EFFECTS OF ESTROGENS AND ANDROGENS ON BONE CELL METABOLISM

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ABSTRACT

This thesis investigates the effect of estrogen deficiency and dihydrotestosterone (DHT) treatment in ovary-intact (sham), oophorectomised (oophx) and osteopenic oophx rats on bone cell metabolism as indicated by bone biochemical markers and mRNA levels of osteoblast and osteoclast genes. The effect of estrogen replacement alone and in combination with DHT, to osteopenic oophorectomised rats was also investigated.

Oophorectomy resulted in an increase in bone turnover as indicated by bone biochemical markers and mRNA levels of osteoblast and osteoclast genes. DHT treatment increased bone formation as indicated by serum alkaline phosphatase (ALP) in sham, oophx and osteopenic oophx rats. Bone resorption as measured by urine deoxypyridinoline was suppressed only in osteopenic oophx rats and this was associated with a decrease in serum osteocalcin. DHT treatment decreased urine calcium excretion in sham, oophx and osteopenic oophx rats which was associated with an increase in the renal tubular reabsorption of calcium. DHT treatment increased the mRNA levels of the osteoblast genes at all stages of osteoblast differentiation in sham rats which was accompanied by an increase in bone resorption as indicated by calcitonin receptor (CTR) mRNA levels. In contrast, in oophx rats administered DHT immediately following operation the mRNA levels of type 1α collagen, osteocalcin and CTR were suppressed. In osteopenic oophx rats, DHT administration at 15 weeks post-operation decreased the mRNA levels of type 1α collagen and ALP while CTR mRNA levels were increased. Estradiol (E_2) treatment in osteopenic oophx rats increased the mRNA levels of

type 1α collagen. Estradiol (E_2) in combination with DHT acted synergistically to increase serum ALP and increased the mRNA levels of ALP while suppressing the mRNA levels of osteocalcin and CTR.

This study provides insight into the mechanism by which DHT and estrogen effect osteoblast and osteoclast bone cell activities. This study also demonstrates the differential effects of DHT on bone cell activities in estrogen sufficient and estrogen deficient rats, suggesting that there is an interaction of estrogens and androgens on bone cell metabolism in the female rat.

DECLARATION

This thesis contains no material which has been accepted for the award of any other degree or diploma in any university or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another 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 loan and photocopying.

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CHAPTER 1

SEX STEROID HORMONES AND BONE METABOLISM

1.1 INTRODUCTION

Following the menopause women suffer accelerated bone loss, which may lead to the development of osteoporosis (Albright et al 1941). Osteoporosis describes a disease state in which 'the amount of bony tissue in the bone is below the young normal range determined at the same skeletal site in subjects of the same sex' (Nordin 1987). This bone loss increases the risk of fracture particularly at the wrist, vertebrae and hip and treatment of these fractures incurs a major cost to the health system. Estrogen replacement therapy is a widely used treatment to prevent further bone loss following the menopause, however it does not restore bone lost prior to treatment.

The bone loss that occurs following the menopause is characterised by an increase in bone turnover, with an imbalance in the processes of bone resorption and bone formation (Turner et al 1994). Estrogen treatment in post menopausal women suppresses the elevated bone turnover rate and prevents further bone loss (Lafferty et al 1964, Riggs et al 1972, Lindsay et al 1976). The loss of circulating ovarian hormones following the menopause also has an effect on calcium homeostasis. Post menopausal women have decreased reabsorption of calcium in the kidney resulting in increased excretion of calcium in the urine (Nordin et al 1991, Nordin et al 1994). Furthermore, intestinal calcium absorption

decreases following the menopause (Heaney et al 1989). All of these described changes in bone metabolism and calcium homeostasis are reversed following hormone replacement therapy.

Androgens are thought to stimulate bone formation due to their anabolic properties Testosterone levels have been found to decrease following the menopause and are strongly correlated with bone loss (Nordin et al 1985). Androgens have been shown to be beneficial in treating postmenopausal bone loss and other symptoms associated with the menopause, however their use is limited due to their undesirable side effects (Mauvais-Jarvis et al 1981).

Testosterone, the principal circulating androgen, is converted to dihydrotestosterone (DHT) via the enzyme 5α-reductase and can be aromatised to estrogen (Mooradian et al 1987). DHT is a 5α-reduced androgen which cannot be further converted to estrogen and has been shown to be two to three times more potent than testosterone as it has a higher affinity for the androgen receptor (Mauvais-Jarvis et al 1981).

The oophorectomised rat is a well characterised model of postmenopausal bone loss (Kalu et al 1991a, Yamazaki and Yamaguchi 1989, Wronski et al 1986). The studies described in this thesis were based on the hypothesis that anabolic steroids can stimulate bone formation by promoting osteoblast differentiation and gene expression resulting in bone accretion in the oophorectomised osteopenic rat. DHT will be used to ensure that the observed effects are due to the androgenic properties of DHT without the interference of estrogen which is well established to have an effect on bone (Lindsay 1988).

1.2 BONE

The skeletal system consisting of bone and cartilage, provides the support and attachment site for muscles, protects the organs, houses the bone marrow and provides the extracellular fluid with calcium ions which are essential for every day life. The long bones consist of the epiphysis, the diaphysis (or midshaft) and the metaphysis. In growing bone the epiphysis is separated from the metaphysis by the growth plate. The growth plate is a layer of cartilage from where cells proliferate and is the site of matrix formation which is later calcified. The cells of the growth plate are responsible for the longitudinal growth of bone which is calcified and remodelled by the end of the growth period. The external part of the bone consists of a thick dense layer of calcified tissue referred to as cortical bone. This bone is supported by an internal network of thin calcified trabeculae referred to as cancellous or trabecular bone. The periosteal surface refers to the external surface where the bone is in contact with the soft tissue and the endosteal surface refers to the internal surface of bone. Both of these surfaces are lined with osteogenic cells organised in layers. The trabecular bone is surrounded by bone marrow and consists of 15-25% calcified bone (the remainder being bone marrow, blood vessels and connective tissue) while the cortical bone comprises of 80-90% calcified bone. Thus the cortical bone serves mainly as the mechanical and protective function of bone while the trabecular bone serves the metabolic function of bone (Baron 1993).

1.2.1 Bone remodelling

Bone is remodelled continuously throughout adult life in contrast to modelling which occurs during growth to alter the geometry and size of bones (Frost 1979). This process is often referred to as bone turnover which occurs in basic multicellular units (BMU) and consists of cycles of resorption of existing bone by osteoclasts followed by formation of new bone by osteoblasts (Frost 1979). These two processes are tightly coupled and ensure the skeleton is renewed while its anatomical and structural integrity is maintained. The retraction of the osteoblastic lining cells of the bone by a mechanism which is poorly understood, signals the osteoclast to adhere to the bone surface. The osteoclast resorbs the bone by use of acids and proteolytic enzymes. Once resorption is complete and the osteoclasts vacate the remodelling space, the resorption pit is lined with macrophage-like uncharacterised mononuclear cells and this is referred to as the 'reversal phase'. A cement line consisting of a mixture of glycosaminoglycans and glycoproteins is formed which marks the limit of bone resorption and 'cements' together the old and new bone. Following the reversal phase, osteoblasts adhere to the resorption site and synthesise new bone in the form of osteoid, a matrix of collagen and structural proteins which are then mineralised. Once the osteoblasts have completed the synthesis of new bone, terminally differentiated osteoblasts referred to as 'lining cells' cover the surface of the bone leaving it indistinguishable from the bone prior to remodelling (Baron 1993).

1.2.1.1 The Osteoblast

Osteoblasts originate from local pluripotent mesenchymal stem cells of the bone marrow (Manolagas and Jilka 1995). These cells are always found in groups along the surface of the bone, function together and are connected by short protrusions (Holtrop 1990). Osteoblasts are cuboidal in shape and are characterised by a round nucleus at the base of the cell, a strongly basophilic cytoplasm and a prominent golgi complex located between the nucleus and the apex of the cell (Baron 1993). The osteoblast on the bone surface is in contact with the osteocyte processes in their canniculi. Their plasma membrane is rich in alkaline phosphatase which is used as a biochemical marker of bone formation. Furthermore the osteoblast has the ability to produce cytokines and colony-stimulating factors such as interleukin-6 (IL-6), interleukin-11 (IL-11), granulocyte-macrophage colony-stimulating factor (GM-CSF) and macrophage colony-stimulating factor (M-CSF) (Manolagas and Jilka 1995) which have been implicated in osteoclast development (Chapter 1.2.1.4).

The osteoblasts synthesise and secrete a number of unique proteins which can be used as markers of cellular activity and therefore bone formation. The three major biochemical markers currently measured are procollagen type I carboxyterminal extension peptide (PICP), osteocalcin and alkaline phosphatase. More than 90% of the organic matrix of bone consists of type I collagen which is synthesised as a procollagen precursor molecule. PICP and amino-terminal extension peptide are produced from the cleavage of the procollagen molecule by specific endonucleases (Morris 1994). The measurement of PICP has been suggested to better reflect osteoblast activity than the measurement of the amino-terminal extension peptide (Ebling et al 1992) and serum levels of PICP correlate

positively with histomorphometric indices of bone formation but not bone resorption in postmenopausal osteoporotic women (Hassager et al 1991). To date however, assays are available for the measurement of PICP in human serum but not in serum from other species such as the rat.

Osteocalcin or bone Gla protein (BGP) is a unique product of osteoblasts and odontoblasts and is the most abundant non-collagenous protein in bone, although its precise function remains unknown. Osteocalcin contains three gamma-carboxyglutamic acid residues (Gla) which bind calcium and determine the binding of osteocalcin to hydroxyapatite, the major form of mineral in bone (Morris et al 1993). The synthesis of osteocalcin is thought to occur during the mineralisation of bone (Aubin et al 1995) and a small proportion of newly synthesised osteocalcin is released into the circulation (Morris et al 1993). The serum levels of osteocalcin are a sensitive marker of bone formation correlating with histomorphometric indices of bone formation but not with indices of resorption (Podenphant et al 1987).

Osteoblasts synthesise and store a specific isoenzyme of alkaline phosphatase in membrane vesicles, some of which is released into the circulation (Moss 1982). Only half of the total circulating levels of alkaline phosphatase however are derived from bone in adults with the remaining contributions from the liver and intestine (Morris et al 1993). The measurement of bone alkaline phosphatase in serum is difficult as the two major isosymes from liver and bone are similar as they are products of the same gene and are modified post-translocation. Recently, commercial reagents have become available for the measurement of bone specific alkaline phosphatase in human serum but not in other species.

1.2.1.2 The Osteocyte

Osteocytes are osteoblasts that have become surrounded by mineralised matrix (Holtrop 1990, Puzas 1993) and approximately 10-20% of osteoblasts become osteocytes (Puzas 1993). As additional mineralised matrix is laid down on the bone surface, the osteocyte is located deeper in the bone while being smaller in size. The young osteocyte conserves most of the structural characteristics of the osteoblast, while an older osteocyte located deep in the bone is smaller with an accumulation of glycogen in its cytoplasm. The osteocyte is connected with neighbouring osteocytes and with osteoblasts on the bone surface by means of cell processes that travel through the caniculi in the bone matrix. The caniculi are remnants of cellular processes that extended from the osteoblast and form an extensive array of connecting tubules enabling the transport of nutrients and possibly enabling communication. Osteocytes are phagocytized and digested with the other components of bone by osteoclasts during bone resorption implicating a possible role of osteocytes in locally activating bone turnover (Holtrop 1990, Puzas 1993, Baron 1993).

1.2.1.3 The Osteoclast

The osteoclast is a giant multinucleated cell of approximate size 50-100μm derived from fusion of mononuclear hemopoietic precursors (Baron 1989). These cells are usually found in low numbers of one or two osteoclasts within a lacuna (Howship's lacuna, a result of the osteoclast's resorptive activity) which is at the interface between soft and calcified tissues in an area where the bone matrix is fully mineralised (Baron 1989, Baron 1993). Osteoclasts are characterised by their number of nuclei (2-10), a cytoplasm containing a large number of vacuoles, a basolateral membrane rich in HCO_3^-/Cl^- exchangers and Na^+/H^+ exchangers and the presence of a ruffled border (Suda et al 1992, Baron 1993). The

ruffled border is formed by deep foldings of the plasma membrane in the area facing the bone matrix with dense patches on either side referred to as the 'sealing zone' (Baron 1993). It is within this compartment that the osteoclast resorbs the bone mineral by acidification and lysosomal enzymes. The osteoclast is enriched in lysosomal enzymes such as tartrate-resistant acid phosphatase which is synthesised and transported in vesicles which fuse with the plasma membrane at the ruffled border releasing their contents into the bone-resorbing compartment (Baron 1989). In addition, the osteoclast is also able to acidify the bone-resorbing compartment which aids in the dissolution of bone mineral (Baron 1989, Suda et al 1992). The hydrogen ions are provided by carbonic anhydrase which catalyses the hydration of carbon dioxide to bicarbonate and by the coupling of the passive transport of chloride ions into the resorption lacuna with the secretion of protons (Suda et al 1992).

A number of biochemical markers can be measured which reflects osteoclastic bone resorption including urine hydroxyproline, urine pyridinium cross-links and tartrate-resistant acid phosphatase. Hydroxyproline is derived from the degradation of various forms of collagen of which 10-20% is excreted in the urine. Urine hydroxyproline is not reutilised for collagen synthesis and is therefore useful to detect marked increases in bone resorption but lacks sensitivity as bone is not the sole source of collagen (Morris et al 1993). Pyridinoline and deoxypyridinoline are cross-links which stabilise the collagen chains within the extracellular matrix and are released from the bone matrix during bone resorption (Delmas 1993). Pyridinoline and deoxypyridinoline are excreted in the urine in free form and in peptide bound form and are not metabolised invivo before their urinary excretion. The urinary levels of cross-links have been demonstrated to correlate with bone

resorption as measured by calcium kinetics (Eastell et al 1990) and bone histomorphometry (Delmas et al 1991). The measurements of pyridinoline and deoxypyridinoline are more specific markers of bone resorption than hydroxyproline as deoxypyridinoline is present only in bone collagen while pyridinoline is present mainly in bone with minute amounts in other connective tissues. A potential biochemical marker of bone resorption which can be measured in serum is plasma tartrate-resistant acid phosphatase (TRAP). Osteoclasts contain a large amount of TRAP which is identical to the serum type 5b isoenzyme and is released into the circulation. Plasma TRAP is elevated following oophorectomy (Stepan et al 1987) and a high correlation between serum TRAP activity and urine hydroxyproline/creatinine ratio was found in a large population of normal volunteers (Stepan et al 1985). The use of TRAP as a clinical indicator of bone resorption however, requires further investigation.

1.2.1.4 Role of cytokines in bone resorption

M-CSF and GM-CSF have been demonstrated to stimulate haematopoietic stem cells to differentiate into monocytic precursors and then progress along the monocyte pathway prior to the branching of the differentiation decision to pre-osteoclasts (Zheng et al 1991a, Manolagas 1995). M-CSF and GM-CSF actually inhibit the monocytic precursors to develop into osteoclast precursors and have no effect on osteoclast formation. Supportive evidence for this role of M-CSF is that mutation of this gene results in the formation of osteopetrosis in the mouse (Felix et al 1994) which is a disease characterised by excessive accumulation of bone caused by reduced bone resorption as a result of defective osteoclasts (Marks 1973).

The cytokines interleukin 1 (IL-1), tumour necrosis factor (TNF), IL-6 and (IL-11) stimulate osteoclast development but require the presence of stromal osteoblastic cells (Manolagas 1995). IL-1 and TNF are produced primarily by monocytes and macrophages. In contrast, IL-6 and IL-11 are produced primarily by bone marrow stromal cells and osteoblastic cells in response to a number of factors including the cytokines, IL-1 and TNF (Manolagas 1995), the growth factors, transforming growth factor β (TGF-β), insulin-like growth factor-1 (IGF-1) and platelet-derived growth factor (PDGF), the steroid hormone, 1,25 dihydroxyvitamin D ($1,25(OH)_2D_3$) and the polypeptide hormones, parathyroid hormone (PTH) and parathyroid hormone-related peptide (PTH-rP) (Manolagas 1995). IL-6 stimulates the early stages of haematopoiesis and the early stages of osteoclastogenesis. IL-6 also synergizes with interleukin 3 (IL-3) to stimulate the development of GM-CSFs, the haematopoietic precursors of osteoclasts and stimulates the early formation of osteoclast precursors present in CFU-GM colonies (Manolagas 1995, Manolagas and Jilka 1995). In addition, IL-6 also increases osteoclast formation and bone resorption in fetal mouse bone in vitro and stimulates bone resorption in combination with IL-1 in vivo (Manolagas 1995, Manolagas and Jilka 1995). These cytokines have been implicated in the bone loss following hormone deficiency in males and females (Chapter 1.5.2.1, 1.4.4).

1.2.1.5 Role of osteoblast cells in osteoclast development

Takahasi and colleagues (1988) demonstrated that osteoblasts were required for the development of osteoclasts from osteoclast progenitors. It was later identified that cell to cell contact between osteoblastic stromal cells and pre-osteoclasts in the presence of $1,25(OH)_2D_3$ was required (Suda et al 1992). Interestingly, osteoblasts rather than osteoclasts are the target cells for bone-resorbing hormones such as parathyroid hormone

and $1,25(OH)_2D_3$. These hormones induce the osteoblasts to produce an osteoclast differentiation-inducing factor (Suda et al 1992). In contrast, calcitonin acts directly on osteoclasts through a receptor-mediated process to inhibit bone resorption (Zheng et al 1991b).

1.2.2 Gene expression in osteoblasts

In vitro studies of mRNA levels by Northern blot analyses demonstrate the diversity of the osteoblast cell. The c-*fos* gene is expressed by the most immature proliferating osteoblasts while the collagen gene is expressed during the down regulation of proliferation and the early stages of differentiation. The alkaline phosphatase and osteopontin gene expression occurs at a later stage of maturation, at a time of matrix development and osteocalcin gene expression occurs at a further stage when osteoblasts are mineralising tissue (Owen et al 1990) (Figure 1.1). Similar patterns of osteoblast gene expression during development have also been identified by in situ hybridisation techniques in sheep (Zhou et al 1994), mice (Nakase et al 1994) and in rats (Heersche et al 1992). Supportive evidence for this model of gene expression in osteoblast development is the expression of c-*fos* following fracture of the tibiae in rats which is associated with the formation of bone tissue in the calluses resulting from the fracture. The expression of the c-*fos* gene was paralleled by the expression of the alkaline phosphatase gene which was followed by the expression of osteopontin and osteocalcin genes from 7 to 10 days following fracture (Ohta et al 1991).

