	0.11(0.02)	0.11(0.01)

Values are mean (SEM) for trabecular perimeter (Peri), osteoclast extent (Oc.E) double labelled extent (dLE) and turnover ratio (ToR) measured in the metaphyseal region of the distal femur in non-operated rats (0 days) and sham operated (Sham) and oophorectomised rats (Oophx) at 9, 15, 18, 21, 24, 28, and 30 days post operation at 7 months of age. Φ Significant change with time. # Significantly different to Sham with time. * Significantly different to Sham.

3.3.1.3 Epiphysis

In the epiphysis BV/TV (Fig 3.3A) also fell in both groups (Sham $P<0.001$, Oophx $P<0.0001$), with no difference between operative groups over time. The reduction in BV/TV was due solely to reduced Tb.Th (Fig 3.3C) (Sham $P<0.0001$, Oophx $P<0.0001$), which was not different between operative groups. Tb.N (Fig 3.3B) was stable in the Sham group, and rose in the Oophx group ($P<0.01$).

Peri did not change in either group (Table 3).

Figure 3.3A Temporal changes in trabecular bone volume (BV/TV, %) following oophorectomy (Oophx, ■ , solid line) or sham-operation (Sham, □ , broken line) in the epiphysis of the distal femur in the rat, expressed as mean ± SEM. Φ Significant change with time (P<0.05). # Significantly different to Sham with time (P<0.0001). * Significantly different to Sham (P<0.05).

Figure 3.3B Temporal changes in trabecular number (Tb.N, /mm) following oophorectomy (Oophx, ■ , solid line) or sham-operation (Sham, □ , broken line) in the epiphysis of the distal femur in the rat, expressed as mean ± SEM. Φ Significant change with time (P<0.05). # Significantly different to Sham with time (P<0.01). * Significantly different to Sham (P<0.05).

Figure 3.3C Temporal changes in trabecular thickness (Tb.Th, um) following oophorectomy (Oophx, ■ , solid line) or sham-operation (Sham, □ , broken line) in the epiphysis of the distal femur in the rat, expressed as mean ± SEM. Φ Significant change with time (P<0.05). # Significantly different to Sham with time (P<0.01). * Significantly different to Sham (P<0.05).

Epiphysis

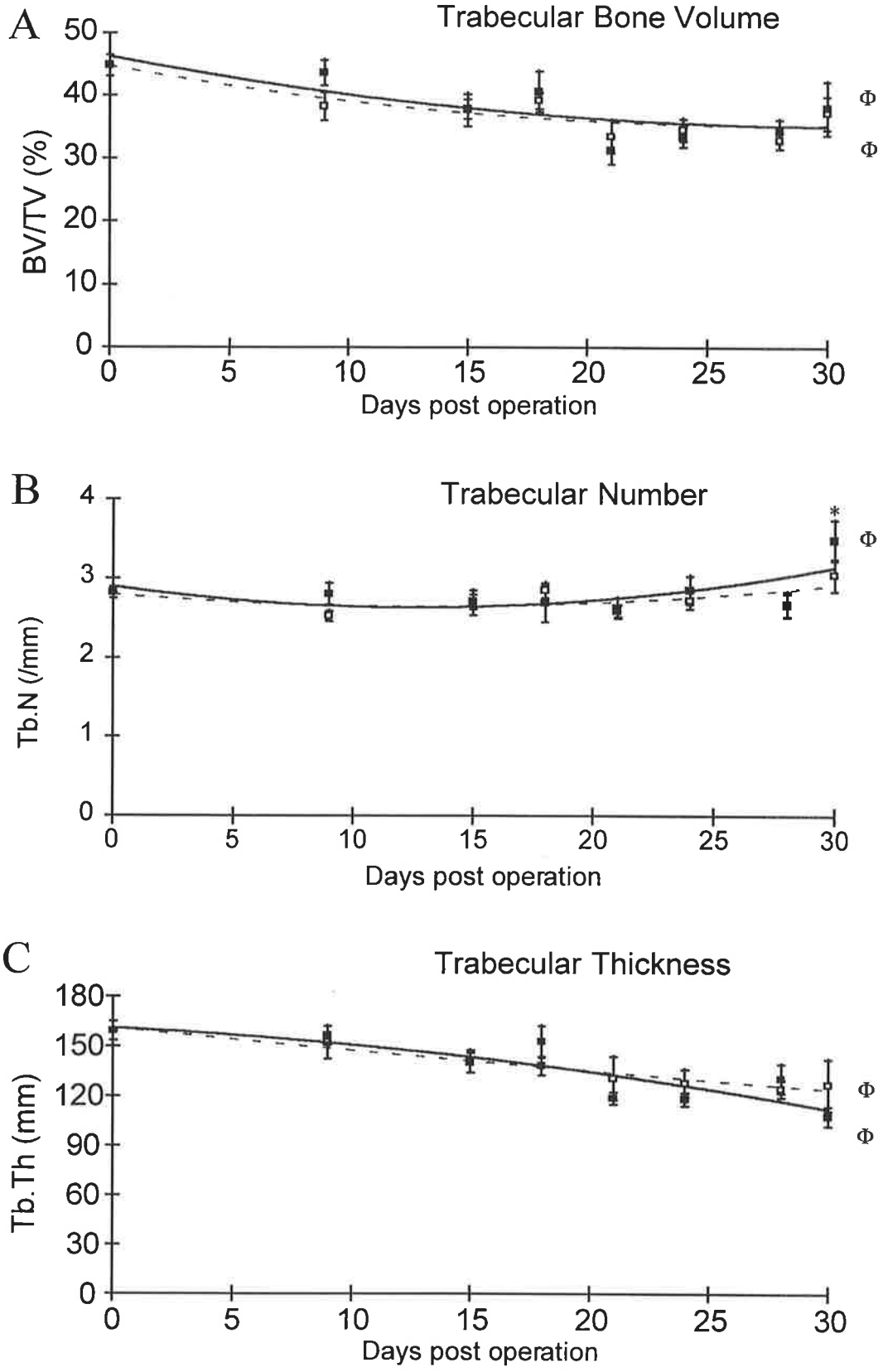

A
Trabecular Bone Volume
BV/TV (%)
50
40
30
20
10
0
0
5
10
15
20
25
30
Days post operation
Φ
Φ
B
Trabecular Number
Tb.N (/mm)
4
3
2
1
0
*
Φ
C
Trabecular Thickness
Tb.Th (mm)
180
150
120
90
60
30
0
Φ
Φ

Table 3.3 Results of epiphyseal morphometric analysis, by days post oophorectomy

Days post operation		0	9	15	18	21	24	28	30
Peri (um)									
	Sham	61278(2776)	51567(5530)	52273(2695)	56354(3822)	58825(2645)	55669(2573)	55933(1999)	53234(4188)
	Oophx		61290(3018)	53417(2715)	49957(6168)	58799(2110)	60599(6113)	60390(4180)	63503(4553)
Oc.E (um)									
	ShamΦ	8492(1279)	6495(973)	7266(774)	4687(421)	5030(672)	3336(352)	5075(1219)	4360(815)
	Oophx#		8948(1163)	9690(1222)	6921(892)	9827(1453)*	11004(1330)*	9314(591)*	9575(815)*
dLE (um)									
	ShamΦ	5482(629)	4694(1114)	5424(1396)	7399(1443)	10446(607)	9871(1644)	9212(1312)	7017(1680)
	OophxΦ#		7542(1756)	6161(660)	7280(1482)	11247(1214)	12487(2036)	17125(1038)*	19649(1752)*
ToR									
	ShamΦ	0.33(0.09)	0.26(0.04)	0.35(0.21)	0.13(0.04)	0.07(0.01)	0.06(0.01)	0.10(0.02)	0.14(0.03)
	OophxΦ		0.22(0.03)	0.31(0.09)	0.25(0.05)	0.16(0.03)	0.16(0.03)	0.10(0.02)	0.09(0.01)

Values are mean (SEM) for trabecular perimeter (Peri), osteoclast extent (Oc.E) double labelled extent (dLE) and turnover ratio (ToR) measured in the epiphyseal region of the distal femur in non-operated rats (0 days) and sham operated (Sham) and oophorectomised rats (Oophx) at 9, 15, 18, 21, 24, 28, and 30 days post operation at 7 months of age. Φ Significant change with time. # Significantly different to Sham with time. * Significantly different to Sham.

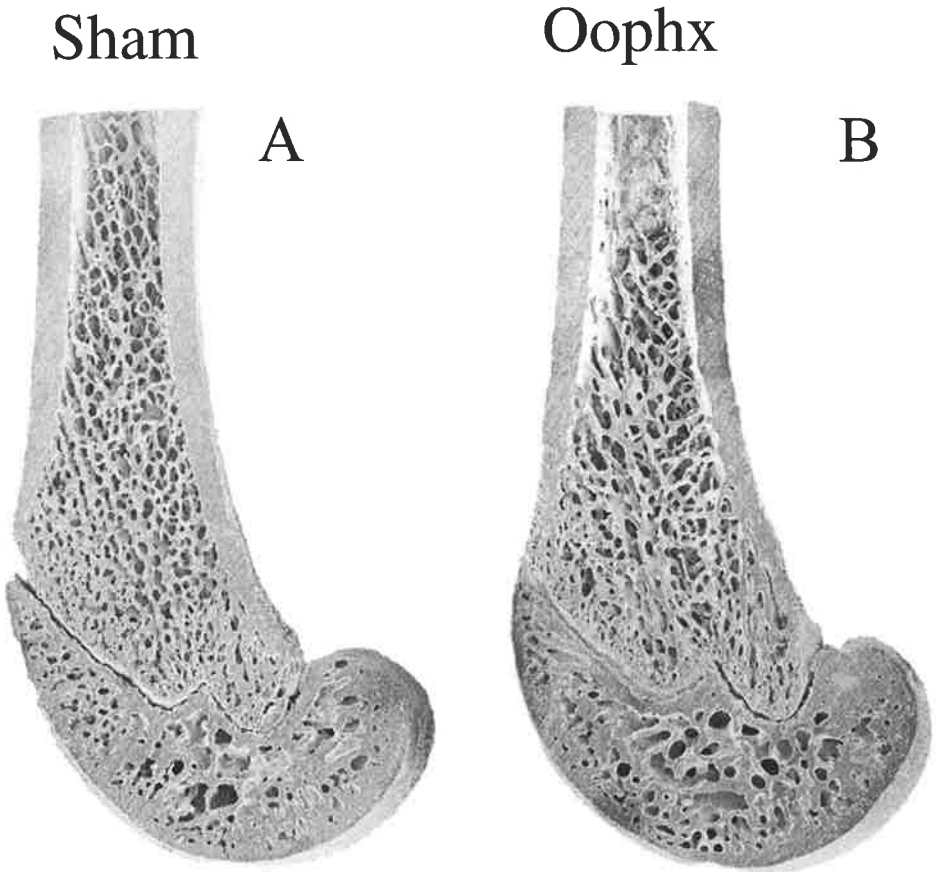


Figure 3.4 The effect of 30 days of ovarian hormone deficiency on the trabecular bone of the distal femur. Ovary intact (A) femur displays a rodust trabecular network extending to the margins of the sample. Following oophorectomy (B) bone loss was maximal in the diaphysis, considerable in the metaphysis and absent in the epiphysis.

3.3.2 Osteoclast surface

In the diaphysis Oc.S (Fig 3.5A) remained constant in the Sham group but rose in the Oophx group ($P<0.005$) and was elevated relative to Sham groups ($P<0.01$) from day 9.

In the metaphysis Oc.S (Fig 3.5B) rose in the Oophx group ($P<0.001$) and decreased in the Sham group ($P<0.005$) resulting in significantly greater levels in the Oophx group ($P<0.0001$) from day 9.

In the epiphysis there was no oophorectomy-induced rise in Oc.S (Fig 3.5C). However a significant decrease in the Sham group ($P<0.001$) resulted in a relative increase in Oc.S in the Oophx group ($P<0.0001$) delayed until day 18.

3.3.3 Osteoclast extent

In the diaphysis Oc.E (Table 3.1) fell in both groups (Sham $P<0.005$, Oophx $P<0.05$) with no difference between operative groups over time, in contrast to Oc.S.

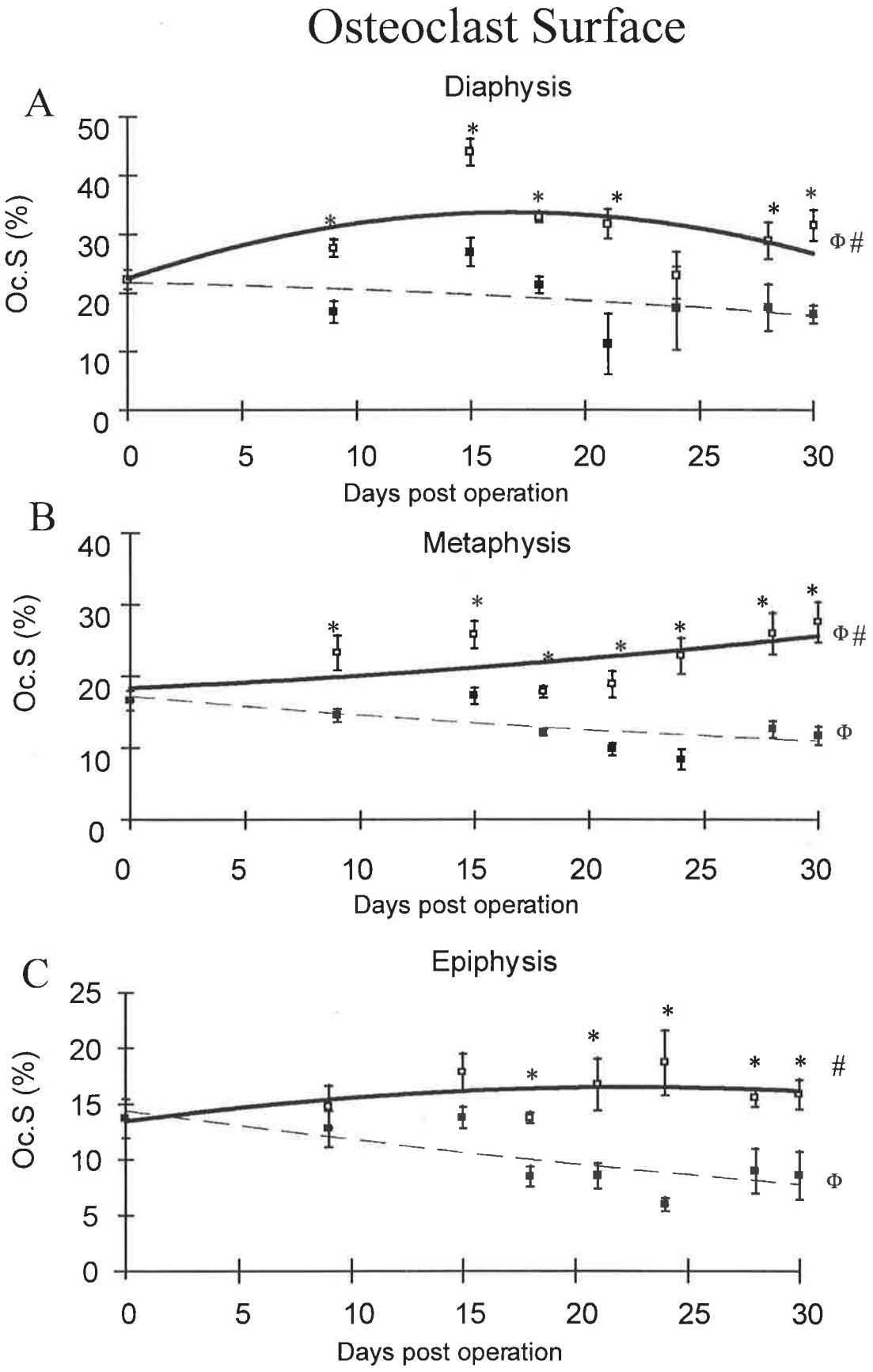
In the metaphysis Oc.E (Table 3.2) decreased in the Sham group ($P<0.05$) and increased in the Oophx group ($P<0.01$), resulting in significantly greater levels in Oophx ($P<0.0001$) over time.

In the epiphysis Oc.E (Table 3.3) did not change in Oophx, but decreased in Sham ($P<0.0005$) resulting in greater values in Oophx ($P<0.0001$) from day 21.

Figure 3.5A Temporal changes in osteoclast surface (Oc.S, %) following oophorectomy (Oophx, ■ , solid line) or sham-operation (Sham, □ , broken line) in the diaphysis of the distal femur in the rat, expressed as mean ± SEM. Φ Significant change with time (P<0.05). # Significantly different to Sham with time (P<0.01). * Significantly different to Sham (P<0.05).

Figure 3.5B Temporal changes in osteoclast surface (Oc.S, %) following oophorectomy (Oophx, ■ , solid line) or sham-operation (Sham, □ , broken line) in the metaphysis of the distal femur in the rat, expressed as mean ± SEM. Φ Significant change with time (P<0.05). # Significantly different to Sham with time (P<0.01). * Significantly different to Sham (P<0.05).

Figure 3.5C Temporal changes in osteoclast surface (Oc.S, %) following oophorectomy (Oophx, ■ , solid line) or sham-operation (Sham, □ , broken line) in the epiphysis of the distal femur in the rat, expressed as mean ± SEM. Φ Significant change with time (P<0.05). # Significantly different to Sham with time (P<0.01). * Significantly different to Sham (P<0.05).


Osteoclast Surface
A
Diaphysis
Oc.S (%)
50
40
30
20
10
0
0
5
10
15
20
25
30
Days post operation
Φ#
B
Metaphysis
Oc.S (%)
40
30
20
10
0
0
5
10
15
20
25
30
Days post operation
Φ#
Φ
C
Epiphysis
Oc.S (%)
25
20
15
10
5
0
0
5
10
15
20
25
30
Days post operation
#
Φ

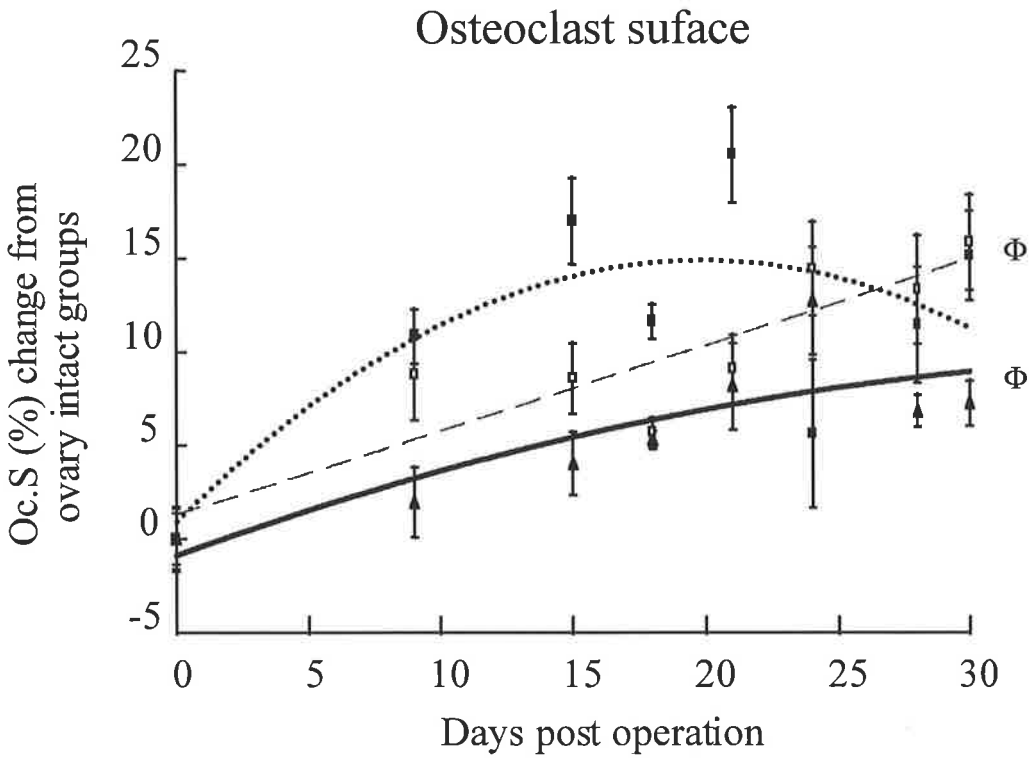


Figure 3.6 Oophorectomy induced changes from ovary-intact groups in osteoclast surface (Oc.S, %) in the diaphysis (DIA, dotted line, ■), metaphysis (META, dashed line, □) and epiphysis (EPI, solid line, ▲) of the distal femur in the rat, expressed as mean ± SEM. Φ Significantly different to DIA with time ($P<0.05$). # Significantly different to META with time ($P<0.05$).

3.3.4 Fluorochrome double labelled surface

In the diaphysis dLS (Fig 3.7A) did not change in the Sham group but rose in the Oophx group ($P<0.0001$) with values greater in the Oophx group ($P<0.001$) from day 9.

In the metaphysis dLS (Fig 3.7B) rose in both groups (Sham $P<0.05$, Oophx $P<0.0001$) with values greater in the Oophx group ($P<0.0001$) from day 21.

In the epiphysis dLS (Fig 3.7C) rose in both groups (Sham $P<0.005$, Oophx $P<0.0001$) with values greater in the Oophx group ($P<0.0001$) from day 28.

3.3.5 Fluorochrome double label extent

In the diaphysis dLE (Table 3.1) did not change in either group in contrast to dLS, with the Sham group lower at both day 9 ($P<0.01$) and day 30 ($P<0.05$).

In the metaphysis dLE (Table 3.2) increased in both groups (Sham $P<0.001$, Oophx $P<0.0001$) with values greater in Oophx ($P<0.0001$) over time.

In the epiphysis dLE (Table 3.3) increased in both groups (Sham $P<0.05$, Oophx $P<0.0001$), as with dLS this was greater in the Oophx group ($P<0.0001$) from day 28.

3.3.6 Mineral apposition rate

In the diaphysis MAR (Fig 3.8A) did not change in either group.

In the metaphysis MAR (Fig 3.8B) rose in the both groups (Sham $P<0.005$, Oophx $P<0.001$), with no difference between operative groups.

In the epiphysis MAR (Fig 3.8C) rose in Sham ($P<0.05$) but did not change in Oophx, with no difference between operative groups.

Figure 3.7A Temporal changes in fluorochrome double labelled surface (dLS, %) following oophorectomy (Oophx, ■ , solid line) or sham-operation (Sham, □ , broken line) in the diaphysis of the distal femur in the rat, expressed as mean ± SEM. Φ Significant change with time (P<0.05). # Significantly different to Sham with time (P<0.01). * Significantly different to Sham (P<0.05).

Figure 3.7B Temporal changes in fluorochrome double labelled surface (dLS, %) following oophorectomy (Oophx, ■ , solid line) or sham-operation (Sham, □ , broken line) in the metaphysis of the distal femur in the rat, expressed as mean ± SEM. Φ Significant change with time (P<0.05). # Significantly different to Sham with time (P<0.01). * Significantly different to Sham (P<0.05).

Figure 3.7C Temporal changes in fluorochrome double labelled surface (dLS, %) following oophorectomy (Oophx, ■ , solid line) or sham-operation (Sham, □ , broken line) in the epiphysis of the distal femur in the rat, expressed as mean ± SEM. Φ Significant change with time (P<0.05). # Significantly different to Sham with time (P<0.01). * Significantly different to Sham (P<0.05).

Double Labelled Surface

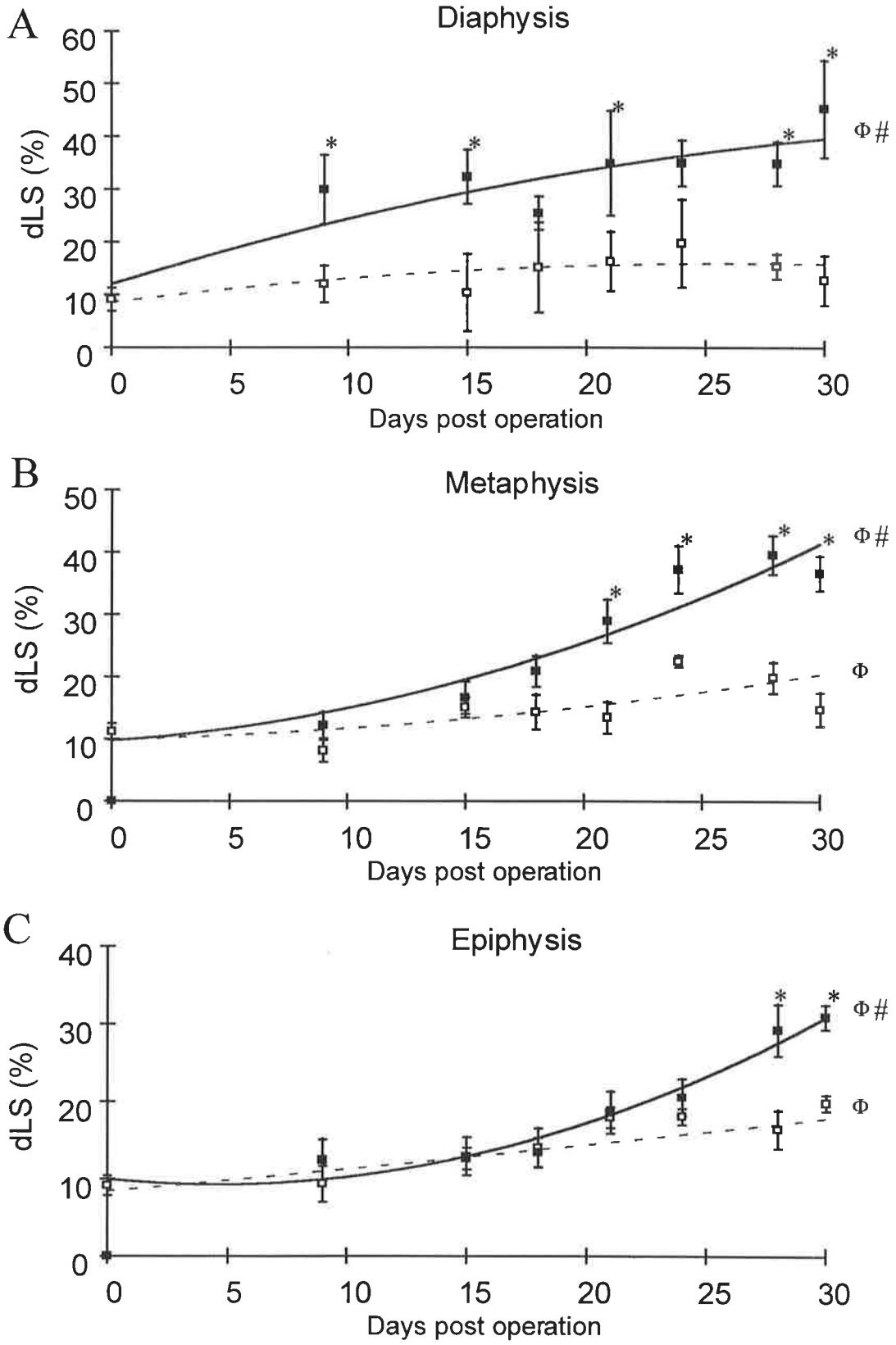
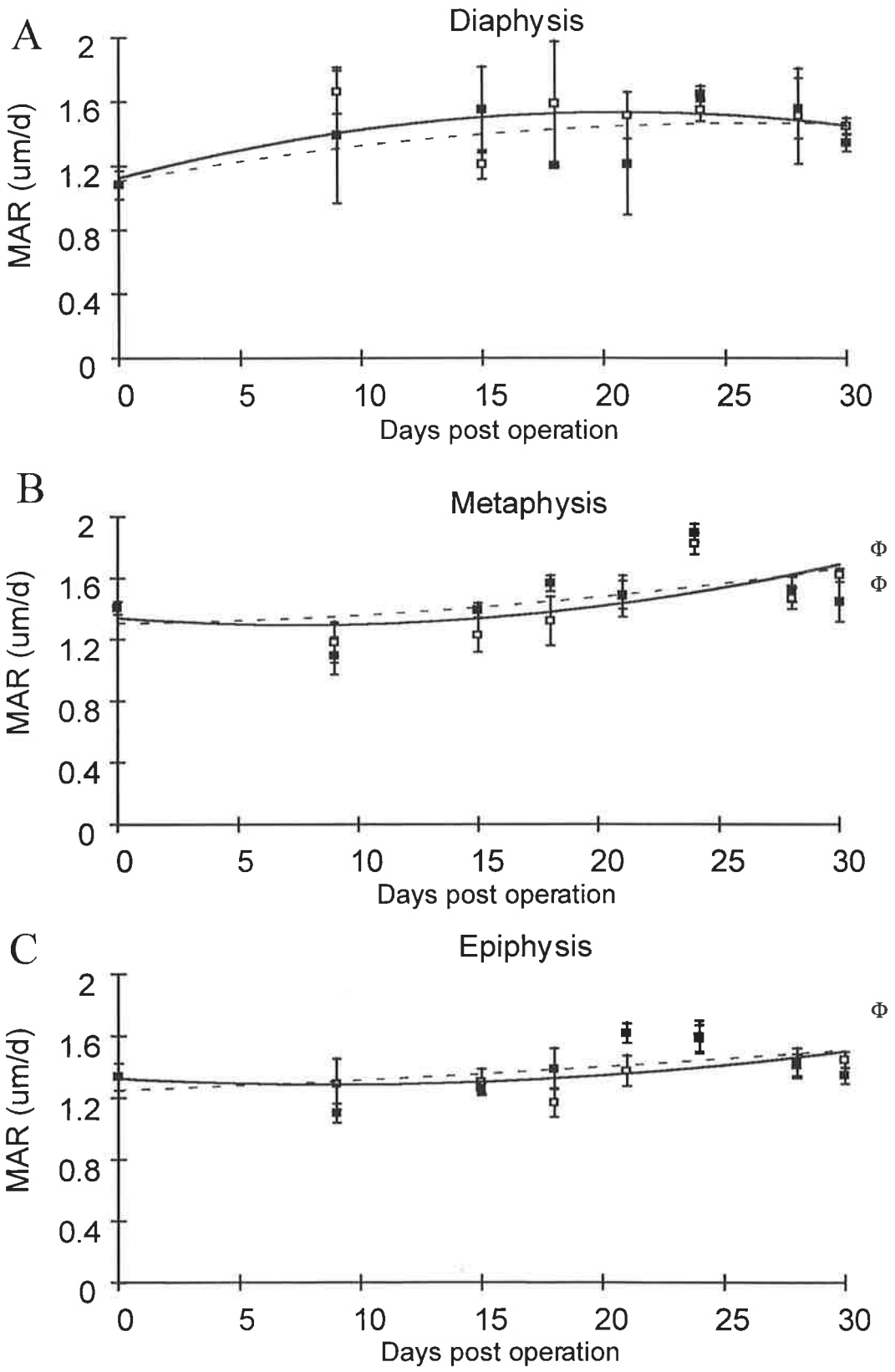


Figure 3.8A Temporal changes in mineral apposition rate (MAR, um/d) following oophorectomy (Oophx, ■ , solid line) or sham-operation (Sham, □ , broken line) in the diaphysis of the distal femur in the rat, expressed as mean ± SEM. Φ Significant change with time (P<0.0001). * Significantly different to Sham (P<0.05).

Figure 3.8B Temporal changes in mineral apposition rate (MAR, um/d) following oophorectomy (Oophx, ■ , solid line) or sham-operation (Sham, □ , broken line) in the metaphysis of the distal femur in the rat, expressed as mean ± SEM. Φ Significant change with time (P<0.0001). * Significantly different to Sham (P<0.05).

Figure 3.8C Temporal changes in mineral apposition rate (MAR, um/d) following oophorectomy (Oophx, ■ , solid line) or sham-operation (Sham, □ , broken line) in the epiphysis of the distal femur in the rat, expressed as mean ± SEM. Φ Significant change with time (P<0.0001). * Significantly different to Sham (P<0.05).

Mineral Apposition Rate



3.3.7 Bone formation rate

In the diaphysis BFR (Fig 3.9A) remained constant in Sham but rose in the Oophx group ($P<0.0001$) with values greater in the Oophx group ($P<0.001$) from day 9.

In the metaphysis BFR (Fig 3.9B) increased in both groups (Sham $P<0.005$, Oophx $P<0.0001$) with values greater in the Oophx group ($P<0.0001$) from day 21.

In the epiphysis BFR (Fig 3.9C) increased in both groups (Sham $P<0.005$, Oophx $P<0.0001$) with values greater in the Oophx group ($P<0.005$) from day 28.

3.3.8 Turnover ratio

In the diaphysis ToR (Table 3.1) decreased in both groups (Sham $P<0.005$, Oophx $P<0.05$) with no difference between operative groups.

In the metaphysis ToR (Table 3.2) also decreased in both groups Sham $P<0.0001$, Oophx $P<0.0001$) with no difference between operative groups.

In the epiphysis this pattern for ToR (Table 3.3) was repeated, (Sham $P<0.0001$, Oophx $P<0.0001$).

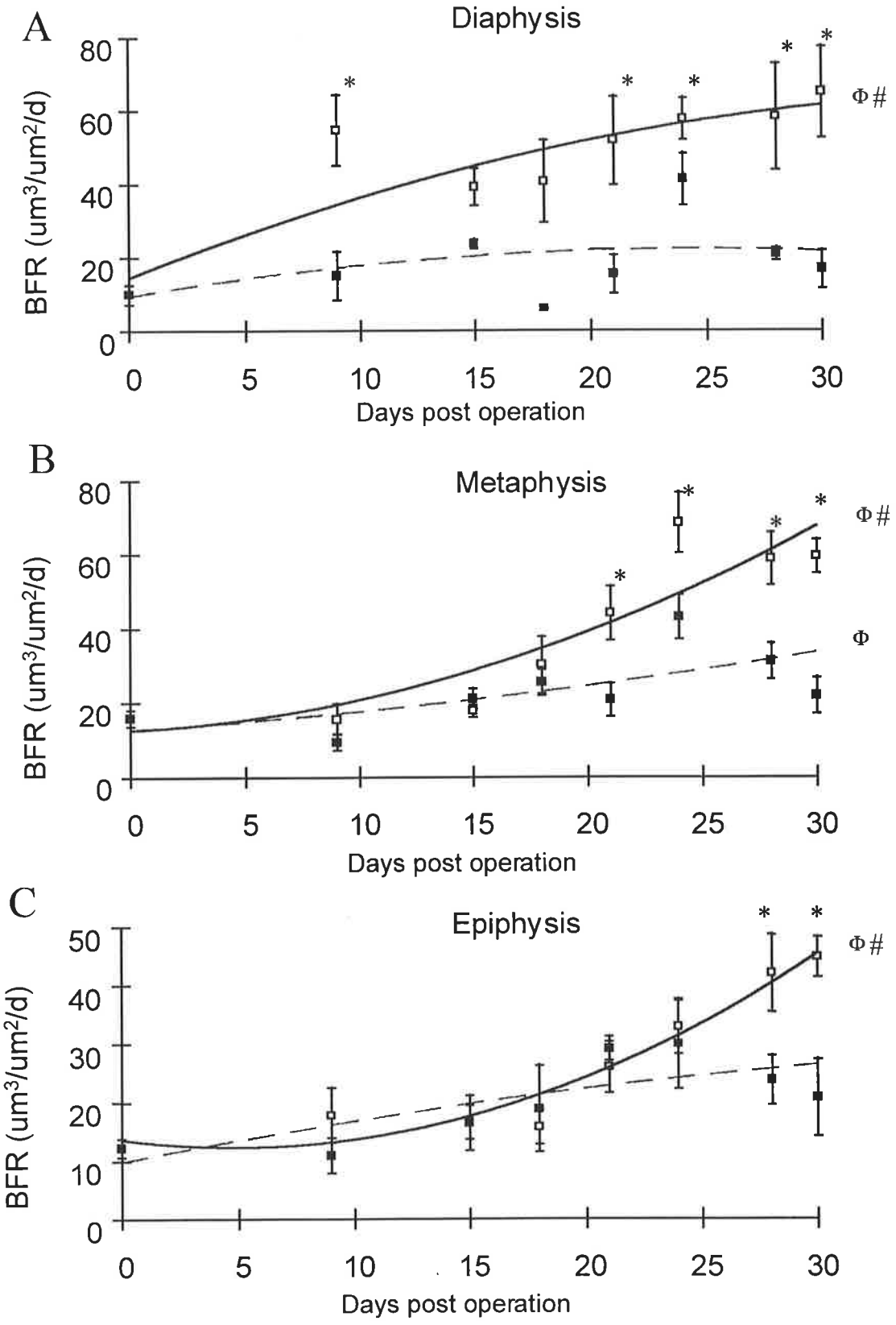
The general pattern of change with time post operation in all regions represented a shift from resorption to formation.

Figure 3.9A Temporal changes in bone formation rate (BFR, $um^3/um^2/d$) following oophorectomy (Oophx, ■ , solid line) or sham-operation (Sham, □ , broken line) in the diaphysis of the distal femur in the rat, expressed as mean ± SEM. Φ Significant change with time (P<0.0001). * Significantly different to Sham (P<0.05).

Figure 3.9B Temporal changes in bone formation rate (BFR, $um^3/um^2/d$) following oophorectomy (Oophx, ■ , solid line) or sham-operation (Sham, □ , broken line) in the metaphysis of the distal femur in the rat, expressed as mean ± SEM. Φ Significant change with time (P<0.0001). * Significantly different to Sham (P<0.05).

Figure 3.9C Temporal changes in bone formation rate (BFR, $um^3/um^2/d$) following oophorectomy (Oophx, ■ , solid line) or sham-operation (Sham, □ , broken line) in the epiphysis of the distal femur in the rat, expressed as mean ± SEM. Φ Significant change with time (P<0.0001). * Significantly different to Sham (P<0.05).

Bone Formation Rate



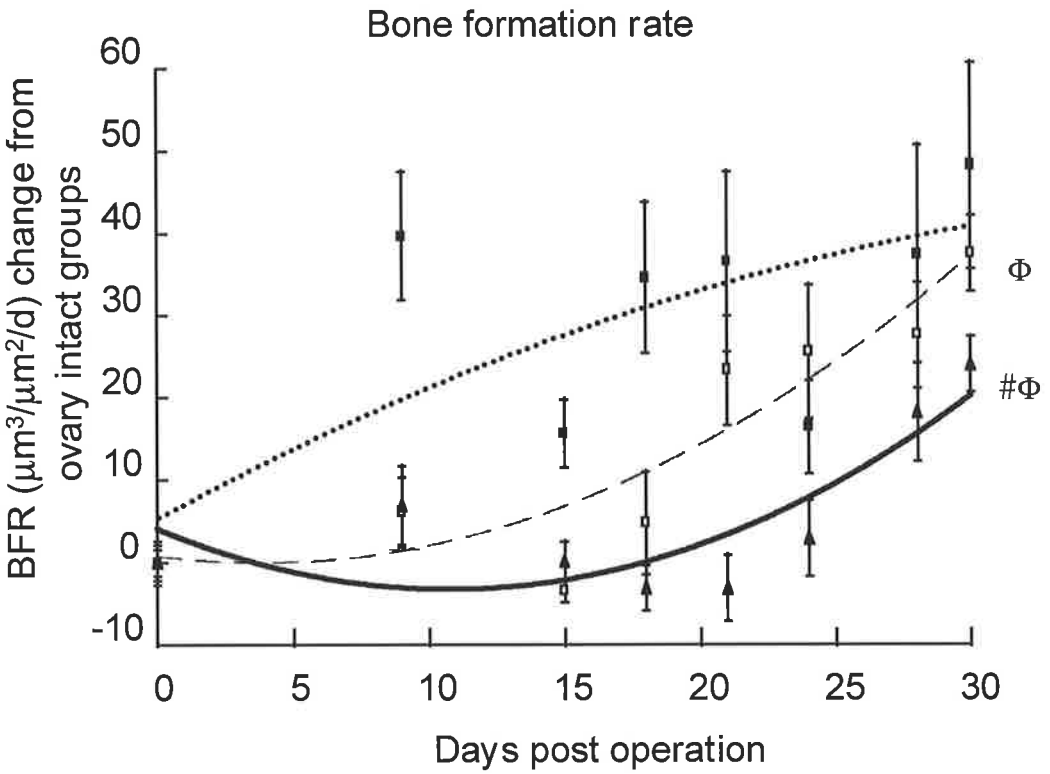


Figure 3.10 Oophorectomy induced changes from ovary-intact groups in bone formation rate (BFR, $um^3/um^2/d$) in the diaphysis (DIA, dotted line, ■), metaphysis (META, dashed line, □) and epiphysis (EPI, solid line, ▲) of the distal femur in the rat, expressed as mean ± SEM. Φ Significantly different to DIA with time ($P<0.05$). # Significantly different to META with time ($P<0.05$).