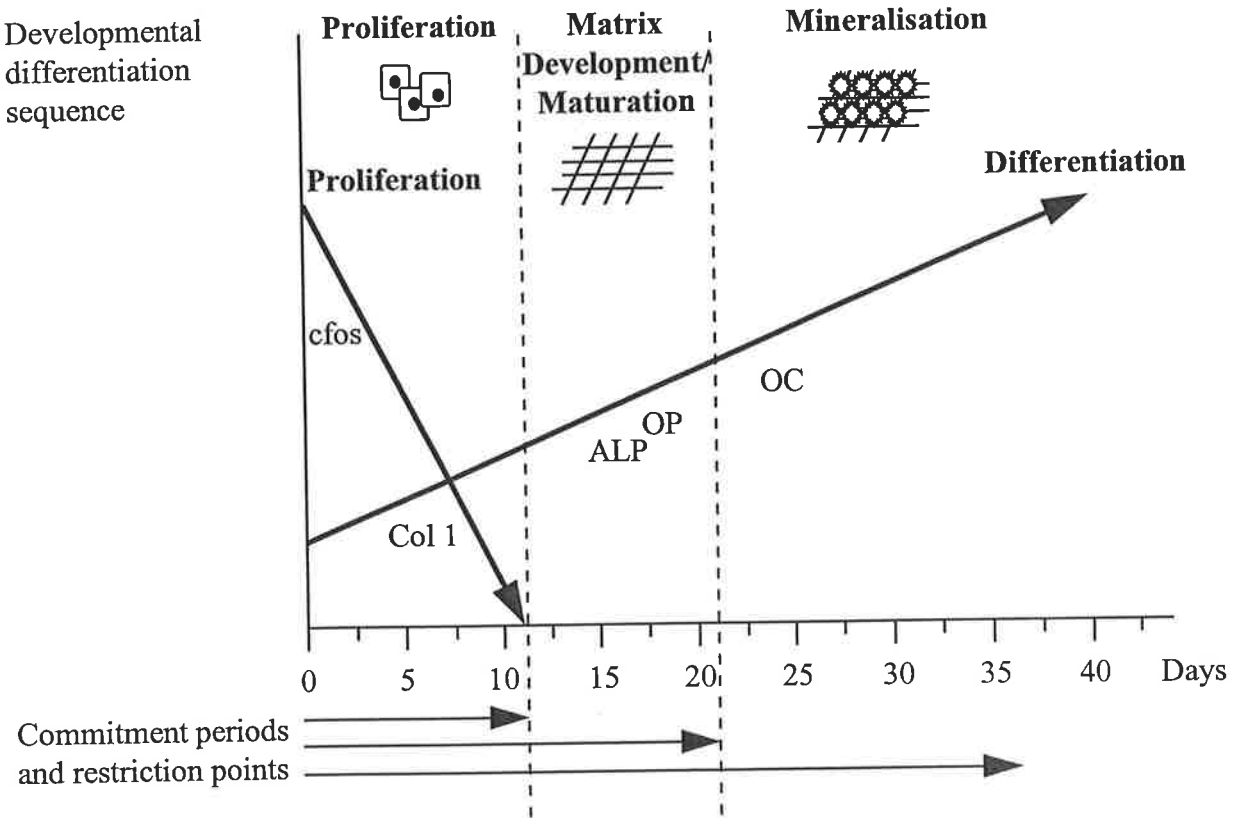


Figure 1.1 Proposed sequence of gene expression during development of the rat osteoblast and the relationship between proliferation and differentiation. Col 1, Type 1α collagen; ALP, Alkaline phosphatase; OP, Osteopontin; OC, Osteocalcin (Owen et al 1990).

The sequence of osteoblast development is separated by two commitment points to which it is proposed cells can progress but cannot pass without the further appropriate signals. The first restriction point is when proliferation is down regulated and the initiation of the expression of genes that are associated with the extracellular matrix maturation and the second is when mineralisation occurs (Owen et al 1990). The model proposed by Owen and colleagues (1990) indicates that the genes involved in the production of the extracellular matrix must be expressed during the proliferative stage in order for the progression of differentiation of the osteoblast to occur. Evidence to support this model is abnormal bone development and bone tumours that occur when the expression of the c-*fos* gene is deregulated, indicating the specific role of this gene to promote the progression of normal skeletal development (Ruther et al 1987). Owen and colleagues also postulated that the production of an organised bone specific extracellular matrix contributes to the shutdown of the proliferation stage. The completion of the extracellular matrix and its ability to support mineralisation is essential for completion of the osteoblast phenotype (Owen et al 1990).

In situ hybridisation techniques have been able to identify gene expression in vivo in localised osteoblasts at different stages of development. In adult bone tissue, osteopontin mRNA is strongly expressed by osteocytes and hypertrophic chondrocytes on the bone formation surface but not in cuboidal osteoblasts. In contrast, periosteal and endosteal cuboidal osteoblasts have been demonstrated to strongly express osteocalcin mRNA, but osteoblasts in primary spongiosia near the growth plate do not (Ikeda et al 1992). These observations are all consistent with the model of gene expression during development and maturation of the osteoblast as proposed by Owen and colleagues (1990).

1.2.2.1 Factors that affect osteoblast gene expression

Noda and Rodan (1987) demonstrated that TGFβ1 increases type 1α procollagen, alkaline phosphatase and osteonectin mRNA levels in rat osteosarcoma cells, indicating the possible role of growth factors in osteoblast development. The glucocorticoid dexamethasone decreases collagen mRNA levels in rat bone marrow cells (Yao et al 1994). The mRNA levels of c-*fos* in MC3T3-E1 cells are stimulated by bone morphogenetic proteins (bioactive proteins) (BMP), TGF-β1, IGF-I, insulin-like growth factor II (IGF-II) and fetal bovine serum (FBS) (Ohta et al 1992a). BMPs have also been identified to inhibit proliferation but to promote differentiation of osteoblast precursors into mature osteoblasts as indicated by increased levels of alkaline phosphatase and osteocalcin mRNA (Vukicevic et al 1990, Yamaguchi et al 1991). In contrast, TGFβ1, IGF-I, IGF-II or FBS are unable to increase osteocalcin mRNA, however TGFβ1 and FBS suppress alkaline phosphatase mRNA levels (Ohta et al 1992a).

1.3 POST-MENOPAUSAL BONE LOSS

Following the menopause and the decline in circulating estrogens (Lindsay 1988, Ohta et al 1992), women suffer bone loss which may lead to the development of osteoporosis (Albright et al 1941). Osteoporosis is a disease state where a thinning of the bone occurs which can lead to fractures. The magnitude of bone loss varies at different sites as cortical bone is lost at a rate of approximately 2% per year whereas the loss of trabecular bone from the centre of the lumbar vertebrae is approximately 8% per year (Genant et al 1982). The bone loss occurs as a result of an imbalance between the processes of bone resorption and

bone formation with an increase in the rate of bone resorption (Eriksen et al 1990, Turner et al 1994). Evidence exists to suggest that there is a reduction in mean wall thickness, that is the thickness of bone at the completed remodelling site, in osteoporotic patients compared to controls, leading to a pronounced remodelling imbalance (Eriksen et al 1990, Cohen-Solal et al 1991). Furthermore, although not significant, osteoporotic patients have an increased osteoclast activation frequency and a slight increase in the depth of the resorption pits (Eriksen et al 1990). In the case of osteoporosis, it has been suggested that every resorption cavity is not completely refilled, leading to pronounced thinning of trabeculae and eventual perforation by resorption and subsequent removal (Eriksen et al 1990).

Post menopausal bone loss can be prevented by treatment with estrogen (Lafferty et al 1964, Lindsay et al 1976, Riggs et al 1972) and a combination of estrogen and calcium (Ettinger et al 1987), however the bone lost prior to treatment cannot be restored. The prevention of bone loss following estrogen treatment is associated with a decrease in bone resorbing surfaces (Riggs et al 1972) and a secondary decrease in bone formation (Lafferty et al 1964) presumably due to the tight coupling between bone resorption and bone formation. Cessation of estrogen treatment results in bone loss similar to that observed prior to treatment (Lindsay 1988).

The bone loss following the menopause is associated with an increase in bone turnover with increases in the biochemical markers of bone resorption urine hydroxyproline (Nordin and Polley 1987, Lindsay 1988) and urine deoxypyridinoline (Ebling et al 1996, Ohta et al 1996) and the bone formation markers alkaline phosphatase and osteocalcin (Lindsay

1988). Following the menopause increases in serum calcium and phosphate and a rise in urine calcium are observed which is attributed to a decrease in the renal tubular reabsorption of calcium (Nordin et al 1991, Nordin et al 1994) with suggestions that estrogen deficiency decreases renal sensitivity to PTH (Adami et al 1992). This suggests that estrogen has a direct effect on the kidney and supportive evidence for this action is the identification of estrogen receptors in the kidney (Hagenfeldt and Eriksson 1988). The observed changes in the biochemical variables following the menopause are corrected with estrogen treatment (Lindsay 1988, Adami et al 1992).

The role of estrogens in the regulation of calcitropic hormones is to date, not well established. Calcium absorption decreases at the menopause (Heaney et al 1989) without changes in serum 1,25 dihydroxyvitamin D ($1,25(OH)_2D_3$), the major regulator of calcium absorption (Prince 1994). Estrogen receptors have also been identified in the intestine (Thomas et al 1993) and therefore the decrease in calcium absorption following the menopause may be due to a decrease in the responsiveness of intestinal calcium absorption to $1,25(OH)_2D_3$ in the absence of estrogen (Prince 1994), however this hypothesis requires further investigation. The effect of the menopause on the calciotropic hormone parathyroid hormone (PTH) is inconclusive as some studies suggest no change (Adami et al 1992) while other studies suggest a decrease in serum levels (Cheema et al 1989, Scharla et al 1990). PTH increases the serum levels of calcium by a number of actions including, acting on the bone to increase bone resorption, acting on the kidney to conserve calcium and by stimulating the production of 1-hydroxylase in the kidney, which converts 25 hydroxyvitamin D into its active form $1,25(OH)_2D_3$. It is therefore not surprising that PTH may be affected by estrogen deficiency as all of these processes are altered following the

menopause. However since estrogen has a major effect on many of the organs and hormones that affect extra-cellular fluid calcium levels which in turn determines PTH production, this may explain the diverse reports of the effect of the menopause on PTH levels.

1.4 ESTROGENS

1.4.1 Estrogen receptors in osteoblasts

Estrogen receptors have been identified in osteoblasts in normal human osteoblast-like cells (Eriksen et al 1988) and in osteoblasts derived from rat bone (Hoshino et al 1995, Komm et al 1988). Functional estrogen receptors are required for maintenance of bone as estrogen receptor knockout mice have significant bone loss (Korach 1994) and mutation of the estrogen receptor in a male patient resulted in significant bone loss leading to osteoporosis, unfused epiphyses and continuing linear growth in adulthood (Smith et al 1994).

1.4.2 Estrogen effects on osteoblasts

The effect of estrogen on osteoblasts in culture is controversial. Estrogen has been demonstrated to increase osteoprogenitor cell proliferation (Ernst et al 1988), to directly stimulate proliferation of pure rat osteoblast-like cells (Ernst et al 1988) and to increase creatinine kinase activity and [^{3}H]thymidine incorporation into DNA in rat epiphyseal cartilage cells (Somjen et al 1989). Furthermore, estrogen enhances the potential matrix formation in osteoblast-like cells indicated by an increase in type α_1(I)-procollagen mRNA

levels (Ernst et al 1988) and to increase differentiation of osteoblast-like cells as indicated by a stimulation in alkaline phosphatase activity per cell number (Gray et al 1987). In contrast, estrogen treatment has been demonstrated to transiently inhibit proliferation of rat UMR-106-06 osteoblast-like osteosarcoma cells (Gray et al 1987). Keeting and colleagues (1991) failed to show an effect of estrogen on mature human osteoblast cells. The conflicting effects of estrogen on osteoblasts may be due to the different levels of maturity of the transformed osteoblast cell lines used in these studies, as the UMR-106-06 cell line for example, is less mature than other cell lines. The effect of estrogen on osteoblasts therefore may be dependent on the maturity of the osteoblast and this is reflected in the study by Gray and colleagues (1987) where the alkaline phosphatase response to estrogen was shown to occur during the rapid proliferative phase of the osteoblast-like cell cultures but not during the early days and late stages of the culture when the cells were confluent. Similarly the cells used in the study of Keeting and colleagues (1991) were also of a mature phenotype with a low growth rate and were well differentiated as determined by alkaline phosphatase content and osteocalcin release. These data indicate that estrogen may inhibit proliferation of mature osteoblasts but increase bone formation in addition to its primary effect to inhibit bone resorption, however this effect is in contrast to the effect of estrogen on bone formation in vivo as estrogen treatment decreases bone formation (Wronski et al 1988, Kalu et al 1991b).

The effect of estrogen on bone cells has been shown to be sex specific as diaphyseal bone cells derived from male rats is unaffected by the addition of estrogen to the media. In contrast, epiphyseal bone cells derived from both male and female rats respond to estrogen (Somjen et al 1989).

1.4.3 Estrogen effects on osteoclasts

Estrogen decreases resorption activities as indicated by [^{3}H]proline-labelled rat bone particle assay in avian osteoclasts and osteoclast-like cells (Oursler et al 1993a) which was associated with a decrease in the number of pits formed per avian osteoclast but not the mean pit area. In addition, estrogen decreases the expression of the lysosomal-associated genes, lysosome and LEP 100 (an integral membrane protein of lysosomes) (Oursler et al 1993b). It has been suggested that estrogen has the potential to modulate the transcription of the proto-oncogenes c-*fos* and c-*jun* as estrogen increases the mRNA levels of these genes in avian osteoclasts (Oursler et al 1991).

1.4.4 Effect of estrogens on cytokines

IL-6 has been implicated in osteoclast development (Chapter 1.2.1.4) and is thought to be involved in the bone loss following oophorectomy. Estrogen deficiency results in an increase in production of IL-6 by cultured bone marrow cells in response to either $1,25(OH)_2D_3$ or PTH and withdrawal of estrogen in cultures increases IL-6 production (Passeri et al 1993). Osteoclast formation in response to IL-6 in combination with soluble IL-6 receptor or IL-11 is greater in bone marrow cultures derived from oophorectomised rats than controls (Jilka et al 1994). Thus, not only the production of osteoclast precursors but their responsiveness to IL-6 and other cytokines appears to be enhanced in estrogen deficiency. It is of interest that IL-6 does not appear to play a part in the development of osteoclast precursors in marrow or influence the number of osteoclasts in trabecular bone in animals with sufficient sex steroids (Jilka et al 1992) and therefore in normal conditions, IL-6 is either inactive or its production is below the sensitivity required for osteoclastogenesis (Manolagas and Jilka 1995). Further evidence for the role of IL-6 as the

mediator of bone loss in estrogen deficiency is that IL-6 deficient mice do not have an increase in GM-CFU formation and osteoclastogenesis following oophorectomy and are protected from subsequent bone loss.

IL-1 may also contribute to the pathologic bone loss in estrogen deficiency as its production is increased following oophorectomy in the rat (Manolagas and Jilka 1995). Treatment of oophorectomised rats with an IL-1 inhibitor which directly blocks IL-1 at the level of its receptor, decreases the bone loss following oophorectomy in mice (Kitazawa et al 1994) and in rats (Kimble et al 1994, Kimble et al 1995). The decrease in bone loss following treatment with an IL-1 antagonist was associated with decreases in the number of osteoclasts on endocortical surfaces and decreases in urine deoxypyridinoline excretion (Kimble et al 1995). Furthermore, addition of IL-1 receptor antagonist in combination with TNF binding protein (a TNF inhibitor), has a synergistic effect on inhibiting bone resorption in oophorectomised rats with total prevention of bone loss (Kimble et al 1995).

Oophorectomy also results in an increase in IGF-1 and IGF binding protein 3 (IGF-BP3) (Kalu et al 1994). IGF-1 has been demonstrated to increase IL-6 production by mouse long bones (Slootweg et al 1993) and therefore could have implications in the increased osteoclastogenesis that occurs following oophorectomy. This increase in IGF-1 can be reversed with estrogen treatment.

1.5 ANDROGENS

Testosterone is the major circulating androgen. Testosterone is transported in the blood bound to albumin and sex hormone binding globulin (SHBG) (Schweikert and Romalo 1990). SHBG is a hepatic secreted protein which also binds other 17β-hydroxylated steroids such as estradiol and its secretion is enhanced by estrogens and suppressed by androgens. The protein-bound portion of the steroid is in dynamic equilibrium with the free or unbound fraction which can enter target cells (Schweikert and Romalo 1990). The testosterone available to the tissues is the sum of testosterone weakly bound to albumin and that unbound to protein. Testosterone is metabolised in the liver by oxidation to 4-androstenedione and by reduction to 5-α or 5-β-reduced, 3,15, and 16 hydroxylated compounds (Mooradian et al 1987). In addition to the androgenic effects of testosterone, it may also have biological effects as an anti-estrogen by competing with estrogen for the estrogen receptor (Zava and McGuire 1978, Casey and Wilson 1984). In reproductive tissues, testosterone is reduced to dihydrotestosterone (DHT) via the enzyme 5α-reductase and can be aromatised to estrogen (Mooradian et al 1987). Unlike testosterone, DHT cannot be further aromatised to estrogen and is two to three times more potent than testosterone and has a higher affinity for the androgen receptor (Mauvais-Jarvis et al 1981, Chang et al 1988).

1.5.1 Effect of androgens on bone cells in culture

1.5.1.1 Androgen receptors in osteoblasts

Androgen receptors have been identified in osteoblastic cell lines derived from human and rat origin (Colvard et al 1989, Orwoll et al 1991, Masuyama et al 1992) and also in bone marrow-derived murine stromal cells (Bellido et al 1993). These receptors have high binding affinity for all androgens and are of similar structure in humans and in the rat, and thus the rat is an appropriate model to investigate the actions of androgens on bone cells (Chang et al 1988). Human bone cells from male and female donors have similar concentrations of estrogen and androgen receptors, suggesting that both sex steroids play an important role in the maintenance of bone mass in both sexes (Colvard et al 1989). Androgens therefore, are also important in postmenopausal women whose serum estrogen levels can be low while serum testosterone is reduced only slightly (Meldrum et al 1981).