3.3.9 Inter-region comparison

The ovariectomy-induced decrease in BV/TV in the diaphysis was significantly greater than in the epiphysis ($F=7.21$, $P<0.01$). There was also a trend for bone loss to be greater in the metaphysis compared to the epiphysis ($F=3.12$, $P<0.08$). There was no difference between loss of BV/TV in the diaphysis and the metaphysis.

Tb.N was significantly reduced in the diaphysis ($F=5.81$, $P<0.05$) and metaphysis ($F=5.96$, $P<0.05$) compared to the epiphysis. There was also a trend for a greater reduction in the diaphysis compared to the metaphysis ($F=3.16$, $P<0.08$).

Tb.Th was significantly reduced in the diaphysis compared to the metaphysis ($F=4.15$, $P<0.05$).

The time for the response of Oc.S (Fig 3.6) to ovariectomy in the diaphysis was less than for both the metaphysis ($F=7.09$, $P<0.01$) and the epiphysis ($F=4.63$, $P<0.05$). There was no significant difference between the metaphysis and epiphysis.

The time for the response of BFR (Fig 3.10) to ovariectomy was significantly less in the diaphysis than for both the metaphysis ($F=6.02$, $P<0.05$) and the epiphysis ($F=5.93$, $P<0.05$), while the metaphysis was significantly less than for the epiphysis ($F=5.55$, $P<0.05$).

3.3.10 Scanning electron microscopy

In the ovary intact femur 30 days after sham operation, scanning electron micrography (Fig 3.4A) revealed a robust cancellous network in the epiphysis. The metaphysis also contained a robust trabecular network, extending to the margins of the sample. The density of the trabecular network did diminish toward the proximal aspect of the medullary cavity.

Oophorectomy (Fig 3.4B) did not have an effect on the bone of the epiphysis, with a similar appearance to the ovary intact femur. In contrast, the diaphysis was markedly osteopenic with trabeculae virtually absent from the proximal aspect of the medullary cavity, consistent with ovarian hormone related bone loss.

3.4 Discussion

3.4.1 Regional comparison of the development of osteopenia following ovarian hormone deficiency

The response of trabecular bone to ovarian hormone deficiency has generally been studied using single sample areas (Wronski *et al.,* 1985, Shen *et al.,* 1995) or multiple areas from disparate parts of the skeleton (Wronski *et al.,* 1989). When multiple sites are sampled within a single bone significant variation has been reported in the magnitude of osteopenia resulting from ovarian hormone deficiency (Durbridge *et al.,* 1990, Westerlind *et al.,* 1997). Moreover, the femoral epiphyseal trabecular bone has been reported to be resistant to the initial development of osteopenia (Martin and Zissimos, 1991, Ikeda *et al.,* 1996). By sampling three sites within the femur the extent of variation in response to ovarian hormone deficiency has been further quantified. Thirty days of ovarian hormone deficiency reduced BV/TV compared to ovary intact animals by 71% in the diaphysis, 35% in the metaphysis, while the epiphysis showed no decline. These data confirm previous reports that the femoral epiphysis is resistant to the short term consequences of ovarian hormone deficiency and further, suggest a graded propensity for the development of osteopenia in the distal femur, increasing proximally.

3.4.2 Regional comparison of the development of osteopenia following ovarian hormone deficiency: Effects of the remodelling transient

In a study of short term effects following oophorectomy, the impact of a remodelling transient must be considered. The steady state contributions of oestrogen deficiency and those transient effects due to altered activation frequency cannot be separated during the first remodelling cycle following oophorectomy (Heaney, 1994). The

remodelling period has been estimated in the caudal vertebra of 3 month old rats to last for 38 days (Baron *et al.,* 1984). Thus the osteopenia recorded during this initial period following oophorectomy is likely to be dominated by the remodelling transient. In the diaphysis and metaphysis 72% and 73% of total bone loss occurred in the first 9 days respectively, suggesting that resorption increased before day 9 in these two regions. This time course is consistent with previous reports showing increased resorption in the femoral metaphysis 6 days after oophorectomy (Sims *et al.,* 1996). Further, these data demonstrate that the remodelling transient is the dominant factor in the development of osteopenia in these regions at this time post operation. BMC measurements in the distal femur, have shown that the relative osteopenia detected at 35 days post oophorectomy was no more pronounced at 540 days (Kimmel and Wronski, 1990), consistent with the large, rapid loss of bone during the period of the remodelling transient.

It should also be noted that at day 15, BV/TV in the diaphysis was 0.53%. The scarcity of bone in this region so early after oophorectomy reduces the suitability of this region for study of ovarian hormone deficient bone loss, while emphasising its suitability for study of the remodelling transient.

3.4.3 Structural changes in trabecular bone during the remodelling transient

Tb.Th was reduced in all regions. However, the reduction was independent of operation group, and therefore cannot be considered an ovarian hormone dependent occurrence, and is most likely an effect of operative procedures (3.4.6). While this thinning of trabeculae is potentially temporary, perforation events generated during this early period are permanent. Perforation of a trabecular strut at any point along its axis triggers the removal of the entire element (Parfitt *et al.,* 1983, Yoshida *et al.,*

1991), as a result of its inability to transfer mechanical load (Mosekilde 1990). The mechanism of ovarian hormone deficient bone loss is dominated by the perforation and removal of entire trabecular units (reviewed by Kalu, 1991, Frost and Jee, 1992). In the present study, oophorectomy reduced Tb.N by 50% in the diaphysis and by 25% in the metaphysis, while no loss was recorded in the epiphysis. The deficit in BV/TV produced by the initial perforation event is therefore greatly magnified as the removal of the entire strut proceeds. Following ovarian hormone deficiency turnover is accelerated, with resorption exceeding formation. Perforation creates an additional physical imbalance in turnover, due to the absence of template for later bone formation at the site of the perforation (Reeve *et al.,* 1987). The retention of template in the epiphysis during this initial period therefore confers long term advantages to this region (4.4.2).

3.4.4 Influence of local mechanical strain on the development of osteopenia

Oophorectomy significantly reduced Tb.N in the diaphysis and metaphysis, but not in the epiphysis. Clearly the resistance to perforation is the result of a factor, or factors local to the region. The influence of mechanical strain has previously been claimed to explain the resistance of the epiphysis to bone loss (Westerlind *et al.,* 1994, Turner *et al.,* 1994b). External mechanical loading has been shown to decrease osteoclast proliferation (Rubin *et al.,* 1996) and function (Lafage *et al.,* 1995) and stimulate formation (Forwood and Turner, 1995). Mechanical strain, presenting locally within each trabeculae, may also influence the likelihood of perforation, with the less loaded units being removed first (Frost, 1992, Mosekilde, 1990). Finite element analysis has shown strain to be higher in the trabeculae of the epiphysis compared to the more proximal regions (Westerlind *et al.,* 1997). This may predispose the trabeculae in low

strain regions, such as the diaphysis, to perforation before those of the epiphysis, as evident in the present study. This preferential loss of less strained trabeculae is supported by the loss of horizontal trabeculae in the vertebral bodies (Mosekilde, 1990) and the disruption of the normal pattern of oophorectomy induced bone loss in the tibia of the rat when performed in concert with hemicordectomy (Yoshida *et al.*, 1991). Westerlind *et al.*, (1997) further suggested that local mechanical strain was responsible for maintaining the local bone balance independent of the rate of turnover. Thus the resistance of epiphyseal trabeculae to ovarian hormone deficient bone loss is likely to be the result of higher local mechanical strain, which impedes the perforation of trabeculae, in addition to the architectural protection from perforation due to thicker trabeculae. These conclusions are consistent with the findings of previous studies of femoral epiphyseal bone loss (Westerlind *et al.*, 1997).

3.4.5 Regional comparison of the time course for increased bone cell activity following oophorectomy

It has been reported that oophorectomy initiates rapid increases in bone turnover in the metaphysis of the distal femur of the rat (Sims *et al.*, 1996, Lane *et al.*, 1998). This finding is confirmed and expanded to include the diaphysis and epiphysis. The time course of these changes however was not uniform. The rise in resorption was significantly more rapid in the diaphysis than in the metaphysis and epiphysis, with the response being similar in the latter two regions. The speed of the increase in bone formation was significantly different between all regions, being most rapid in the diaphysis and slowest in the epiphysis.

These findings are consistent with differences in the time course of increased bone cell activity recorded between the proximal tibial metaphysis (Wronski *et al.*, 1988)

and the first lumbar vertebrae (Wronski *et al.,* 1989), with the latter region exhibiting a delayed response. Thus factors local to particular regions must modify the time course of alterations to bone turnover subsequent to oestrogen deficiency. The slower response in the epiphysis is clearly a factor in the initial resistance to osteopenia exhibited by this region. However, observation over an extended period would be required to ascertain whether resistance to osteopenia was complete or related to the time of exposure to increased turnover. Thus not only does architectural variation provide a physical protection the epiphyseal bone from initial loss, but a delay in the increase in bone cell activity provides a temporal protection during this early period.

3.4.6 Trabecular thinning in oophorectomised and ovary intact groups: Lack of evidence for age related mechanism

Tb.Th decreased in each area independent of oestrogen status in contrast to previous studies (Dempster *et al.,* 1995, Sims *et al.,* 1996). In ovary intact groups this occurred despite stable or decreasing Oc.S and stable or increasing BFR. The reduction in Tb.Th is unlikely to be the result of age related loss. From Chapter 7 it is known that in 7 month old rats, age related loss of trabecular bone is limited to approximately 1.2% per month in the diaphysis and 0.3% per month in the metaphysis and epiphysis. The reduction in BV/TV observed in the ovary intact groups in this study was one or two orders of magnitude higher, pointing to pathology rather than normal aging. The process of age related bone loss has been associated with decreased osteoblast extent and/or function (Melsen *et al.,* 1978, Eriksen, 1986). The stable or increasing values for dLS and MAR in this study suggests that the decrease in trabecular thickness is not occurring by the same mechanism. Further, in the ovary intact groups these changes occurred during a period when Oc.S was decreasing or stable, suggesting the possible

role of increased resorption depth in this process. Given the conflicting bone cell activity in the ovary intact groups, the decrease in Tb.Th was most likely the result of a temporary aberration due to the operative procedure. If so the changes would not be permanent and further cycles of remodelling should return Tb.Th to baseline levels. Long term observation is required to determine whether these changes are sustained.

3.4.7 Regional comparison of changes in the proliferation of bone cells following oophorectomy

The comparison of surface referent percentage values for bone cell indices between oophorectomised and ovary intact groups may be misleading due to the reduction of trabecular bone surface following oophorectomy. The loss of trabecular perimeter during bone loss magnifies percentage based indices of bone turnover, making comment upon proliferation inappropriate in regions subject to bone loss (Parfitt *et al.,* 1983). While oestrogen deficiency is known to extend osteoclast lifespan *in vitro* and *ex vivo* (Hughes *et al.,* 1996), the increase in linear extent of bone cells in ophorectomised rats is consistent with increased proliferation with oestrogen deficiency known to occur *in vivo* (1.10.1.1).

Increases in the linear extent of bone cells following oophorectomy were consistent with the analysis of percentage values in the metaphysis and epiphysis, displaying an increase in proliferation due to ovarian hormone deficiency. It must be noted that in the epiphysis this rise was not absolute but relative to a fall in the ovary intact group, in a similar manner to Oc.S in this region.

In the diaphysis however, analysis of Oc.E and dLE showed no difference between the operative groups over time. Further, Oc.E decreased over time in both groups. This lack of an absolute increase in bone cell proliferation in the diaphysis

following oophorectomy is consistent with suggestions that differences in progenitor populations can affect regional responses to ovarian hormone deficiency (Turner *et al.*, 1994b). It is however inconsistent with the bone loss seen in both groups during the study.

It is possible that osteoclast proliferation had occurred and returned to ovary intact levels by day 9. This is unlikely without a subsequent and prolonged increase in osteoblast proliferation. dLE was greater in this region only at day 9 and day 30 after oophorectomy. The loss of template resulting from such dramatic bone loss may have made a more prolonged increase in formation physically impossible, with only 24% of the trabecular surface inactive at day 15. However the formation period in the rat has been calculated to take 33 days and it is unlikely that all evidence of increased formation was halted by perforation events by day 15. Thus there appears to be some inconsistency between cellular proliferation and bone loss in the diaphysis, suggesting altered physiology between this region and those more distal regions.

3.4.8 Influence of local trabecular structure on the development of osteopenia

The decline in the linear extent of osteoclasts in the diaphysis indicates a decrease in osteoclast proliferation, and is thus difficult to reconcile with the observed bone loss. This contradiction may be explained in part by differences in structure between the regions. At operation the most prominent structural difference between the three regions was Tb.Th, baseline values were 64 um in the diaphysis, 90 um in the metaphysis and 159 um in the epiphysis. The stochastic probability of perforation is known to be greatest in the region with the thinnest trabeculae (Parfitt 1992, Thomsen *et al.*, 1996). Given similar resorption depth in all regions, the reduced thickness of

trabeculae in the diaphysis would predispose this region to bone loss by perforation to greater extent compared to the metaphysis and epiphysis.

Interestingly, local structural differences may explain the greater propensity for perforation in the diaphysis as a whole but they do not explain the initiation of greater osteopenia following oophorectomy. Once bone loss is initiated in the diaphysis of oophorectomised animals, a similar linear extent of osteoclasts was activated on a constantly diminishing surface. This increased turnover per unit of existing bone surface, in effect producing a situation similar in nature to that evident in the metaphysis and epiphysis. Thus a surface based increase in turnover may be sufficient to explain the continuation of bone loss in this region. This does not explain the initial trabecular perforation which reduced the bone surface to drive the surface based increase in turnover. As the linear extent of osteoclasts in both operative groups was similar throughout the study, bone turnover could only be elevated following oophorectomy as a result of bone loss. Therefore, the oophorectomy induced increase in bone turnover and the resultant effects of the remodelling transient (4.4.2) in this region result from a loss of bone which is independent of increased proliferation. This perforation of trabeculae in the diaphysis, without an increase in osteoclast proliferation, indicates the involvement of increased resorption depth, consistent with the speed of bone loss during the first 9 days. Thus in the diaphysis, ovarian hormone deficiency may stimulate in increase in resorption depth during the fisrt 9 days following operation, thereby stimulating increased turnover despite no apparent increase in proliferation of osteoclastsic or osteoblastic cells.

3.4.9 Comparison of turnover ratio between operative groups: Implications for resorption depth

The osteoblastic response to oophorectomy can be separated into dLS and MAR, with the former more representative of proliferation and the latter more representative anabolic activity (Parfitt, 1995). While these generalisations restrict comment to speculation. Given this, the present study indicates that the majority of the response comes from increased proliferation with only minor contributions from increased activity. While it is clear that osteoclast proliferation is increased in the metaphysis and epiphysis following oophorectomy, the contributions of increased resorption depth, particularly in the diaphysis, remain unclear. The osteoclastic response cannot be analysed in the same manner as the osteoblastic response due to the lack of a direct method for estimating resorption depth in this model. However changes in osteoclast function may be indirectly assessed. Estimates of turnover ratio were produced in this study to examine whether regional variations in resorption depth were involved in the variations in bone loss observed. This ratio assumes that bone loss is proportional to the balance between net resorption and net formation. It accounts for the extent of bone surface undergoing resorption, as well as the extent of bone surface undergoing formation and the amount formed at these sites, thus the only missing factor is resorption depth (2.14). There was no difference in ToR between operative groups in all three regions, despite the osteopenia resulting from oophorectomy in the diaphysis and metaphysis. Therefore, the bone loss can only be accounted for by the factor outside the equation, i.e. resorption depth. Further, these calculations suggest that a rapid increase in resorption depth occurs following oophorectomy. Similar findings have been demonstrated in the lumbar vertabrae of oophorectomised minipigs, with a 25% increase in final resorption depth was recorded following operation (Mosekilde *et*

al., 1993, Boyce *et al.,* 1995), using the lamellar counting method (Eriksen *et al.,* 1984).

An increase in resorption depth soon after oophorectomy is consistent with the rapid bone loss in the first 9 days after operation and is supported by computer modeling studies (Garrahan *et al.,* 1990). Increases in activation frequency are unable to produce large, permanent alterations to trabecular bone volume (Heaney, 1994, Hilliam and Skerry, 1995), with computer modeling showing that a doubling of activation frequency causes transient, mainly reversible bone loss. However when this is accompanied by an increase in resorption depth, the irreversible component is more pronounced (Thomsen *et al.,* 1994). Further support for an increase in resorption depth is the perforation of trabeculae in the diaphysis prior to an increase in turnover. This increase in resorption depth is likely the result of increased production of lytic enzymes in osteoclasts, shown to result from oestrogen deficiency in vivo (Pacifi, 1996, Jilka, 1998).

3.4.10 Influence of increased resorption depth on the development of osteopenia

An increase in resorption depth would increase the likelihood of trabecular perforation in all areas, however areas with thinner trabeculae are more likely to undergo perforation (Parfitt *et al.,* 1983, Compston *et al.,* 1989). Such a process is consistent with the regional difference in the magnitude of osteopenia that develops following oophorectomy in the distal femur of the rat (3.4.1). It also appears that the osteopenic effect of the increase in resorption depth is sufficient to remove bone from the diaphysis, already susceptible to perforation due to its structure (3.4.8), without an increase in osteoclast proliferation. In the epiphysis the greater thickness of trabeculae would impede perforation resulting from an increase in both resorption depth and

osteoclast proliferation (3.4.3), further enhancing template retention and therefore long term resistance to bone loss.

To support the existence of an increase in resorption depth using this indirect method osteopenia must be greater following oophorectomy, which is not the case in the epiphysis. It is therefore possible that the increase in resorption depth is confined to the metaphysis and diaphysis, or is greatly diminished in the epiphysis. Consistent with such a hypothesis is the similarity of trabecular thinning in the epiphysis in both operative groups. Any substantial increase in resorption depth would result in a decrease in Tb.Th, suggesting that resorption depth is not increased in this region following oophorectomy. The hypothesis of regional variation in resorption depth following oophorectomy is supported by a study reporting that bone balance is modulated by local mechanical strain (Westerlind *et al.,* 1997). They demonstrate that high strain environments such as the epiphysis oppose the osteopenic effects of the changes in bone cell activity induced by oophorectomy without diminishing the increase in turnover. Data from the present study suggests that high strain prevents the increase in resorption depth following oophorectomy, impeding the development of osteopenia.

From these observations it is suggested that ovarian hormone withdrawal initiates an increase in bone resorption, involving both greater depth and extent of osteoclastic activity which is modulated by local factors such as mechanical strain. In the diaphysis, where trabeculae are thin and strain is low, osteopenia develops without proliferation, suggesting that the increase in resorption depth may come before proliferation, and further, that excessive bone loss may inhibit proliferation at a local level. In the epiphysis, where trabeculae are thick and mechanical strain is high, the increase in resorption depth is diminished or blocked and proliferation is delayed.

Chapter 4

Long term variation following oophorectomy in two regions of the distal femur of the rat.

4.1 Introduction

Bone turnover is elevated in the femoral epiphysis 11.5 months post oophorectomy (Westerlind *et al.,* 1997), it is however unknown whether the increased activation of bone cells endures from the time of oophorectomy or is diphasic as seen in the proximal tibiae (Wronski *et al.,* 1989b). The previous chapter demonstrated that Oc.S was elevated relative to ovary-intact animals in the epiphysis by day 18 and BFR elevated by day 28, with no associated bone loss in the first 30 days following oophorectomy. However the short period of elevated turnover recorded in that study cannot confirm the long term immunity of this region to ovarian hormone deficient bone loss. Mechanical strain has been shown to be a dominant factor in the resistance of this region to osteopenia (Westerlind *et al.,* 1997), however its effect on age related bone loss has not been evaluated. Further, there was a reduction in trabecular thickness in all regions of trabecular bone over this 30 day period, independent of ovarian hormone status. Observations over a number of remodelling cycles would distinguish whether this was a permanent alteration to bone structure or a temporary effect of operative procedures.

In order to address these questions we have monitored the effects of oophorectomy in two regions of the distal femur of the rat up to 150 days after operation, recording bone cell activity and trabecular bone morphometry in the metaphyseal and epiphyseal trabecular compartments.

4.2 Materials and Methods

4.2.1 Animals

112 Female Sprague-Dawley rats 6 months old were maintained on commercial rat chow (2.2) and tap water *ad libitum*. All procedures involving the animals were approved by the IMVS Animal Ethics Committee.

4.2.2 Surgery

At 7 months of age 6 animals were killed as a baseline group and the remainder allocated to either oophorectomy or sham operation, via the dorsal approach (2.4.3). All rats received demeclocycline and calcein (2.2), 6 and 2 days respectively, prior to killing. At 30, 36, 42, 48, 66, 90, 120 and 150 days post operation, 6 Oophx and 6 Sham rats were killed and the femora were excised.

4.2.3 Histomorphometry

The distal 20 mm of the right femora were bisected in the sagittal plane and processed into MMA resin (2.9.1.2). One half of the left femora of a randomly chosen sample from each group killed at 150 days were used for scanning electron microscopy (2.8). Sections were partitioned into two regions. Due to the paucity of bone in the diaphysis, this region was not sampled for long term analysis. The metaphysis and epiphysis were sampled (2.11.1). BV/TV, Tb.N, Tb.Th and Peri were calculated (2.14) from von Kossa stained sections (2.9.3.1).

dLS and interlabel distance were estimated from unstained sections (2.11.3) and Oc.S was estimated (2.12.2.3) from VK/HE sections (2.9.3.2).

4.2.4 Calculations and statistics

MAR, BFR, Oc.E, dLE and ToR were calculated (2.14). Intra-region analysis was conducted using repeated measures analysis of variance with contrast to compare the mean values for each variable for the two operative groups at each time point. Change through time was estimated by regression analysis assessed for each operative group in that region. Inter-region analysis was conducted by analysis of variance with contrast on slopes of the regression equations on time for the individual Oophx values and the mean Sham value for each time point. Due to difference in baseline values between regions, static trabecular bone variables were normalised by expressing the resultant individual Oophx values as a percentage of the Sham mean for that time point and region. Statistics were performed using PC-SAS (SAS Institute, Cary NC). $P<0.05$ was considered significant for all analyses.

4.3 Results

4.3.1 Trabecular bone structure

4.3.1.1 Metaphysis

In the ovary-intact groups, BV/TV (Fig. 4.1A) fell with increasing age ($P<0.0001$), due to a decrease in Tb.N (Fig. 4.2A) ($P<0.0001$), with Tb.Th remaining stable (Fig. 4.3A). Oophorectomy produced greater bone loss ($P<0.0001$), falling to 27 % of the Sham values at day 150. The reduction was due to a decrease in Tb.N ($P<0.0001$), with Tb.N lower in the Oophx group compared to Sham ($P<0.0001$) from day 30. The decrease in Tb.N within Oophx occurred earlier than in Sham, with 62 % of total loss occurring by day 66 in Oophx, compared to 30 % of total loss at the same time point in Sham. Tb.Th was stable in Oophx over the 150 days.

Peri (Table 4.1) decreased in both groups (Sham $P<0.0005$, Oophx $P<0.0001$), the decrease was greater in Oophx ($P<0.001$) from day 36.

4.3.1.2 Epiphysis

In the ovary-intact groups, BV/TV (Fig. 4.1B) again fell with increasing age ($P<0.05$), due to a reduction in Tb.N (Fig. 4.2B) ($P<0.0001$), with Tb.Th remaining stable (Fig. 4.3B). Oophorectomised groups also lost bone ($P<0.0001$), however there was no consistent difference between operative groups. The fall in BV/TV was due to a reduction in Tb.N ($P<0.0001$), with Tb.N being lower in Oophx ($P<0.001$) over time. Tb.Th increased in the Oophx group ($P<0.0001$), to be greater than Sham ($P<0.05$) over time. Peri (Table 4.2) was stable in the Sham group but fell in Oophx ($P<0.0001$) to be lower than Sham ($P<0.005$) from day 120.

4.3.1.2

The development of osteopenia following oophorectomy was significantly more rapid in the metaphysis than the epiphysis ($P<0.0001$), as was the loss of trabeculae ($P<0.0001$), with no difference between changes in Tb.Th. The decrease in Peri in the metaphysis following oophorectomy was significantly more rapid than in the epiphysis ($P<0.0001$).

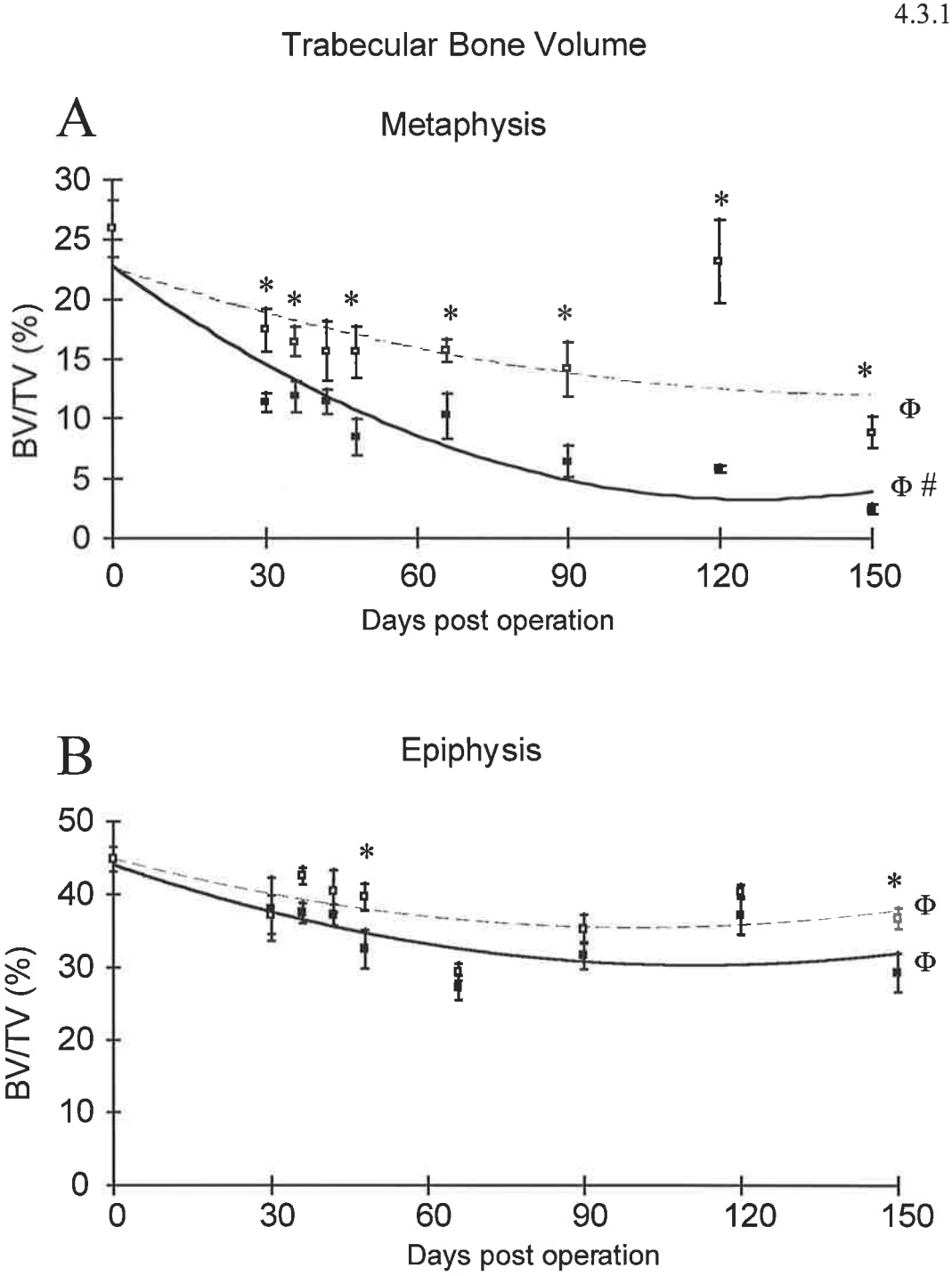


Figure 4.1 Temporal changes in trabecular bone volume (BV/TV, %) following oophorectomy (Oophx ■ , solid line) or sham-operation (Sham □, broken line) in the metaphysis (A) and epiphysis (B) of the distal femur in the rat, expressed as mean ± SEM. Φ Significant change with time ($P<0.05$). # Significantly different to Sham with time ($P<0.0001$). * Significant difference between operative groups ($P<0.05$).

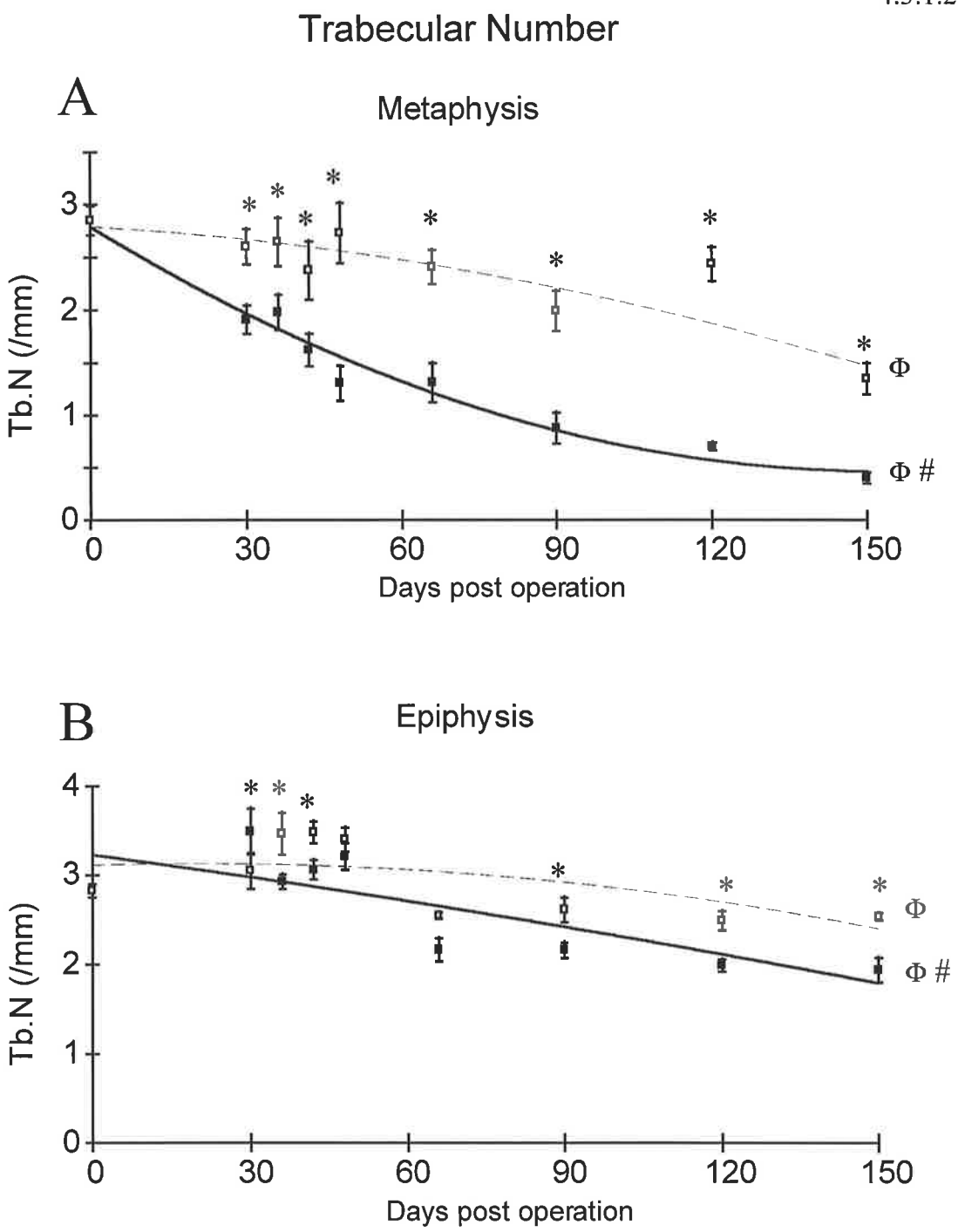


Figure 4.2 Temporal changes in trabecular number (Tb.N, /mm) following oophorectomy (Oophx ■ , solid line) or sham-operation (Sham □, broken line) in the metaphysis (A) and epiphysis (B) of the distal femur in the rat, expressed as mean ± SEM. Φ Significant change with time (P<0.0001). # Significantly different to Sham with time (P<0.001). * Significant difference between operative groups (P<0.05).

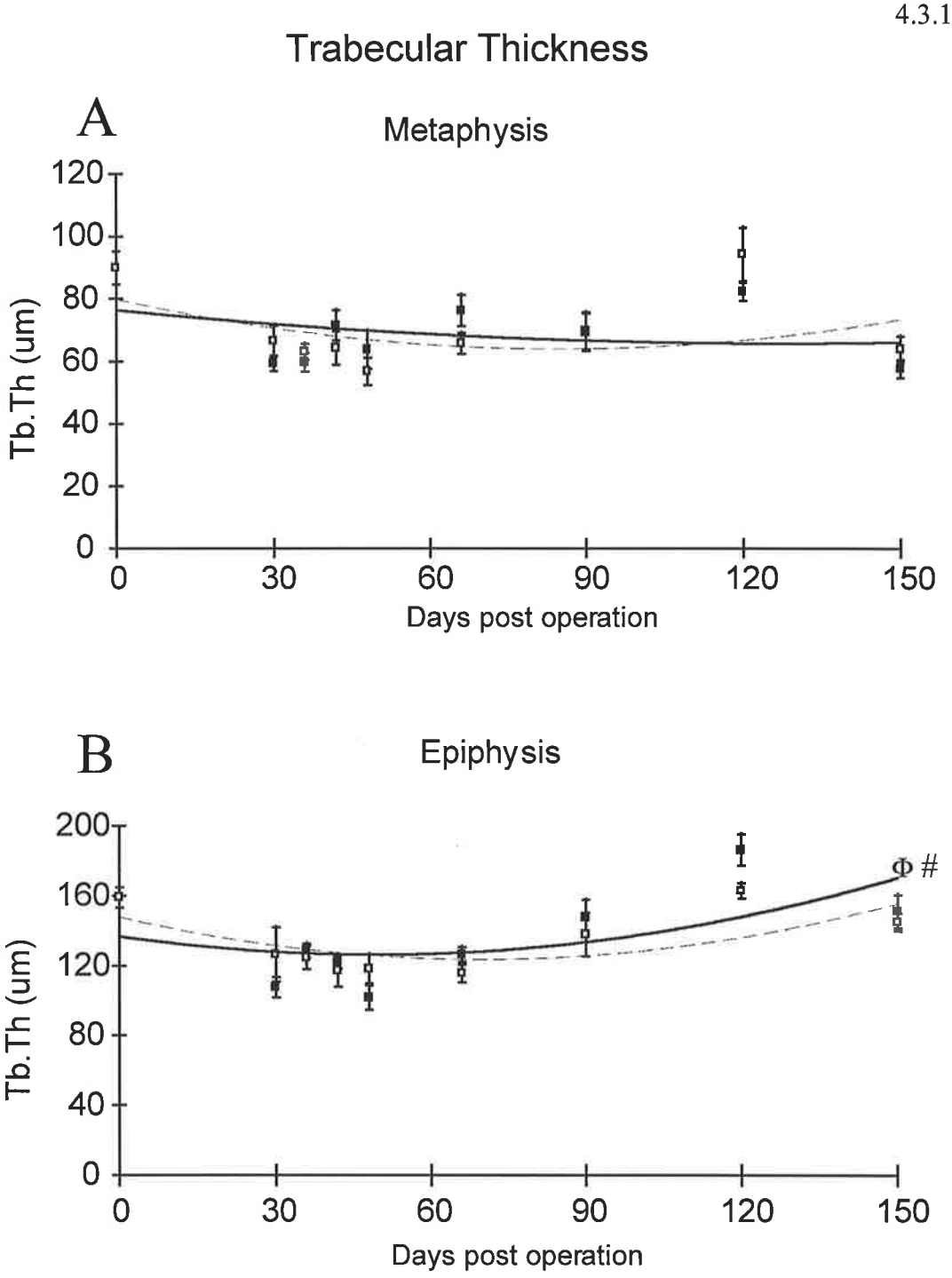


Figure 4.3 Temporal changes in trabecular thickness (Tb.Th, um) following oophorectomy (Oophx ■ , solid line) or sham-operation (Sham □, broken line) in the metaphysis (A) and epiphysis (B) of the distal femur in the rat, expressed as mean ± SEM. Φ Significant change with time ($P<0.0001$). # Significantly different to Sham with time ($P<0.05$).

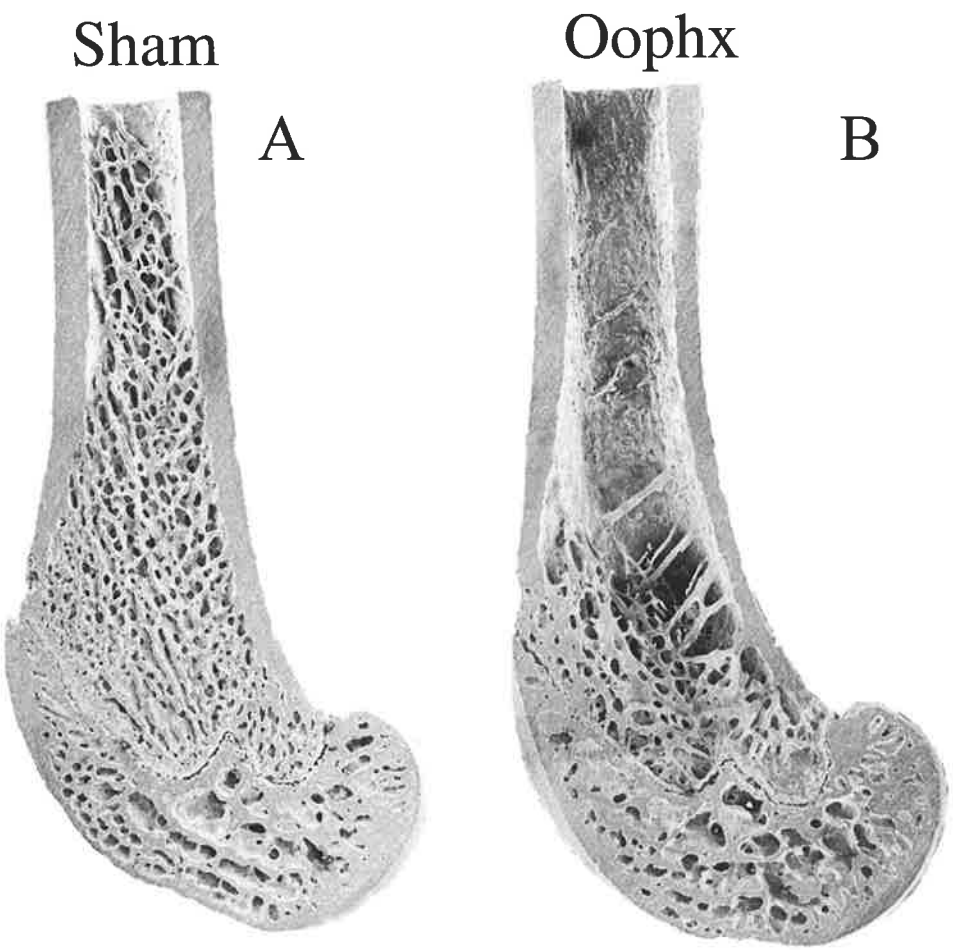


Figure 4.4 The effect of 150 days of ovarian hormone deficiency on the trabecular bone of the distal femur. In the metaphysis bone loss is evident in the ovary intact bone (A) and greatly increased by oophorectomy (B), however is absent in the epiphysis.

Table 4.1 Results of metaphyseal morphometric analysis, by days post oophorectomy

Days post		0	30	36	42	48	66	90	120	150
Peri										
	ShamΦ	74774(2989)	73022(6259)	77313(8618)	684618780)	74861(8017)	72740(5156)	67582(6143)	81302(8820)	41697(5786)
	OophxΦ		64799(4906)	58517(5193)*	40665(4878)*	37566(5864)*	41213(5667)*	28002(5336)*	24469(735)*	11873(1725)*
Oc.E										
	ShamΦ	12389(1041)	8636(1299)	6253(1115)	9488(1768)	9351(1296)	11432(1674)	8479(1033)	6567(792)	5129(863)
	OophxΦ		16587(2747)*	14416(1202)*	8936(1839)	7756(1949)	8504(968)	7475(1304)	4414(154)	2432(344)
dLE										
	ShamΦ	8262(894)	9328(839)	12493(2140)	9274(3261)	7711(1610)	10706(802)	9706(3165)	15278(3817)	13362(1907)
	OophxΦ		23642(2038)*	14890(1683)	16888(2140)*	13106(1997)*	14417(1382)	9195(1808)	11032(518)	5278(1548)*
ToR										
	Sham	0.28(0.04)	0.12(0.03)	0.10(0.02)	0.20(0.02)	0.21(0.04)	0.29(0.04)	0.23(0.05)	0.15(0.02)	0.10(0.01)
	Oophx		0.11(0.01)	0.20(0.06)	0.09(0.02)	0.09(0.02)	0.14(0.02)	0.23(0.02)	0.13(0.01)	0.12 (0.04)

Values are mean (SEM) for trabecular bone perimeter (Peri, um), osteoclast extent (Oc.E, um), fluorochrome double label extent (dLE, um) and turnover ratio (ToR) measured in the metaphyseal region of the distal femur in non-operated rats (0 days) and sham operated (Sham) and oophorectomised rats (Oophx) at 30, 36, 42, 48, 66, 90, 120, and 150 days post operation at 7 months of age. Φ Significant change with time ($P<0.01$). # Significantly different to Sham with time ($P<0.01$). * Significantly different to Sham ($P<0.05$).

Table 4.2 Results of epiphyseal morphometric analysis, by days post oophorectomy

Days post operation		0	30	36	42	48	66	90	120	150
Peri										
	Sham	61278(2776)	53234(4188)	73902(5896)	66188(6464)	61202(3422)	56252(2006)	58329(2190)	60959(2987)	60231(2489)
	OophxΦ#		63503(4553)	62677(3732)	62169(4904)	59427(3004)	48250(4762)	52484(3883)	47603(1477)*	47521(2941)*
Oc.E										
	Sham	8492(1279)	4360(815)	4666(516)	5532(1017)	6696(702)	2977(919)	5523(424)	4584(495)	5301(676)
	OophxΦ#		9575(815)*	10698(924)*	10226(1920)*	10531(1070)*	8036(848)*	10185(1311)*	4582(534)	5665(853)
dLE										
	ShamΦ	5482(628)	7017(1680)	11827(2017)	8513(2513)	6541(1185)	8507(1229)	8207(2270)	11202(2132)	18103(1415)
	OophxΦ#		19649(1752)*	1655(2285)	25393(2206)*	21297(1878)*	18651(1857)*	19001(2068)*	21720(1735)*	26296(1694)*
ToR										
	ShamΦ	0.26(0.04)	0.14(0.03)	0.07(0.01)	0.18(0.11)	0.25(0.06)	0.10(0.03)	0.28(0.12)	0.15(0.4)	0.08(0.02)
	OophxΦ		0.09(0.01)	0.12(0.02)	0.06(0.01)	0.09(0.01)*	0.11(0.01)	0.14(0.02)*	0.07(0.01)	0.05(0.01)

Values are mean (SEM) for trabecular bone perimeter (Peri, um), osteoclast extent (Oc.E, um), fluorochrome double labelled extent (dLE, um) and turnover ratio (ToR) measured in the epiphyseal region of the distal femur in non-operated rats (0 days) and sham operated (Sham) and oophorectomised rats (Oophx) at 30, 36, 42, 48, 66, 90, 120, and 150 days post operation at 7 months of age. Φ Significant change with time ($P<0.005$). # Significantly different to Sham with time ($P<0.01$). * Significantly different to Sham ($P<0.05$).

4.3.2 Osteoclast surface

In the metaphysis Oc.S (Fig. 4.5A) decreased with age ($P<0.0005$) and increased following oophorectomy ($P<0.05$) resulting in greater levels in the Oophx group ($P<0.01$) from day 30.

In the epiphysis Oc.S (Fig. 4.5B) was stable with age, oophorectomy induced an initial rise in Oc.S ($P<0.005$) and was elevated compared to Sham over time ($P<0.0001$). However, Oophx values fell to Sham values by day 120.

The response of Oc.S following oophorectomy was not different between regions.

4.3.3 Osteoclast extent

In the metaphysis Oc.E (Table 4.1) decreased with age ($P<0.0005$), and with oophorectomy ($P<0.0001$). The decrease was greater in the Oophx group ($P<0.0001$) although values were higher in Oophx at day 30 and 36 ($P<0.0001$).

In the epiphysis Oc.E (Table 4.2) was stable with age, oophorectomy induced an initial rise ($P<0.0001$) and was greater than Sham over time ($P<0.005$). However, as with Oc.S, Oophx values fell to Sham values by day 120.

The response of Oc.E to oophorectomy was greater in the epiphysis ($P<0.005$).

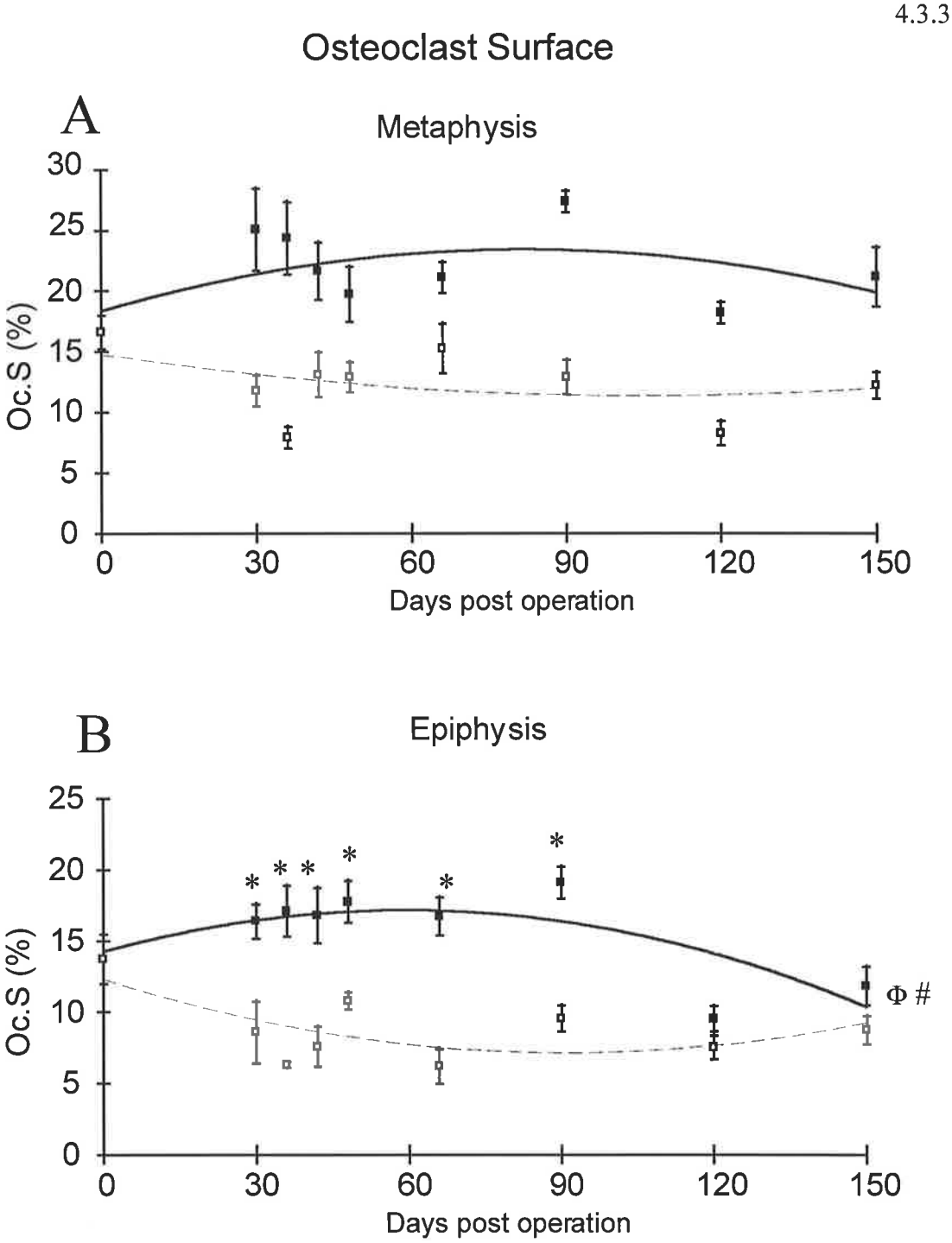


Figure 4.5 Temporal changes in osteoclast surface (Oc.S, %) following oophorectomy (Oophx ■ , solid line) or sham-operation (Sham □, broken line) in the metaphysis (A) and epiphysis (B) of the distal femur in the rat, expressed as mean ± SEM. Φ Significant change with time ($P<0.05$). # Significantly different to Sham with time ($P<0.01$). * Significant difference between operative groups ($P<0.05$).

4.3.4 Fluorochrome double labelled surface

In the metaphysis dLS (Fig. 4.6A) increased with age ($P<0.0001$) and oophorectomy ($P<0.0001$), the rise was greater in Oophx ($P<0.005$) from day 30.

In the epiphysis dLS (Fig. 4.6B) also increased with age ($P<0.0001$) and oophorectomy ($P<0.0001$), the rise was greater in Oophx ($P<0.0001$) from day 30.

The increase in dLS following oophorectomy was not different between regions.

4.3.5 Fluorochrome double label extent

In the metaphysis dLE (Table 4.1) increased with age ($P<0.01$), and oophorectomy induced an initial rise to day 48 ($P<0.0001$) however, unlike dLS, Oophx values fell to below Sham levels by day 150.

In the epiphysis dLE (Table 4.2) increased with age ($P<0.0001$) and oophorectomy ($P<0.0001$), the rise was greater in Oophx ($P<0.0001$) from day 30.

The response of dLE to oophorectomy was greater in the epiphysis ($P<0.0005$).

4.3.6 Mineral apposition rate

In the metaphysis MAR (Fig. 4.7A) was stable with age to day 48 (9 months of age) then fell to below baseline levels in both groups between days 66 and 150 (Sham $P<0.0001$, Oophx $P<0.0001$). There was no consistent difference between operative groups.

In the epiphysis this pattern was repeated (Fig. 4.7B), with both groups falling at the same age (Sham $P<0.0001$, Oophx $P<0.0001$), to remain below baseline levels from day 66 onwards.

The response of MAR to oophorectomy was not different between regions.

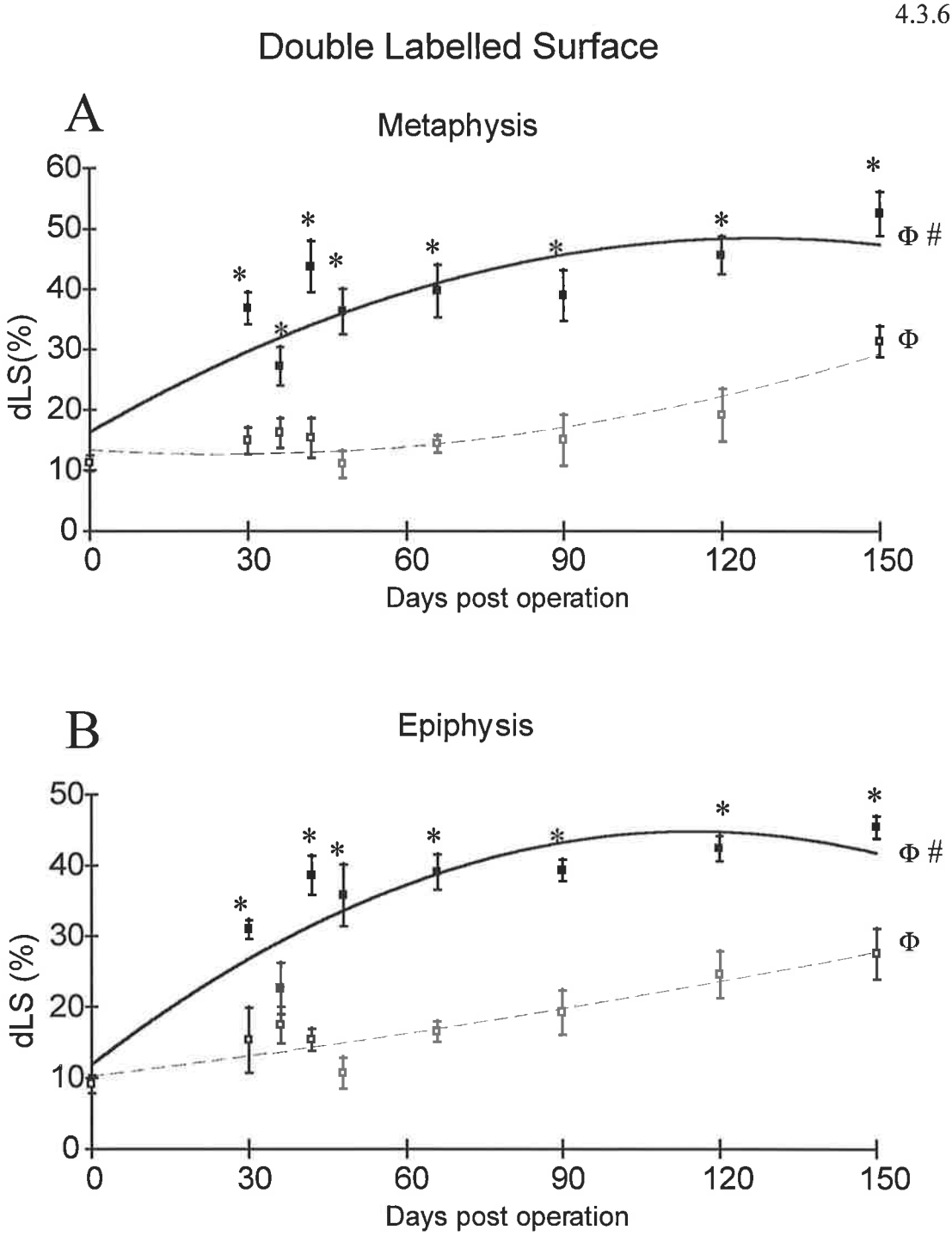


Figure 4.6 Temporal changes in fluorochrome double labeled surface (dLS, %) following oophorectomy (Oophx ■ , solid line) or sham-operation (Sham □, broken line) in the metaphysis (A) and epiphysis (B) of the distal femur in the rat, expressed as mean ± SEM. Φ Significant change with time (P<0.0001). # Significantly different to Sham with time (P<0.01). * Significant difference between operative groups (P<0.05).

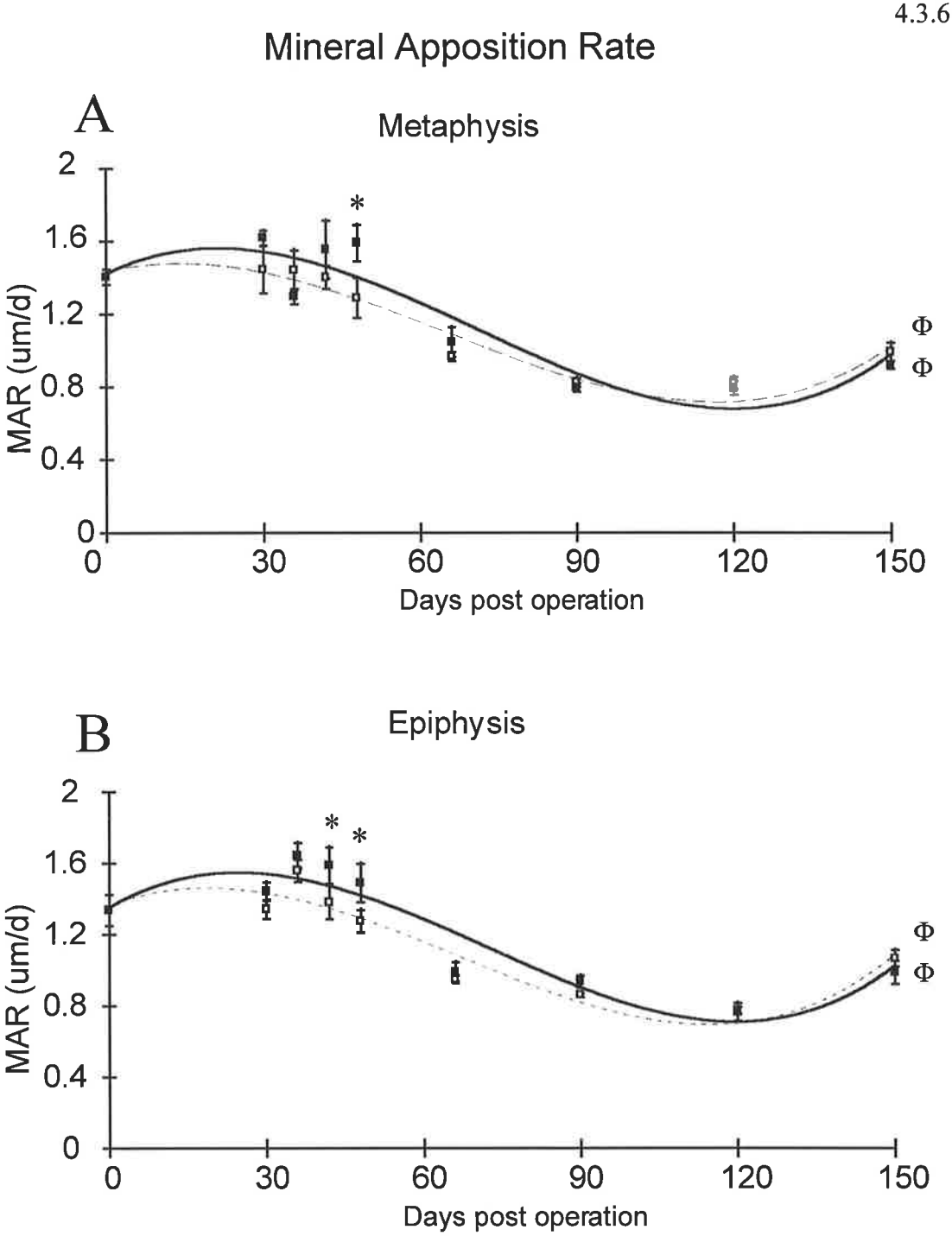


Figure 4.7 Temporal changes in mineral apposition rate (MAR, um/d) following oophorectomy (Oophx ■, solid line) or sham-operation (Sham □, broken line) in the metaphysis (A) and epiphysis (B) of the distal femur in the rat, expressed as mean ± SEM. Φ Significant change with time ($P<0.0001$). * Significant difference between operative groups ($P<0.05$).

4.3.7 Bone formation rate

In the metaphysis BFR (Fig. 4.8A) was stable in Sham and increased in Oophx ($P<0.01$), resulting in greater levels in the Oophx group ($P<0.0001$) from day 30. In the epiphysis BFR (Fig. 4.8B) increased with age ($P<0.0001$) and oophorectomy ($P<0.0001$), the rise was greater in Oophx ($P<0.005$) from day 30.

The response of BFR to oophorectomy was not different between regions.

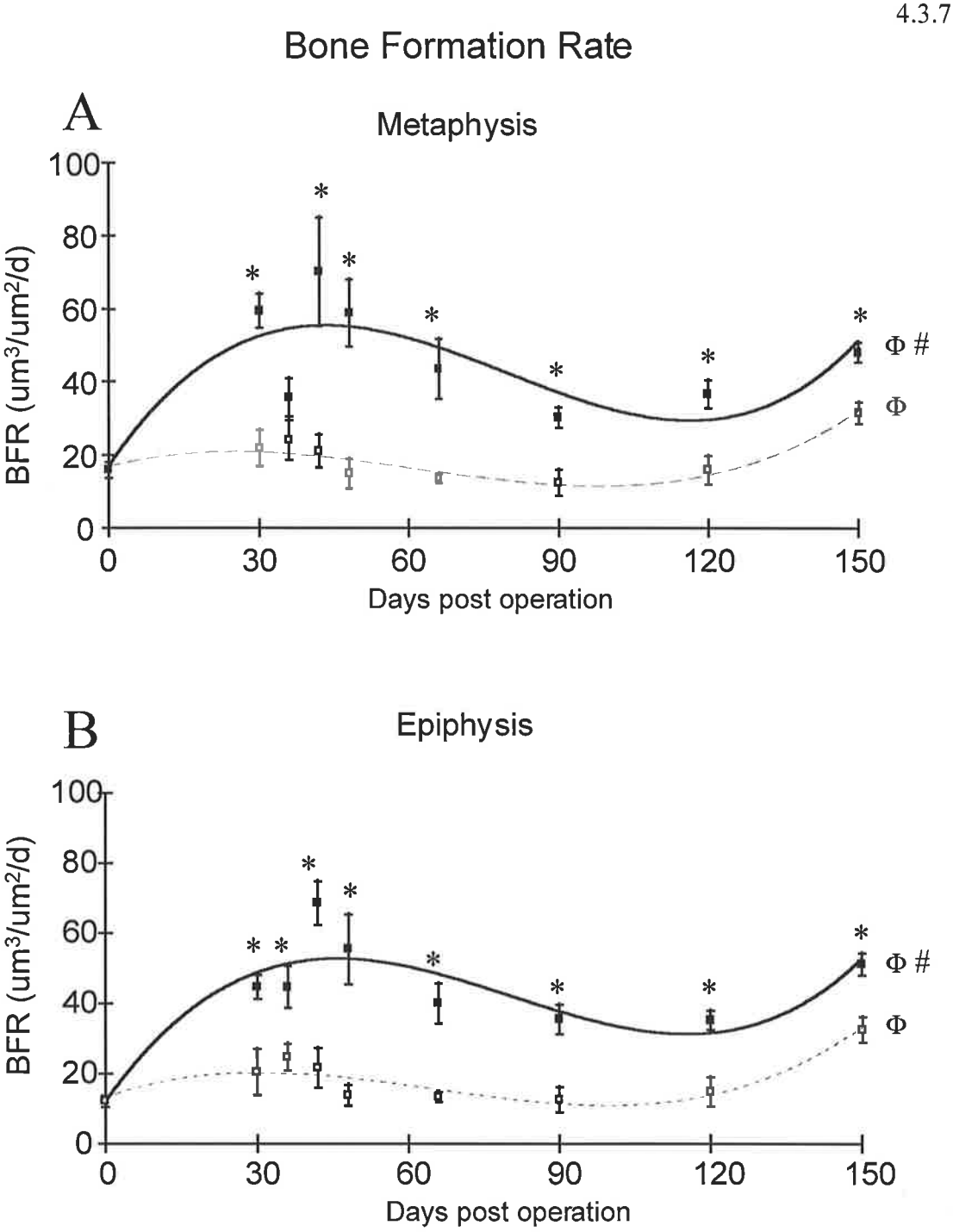


Figure 4.8 Temporal changes in bone formation rate (BFR, $um^3/um^2/d$) following oophorectomy (Oophx ■, solid line) or sham-operation (Sham □, broken line) in the metaphysis (A) and epiphysis (B) of the distal femur in the rat, expressed as mean ± SEM. Φ Significant change with time ($P<0.0001$). # Significantly different to Sham with time ($P<0.01$). * Significant difference between operative groups ($P<0.05$).

4.3.8 Turnover ratio

In the metaphysis ToR (Table 4.1) was stable with age and oophorectomy, with no difference between operative groups.

In the epiphysis ToR (Table 4.2) declined with age and oophorectomy (Sham $P<0.005$, Oophx $P<0.001$), with no consistent difference between operative groups.

The change in ToR was significantly more rapid in the epiphysis ($P<0.0001$).

4.3.9 Scanning electron microscopy

In the ovary intact femur 150 days after sham operation, scanning electron micrography (Fig 4.4A) revealed a robust trabecular network in the epiphysis. The metaphysis also contained a robust trabecular network, however this became markedly osteopenic in the proximal aspect of the medullary cavity, consistent with age related bone loss.

Oophorectomy (Fig 4.4B) did not have an effect on the bone of the epiphysis, with a similar appearance to the ovary intact femur. In contract, the metaphysis was markedly osteopenic with trabeculae virtually absent from the entire length of the central region of the medullary cavity, consistent with ovarian hormone related bone loss.

4.4 Discussion

4.4.1 Comparison of age related changes in bone and bone cell activity

In ovary intact animals age related bone loss occurred in both regions. BV/TV reduced 66% and 18% in the metaphysis and epiphysis, respectively, due solely to a 53% and 10% fall in Tb.N. Operation at 7 months of age clearly had an osteopenic effect on the trabecular bone of the distal femur, with an initial reduction in Tb.Th recorded in all areas (3.4.6). However this was temporary, with Tb.Th stable over time in both groups in the metaphysis and increasing in the Oophx epiphysis, relative to a stable Sham group. Thus the epiphysis is resistant to age related bone loss compared to the metaphysis, with a significantly slower rate of bone loss during this period.

This loss did not correspond to a change in osteoclastic activity but was coincident with a decline in MAR, independent of ovarian status. MAR has previously been shown to decline with age in the femoral neck (Li *et al.,* 1997), the proximal tibial metaphysis (Wronski *et al.,* 1989) and the lumbar vertebrae (Wronski *et al.,* 1989b) of the rat and the iliac crest of postmenopausal women (Parfitt *et al.,* 1995). Computer simulation has shown that decreased formation balance, as seen here with a decline in MAR, accounts for the largest component of postmenopausal bone loss (Thomsen *et al.,* 1994). The decline in MAR is also likely to be a factor in the acceleration of bone loss seen in the metaphysis of ovary-intact groups, with 70% of total loss occurring after day 66. These data indicate that trabeculae are lost in this region with both estrogen deficiency and age, however by different mechanisms, estrogen deficiency intensifies resorption while aging diminishes formation. Any effect of the decline in MAR in the metaphysis of estrogen deficient animals was likely masked by the prominent osteopenia already evident in this region. This early phase of age related bone loss may represent terminal destructive modeling (Turner *et*

al., 1994c), whereby trabecular architecture is matched to local mechanical strain. The slow phase of bone loss seen in humans, resulting in reduced trabecular thickness (Parfitt, 1988) may not be evident in the rat due to its short life span, providing insufficient time for the development of osteoblast insufficiency (Wronski, 1989). Furthermore, laboratory rats gain significant weight due to sedentary behaviour and ad libitum feeding (Sontag, 1992). Weight gain has been shown to exert a protective effect on ovarian hormone deficient bone loss (Wronski, 1987) and may contribute to maintenance of bone volume with age.

4.4.2 Comparison of osteopenia induced by the remodelling transient and steady state changes

The previous chapter reported variation in the development of osteopenia immediately following oophorectomy, displaying the differing effect of the remodelling transient in three regions of trabecular bone in the distal femur. During the first 30 days following oophorectomy showed no loss of bone was evident in the epiphysis with a graded response increasing proximally. Extended measurement in two regions of this bone has enabled the steady state effects of ovarian hormone deficiency to be evaluated.

The development of osteopenia in the metaphysis occurred in an exponential fashion, with an initial rapid phase in the first 48 days, encompassing the remodelling transient, which accounted for 74% of the total bone loss. Following this period of rapid bone loss, BV/TV continued to decline throughout the study. The rate of bone loss fell over 6-fold, from 0.36%/d in the first 48 days, to 0.06%/d over the remaining period, with BV/TV reduced to 9% of baseline at day 150. This diphasic pattern is consistent with trabecular bone loss in both postmenopausal women (Stepan *et al.,*

1987, Nordin *et al.,* 1990) and the distal femur (Peng *et al.,* 1997) and the proximal tibiae (Wronski *et al.,* 1989b) of the oophorectomised rat.

The epiphysis in contrast, did not develop osteopenia relative to the ovary-intact groups over the period of the study. Thus this region is resistant to both the remodelling transient and the steady state alterations of bone turnover initiated by ovarian hormone deficiency. The perforation and removal of trabeculae so prominent in the metaphysis following oophorectomy is greatly diminished in the epiphysis despite increased bone cell activity. The varied effect of ovarian hormone deficiency on the development of osteopenia in the two regions is clearly evident in the scanning electron micrographs (Fig. 4.2). Significant loss was apparent in the metaphyseal region by day 150, whilst the epiphyseal region appears unaltered. Resistance of the trabecular bone of the femoral epiphysis to ovarian hormone deficiency induced osteopenia has been reported previously in both short (Martin and Zissimos, 1991, Ikeda *et al.,* 1996) and long term studies (Yamazaki and Yamaguchi 1989, Westerlind *et al.,* 1994, Westerlind *et al.,* 1997). This variation in the osteopenic response to ovarian hormone deficiency has also been suggested previously by the altered rates of bone loss between different skeletal sites (Kalu *et al.,* 1989, Durbridge *et al.,* 1990, Wronski *et al.,* 1989a, Tanaka *et al.,* 1999).

4.4.3 Steady state changes in trabecular bone structure following oophorectomy

Osteopenia developed in the metaphysis following oophorectomy by trabecular perforation with no permanent change in Tb.Th, as previously reported in both postmenopausal women (Parfitt, 1992) and oophorectomised rats (Kalu, 1991, Dempster *et al.,* 1995, Sims *et al.,* 1996). Thus the reduction in Tb.Th immediately following operation in all regions in the previous chapter was not a permanent

alteration to structure, nor was it dependent of ovarian hormone status. These facts, combined with the lack of evidence for an age related effect (3.4.5), suggests this reduction in thickness as a result of the operative procedures.

Those trabeculae that were thinned but not perforated were able to accrue bone mineral independent of ovarian hormone status, returning Tb.Th to baseline values at later times. The importance of such a retention of template as evident in the epiphysis, to the outcome of ovarian hormone deficiency is demonstrated in the maintenance of BV/TV in this region following oophorectomy.

Trabeculae were lost from the epiphysis however, between day 48 and 66, coincident with an age related decline in MAR. However this was offset by a subsequent increase in Tb.Th between day 66 and 150. This thickening of trabeculae following a reduction in Tb.N suggests that epiphyseal trabecular bone maintains BV/TV by a process that compensates for the loss of trabeculae by increasing the thickness of the remaining trabeculae, as seen following oophorectomy in the mandible (Tanaka *et al.,* 1999). The ability to increase Tb.Th independent of oestrogen status was also observed in the epiphysis of rats oophorectomised at 3 weeks of age (7.3.4.3), as a response to increasing body weight. Similar observations have been made in the iliac crest of postmenopausal women, where thickening of trabeculae with age was suggested to result from an increase in the load on remnant trabeculae following perforation and disruption of the trabecular network (Vesterby *et al.,* 1989). The increase in Tb.Th is compatible with Frost's theory of skeletal response to increasing load (Frost *et al.,* 1992). These observations, and the substantial thickening of trabeculae demonstrated in oophorectomised rats following treatment with PGE_2 (Tang *et al.,* 1992) and PTH (Meng *et al.,* 1996) suggest the ability to

accrue bone is not impeded by ovarian hormone deficiency, particularly in regions of high strain.

4.4.4 Regional comparison of changes in osteoclast activity following oophorectomy

4.4.4.1 Surface percentage data

The duration of elevated bone cell activity following oophorectomy is uncertain. In long bone metaphyses of the proximal tibia, longitudinal studies have shown the increase in resorption to be temporary, after which a new steady state is reached, independent of ovarian hormone status in the proximal tibia (Wronski *et al.,* 1998a, Wronski *et al.,* 1989b) and distal femur (Peng *et al.,* 1997). However in the femoral epiphysis elevated resorption has been reported at 90 days (Martin and Zissimos, 1991) and 11.5 months (Westerlind *et al.,* 1997) postoophorectomy, suggesting a sustained increase in resorption. However no longitudinal study has evaluated of the pattern of bone cell activity in the epiphysis following oophorectomy.

In the present study the changes in osteoclast surface following oophorectomy, when analysed over time, were uniform across the two regions. However in the epiphysis, Oc.S declined to ovary-intact levels after day 90, but remained elevated in the metaphysis throughout the study. These data indicate that the initial increase in resorption in the epiphysis is temporary, in a similar fashion to the proximal tibial metaphysis. The increase in resorption reported in the femoral epiphysis 11.5 months after oophorectomy (Westerlind *et al.,* 1997) suggests a diphasic pattern of bone cell activity in this region. Increases in bone cell activity at extended periods following oophorectomy have been reported previously. In the proximal tibial metaphysis, mineralising surface and osteoblast surface increased 12 months after oophorectomy,

Oc.S also rose at this time however this was no greater in the oophorectomised groups (Wronski *et al.,* 1989b).

The reduction in Oc.S in the epiphysis occurred independently of dLS which was elevated relative to ovary-intact animals at all times post oophorectomy. This is again a similar pattern to the proximal tibial metaphysis, where Oc.S returned to ovary-intact levels by day 120 while dLS remained elevated throughout the 540 days of the study (Wronski *et al.,* 1989b). The similarity of the time course of altered levels of resorption between the femoral epiphysis and the proximal tibial metaphysis, suggests a consistency of osteoclast activity following oophorectomy in the appendicular skeleton of the rat. Given this consistency of response, it is likely that Oc.S in the femoral metaphysis would return to ovary-intact levels at a later time point. The prolongation of the response in the metaphysis may involve the magnitude of bone loss in this region (4.4.4.2).

4.4.4.2 Linear extent data

As in the diaphysis in the previous chapter (3.4.7), reductions in trabecular perimeter during the study necessitated the analysis of linear extent parameters. In the epiphysis, despite reduction in Peri following oophorectomy, the pattern of changes in Oc.E followed that of Oc.S, indicating a temporary increase in osteoclast proliferation lasting approximately 90 days. The metaphysis however reveals a different pattern to that described by the surface percentage data. While Oc.S was permanently elevated by oophorectomy, Oc.E was elevated compared to the ovary-intact animals only until day 36. This observation is consistent with both the initial rise in Oc.S and the diminishing rate of bone loss observed later after oophorectomy, with 60% of total bone loss complete by this time. Thus it can be seen that in terms of the pattern of

osteoclast proliferation, the response to ovarian hormone deficiency contrasts surface based estimations, with a larger and more prolonged effect occurring in the epiphysis.

In the metaphysis Oc.E was elevated following oophorectomy only until day 36, despite the continued loss of bone. In this region the immediate effects of ovarian hormone deficiency produce an enlarged and more vigorous osteoclast population and subsequently, osteopenia. In a similar manner to the diaphysis in the previous chapter (4.4.7), osteopenia concentrates this population on a continually diminishing surface of bone. This in turn magnifies the probability of perforation in remaining trabeculae (Parfitt *et al.*, 1984, Reeve 1987b) and prolongs the period over which Oc.S is elevated compared to ovary-intact levels. Thus bone loss proceeds without an increase in osteoclastogenesis. In the epiphysis ovarian hormone deficiency also produces an enlarged osteoclast population. However in contrast to the metaphysis, local factors such as mechanical strain (3.4.3), greater thickness of trabeculae (3.4.7) and possibly less vigorous resorption (4.4.6) act to resist the development of osteopenia and effects of the increased osteoclast population.

4.4.5 Regional comparison of the changes in osteoblast activity following oophorectomy

4.4.5.1 Surface percentage data

The changes in dLS following oophorectomy were uniform across the two regions. dLS was elevated following oophorectomy at all time points. The changes in BFR following oophorectomy were also uniform across the two regions, again being elevated following oophorectomy at all time points, and remained elevated in the epiphysis despite the reduction in Oc.S by day 120. The increase in formation was prolonged in the epiphysis despite the close temporal relationship between the increase

in resorption and formation initiated by oophorectomy observed here and previously (Wronski *et al.,* 1988, Dempster *et al.,* 1995, Sims *et al.,* 1996). In the proximal tibial metaphysis mineralising surface was elevated 540 days after oophorectomy despite Oc.S returning to ovary-intact levels by 120 days (Wronski *et al.,* 1989b). This may be explained in part by the greater time taken for completion of the formation process (Baron *et al.,* 1984), the magnification of surface referent parameters by continuing osteopenia (3.4.6) and the existence of a homeostatic mechanism acting to maintain mechanical strain within a predetermined physiological range (Frost, 1987). The short life span of rats has also been suggested as an explanation (Wronski *et al.,* 1989b), providing insufficient time for the development of age related decreases formation reported in postmenopausal women (Parfitt *et al.,* 1995).

4.4.5.2 Linear extent data

In the epiphysis, in a similar manner to resorption estimates, the pattern of changes in dLE followed that of dLS, with a sustained elevation in dLE following oophorectomy. In the metaphysis however, in a similar manner to resorption estimates, dLE displayed a different pattern following oophorectomy to that of dLS. Values were greater in the Oophx group to day 48, with dLE declining to be lower than Sham by day 150.

Thus in terms of bone cell proliferation, the response to oophorectomy in the metaphysis is transient, in contrast to surface percentage data. This reduction in the total population of bone cells may result from the extensive bone loss that occurs in this region following oophorectomy. During a normal cycle of bone remodelling, formation is driven by the previous episode of resorption, balancing these two processes at a given site (Parfitt *et al.,* 1979). However in the absence of ovarian hormones, trabecular perforation reduces the surface of bone available for the bone

formation response (Reeve *et al.,* 1987). The reduction in bone surface, combined with the greater time needed for completion of the formative process (Baron *et al.,* 1984), in turn reduces the surface available for resorption.

As bone loss continues and an increasing amount of surface is still involved in formation, the availability of inactive bone surface may become a limiting factor in the rate of bone resorption. In this region osteoclastogenesis returned to ovary-intact levels from day 42. At this time 65% of remaining surface was involved in remodelling and BV/TV had reduced to 44% of baseline. Furthermore, the total percentage of active trabecular bone, in the metaphysis rose from 27% at baseline to 73% at day 150, consistent with the limiting nature of inactive bone surface at these later times post oophorectomy. The decreasing number of sites undergoing resorption in turn reduces the number of sites subsequently undergoing formation and thereby the outright turnover of bone, and the requirement for bone cell proliferation diminishes. Such a reduction in turnover rate is reflected in the reduced rate of bone loss seen in the latter times post-oophorectomy in the metaphyseal region.

These data indicate that bone cell proliferation is at least in part under local regulation. Ovarian hormone deficiency stimulates bone cell proliferation and thus turnover on all trabecular surfaces. However increasing osteopenia appears to exert a negative, local control on proliferation. Thus in the epiphysis, where osteopenia does not develop, bone cell proliferation remains elevated, while it decreases in the osteopenic metaphysis. This local control of bone cell proliferation is consistent with a mechanism of regulation involving the release of paracrine factors from bone during resorption. Rat bone has been shown to contain factors with powerful effects on bone cell proliferation embedded in the matrix such as IL-1α, TNF and TGFβ (Pacifi, 1996, Jilka, 1998). Thus the reduced release of these factors as the rate of bone loss

diminishes may act in a local manner to reduce proliferation. Elucidation of the identity and function of such compounds would provide potential therapeutic interventions for the control of increased bone turnover following the menopause.

4.4.6 Influence of changes in mineral apposition rate on trabecular structure

MAR was unaffected by both operation and region, but underwent a sharp decline at around 9 months of age in all groups. The decrease was coincident with a decrease in Tb.N in the epiphyses of both operative groups and the metaphyses of the ovary-intact groups. Importantly, dLE increased and Oc.E was steady or decreased in all three groups over this time. Age related bone loss is characterised as occurring by an osteoblast mediated process involving decreased cell proliferation and/or anabolic activity (Melsen *et al.,* 1978, Eriksen, 1986). The increase in dLE in the epiphysis during this period excludes a problem with cell proliferation. The decline in MAR at around 9 months of age suggests an age dependent change in osteoblast activity and not osteoblast proliferation as a factor in long term trabecular bone loss in the rat. MAR also fell with age in the femoral neck in both oophorectomised and ovary-intact rats (Li *et al.,* 1997). This reduction in MAR with age is consistent with observations in normal postmenopausal women (Recker *et al.,* 1988). These observations are compatible with computer simulations suggesting that long term loss of trabecular bone is dominated by a reduction in formation not an increase in resorption (Thomsen *et al.,* 1994). However decreased wall thickness has been reported in the iliac crest of postmenopausal women, leading to the suggestion that defective osteoblast recruitment is a factor in bone loss during this period (Parfitt *et al.,* 1995). This does not appear to be factor in the oophorectomised rat with dLS remaining elevated in the proximal tibial metaphysis 540 days after oophorectomy, despite Oc.S returning to

ovary-intact levels by day 120. These observations may be misleading due to various factors (4.4.4.1), however they suggest that the oophorectomised rat may have limitations in its usefulness for examining the mechanisms involved in long term bone loss in the human.