1.5.1.2 Androgen metabolism in bone cells

Bone cells derived from humans (Schweikert et al 1980) and rats (Vittek et al 1974) are able to metabolise testosterone to DHT and estrogen. In fact, patients with 5α-reductase abnormalities have retardation in the maturation of bone, suggesting that the maturation of bone, in particular the epiphyses, is mediated by DHT rather than testosterone (Fisher et al 1978).

1.5.1.3 Effect of Androgens on osteoblasts in-vitro

DHT stimulates the proliferation of mouse calvarial cells (Kasperk et al 1989), rat bone cells (Somjen et al 1991) and MC3T3-E1 osteoblast-like cells (Masuyama et al 1992).

DHT increases [^{3}H]thymidine incorporation into these cells in a dose dependent manner (Gray et al 1992, Kasperk et al 1989, Somjen et al 1991), indicating increased DNA synthesis, and [^{3}H]proline incorporation, an index of collagen production (Gray et al 1992) while also increasing creatinine kinase production, indicating increased cell metabolism (Somjen et al 1991). In addition, DHT increases the number of differentiated bone cells as indicated by positive staining for alkaline phosphatase (Kasperk et al 1989). The actions of DHT on bone cells suggest that androgens act directly on osteoblastic cells through a receptor mediated mechanism (Masuyama et al 1992).

It has been proposed that androgens directly affect proliferation and differentiation of osteoblastic cells by; 1) modulating the production of growth factors, 2) changing the responsiveness of bone cells to growth factors and/or 3) affecting the binding of growth factors to bone cells (Kasperk et al 1990). Evidence exists to suggest that androgens act by each of these mechanisms. Firstly, DHT increases the amounts of both TGFβ and TGFβ_2 mRNA in human bone cell cultures. TGFβ has been demonstrated to stimulate bone cell differentiation as indicated by increased levels of alkaline phosphatase and type 1α collagen (Noda and Rodan 1987). Secondly, DHT treatment enhanced the bone cell response to fibroblast growth factor (FGF) and IGF-II which is not mediated by the increased levels of TGFβ following androgen treatment. Thirdly, DHT acts to increase the IGF-II receptor binding in osteoblastic cells, which is largely due to an increase in receptor number rather than IGF-II receptor affinity (Kasperk et al 1990). Further supportive evidence for a direct effect of androgens on bone cells is the action of Stanzolol, a synthetic anabolic steroid, to increase osteoblast cell number, 1,25$(OH)_2$ D_3-dependent osteocalcin

synthesis, indicative of bone formation and the synthesis of alkaline phosphatase, an enzyme utilised by osteoblasts (Vaishnav et al 1988).

Androgens can directly stimulate mineralisation of human osteoblast-like osteosarcoma cells in culture as indicated by calcium accumulation in the extra-cellular matrix. Mineralisation occurs approximately 10 days following androgen treatment and is associated with an increase in the number of androgen receptors and androgen receptor mRNA, which possibly contributes to the DHT stimulation of mineralisation in osteoblasts (Takeuchi et al 1994).

The response of bone cells to androgens is modulated by local and systemic factors such as $1,25(OH)_2$ D_3. These actions differ depending on the origin of the bone cells. For instance, with cells derived from the long bones of rats, $1,25(OH)_2$ D_3 acts synergistically with testosterone to increase proliferation, while in calvarial cells $1,25(OH)_2$ D_3 abolishes the effect of DHT (Gray et al 1992). This differential effect of $1,25(OH)_2$ D_3 in combination with androgens in different bone cell cultures may have arisen due to treatment of the cultures with different androgens. Testosterone can be metabolised to estrogen which is well established to increase proliferation in osteoblast cells (Ernst et al 1988, Somjen et al 1989). Thus treatment with testosterone may have resulted in both an estrogenic and androgenic effects on the cells derived from the long bones in comparison to the calvarial cells which were treated with DHT which cannot be further metabolised.

1.5.1.4 Effect of androgens on osteoblast gene expression

To date only a few studies have investigated the effect of androgens on the expression of some of the genes associated with osteoblast development. Bodine et al (1995) demonstrated that testosterone, DHT or androstendione had no effect on the mRNA levels of c-*fos*, c-*jun* or TGFβ1 in human osteoblast cells in-vitro. Interestingly, dehydroepiandrosterone (DHEA) and its metabolite dehydroepiandrosterone sulphate (DHEAS) rapidly and significantly reduced the mRNA levels of c-*fos* (Bodine et al 1995). This mechanism of DHEAS to decrease c-*fos* mRNA is not fully understood as no receptor for DHEAS has been identified in rat bone and this action could not be due to its metabolism to testosterone, DHT or estrogen as the effect observed was too rapid to allow time for the metabolism to occur. In human-derived osteoblast-like osteosarcoma cells the androgens testosterone and DHT, increased mRNA levels of type α_1(I)-procollagen and TGFβ (Benz et al 1991). It has been proposed that increased expression of type α_1(I)-procollagen would increase bone matrix synthesis by osteoblasts, resulting in increased bone density (Benz et al 1991) as is observed with postmenopausal women following treatment with synthetic androgens (Chapter 1.5.3).

1.5.1.5 Effect of androgens on osteoclasts in vitro

Androgen receptors have been identified in bone marrow stromal cells (Bellido et al 1993) and in osteoclast-like mutlinucleated cells (Mizuno et al 1994), suggesting a role of androgens in bone resorption. DHT however, has been shown to have no affect on osteoclasts in culture with or without the presence of osteoblast cells (Tobias and Chambers 1991). Similarly, DHT has no effect on osteoclasts derived from fetal rat long bones as determined by ^{45}calcium release (Caputo et al 1976). In contrast, DHT at high

doses in osteopenic oophorectomised rats decreases osteoclast surface and number resulting in decreased bone resorption (Chapter 1.7). Perhaps the action of androgens is by an indirect mechanism through other calcium regulating hormones or cytokines which are not present in the cell cultures. Supportive evidence for this hypothesis is the action of DHT to inhibit the cyclic adenine monophosphate (cAMP) response to PTH, a potent bone resorbing agent, in human osteoblast-like cells (SaOS-2) (Fakayama and Tashjian 1989). In addition, testosterone and DHT inhibit the production of interleukin-6 (IL-6) by murine bone marrow-derived stromal cells (Bellido et al 1995), through the androgen specific receptor to inhibit expression of the IL-6 gene. IL-6 has been identified as a mediator of bone loss (Managolas and Jilka, 1995) and in estrogen and androgen deficiency, upregulation of the IL-6 gene occurs with increased osteoclastogenesis and therefore increased bone resorption (Bellido et al 1995). At present the action of androgens on bone resorption remains uncertain and requires further investigation.

1.5.2 Effect of androgens on bone in animals

1.5.2.1 Androgen Deficiency in male rats

Androgens have been shown to be important in the preservation of the rat skeleton since castration in male rats results in the loss of both cortical and trabecular bone with the development of osteopenia of the lumbar vertebrae, tibia and femur (Gunness and Orwoll 1995, Danielsen et al 1992, Vanderschueren et al 1992). This bone loss occurs in young (Turner et al 1990a), adult (Danielsen et al 1992) and aged rats (Vanderschueren et al 1992, Vanderschueren et al 1993a) following orchidectomy, however bone loss is detectable 5

weeks following orchidectomy in young rats (Turner et al 1990a), 3 months in adult rats (Danielsen et al 1992) and 4 months in aged rats (Vandershueren et al 1992). Although bone loss is not observed until after 3 months following orchidectomy in adult and aged rats, bone turnover is elevated at only 5 weeks following orchidectomy (Schot et al 1993). The delay in detection of the bone loss observed following orchidectomy in adult and aged rats is possibly due to the decrease in bone metabolism rate that occurs with age in the rat (Kalu et al 1989).

Associated with the loss of bone following orchidectomy is an increase in bone turnover with the suggestion that an imbalance exists between bone formation and resorption. The mechanism by which orchidectomy decreases bone volume differs between trabecular and cortical bone. Following orchidectomy, trabecular bone volume decreases in association with increases in bone formation rate, osteoblast and osteoid surface, mineralising surfaces, mineral apposition rate, as well as an increase in the osteoclast surface and number (Vanderschueren et al 1992, Gunness and Orwoll 1995). In contrast, the cortical bone volume decreases due to marked decreases in wall thickness and collagen content (Danielsen et al 1992). A decrease in bone formation at the periosteal surface is also observed following orchidectomy, however the bone formation rate at the endosteal surface is unaffected. Mineralising surfaces at the periosteal surface decrease while mineral apposition rate remains unchanged (Gunness and Orwoll 1995), indicating that the bone loss arises due to a decrease in bone cell number and not bone cell activity. The effect of orchidectomy on trabecular and cortical bone demonstrates the different responses of these bone tissues to androgens.

Biochemical markers of bone turnover are increased in association with the bone loss following orchidectomy. Serum osteocalcin, a marker of bone formation, is elevated one month following orchidectomy, which is no longer significant after 3 months compared to controls (Vandershueren et al 1992, Vandershueren et al 1994). In contrast, alkaline phosphatase, an enzyme utilised by osteoblasts, is not affected by orchidectomy. Bone resorption markers urine pyridinoline and deoxypyridinoline are also increased in relation to sham rats following orchidectomy (Vandershueren et al 1992). The increase in bone resorption markers occurs before the rise in osteocalcin since bone resorption is coupled to and precedes bone formation. Similar patterns in these biochemical markers of bone turnover are observed following estrogen deficiency in female rats (Chapter 1.6.1, Sims et al 1996a).

1.5.2.2 Androgen replacement in orchidectomised rats

The bone loss observed following orchidectomy in male rats can be prevented by treatment with testosterone, DHT, 1 dehydrotestosterone (DeHT) and nandrolone decanoate (Wakely et al 1991, Schot et al 1993, Vandershueren et al 1992). Treatment of young castrated rats with low doses of testosterone partially prevents cancellous bone loss, while treatment with medium doses of testosterone totally prevents bone loss and treatment with high doses of testosterone not only prevents the bone loss associated with orchidectomy, but results in increased bone volume (Wakely et al 1991). The synthetic androgen nandrolone decanoate decreases bone turnover in rats 5 weeks following orchidectomy and completely prevents bone loss in rats 4 months following orchidectomy (Schot et al 1993). Testosterone and nandrolone decanoate are more effective in preventing the bone loss following orchidectomy on a weight basis (Wakely et al 1991, Vandersheuren et al 1992), possibly

because they can be aromatised to estrogen, unlike DHT. Estrogen treatment has been demonstrated not only to conserve bone mass in orchidectomised rats but to increase cancellous bone mass compared to controls (Vandersheuren et al 1992). It has been suggested that estrogen exerts this positive effect on bone mass by strong inhibition of bone resorption (Vandersheuren et al 1992). Testosterone and estrogen may therefore act synergistically to increase bone mass. The decrease in bone loss with androgen treatment is associated with a reduction in the bone turnover indices; osteoclast number, the amount of bone surface covered by active osteoclasts and osteoblasts and bone formation rate (Wakely et al 1991, Vandersheuren et al 1992).

A study by Kapur and Reddi (1989) using a bone matrix-induced endochondral bone development model demonstrated that DHT promotes mineralisation of bone in young 3 week old castrated rats. The increase in bone mineralisation was preceded by an increase in alkaline phosphatase with peak levels occurring at 10 days following treatment with subsequent peak calcium mineralising activity occurring at 12 days (Kapur and Reddi, 1989). At 21 days following treatment, alkaline phosphatase levels were unaffected, however mineralisation reflected by calcium levels were high.

The described changes in bone following orchidectomy in male rats was not accompanied by changes in the calciotropic hormone $1,25(OH)_2D_3$ or nephrogenous cAMP (Vandersheuren et al 1992).

1.5.2.3 Estrogen treatment in orchidectomised rats

Estrogens have been demonstrated to suppress the increased bone turnover in osteopenic orchidectomised rats in addition to increasing cancellous bone volume (Vandersheuren et al 1992). This increase in cancellous bone volume is a result of the inhibitory action of estrogen on bone resorption. This effect of estrogen has also been demonstrated in normal male rats with no evidence of an anabolic action (Wakley et al 1997). Androgen receptors in androgen-resistant, testicular feminised (Tfm) rats have a deficiency in binding androgens caused genetically by a single point mutation in the steroid binding domain of the androgen receptor (Yarbrough et al 1990). As a result, the androgens can not be utilised by the tissues due to the absence of a functional androgen receptor, and are therefore aromatised to DHT and testosterone (Bruch et al 1992) which is further aromatised to estrogen, thus these rats have higher concentrations of circulating estrogens compared to normal male rats (Vandersheuren et al 1993b). Tfm rats have bone structures similar to that of female rats, with shorter and thinner femora and decreased body weight compared to normal male rats, thus suggesting that androgen receptor activation is required for full skeletal growth and weight gain in male rats. Interestingly, Tfm rats maintain trabecular bone volume with a normal rate of bone formation, osteoblast surface and serum osteocalcin levels (Vandersheuren et al 1993b), unlike young and old orchidectomised rats which develop cancellous osteopenia (Section 1.5.2.1). The preservation of the skeleton in Tfm is possibly due to the changes in the steroid profile of these rats as the high levels of estrogen would account for the development of the feminine bone structures in these rats.

Androgen effects on bone cells may be sex specific as diaphysial bone cells derived from male rats respond to androgens in culture while bone cells derived from females do not.

This sex-specific response of diaphyseal bone can be abolished in perinatal rats by gonadectomy, thus allowing bone cells from either male of female rats to respond to both estrogens and androgens (Somjen et al 1994).

1.5.3 Effect of androgens on bone in humans

Hypogonadism in males results in bone loss clearly indicating the importance of androgens in the maintenance of bone in males (Francis et al 1986). Testosterone production and clearance both decline substantially with age associated with the slowly declining levels of available testosterone after the age of 50 (Mooradian et al 1987). Treatment with testosterone increases bone formation by increasing bone forming surfaces as determined from iliac crest biopsies (Francis et al 1986, Nordin et al 1981).

Androgens also appear to play an important role in the maintenance of bone in women, as testosterone levels have been found to decrease following the menopause and are strongly correlated with bone loss (Nordin et al 1985). In addition, the adrenal androgens precursors DHEA and DHEAS decline with age (Meldrum et al 1981). Their effects can be observed prior to the menopause in women who are still menstruating but are becoming testosterone deficient due to a decrease in available testosterone (Steinberg et al 1989). Patients suffering from hip fractures have been demonstrated to have significantly lower levels of free estrogen and testosterone with higher levels of sex-hormone binding globulin (SHBG) and is attributed to the difference in the body size of the hip fracture patients,

which were more slender compared to normal premenopausal controls (Davidson et al 1982).

In premenopausal women suffering from hirsutism with excess serum levels of 3α-androstanediol glucuronide, (a peripheral marker of intracellular androgen metabolism), androstenedione, (the major precursor of 3α-androstanediol glucuronide), DHEAS and testosterone, trabecular bone density was significantly higher and cortical bone density was insignificantly higher compared to normal premenopausal controls (Buchanan et al 1988). which indicates the positive action of androgen on bone density in women.

Many trials for the use of androgens as a treatment for postmenopausal osteoporosis have been conducted with the aim of replacing the bone lost following the menopause. Nandrolone decanoate, a synthetic androgen, has been demonstrated to increase bone mineral density in the forearm (Need et al 1987) and bone mineral content of the distal forearm in post menopausal women (Johansen et al 1989) and at the radius in osteoporotic men and women suffering from vertebral crush fractures (Geusens and Dequeker 1986).

The positive effect of androgens on bone mineral density of the forearm was accompanied by an increase in calcium absorption with no effect observed on urine hydroxyproline, a marker of bone resorption (Need et al 1987). These data suggest that androgens may act directly to increase bone mineral density by increasing bone formation. To date this action of androgens in postmenopausal women has not been confirmed as analysis of bone formation markers such as alkaline phosphatase have been shown to be unaffected by androgen treatment (Need et al 1987, Johansen et al 1989), while other studies demonstrate

a decrease in alkaline phosphatase following treatment (Riggs et al 1972, Chestnut et al 1983). Caution must be taken in the interpretation of these data as all of these measurements were of total alkaline phosphatase. Since alkaline phosphatase is also synthesised by the liver, the measurement of total alkaline phosphatase could possibly be obscured by an affect of androgens on the liver. Measurement of liver enzymes known to be affected by alterations in liver function such as alanine amino transferase would provide useful information to the affect of androgens on alkaline phosphatase.

Johanssen and co-workers (1989) demonstrated that nandrolone decanoate, combined with oral calcium supplementation, increased bone mineral content with no effect on bone formation as indicated by osteocalcin, a protein synthesised by osteoblasts and thus a specific marker of bone formation (Brown et al 1984). The increase in bone mineral content was attributed to a direct suppression of bone resorption, however the calcium supplements administered with nandrolone decanoate may have obscured the results due to the anti-resorptive actions of calcium (Wright and McMillan 1994). Riggs and colleagues (1972) demonstrated a decrease in bone resorbing surfaces, although not significant, following nandrolone decanoate treatment in postmenopausal osteoporotic women and Lafferty and colleagues (1964) demonstrated a reduction in isotopic resorption rate following Dianabol therapy (a synthetic androgen).

In addition to a direct effect on bone, the administration of Stanozolol, another synthetic androgen, in postmenopausal osteoporotic women results in an increase in total body calcium and serum creatinine, consistent with increased muscle mass, possibly indirectly contributing to the stimulation of bone formation (Chestnut et al 1983). Need and

colleagues (1987) also identified the increase in serum creatinine following nandrolone decanoate treatment in postmenopausal osteoporotic women which was associated with increased skin thickness (Need et al 1987).

Androgens have also been demonstrated to promote a positive calcium balance by decreasing urine calcium (Riggs et al 1972, Chestnut et al 1983, Need et al 1987) while increasing the tubular reabsorption of calcium at the kidney (Need et al 1987). Whether this is a direct effect of androgens on the kidney or by an indirect mechanism through parathyroid hormone is yet to be established. Serum immunoreactive parathyroid hormone has been shown to be unaffected (Riggs et al 1972) while other studies have demonstrated a decrease following androgen treatment (Chestnut et al 1983). A direct effect of androgens on the kidney to decrease urinary calcium would lead to an increase in serum calcium which would account for the decrease observed in parathyroid hormone following treatment. A decrease in serum calcium has been identified in osteoporotic postmenopausal women following treatment with nandrolone decanoate (Need et al 1987).

Treatment of postmenopausal osteoporotic women for three years with a combination of hormone replacement therapy (HRT) and nandrolone decanoate results in an increase in lumbar bone mineral content of 3.5% and this effect is not observed with HRT alone (Erdstieck et al 1994). The favourable addition of nandrolone decanoate is still present one year after cessation of treatment, demonstrating the positive effect of androgens on bone. In agreement with these findings Raisz and colleagues (1996) demonstrated that the addition of a small dose of oral androgen (methyltestosterone) in combination with estrogen replacement therapy can reverse the inhibitory effects of estrogen on the markers

of bone formation without altering the inhibitory effects of estrogen on bone resorption. In fact, the addition of oral androgen resulted in an increase in the bone formation markers osteocalcin, bone specific alkaline phosphatase and C-terminal procollagen peptide (Raisz et al 1996). These data suggest that combined androgen and estrogen treatment may possibly have a positive effect on bone density by stimulation of bone formation by androgens, in addition to an inhibition of bone resorption by estrogen, although androgens may also act to inhibit bone resorption. Further studies are required to confirm this action and to investigate whether this effect is sustained for longer treatment periods.

Clearly the mechanism by which androgens increase bone density and bone mineral content in postmenopausal women remains unclear with suggestions of an anabolic effect on bone formation and/or an inhibitory effect on bone resorption which requires further investigation.

1.6 THE OOPHORECTOMISED RAT MODEL OF POSTMENOPAUSAL BONE LOSS

1.6.1 The effect of oophorectomy on rat bone

The oophorectomised rat is a widely used and well established model of postmenopausal bone loss, since the bone loss which occurs in both of these states is similar in many ways (Kalu et al 1991a, Wronski et al 1986, Yamazaki and Yamaguchi 1989). Oophorectomy in the rat results in a decrease in bone calcium per unit volume of bone (Saville 1969)

resulting in osteopenia in the tibia (Wronski et al 1986, Turner et al 1989), femur (Yamazaki and Yamaguchi 1989, Kalu et al 1989) and lumbar vertebrae (Wronski et al 1986, Kalu et al 1989). The oophorectomy induced bone loss varies at different sites with a greater loss of trabecular than cortical bone (Yamazaki and Yamaguchi 1989).

The trabecular bone loss following oophorectomy is due to rapid osteoclastic perforation of the trabecular plates and their subsequent removal, suggested to occur where mechanical stress is lowest (Yoshida et al 1991). The loss of trabecular bone and connectivity occurs without prior thinning of the trabecular plates (Dempster et al 1995). Associated with the bone loss following oophorectomy is an increase in the percentage of total trabecular surface covered by osteoclasts in the tibial diaphysis (Turner et al 1989, Wronski et al 1989a) and periosteal and endosteal bone formation is also increased following oophorectomy (Turner et al 1989).

The bone loss following oophorectomy in the tibiae and femur is characterised by a rapid phase of bone loss occurring during the first 3 months following oophorectomy (Wronski et al 1989a) with a decrease in bone volume detectable at 15 days post-oophorectomy (Dempster et al 1995, Sims et al 1996a) after which the trabecular bone volume stabilises at approximately 25-30% of control rats (Wronski et al 1989a). This is followed by a slow late phase of bone loss at approximately 9 months post-operation which is attributed to aging as this bone loss is also observed in control rats (Wronski et al 1989a). The loss of bone is associated with an increase in bone resorption within 5 days following oophorectomy as identified by increased osteoclast surface (Dempster et al 1995, Sims et al 1996a) and in the biochemical markers urine hydroxyproline (Sims et al 1996a) and urine

pyridinoline (Frolick et al 1996). Bone formation however, is not elevated until 10-12 days post-oophorectomy as indicated by increased bone formation rate (Dempster et al 1995), mineral apposition rate, double labelled surface (Sims et al 1996a) and the biochemical markers alkaline phosphatase and osteocalcin (Sims et al 1996a). These data are consistent with the model that the effect of estrogen deficiency on bone cell activity is to stimulate bone resorption which is followed approximately 5 days later by an increase in bone formation, due to the coupling between these two processes.

In contrast, the vertebrae undergoes the same biphasic pattern of bone loss following oophorectomy as seen in the tibia, however the initial phase of bone loss does not occur until 60 days post-oophorectomy with 270 days required for loss of 50% of trabecular bone compared with 30-60 days in the tibiae. In the vertebrae age-related bone loss is not observed (Wronski et al 1989b).

Associated with oophorectomy in the rat is an increase in body weight (Tarttelin and Gorski 1973) which has been suggested to affect the development of osteopenia. Wronski and colleagues (1987) demonstrated only a partial protection against the development of osteopenia in oophorectomised rats which was proposed to occur through stimulation of bone formation by mechanical stress in weight bearing bones.

The increase in bone turnover following oophorectomy is associated with an increase in the biochemical markers of bone resorption urine hydroxyproline (Morris et al 1992) and urine pyridinoline (Frolick et al 1996) and in the markers of bone formation alkaline phosphatase and osteocalcin (Morris et al 1992, Ismail et al 1988). A positive correlation between urine

hydroxyproline excretion and serum ionised calcium has been identified following oophorectomy in the rat and is consistent with a direct effect of the estrogen deficiency on bone cells, stimulating resorption (Morris et al 1992).

Oophorectomy in adult rats results in a reduced calcium balance compared to control rats. The decrease in calcium balance is due in the short-term to an increase in intestinal calcium secretion and urine excretion and in the long-term to a reduction in intestinal calcium absorption (O'Loughlin 1997).

Although the bone loss in rats following oophorectomy shares the characteristics described above with the bone loss following the menopause in women, it must be noted that there are a few important differences that must be taken into consideration. Osteopenic oophorectomised rats do not experience fractures as postmenopausal women do which are the 'hallmark' of postmenopausal osteoporosis and in addition, the cortical bone of rats lacks Harvesian systems. The effect of oophorectomy in the rat on urinary calcium excretion is to date unclear as some studies demonstrate no change (Morris et al 1992, Yamazaki and Yamaguchi 1989) while more recent studies have identified an increase following oophorectomy (Morris et al 1995) which is consistent with the postmenopausal woman. Apart from these differences the oophorectomised rat is an appropriate and convenient model for postmenopausal bone loss, in particular trabecular bone and in the study of factors that affect bone turnover and bone loss as the effects of oophorectomy on bone in the rat closely parallels that of women following the menopause.

1.6.2 Effect of estrogen replacement in oophorectomised rats

The bone loss that occurs following oophorectomy in the rat can be prevented with estrogen replacement (Wronski et al 1988, Kalu et al 1991b, Abe et al 1993, Frolik et al 1996) as is observed with hormone replacement in women (Lindsay 1988). Estrogen exerts this bone preserving effect by depressing the raised bone turnover following oophorectomy with decreases in both bone resorption and bone formation as indicated by quantitative histomorphometric techniques (Wronski et al 1988, Abe et al 1993, Turner et al 1987) and by biochemical markers of bone turnover (Kalu et al 1991b, Frolik et al 1996). Histomorphometric techniques have identified decreases in bone formation rate, mineral apposition rate, osteoblast surface and osteoclast surface in trabecular bone (Wronski et al 1988, Abe et al 1993) and in cortical bone (Turner et al 1987). Estrogen treatment in oophorectomised rats decreases the bone resorption markers tartrate-resistant acid phosphatase, urine hydroxyproline (Kalu et al 1991b) and urine pyridinoline (Frolik et al 1996) while decreasing the bone formation markers alkaline phosphatase and osteocalcin (Kalu et al 1991b, Frolik et al 1996). These decreases can be observed from 7 days following treatment (Frolik et al 1996, Sims et al 1996b). Estrogen replacement however does not restore the bone lost prior to treatment (Abe et al 1993) and withdrawal of treatment results in a rapid loss of cancellous bone (Wronski et al 1993). Progesterone has also been implicated in preventing bone loss following oophorectomy (Aitken et al 1972).

In addition to its effect on bone, estrogen has been demonstrated to increase intestinal calcium absorption in normal rats (Arjmandi et al 1994) indicating a possible role to restore the impaired intestinal calcium absorption observed following oophorectomy in the rat.

Other agents such as bisphosphonates (Wronski et al 1993), tamoxifen and human calcitonin (Abe et al 1993) also suppress the increased bone turnover following oophorectomy by inhibiting bone resorption, however not as effectively as that observed with estrogen treatment. Parathyroid hormone given in small doses has been shown to have an anabolic effect on bone resulting in increased bone volume in intact (Jerome 1994) and oophorectomised rats (Hock et al 1988).

1.6.3 Effect of oophorectomy on bone cell gene expression

1.6.3.1 Effect of oophorectomy on osteoclast gene expression

Following oophorectomy, marked increases of 3 to 4 fold in the mRNA levels of the carbonic anhydrase II (CAII) and tartrate-resistant acid phosphatase (TRAP) genes are observed from 1 week post-operation which are sustained up to 8 weeks post-operation. This increase in mRNA levels of bone-resorbing enzymes was suppressed by the administration of estradiol from 18 hours following treatment (Zheng et al 1994). The mechanism by which this occurs is unclear. Whether there is a direct effect of estrogens on osteoclasts to suppress gene transcription of CAII and TRAP or by an indirect effect to inhibit production by osteoblasts, stromal cells or monocytes of cytokines, the mediators of bone resorption, remains to be established. In contrast, Petersen and colleagues (1992) were unable to identify any changes in TRAP mRNA following oophorectomy in the proximal femur, proximal tibia or calvaria in the rat, thus further investigation of the role of estrogen on osteoclast gene expression is required.

1.6.3.2 Effect of oophorectomy on osteoblast gene expression

In osteoblasts, oophorectomy has been demonstrated to increase the mRNA levels of the genes for c-*myc*, type 1α collagen and osteocalcin in vitro and in vivo 3 weeks post-operation in rat trabecular bone (Salih et al 1993, Ikeda et al 1993). In situ hybridisation techniques have identified this increase in collagen mRNA following oophorectomy to occur in osteoblasts on the bone surface at the metaphysis and epiphysis (Ikeda et al 1996). Estrogen deficiency also results in a decrease in the mRNA levels of TGFβ in bone cells (Petersen et al 1992, Noda et al 1989).

1.7 EFFECT OF ANDROGENS ON BONE IN INTACT AND OOPHORECTOMISED FEMALE RATS

It has been suggested that adrenal androgen deficiency may lead to osteopenia. Adrenalectomy in female rats causes femoral trabecular bone loss similar to that observed following oophorectomy which can be halted by treatment with nandrolone decanoate (Durbridge et al 1990). Furthermore, treatment of intact female rats with the potent anti-androgen flutamide, lowers skeletal calcium content and weight of femoral bone per unit volume thus resulting in osteopenia. The suggested mechanism for this bone loss is that flutamide depresses bone formation in contrast to buserelin, (a gonadotropin releasing hormone antagonist inhibiting ovarian estrogen secretion) which causes osteopenia by stimulating bone breakdown (Goulding and Gold 1993). Further supportive evidence is the lack of flutamide to significantly elevate bone resorption as indicated by no change in urine hydroxyproline, a biochemical marker of bone resorption and total-body radio-labelled

calcium with only a slight increase in urinary radio-labelled calcium (Goulding and Gold 1993).

The bone loss that occurs in rats which have been adrenalectomised and oophorectomized does not differ significantly from the bone loss arising from adrenalectomy or oophorectomy alone (Durbridge et al 1990). Similarly the bone loss in rats treated with both flutamide and buserelin in comparison to rats treated with either flutamide or buserelin alone also did not differ (Goulding and Gold 1993). It has been proposed that the rise in the rate of bone formation stimulated by estrogen deficiency may counteract the decrease in bone formation rate in androgen deficiency (Goulding and Gold 1993).

Treatment with nandrolone decanoate in oophorectomised and adrenalectomised animals at 9 weeks post-operation when osteopenia is established prevents further bone loss, however treatment with dehydroepiandrosterone by intraperitoneil injection twice a week has no effect (Durbridge et al 1990). In contrast, a study by Turner et al (1990b) demonstrated that treatment of intact and oophx rats with dehydroepiandrosterone, by control release pellets, results in a reduction of the cancellous bone osteopenia following oophorectomy, however with no effect on cortical bone, further highlighting the importance of adrenal androgens in the maintenance of trabecular bone in female rats. The results of these two studies may differ due to the different serum concentrations of DHEAS achieved following treatment as a result of different administration protocols.

When nandrolone decanoate is administered to three month old oophorectomised rats at the time of operation, that is when the animals have a high rate of bone turnover due to

oophorectomy, and to adult oophorectomised rats, the oophorectomy-induced increase in serum osteocalcin levels and loss of trabecular bone is inhibited with a dose related increase in cortical width, femoral length and calcium content. Thus in the growing and adult rat, nandrolone has an anti-catabolic effect on increased bone turnover and bone loss due to estrogen deficiency, and an anabolic effect on longitudinal and periosteal bone growth (Schot et al 1993). In adult 19 month old oophorectomised rats treatment with nandrolone decanoate prevents the oophorectomy-induced trabecular bone loss and cortical bone loss, in addition to increasing metaphyseal trabecular bone density. Furthermore, nandrolone decanoate treatment increased femur length and femur dry weight and decreased the serum osteocalcin levels which were raised as a result of oophorectomy. This indicates an anti-catabolic action as well as an anabolic effect of nandrolone decanoate on bone metabolism in older rats (Schot et al 1993).

Tobias and colleagues (1994) investigated the effect of androgens on specific skeletal sites in the osteopenic oophorectomised rats (a model with a low rate of bone turnover). DHT was administered to rats 90 days following oophorectomy in order to allow osteopenia to develop and resulted in a dose-related increase in bone volume reflected by an increase in both trabecular number and thickness. The effect of DHT on trabecular thickness appeared stronger as it was increased by all doses of DHT and trabecular thickness but not number, was restored in treated rats to that of control rats. In contrast, the number of trabeculae were preserved in the oophx rats following treatment but were not restored to that of control rats. It has been suggested that androgens are able to increase the trabecular bone volume of the skeleton by increasing the size of existing trabeculae, rather than increasing the production of new trabeculae. DHT treatment also produced a stimulatory effect on

bone formation at the growth plate, endocortical and periosteal surfaces, suggesting that DHT exerts an anabolic effect on the skeleton as a whole. In addition Coxam and collegues (1996) demonstrated increases in bone formation rates at the periosteal surface and in trabecular bone producing an increase in bone mineral density and calcium content. The increases in bone formation rate was attributed to increases in the active bone forming surface as indicated by increased double labelled surface and mineralising surface rather than due to changes in mineral apposition rate.

At high doses of DHT (1mg/kg body weight daily) osteoclast surface and number are suppressed. It is of interest, that at these high doses of DHT, the bone formation rate still did not differ from oophorectomised control rats at 60 days post-treatment. The inhibition of bone resorption at the high dose however, may result in a reduction of bone formation due to the coupling between these two processes at a later time. Thus, DHT increases cancellous bone volume in osteopenic oophorectomised rats in association with both anabolic and antiresorptive actions (Tobias et al 1994) and further highlights the importance of androgens in the maintenance of bone in female rats.

Since androgens have an anabolic action on bone formation and estrogens have an inhibitory effect on bone resorption, combination treatment of these steroids therefore may prove to be of greater benefit to the bone than either treatment alone. Coxam and collegues (1996) demonstrated that DHT treatment in combination with estrogen to osteopenic oophorectomised rats increased bone mineral content, total bone calcium content and endochondral bone growth. In cortical bone, DHT in combination with estrogen increased the bone formation rate and mineral apposition rate but was less effective at increasing

these bone parameters in trabecular bone. Furthermore, DHT in combination with estrogen resulted in the highest bone calcium content per weight basis, suggesting a synergistic effect of androgens and estrogens on bone mass. Interestingly, it appeared that the addition of DHT in combination with estrogen had the greatest effect at overcoming the suppressive effects of estrogen on bone formation in cortical bone rather than trabecular bone. The results from this study suggest that androgens promote bone formation and bone growth and partially reverse the suppressive effects of estrogen on bone formation, particularly in the cortical bone.

1.8 AIMS AND HYPOTHESES

1.8.1 The effect of oophorectomy on osteoblast and osteoclast gene expression.

While the histomorphometric and biochemical effects of oophorectomy on bone metabolism are well documented, there is little documentation on the effect of estrogen deficiency on gene expression in osteoblasts or osteoclasts. It has been identified that the loss of bone following estrogen deficiency in female rats is associated with an increase in bone turnover, presumably with the rate of bone resorption exceeding the rate of bone formation. This has been identified by histomorphometric techniques and by measurement of bone biochemical variables.

Hypothesis 1: Oophorectomy in the rat has been previously shown to result in an increase in the mRNA levels of the osteoclast genes carbonic anhydrase II and tartrate-resistant acid phosphatase. Thus, we hypothesise that the mRNA levels of calcitonin receptor, specific

receptors present on osteoclasts, will increase following oophorectomy. Similarly, since oophorectomy is associated with an increase in bone formation as well as an increase in bone resorption, we therefore hypothesise that the mRNA levels of c-*fos*, type 1α collagen, alkaline phosphatase, osteopontin and osteocalcin will be increased in the estrogen deficient state.

1.8.2 The effect of androgens on the biochemical markers of bone turnover and on osteoblastic and osteoclastic gene expression in sham, oophorectomised and osteopenic oophorectomised rats.

Evidence exists to suggest that androgens stimulate bone formation in female rats. Little is known on the effect of androgens in both sham and oophorectomised rats on the biochemical markers of bone turnover, calcium homeostasis or gene expression in osteoclasts and in osteoblasts. Osteoblasts have a diversity of phenotypes associated with various stages of the bone mineralisation process but it is not known at what stage of osteoblast development anabolic steroids may be acting.

Hypothesis 2: Since androgens have been demonstrated to increase bone formation, we hypothesise that DHT will increase the biochemical markers alkaline phosphatase and osteocalcin in sham, oophorectomised and osteopenic oophorectomised rats. In vivo, anabolic steroids have both anabolic and anti-catabolic activities on bone formation and bone resorption respectively. We therefore hypothesise that DHT will increase the mRNA levels of all osteoblastic genes and at high doses will inhibit the mRNA levels of the calcitonin receptor gene. Androgens can stimulate proliferation and mineralisation of osteoblasts in vitro and therefore a sequence of increased c-*fos* and type 1α collagen

expression, followed by alkaline phosphatase and osteopontin expression, and finally osteocalcin expression following DHT treatment would be consistent with a model of androgens stimulating proliferation and maturation of early osteoblasts. The effect of DHT will be tested in both oophorectomised rats with a high rate of bone turnover and in osteopenic oophorectomised rats with a rate of bone turnover that does not differ from control rats. We hypothesise that the anabolic effect of DHT on bone formation will be more prominent in the osteopenic oophorectomised rats due to the lower rate of bone turnover prior to treatment.