4.4.7 Comparison of turnover ratio between operative groups, implications for resorption depth and mechanical strain

As in the previous chapter (3.4.8) the ratio of Oc.S to BFR was calculated in order to investigate the possible role of increased resorption depth in the development of ovarian hormone deficient osteopenia, as recorded in a pig model (Mosekilde *et al.,* 1993, Boyce *et al.,* 1995). It was suggested that resorption depth increased following oophorectomy, increasing the likelihood of perforation events in the proximal regions, with the greater mechanical strain and thickness of epiphyseal trabeculae protecting this region from perforation. In the present study, there was no difference in ToR between operative groups, despite differences in osteopenia. This supports a model of a sustained increase in resorption depth occurring following oophorectomy in the metaphysis, which continues to play a role in the development of osteopenia. As in Chapter 3 (3.4.10), this ratio can only infer an increase in resorption depth where a loss of bone has occurred, excluding the epiphysis. Thus in the epiphysis it is possible that local factors may diminish the increase in resorption depth triggered by ovarian hormone deficiency.

From these observations it is suggested that ovarian hormone deficiency removes a physiological regulation of bone cell proliferation on all trabecular surfaces and that local strain conditions regulate the vigor of resorption produced by this enlarged population of osteoclasts. The magnitude of osteopenia that develops also plays a local

role in matching the rate of turnover, albeit at an increased level, to the amount of bone via an inhibition of proliferation.

Chapter 5

Effects of 17β-oestradiol administration on bone cell activity and trabecular structure

5.1 Introduction

Antiresorptive agents such as oestrogens, bisphosphonates and calcitonin are employed as treatment regimes in osteopenic patients for their ability to inhibit bone resorption. Linked to this reduction in resorption however, is a subsequent reduction in formation, resulting in a net decrease in bone turnover (Lufkin *et al.,* 1992, Vedi *et al.,* 1996). Consistent with the reduction in turnover, short term trials have reported increased BMD relative to untreated controls (reviewed in Eriksen *et al.,* 1996), associated with the contraction of the remodelling space (Steiniche *et al.,* 1989). Interestingly, some long term clinical trials have shown continued increases in BMD using these agents (Storm *et al.,* 1990, Liberman *et al.,* 1995, Eiken *et al.,* 1996), leading to the suggestion that these agents have a long term anabolic effect on osteoblasts (McClung, 1996). The anabolic potential of oestradiol has been investigated in acute studies (Abe *et al.,* 1992), however this has not been shown conclusively under normal physiological conditions, with some evidence for reduction in resorption depth (Ettinger *et al.,* 1985, Vedi and Compston, 1996).

More important from a clinical perspective, the fracture prevention efficacy of these treatments is greater than would be predicted from the relationship of BMD to fracture rate in the general population (Cumings *et al.,* 1996, Riggs *et al.,* 1996). The factors responsible for the additional reduction in fracture rate are unknown (Lufkin *et al.,* 1992). However, high bone turnover has recently been identified as an independent risk factor for fracture (Garnero *et al.,* 1996, Delmas, 1997, Melton III *et al.,* 1997) and is associated with lower BMD in postmenopausal women (Ravn *et al.,* 1996). Thus reducing turnover may reduce fractures independent of other factors. In addition, the possible contribution of architectural changes resulting from

antiresorptive treatment to this fracture prevention has not been comprehensively studied (Parfitt, 1991).

Previous chapters have investigated the effects of ovarian hormone deficiency on femoral trabecular bone, demonstrating the contribution to osteopenia of the initial increase in turnover (Chapter 3) and the steady state effects of high turnover (Chapter 4). The metaphysis experiences rapid and permanent osteopenia resulting from trabecular perforation coincident with high bone turnover. In contrast, it has been established that the oestrogen deficient increases in bone turnover do not produce osteopenia in the trabecular bone of the epiphysis. This observation is consistent with the hypothesis that high mechanical strain maintains bone independently of oestrogen deficient increases in turnover (Westerlind *et al.,* 1997). Having shown variation in response to the ovarian hormone deficient increases in turnover, the present chapter investigates the effect of decreased bone turnover induced by oestradiol supplementation in these two regions. 17β-oestradiol (E_2) was administered to oophorectomised and ovary-intact animals, and the changes in bone cell activity and trabecular structure were characterised in the distal femoral metaphysis and epiphysis 105 days after operation. In addition to its effects on the rate of bone turnover, the potential anabolic nature of oestradiol on osteoblasts was also investigated.

5.2 Materials and Methods

5.2.1 Animals

The studies in the present chapter and those of Chapter 6 were conducted as a single study group. However, for reasons of ease of presentation and interpretation the oestradiol component has been presented separately from the dietary calcium restriction component. It must be noted that the 0.2% Ca fed group is reproduced in

this chapter and Chapter 6, as a result no comparison was conducted between the groups in the two chapters.

40 female Sprague-Dawley rats at six months of age were fed a semi-synthetic diet containing 0.2% Ca, 0.3% P and 4000IU/kg vitamin D_3 (2.2) throughout the study, with 4 weeks acclimatisation to the diet prior to operation. All procedures involving the animals were approved by the IMVS Animal Ethics Committee.

5.2.2 Experimental protocol

At 7 months of age all animals were allocated to either oophorectomy or sham operation via the ventral approach (2.4.3). 10 from each operative group were allocated to E_2 supplementation, and received 17β oestradiol implants at the time of operation (2.5). Treatment was maintained for 105 days. All rats received fluorochrome injections prior to collection (2.3). Rats were killed by exsanguination and the right femora were excised.

5.2.3 Histomorphometry

The distal 20 mm of the right femora were bisected in the sagittal plane, and processed into MMA resin (2.9.1.2). One half of the left femora of a randomly chosen sample from each operative group were used for scanning electron microscopy (2.8). Bone cell activity and trabecular bone morphology (2.14) were estimated in two regions, the metaphysis and epiphysis (2.11.1).

5.2.4 Calculations and statistics

All results were analysed using two-way ANOVA for operation and E_2 supplementation, significant results were further analysed using one-way ANOVA

within operative or supplement group. All ANOVA were followed by Bonferroni/Dunn post hoc analysis. Spearman correlation values of Tb.Th with Oc.S and BFR were also performed and presented (slope, significance of difference from slope = 0). Each analysis was conducted using Super ANOVA (Abacus Concepts, CA, USA).

Comparison between regions was conducted with relative values. In order to calculate these values, the difference of an individual value from the mean of the respective Sham group by was divided the Sham mean.

ie. $\frac{[\text{value - Sham mean}]}{\text{Sham mean}}$

The effect of oestradiol supplementation on dietary calcium induced bone loss was assessed by comparing the groups in the present study with those consuming 0.8% Ca diet in the previous chapter. The group chosen was 120 days post oophorectomy, conducted at the same age as all mature oophorectomy studies in this thesis, 7 months. These rats were therefore 15 days older than those in the present study, considering the constant bone loss in aging rats an older group was considered conservative for comparison. These animals were included in analysis only where stated. $P < 0.05$ was considered significant.

5.3 Results

5.3.1 Bone cell activity

5.3.1.1 Resorption indices

In the ovary-intact groups Oc.S (Fig 5.1) decreased following E_2 supplementation (Meta $P<0.0005$, Epi $P<0.01$).

Oophorectomy increased Oc.S (Meta $P<0.0001$, Epi $P<0.005$). E_2 supplementation of Oophx returned Oc.S to ovary-intact levels, and significantly reduced Oc.S compared to non-supplemented groups (Meta $P<0.0001$, Epi $P<0.0001$).

In the ovary-intact groups Oc.E (Fig 5.2) decreased following E_2 supplementation (Meta $P<0.005$, Epi $P<0.05$).

Oophorectomy did not alter Oc.E. In the metaphysis there was no difference in Oc.E between the two oophorectomised groups and the Sham group.

In the epiphysis Oc.E decreased following E_2 supplementation compared to non-supplemented groups ($P<0.0005$).

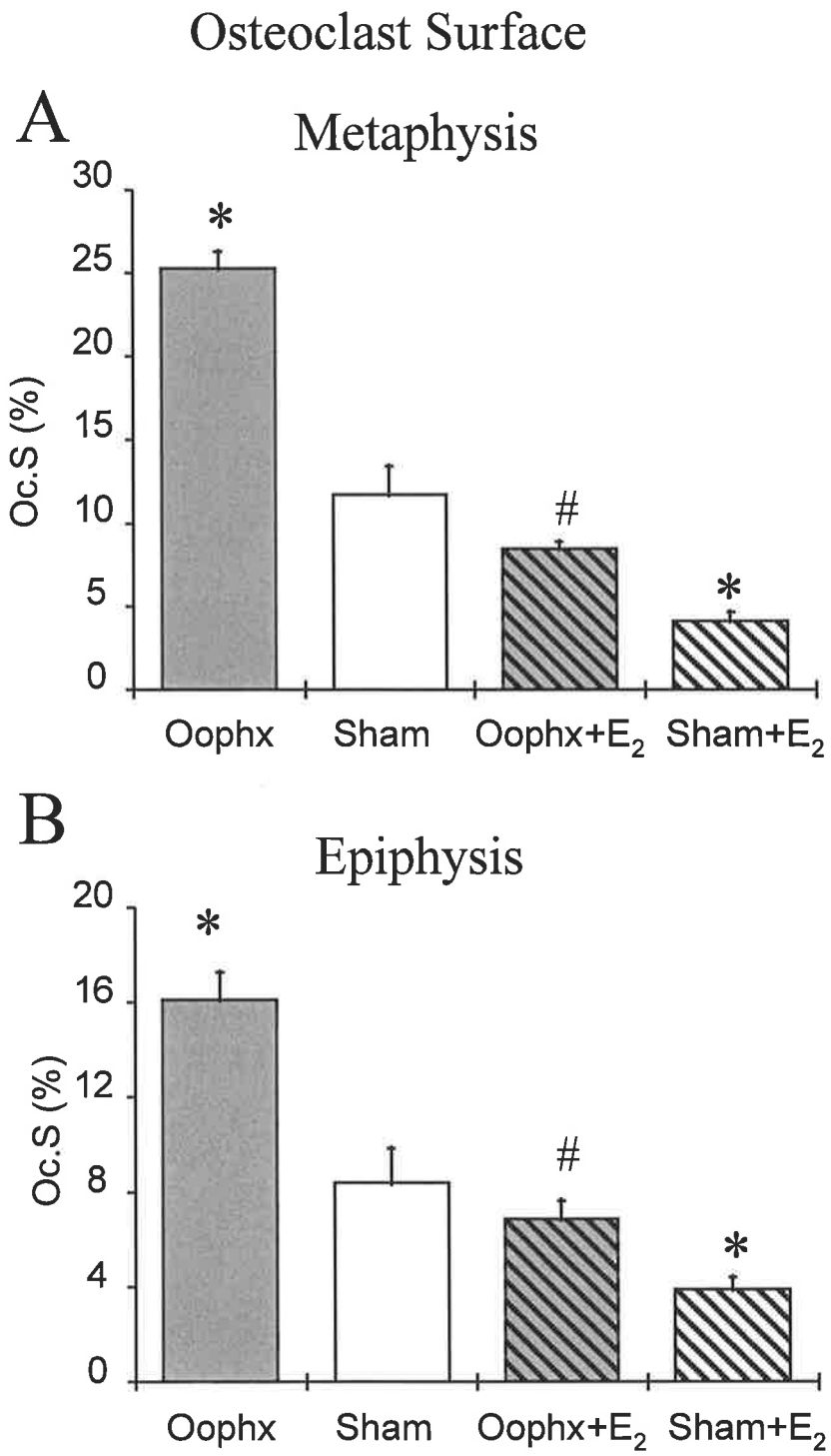


Figure 5.1 Effect of 17β oestradiol supplementation (E_2, hatch) on osteoclast surface (Oc.S, %) in the metaphysis (A) and epiphysis (B) of the distal femur of the rat in sham operated (white bars) and oophorectomised (grey bars) rats. # Significant effect of E_2 in Oophx, * Significant difference from Sham.

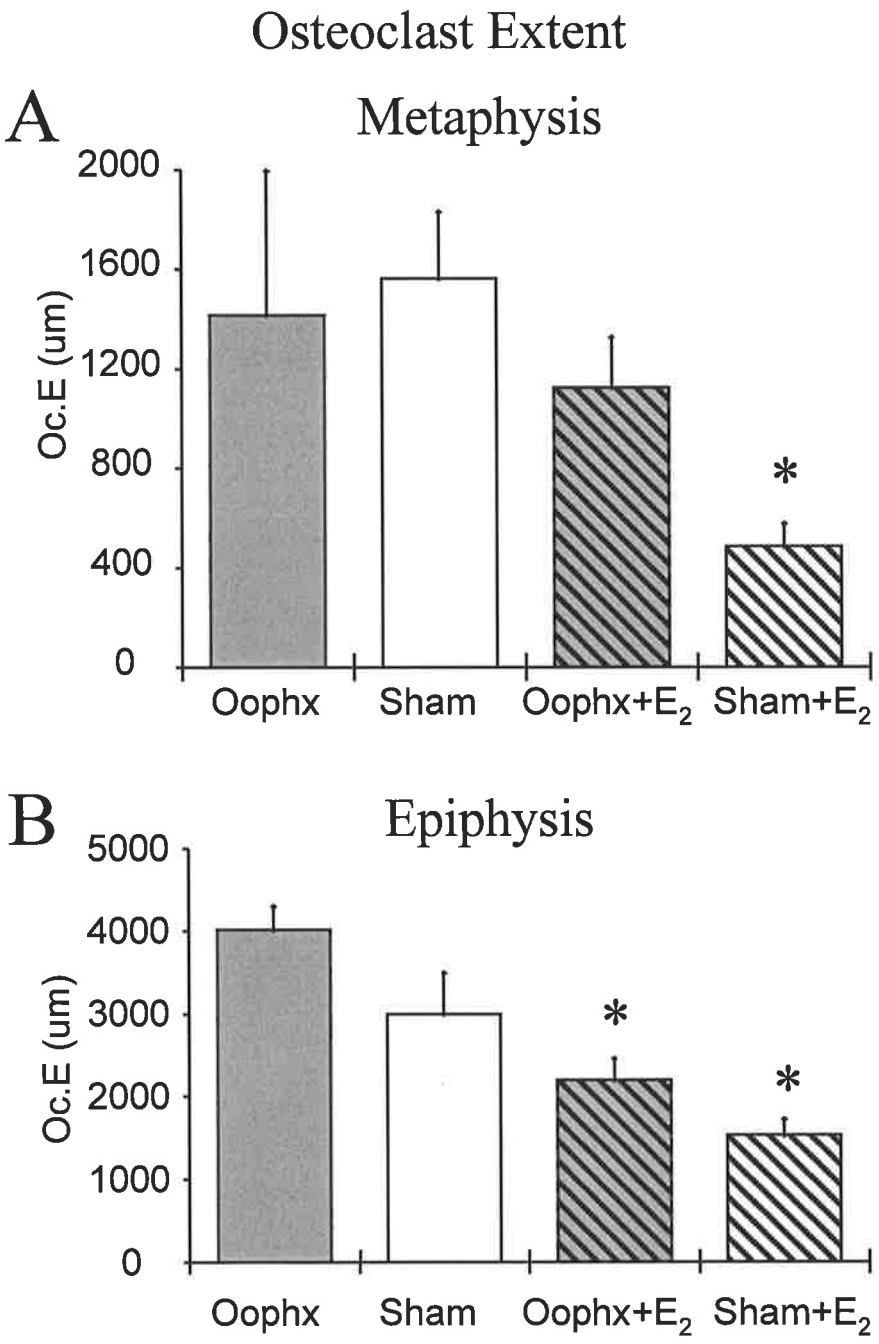


Figure 5.2 Effect of 17β oestradiol supplementation (E_2, hatch) on osteoclast extent (Oc.E, um) in the metaphysis (A) and epiphysis (B) of the distal femur of the rat in sham operated (white bars) and oophorectomised (grey bars) rats. # Significant effect of E_2 in Oophx, * Significant difference from Sham.

5.3.1.2 Formation indices

In the ovary-intact groups E_2 supplementation decreased dLS (Fig 5.3) following (Meta P<0.0001, Epi P<0.0001).

Oophorectomy increased dLS (Meta P<0.0005, Epi P<0.005). E_2 supplementation reduced dLS below ovary-intact levels (Meta P<0.0001, Epi P<0.05), and significantly reduced dLS compared to non-supplemented groups (Meta P<0.0001, Epi P<0.0001).

In the ovary-intact groups E_2 supplementation decreased dLE (Fig 5.4) (Meta P<0.001, Epi P<0.0001).

Oophorectomy did not alter dLE. E_2 supplementation reduced dLE below ovary-intact levels (Meta P<0.01, Epi P<0.005). In the metaphysis, as with Oc.E, there was no difference in dLE between the two oophorectomised groups and the Sham group. In the epiphysis dLE decreased following E_2 supplementation compared to non-supplemented groups (P<0.0001).

In the ovary-intact groups E_2 supplementation decreased MAR (Fig 5.5) (Meta P<0.0001, Epi P<0.0001).

Oophorectomy decreased MAR in the metaphysis (P<0.01) with no change in the epiphysis. E_2 supplementation decreased MAR below ovary-intact levels (Meta P<0.0001, Epi P<0.01, and significantly reduced MAR compared to non-supplemented groups (Meta P<0.0005, Epi P<0.01).

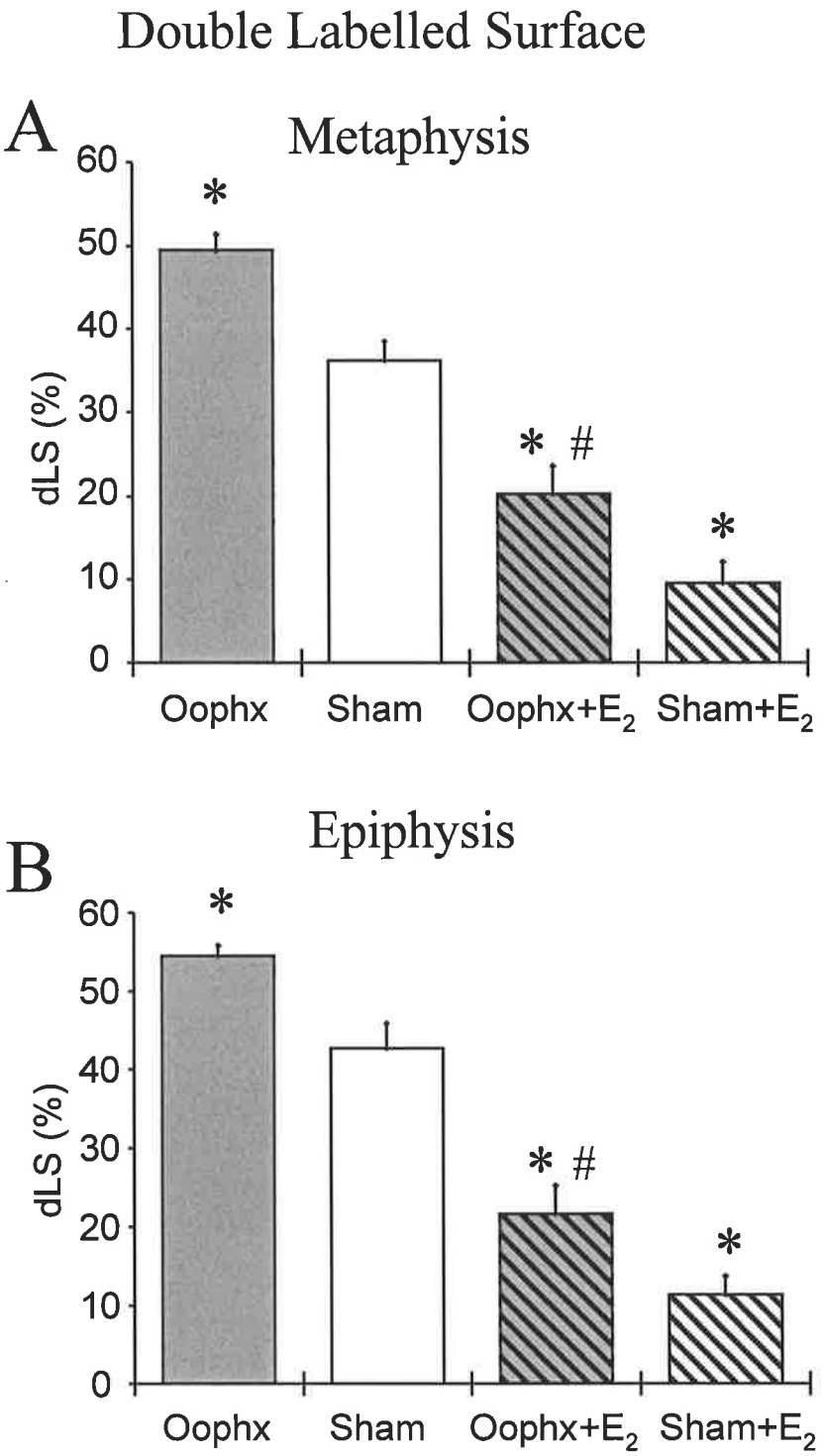


Figure 5.3 Effect of 17β oestradiol supplementation (E_2, hatch) on double labelled surface (dLS, %) in the metaphysis (A) and epiphysis (B) of the distal femur of the rat in sham operated (white bars) and oophorectomised (grey bars) rats. # Significant effect of E_2 in Oophx, * Significant difference from Sham.

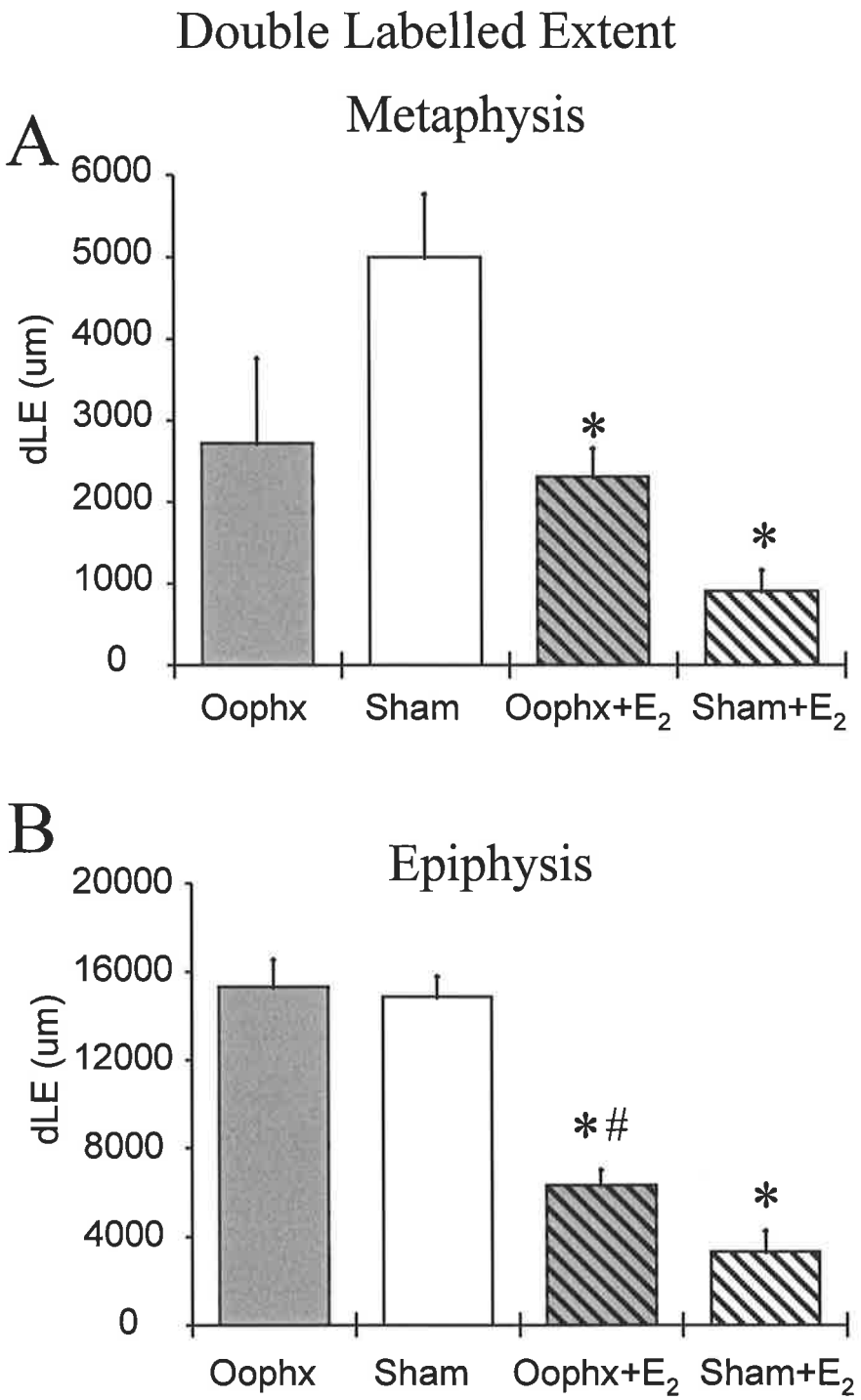


Figure 5.4 Effect of 17β oestradiol supplementation (E_2, hatch) on double labelled extent (dLE, um) in the metaphysis (A) and epiphysis (B) of the distal femur of the rat in sham operated (white bars) and oophorectomised (grey bars) rats. # Significant effect of E_2 in Oophx, * Significant difference from Sham.

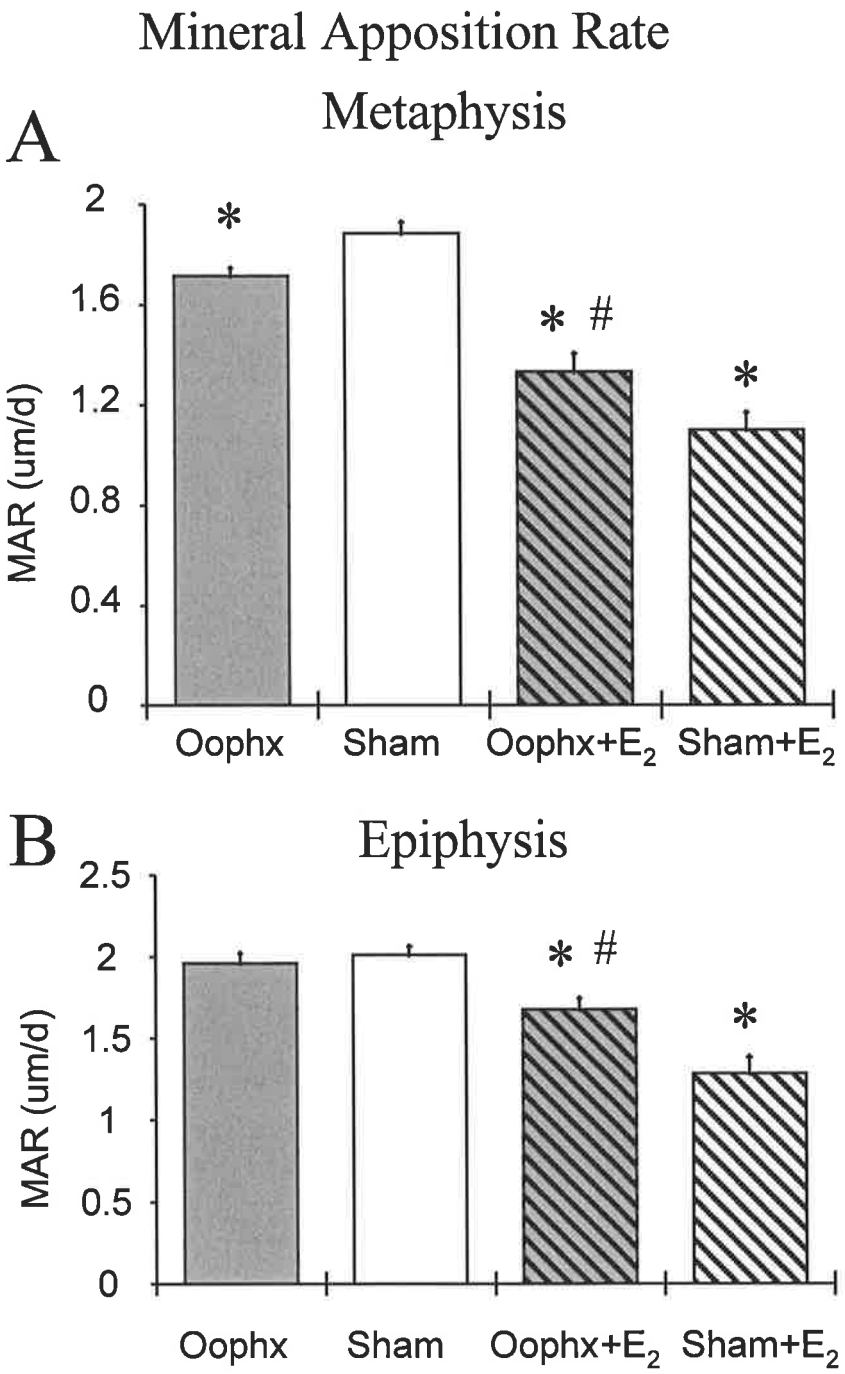


Figure 5.5 Effect of 17β oestradiol supplementation (E_2, hatch) on mineral apposition rate (MAR, um/d) in the metaphysis (A) and epiphysis (B) of the distal femur of the rat in sham operated (white bars) and oophorectomised (grey bars) rats. # Significant effect of E_2 in Oophx, * Significant difference from Sham.

5.3.1.2

In the ovary-intact groups E_2 supplementation decreased BFR (Fig 5.6) (Meta $P<0.0001$, Epi $P<0.0001$).

Oophorectomy increased BFR (Meta $P<0.005$, Epi $P<0.005$). E_2 supplementation reduced BFR below ovary-intact levels (Meta $P<0.0001$, Epi $P<0.005$), and significantly reduced BFR compared to non-supplemented groups (Meta $P<0.0001$, Epi $P<0.0001$).

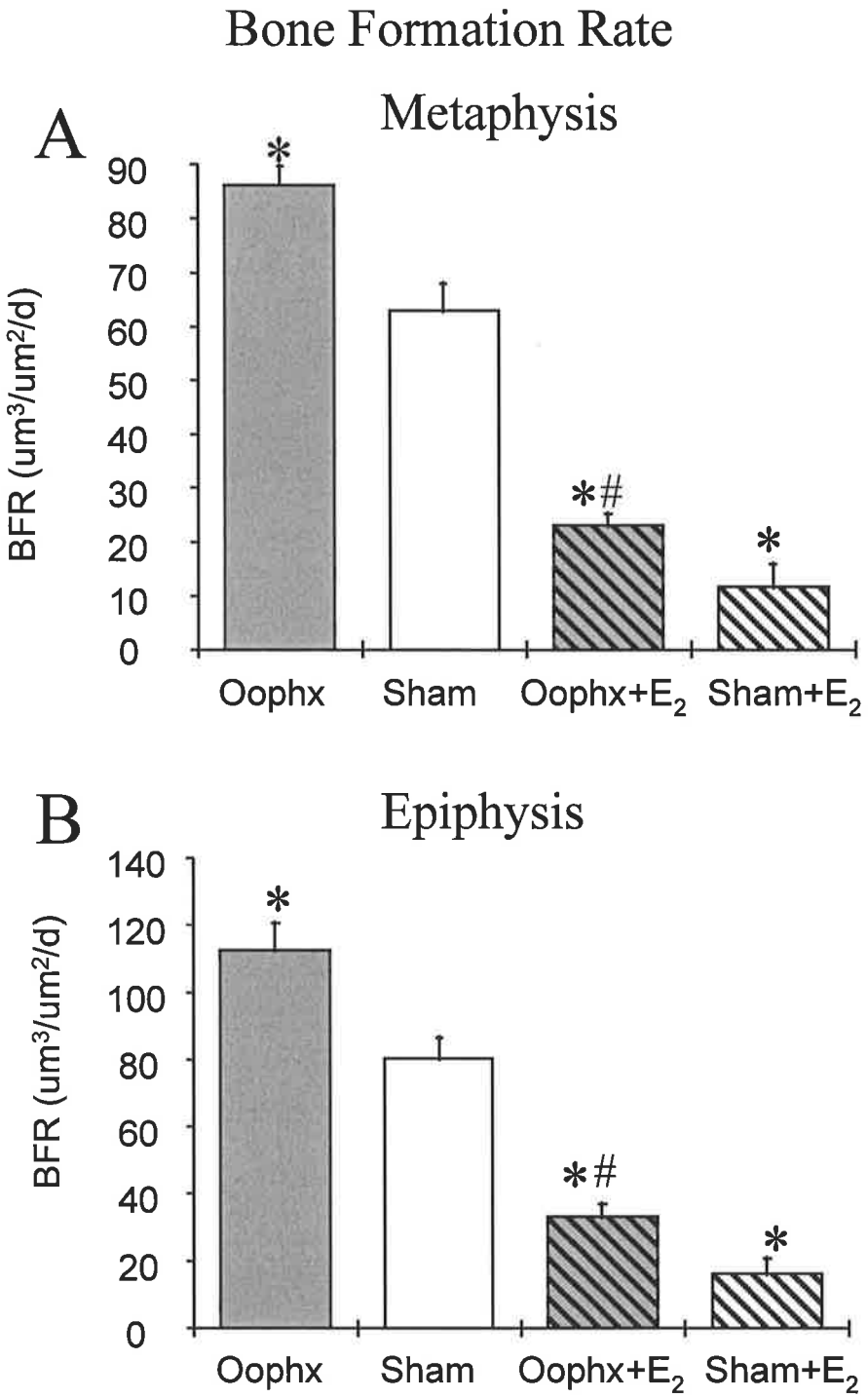


Figure 5.6 Effect of 17β oestradiol supplementation (E_2, hatch) on bone formation rate (BFR, $um^3/um^2/d$) in the metaphysis (A) and epiphysis (B) of the distal femur of the rat in sham operated (white bars) and oophorectomised (grey bars) rats. # Significant effect of E_2 in Oophx, * Significant difference from Sham.

5.3.2 Trabecular bone structure

In the ovary-intact groups E_2 supplementation did not alter BV/TV (Fig 5.7).

Oophorectomy decreased BV/TV (Meta P<0.005, Epi P<0.005). E_2 supplementation prevented the oophorectomy induced decrease in BV/TV. BV/TV was significantly greater following E_2 supplementation in oophorectomised groups (Meta P<0.05, Epi P<0.005). There was no difference in BV/TV between the three oestradiol sufficient groups.

In the ovary-intact groups E_2 supplementation increased Tb.Th (Fig 5.8) (Meta P<0.005, Epi P<0005).

Oophorectomy decreased Tb.Th in the metaphysis (P<0.005) but not the epiphysis. E_2 supplementation returned Tb.Th to ovary-intact levels, and significantly thickened trabeculae compared to non-supplemented groups in the metaphysis (P<0.005). In the epiphysis there was no difference in Tb.Th in the two Oophx groups and Sham.

In the oestradiol sufficient groups, there was an increase in Tb.Th as bone cell activity decreased, with Tb.Th negatively correlated to both Oc.S (Meta -0.64, P<0.001, Epi -0.42, P<0.01) and BFR (Meta -0.66, P<0.001, Epi -0.60, P<0.001).

In the ovary-intact groups E_2 supplementation significantly decreased Tb.N (Fig 5.9) in the epiphysis (P<0.001) but not the metaphysis.

Oophorectomy decreased Tb.N (Meta P<0.05, Epi P<0.005). E_2 supplementation returned Tb.N to ovary-intact levels, and significantly increased Tb.N compared to non-supplemented groups in the epiphysis (P<0.05), with a strong trend in the metaphysis (P<0.06). However in the metaphysis, Tb.N of the Oophx+E_2 group was not different from both Oophx and Sham.

In the ovary-intact groups E_2 supplementation did not alter Peri (Fig 5.10). Oophorectomy decreased Peri (Meta $P<0.05$, Epi $P<0.05$). E_2 supplementation returned Peri to ovary-intact levels. In the metaphysis E_2 supplementation significantly increased Peri compared to the non-supplemented group ($P<0.05$). In the epiphysis Peri of the Oophx+E_2 group was not different from both Oophx and Sham groups.

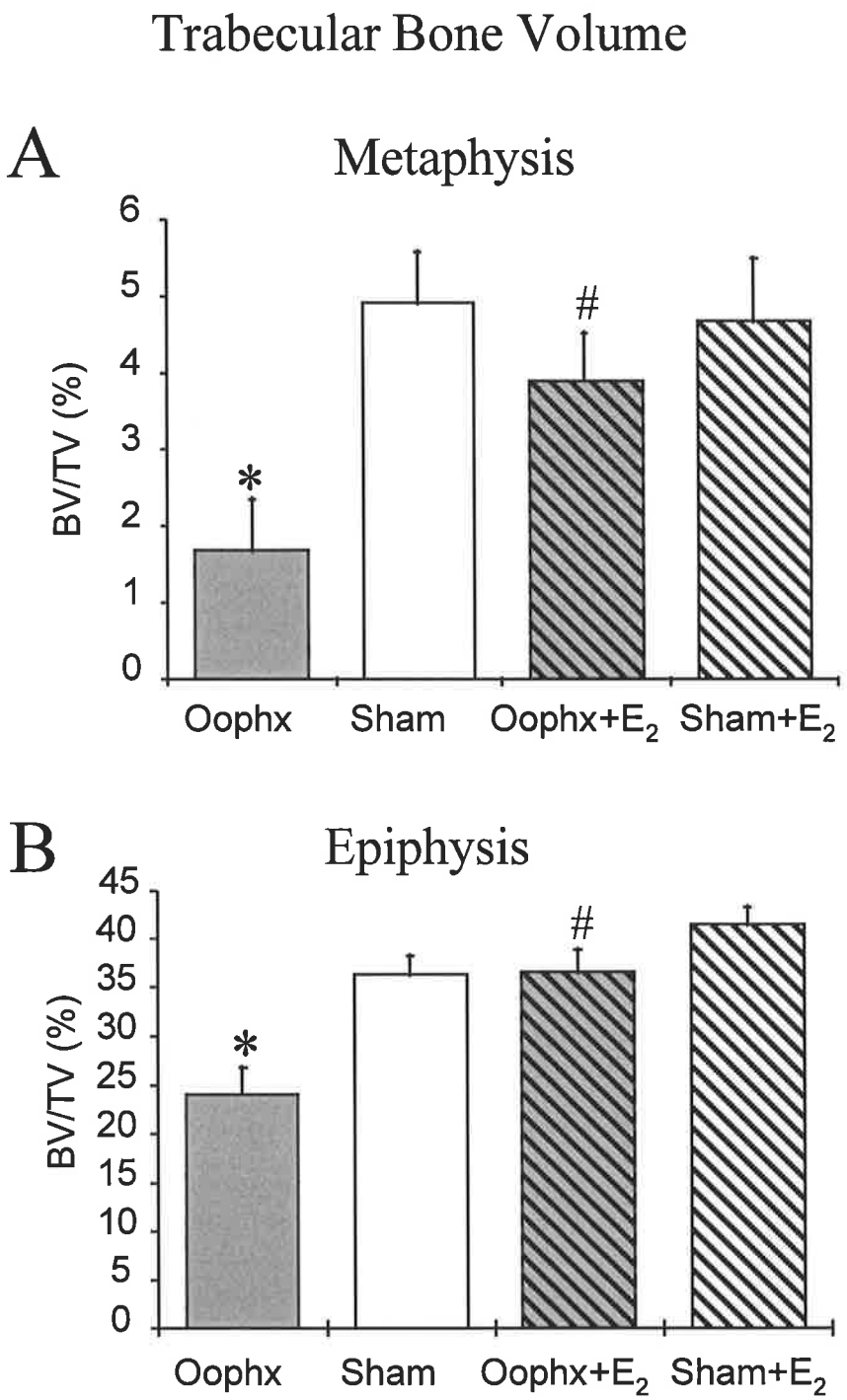


Figure 5.7 Effect of 17β oestradiol supplementation (E_2, hatch) on trabecular bone volume (BV/TV, %) in the metaphysis (A) and epiphysis (B) of the distal femur of the rat in sham operated (white bars) and oophorectomised (grey bars) rats. # Significant effect of E_2 in Oophx, * Significant difference from Sham.