1.8.3 The effect of estrogen alone and in combination with DHT on the biochemical markers of bone turnover and osteoblastic and osteoclastic gene expression in osteopenic oophorectomised rats.

Estrogen treatment has been demonstrated to inhibit the increase in bone turnover following oophorectomy, primarily by inhibiting bone resorption. There is potential for a combination treatment of androgens and estrogens to prevent and restore the bone loss following estrogen deficiency.

Hypothesis 3: DHT combined with estrogen treatment has the ability to overcome the suppressive effects of estrogen treatment on bone formation. We hypothesise that DHT treatment in combination with estrogen will result in a suppression of osteoclast biochemical markers and gene expression with no suppression and perhaps stimulation of the osteoblast biochemical markers and gene expression in osteopenic oophorectomised rats.

CHAPTER 2

MATERIALS AND METHODS

2.1 INTRODUCTION

Many studies have described the extraction of RNA from a wide range of soft tissues for gene expression studies, however few have extracted RNA from bone. Since bone is a hard tissue, this makes the extraction of pure, undegraded RNA difficult. Ohta and colleagues (1991) extracted total RNA from rat external and periosteal hard calluses and from the midshaft region of the tibia using the acid-guanidinum-thiocynate-phenol-chloroform method (Chomczynski and Sacchi 1987). On using this method for the extraction of RNA from rat tibia and femur we found that it was contaminated with proteoglycans, which also affected the migration of RNA through agarose gels during electrophoresis. In addition, the RNA was possibly partially degraded in some samples. The following chapter describes the modification of the RNA method described by Ohta and colleagues (1991) for the removal of contaminating proteoglycans to yield pure, undegraded RNA from mature rat bones for use in Northern blot analysis. This chapter also describes the determination of the best collection procedure of rat bones to yield the largest amount of RNA and the determination of the amount of RNA to be analysed by northern blot analysis to allow detection of the messenger RNA (mRNA) levels of osteoblastic genes. This chapter also describes a relatively 'new' technique where by products obtained from reverse transcription-polymerase chain reaction (RT-PCR) (Fleet

and Wood 1994) can be semi-quantified by Southern blot analysis. This technique was employed to analyse the mRNA levels of the osteoclast gene, calcitonin receptor. Also described are the surgical procedures used on animals, specimen collection procedures and the techniques for the measurement of biochemical variables associated with bone turnover and calcium homeostasis used for all experiments.

2.2 MATERIALS

All chemicals and steroids used in the experiments were molecular biology grade reagent purchased from Sigma Chemical Company (Milwaukee, USA) unless otherwise stated.

2.3 ANIMALS

All animals used in the experiments were virgin Female Sprague Dawley rats at 8 months of age which were obtained from the Gilles Plains Animal Resource Centre (Gilles Plains, South Australia). The animals were housed at 26°C with a 12 hour light-dark cycle.

2.3.1 Diet

Each rat was meal fed 20g per day commercial rat chow containing 0.7% calcium, 0.6% phosphorus and 200U/Kg vitamin D (Milling Industries Pty Ltd., South Australia). Tap water was supplied ad libitum. It was important to restrict dietary intake in oophorectomised rats as increased food intake and subsequent weight gain occurs

following oophorectomy (Tarttelin and Gorski 1973) and in an attempt to avoid differences between the two groups due to differences in weight, the sham and oophx rats were fed equal amounts of food (20g per rat) provided daily at 9am. Animal ethics approval was obtained from the Institute of Medical and Veterinary Science and The University of Adelaide Animal Ethics Committees prior to commencement of all experiments.

2.3.2 Sham and oophorectomy operations

All operations and invasive procedures were performed under halothane anaesthesia in aseptic conditions. Prior to these procedures each rat was anaesthetised in 2% halothane (Zenera Limited, Macclesfield, UK) in oxygen:nitrous oxide (2:1) and the extent of anaesthesia was confirmed by lack of response from the rat to pinching of the sole of the back foot. Prior to operative procedures, hair was removed from a small area of the lower back by use of hair clippers and iodine antiseptic (Betadine, Faulding Pharmaceuticals, Adelaide, Australia) was applied liberally to the area. A small incision was made in the skin at the dorsal region of the lower back and the skin and muscle layer were separated by use of surgical scissors. A blunt incision was then made in the muscle layer above the left ovary, and the ovarian duct and ovary were located and exposed by use of tweezers. The ovaries were examined for abnormalities before being carefully replaced in sham operations. In the oophorectomy operations, the ovarian duct was clamped immediately below the ovary and occluded with braided silk (Ethicon, Johnson and Johnson, Sydney, Australia). The ovary was removed with a scalpel and the procedure was repeated for the right ovary. The external excision in the skin for both operations was closed with michelle clips (Mikron Wound Clips, Becton and Dickinson, New Jersey, USA) and iodine was applied in aid to prevent infection. The rats regained consciousness under an infra-red heat

lamp and were closely monitored for one week following operation. The wounds were examined daily and re-stapled if required.

2.3.3 Administration of Dihydrotestosterone and Estradiol

17β-Hydroxy-5α-androstan-3-one (DHT) and 1,3,5[10]Estretriene-3,17β-diol (Estradiol) were administered by silastic implants (Dow-Corning Medical Silastic tubing, Dow-Corning, Midland, Michigan). Preparation of all silastic implants were carried out in a laminar flow hood using standard sterile techniques. The tubing was cut to the required length and one end tied with braided silk. The tubing was sterilised in 70% ethanol for 30 minutes, allowed to dry and filled with either DHT or estradiol by use of a sterilised positive displacement pipette (SMI, American Dade, Florida, USA). The end of the tubing was secured with braided silk. All implants were administered to the animals surgically under halothane anaesthesia (Section 2.3.2). An incision was made in the lower dorsal region of the rat and a second blunt incision was made in the muscle layer overlying the left ovary. The silastic implant was inserted into the fat layer directly underlying the incision.

2.3.4 Blood Sample Collection

All blood samples were taken from the tail vein under halothane anaesthesia. This was achieved by removing a small portion of the tip of the tail (1 to 2mm in length) and collecting 2-2.5mLs of blood. The whole blood was placed on ice for 30 minutes to allow clotting and centrifuged at 4,000 rpm for 10 minutes to collect serum. All serum samples were stored at -70°C until required for analysis. Whole blood requiring analysis was collected into tubes containing 50μL of lithium and calcium heparin (2000 IU/L) (Ciba-

Corning, Halstead Essex, United Kingdom), and placed on ice until analysis to prevent clotting.

2.3.5 Urine Collection

Urine samples were collected by use of metabolic cages. The animals were fasted for 24 hours prior to being placed in metabolic cages and a urine specimen was collected during the following 24 hours. All urine samples were acidified with approximately 250μL of hydrochloric acid (BDH Chemicals, Kilsyth, Australia) and stored at -20°C until required for analysis.

On completion of each experiment the animals were exsanguinated by cardiac puncture under halothane anaesthesia and sacrificed by cervical dislocation.

2.4 BLOOD AND URINE BIOCHEMISTRY

2.4.1 Urine Creatinine

Urine creatinine was analysed before the samples were acidified on the day of collection as creatinine in rat urine degrades with time and upon freezing and acidification (unpublished observation). Urine creatinine was measured by the method of Fabing and Eringhausen (1971). Creatinine reacts with alkaline picrate to form a product red in colour which is directly proportional to the creatinine concentration and is measured photometrically at 500 to 520nm. Urine creatinine was measured on a Chemical Chemistry analyser (Kone

Progress Plus, Kone Corporation, Ruukintie, Finland) using reagents manufactured by Trace Scientific (Victoria, Australia).

2.4.2 Urine Hydroxyproline

Urine hydroxyproline was measured by the method of Bergman and Loxley (1970). The urine samples were hydrolysed at 120°C overnight in 1mL of hydrochloric acid. The hydrolysate was neutralised and then oxidised with chloramine T reagent (20mM chloramine trihydrate (Merck, Frankfurt, Germany), 60mM sodium acetate, 0.13M sodium citrate and 18% propan-2-ol). The hydrolysate was then decarboxylated to produce a pyrrole derivative which was detected and quantified via a reaction with Erlich's reagent (5.75M p-dimethyl-aminobenz-aldehyde, 40mM hydrochloric acid and 73% propan-2-ol (BDH Chemicals, Kilsyth, Australia)) by incubating at 65°C for 20 minutes followed by a further incubation at 25°C at room temperature. The product was measured at an absorbance of 562nm on a spectrophotometer (Gilford Instrument Laboratories Inc., Oberlin, Ohio). The minimal detectable concentration of the assay was 20μmol/L and the interassay coefficient of variation at 182μmol/L was 6.4%.

2.4.3 Urine Deoxypyridinoline

Free urinary deoxypyridinoline (Dpd) was measured by competitive enzyme immunoassay using a commercially available kit (Pyrilinks-D, Metra Biosystems Inc., CA, USA). Prior to measurement, the acidified samples were neutralised 1:5 with 1N sodium hydroxide (BDH Chemicals, Kilsyth, Australia). The deoxypyridinoline in the samples competes with conjugated Dpd-alkaline phosphatase for the monoclonal anti-Dpd antibody bound to the well. The reaction is detected with a p-nitrophenyl phosphate substrate and the

absorbance is measured at 405nm which is inversely proportional to the amount of Dpd in the sample. The minimal detectable concentration of the assay was 1.1nM and the interassay coefficient of variation at 30nM was 4.6%.

2.4.4 Urine Calcium and Phosphate

All urine samples were adjusted to a pH of less than two with hydrochloric acid before being analysed for urine calcium and phosphate to promote the dissociation of calcium and phosphate ions. Urine calcium was measured by the method of Morrehead and Biggs (1974). Cresolphthalein complexone reacts with calcium and magnesium in alkaline solution to form a deeply coloured complex which can be measured photometrically at between 540 and 600nm.

Urinary inorganic phosphorus was measured based on the method of Daly and Ertingshausen (1972). Phosphate reacts with ammonium molybdate in the presence of sulphuric acid to produce unreduced phosphomolybdate complexes which can be measured at 340nm and is directly proportional to the concentration of inorganic phosphorus present in the sample.

Urine calcium and phosphate were measured on a Chemical Chemistry analyser (Kone Progress Plus, Kone Corporation, Ruukintie, Finland) using reagents manufactured by Trace Scientific (Victoria, Australia).

2.4.5 Whole Blood Ionised Calcium

Whole blood ionised calcium was measured by ion specific electrode (Calcium-pH analyser, Ciba-Corning 634, Halstead Essex, United Kingdom) on the day of collection.

2.4.6 Serum Electrolytes

Serum sodium, potassium, chloride and bicarbonate were analysed on the day of collection by ion specific electrodes (Fast 4 electrolyte system, Ciba-Corning 664, Massachusetts, USA).

2.4.7 Serum Osteocalcin

Serum osteocalcin was measured by radioimmunoassay based on the method of Price and Nishimoto (1980) as described by Morris et al (1990). Bovine osteocalcin antisera were raised in rabbits by multiple injections of osteocalcin/bovine-serum-albumin conjugate. Standard concentrations were established by quantitative amino acid analysis. Parallel displacement of bovine osteocalcin standards by rat osteocalcin confirmed cross reactivity. The minimum detectable concentration of the assay was 1.1μg/L and the interassay coefficient of variation at 14.4μg/L was 15%.

2.4.8 Serum 1,25 dihydroxyvitamin D_3

1,25 dihydroxyvitamin D_3 ($1,25(OH)_2D_3$) was measured based on the method of Taylor et al (1980). Vitamin D_3 and all metabolites are extracted from the serum with cyclohexane and ethyl acetate (1:1) (BDH Chemicals, Kilsyth, Australia). The $1,25(OH)_2D_3$ is separated from interfering substances and other metabolites by chromatography on Sephadex LH 20 (Pharmacia Biotech, Uppsala, Sweden) and high-pressure liquid

chromatography. $1,25(OH)_2D_3$ is then analysed by a competitive protein binding assay. The minimum detectable concentration of the assay was 10 pmol/L and the interassay coefficient of variation at 75 pmol/L was 15%.

2.4.9 Serum Dihydrotestosterone

Serum dihydrotestosterone (DHT) (pmol/L) was measured by radioimmunoassay using a commercially available kit (Diagnostic System Laboratories Inc., Webster, Texas, USA). The serum samples were oxidised with potassium permanganate to remove most of the testosterone as measurements of DHT concentrations are confounded by antibody cross-reactivity to testosterone. Following the oxidation/extraction procedure the cross-reactivity with testosterone was 0.02%. The minimum detectable concentration of the assay was 14pmol/L and the interassay coefficient of variation at 106pmol/L was 8.5%.

2.4.10 Serum Estradiol

Serum estradiol was measured by Microparticle Enzyme Immunoassay technology using a commercially available kit (Abbott Laboratories, Irving Texas, USA) performed on an automated chemical analyser (Abbott IMx, Abbott Diagnostics, AnSan-City, Korea). This technology measures serum estradiol by using a solution of suspended submicron sized latex particles coated with anti-estradiol. An estradiol-alkaline phosphatase (ALP) conjugate is added to the antigen-antibody complex which binds to the unoccupied antibody binding sites. The estradiol-ALP conjugate complex is detected by the addition of 4-methylumbelliferyl phosphate which is hydrolysed to 4-methylumbelliferone by ALP producing fluorescent light and is inversely proportional to the concentration of estradiol in

the serum. The minimal detectable concentration of the assay was 50pmol/L and the interassay coefficient of variation at 513pmol/L was 6.2%.

2.4.11 Serum Parathyroid Hormone

Parathyroid hormone (PTH) was measured by a rat specific two-site immunoradiometric assay (IRMA) (Nichols Institute, San Juan Capritrano CA, USA). This assay measures biologically active intact PTH (1-84 amino acids) and the N-terminal PTH (1-34 amino acids). PTH was determined only in blood samples that were collected from the tail-vein of the rat, as blood samples collected from cardiac punctures have very high concentrations of PTH possibly due to the direct drainage into the heart from the site of production (unpublished observation, Mason RA and Morris HA, 1994). The minimum detectable concentration of the assay was 1.0pg/mL and the interassay coefficient of variation at 50pg/mL was 4.0%.

2.4.12 Serum Alanine Aminotransferase

Serum alanine aminotransferase (ALT) was measured by an automated chemical analyser (Cobias Bio, Roche Ltd., Basle Switzerland) by the method of Wroblewski and LaDue (1956) as modified by Bergmeyer et al (1978). Alanine aminotransferase catalyses the conversion of the substrates L-alanine and α-ketoglutarate to pyruvate and glutamate. Pyruvate is converted by lactate dehydrogenase to lactate with the oxidation of NADH to NAD. The reaction is monitored by measurement of the decrease in absorbance at 340nm as NADH is converted to NAD which is proportional to the activity of ALT in the serum.

2.4.13 Serum Alkaline Phosphatase

Alkaline phosphatase was measured using the adenine monophosphate method based on the recommendations of the International Federation of Clinical Chemistry (AMP-IFCC) (Tietz et al 1983). Alkaline phosphatase catalyses the conversion of the substrate 4-nitrophenylphosphate to 4-nitrophenoxide and phosphate. The 4-nitrophenoxide has an intense yellow colour and its production can be monitored by measuring the increase in absorbance at 405nm which is proportional to the activity of ALP in the serum. This assay measures all isoforms of alkaline phosphatase. Serum alkaline phosphatase was measured on a Chemical Chemistry analyser (Kone Progress Plus, Kone Corporation, Ruukintie, Finland) using reagents manufactured by Trace Scientific (Victoria, Australia).

2.4.14 Serum Albumin

Albumin was measured by the method of Doumas et al (1972). Albumin binds with bromocresol green at pH 4.2 and causes a shift in the absorption spectra of the yellow bromocresol green dye. This shift in the absorption spectra is directly proportional to the amount of albumin present and can be measured at a wavelength between 580 and 630nm. Serum albumin was measured on a Chemical Chemistry analyser (Kone Progress Plus, Kone Corporation, Ruukintie, Finland) using reagents manufactured by Trace Scientific (Victoria, Australia).

2.4.15 Serum Total Protein

Total protein was measured by the biuret method by Goodwin et al (1965) and Flack and Woollen (1984). The peptide bond of protein reacts with copper II ions in alkaline solution to form a blue complex with each copper ion complexing with 5 or 6 peptide bonds. This

reaction is stabilised with tartrate and iodide is added to prevent auto-reduction of the alkaline copper complex. The complex formed is directly proportional to the amount of total protein present and is measured at 520 to 560nm. Serum total protein was measured on a Chemical Chemistry analyser (Kone Progress Plus, Kone Corporation, Ruukintie, Finland) using reagents manufactured by Trace Scientific (Victoria, Australia).

2.4.16 Serum Creatinine, Calcium, Phosphate

Serum creatinine, calcium and phosphate were measured by the methods described for urine analyses (Chapter 2.4.1, 2.4.4).

2.5 CALCULATIONS

Urinary calcium, phosphate and hydroxyproline were expressed as a ratio to urine creatinine. Creatinine excretion (mmol/day) was calculated by multiplying urine creatinine (mmol/L) by 24 hour urine volume (L/day). Urinary excretion of calcium (mmol/LGF) and phosphate (mmol/LGF) were calculated from the molar ratios to urine creatinine multiplied by the serum creatinine (mmol/L) and therefore expressed per litre of glomerular filtrate.

Anion gap was calculated from the difference between the sum of the serum sodium and potassium and the sum of the serum chloride and bicarbonate. The maximum tubular reabsorption of phosphate (TmP) and the maximum tubular reabsorption of calcium (TmCa) were calculated by the formulae described by Marshall (1976) as follows:

$$TmP = \frac{\text{Serum Phosphate - Phosphate Excretion}}{1 - 0.1 \times \log_e \frac{\text{(Serum Phosphate)}}{\text{(Phosphate Excretion)}}}$$

$$TmCa = \frac{\text{Ultrafiltrable Calcium - Calcium Excretion}}{1 - 0.08 \times \log_e \frac{\text{(Ultrafiltrable Calcium)}}{\text{(Calcium Excretion)}}}$$

however the calculated serum ultrafiltrable calcium was used as the measure of the filtered load of plasma calcium. Ultrafiltrable calcium was calculated from the serum calcium fractions which were calculated from the serum levels of calcium, albumin, globulins, bicarbonate and anion gap as described previously (Nordin et al 1989):

$$\text{Ultrafiltrable Calcium} = \text{Ionised calcium } [Ca^{2+}] + \text{Complexed Calcium}$$

$$\text{Ionised Calcium} = \text{Total Calcium - Protein-bound Calcium - Complexed Calcium}$$

$$\text{Protein-bound Calcium} = \frac{0.0169[Ca^{2+}]Alb}{1 + 0.0169[Ca^{2+}]} - \frac{0.0064[Ca^{2+}]\,Glob}{1 + 0.0064[Ca^{2+}]}$$

$$\text{Complexed Calcium} = \frac{0.0105[Ca^{2+}]AnGaP}{1 + 0.0105[Ca^{2+}]} + \frac{0.0136[Ca^{2+}]\,Bicarb}{1 + 0.0136[Ca^{2+}]}$$

where Alb is serum albumin in g/L, Glob is serum globulin (g/L), AnGap is serum anion gap (mmol/L) and Bicarb is serum bicarbonate (mmol/L).