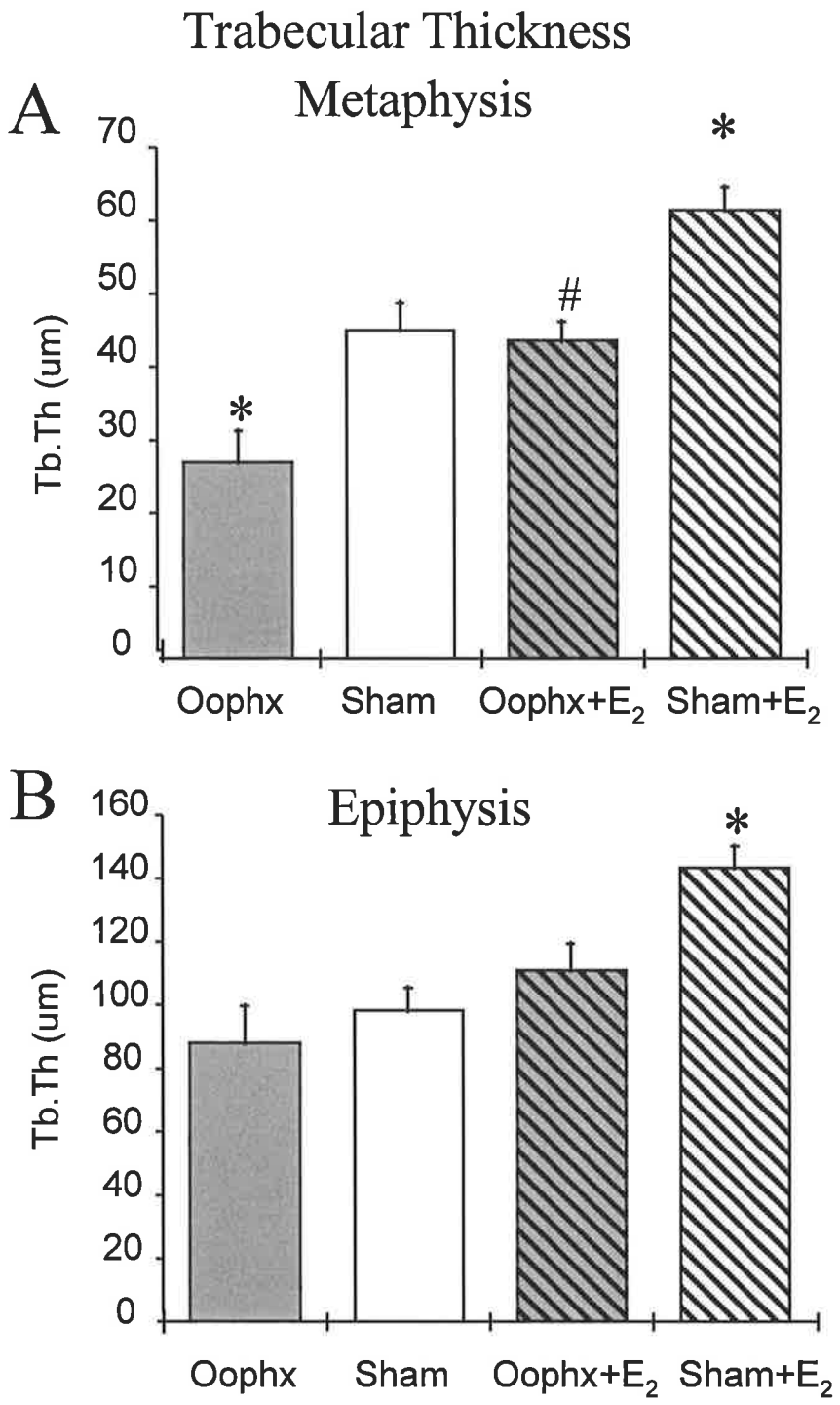


Figure 5.8 Effect of 17β oestradiol supplementation (E_2, hatch) on trabecular thickness (Tb.Th, um) in the metaphysis (A) and epiphysis (B) of the distal femur of the rat in sham operated (white bars) and oophorectomised (grey bars) rats. # Significant effect of E_2 in Oophx, * Significant difference from Sham.

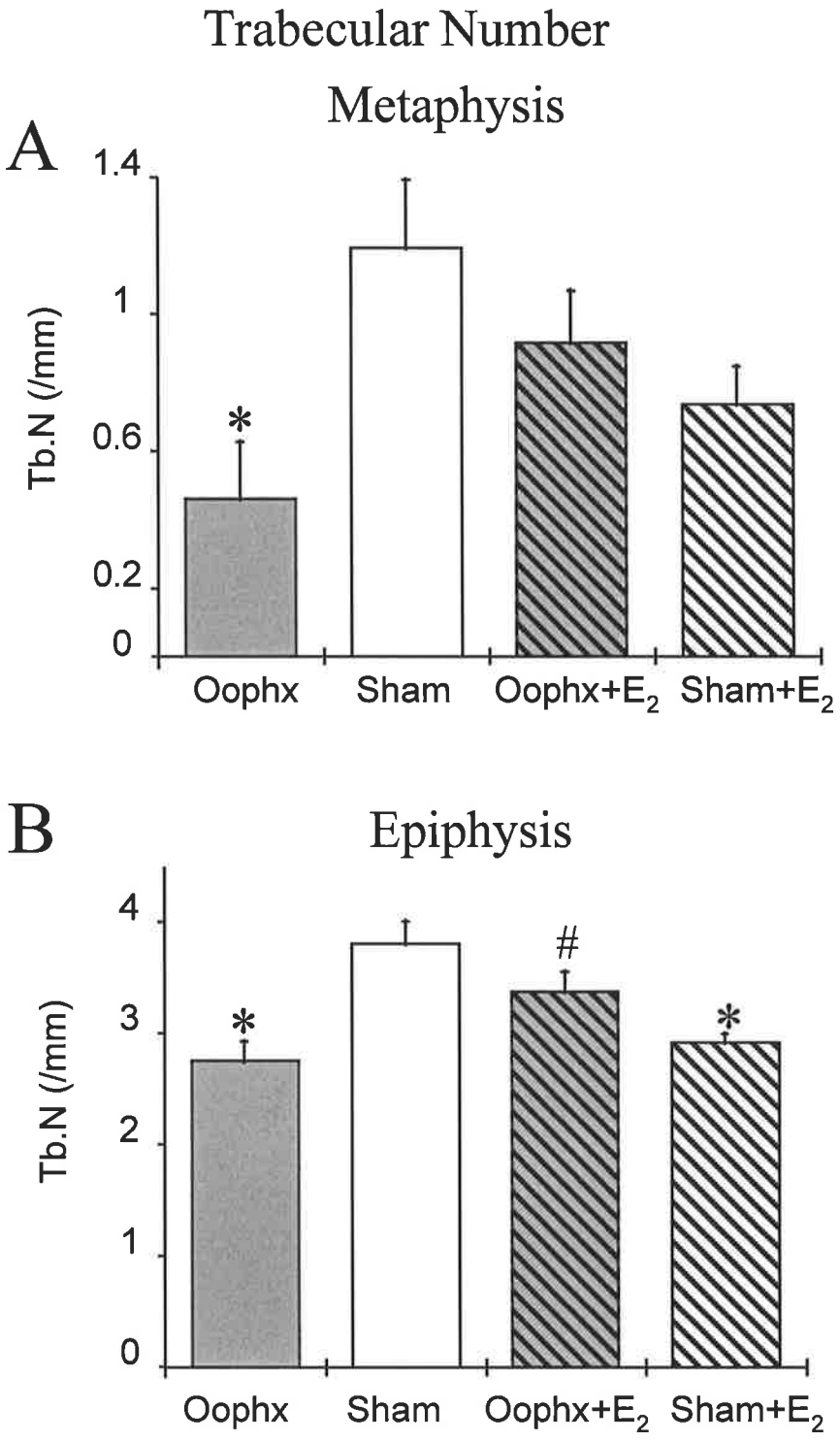


Figure 5.9 Effect of 17β oestradiol supplementation (E_2, hatch) on trabecular number (Tb.N, /mm) in the metaphysis (A) and epiphysis (B) of the distal femur of the rat in sham operated (white bars) and oophorectomised (grey bars) rats. # Significant effect of E_2 in Oophx, * Significant difference from Sham.

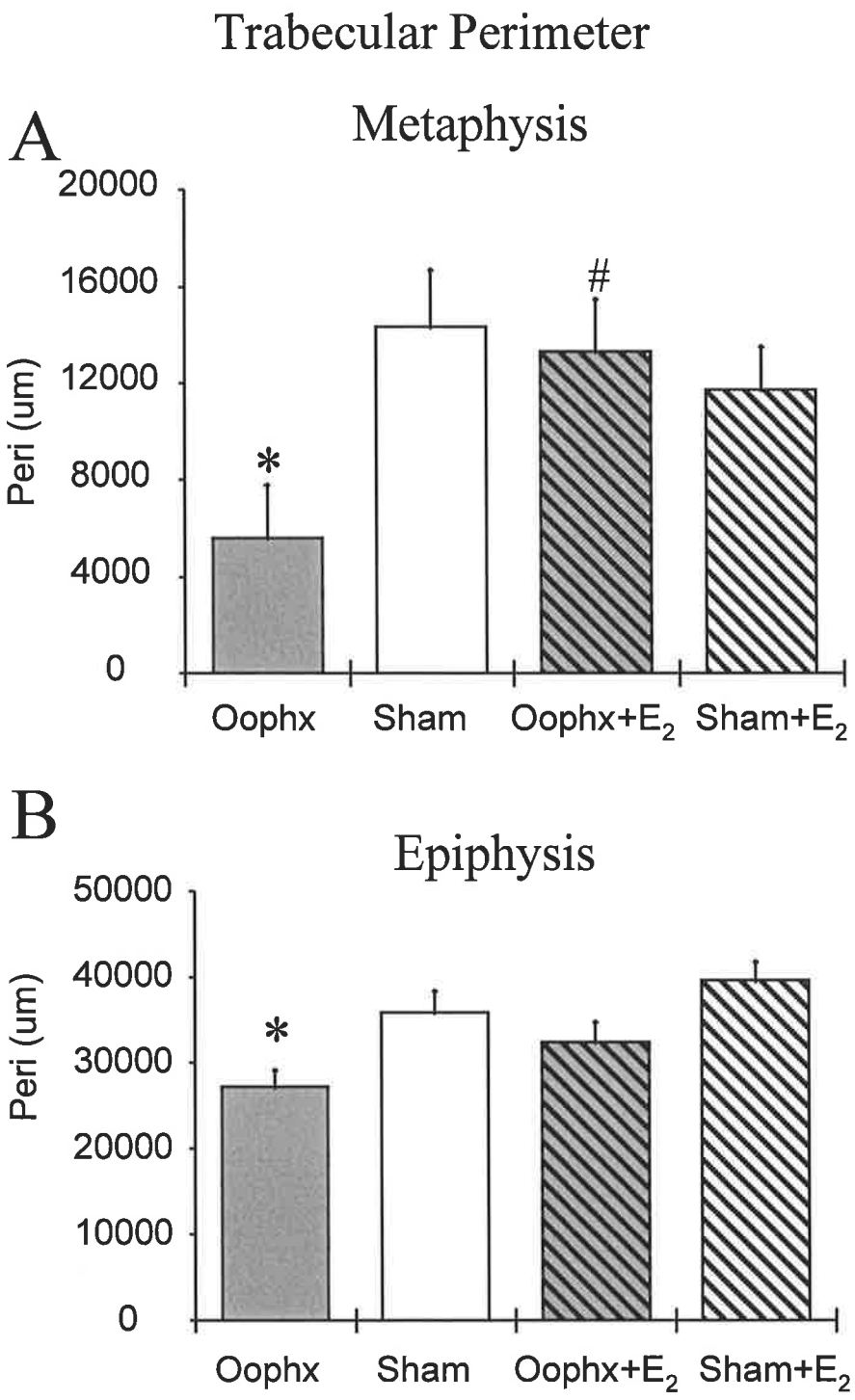


Figure 5.10 Effect of 17β oestradiol supplementation (E_2, hatch) on trabecular perimeter (Peri, um) in the metaphysis (A) and epiphysis (B) of the distal femur of the rat in sham operated (white bars) and oophorectomised (grey bars) rats. # Significant effect of E_2 in Oophx, * Significant difference from Sham.

5.3.3 Inter-region comparison

There was no difference between the two regions in the relative changes from Sham following E_2 supplementation in any measured parameter. In the Oophx group however, there was a strong trend for greater bone loss in the metaphysis ($P<0.06$), with Tb.Th ($P<0.05$) and Tb.N ($P<0.05$) both reduced to a greater extent. These changes were consistent with a greater increase in Oc.S in Meta ($P<0.01$), with no increase in dLS, MAR or BFR. Oc.E and dLE were not different between regions.

5.3.4 The effect of oestradiol supplementation on dietary calcium induced bone loss

In ovary-intact groups, the reduction in BV/TV between the 0.8% (4.3.1) and 0.2% Ca groups (5.3.2) was significant in both regions (Meta $P<0.0001$, Epi, $P<0.05$). E_2 supplementation in the metaphysis did not change BV/TV, with Sham+E_2 reduced compared with the 0.8% Ca group ($P<0.0001$). In contrast, in the epiphysis calcium restriction-induced bone loss was completely corrected. There was no difference between Sham+E_2 and 0.8% Ca groups.

In the metaphysis Tb.Th was greater in Sham 0.8% Ca than both 0.2% Ca groups (Sham $P<0.0001$, Sham+E2 $P<0.001$). Tb.N was also greater in Sham 0.8% Ca than both 0.2% Ca groups (Sham $P<0.005$, Sham+E2 $P<0.0001$).

The correction in BV/TV in the epiphysis was due to an increase in Tb.Th. Tb.Th was not different between Sham+E_2 and Sham 0.8% Ca, with both thicker than Sham 0.2% Ca (Sham+E_2 $P<0.005$, Sham 0.8% Ca $P<0.0005$). Tb.N was greater in both 0.2% Ca groups, compared to 0.8% Ca (Sham $P<0.0005$, Sham+E2 $P<0.01$).

5.3.5 Scanning electron microscopy

In the ovary-intact, 0.2% Ca fed sample (Fig 5.11A), the metaphyseal trabecular network is considerably shortened compared with 0.8% Ca fed animals (Fig 4.4), with no difference in the epiphysis. The metaphysis is not substantially different in the ovary-intact, E_2 supplemented sample (Fig 5.11C), although the trabecular network does extend to the margin of the sample, consistent with retention of BV/TV in this region. No difference in the epiphysis is evident.

Oophorectomy markedly reduced the size of the metaphyseal trabecular network (Fig 5.11B), compared to both ovary-intact samples. The preservation of trabeculae in the metaphysis is markedly evident in the oophorectomised, E_2 supplemented sample, consistent with the similarity of BV/TV in the three oestradiol replete groups (Fig 5.11D). The epiphyseal network does not appear altered throughout the samples.

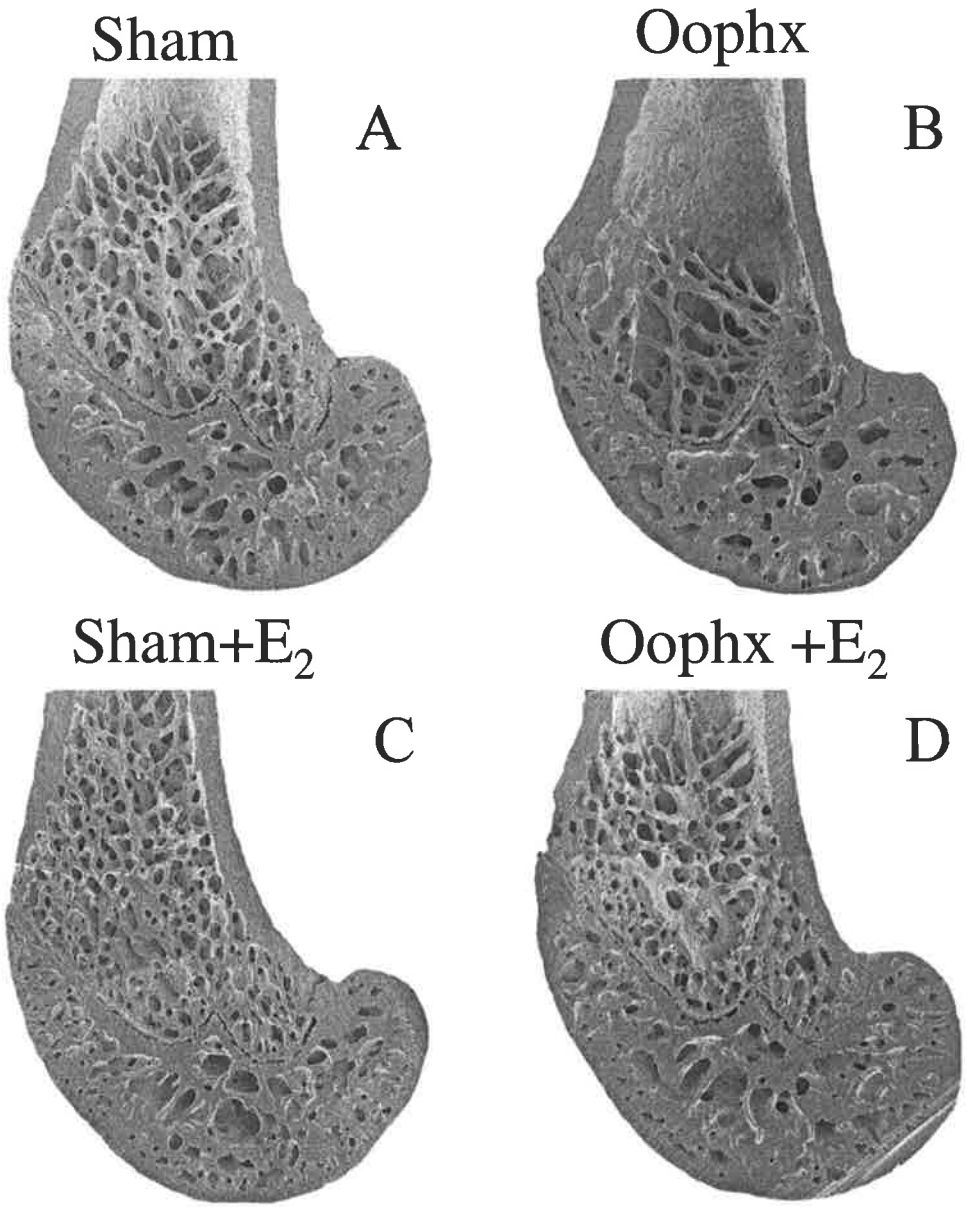


Figure 5.11 The effect of 17β oestradiol (E_2) supplementation on oophorectomy-induced osteopenia in the distal femur. In the metaphysis considerable bone loss is evident in the ovary intact bone (A) with increased loss following oophorectomy (B). E_2 supplementation produced a slight increase in ovary intact bone (C), with considerable retention of bone in the oophorectomised bone (D). The epiphysis remained relatively unaffected by both oophorectomy and E_2 supplementation.

5.4 Discussion

5.4.1 The effect of 17β oestradiol supplementation on bone cell activity

5.4.1.1 Surface percentage data

Antiresorptive therapy is employed in the treatment of osteoporotic patients to inhibit the increase in bone resorption which follows the menopause (Stephan *et al.,* 1987). Similarly in the present study, E_2 supplementation prevented the oophorectomy induced increase in Oc.S in both regions, consistent with human trials (Ettinger *et al.,* 1985, Vedi and Compston 1996), and previous studies in the oophorectomised rat (Wronski *et al.,* 1988b, Wronski *et al.,* 1989c, Kalu 1991, Kalu *et al.,* 1991b, Sims *et al.,* 1996). This reduction was also evident with E_2 supplementation in the ovary-intact group, suggesting that supraphysiological levels of oestradiol were produced, and are capable of exerting a physiological action.

This decrease in turnover contracts the remodelling space, reducing the total volume of Howsips lacunae, producing an increase in bone volume, as recorded in short term human trials (reviewed in Eriksen *et al.,* 1996). Interestingly, some long term human trials (Storm *et al.,* 1990, Liberman *et al.,* 1995, Eiken *et al.,* 1996) have reported slower but continued increases in BMD, leading to the suggestion of an anabolic effect of oestradiol on osteoblasts (McClung, 1996). The anabolic nature of oestrogens is a controversial topic (1.10.2.1). Evidence suggesting a short term anabolic effect has been produced from ovary-intact rats using doses of oestradiol following bisphosphonate treatment (Abe *et al.,* 1992, Chow *et al.,* 1992) The investigation of such a claim is important for the understanding of the pathogenesis of osteoporosis. Further, an understanding of the anabolic potential of oestradiol on osteoblasts would prove an important consideration for the clinical application of this compound. If oestradiol were shown to be anabolic, its use may be expanded beyond

that of antiresorptive therapy early after menopause, to include the management of age related bone loss, generally attributed to declining osteoblast function (Parfitt, 95, Thomsen *et al.,* 1996).

The high levels of oestradiol administered in this study may complicate comparison with human trials, however the reduction in BFR evident following E_2 supplementation resulted from decreases in both dLE and MAR. These two indices are strong indicators of osteoblast proliferation and anabolic activity, respectively (Parfitt, 95), thus an anabolic effect of 17β oestradiol on osteoblasts cannot be supported. This lack of evidence for an anabolic response by osteoblasts to E_2 supplementation at this level of supplementation is in agreement with previous studies in both humans (Steiniche *et al.,* 1989, Storm *et al.,* 1990) and the rat model (Wronski *et al.,* 1988b, Takano-Yamamoto and Rodan, 1990, Kalu *et al.,* 1991).

5.4.1.2 Linear extent data

In the ovary-intact animals, Oc.E and dLE were reduced by E_2 supplementation in both regions, demonstrating the inhibition of bone cell proliferation characteristic of antiresorptive treatments (1.10.2). In the oophorectomised, non-oestradiol supplemented group Oc.E and dLE were not elevated in either region at this time post operation. This finding is consistent with findings from Chapter 4, with Oc.E returned to ovary-intact levels in both regions after day 90 (4.3.3) and dLE returned to ovary-intact levels by day 48 in the metaphysis (4.3.5). In contrast, the sustained increase in dLE in the epiphysis previously demonstrated was absent, consistent with the effects of decreased dietary calcium (6.4.7) used in this study.

5.4.2 The effect of 17β oestradiol supplementation on the development of osteopenia following oophorectomy

In the metaphysis oophorectomy resulted in severe osteopenia as demonstrated throughout these studies. The epiphysis also experienced bone loss following oophorectomy, which was associated with the decreased dietary calcium as demonstrated in the subsequent chapter. In both regions, this bone loss was not evident following E_2 supplementation, emphasising the central role of this hormone in the pathogenesis of ovarian hormone deficient bone loss (Lane *et al.,* 1999). The prevention of osteopenia following E_2 supplementation in the metaphysis and epiphysis of oophorectomised animals also emphasises the importance of the increase in turnover to this process. Inhibition of this increase in bone cell activity blocks any loss of bone.

However, given the length of the present study and its single sample time, it is not possible to evaluate accurately whether bone loss was prevented completely by E_2 supplementation, or whether any initial bone loss was later restored. Chapter 3 demonstrated the impact of the remodelling transient on metaphyseal osteopenia producing rapid and permanent bone loss as early as 15 days post oophorectomy due to trabecular perforation (3.3.1). The lack of oophorectomy induced bone loss in the present study suggests that E_2 supplementation prevented the initial increase in turnover and therefore bone loss. In particular this finding highlights the importance of controlling bone turnover in preserving trabecular bone in regions of low strain. Further, it demonstrates that the trabecular bone of the epiphysis is resistant to reduced turnover, and thus able to maintain BV/TV despite the contraction of the remodelling space.

5.4.3 The effect of low bone turnover on trabecular bone structure

BV/TV was not different between the three oestradiol sufficient groups, in both the metaphysis and epiphysis, implying a uniform response to the presence of this hormone. However, there were significant effects on the structure of the remaining bone brought about by the differences in bone cell activity between the three groups. The initial structural outcome of such a contraction of the remodelling space is an increase in Tb.Th (Watts *et al.,* 1990). This change was inversely proportional to bone turnover, with an average increase in Tb.Th of 48% in the E_2 supplemented groups. An increase such as this may be viewed as evidence of anabolic activity, however in this case it is not. BV/TV was not increased by E_2 supplementation, and the increase in Tb.Th was proportional to decreasing bone turnover, reflective of decreased osteoblast proliferation, and not an increase in osteoblast function. Presumably, this process would cease once a new steady state has been reached (Riggs *et al.,* 1996).

An increase in Tb.Th of this magnitude may change the local strain environment, eliciting a response from the homeostatic mechanism. Mechanical strain has been suggested as the factor which modulates homeostatic maintenance of the skeleton (Frost, 1990, Frost, 1994), modifying trabecular structure to maintain strain within a physiological range of approximately 1500 microstrain across mammalian species (Rubin and Lanyon 1982, Rubin 1984, Lanyon, 1992).

Such alterations in structure resulting from local strain changes have been demonstrated previously. In the case of an expansion in the remodelling space, as seen after oophorectomy, trabeculae in the epiphysis were thinned (3.3.1.1), increasing the strain environment. This effect was corrected during subsequent remodelling cycles by returning Tb.Th to baseline values (4.3.1.1). In the diaphysis and metaphysis trabeculae were perforated, the lack of template limited the remodelling response. In

the present study however, the contraction of the remodelling space following E_2 supplementation which thickened trabeculae and decreased the strain environment, was compensated by decreasing Tb.N.

The loss of trabeculae is inconsistent with the low levels of bone cell activity recorded in this study. This suggests that the homeostatic response was completed before the completion of the study, with Oc.S rising to produce the reduction in Tb.N. Alternatively, an increase in resorption depth is implicated. In previous chapters, it was calculated that the increase in bone turnover following oophorectomy is associated with a increased resorption depth which has been shown to induce perforation in the metaphysis. In the present study a reduction in turnover is also associated with perforation of trabeculae. This perforation cannot be linked to the change in dietary calcium, which produced alterations to Tb.Th (6.3.1.1). These data support the theory that bone balance is maintained by a mechanically governed system, independent of prevailing bone turnover (Westerlind *et al.,* 1997), as seen in the epiphysis after oophorectomy. Based on the present data, the dominant effector of this homeostatic system appears to be alterations to resorption depth, which presumably can be reduced when osteoclast activity is increased in high strain regions or increased in the present case of decreased turnover in low strain regions.

5.4.4 Regional comparison of the effect of 17β oestradiol supplementation on the development of osteopenia due to decreased dietary calcium

Oestradiol supplementation prevented the osteopenia resulting from ovarian hormone deficiency in both regions, however its effect on osteopenia resulting from dietary calcium restriction was site specific. All animals were placed on a 0.2% Ca diet for a 4 week preoperative acclimatisation period prior to operation, sufficient time for dietary

calcium related bone loss prior to commencement of E_2 supplementation (Shen *et al.*, 1995b). The mean metaphyseal BV/TV in the Sham group was 5.6%, E_2 supplementation did not alter this loss with the Sham+E_2 BV/TV was 4.7%. However, BV/TV in age equivalent animals fed the 0.8% Ca diet (4.3.1.1) was 23%, demonstrating that osteopenia did occur due to calcium restriction in the present study. Thus E_2 supplementation cannot counteract the loss of bone resulting from a decrease in dietary calcium in this region. Such an observation is consistent with a predominantly metabolic role for the low strain regions of trabecular bone, such as the femoral metaphysis (Wu *et al.*, 1990).

In contrast, in the epiphysis BV/TV in animals fed the 0.8% Ca diet (4.3.1.2) was 40% compared to 42% in Sham+E_2 animals in the present study, while the Sham group was significantly, although not substantially, lower than both with BV/TV of 36%. Thus the mechanically important epiphyseal bone is retained in preference to the metaphysis, with BV/TV in the epiphysis increasing to non-calcium restricted levels following E_2 supplementation.

These regional differences in recovery from dietary calcium induced bone loss may also relate in part to the architectural consequences of this loss. In the metaphysis both Tb.Th and Tb.N were reduced by decreasing dietary calcium. This perforation would produce an uncorrectable architectural barrier to restoration in this region. While, the lack of correction of Tb.Th following E2 supplementation may reflect a continuing calcium requirement in 0.2% Ca fed animals. In the epiphysis, osteopenia following to calcium deficiency was due to reduced Tb.Th alone which was restored during the oestradiol stimulated contraction of the remodelling space, with no difference in Tb.Th between Sham animals fed 0.8% Ca and Sham+E_2 fed 0.2% Ca.

Tb.N however, was greater in the 0.2% Ca groups, an anomalous result discussed in Chapter 6 (6.4.3).

5.4.5 The effect of low bone turnover on trabecular bone structure: Implications for fracture rate

This study has shown significant alterations to trabecular bone structure resulting from antiresorptive therapy, with minor alterations to trabecular bone volume. In a similar manner, substantial decreases in fracture rate have been reported in human trials, with modest increases in BMD. A recent study calculated that only 43% of the reduction in vertebral fracture rate was accounted for by change in BMD following 3 years of oestradiol treatment (Cummings *et al.,* 1996). Whilst an increased period for bone formation brought about by reduced turnover has been linked to improved bone quality (Dempster, 1997) and reduction in turnover rate is liked with reduction in fracture (Garnero *et al.,* 1996, Delmas, 1997, Melton III *et al.,* 1997). A significant proportion of the remaining reduction in fracture rate may be related to the structural changes resulting from the fall in turnover.

Contraction of the remodelling space increases bone volume due to increases in Tb.Th (Eriksen, 1986). An association between Tb.Th and strength has been demonstrated in osteopenic, oophorectomised rats treated with intermittent PTH (Meng *et al.,* 1996). Increased BV/TV and bone strength in the distal femur was the result of trabecular thickening only with no increase in Tb.N, with the strength of the distal femur being strongly correlated to Tb.Th. This relationship is consistent with both the structural changes seen in this study and the reduction in fracture seen in human trials. Therefore antiresorptive treatment may have a double benefit to the patient. Firstly the decrease in resorption, reducing the probability of perforation

(Parfitt, 1984) and increasing bone volume due to the contraction of the remodelling space. Secondly, the increase in strength due to the thickening of trabeculae upon contraction of the remodelling space. These changes appear to be enhanced in the high strain regions, but also appear to provide benefit to the regions of lower strain that are sensitive to oestrogen deficiency. Thus, anabolic therapy to osteoporotic patients may be able to significantly increase strength even after substantial bone has been lost by perforation. This fact, which is beginning to emerge from clinical trails may alter the treatment regimes applied to osteopenic patients.

Chapter 6

Effect of dietary calcium restriction on oophorectomy induced bone loss

6.1 Introduction

Low dietary calcium intake (reviewed in Dawson-Hughes, 1991, reviewed in Nordin, 1997) and oestrogen deficiency (Prior *et al.,* 1990) are both potent risk factors for osteoporotic fracture. The previous chapters demonstrated the central role of ovarian hormones in the maintenance of skeletal tissue, and the regional variation in response to withdrawal of these hormones. The present chapter investigates the impact of reduced dietary calcium intake on this process.

In addition to its mechanical functions, bone acts as the body's store of calcium and other inorganic compounds. Calcium forms an integral part in many physiological processes, its importance reflected in the precision with which its serum concentration is regulated under normal conditions (Kurokawa, 1994). When dietary calcium intake is insufficient a compensatory mobilisation from bone is required. This is achieved in part by an increase in resorption and loss of bone (Bushinsky and Lechleider, 1987). In previous chapters ovarian hormone deficiency has been shown to stimulate increased resorption and bone loss in metaphyseal trabecular bone, with no permanent changes in the epiphysis. These alterations to bone turnover and trabecular architecture are again investigated in these two regions, in relation to the additional impact of decreasing dietary calcium.

The trabecular bone of the epiphysis is clearly resistant to high turnover osteopenia induced by oophorectomy, suggesting mechanical sensitivity of this pathway. However, calcium deficient osteopenia may not to be regulated in the same manner. The high local strain that protected the epiphysis from osteopenia following oophorectomy may not preserve bone during calcium deficiency. Osteopenia following oophorectomy has been shown to exert a reduced effect on the loaded bone of the long bone cortices (reviewed in Kalu 1991, reviewed in Frost and Jee , 1992),

while calcium deficency induced bone loss effects both less loaded trabecular bone and the cortices (Shen *et al.,* 1995). Further, it has been demonstrated that the osteopenic actions of calcium deficiency and oestrogen deficiency are additive (Kalu *et al.,* 1989, Shen *et al.,* 1995), increasing the likelihood of epiphyseal bone loss.

6.2 Materials and Methods

6.2.1 Animals

60 female Sprague-Dawley rats were maintained on commercial rat chow (2.2) (0.8% calcium, 0.4% available phosphorus and 2000 IU/kg vitamin D_3) until six months of age. At this time the animals were randomly allocated to a semi-synthetic diet (2.2) containing either 0.2% or 0.04% Ca, 0.3% P and 4000IU/kg vitamin D_3 was randomly allocated, and used throughout the study period. The IMVS Animal Ethics Committee approved all procedures involving the animals.

6.2.2 Surgery

At 7 months of age, following a dietary acclimatisation period of 4 weeks, 10 animals from each dietary group were allocated to either oophorectomy or sham operation via the ventral approach (2.4.3). All rats received fluorochrome injections prior to killing (2.3). Rats were killed by exsanguination and the right femora were excised.

6.2.3 Histomorphometry

The distal 20 mm of the right femora were bisected in the sagittal plane and processed into MMA resin (2.9.1.2). One half of the left femora of a randomly chosen sample from each group were used for scanning electron microscopy (2.8). Bone cell activity

and trabecular bone morphology (2.14) were estimated in two regions, the metaphysis and epiphysis (2.11.1).

6.2.4 Calculations and statistics

All results were analysed using two-way ANOVA for operation and diet, significant results were further analysed using one-way ANOVA within operative or dietary group. All ANOVA were followed by Bonferroni/Dunn post hoc analysis. All analysis was conducted using Super ANOVA (Abacus Concepts, CA, USA).

The effect of dietary calcium induced bone loss was further assessed by comparing the groups in the present study with those consuming 0.8% Ca diet in Chapter 4. The group chosen was 120 days post oophorectomy, conducted at the same age as all mature oophorectomy studies in this thesis, 7 months. These rats were therefore 15 days older than those in the present study, considering the constant bone loss in aging rats an older group was considered conservative for comparison. This component was again analysed by two-way ANOVA for diet and operation. Contrasts were used to assess differences between particular groups following significant results in the ANOVA. These animals were included in analysis only where stated. All analysis was conducted using Super ANOVA (Abacus Concepts, CA, USA). $P < 0.05$ was considered significant.

6.3 Results

6.3.1 Trabecular bone structure

6.3.1.1 Metaphysis

Decreasing dietary calcium reduced BV/TV (Fig 6.1A) only in Sham ($P<0.05$). The 40% reduction was due to a nonsignificant 26% reduction in Tb.Th (Fig 6.3A) ($P<0.08$), with no change in Tb.N (Fig 6.2A). In Oophx calcium restriction produced a nonsignificant 60% reduction in BV/TV.

Oophorectomy resulted in bone loss in all dietary groups (0.2% $P<0.005$, 0.04% $P<0.01$). This was the result of a fall in Tb.N (0.2% $P<0.05$, 0.04% $P<0.01$), with a reduction in Tb.Th in 0.2% ($P<0.005$).

Peri (Fig 6.4A) was not affected by dietary calcium restriction. Oophorectomy reduced Peri in both dietary groups (0.2% $P<0.005$, 0.04% $P<0.01$)

6.3.1.2 Epiphysis

BV/TV (Fig 6.1B) was not affected by decreasing dietary calcium in both operative groups.

Oophorectomy did produce bone loss in both the 0.2% ($P<0.005$) and 0.04% Ca groups ($P<0.005$). This was due to reduced Tb.N (Fig 6.2B) (0.2% $P<0.005$, 0.04% $P<0.0005$) ($P<0.005$), with no difference in Tb.Th (Fig 6.3B).

Peri (Fig 6.4B) was not affected by dietary calcium restriction. Oophorectomy reduced Peri in both dietary groups (0.2% $P<0.005$, 0.04% $P<0.005$)

Trabecular Bone Volume

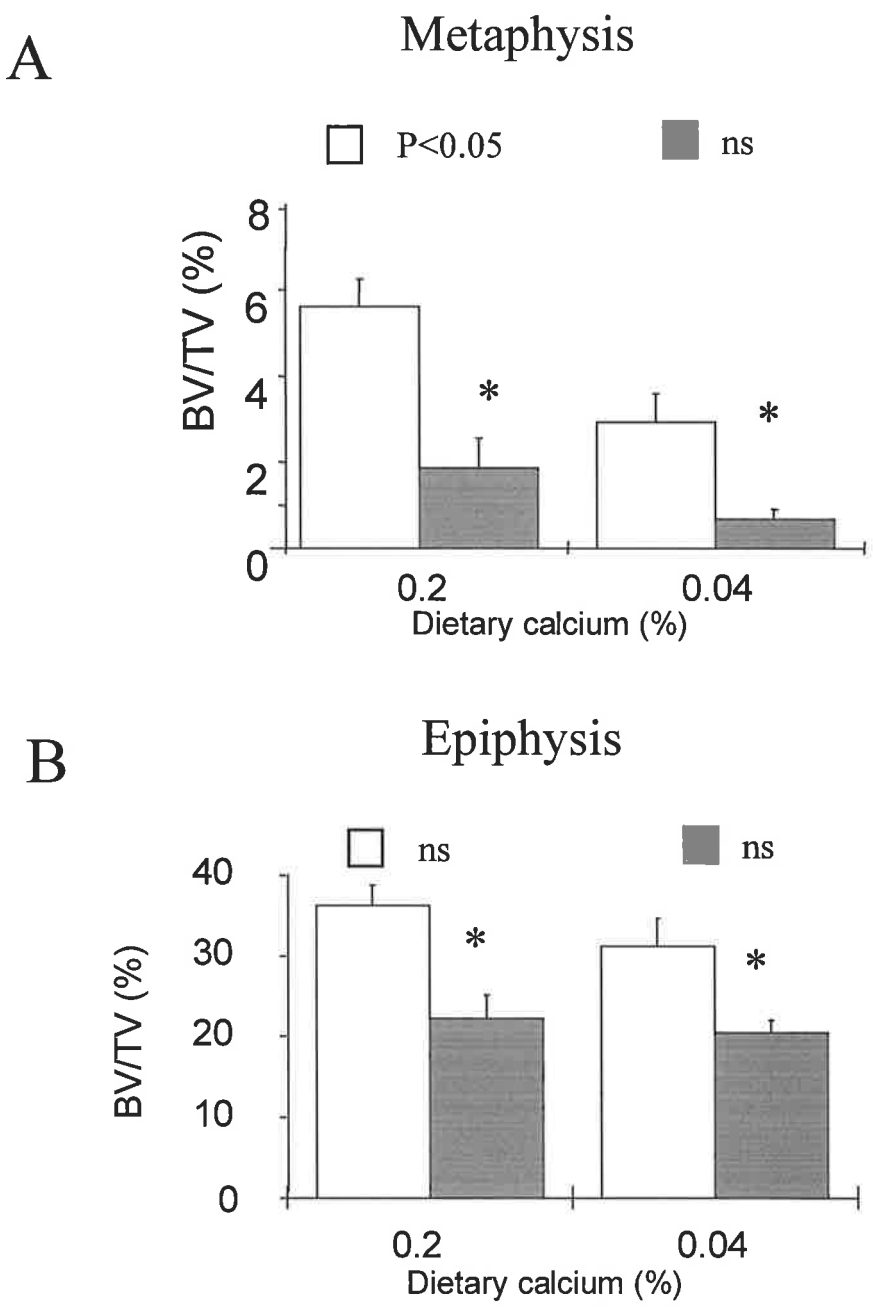


Figure 6.1 Effect of increasing dietary calcium restriction on trabecular bone volume (BV/TV, %) in the metaphysis (A) and epiphysis (B) of the distal femur of the rat in sham operated (white bars) and oophorectomised (grey bars) rats. P< one-way ANOVA within operation group, * significant difference between operation group.

Trabecular Number

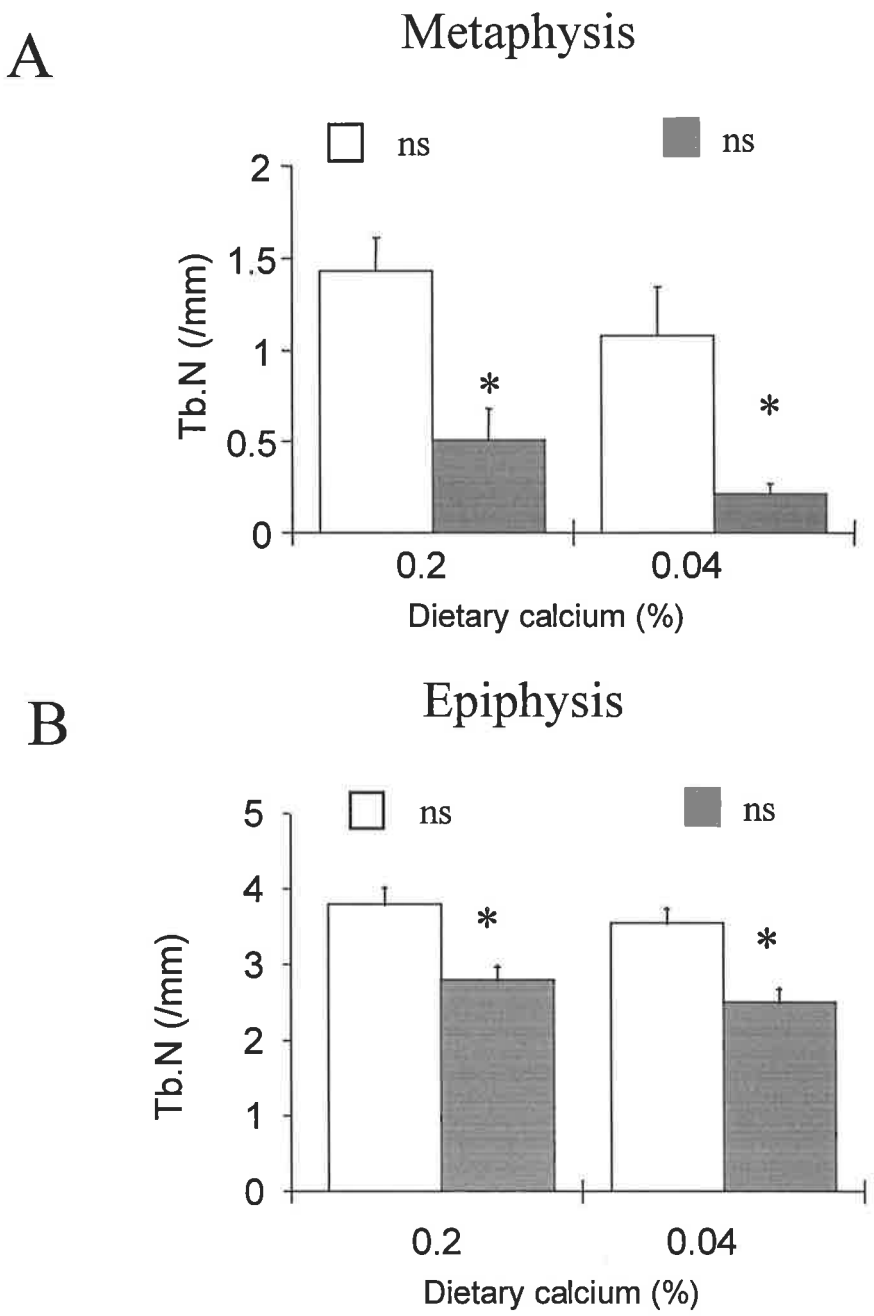


Figure 6.2 Effect of increasing dietary calcium restriction on trabecular number (Tb.N, /mm) in the metaphysis (A) and epiphysis (B) of the distal femur of the rat in sham operated (white bars) and oophorectomised (grey bars) rats. P< one-way ANOVA within operation group, * significant difference between operation group.

Trabecular Thickness

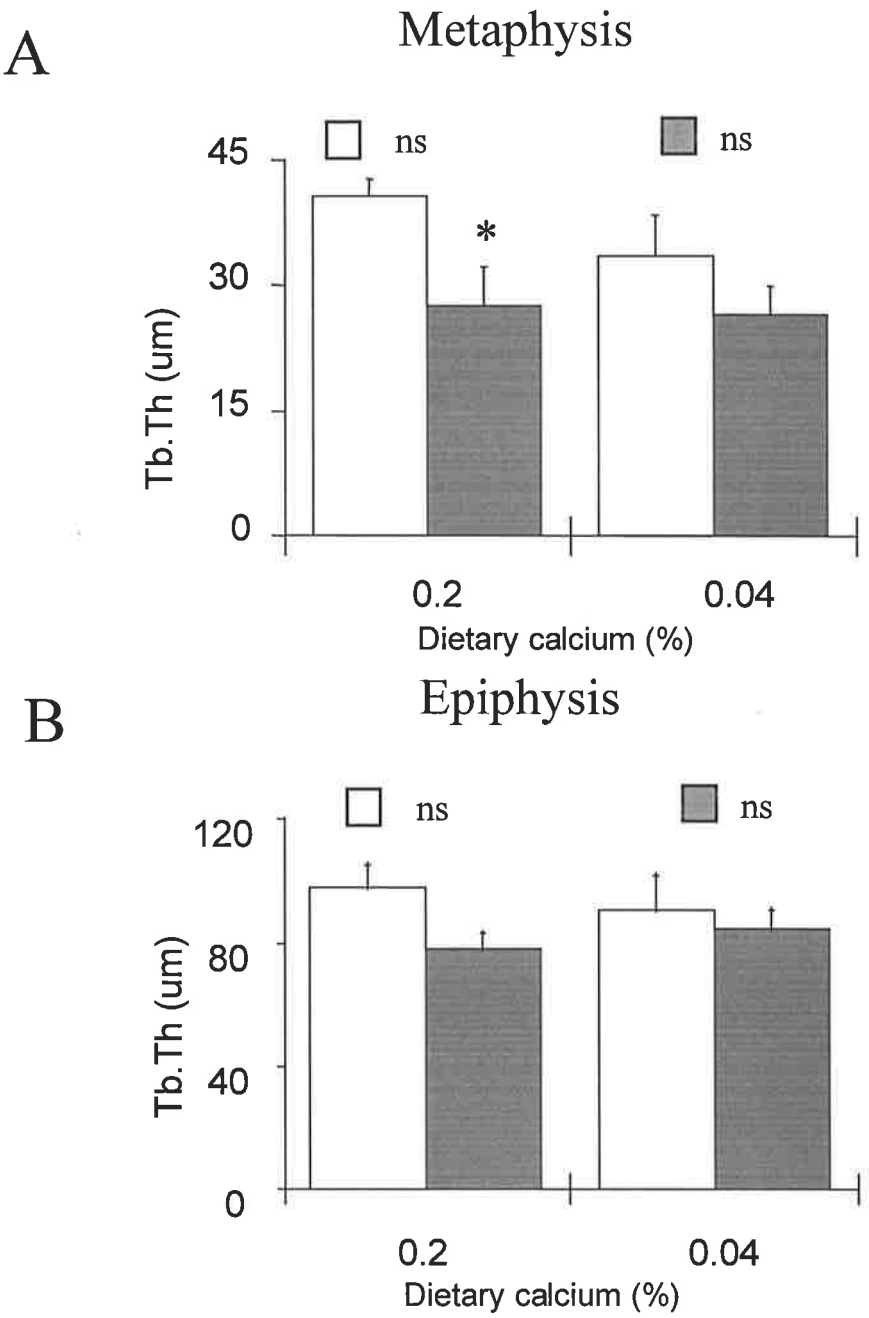


Figure 6.3 Effect of increasing dietary calcium restriction on trabecular thickness (Tb.Th, um) in the metaphysis (A) and epiphysis (B) of the distal femur of the rat in sham operated (white bars) and oophorectomised (grey bars) rats. P< one-way ANOVA within operation group, * significant difference between operation group.

Trabecular Perimeter

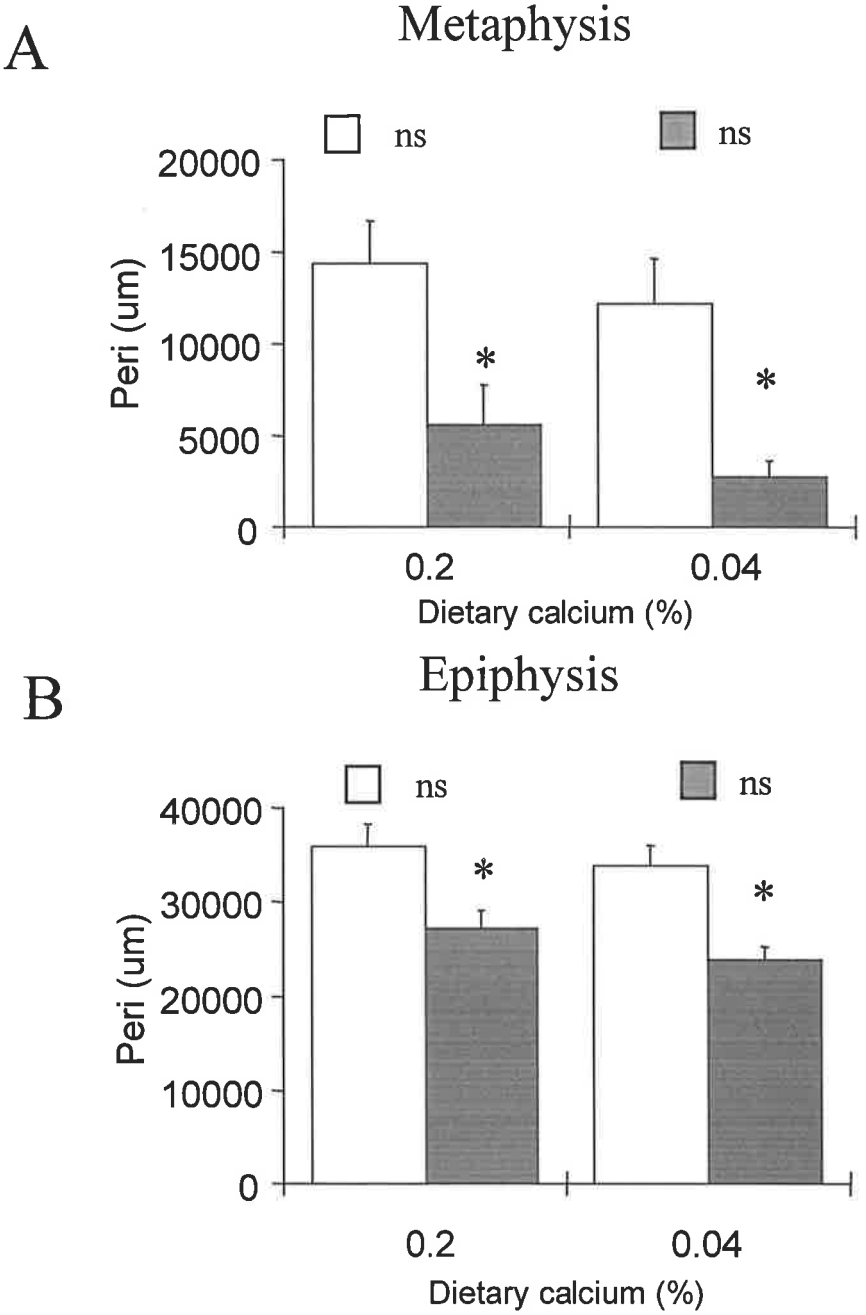


Figure 6.4 Effect of increasing dietary calcium restriction on trabecular perimeter (Peri, um) in the metaphysis (A) and epiphysis (B) of the distal femur of the rat in sham operated (white bars) and oophorectomised (grey bars) rats. P< one-way ANOVA within operation group, * significant difference between operation group.

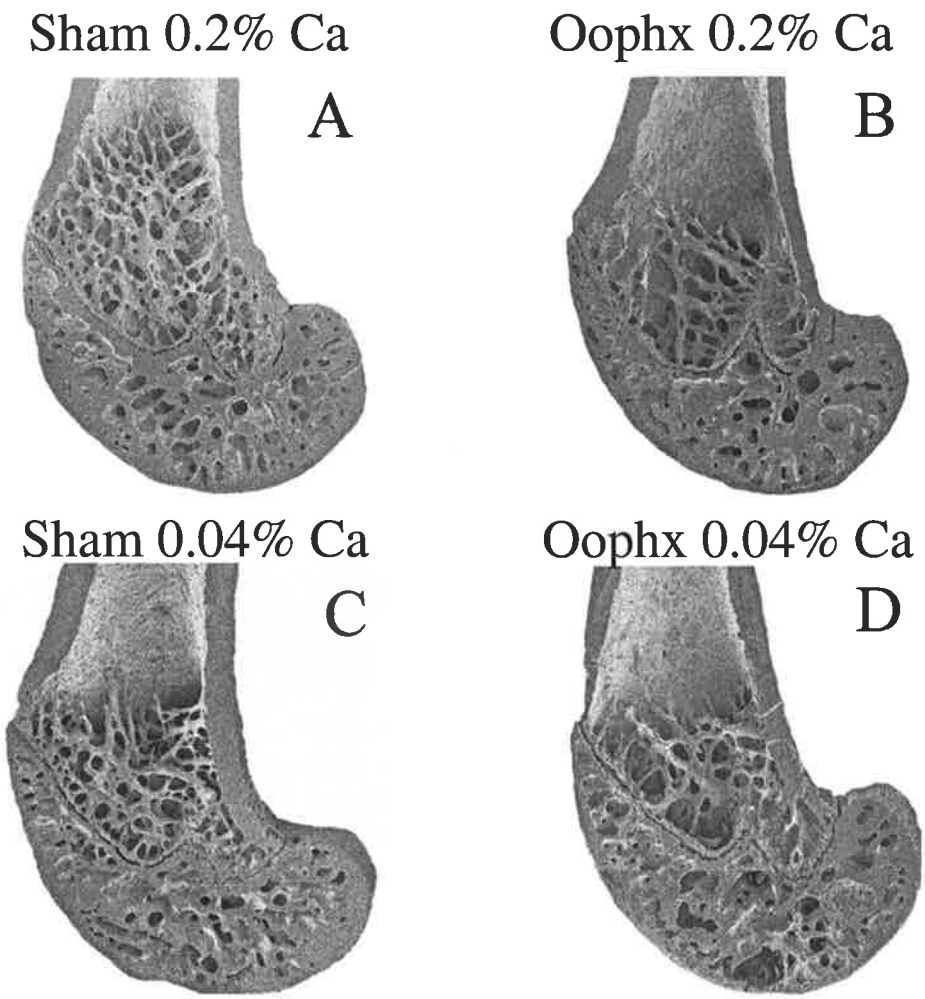


Figure 6.4 The effect of dietary calcium restriction on oophorectomy-induced osteopenia in the distal femur. In the metaphysis considerable bone loss is evident in the ovary intact bone (A) with increased loss following oophorectomy (B). Dietary calcium restriction produced a further decrease in ovary intact bone (C), with further loss of bone in the oophorectomised bone (D). The epiphysis remained relatively unaffected by both oophorectomy and a slightly more open trabecular network is evident in the calcium restricted, oophorectomised epiphysis.

6.3.2 Osteoclast surface

In the metaphysis Oc.S (Fig 6.6A) increased with decreasing dietary calcium in Sham ($P<0.05$), with no change in Oophx. Oophorectomy resulted in increased Oc.S in both dietary groups (0.2% $P<0.0001$, 0.04% $P<0.05$).

In the epiphysis Oc.S (Fig 6.6B) was not affected by decreasing dietary calcium. Oophorectomy resulted in increased Oc.S in both dietary groups (0.2% $P<0.005$, 0.04% $P<0.0005$).

6.3.3 Osteoclast extent

In the metaphysis Oc.E (Fig 6.7A) was not affected by decreasing dietary calcium. Oophorectomy resulted in a significant decrease in Oc.E in the 0.04% group only ($P<0.05$).

In the epiphysis Oc.E (Fig 6.7B) was not affected by decreasing dietary calcium or oophorectomy.

Osteoclast Surface

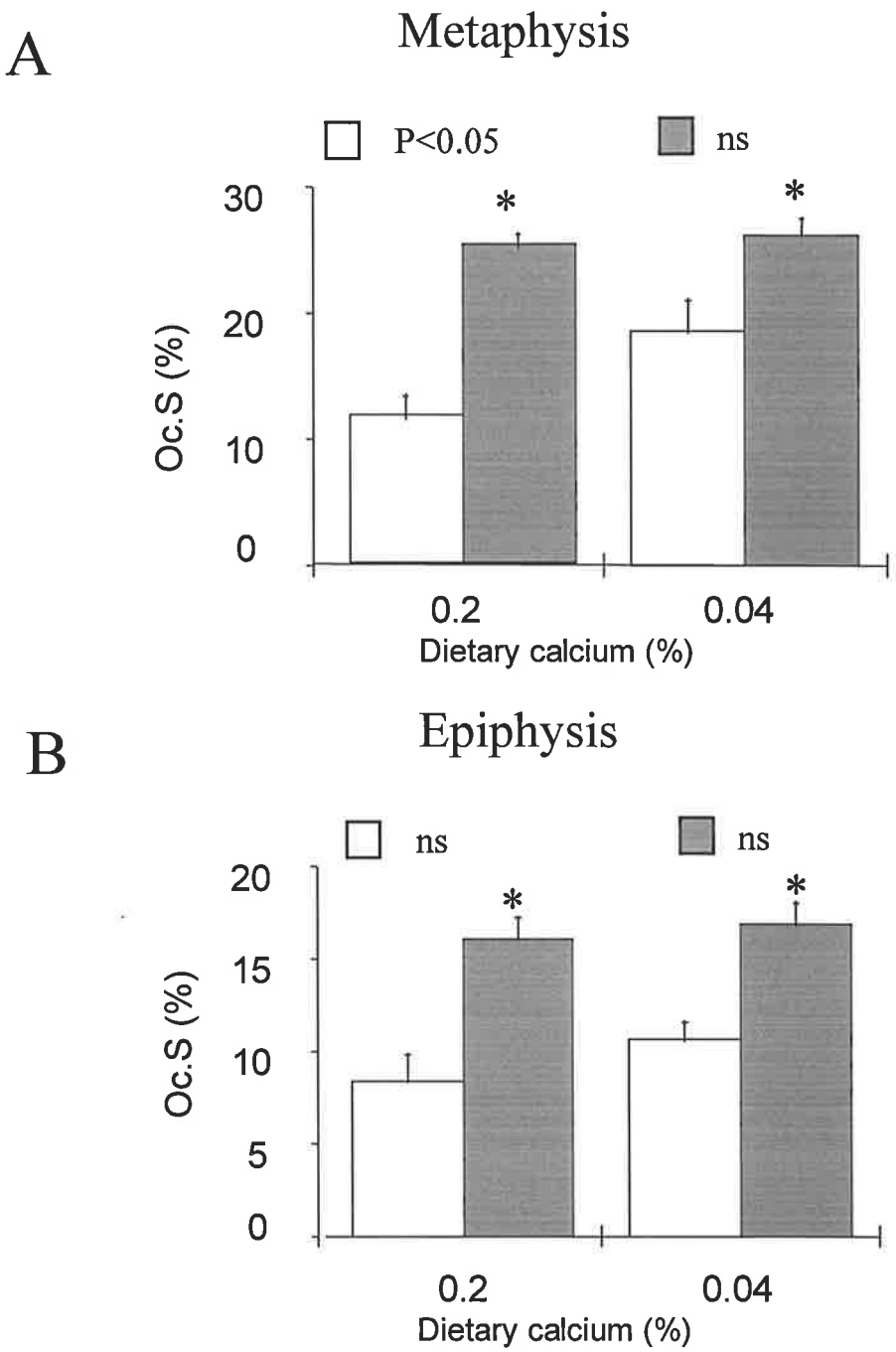
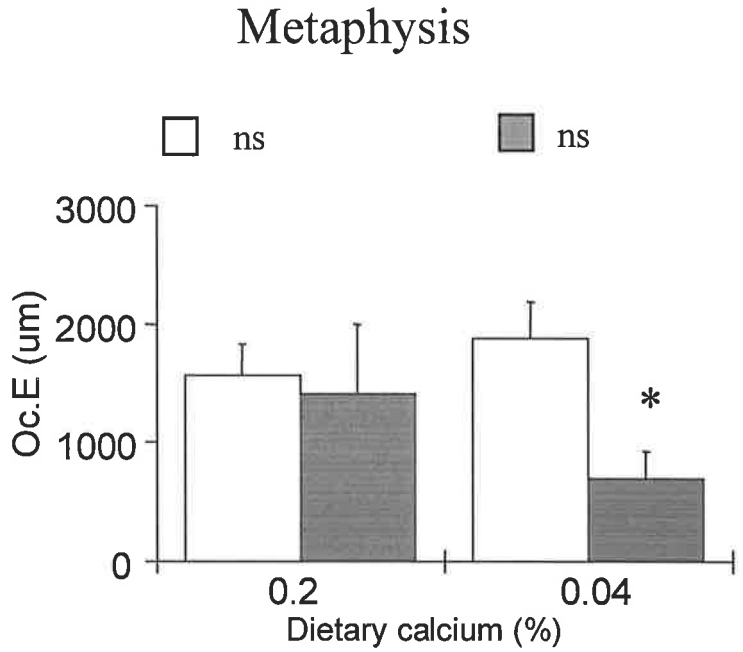


Figure 6.6 Effect of increasing dietary calcium restriction on osteoclast surface (Oc.S, %) in the metaphysis (A) and epiphysis (B) of the distal femur of the rat in sham operated (white bars) and oophorectomised (grey bars) rats. P< one-way ANOVA within operation group, * significant difference between operation group.

Osteoclast Extent

A



B

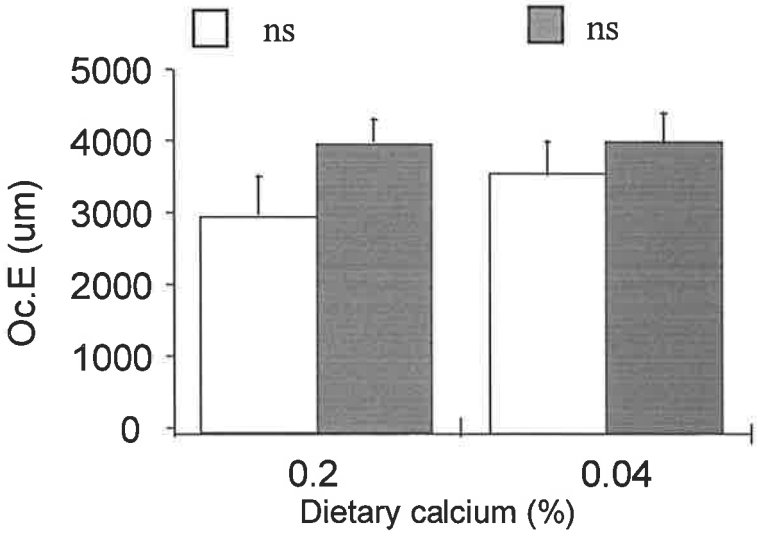


Figure 6.7 Effect of increasing dietary calcium restriction on resorption extent (Oc.E, um) in the metaphysis (A) and epiphysis (B) of the distal femur of the rat in sham operated (white bars) and oophorectomised (grey bars) rats. P< one-way ANOVA within operation group, * significant difference between operation group.

6.3.4 Fluorochrome double labelled surface

In the metaphysis dLS (Fig 6.8A) increased with decreasing dietary calcium in Sham ($P<0.05$), with no change in Oophx. Oophorectomy resulted in increased dLS in the 0.2% group (0.2% $P<0.0005$), with no change in the 0.04% group.

In the epiphysis dLS (Fig 6.8B) increased with decreasing dietary calcium in Sham only ($P<0.0005$). Oophorectomy resulted in increased dLS in both dietary groups (0.2% $P<0.005$, 0.04% $P<0.01$).

6.3.5 Fluorochrome double labelled extent

In the metaphysis dLE (Fig 6.9A) was not affected decreasing dietary. Oophorectomy resulted in a significant decrease in dLE in the 0.02% group only ($P<0.05$).

In the epiphysis dLE (Fig 6.9B) was not affected by decreasing dietary calcium in either operative group. Oophorectomy did not alter dLE, despite a nonsignificant 16% difference in the 0.2% group ($P<0.07$).

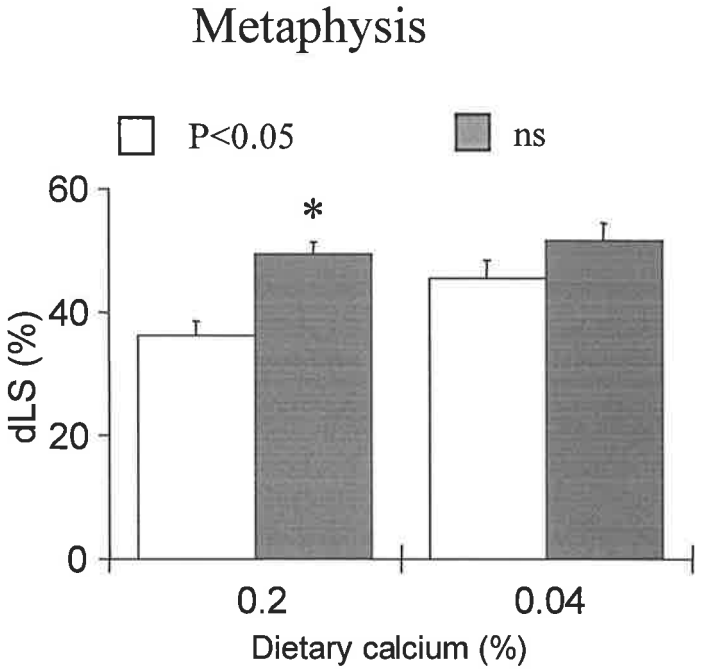
6.3.6 Mineral apposition rate

In the metaphysis MAR (Fig 6.10A) increased with decreasing dietary calcium in Oophx ($P<0.05$), with no change in Sham. Oophorectomy did not alter MAR in any dietary group.

In the epiphysis MAR (Fig 6.10B) was not affected by decreasing dietary calcium or oophorectomy.

Double labelled Surface

A



B

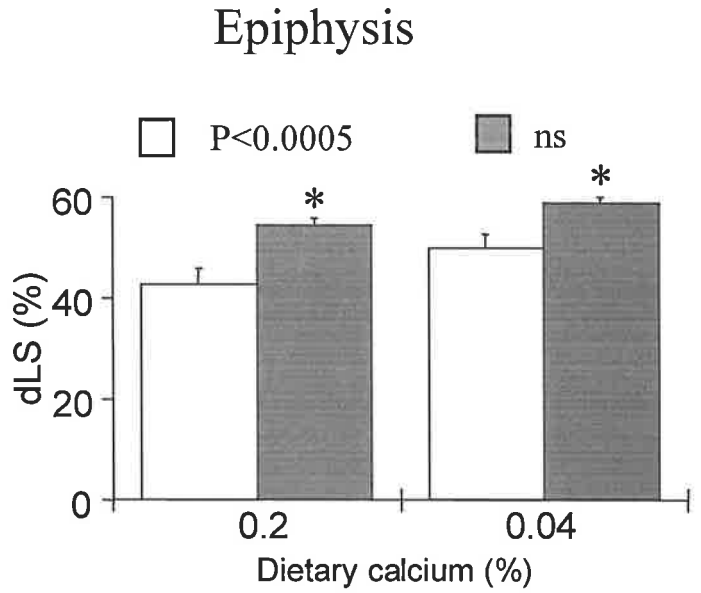


Figure 6.8 Effect of increasing dietary calcium restriction on fluorochrome double labelled surface (dLS, %) in the metaphysis (A) and epiphysis (B) of the distal femur of the rat in sham operated (white bars) and oophorectomised (grey bars) rats. P< one-way ANOVA within operation group, * significant difference between operation group.

Double labelled Extent

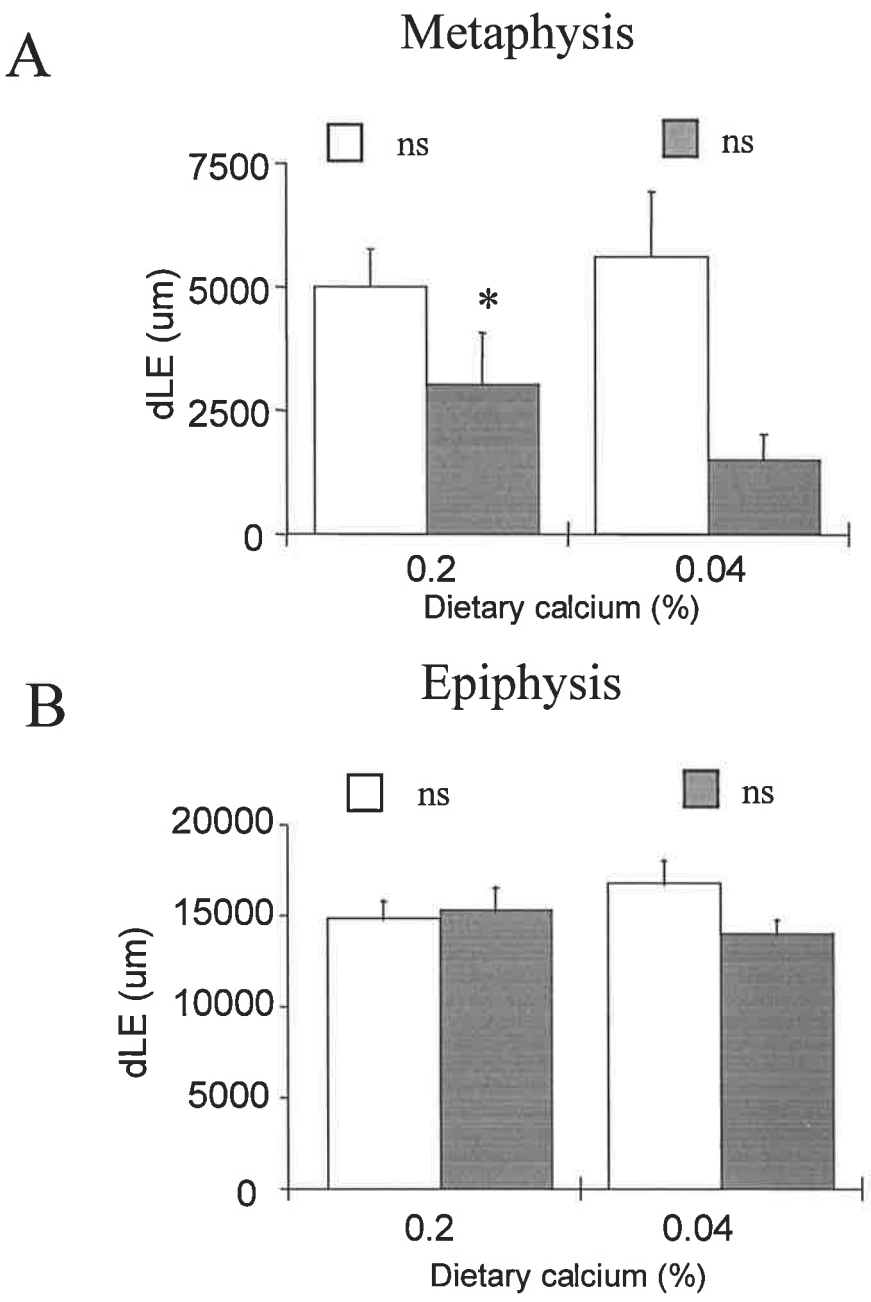


Figure 6.9 Effect of increasing dietary calcium restriction on fluorochrome doubled labelled extent (dLE, um) in the metaphysis (A) and epiphysis (B) of the distal femur of the rat in sham operated (white bars) and oophorectomised (grey bars) rats. P< one-way ANOVA within operation group, * significant difference between operation group.

Minereal Apposition Rate

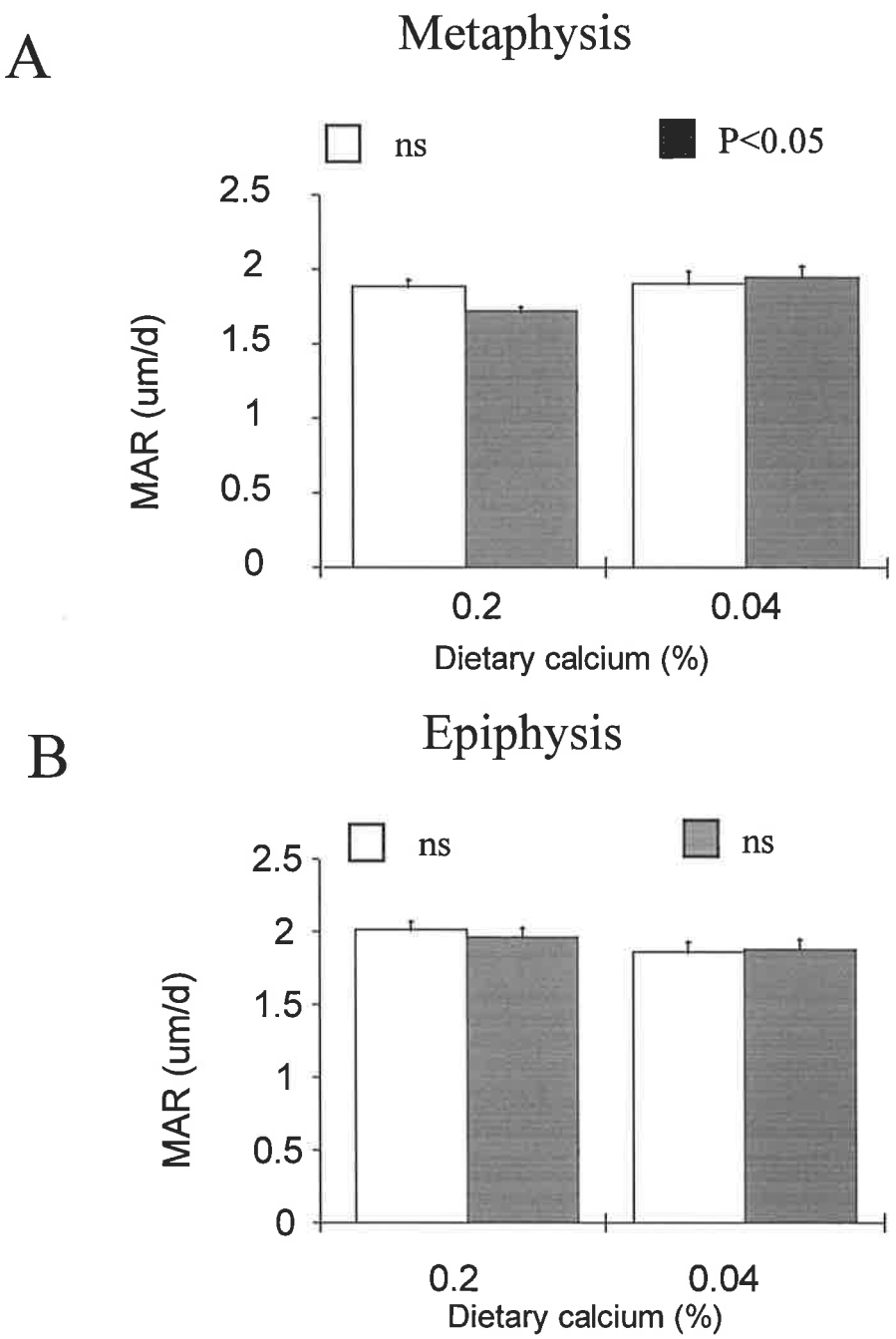


Figure 6.10 Effect of increasing dietary calcium restriction on mineral apposition rate (MAR, um/d) in the metaphysis (A) and epiphysis (B) of the distal femur of the rat in sham operated (white bars) and oophorectomised (grey bars) rats. P< one-way ANOVA within operation group, * significant difference between operation group.

6.3.7 Bone formation rate

In the metaphysis BFR (Fig 6.11A) increased with decreasing dietary calcium in Sham ($P<0.01$) with a nonsignificant 15% change in Oophx ($P<0.08$). Oophorectomy resulted in increased BFR in the 0.2% group ($P<0.005$), with no change in the 0.04% group.

In the epiphysis BFR (Fig 6.11B) was not affected by decreasing dietary calcium. Oophorectomy resulted in increased BFR in both dietary groups (0.2% $P<0.005$, 0.04% $P<0.05$).

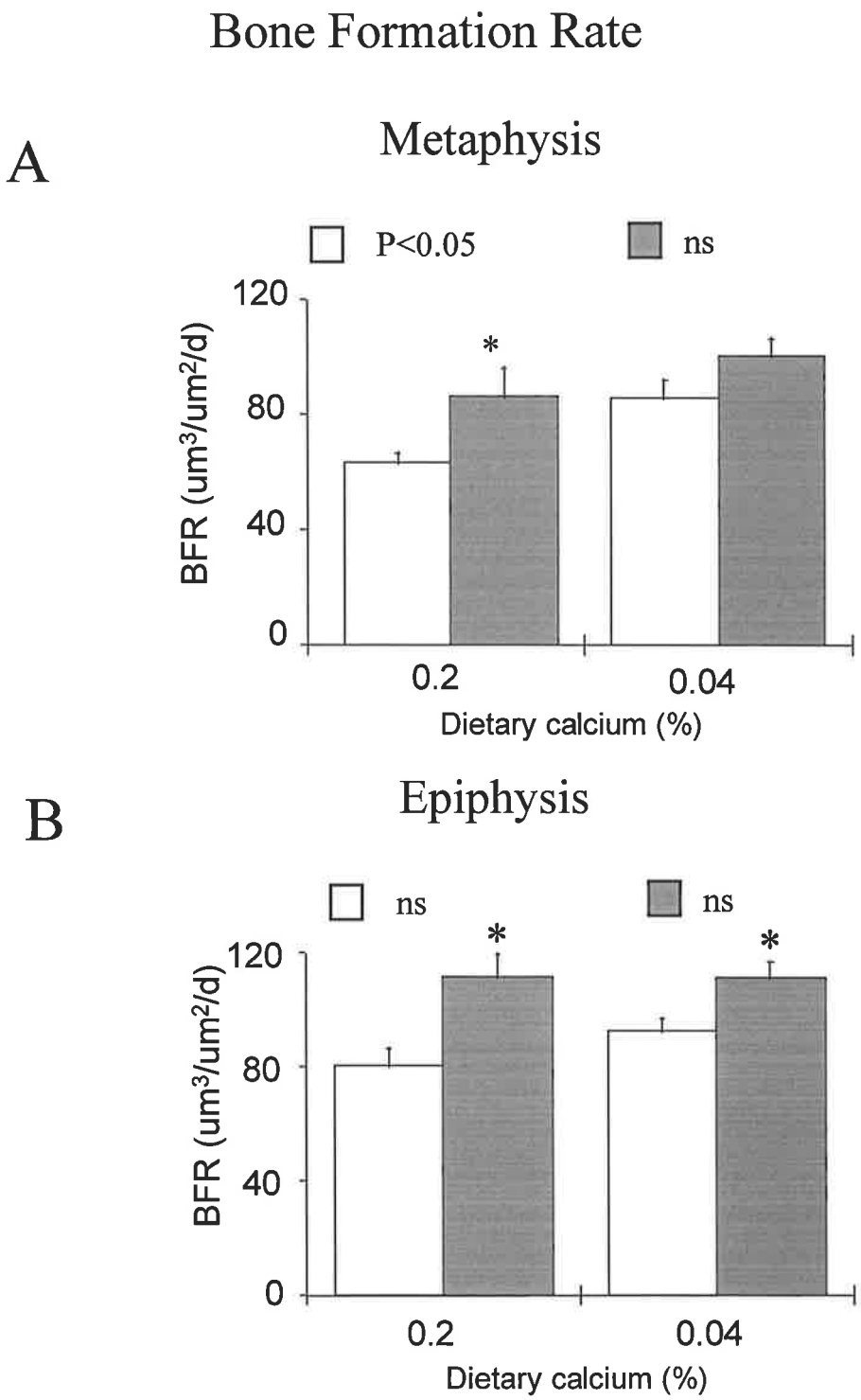


Figure 6.11 Effect of increasing dietary calcium restriction on bone formation rate (BFR, $um^3/um^2/d$) in the metaphysis (A) and epiphysis (B) of the distal femur of the rat in sham operated (white bars) and oophorectomised (grey bars) rats. P< one-way ANOVA within operation group, * significant difference between operation group.

6.3.8 Comparison with age equivalent animals consuming 0.8% Ca diet

In the metaphysis BV/TV of both operative groups was greater in the 0.8% group (Sham P<0.0001, Oophx P<0.0001) compared to both the 0.2 and 0.04% groups. The retention of BV/TV was due to greater Tb.N (Sham P<0.0001, Oophx P<0.05) and Tb.Th (Sham P<0.0001, Oophx P<0.0001).

In the epiphysis BV/TV of both operative groups was greater in the 0.8% group (Sham P<0.0001, Oophx P<0.0001) compared to both the 0.2 and 0.04% groups. The difference was due to greater Tb.N (Sham P<0.0001, Oophx P<0.05) and Tb.Th (Sham P<0.0001, Oophx P<0.0001).

In the metaphysis compared to the 0.8% group, Oc.S in the 0.04% group was increased in both operative groups (Sham P<0.05, Oophx P<0.0005), with an increase in the 0.2% group only in Oophx (P<0.005).