Serum levels of dihydrotestosterone (DHT) measured in pg/mL were converted to pmol/L by using the following formula;

$$pmol/L = 3.44 \times pg/mL$$

Bone biochemical variables hydroxyproline/creatinine, alkaline phosphatase and osteocalcin were corrected by subtracting the pre-operative baseline measurements from the experimental values for each rat to correct for individual variation observed in these measurements.

2.6 STATISTICAL ANALYSES

2.6.1 One Way Analysis of Variance

One way analysis of variance was used to analyse the effect of operation in rats receiving vehicle alone and to analyse the effect of DHT treatment at individual experimental time points. The data were analysed in Mintab, version 9.2 run on a personal computer. A value of $P<0.05$ was considered significant.

2.6.2 Repeated Measures Analysis of Variance

Multiple comparisons of mean values were made by repeated measures analysis of variance. When the assumption of equal correlations between biochemical analyses at each time point was violated, a conservative adjustment to the degrees of freedom was made using the following formula described by Greenhouse and Geisser (1959):

degrees of freedom (adjusted) = $\frac{\text{within subject degrees of freedom}}{\text{(no. of within subject groups - 1)}}$

The F value was then checked for statistical significance. The data were analysed in SAS version 6.10 run on a personal computer. A value of $P<0.05$ was considered significant.

2.6.3 Tukey's Post Hoc Test

Tukey's post hoc test was used to identify significant differences between mean values (Pagano 1986).

2.6.4 Regression Analyses

Regression analyses were used to analyse continuous data. The data were analysed in Minitab, version 9.2 run on a personal computer. A value of $P<0.05$ was considered significant.

2.6.5 Two-way analysis of variance

Two-way analysis of variance was used to analyse the effect of DHT, estradiol and an interaction between DHT and estradiol treatment. The data were analysed in Minitab, version 9.2 run on a personal computer. A value of $P<0.05$ was considered significant.

2.7 MOLECULAR BIOLOGY TECHNIQUES

All molecular biology procedures were carried out using standard sterile techniques. All consumables and solutions were autoclaved at 120°C prior to use. All procedures that required the use of transformed cells were carried out in a specified C1 laboratory and standard C1 techniques and disposal methods were performed.

2.7.1 Materials and Preparation of Reagents

2.7.1.1 Antibiotics

Stock solutions of ampicillin were prepared in deionised water to a concentration of 100mg/mL and sterilised by filtration through an 0.2µm Minisart (Sartorius AG, Göttingen, Germany). Tetracycline was prepared in 50% ethanol and 50% water to a concentration of 12.5mg/mL. All antibiotics were protected from the light and stored at -20°C.

2.7.1.2 RNase A

RNase A was prepared in 10mM Tris-HCl, pH 8.0 and 15mM sodium acetate to a concentration of 10mg/mL. The solution was heated at 100°C for 15 minutes to destroy any DNase activity and stored at -20°C.

2.7.1.3 RNase inhibiting solution

Solution D, an RNase inhibiting solution, was prepared by dissolving 4M guanidine isothiocynate (Gibco RBL Life Technologies Inc., MD, USA), 25mM sodium citrate, pH 7 (BDH Chemicals, Kilsyth, Australia) and 0.5% sarcosyl at 60°C. Solution D was stored at 4°C and 2-mercaptoethanol was added to a concentration of 0.1M prior to use.

2.7.1.4 Phenol

All procedures using phenol were carried out in a fume hood. Crystalline phenol (BDH Chemicals, Kilsyth, Australia) for DNA extraction was heated in a water bath at 60°C until dissolved. The phenol was saturated in an equal volume of 10mM Tris and 1mM EDTA, pH 8.0 (TE buffer), mixed and the layers allowed to separate. On separation most of the upper layer was removed and the phenol was redistilled until the pH was approximately 8.0 as indicated by pH indicator paper (Whatman International Ltd., Maidstone, England). 8-hydroxyquinoline (Ajax Chemicals, Sydney, Australia) was added to a final concentration of 500mg/L as an indicator of oxidation and the phenol was stored at 4°C. Phenol for RNA extraction was prepared as above, however the phenol was redistilled twice in deionised water.

2.7.1.5 Formamide

Formamide (BDH Chemicals, Kilsyth, Australia) was deionised by adding 5% of ion exchange resin (AG-501-X8 resin, BioRad, California, USA) and stirring at room temperature for 30 minutes. The formamide was filtered twice with Whatman paper, No. 1 (Whatman International Ltd., Maidstone, England) and was stored at 4°C.

2.7.1.6 Loading Buffers for Electrophoresis

For electrophoresis in agarose gels, urea loading buffer, three times concentrated (3xULB) was prepared by dissolving 4M urea (BDH Chemicals, Kilsyth, Australia), 50% sucrose (BDH Chemicals, Kilsyth, Australia), 50mM EDTA and 0.1% bromophenol blue at 37°C for 30 minutes. 5 x RNA loading buffer consisted of 0.5% sodium dodecyl sulphate (SDS), 25% Glycerol (BDH Chemicals, Kilsyth, Australia), 25mM EDTA and 0.025% bromophenol blue. All loading buffers were stored at -20°C.

2.7.1.7 cDNA probes

The messenger RNA probe for c-*fos* (Curran et al 1987) was kindly donated by Dr. Gary Wittert, Royal Adelaide Hospital, Australia. Messenger RNA probes for rat type 1α collagen ($p\alpha_1R1$) (Genovese and Kream 1984), rat bone/liver/kidney alkaline phosphatase (pRAP-54) (Thiede et al 1988), rat osteopontin (pROPN) (Yoon et al 1987), and rat osteocalcin (pROC-918) (Yoon et al 1988) were kindly donated by Dr. Jon N. Beresford, Bath Institute for Rheumatic Diseases, Bath, Avon. The messenger RNA probe for rat glyceraldehyde 3-phosphate dehydrogenase (GAPDH)(Tso et al 1985, Fort et al 1985) was kindly donated by Dr. Brian May, Department of Biochemistry, The University of Adelaide, Australia, and the messenger RNA probe for human carbonic anhydrase II (Zheng et al 1994) and rat tartrate resistant acid phosphatase (Ek-Rylander et al 1991) was kindly donated by Dr. M.H. Zheng, The University of Western Australia, Australia. All probes were in the form of cDNA and were received contained in plasmids (Table 2.1).

cDNA Probe	Size (kb)	Host Plasmid	Restriction site of insertion in plasmid	Antibiotic Resistance
Rat c-*fos*	2.0	pGEM4 (Promega)	*Eco* R1	Ampicillin
Rat Type 1α Collagen	1.6	pUC	*Pst* 1	Ampicillin
Rat Alkaline Phosphatase	2.5	pBS+ (pBlueScript)	*Eco* R1	Ampicillin
Rat Osteopontin	1.3	pBS+ (pBlueScript)	*Eco* R1	Ampicillin
Rat Osteocalcin	0.52	pBS+ (pBlueScript)	*Eco* R1	Ampicillin
RatTartrate-resistant Acid Phosphatase	0.83	pGEM4 (Promega)	*Eco* R1, *Pst* 1	Ampicillin
Human Carbonic Anhydrase	1.4	pGEM4 (Promega)	*Eco* R1	Ampicillin
GAPDH	1.8	pBR322	*Pst* 1	Tetracycline

Table 2.1: Host plasmids for cDNA probes, their restriction site of insertion in the host plasmid and the antibiotic resistance of the host plasmid.

2.7.2 Preparation of cDNA probes

2.7.2.1 Transformation of plasmids into competent cells

Plasmids were transformed into chemically competent *E*coli DH5α-RecA$^-$ cells kindly donated by the Biochemistry Department, The University of Adelaide, Australia. Plasmids (2.7.1.7) were reconstituted in 10mM Tris-HCl and 1mM EDTA, pH 7.5 (TE buffer) to provide a stock solution of concentration of 100ng/mL. The stock solutions were stored at -70°C until required. 5ng of plasmid was added to 20μL of chemically competent DH5α-Rec A$^-$ cells and incubated on ice for 20 minutes. The cells were heat shocked by incubating at 42°C for exactly 2.5 minutes and then incubated on ice for a further 2 minutes. The cells were allowed to recover in 1mL of L-Broth (0.01% sodium chloride (BDH Chemicals, Kilsyth, Australia), 0.005% bacto yeast extract (Difco Laboratories, Michigan, USA) and 0.01% Bactotryptone (Difco Laboratories, Michigan, USA)) and 20mM Glucose (BDH Chemicals, Kilsyth, Australia) at 37°C for 30 minutes in a shaking water bath, after which the cells were recovered by centrifugation at 13,000 rpm and the supernatant was removed. The remaining pellet was plated onto agar plates (15g/L bactoagar (Difco Laboratories, Michigan, USA) in L-Broth) containing either 50μg/mL ampicillin (2.7.1.1) or 12.5μg/mL tetracycline (2.7.1.1) depending on the antibiotic resistance of the plasmid to be selected (Table 2.1). The agar plates were inverted to prevent condensation forming on the agar and incubated at 37°C overnight. The above procedure was repeated for each plasmid containing the cDNA probe of interest.

2.7.2.2 Plasmid Screening Procedure

To determine whether the clones contained the plasmids of interest, the plasmids were isolated by inoculating 100μL of L-Broth with a small isolated colony from each antibiotic agar plate. The culture was incubated at 65°C for a total of 25 minutes to lyse the host cells. The cells were collected by centrifugation and the remaining supernatant was analysed for the presence of the plasmids by electrophoresis in a 1% agarose gel (Promega Company, Annadale, Australia) in 1 x TAE (2mM Tris-acetate, 0.1mM EDTA) , pH 8.2 at 75 volts for 30 minutes. The agarose gel was stained in 1mg/L ethidium bromide and photographed under ultra-violet (UV) light. The molecular weight was approximated by comparison with 1μg of a DNA molecular weight standard (SSP1/Eco R1, Bresatec, Adelaide, Australia) (Table 2.2).

Fragment Number	Size (Kb) Mean (SD)	Amount (ng)
1	8.51 (0.07)	97.5
2	7.35 (0.06)	84.3
3	6.11 (0.06)	70.0
4	4.84 (0.03)	55.5
5	3.59 (0.01)	41.2
6	2.81 (0.01)	32.2
7	1.95 (0.01)	22.4
8	1.86 (0.02)	21.3
9	1.51 (0.01)	17.3
10	1.39 (0.02)	15.9
11	1.16 (0.01)	13.3
12	0.98 (0.01)	11.2
13	0.72 (0.00)	8.3
14	0.48 (0.01)	5.5
15	0.36 (0.01)	4.1

Table 2.2: DNA molecular weight standard. The lengths and amount of the 15 fragments produced when SPP1- bacteriophage DNA is digested with Eco R1 (Ratcliff 1979).

2.7.2.3 Growth of Bacterial Cultures

Once it was determined that each of the plasmids were of the correct size, a single colony was selected from each antibiotic agar plate for each plasmid and used to inoculate a 'starter' culture of 20mL of L-broth. The cultures were incubated at 37°C in a shaking water bath for 6 hours after which the 'starter' cultures were used to inoculate 500mLs of L-Broth containing either 50μg/mL ampicillin (2.7.1.1) or 12.5μg/mL tetracycline (2.7.1.1) depending on the resistance of the plasmid for selection (Table 2.1). The plasmid pBR322 containing the GAPDH cDNA probe required amplification in chloramphenicol. Cultures were grown to a A_{600}nm of 0.4-0.6 after which chloramphenicol (34mg/mL in 100% ethanol) was added to a final concentration of 170μg/mL. All cultures were incubated at 37°C in a shaking water bath overnight. Glycerol stocks of each of the cultures were produced to allow additional growth of transformed cell cultures without the requirement to repeat the transformation (2.7.2.1) and screening procedures (2.7.2.2). This was achieved by adding 300μL of culture to 300μL of 80% Gycerol and was stored at -70°C.

2.7.2.4 Isolation of plasmid DNA

The cells were collected by centrifuging the cultures at 5,000rpm at 4°C. The supernatant was removed and the pellets drained. The cells were resuspended in 5mLs of TES (25mM tris, 10mM EDTA, 15% (weight/volume) sucrose) and 1mL of lysozyme (12mg/mL freshly dissolved in TES). The cells were incubated at 4°C for 40 minutes before being lysed with 12mLs of 0.2M sodium hydroxide (NaOH, BDH Chemicals, Kilsyth, Australia) and 1% sodium dodecyl sulphate, and incubated on ice for a further 10 minutes. 7.5mLs of 3M sodium acetate, pH 4.6 was added to neutralise the NaOH and the cells were replaced on ice for a further 10 minutes. The cell debris was removed by centrifugation at 15,000

rpm for 15 minutes at 4°C. The supernatant was removed and digested with 100μL of 10mg/mL RNase (2.7.1.2) and incubated at 37°C for 30 minutes. The plasmid DNA was extracted twice with 1 volume of TE buffer saturated phenol (2.7.1.4), and 1 volume of 24:1 chloroform/isoamyl alcohol (Ajax Chemicals, Auburn, New South Wales, Australia). The aqueous phase was recovered by centrifugation at 13,000 rpm for 5 minutes and the DNA was recovered by precipitation with 2 volumes of ethanol at room temperature for 5 minutes. The DNA was recovered by centrifugation at 13,000 rpm for 10 minutes, washed with 1mL of 70% ethanol, dried under vacuum (Speed-vac, Savant Instruments Inc., New York, USA) and redissolved in 1mL of TE. The plasmid was electrophoresed in a 1% agarose gel containing 1 x TAE at 80 volts for 30 minutes, stained in ethidium bromide and viewed under ultra-violet light. On viewing the gels it was noted that the plasmids were contaminated with a large amount of RNA that was not removed in the extraction procedure. This could potentially interfere with the restriction enzymes used to excise the cDNA from the plasmids resulting in partial digestion. To remove the RNA, the samples were incubated with RNase (2.7.1.2) at 37°C for 2 hours. The DNA was extracted with 1 volume of TE buffer saturated phenol (2.7.1.4) and 1 volume of 24:1 chloroform/isoamyl alcohol and precipitated with ethanol. The pellet was recovered by centrifugation, washed with 70% ethanol and reconstituted in TE buffer.

The concentration of plasmid DNA was determined by measuring the absorbance at A_{260}nm on a spectrophotometer (Beckman Instruments, California, USA). The DNA concentration was calculated using the following formula:

$$1.0\ A_{260nm} = 50\mu g\ DNA$$

(Sambrook et al 1989).

2.7.2.5 Isolation of cDNA

The plasmids were digested with the appropriate restriction enzyme which would result in complete release of the cDNA from the host plasmid (Table 2.1). The amount of plasmid, restriction enzyme and reaction buffer (100mM tris-acetate, pH 7.5, 100mM magnesium acetate, 500mM potassium acetate) required were determined according to manufacturer recommendations (Pharmacia Biotech, Uppsala, Sweden) and incubated at 37°C for 2 hours. The DNA fragments were separated from the plasmid DNA by electrophoresis in a 1% TAE agarose gel at 80 volts for 30 minutes, which was stained in ethidium bromide, and viewed under UV light. The cDNA of each probe was confirmed by length using a molecular weight standard (SPP1-Eco R1, Bresatec, Adelaide, Australia)(Table 2.2) and was excised from the gel. The cDNA was extracted from the agarose gel using a gel extraction kit (Quaigen GmbH, Hilden, Germany). The agarose was solubilised in the presence of glass particles and in a high salt buffer at 50°C for 10 minutes. In these conditions the DNA binds to the glass particles. The DNA was washed twice in a high salt buffer to remove any remaining agarose and twice in a 60% ethanol solution to remove the salt present. The glass particles were air dried and the DNA was eluted in TE buffer. 1μL of the extracted cDNA was electrophoresed on a 1% agarose gel in 1 x TAE buffer at 80 volts for 30 minutes and the concentration was estimated by comparison of the intensity of the bands of the cDNA probe with 1μg of SPP-1/Eco R1 molecular weight marker (Table 2.2).

2.7.3 Extraction of Total RNA from Bone

2.7.3.1 Collection of Rat Bones for Extraction of RNA

The rats were sacrificed by cervical dislocation under halothane anaesthesia and one femur and both tibiae were excised. The surrounding soft tissue and the ends of the bones were removed just below the epiphyses and the cavity of the bones were flushed with normal ice cold saline to remove the bone marrow (Appendix E.1). The bones were homogenised in 10mLs of Solution D (2.7.1.3) in a 50mL sterile tube (Corning Incorporated, New York, USA) by use of an Ultra-Tarrax (Janke and Kunkel, Staufen, Germany).

2.7.3.2 Extraction of RNA from Bone

The homogenised bone was centrifuged at 5,000rpm at 4°C to separate the bone mineral and lysate. The RNA was extracted from the lysate based on the method of Chomczynski and Sacchi (1987) as described by Ohta et al (1991). The DNA and proteins were removed by mixing by inversion with 1 volume of water saturated phenol (2.7.1.4) and 0.2 volume of 24:1 chloroform/isoamyl alcohol followed by a 15 minute incubation on ice. The aqueous layer containing the RNA was recovered by centrifugation at 14,000 rpm for 5 minutes. The RNA was precipitated with 1 volume of isopropanol overnight at -20°C. The RNA was recovered by centrifugation at 15,000rpm for 15 minutes, the isopropanol was removed and the RNA pellet was drained and air-dried for 5 minutes. The RNA was re-dissolved in 450μL of TE buffer and 50μL of 3M sodium acetate, pH 5.2. The RNA was re-extracted with 1 volume of water saturated phenol (2.7.1.4) and 1 volume of 24:1 chloroform/isoamyl alcohol followed by an additional re-extraction with 1 volume of 24:1 chloroform/isoamyl alcohol to remove any phenol remaining in the aqueous layer. The RNA was precipitated

RNA was precipitated with 4 volumes of 4M sodium acetate (BDH Chemicals, Kilsyth, Australia), pH 7.0, at -20°C overnight, washed with 500µL of 70% ethanol, dried under vacuum and dissolved in 200µL of TE buffer. The integrity of the RNA was determined by electrophoresis. This was achieved by adding 3µL of 3xULB (2.7.1.6) to 5µL of each sample and heating at 65°C for 5 minutes to denature the ribosomal RNA. The samples were cooled on ice and separated in a 1% agarose gel in 1 x TAE buffer by electrophoresis at 80 volts for 30 minutes. The gel was viewed under UV light to check the integrity of the RNA by the presence of the three RNA ribosomal bands 26s, 18s and 4s. A 2:1 ratio of the 26s ribosomal band to the 18s ribosomal band also indicates the RNA is undegraded (Sambrook et al 1989).