In the epiphysis Oc.S increased with decreasing dietary calcium in the Oophx group (P<0.001), with no change in Sham. In Oophx the 0.2% and 0.04% groups were increased compared to the 0.8% group (0.2% P<0.01, 0.04% P<0.001).

In the metaphysis, compared to the 0.8% group, BFR was increased in the 0.2% (Oophx P<0.0001, Sham P<0.0001) and 0.04% groups (Oophx P<0.0001, Sham P<0.0001).

In the epiphysis BFR increased with decreasing dietary calcium in the both operative groups (Oophx P<0.0001, Sham P<0.0001). Compared to the 0.8% group, BFR was increased in the 0.2% (Oophx P<0.0001, Sham P<0.0001) and 0.04% groups (Oophx P<0.0001, Sham P<0.0001).

6.3.9 Scanning electron microscopy

In the ovary-intact, non calcium restricted sample (Fig 6.5A), the metaphyseal trabecular network is considerably shortened compared with 0.8% Ca fed animals (Fig 4.4), with no difference in the epiphysis. The metaphysis is further shortened in the 0.04% Ca fed sample (Fig 6.5C), consistent with reduction in BV/TV in this region, again no difference in the epiphysis is evident.

Oophorectomy markedly reduced the size of the metaphyseal trabecular network (Fig 6.5B), compared to both ovary-intact samples. The contraction of the metaphysis is greatest in the 0.04% Ca fed, oophorectomised sample, consistent with the additive effects of calcium and ovarian hormone deficiency (Fig 6.5D). The epiphyseal network also appears more open, consistent with bone loss in this region.

6.4 Discussion

6.4.1 Regional comparison of the development of osteopenia following dietary calcium deficiency

Osteopenia resulting from dietary calcium deficiency has been well characterised in the metaphyseal region of long bones in the rat, with loss of trabecular bone a consistent finding in the distal femur (reviewed in Kalu *et al.,* 1989) and proximal tibiae (Shen *et al.,* 1995). In keeping with previous studies, reduced dietary calcium resulted in significant bone loss from the femoral metaphysis. Further, the data presented in this chapter demonstrate that the femoral epiphysis does not experience significant bone loss under these conditions. This finding is consistent with previous chapters that demonstrate the strain mediated differences in osteopenia following oophorectomy, and suggests an interaction between strain and calcium-induced bone loss. An inverse relationship between local mechanical strain and metabolic susceptibility would result in the maintenance of mechanically important regions of the skeleton, while still enabling appropriate bone dissolution to occur in response to calcium homeostatic requirements.

6.4.2 Effect of oophorectomy on regional development of osteopenia following dietary calcium deficiency

It has been demonstrated that calcium deficiency and oestrogen deficiency act in an additive manner on bone (Kalu *et al.,* 1989, Shen *et al.,* 1995). This is evident in the metaphysis of the combined oestrogen and calcium deficient groups where osteopenia was severe, with BV/TV reduced to below 2% in both groups. In the epiphysis, oophorectomy alone produced a significant reduction in BV/TV of 33% and 35% in the 0.2 and 0.04% Ca groups respectively. This is the first evidence in this thesis of

osteopenia in this region, and provides direct evidence of the additive effect of ovarian hormone deficiency and dietary calcium deficiency. This result is in contrast to the lack of osteopenia following long term oophorectomy on 0.8% Ca chow diet (4.3.1.2). This finding suggests that high calcium intake can reduce the osteopenia resulting from oestrogen deficiency, providing evidence for the clinical benefit of calcium supplementation in perimenopausal (Elders *et al.,* 1991) and postmenopausal women (Chapuy *et al.,* 1992, Recker *et al.,* 1996). However, the chow diet could not protect the metaphysis from oophorectomy induced osteopenia (4.3.1.1), suggesting the benefit may be greatly reduced in regions of minimal mechanical strain, where antiresorptive therapy may be more beneficial (5.4.4).

Given that 99% of whole body calcium is in bone, calcium balance equates to bone balance. Thus an oestrogen related decrease in calcium balance would result in bone loss. The elevated response to ovarian hormone deficiency when consuming a low calcium diet, as evident by the osteopenia in the epiphysis, may result from a decrease in intestinal calcium absorption secondary to oestrogen deficiency (Kalu *et al.,* 1989, O'Loughlin and Morris, 1998). Consistent with a decrease in calcium absorption following the menopause (Heaney *et al.,* 1989), oophorectomy has been shown to impair calcium balance by reducing net intestinal calcium absorption, in young (O'Loughlin and Morris, 1994) and mature rats (Kalu *et al.,* 1989, O'Loughlin and Morris, 1998). This impairment has been shown to be independent of the level of dietary calcium (O'Loughlin and Morris, 1998). Thus it is likely that oophorectomised groups are under added calcium restriction due to an inability to absorb the same amount of calcium as ovary-intact groups, enhancing ovarian hormone deficient bone loss.

This added calcium restriction has required the mobilisation of calcium from the epiphysis of oophorectomised animals, while this mobilisation is not required in ovary-intact groups at this level of restriction, due to sufficient intestinal calcium absorption. The likelihood of an interaction between calcium and mechanical usage, and its modulation by oestrogen is evidenced by the changes in bone accretion during various stages of life. The homeostatic control of bone formation maintains local strain within physiological limits (Rubin and Lanyon 1982, Rubin 1984, Lanyon, 1992). However, this limit is likely to be modulated by oestrogen levels, such that more bone is added at puberty (Gilsanz *et al.,* 1988) and during pregnancy (Heaney and Skillman, 1971) when oestrogen levels rise, and bone is lost at menopause and lactation (Brommage and DeLuca, 1985) when levels fall.

6.4.3 Effect of reducing dietary calcium intake on trabecular structure

In the metaphysis oophorectomy produced the characteristic pattern of osteopenia with perforation of trabeculae in all dietary groups, with an additional change in trabecular thickness in the 0.2% Ca group. In contrast, dietary calcium restriction alone tended to effect Tb.Th. In ovary-intact groups, when compared to age equivalent groups consuming 0.8% Ca, the decrease in Tb.Th was substantial, reducing by around 50% in the calcium restricted groups, with a further 50% reduction in Tb.N. The decrease in Tb.Th may be involved in the greater perforation in these groups, with thinner trabeculae more likely to be perforated (Parfitt 1992, Thomsen *et al.,* 1996). Thus in the ovary-intact metaphysis, perforation may be a secondary consequence of trabecular thinning.

In Chapter 4 it was calculated that an increase in resorption depth followed oophorectomy, contributing to trabecular perforation and rapid bone loss. In contrast,

the perforation following calcium restriction in ovary-intact groups appears to result from trabecular thinning, consequent to increased Oc.S, without an increase in resorption depth. This scenario demonstrates that continued calcium restriction can produce a similar pattern of perforation to that seen soon after ovarian hormone deficiency. It is interesting to note that the increase resorption depth of ovarian hormone deficiency would suggest that it would be best treated with antiresorptive therapy, whilst the trabecular thinning of calcium restriction would suggest a better response to anabolic treatment. Thus combined therapy may be of value in postmenopausal women to offset the synergistic effects of menopause and poor calcium balance.

In the epiphysis oophorectomy resulted in bone loss due to a decrease in Tb.N, consistent with the pattern observed in the metaphysis throughout this thesis. In contrast, calcium restriction did not alter bone structure, with no change in Tb.Th or Tb.N. Thus with a calcium restriction from 0.2% to 0.04% Ca the epiphysis is not involved in the supply of extracellular calcium. However, comparison to age equivalent animals consuming 0.8% Ca diet reveals a reduction in Tb.Th of around 50%, independent of operation. This suggests that a calcium restriction from 0.8% to 0.2% Ca does involve the epiphysis in the supply of extracellular calcium. It has been suggested that the increased response to oophorectomy on low dietary calcium may involve reduced intestinal calcium absorption (6.4.2). Thus, another factor involved in the loss of trabeculae from the epiphysis may be this calcium related reduction in Tb.Th. Throughout this thesis the greater thickness of epiphyseal trabeculae has been shown to confer an architectural resistance to ovarian hormone deficient bone loss. In the present study, as in the metaphysis of ovary-intact animals, the calcium restriction

induced reduction in Tb.Th may have reduced this architectural protection, leaving the epiphyseal trabeculae more prone to perforation.

In an anomalous result, trabecular number rose in both operative groups with calcium restriction, when compared to age equivalent animals consuming a 0.8% Ca diet. The reduction in Tb.N, a derived structural estimator (2.14), is inconsistent with the primary structural changes measured in this region, Peri and sample area and bone area (2.11.2). Given that the area of the sample region was uniform (data not shown) alterations in BV/TV [BV/TV = Bone Area/Total Area] and Peri are the only directly measured structural changes in these bones.

Analysis of these two indices reveals significant reductions in Peri with minimal reductions in BV/TV in the epiphysis, most evident in Sham. For example, from the 0.8% Ca to the 0.2% Ca group, BV/TV is reduced by 9% while Peri is reduced by 44%. This exaggerated reduction in Peri has the result of skewing the calculations for Tb.N [(500xPeri)/Total Area] toward lower values, whilst increasing the values of Tb.Th [(2xBone Area)/Peri]. However the observed change in Tb.N is the opposite. Thus the measured changes in Peri are in direct contrast to the changes in the derived estimates of Tb.N and Tb.Th. While a reduction in Tb.Th and an increase in Tb.N in a region such as the epiphysis, with very thick trabeculae may be viewed as a process involving the conversion of thick trabeculae into multiple thinner units, this would result in an increase in Peri, and thus cannot be supported. The production of *de novo* trabeculae at this age is very unlikely without a potent anabolic such as PTH (Meng *et al.,* 1996). The unlikely occurrence of a component of the semi-synthetic diet possessing this effect would require further study beyond the scope of this thesis.

6.4.4 Effect of reducing dietary calcium on osteoclast activity: Surface percentage data

In the metaphysis, calcium restriction increased resorption in ovary-intact groups. This increase in Oc.S suggests a continued metabolic demand for calcium mobilisation from this region, consistent with long term calcium balance studies (O'Loughlin and Morris, 1998). The additional increase due to oophorectomy was similar across the dietary groups, demonstrating that the long term oestrogen deficient increase in resorption evident in Chapter 5 is independent of diet. However, in Oophx calcium restriction did not elevate Oc.S. This lack of a response may reflect the already elevated turnover produced by oophorectomy and the 0.2% Ca diet, and may represent a near maximal osteoclastic response, with approximately 25% of remaining surface covered in osteoclasts, and over 75% of surface actively remodelling. However the magnitude of bone loss in this region makes interpretation of surface referent data difficult.

In the epiphysis, decreasing dietary calcium in the ovary-intact groups did not stimulate Oc.S significantly. This is consistent with in the lack of osteopenia in this group. This diminished sensitivity to calcium restriction is consistent with the structural role of this region and the greater metabolic role of the metaphysis. If mechanical strain is the local factor governing the sensitivity to metabolic demand, there may be a similar pathway involved in the resistance to both calcium and oestrogen deficient bone loss, acting in the epiphysis but not in the metaphysis. Such a pathway would be of considerable clinical interest. Oophorectomy, in contrast, increased resorption in both groups, consistent with the osteopenia in this operative group. As demonstrated in Chapter 4, Oc.S was reduced by day 120 following oophorectomy in animals receiving 0.8% Ca (4.3.2). The added demand of calcium

restriction appears to produce an elongation of the period of increased resorption following oophorectomy, with values still high at 105 days, although this cannot be demonstrated conclusively from these data.

6.4.5 Effect of reducing dietary calcium on osteoblast activity: Surface percentage data

In the metaphysis BFR changed in ovary-intact groups in a similar manner to Oc.S, increasing with calcium restriction. This resulted from an increase in dLS, with no change in MAR, suggesting alterations to osteoblast proliferation not anabolic activity (Parfitt *et al.,* 1995). Thus the cellular response to calcium restriction is similar to that of ovarian hormone restriction in that osteopenia is coincident with increased turnover. However, the microarchitectural changes that occur as a result are different. Calcium restriction produces a reduction in Tb.Th rather than the perforation that typically follows ovarian hormone deficiency. This is consistent with a relative lessening in the vigour of resorption following the calcium restriction, with resorption depth not increased to the level that produces the perforation evident following oophorectomy.

In Oophx, BFR was not increased by calcium restriction. This was consistent with a lack of increase in dLS, despite greater MAR. While this suggests a lessening of the osteoblastic proliferative response in the Oophx group, such surface referent estimates of bone cell activity are strongly effected by the magnitude of the osteopenia in this region. Bone volume in Oophx was less than 5% and 3% in both 0.2% and 0.04% Ca groups, respectively, with approximately 75% of the remaining bone surface involved in bone turnover in the oophorectomised, calcium restricted groups. Indeed, taking reversal period and mineralisation lag time into account this percentage is probably conservative. Such an intensity of bone cell activity in these

groups may physically limit the extent of the osteoblastic response to the physiological stresses, while not reflecting a problem with osteoblastic function. This physical limitation to bone cell activity may also account for the lack of increase in Oc.S due to calcium restriction following oophorectomy in this region. Further this may also account for the lack of increased turnover following oophorectomy in the 0.04% Ca group. Bone cell activity in the ovary-intact group at this level of calcium restriction involved 67% of the bone surface, while in Oophx 85% was active. Taking into account the error involved in making estimates over such small perimeters, it is likely that both measurement error and physical limitation of bone cell proliferation diminished the demonstration of any post-oophorectomy increases in bone turnover.

In the epiphysis, BFR in ovary-intact groups did not increase with calcium restriction, in contrast to the metaphysis. This lack of increased BFR is consistent with the lack of response in Oc.S. Thus the bone cell activity of the epiphysis is not affected by this alteration in dietary calcium at this time post restriction. This could be interpreted as a complete resistance to calcium restriction, consistent with the maintenance of the mechanically important trabeculae of this region. However this is not consistent with the reduction in Tb.Th between the groups in the present study and those consuming the 0.8% Ca diet, this is discussed below (6.4.6).

In Oophx, BFR increased with calcium restriction, as did Oc.S, consistent with a long term increase in turnover with decreasing calcium in this operative group. Thus the epiphysis of calcium restricted, ovarian hormone deficient animals is subject to osteopenia, and long term increases in turnover. The contrast with ovary-intact animals, with no elevation in turnover at this time, suggests an ovarian hormone related mechanism, and thus the oestrogen related decrease in calcium balance may be involved in the increase in bone cell activity in this region.

6.4.6 Effect of reducing dietary calcium on bone cell activity: Comparison to chow fed animals

In order to assess whether calcium restriction did affect the epiphysis, the calcium restricted groups of this region were compared to age equivalent animals fed 0.8% Ca diet. In ovary-intact groups from the present study, Oc.S was not elevated even when compared to chow fed animals of the same age. The lack of an increase in Oc.S at this time is consistent with the lack of osteopenia within the ovary-intact groups in this region.

In contrast, BFR was increased in both restricted groups compared to 0.8% Ca fed animals, despite the constant values for Oc.S. This imbalance in turnover suggests a positive bone balance in the Sham epiphysis, and may be the result of previously high levels of resorption in this region. The latter would explain the decrease in Tb.Th of around 50% when restricted groups are compared to 0.8% Ca fed groups, in the epiphysis. The lack of an increase in Oc.S in Sham in this region is thus most likely due to the time of sampling after the commencement of calcium restriction, having risen previously, thinning trabeculae and then returned to baseline levels. A similar return to baseline levels has been demonstrated previously (4.3.2). Given this assumption, following calcium restriction resorption rises in both regions, causing generalised trabecular thinning. In the epiphysis this rise in resorption is corrected in ovary-intact groups by day 105, enabling subsequent formation to begin restoration of Tb.Th. In oophorectomised groups calcium malabsorption contributes to perforation of already thinned trabeculae. In the metaphysis the continued metabolic demand and the perforation of trabeculae result in permanent and virtually complete bone loss.

6.4.7 Effect of reducing dietary calcium on bone cell activity: Linear extent data

The magnitude of bone loss in the metaphysis magnifies the surface referent indices of bone turnover and provides potentially false estimates of proliferation (Parfitt, 1983). This is evident in the contrasting patterns of Oc.S and Oc.E in the metaphysis caused by the significant reduction in bone perimeter. While Oc.S increased significantly as a result of both calcium restriction and oophorectomy, suggesting increased osteoclast proliferation, Oc.E was stable or decreased, strongly indicative of reduced proliferation. The pattern is the same for dLS and dLE. The increased turnover evident in the metaphysis is therefore occurring with a reduced proliferation of bone cells. This supports the view that the lessened response following oophorectomy in the 0.04% Ca group restriction was the result of physical limitations of bone surface availability not a regional difference in proliferation potential. Consistent with the strong adaptation to calcium restriction in the first 47 following dietary change (O'Loughlin and Morris, 1998), the greater turnover on this remnant of metaphyseal trabecular bone at this time may indeed be the result of normal metabolic demand, having restored calcium balance by increased calcium absorption in the gut and reduced calcium excretion in the faeces and urine.

In the epiphysis, trabecular perimeter also decreased, but only following oophorectomy, although to a lessened extent than in the metaphysis. The relative retention of trabecular perimeter in the epiphysis contributed to a different pattern of proliferation than in the metaphysis. Both Oc.E and dLE were unaffected by ovarian hormone or calcium deficiency, consistent with uniform rates of proliferation. The reduction in perimeter in Oophx however, contributed to an increase in turnover as evident in the percentage of bone surface involved in turnover, up to 77%. Thus increased bone turnover at this time may be a secondary result of previous bone loss.

Chapter 7

The effect of prepubertal oophorectomy on trabecular structure during growth and aging

7.1 Introduction

The mature skeleton is highly responsive to changes in circulating oestrogen levels, with bone loss following the menopause or oophorectomy and maintenance of bone mineral content with hormone replacement therapy. A similar relationship also exists in the rat (Kalu, 1991). The femoral trabecular osteopenia following oophorectomy in the rat is extensive, particularly in the distal metaphyseal-diaphyseal region, which is prevented by oestrogen replacement (Sims *et al.,* 1996b). This sensitivity to oestrogen deficiency in the distal femur decreases distally, with the epiphysis resistant to oophorectomy induced osteopenia when rats consume a high dietary calcium intake. The strong relationship between bone and oestrogen in the mature skeleton suggests that skeletal development maybe influenced by this hormone. The presence of oestrogen during growth may result in the production of a low-strain, oestrogen sensitive quanta of bone that is highly metabolically active during periods when oestrogen levels vary and calcium is required to be mobilised such as during reproduction and lactation (Zeni *et al.,* 1999). Such bone would be lost following oophorectomy and maintained by oestradiol replacement.

The bone of the epiphysis however, develops under the influence of increasing mechanical demands due to continued weight gain during this period (Sontag, 1992). The presence of high mechanical strain during growth may result in the strain sensitising of the bone, and produce a high-strain, oestrogen insensitive quanta of bone that will not be lost following oophorectomy. Were this hypothesis valid, the diaphyseal trabecular bone of the distal femur would fail to develop without the presence of oestrogen, while the epiphyseal bone would be unaffected.

Ovarian hormone deficiency increases osteoclast activity and results in perforation and complete removal of trabeculae. The susceptibility of a region to

perforation relates not only to the activity of osteoclasts but also the thickness of individual trabeculae (Parfitt, 1992). The resistance of the epiphysis to ovarian hormone deficiency therefore, may result indirectly from the structural differences arising during development and may not be the result of strain sensitisation of trabeculae. The greater thickness of epiphyseal trabeculae impeded perforation during the remodelling transient and protected this region from permanent bone loss during this early period following oophorectomy. In contrast, the thinner trabeculae of the diaphysis and metaphysis were rapidly perforated and permanently removed (3.4.8). This structural variation may relate to mechanical stimuli during development, with thicker trabeculae forming in regions of higher stain. If this hypothesis were valid the absence of oestrogen during development would not affect Tb.Th, particularly in the epiphysis, with Tb.Th more closely associated with body weight than ovarian hormone status.

The hypotheses investigated in this chapter, that diaphyseal bone is oestrogen dependent and the epiphyseal bone is oestrogen independent, as well as the body weight dependence of Tb.Th, were tested in rats deprived of ovarian hormones by prepubertal oophorectomy. Static histomorphometric indices were analysed to assess the structural alterations occurring during growth and aging in three regions of the distal femur. These were compared to normal developmental patterns of sham operated animals from 3 to 78 weeks of age.

7.2 Materials and Methods

7.2.1 Animals

72 juvenile female Sprague-Dawley rats (3 weeks of age) were maintained on commercial rat chow (2.2) and tap water *ad libitum*. All procedures involving animals were approved by the IMVS Animal Ethics Committee.

7.2.2 Surgery

At 3 weeks of age 6 animals were killed as a baseline group and the remainder allocated to either oophorectomy or sham operation, via the ventral approach (2.4.2). At 3, 6, 12, 20, 28, 52 and 78 weeks of age, 6 Oophx and 6 Sham rats were weighed, killed and the femora excised.

Serum oestradiol was measured at collection in 33/36 Oophx and in all 36 Sham rats (2.13).

7.2.3 Histomorphometry

Femoral length was determined and the distal end of the right femur was cut transversely at 1/3 of the total length (2.9). Sections were partitioned into three regions, the epiphysis, the metaphysis and the diaphysis (2.13). BV/TV, Tb.N and Tb.Th were calculated (2.14) from von Kossa stained sections (2.11.4.1). Growth plate thickness was estimated from VK/HE stained sections (2.12.5).

7.2.4 Scanning electron microscopy

Scanning electron micrographs (2.8) were produced from random samples of both Sham and Oophx distal femora at 3, 20 and 78 weeks, using the left femur.

7.2.5 Calculations and statistics

All results within a region were analysed using two-way ANOVA for operation and age, significant results were further analysed using one-way ANOVA within operative group. All ANOVA were followed by Bonferroni/Dunn post hoc analysis. Spearman correlation values of Tb.Th with body were also performed. All analysis was conducted using Super ANOVA (Abacus Concepts, CA, USA).

7.3 Results

7.3.1 Serum oestradiol levels

Serum oestradiol (Fig 1) was significantly higher in Sham than in Oophx rats, all of which had values under 30 pmol/L. In ovary intact animals, oestradiol values were highest at 52 weeks, and remained higher than Oophx at 78 weeks.

7.3.2 Epiphyseal growth plate thickness

In the ovary intact groups growth plate thickness (Fig 7.2A) fell rapidly to 20 weeks, reducing 64% during this time. Growth plate thinning continued with age at a reduced rate, with the growth plate 75% thinner at 78 weeks compared to prepubertal levels. Oophorectomy significantly delayed the thinning of the growth plate ($P<0.005$), with values greater from 6 to 28 weeks, inclusive. However, the growth plate had returned to Sham thickness by 52 weeks.

7.3.3 Femoral length

In the ovary intact groups femoral length (Fig 7.2B) increased rapidly to 20 weeks, with 88% of total length attained by this time, growth continued at a reduced rate with length increasing by more than 2 fold during the study.

Oophorectomy did not affect femoral length during growth. However, femora of Oophx were significantly longer after growth ($P<0.01$), with the femur 5% longer in the Oophx group at 78 weeks ($P<0.05$).

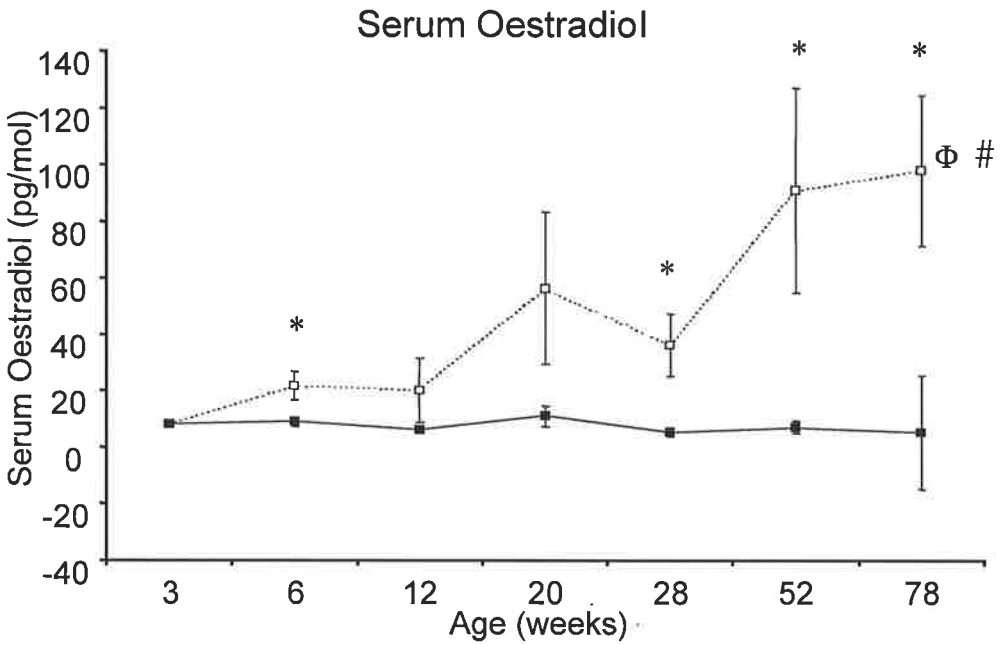


Figure 7.1 Temporal changes in serum oestradiol (pmol/L) following prepubertal oophorectomy (Oophx, ■ , solid line) or sham-operation (Sham, □, broken line) in the rat, expressed as mean ± SEM. Φ Significant change with time (P<0.0001). # Significantly different to Sham with time (P<0.005). * Significantly different to Sham (P<0.05).

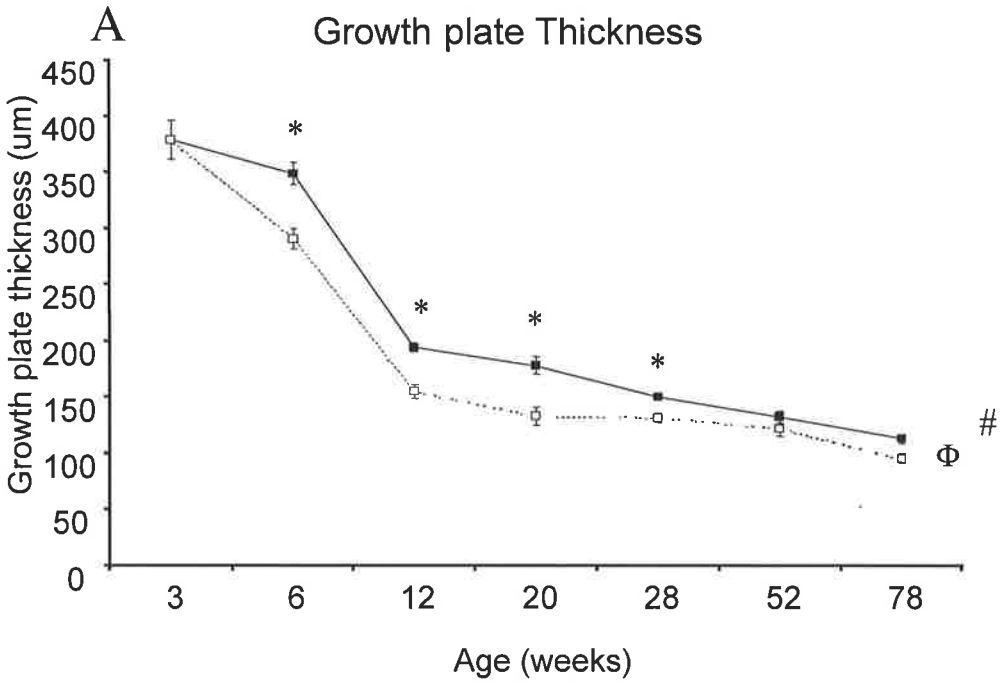


Figure 7.2A Temporal changes in epiphyseal growth plate thickness (um) following prepubertal oophorectomy (Oophx, ■ , solid line) or sham-operation (Sham, □ , broken line) in the distal femur in the rat, expressed as mean ± SEM. Φ Significant change with time (P<0.0001). # Significantly different to Sham with time (P<0.005). * Significantly different to Sham (P<0.05).

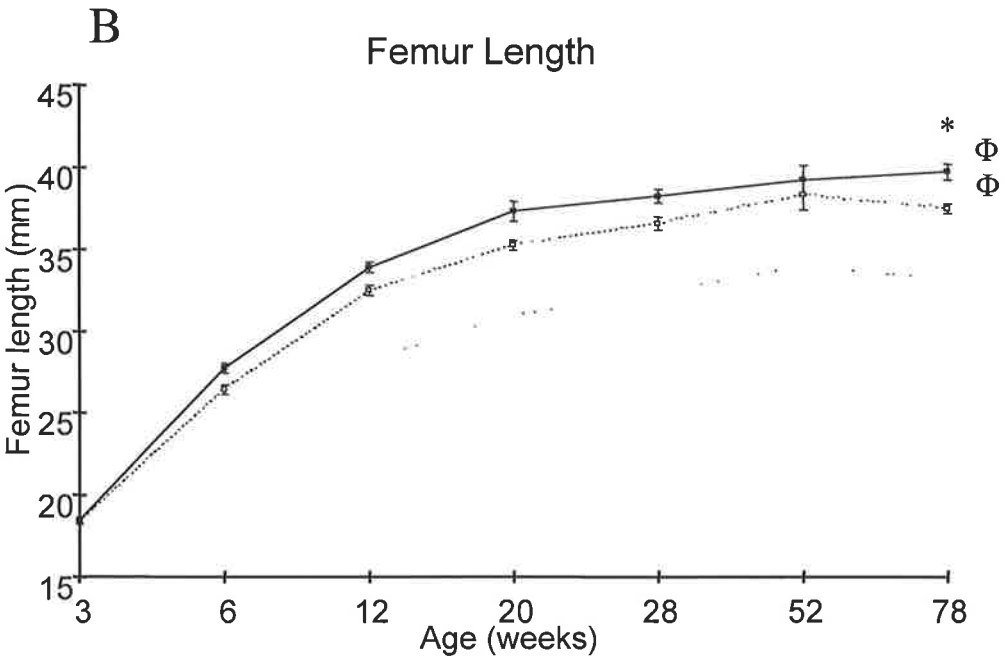


Figure 7.2B Temporal changes in femoral length (mm) following prepubertal oophorectomy (Oophx, ■ , solid line) or sham-operation (Sham, □ , broken line) in the rat, expressed as mean ± SEM. Φ Significant

7.3.4 Body weight

In the ovary intact groups body weight (Fig 7.3) increased over 2.5 fold with age. Oophorectomised animals gained more weight than Sham and were heavier from 6 weeks, with a 53% increase in weight at 78 weeks.

Tb.Th was significantly correlated to body weight, independent of age, in the epiphysis (Sham $r=0.74$, $P<0.001$, Oophx $r=0.71$, $P<0.01$) and the metaphysis of ovary intact groups ($r=0.63$, $P<0.01$). In contrast there was no correlation of Tb.Th

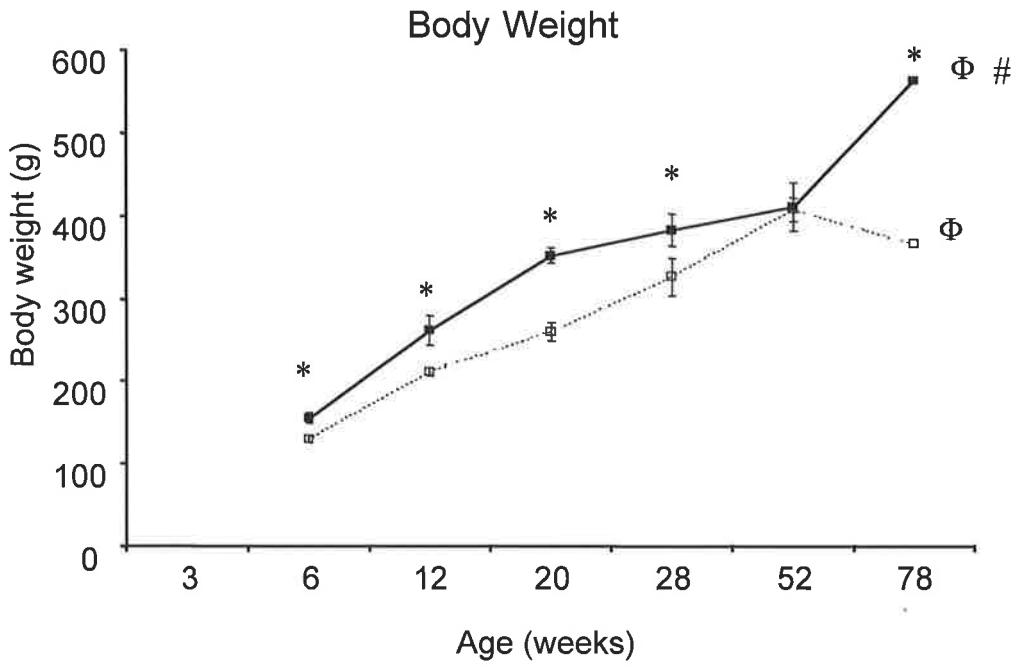


Figure 7.3 Temporal changes in body weight (g) following prepubertal oophorectomy (Oophx, ■ , solid line) or sham-operation (Sham, □ , broken line) in the rat, expressed as mean ± SEM. Φ Significant change with time ($P<0.0001$). # Significantly different to Sham with time ($P<0.01$). * Significantly different to Sham ($P<0.05$).

7.3.5 Trabecular bone structure

7.3.5.1 Diaphysis

In the ovary intact animals BV/TV (Fig 7.4A) increased rapidly with growth to 20 weeks ($P<0.05$), peaking at 13%. The growth phase was characterised by a rapid, one step rise in Tb.N (Fig 7.4B) following puberty, increasing nearly 6 fold between 6 and 12 weeks. Tb.Th (Fig 7.4C) also increased steadily with age ($P<0.0001$) to peak at 59 um at 20 weeks. From 20 weeks BV/TV declined with age ($P<0.0001$).

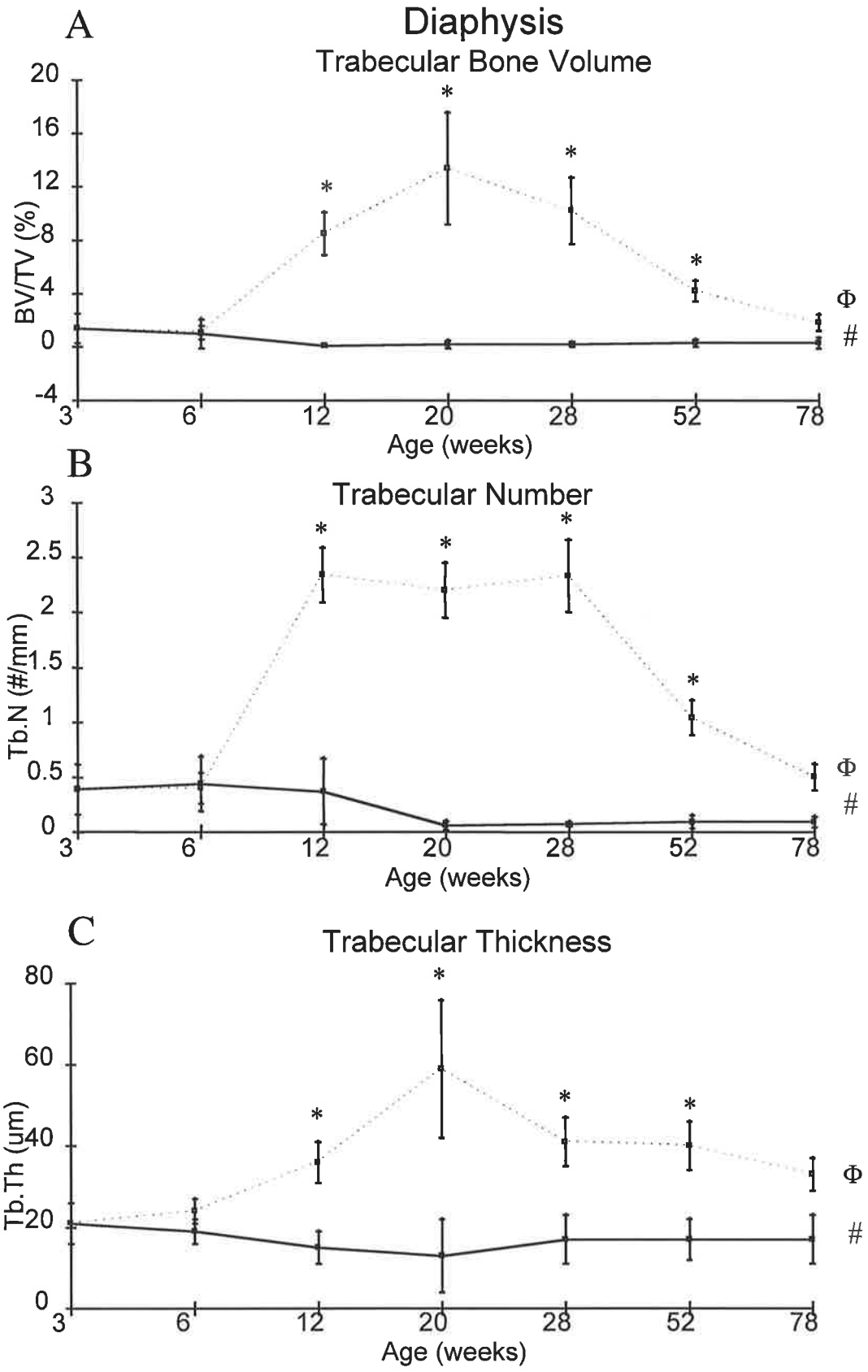
The aging phase was characterised by a steady decline in Tb.Th ($P<0.0001$), again returning to peripubertal levels by 78 weeks. Tb.N remained stable to 28 weeks after which it fell ($P<0.0001$) to prepubertal levels by 78 weeks.

Oophorectomy prevented the supply of trabecular bone to this region. There was no increase in any trabecular parameter, with the region remaining virtually vacant of bone at all time points.

Figure 7.4A Temporal changes in trabecular bone volume (BV/TV, %) following prepubertal oophorectomy (Oophx, ■ , solid line) or sham-operation (Sham, □ , broken line) in the diaphysis of the distal femur in the rat, expressed as mean ± SEM. Φ Significant change with time (P<0.001). # Significantly different to Sham with time (P<0.0001). * Significantly different to Sham (P<0.05).

Figure 7.4B Temporal changes in trabecular number (Tb.N, /mm) following prepubertal oophorectomy (Oophx, ■ , solid line) or sham-operation (Sham, □ , broken line) in the diaphysis of the distal femur in the rat, expressed as mean ± SEM. Φ Significant change with time (P<0.0001). # Significantly different to Sham with time (P<0.0001). * Significantly different to Sham (P<0.05).

Figure 7.4C Temporal changes in trabecular thickness (Tb.Th, um) following prepubertal oophorectomy (Oophx, ■ , solid line) or sham-operation (Sham, □ , broken line) in the diaphysis of the distal femur in the rat, expressed as mean ± SEM. Φ Significant change with time (P<0.05). # Significantly different to Sham with time (P<0.0001). * Significantly different to Sham (P<0.05).


A
Diaphysis
Trabecular Bone Volume
BV/TV (%)
20
16
12
8
4
0
-4
3
6
12
20
28
52
78
Age (weeks)
Φ
#
B
Trabecular Number
Tb.N (#/mm)
3
2.5
2
1.5
1
0.5
0
Age (weeks)
C
Trabecular Thickness
Tb.Th (um)
80
60
40
20
0
Age (weeks)

7.3.5.2 Metaphysis

In the ovary intact animals the growth phase again lasted 20 weeks, with a peak BV/TV (Fig 7.5A) of 32% attained at this time. This resulted from a one step increase in Tb.N (Fig 7.5B) between 3 and 6 weeks, increasing by 69% and a steady increase in Tb.Th (Fig 7.5C) ($P<0.0001$) at all ages.