2.7.3.3 Quantification of RNA

All RNA was quantified by measuring the absorbance of a 1 in 100 dilution of RNA in deionised water on a spectrophotometer (Beckman Instruments, California, USA) at wavelengths of 230λ, 260λ, 280λnm. The concentration of RNA was calculated using the following formulae:

$$\text{RNA concentration (µg/µL)} = \text{Absorbance at } 260\lambda \times \frac{40}{100} \times \text{dilution factor.}$$

The yield of RNA was calculated using the following formulae:

$$\text{Yield of RNA (µg)} = \text{RNA concentration (µg/µL)} \times \text{volume (µL)}$$

To check the purity of the RNA the following ratios were calculated:

$\frac{\text{Absorbance at } 260\lambda}{\text{Absorbance at } 280\lambda} \approx 1.8$ for pure RNA

$\frac{\text{Absorbance at } 260\lambda}{\text{Absorbance at } 230\lambda} \approx 2$

(Sambrook et al 1989)

2.7.4 Northern Blot analysis

2.7.4.1 Separation of RNA on a Denaturing Gel and Transfer to Nylon Membrane

The volume of RNA was calculated from the concentration (2.7.3.3) to provide 15µg of RNA to be analysed by northern blot analysis. The RNA was precipitated with 2 volumes of ethanol and 0.1 volume 3M sodium acetate, pH 5.2, at -20°C overnight, recovered by centrifugation at 13,000rpm for 30 minutes, washed in 70% ethanol and dried under vacuum. This material was redissolved in 50% deionised formamide (2.7.1.5), 18% formaldehyde, 0.01M sodium phosphate buffer, pH 7 (0.1mM di-sodium hydrogen phosphate, 0.1mM sodium di-hydrogen phosphate (BDH Chemicals, Kilsyth, Australia)) and 0.5mM EDTA. The RNA was denatured at 65°C, cooled in iced water and 3µL of 5 x RNA loading buffer (2.7.1.6) was added. RNA samples were separated on a 1.2% formaldehyde denaturing gel by electrophoresis at 80 volts in 0.01M sodium phosphate buffer, pH 7. The buffer was recirculated at approximately 2.5mLs per minute to ensure uniform distribution of ions. The denaturing gel was electrophoresed for 2-3 hours or until the loading buffer had migrated a distance of 6.5-7cm from the loading wells. The gel was

stained with 0.1mg/L ethidium bromide in 0.25M sodium acetate, pH 4.6, for 10 minutes and de-stained in deionised water for 30 to 45 minutes. The gel was photographed under ultra-violet light to check the integrity of the RNA, as described in section 2.7.3.2, before transferring to a nylon membrane. The RNA was transferred to a nylon membrane (Boehringer Mannheim-GmBH, Mannheim, Germany) by capillary action in 20 x SSPE, pH 7.4, (0.36M sodium chloride, 0.02M sodium dihydrogen phosphate (BDH Chemicals, Kilsyth, Australia), 0.002M EDTA), overnight. The RNA was bound to the nylon membrane by exposure to UV radiation for 30 seconds (UV Crosslinker, Ultra-Lum, Adelaide, Australia) which results in the formation of covalent bonds between some of the bases in the RNA and the nylon membrane. The membrane was rinsed in 2 x SSPE, pH 7.4, sealed in a plastic bag and stored at 4°C until required.

2.7.4.2 Pre-hybridisation of Nylon Membrane

The nylon membranes were prehybridised at 42°C for 2 hours in a buffer solution containing 50% formamide, 5 x Denhardt's solution (0.1% bovine serum albumin, 0.1% polyvinylpyrrolidone and 0.1% Ficoll 400), 0.1% SDS, 5 x SSPE, 0.05% sodium tetra-phosphate (BDH Chemicals, Kilsyth, Australia) and 200µg/mL denatured salmon sperm DNA.

2.7.4.3 Hybridisation of Labelled Probe with Nylon Membrane

100ng of the cDNA probes (2.7.2.5) were labelled with [^{32}αP]-dATP (Bresatec, Adelaide, Australia) by random prime extension, based on the method of Feinberg and Vogelstein (1983) using a commercially available kit (Megaprime™ DNA labelling kit, Amersham, Sydney, Australia). The probe and primer were boiled at 100°C in a water bath for 5

minutes to denature the DNA and to allow the binding of the primers. Reaction buffer (Tris-HCl, pH 7.5, 2-mercaptoethanol, magnesium chloride) was added prior to the addition of nucleotides dCTP, dTTP, dGTP and [α^{32}P]-dATP. 1 unit of Klenow enzyme was added to catalyse the reaction followed by a 20 minute incubation at 37°C. The radiolabelled probe was purified by adding 165µL of TNE buffer (50mM Tris, 1mM EDTA, 100mM NaCl), pH 8, to stop the labelling reaction, 10µL of 10mg/mL salmon sperm DNA were added to act as carrier for the labelled probe which was precipitated with 200µL of 4M ammonium chloride, pH 5.2 (BDH Chemicals, Kilsyth, Australia) and 750µL of 100% ethanol. The labelled probe was recovered by centrifugation, washed with 70% ethanol, dried and reconstituted in 200uL of deionised water. The probe was denatured by adding 20uL of 10M NaOH and incubated at 37°C for 10 minutes before addition to the prehybridisation solution. The activity of the purified probe was approximately 3,000 Ci/mmol. The labelled probe was incubated with the nylon membrane at 42°C overnight.

2.7.4.4 Washing the Nylon Membrane

The prehybridisation solution containing the radiolabelled probe was removed. The membrane was washed twice at 42°C in 2.5 x SSPE, pH 7.4, 0.1% SDS and 0.01% tetra-sodium pyrophosphate ($Na_4P_2O_7$, BDH Chemicals, Kilsyth, Australia) for 10 minutes and was then washed twice at 65°C in 5 x SSPE, 0.01% SDS and 0.01% $Na_4P_2O_7$ in a shaking water bath. The membranes were rinsed in 2 x SSPE, pH 7.4, sealed in a plastic bag and were exposed to a PhosphoImager™ screen (Molecular Dynamics, Sunnyvale California, USA) overnight. The membrane was viewed and the signal was quantified by using a

Molecular Dynamics PhosphorImager™ and the ImageQuant program version 5.25 on a personal computer.

2.7.4.5 Removal of Probe from Nylon Membrane

The radiolabelled probe was removed from the nylon membrane to allow the membrane to be reprobed with another radiolabelled mRNA cDNA probe. This was achieved by boiling the membrane in 1% SDS and 0.1 x SSPE, pH 7.4 for 20 minutes. The membrane was rinsed in 2 x SSPE, pH 7.4, sealed in a plastic bag and stored at 4°C until required.

2.7.4.6 Statistical Analysis

All mRNA levels were normalised for GAPDH mRNA levels by dividing the signal obtained from the ImageQuant program (2.7.4.4) for the mRNA species of interest by the signal for the GAPDH mRNA for each rat. The normalised mRNA levels were then expressed as a fold increase relative to the control rats for each individual Northern blot analysis. This was achieved by dividing the normalised signal for the mRNA of each experimental group by the normalised signal for mRNA of the control sample, thus allowing for comparison of mRNA levels determined from different Northern blot analyses. The effect of oophorectomy and the administration of DHT were analysed by using an unpaired t-test for mean values on a personal computer. A value of $P<0.05$ was considered significant.

2.7.5 Development of the Method for Extraction of RNA from Bone and Optimisation of Northern Blot Analysis

A series of experiments were carried out to determine the best method for collecting long bones for the extraction of RNA in order to achieve the greatest yield of RNA. In addition, modifications were made to the RNA extraction procedure to ensure the RNA was of high quality and the amount of total RNA to be analysed by Northern blot analysis was determined to produce optimal detection of the mRNA levels of the genes of interest. The modified procedures for RNA extraction and Northern blot analysis were used for all experiments and are described in chapter 2.7.3 and 2.7.4.

2.7.5.1 Experiment 1 - RNA collection procedure

To determine the best method for collecting rat bones for the extraction of RNA, 6 six month old virgin female sprague dawley rats were divided into 3 groups each consisting of 2 rats. The marrow cavity of the bones in group 1 were flushed through with 10mLs of solution D by use of a sterile syringe and 19-gauge needle (Terumo Corporation, Tokyo, Japan), and collected into a 50mL sterile tube (Corning Incorporated, New York, USA). The bones from group 2 were crushed into 10mLs of solution D by use of surgical pliers and the bones from group 3 were homogenised in solution D by use of an Ultra-Tarrax (Janke and Kunkel, Staufen, Germany). The RNA was extracted as described in 2.7.5.2. The effect of the different collection procedures of the rat bones on the concentration and yield of RNA was determined using a one-tailed unpaired t-test for mean values. Values of $P<0.05$ were considered significant.

2.7.5.2 Experiment 2 - RNA Extraction Protocol

Total RNA was collected from the samples collected in experiment 1 (2.7.5.1) as described in Chapter 2.7.3.2. The RNA was precipitated however by conventional methods as described by Chomczynski and Sacchi (1987) using 3 volumes of 100% ethanol at -20°C overnight, instead of using 3 volumes of 4M sodium acetate, pH 7.0 as described in Chapter 2.7.3.2. The integrity of the RNA was determined as described in Chapter 2.7.3.2 and quantified as described in Chapter 2.7.3.3.

2.7.5.3 Experiment 3 - Determination of Amount of RNA for Northern Blot Analysis

In order to determine the amount of RNA to be analysed by northern blot analysis to allow for detection of mRNA levels of the osteoblastic genes and to give optimal separation, 10, 15 and 20μg were analysed by northern blot analysis for the mRNA levels of the alkaline phosphatase and GAPDH genes.

2.7.5.4 Results and Discussion

2.7.5.4.1 Experiment 1 - RNA Collection Procedure

The bones from Group 3 which were homogenised in solution D, had the greatest concentration and yield of RNA in comparison to the bones in group 1 which were flushed through with solution D (although not significant) and the bones in group 2 which were crushed in solution D ($P<0.05$) (Table 2.3). These data suggest that RNA from bone is contained in a large population of cells which reside deep in the bone, requiring homogenisation rather than crushing alone, for the release of RNA into solution. In addition, the low concentration of RNA achieved by flushing the bone cavity with solution D indicates that only a small proportion of bone cells reside on the inter-cavity bone

surface. The inability to reach significance between group 1 and groups 2 and 3 was due to the low sample numbers particularly as the RNA from one sample in group 1 was lost in the extraction procedure.

2.7.5.4.2 Experiment 2 - RNA Extraction Protocol

The analysis of the RNA by electrophoresis demonstrated that the samples were possibly partially degraded as the 26s, 18s and 4s RNA ribosomal bands were not clearly visible. In addition there was evidence of contamination of a substance that interferes with the migration of the RNA through the agarose gel as indicated by the 'smearing' of the RNA (Figure 2.1). Substances that can produce this effect are carbohydrates such as proteoglycans which are present in bone (Simmons and Grynpas, 1990). The cell/matrix component of bone contains 10% proteoglycans and the bone mineral content comprises of 7% proteoglycans (Simmons and Grynpas, 1990). Proteoglycans cannot be removed by phenol/chloroform extraction and are also precipitated by ethanol. In an attempt to remove the proteoglycans, the RNA was precipitated in a high salt solution (3 volumes of 4M sodium acetate, pH 7) at -20°C overnight (Sambrook et al 1989). This successfully precipitated only the RNA while the proteoglycans remained in solution. The RNA was recovered by centrifugation, washed in 70% ethanol, dried and redissolved in TE buffer. The integrity of the RNA was determined as described in section (2.7.3.2). Following precipitation with 4M sodium acetate the migration of RNA through the gel was not affected and the ribosomal bands were clearly visible (Figure 2.2).

2.7.5.4.3 Experiment 3 - Northern Blot Analysis

15μg of RNA was shown to give the optimal and clearest band for detection of both alkaline phosphatase and GAPDH mRNA for all three samples (Figure 2.3).

Group	Sample Collection into Solution D	RNA (μg/μL)	Yield (μg)
1	Flushed	0.084*	16.8*
2	Crushed	0.192 (0.056)	38.4 (11.2)
3	Homogenised	1.275 (0.255)[a]	255.0 (51.0)[a]

Table 2.3: Concentration of RNA (μg/μL) and yield of RNA (μg) obtained from bone using different collection procedures into solution D, an RNase inhibiting solution. Values are Mean (SE), n=2, except for * which are single values. [a] $P<0.05$ versus Group 2.

Figure 2.1: Example of RNA samples extracted from rat bone, precipitated with 2 volumes of ethanol and separated in a 1% agarose gel in 1 x TAE buffer. The 'smearing' of the RNA samples and the absence of the ribosomal bands indicates possible degradation and/or contamination by carbohydrates.

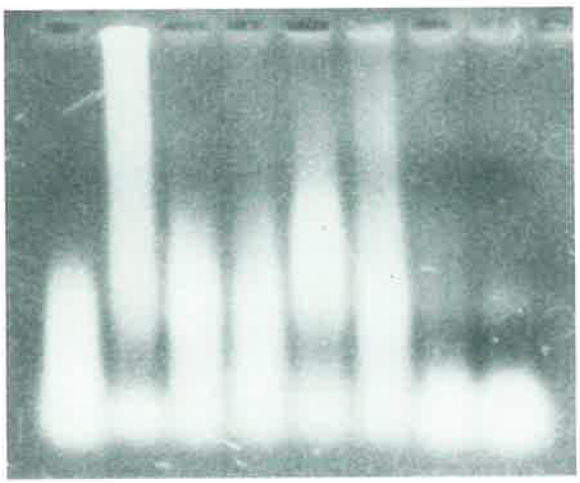


Figure 2.2: Example of RNA samples extracted from bone, precipitated with 3 volumes of 4M sodium acetate and separated in a 1% agarose gel in 1 x TAE buffer. The presence of the three ribosomal bands (28S, 18S and 5S) indicates pure and undegraded RNA.

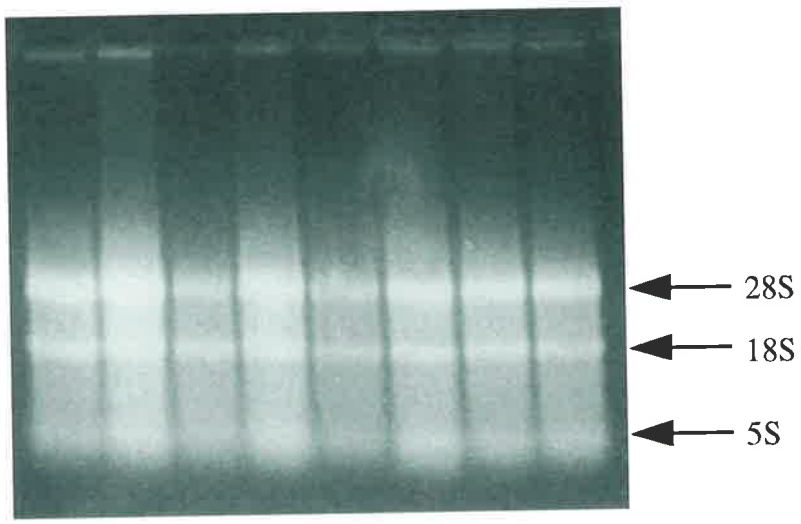
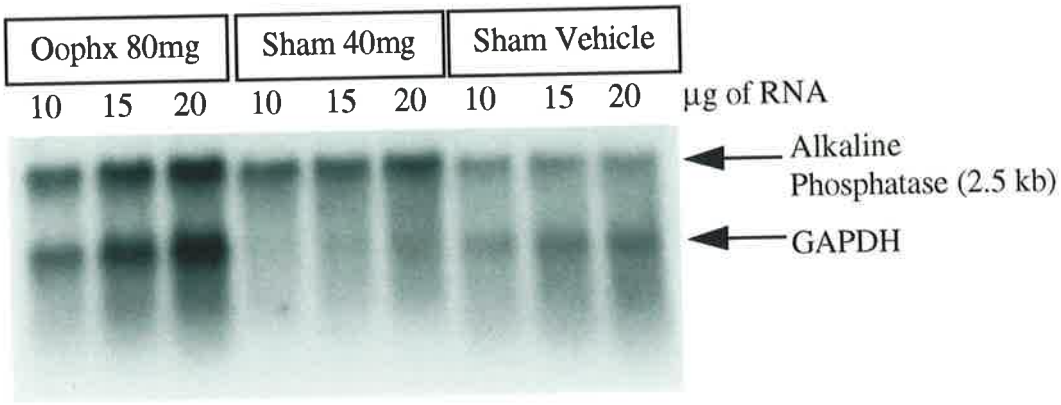

28S
18S
5S

Figure 2.3: Alkaline phosphatase and GAPDH mRNA levels for three samples of 10, 15 and 20μg of total RNA isolated from rat bone and detected by northern blot analysis. 80mg and 40mg refers to dose of DHT per kg body weight administered to rats at the time of operation.


Oophx 80mg
Sham 40mg
Sham Vehicle
10 15 20 10 15 20 10 15 20
µg of RNA
Alkaline Phosphatase (2.5 kb)
GAPDH

2.7.6 Semi-quantitative reverse transcriptase polymerase chain reaction

Semi-quantification of polymerase chain reaction (PCR) products is a relatively 'new' technique. The following protocol is based on the method of Fleet and Wood (1994) and describes the quantification of rat calcitonin receptor (isoform C1a) (CTR) mRNA levels. In this procedure, cDNA is prepared from RNA by reverse transcription. The cDNA is then amplified for the gene of interest using PCR. The PCR products are quantified by Southern blot analysis. To allow quantification of PCR cDNA products, a preliminary experiment is required to determine a PCR product curve for each gene of interest, that is the relationship between the number of cycles of PCR and the amount of PCR product produced. The PCR product curve is exponential with a lag time prior to amplification of the cDNA, a linear portion of amplification and a saturation point. A PCR product curve was determined for both rat CTR mRNA and rat GAPDH mRNA using one total RNA sample isolated from rat bone. From this curve the number of cycles required for linear amplification of the cDNA for both CTR mRNA and GAPDH mRNA were determined. Each sample of interest was then amplified using the same number of PCR cycles, thus allowing for direct comparisons to be made between RNA samples isolated from rats in different treatment groups.