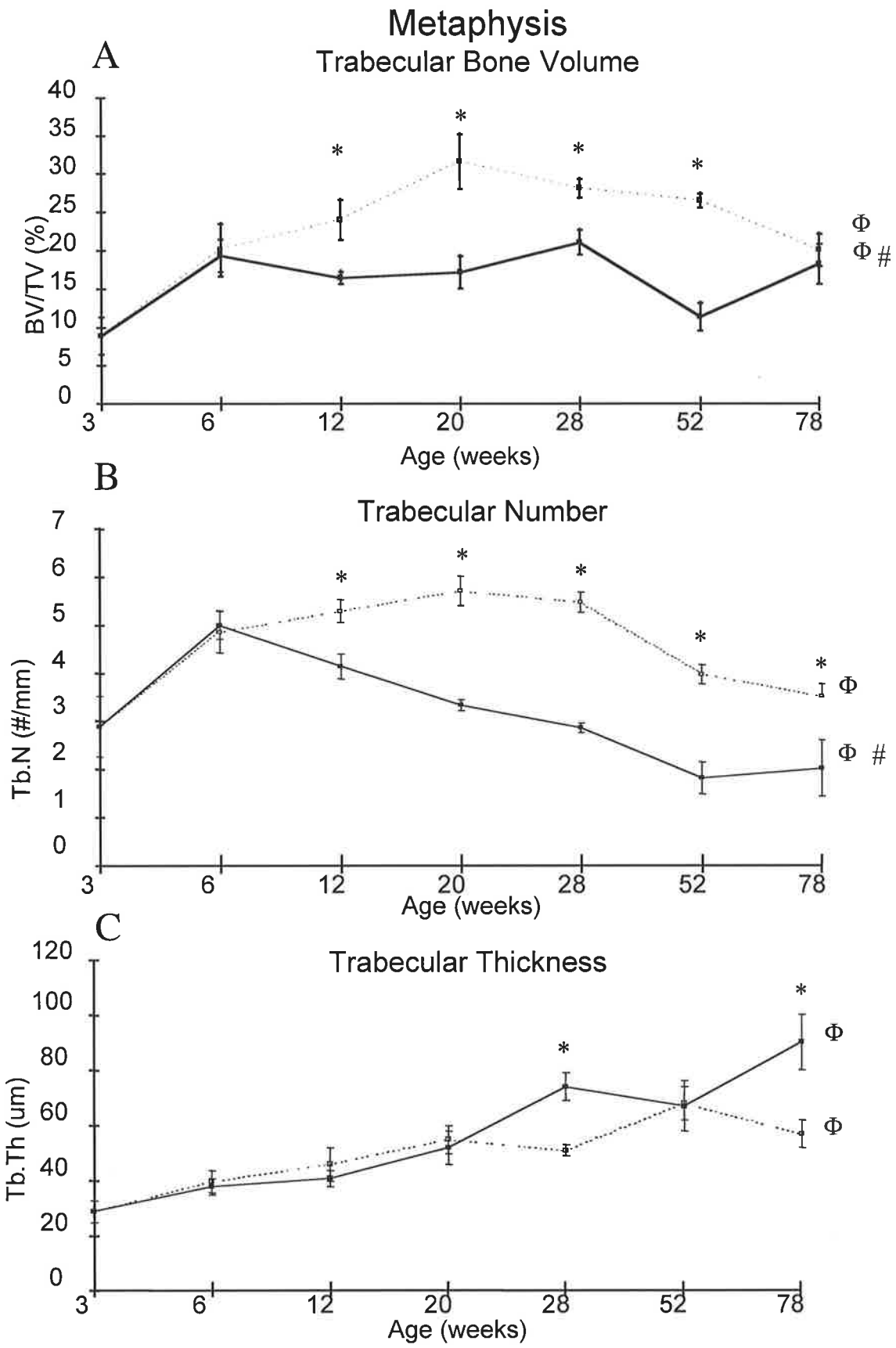
Following the growth phase, BV/TV declined, due to decreasing Tb.N, and was not different to oophorectomised levels by 78 weeks. Tb.Th continued to increase with age, doubling during the study.

Oophorectomised animals developed normally prior to puberty, with BV/TV increasing over 2 fold between 3 and 6 weeks, similar to ovary intact groups. This was achieved primarily by a 73% increase in Tb.N and a smaller increase in Tb.Th. After the normal onset of puberty this level of BV/TV was maintained, but not increased. Tb.N decreased from 6 weeks, returning to prepubertal levels by 78 weeks. Tb.Th however increased continuously to be over 3 fold thicker at 78 weeks, maintaining BV/TV despite decreasing Tb.N.

Figure 7.5A Temporal changes in trabecular bone volume (BV/TV, %) following prepubertal oophorectomy (Oophx, ■ , solid line) or sham-operation (Sham, □ , broken line) in the metaphysis of the distal femur in the rat, expressed as mean ± SEM. Φ Significant change with time (P<0.005). # Significantly different to Sham with time (P<0.0001). * Significantly different to Sham (P<0.05).

Figure 7.5B Temporal changes in trabecular number (Tb.N, /mm) following prepubertal oophorectomy (Oophx, ■ , solid line) or sham-operation (Sham, □ , broken line) in the metaphysis of the distal femur in the rat, expressed as mean ± SEM. Φ Significant change with time (P<0.0001). # Significantly different to Sham with time (P<0.0001). * Significantly different to Sham (P<0.05).

Figure 7.5C Temporal changes in trabecular thickness (Tb.Th, um) following prepubertal oophorectomy (Oophx, ■ , solid line) or sham-operation (Sham, □ , broken line) in the metaphysis of the distal femur in the rat, expressed as mean ± SEM. Φ Significant change with time (P<0.0001). * Significantly different to Sham (P<0.05).


Metaphysis
A
Trabecular Bone Volume
BV/TV (%)
40
35
30
25
20
15
10
5
0
3
6
12
20
28
52
78
Age (weeks)
Φ
Φ #
B
Trabecular Number
Tb.N (#/mm)
7
6
5
4
3
2
1
0
Age (weeks)
Φ
Φ #
C
Trabecular Thickness
Tb.Th (um)
120
100
80
60
40
20
0
Age (weeks)
Φ
Φ

7.3.5.3 Epiphysis

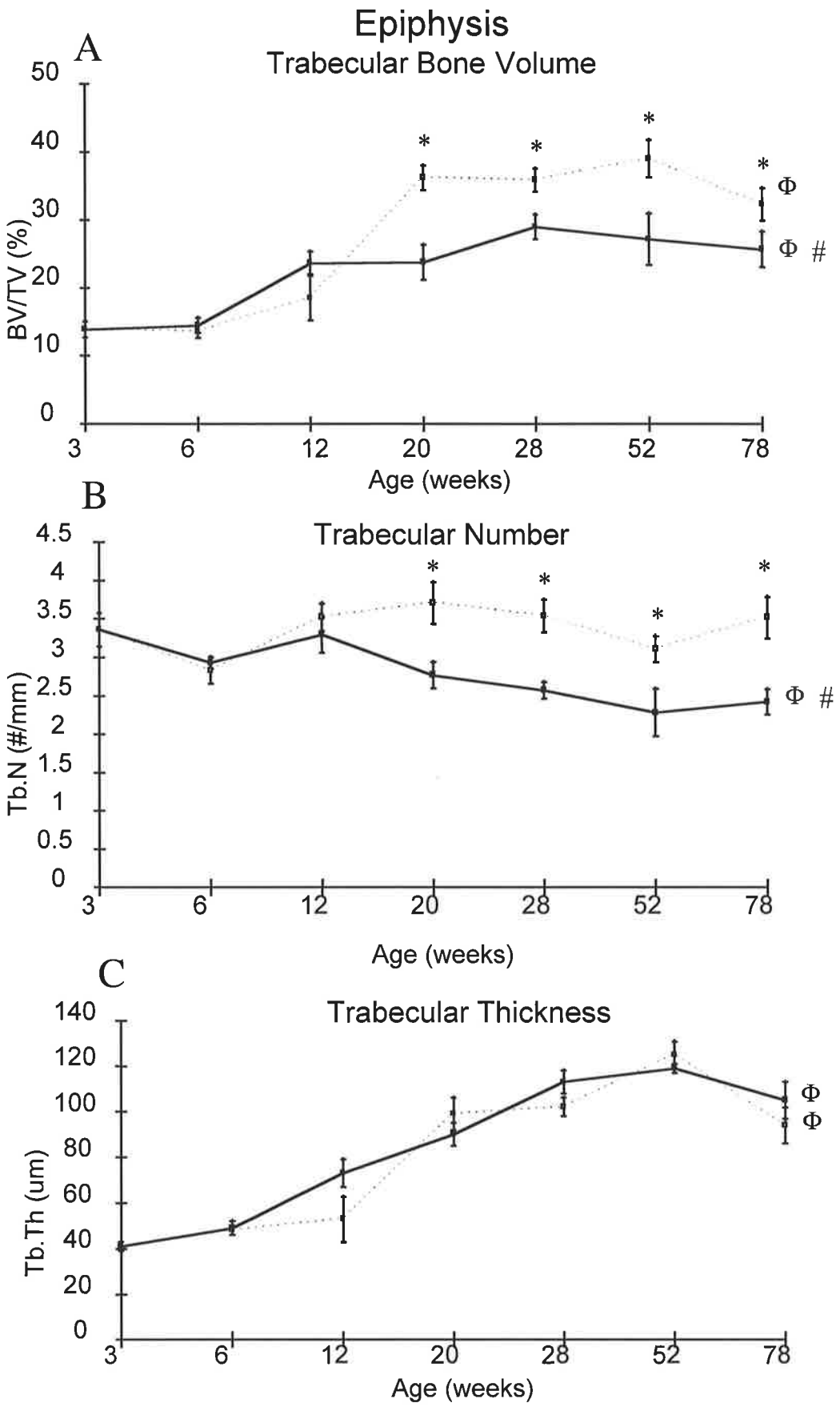
The growth phase again lasted 20 weeks, with a 2.5 fold increase in BV/TV (Fig 7.6A) from 6 weeks. In contrast to the previous regions, the developmental pattern in the epiphysis did not involve increasing Tb.N (Fig 7.6B), which remained constant from 3 weeks. The growth phase came from increasing Tb.Th (Fig 7.6C), which continued throughout the study, to be over 2 fold thicker at 78 weeks.

Oophorectomised groups developed normally up to 12 weeks, with BV/TV, Tb.N and Tb.Th equivalent to ovary-intact groups. From this time onward there was no increase in BV/TV, due to decreasing Tb.N. Tb.Th increased in parallel to ovary intact groups, resulting in maintenance of BV/TV, a similar pattern to the metaphysis of oophorectomised groups.

Figure 7.6A Temporal changes in trabecular bone volume (BV/TV, %) following prepubertal oophorectomy (Oophx, ■ , solid line) or sham-operation (Sham, □ , broken line) in the epiphysis of the distal femur in the rat, expressed as mean ± SEM. Φ Significant change with time (P<0.0005). # Significantly different to Sham with time (P<0.001). * Significantly different to Sham (P<0.05).

Figure 7.6B Temporal changes in trabecular number (Tb.N, /mm) following prepubertal oophorectomy (Oophx, ■ , solid line) or sham-operation (Sham, □ , broken line) in the epiphysis of the distal femur in the rat, expressed as mean ± SEM. Φ Significant change with time (P<0.05). # Significantly different to Sham with time (P<0.01). * Significantly different to Sham (P<0.05).

Figure 7.6C Temporal changes in trabecular thickness (Tb.Th, um) following prepubertal oophorectomy (Oophx, ■ , solid line) or sham-operation (Sham, □ , broken line) in the epiphysis of the distal femur in the rat, expressed as mean ± SEM. Φ Significant change with time (P<0.0001). * Significantly different to Sham (P<0.05).

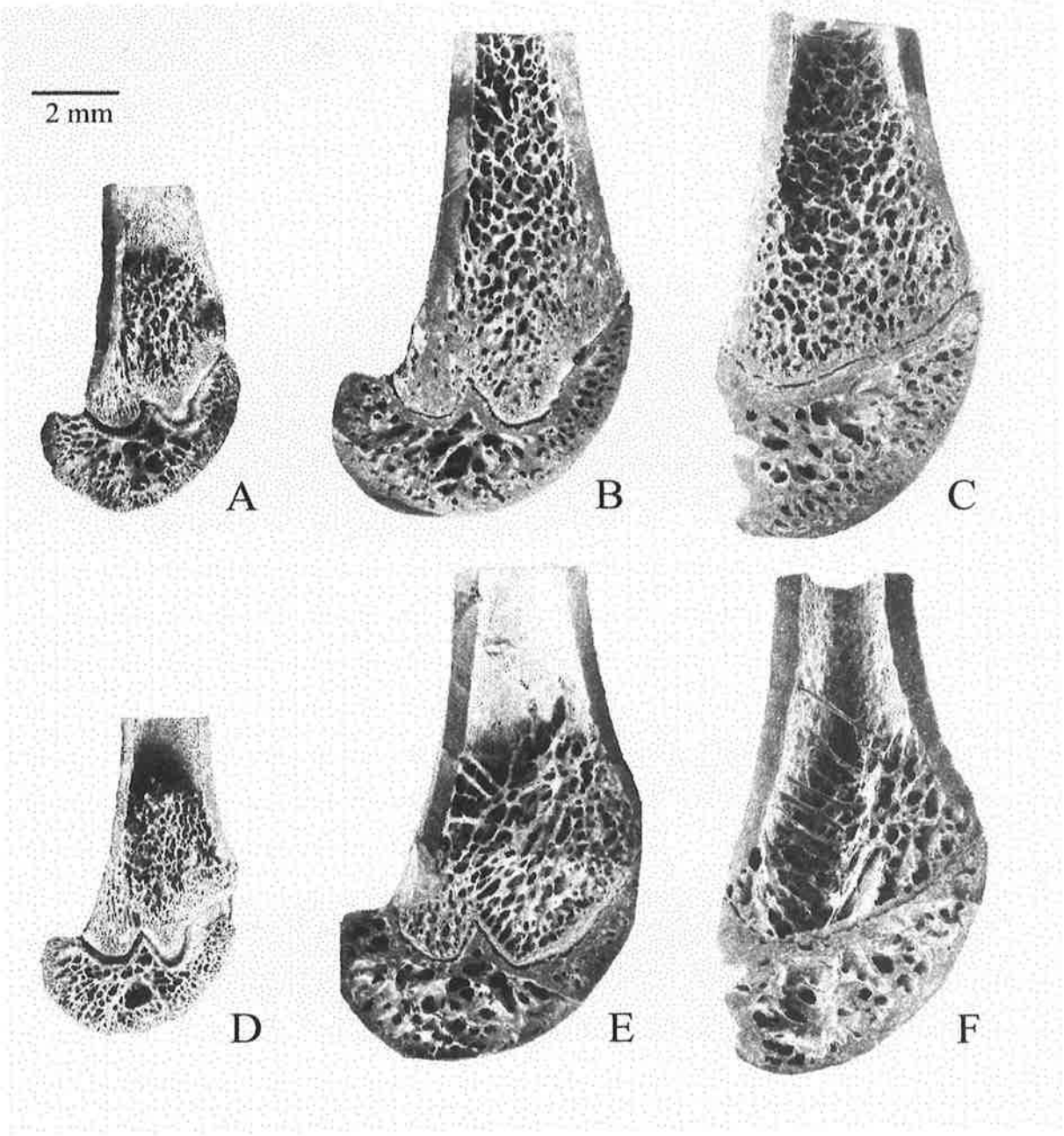

Epiphysis
A
Trabecular Bone Volume
BV/TV (%)
50
40
30
20
10
0
3
6
12
20
28
52
78
Age (weeks)
Φ
Φ #
B
Trabecular Number
Tb.N (#/mm)
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
Age (weeks)
C
Trabecular Thickness
Tb.Th (um)
140
120
100
80
60
40
20
0
Age (weeks)

7.3.6 Scanning electron microscopy

In the ovary intact group scanning electron microscopy revealed the rapid growth between 6 (Fig 7.7A) and 20 (Fig 7.7B) weeks of age. During growth the trabecular region also lengthened to fill the medullary cavity of the distal third of the femur. The epiphysis also expanded radially during this period. The length of this trabecular region was markedly shortened by 78 weeks of age (Fig 7.7C), with little difference in the epiphysis.

Oophorectomy did not alter the appearance of the bone samples at 6 weeks of age (Fig 7.7D) or growth of the femur. The length of the trabecular region in the medullary cavity however, was markedly shorter at 20 weeks (Fig 7.7E), with trabeculae absent from the diaphyseal region at this age. The bone loss from the medullary cavity continued with age, with trabeculae virtually absent from the central region of the medullary cavity at 78 weeks (Fig 7.7F). Trabeculae that remained at this age were well organised in a linear fashion and appeared thicker than those of the age matched ovary intact sample.

Figure 7.7. Scanning electron micrographs showing the effect of prepubertal oophorectomy on growth and development of the distal femur. Ovary intact samples demonstrate the considerable growth between 6 weeks (A) and 20 weeks (B) of age. Age related osteopenia is evident in the proximal margin of the medullary cavity at 78 weeks (C). Prepubertal oophorectomy did not markedly alter growth of the femur, with similar dimensions at all ages. Trabecular bone of the medullary cavity was not affected by oophorectomy at 6 weeks of age (D). In contrast, osteopenia is well developed in the medullary cavity at 20 weeks (E), with trabeculae virtually absent from the diaphyseal region. Osteopenia continued to develop to 78 weeks (F), with trabeculae absent from the central core of the medullary cavity at this age. The epiphysis was less affected by oophorectomy, with a slightly more open pattern at 78 weeks.


2 mm
A
B
C
D
E
F

7.4 Discussion

7.4.1 Influence of oophorectomy on femoral length and body mass during growth and aging

In both operative groups the femur increased in length by around 100% during the study period from around 20 to 40 mm. Early growth was rapid, accounting for around 90% of total length by 20 weeks. This resulted in an increase of approximately 6.2mm in the distal third of the femur between 6 and 12 weeks, all of this occurring above the epiphysis. As a result, the trabeculae sampled in the metaphysis at 6 weeks were sampled in the diaphysis at 12 weeks. Oophorectomy did not significantly alter longitudinal growth during this period, in contrast to the delay in growth plate thinning, and previous studies (Stenstrom *et al.,* 1982). Despite the extent of early growth, the increase in length following this age was significant in both groups. While oophorectomy did not affect femoral length overall, the increase after 20 weeks was greater in Oophx, with a 4% increase by 78 weeks. The lack of an overall increase femoral length may relate to the age of operation, as it has been shown that increases in longitudinal growth are more pronounced the greater the age at which oophorectomy is performed (Stenstrom *et al.,* 1982).

Body weight increased over 3 fold in both groups throughout the study, the increase was greater in Oophx. The greater weight gain in Oophx is consistent with previous studies (Wronski *et al.,* 1987). This weight gain is produced in part by hyperphagia, found to follow ovarian hormone deficiency (Gale and Sclafani, 1977). Even with pair feeding, oophorectomised rats have been shown to gain more body weight (Wronski *et al.,* 1987).

7.4.2 Regional comparison of the development of trabecular structure in ovary intact animals during growth

The growth phase of the femur lasted from 6 weeks, following puberty (Lu *et al.*, 1979), until approximately 20 weeks, with no increase in BV/TV in any region after this age. Each region displayed differing developmental patterns. In the epiphyseal region, where mechanical demand is high even in young animals, BV/TV increased steadily during the growth phase, with a 61% gain during this period. This was the result of a continuous increase in Tb.Th, with Tb.N stable from 3 weeks. This is consistent with the developmental pattern of this region, in which growth is radial from the centre of the epiphysis. As a result the central trabeculae are well formed at an early age, as evident in the scanning electron micrographs of the 6 week animals. With increasing age, these trabeculae remain as new bone is added at the expanding circumference. However it is apparent that the number of trabeculae is not adjusted with age, suggesting that any alterations due to increasing mechanical demand is via alterations to Tb.Th. This is demonstrated by the continued increase in Tb.Th with age.

In the metaphysis, closest to the growth plate, BV/TV increased steadily during growth, similar to the epiphysis, with a gain of 72%. This resulted from early gains in Tb.N between 3 and 6 weeks, around the onset of ovarian hormone production. However following this, the developmental pattern of this region was similar during growth to that of the epiphysis. Despite the rapid increase in femoral length during growth, the number of trabeculae in the metaphysis remained relatively constant. Further gains in BV/TV resulted from increased Tb.Th. Given the similarity of the developmental patterns between these two regions, the metaphysis may experience greater mechanical demands during growth than at later times. The alteration of Tb.Th

rather than Tb.N enabling adaptation to increasing strain. However, the trabeculae of the epiphysis were approximately twice as thick as those of the metaphysis, suggesting the greater mechanical demand in the former region.

In the diaphyseal region BV/TV increased rapidly with age from 6 weeks, increasing 89% until the end of the growth phase at 20 weeks. The relative increase being greater than in the other regions. This was the result of increases in both Tb.N and Tb.Th. In contrast to the other regions, Tb.N increased markedly, rising 88% in a one step manner after 6 weeks, to maximum of 2.2/mm between 12 and 28 weeks. Similar to other regions, Tb.Th increased 64%, steadily from 3 weeks, reaching a maximum thickness of 59um at 20 weeks. The striking increase in Tb.N during growth is consistent with the rate of longitudinal growth during this period.

The lengthening of the bone deposits a fine network of cartilage spicules from the growth plate, which are converted by subsequent remodelling into secondary spongiosa of the metaphyseal region (1.5). This results in a continuous lengthening of the trabecular region in the distal femur, which eventually fills the diaphyseal region. The increase in BV/TV in this region is therefore the result of longitudinal growth. Given the increase in femoral length during the early stages of growth, the trabeculae appearing in the diaphysis at 12 weeks were in the metaphysis at 6 weeks. This lateral transition of sample regions with growth allows analysis of the change in trabeculae as the growth plate recedes. In this case the trabeculae have not thickened, remaining constant around 40um. Tb.N however has reduced by approximately 50%. This displays the process of terminal destructive modelling, which removes trabeculae from the diaphysis as longitudinal growth continues. This is most evident in the tibiae, where the length of the metaphyseal trabecular region does not increase with age, despite gains in tibial length (Turner *et al.,* 1994c). Thus at this early age between 6

and 12 weeks, as the growth plate recedes distally with increasing femoral length, roughly half the trabeculae that remain are perforated and removed before reaching the diaphyseal sample area. The values of Tb.N are mean values calculated from the entire sample region and it is likely that this process occurs in a graded pattern throughout the two regions. However the abrupt termination of the trabecular region in the femur is clearly visible in the scanning electron micrographs at 6 weeks, suggesting that at some point within the diaphysis this process gains in vigour.

7.4.3 Influence of oophorectomy on developmental pattern induced changes in trabecular bone during growth

The patterns of oophorectomy induced bone loss were different between regions, indicating the relative importance of ovarian hormones in their development. The trabecular bone of the diaphysis increased markedly during growth in Sham, however this was completely arrested by oophorectomy. BV/TV in Oophx remained below 1% from 6 weeks onward and was 2% of Sham at 20 weeks, with no increase in Tb.N or Tb.Th at any age. This finding is consistent with the hypothesis that the bone of this region is ovarian hormone dependent. This proximally located bone is reduced extensively following mature oophorectomy as demonstrated in Chapter 3, and does not develop at all in the absence of ovarian hormones, and while this does suggest oestrogen dependence, it may also be related to local mechanical strain. Treadmill exercise has been shown to prevent femoral trabecular bone loss after oophorectomy, but fails to prevent loss of the proximally located metaphyseal trabeculae (Peng *et al.*, 1997), implying that these trabeculae do not transduce mechanical strain. Without the interaction with mechanical input these trabeculae have no protection from osteopenic stimuli, such as oestrogen deficiency. Indeed this may be expanded to dietary calcium

restriction, as this region was not included in the studies in the previous chapter because osteopenia following restriction was too extensive to measure meaningful changes.

The lack of development in the diaphyseal region suggests a strong interaction between local mechanical strain and oestrogen in the development of trabecular bone. The epiphysis in contrast, experiences high strain levels, and this interaction is evident in the gain in bone during growth in Oophx. BV/TV increased 70% during this period, in a process clearly independent of ovarian hormones. It is therefore possible that exposure to local mechanical strain during development is sufficient stimulus for bone formation, independent of ovarian hormones. This hormone independence during development may give rise to the resistance to oophorectomy induced bone loss at mature ages. The independence to oestrogen is not total, with BV/TV 34% lower than Sham in this region at 20 weeks. This difference was the result of a reduction in Tb.N. The increased turnover evident in all regions following oophorectomy (3.4.5) therefore does result in the perforation of some trabeculae in this region. The oophorectomy induced osteopenia in the epiphysis in the present study, which is not evident in mature studies, is consistent with the greater effect of oophorectomy at younger ages (Kalu *et al.,* 1989). This is also consistent with human studies demonstrating earlier menopause as an independent risk factor for osteoporotic fracture.

The development of the metaphysis in ovary-intact animals displayed aspects of both diaphyseal and epiphyseal patterns. In keeping with these observations, the response to oophorectomy also reflected aspects of both of the other regions. As in the epiphysis, BV/TV did increase, by 93% with Tb.Th increasing constantly during growth to be 79% greater at the end of the period. However, BV/TV was stable from 6

weeks onwards to be 45% lower than Sham at 20 weeks, with Tb.N increased at 6 weeks and returned to 3 week levels by 20 weeks, similar to the diaphysis. Thus during development this region may be viewed as an intermediate between the extremes of the strain dependence of the epiphysis and the oestrogen dependence of the diaphysis. Clearly the metaphysis is oestrogen dependent to some extent, with oophorectomy impeding the accrual of bone to a significant degree. The reduced production of trabeculae in Oophx suggests a similar alteration to bone turnover as demonstrated following mature oophorectomy, with increased resorption perforating and removing entire trabecular struts. Increased bone turnover as a result of oophorectomy has been shown to be independent of age (Morris *et al.,* 1992). This reduction would impact upon the supply of spongiosa reaching the diaphyseal region during growth, accounting at least in part for the osteopenia in this region. In contrast, Tb.Th was not affected by oophorectomy, similar to the epiphysis, demonstrating that bone formation was not affected by the absence of oestrogen, a consistent finding throughout these studies. Further, the continuous thickening of trabeculae in both regions demonstrates that positive bone accrual is possible in the absence of ovarian hormones. This is particularly evident in the epiphysis, with a significant increase in BV/TV after 6 weeks, when normal pubertal increase in ovarian hormone production aids in the development of mature skeletal stature. Thus in high strain regions increased ovarian production is not obligatory for trabecular bone development, consistent with the well developed cortical structure in the oophorectomised group.

7.4.4 Regional comparison of the changes in trabecular structure in ovary-intact animals during aging

The divergent developmental patterns evident during growth in the different regions of the distal femur continued during aging. This was most pronounced in the diaphysis, where all the bone gained up to 20 weeks was removed, with BV/TV returning to prepubertal levels by 78 weeks of age. This reduction resulted from a nonsignificant 44% reduction in Tb.Th and a 77% decrease in Tb.N. This reduction may be viewed as a continuation of the terminal destructive modelling evident in the transition of trabeculae from the metaphysis to diaphysis during growth. This removal of bone from the diaphysis was gradual and continuous with increasing age, despite the gain in body weight during this period. This is strong evidence for the lack of mechanical demand experienced by this region, and is consistent with the extent of oophorectomy induced bone loss experienced in this region.

The epiphysis, in contrast to the diaphysis, did not lose bone gained during growth. This is consistent with the mechanical function of this region. However it is not consistent with the continued gain in body weight with age. The lack of adaptation of BV/TV, and particularly Tb.Th, to the increase in body weight during this period suggests that the structure adopted during the growth phase may be conservative, and therefore does not require modification during the increasing weight bearing demands of aging. This conservative nature of trabecular development is consistent with the gain in BV/TV evident in the diaphysis during growth. The majority of trabeculae deposited in this region during longitudinal growth were maintained until growth had ceased. It was only after this period, when mechanical demands would be expected to begin to stabilise, that these trabeculae were removed. The formation of trabeculae in excess of physiological requirements would allow greater potential for adaptation to

both increases in body weight following growth and for calcium homeostatic demands.

As during growth, the changes in the metaphysis with aging again reflect the intermediate function of this region in relation to both mechanical and calcium homeostatic requirements. As a result, this region displays aspects of the developmental pattern of both the diaphysis and epiphysis. Similar to the diaphysis, BV/TV reduced with age, due to a reduction in Tb.N, with Tb.Th stable. A substantial amount of bone was retained however, with BV/TV falling 57%, but still remaining 125% greater than at 3 weeks, consistent with bone retention as evident in the epiphysis. Throughout the aging period Tb.Th remained stable, with all reductions due to trabecular perforation, independent of region or operation. This is consistent with the pattern evident in Chapter 4.

7.4.5 Influence of oophorectomy on aging induced changes in trabecular bone structure

The response of oophorectomised groups to aging was less varied than during growth, with BV/TV maintained in all regions from 20 to 78 weeks of age. In the diaphysis the failure to accrue bone following oophorectomy remained until the end of the study, with BV/TV below 0.5% throughout the period. In contrast, the epiphysis and metaphysis did accrue bone during growth and BV/TV was not significantly different between 20 and 78 weeks of age. This retention was the result of increased Tb.Th in both regions, with Tb.N reduced significantly in the metaphysis by 39% and nonsignificantly in the epiphysis by 13%.

This retention of bone did reveal regional variation when compared to the pattern of ovary-intact groups. In the epiphysis BV/TV in Sham was maintained with

age, and was thus greater than Oophx at all ages. However, in both the metaphysis and diaphysis, Sham BV/TV declined with age to Oophx levels by 78 weeks. This convergence of BV/TV may reflect the conservative development of trabecular bone suggested in the previous section. The osteopenia following oophorectomy may reduce BV/TV to a minimum defined by local mechanical strain. In ovary-intact animals age related bone loss may reduce bone volume until the same minimum is reached. Thus it may be the case that at extreme old age and following oophorectomy, mechanical strain increases in importance as a factor controlling the amount of bone present, not only in high strain regions such as the epiphysis, but also in low strain regions.

7.4.6 Influence of body weight on trabecular bone loss following oophorectomy

The present study investigates the interaction of mechanical strain and ovarian hormones on the development of the distal femur. The hormonal dependence of the trabeculae of the diaphyseal region is clearly demonstrated, with a failure to develop BV/TV at any age following oophorectomy. However, in the absence of an unloading study, the strain dependence of the epiphyseal region is not directly investigated. Given this limitation, indirect evidence of mechanical responsiveness of bone can be gained by investigating the effect of the increase in body weight with age. Increasing body weight results in greater mechanical demands on the weight bearing components of the skeleton, such as the epiphysis. It was hypothesised above that strain is responsible for driving development of bone in this region, and that this stimulus engenders the resistance to ovarian hormone deficiency. Was this hypothesis valid the development of bone in this region should occur in a manner proportional to strain, as produced by body weight. Consistent with this, Tb.Th of both epiphyseal and

metaphyseal regions were significantly correlated with body weight, whilst this relationship was absent in the diaphysis. This is evidence, albeit indirect, that local mechanical strain is the primary developmental stimuli in the epiphysis, thickening trabeculae as mechanical demand increases. Further, the continued adaptation of Tb.Th with aging demonstrates that this mechanism is not confined to development, but continues with age.

Chapter 8

Summary and conclusions

8.1 Summary

This thesis had the broad aim of investigating the relationship of local mechanical strain and bone turnover, using the distal femur of the oophorectomised rat as a model. This model enabled precise control of dietary and hormonal variables, as well as providing a trabecular sample region with a distinct loading profile, with regions of both high and low load.

The first hypothesis related to the estimation of resorption in this model. This study, as suggested by the literature, showed considerable variation in estimates of osteoclast surface between various methods tested. A morphology-based method, assessing osteoclast-like cells opposed to the bone surface (VK-Oc) was chosen for future studies. This method combined greater precision than a Howsips lacunae-based method (Pit-Oc), with significantly reduced variance in estimations of osteoclast surface. Vk-Oc also provided greater accuracy than an enzyme-based method (Acp-Oc), with significantly more sensitive detection of oophorectomy induced increases in osteoclast surface.

The second hypothesis related to the short term effect of local mechanical strain on bone cell activity and osteopenia following oophorectomy. This study clearly demonstrated altered bone cell activity as a function of local mechanical strain. Higher local strain resulting in a delay, but not a cessation, of increased turnover following oophorectomy. The development of osteopenia in the high strain region, the femoral epiphysis, was not evident in the 30 day experimental period, despite extensive loss in the other regions. The loss of bone in the diaphysis was so rapid as to suggest an initiation of bone loss prior to increased osteoclastogenesis in this region, most likely due to increased resorption depth. The exaggerated structural consequences of

increased resorption depth in low strain regions, with thin trabeculae, was also consistent with the observed pattern of osteopenia.

The third hypothesis related to the long term effect of local mechanical strain on bone cell activity and osteopenia following oophorectomy. The elevation of bone turnover was evident as a long term phenomenon in both high and low strain regions. Despite this however, osteopenia did not develop in the high strain region, suggesting a resistance of this region to the elevated turnover resulting from ovarian hormone deficiency. As in the short term study, there was indirect evidence for a sustained elevation in resorption depth in the low strain region, consistent with the differential response to osteopenia between regions. The architectural changes evident were also consistent with such a change in osteoclast activity.

In combination these two studies suggested that oestrogen deficiency elevated turnover on all trabecular surfaces, but that mechanical strain altered the local osteoclastic activity to modulate the resultant osteopenia. This process is also suggested to be influenced by local structural factors such as trabecular thickness.

The fourth hypothesis related to the effect of a reduction in turnover on the hypothesised mechanical homeostatic mechanism. This decrease in turnover was produced by exogenous 17β oestradiol supplementation in both oophorectomised and sham operated animals. Substantial changes in trabecular structure were evident, with trabecular thickness increasing with decreasing turnover, independent of strain, suggesting benefit from antiresorptive therapy even in low strain regions. There was an unexpected reduction in trabecular number, suggesting the possibility of perforation as result of decreased local strain resulting from the excessive trabecular thickening after antiresorptive treatment. The relationship of antiresorptive therapy to recovery from calcium restriction was strain specific, with recovery not possible in low strain

regions, likely due to perforation resulting from calcium deficiency. This was not evident in the high strain region, with recovery almost complete.

The fifth hypothesis related to the interaction of calcium deficiency and strain related resistance to oophorectomy induced bone loss. Oophorectomy did produce bone loss in the high strain region, when combined with dietary calcium restriction, but not alone. This bone loss is evidence of the additive effects of calcium and ovarian hormone deficiency, and may relate to reduced intestinal calcium absorption following oophorectomy. In the low strain region bone loss was evident with both calcium and ovarian hormone deficiency individually, suggesting greater calcium dependence in this region. Dual deficiency produced profound osteopenia, consistent with the additive effects recorded in the high strain region.

The sixth hypothesis related to the influence of strain on the ovarian hormone dependence of skeletal development. Prepubertal oophorectomy had significant effects on long bone development. The ability to increase trabecular number was lost. However the ability to increase trabecular thickness was not affected, and indeed was greater in the osteopenic, ophorectomised animals. The retention of bone during development and aging, was strongly load related, as evidenced by the strong correlation of trabecular thickness and body weight in the regions experiencing weight bearing strains. The high strain region was able to increase BV/TV in the absence of ovarian hormones, via thickening of trabeculae.

8.2 Limitations

It is clear from the text that discussion of possible changes in resorption depth is hypothetical at best, allowing no firm conclusions to be drawn. The circumstantial evidence has not been followed with further investigation. In order to investigate this

in a similar scenario would require the use of a larger animal model in which bone turnover is slower. The more ordered lamellae patterns and resorption profiles would greatly increase the confidence with which resorption depth could be estimated.

Similarly, no direct estimates of bone cell number where made in this thesis. Discussion regarding cell proliferation is thus only suggestive. The possible influence of changes in apoptosis have not been considered. The use of bromodeoxyuridine to label proliferating nuclei (Barou *et al.,* 1998) would offer an opportunity to test the suggestions made in this thesis concerning altered proliferation. Apoptosis could be estimated by DNA fragmentation, such as terminal transferase mediated dUTP nick-end labelling (TUNEL) assays (Stadelmann and Lassmann).

The lack of inclusion of an animal with lessened mechanical input, such as following sciatic neurectomy or tail suspension (reviwed in Vico *et al.,* 1998) would have allowed greater certainty in ascribing load related explanations for alterations in bone physiology between regions. The benefits of such an inclusion are evident in previous studies (Yoshida *et al.,* 1991, Westerlind *et al.,* 1997).

8.3 Future Directions

One of the most important observations made in this thesis is the differential response to immediate ovarian hormone deficiency between the diaphysis and epiphysis. The well defined regions within which these responses occur make gene expression studies a viable method for investigating this phenomenon. The ability to collect discrete tissue samples from the epiphyseal and the diaphyseal/metaphyseal region would facilitate the use of gene chip technology. The well defined temporal sequence of changes in bone cell activity would allow the targeting of specific investigations to particular physiological questions.

Analysis of gene expression profiles prior to 9 days post operation would provide an opportunity to identify genes involved in the hypothesised increase in resorption depth in the diaphysis. Based on observations made in this thesis, diaphyseal samples taken prior to the dramatic bone loss in this region may provide data concerning increased resorption depth without the potential confusion of increased osteoclast proliferation. Further, these samples could also reveal the influence of antiapoptotic action on increased resorption depth in this situation.

Gene expression profiles taken between 9 and 18 days post operation would provide an opportunity to identify genes involved in the delayed increased in bone turnover in the epiphysis following ovarian hormone deficiency. The identification of genes capable of inhibiting turnover in this situation would be of considerable interest to osteoporotic research and treatment, and may also have relevance to other osteolytic diseases such as arthritis. These genes would be highly likely to control the RANKL/OPG ratio, and primary or secondary effects could perhaps be tracked using expression profiles relative to the temporal changes in this ratio.

Expression profiles from later times would provide an opportunity to identify genes involved in the maintenance of bone volume despite elevated turnover, as evident in the epiphysis. The identification of genes modulating the osteopenic response to high turnover would likely include those involved in the mechanical homeostatic system. While the majority of this phenomenon may relate to architectural differences, altered control of osteoclast physiology cannot be ruled out.

The site specific nature of the altered response to ovarian hormone deficiency, within what is otherwise highly similar tissue presents the possibility of specific gene expression differences upon a relatively homogenous expression background.

Subsequent localisation via *in situ* hybridisation would also be simplified by the existence of both "test" and control regions within a single tissue section.

Chapter 9

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Appendix

Published papers and Awards

Awards

Australian Society for Medical Research (ASMR)

State meeting 1997
Ansett student encouragement award, most outstanding oral presentation
Long term variation in bone cell dynamics following adult oophorectomy in the distal femur of the rat

National meeting 1996
AMRAD award, outstanding presentation
Zonal variation in bone cell dynamics following adult oophorectomy in the rat

National meeting 1994
Campion Ma Ploust Award, most outstanding presentation by researcher under 30 years of age
Variation of oophorectomy-induced bone loss is related to body weight

National meeting 1992
Outstanding poster
Femoral histomorphometry in pre-pubertal rats following oophorectomy

Published Papers

Discordance between bone turnover and bone loss: effects of aging and ovariectomy in the rat.
Baldock PA, Need AG, Moore RJ, Durbridge TC, Morris HA
J Bone Miner Res. 14:1442-1448; 1999.

Comparison of three methods for estimation of bone resorption following ovariectomy in the distal femur and the proximal tibia of the rat.
Baldock PA, Moore RJ, Durbridge TC, Morris HA
Bone 24:597-602; 1999.

Variation in the short-term changes in bone cell activity in three regions of the distal femur immediately following ovariectomy.
Baldock PA, Morris HA, Moore RJ, Need AG, Durbridge TC
J Bone Miner Res 13:1451-1457; 1998.

Prepubertal oophorectomy limits the accrual of bone during growth and development
Baldock PA, Morris HA, Moore RJ, Durbridge TC
Calcif Tissue Int 62: 244-249; 1998.

Baldock, P.A., Need, A.G., Moore, R.J., Durbridge, T.C. & Morris, H.A. (1999) Discordance between bone turnover and bone loss: effects of aging and ovariectomy in the rat.
Journal of Bone and Mineral Research, v. 14 (8), pp. 1442-1448

Baldock, P.A., Moore, R.J., Durbridge, T.C. & Morris, H.A. (1999) Comparison of three methods for estimation of bone resorption following ovariectomy in the distal femur and the proximal tibia of the rat. Bone, v. 24 (6), pp. 597-602

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Baldock, P.A., Morris, H.A., Moore, R.J. & Durbridge, T.C. (1998)
Prepubertal oophorectomy limits the accrual of bone during growth and development
Calcified Tissue International, v. 62 (3), pp. 244-249

ERRATUM

page 30, paragraph 2 line 1	immobilsation	to immobilisation
page 37, paragraph 3 line 1	Local	to local
page 119, paragraph 2 line 18	fisrt	to first
page 120, paragraph 1 line 3	While these generalisations restrict comment to speculation. Given this, …	
	to	
	Given these generalisations restrict comment to speculation,…	
page 120, paragraph 1 line 22	vertabrae	to vertebrae
page 178, paragraph 2 line 2	Howsips	to Howship's