2.7.6.1 First Strand Synthesis

First strand cDNA was synthesised from total RNA isolated from an untreated sham-operated rat using a commercially available kit (Gibco BRL, Melbourne, Australia). 2μg of RNA was added to 200ng of oligo-primer dT in a total volume of 8μL and was incubated at 65°C for 5 minutes followed by 5 minutes on ice. Added to the reaction was

375mM potassium chloride, 15mM magnesium chloride ($MgCl_2$). 200 units of Moloney Murine Leukemia Virus Reverse Transcriptase (MMLV-RT) were added to catalyse the reaction (20μL total volume) followed by a 1 hour incubation at 37°C and a 5 minute incubation at 95°C to stop the reaction.

2.7.6.2 Polymerase Chain Reaction Product Curve

6μL of the reaction mixture from the first strand synthesis (2.7.6.1) were submitted to PCR in a total volume of 50μL containing 0.1 pmol of each 5' and 3' primer, 200μmole each of dATP, dCTP, dGTP and dTTP, 20mM $MgCl_2$, DNA polymerase buffer (670mM Tris, pH 8.8, 166mM $(NH_4)_2SO_4$, 2mg/mL gelatin, 4.5% Trixon X-100) and 0.2 units of Taq DNA polymerase (Roche Molecular Systems Inc., New Jersey, USA). The following oligonucleotide primers were derived from previously published sequences:

CTR 1: 5'-ACACCCTGACAGCAACCGAACCT-3' (5' primer complementary to nucleotides 773-794 of the rat C1a isoform).

CTR 2: 5'-GAACCCCCAGCCAAGTAAATAGTA-3' (3' primer complementary to nucleotides 1078-1101). (Sexton et al 1993)

These primers span a region of the gene from the extracellular domain to the 7th transmembrane domain which includes at least three introns.

GAPDH 1: 5'-TCCTGAACCAACTGCTTA-3' (5' primer complementary to nucleotides 475-495)

GAPDH 2: 5'-ACCACCCTGTTGCTGTAGCCA-3' (3' primer complementary to n nucleotides 987-998). (Fort et al 1985)

The primers were synthesised by the Division of Molecular Pathology in the Institute of Medical and Veterinary Science, Adelaide, Australia. Amplification was performed in a Perkin Elmer Cetus Thermal Sequencer (Pelkin-Elmer Corporation, CT, USA). Amplification of CTR mRNA were achieved with cycles of denaturation at 95°C for 60 seconds, annealing at 65°C for 120 seconds and extension at 72°C for 60 seconds. Amplification of GAPDH mRNA was achieved with cycles of denaturation at 94°C for 60 seconds, annealing at 55°C for 30 seconds and extension at 72°C for 60 seconds. To detect for possible contamination, a sample of the PCR reaction constituents and water in place of the cDNA (from the first strand synthesis) was included during each amplification. A PCR product curve for CTR mRNA was achieved by performing 5 PCR reactions all including the same cDNA from first strand synthesis of RNA isolated from an untreated sham rat and removing one PCR tube after 30, 32, 34, 36 and 38 cycles. This was repeated to produce a PCR product curve for GAPDH mRNA with one PCR tube being removed after 18, 20, 22, 24 and 26 cycles.

2.7.6.3 Southern Blot analysis

2.7.6.3.1 Separation of PCR products and transfer to nylon membrane

15μL of the PCR products produced at each cycle were separated in a 2% agarose gel in 1 x TAE, stained in ethidium bromide and viewed under UV light for the presence of PCR products. The cDNA PCR products in the agarose gel were denatured for 1 hour in a buffer solution containing 0.5M NaOH and 1.5M NaCl and neutralised for 30 minutes in a buffer solution containing 1M Tris and 1.5M NaCl, pH 8.0. The PCR products were

transferred to a nylon membrane (Boehringer Mannheim-GmBH, Mannheim, Germany) by capillary action in 20 x SSPE, pH 7.4 overnight and bound to the nylon membrane by exposure to UV radiation for 30 seconds (UV Crosslinker, Ultra-Lum, Adelaide, Australia).

2.7.6.3.2 Prehybridisation and hybridisation of labelled probe with nylon membrane

The nylon membranes were prehybridised as described for northern blot analysis (2.7.4.2). Calcitonin receptor mRNA oligonucleotide probe (5'-GGTCCAACTACACTCTGTGCA-3') complementary to nucleotides 601-1038 (Findlay et al 1996), was labelled with [α^{32}P]-dATP by random prime extension as described for radiolabelling of cDNA probes for northern blot analysis (2.7.4.3). The prehybridisation buffer solution was removed and the nylon membrane was hybridised with 50% formamide, 5 x Denhardt's solution, 0.1% SDS, 1 x SSPE, 0.05% sodium tetra-phosphate and 100µg/mL denatured salmon sperm DNA. The labelled probe was added to the hybridisation solution and incubated at 42°C overnight.

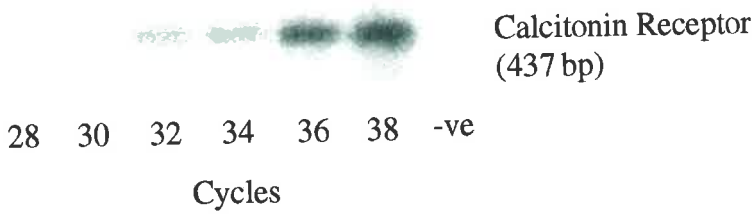
The hybridisation solution containing the radiolabelled probe was removed. The membrane probed with GAPDH cDNA probe was washed as described in 2.7.4.4. The membrane probed with CTR oligonucleotide labelled probe was washed once at 37°C in 5 x SSPE, 0.1% SDS and 0.05% $Na_4P_2O_7$ for 5 minutes. Both membranes were rinsed in 2 x SSPE, pH 7.4 and were exposed to a PhosphoImager™ screen (Molecular Dynamics, Sunnyvale California, USA) overnight. The signal was quantified using the ImageQuant program version 5.25 on a personal computer. The CTR mRNA levels were corrected for GAPDH mRNA as described previously in chapter 2.7.4.6.

2.7.6.4 Semi-quantification of PCR products

The number of PCR cycles required for linear amplification of CTR mRNA (Figure 2.4) and GAPDH mRNA (Figure 2.5) was 35 and 22 cycles respectively. Semi-quantification of mRNA levels of CTR receptor were achieved by performing first strand synthesis on the RNA samples of interest to produce cDNA (2.7.6.1) The cDNA (from first strand synthesis) was amplified using 35 cycles of PCR for CTR and 18 cycles of PCR for GAPDH (2.7.6.2). The PCR products were separated on a 2% agarose gel in 1 x TAE and the mRNA levels for CTR and GAPDH were determined and quantified by southern blot analysis as described in chapter 2.7.6.3.

Figure 2.4: (a) Southern Blot analysis of calcitonin receptor PCR product. The '-ve' lane is a negative control for the PCR reaction. (b) Relationship between PCR product (Relative Volume) and number of PCR cycles for rat calcitonin receptor (isoform C1a) mRNA as determined by RT-PCR and Southern Blot analysis.

A


Calcitonin Receptor
(437 bp)
28 30 32 34 36 38 -ve
Cycles

B

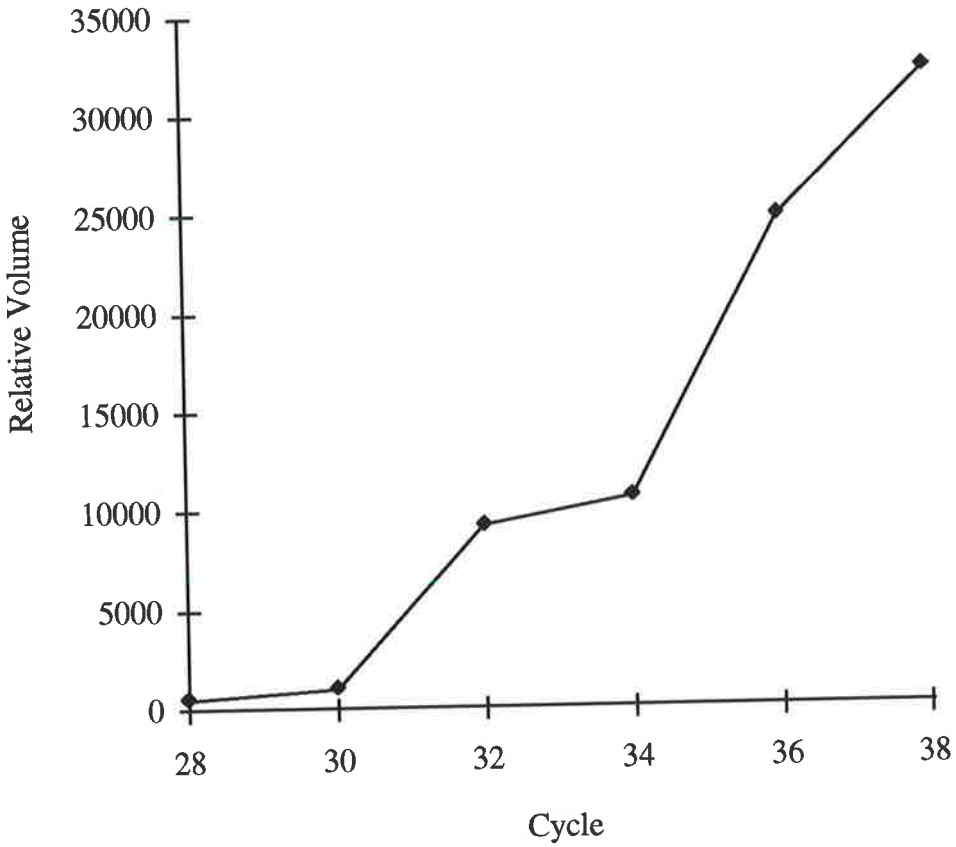
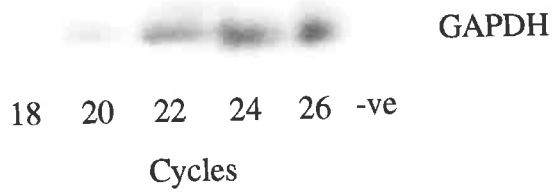
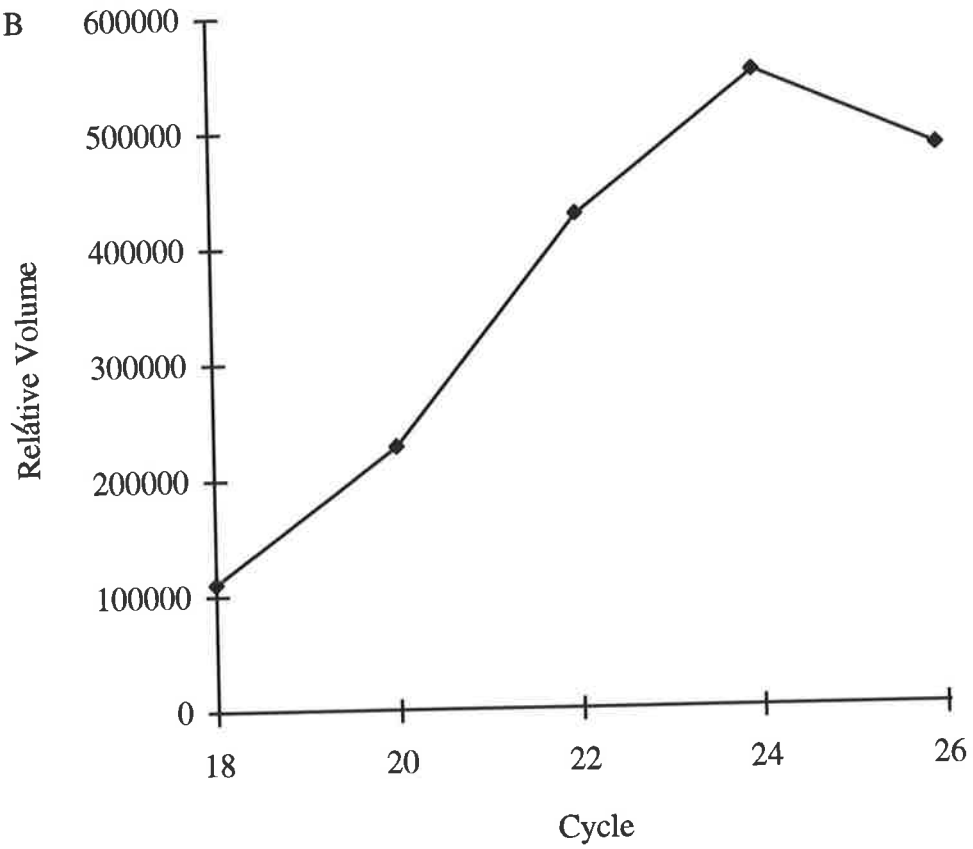

35000
30000
25000
20000
15000
10000
5000
0
Relative Volume
28 30 32 34 36 38
Cycle

Figure 2.5: (a) Southern Blot analysis of GAPDH PCR products. The '-ve' lane is a negative control for the PCR reaction. (b) Relationship between PCR product (Relative Volume) and number of PCR cycles for rat GAPDH mRNA as determined by RT-PCR and Southern Blot analysis.

A


GAPDH
18
20
22
24
26
-ve
Cycles

B


600000
500000
400000
300000
200000
100000
0
Relative Volume
18
20
22
24
26
Cycle

CHAPTER 3

EFFECT OF OOPHORECTOMY AND DHT TREATMENT IN SHAM AND OOPHORECTOMISED RATS ON BONE BIOCHEMICAL MARKERS

3.1 INTRODUCTION

Androgens partially restore bone in both postmenopausal women (Riggs et al 1972, Johanssen et al 1989) and in gonadectomised male (Wakely et al 1991, Vandershueren et al 1992) and female rats (Tobias et al 1994, Coxam et al 1996). While androgen receptors have been identified on human osteoblast-like cells (Colvard et al 1989), it is unclear whether these responses are an effect of androgens on bone cells to stimulate bone formation or to inhibit bone resorption. Evidence exists to suggest that androgens increase forearm mineral density in postmenopausal women by increasing bone formation with no effect on bone resorption (Need et al 1987). In contrast, Johanssen and coworkers (1989) found nandrolone decanoate combined with oral calcium supplementation, also in postmenopausal osteoporotic women, increased bone mineral content with no concominant rise in bone formation. They suggested that androgens have a positive effect on bone by inhibiting bone resorption. Alternatively, androgens may exert an indirect effect on bone by increasing muscle mass and thereby stimulating mechanical forces on the skeleton. Some androgens may be converted within the target cell to estrogen and thus effects may also be exerted via activation of the estrogen receptor.

Adrenalectomised female rats suffer bone loss similar to the oophorectomized rat which is reversed by treatment with nandrolone decanoate (Durbridge et al 1990). 5α-dihydrotestosterone (DHT) partially restores cancellous bone volume in osteopenic oophx rats, reflecting a net gain of bone tissue rather than the prevention of further bone loss (Tobias et al 1994, Coxam et al 1996). Furthermore in female rats, the administration of the androgen antagonist flutamide, induces bone loss independent of estrogen status (Goulding and Gold 1993).

Such data demonstrate the importance of androgens for skeletal integrity in females as well as males. The mechanism of androgens on bone however, remains controversial and in order to differentiate between an anti-resorptive action and stimulation of osteoblast activity, I have investigated the effects of DHT on biochemical indices of bone turnover and calcium homeostasis in sham and oophx rats. Unlike testosterone, DHT is not metabolised to estrogen and therefore the effects observed following such treatment are due to activation of the androgen receptor.

3.2 EXPERIMENTAL PROTOCOL

3.2.1 Experimental Procedure

48 animals were randomly divided into 8 groups and pre-operative fasting blood and urine samples were collected to provide baseline biochemistry. The rats were randomly allocated to either a sham or oophorectomy operation (Chapter 2.3.2) and were administered either vehicle (silastic tubing), 40mg, 80mg or 160mg/kg body weight

dihydrotestosterone by silastic implants of lengths 1.5cm, 2.8cm and 5.6cm respectively, at the time of operation (Chapter 2.3.3). The weight of each rat was recorded and 24 hour fasting blood (Chapter 2.3.4) and urine samples (Chapter 2.3.5) were collected once a week for 8 weeks.

3.2.2 Biochemical Analyses

Urine volumes were recorded. Urine was analysed for hydroxyproline (Chapter 2.4.2), free deoxypyridinoline (Chapter 2.4.3), creatinine (Chapter 2.4.1), calcium and phosphate (Chapter 2.4.4). Serum was analysed for DHT (Chapter 2.4.9), osteocalcin (Chapter 2.4.7), alkaline phosphatase (Chapter 2.4.13), alanine aminotransferase (Chapter 2.4.12), ionised calcium (Chapter 2.4.5), sodium, potassium, chloride, bicarbonate (Chapter 2.4.6), calcium, phosphate, creatinine (Chapter 2.4.16), albumin (Chapter 2.4.14), and total protein (Chapter 2.4.15). Preliminary analyses of parathyroid hormone (Chapter 2.4.11) and 1,25 dihydroxyvitamin D_3 (Chapter 2.4.8) were carried out on selected serum samples to determine if further analyses were warranted. Urinary hydroxyproline, calcium, phosphate and creatinine excretion were determined in 24 hour fasting urine specimens (Chapter 2.5). TmCa and TmP were calculated as described in Chapter 2.5.

3.2.3 Statistical Analyses

Statistical analyses for bone biochemical variables were performed on corrected data (Chapter 2.5). To determine the effect of oophorectomy in rats receiving vehicle alone, one way analysis of variance was performed (Chapter 2.6.1). The effect of oophorectomy, DHT administration, time and interactions of these variables were determined using multiple comparisons of mean values (Chapter 2.6.2). The data were further analysed

using a Tukey's post hoc test (Chapter 2.6.3). Regression analysis was used to determine the relationship between weight and serum creatinine (Chapter 2.6.4).

3.3 RESULTS

3.3.1 Serum Dihydrotestosterone

Serum DHT levels were unaffected by operation and correlated with dose administered in both sham and oophx rats ($P<0.001$). Serum DHT levels were decreased at weeks 8 compared to week 2 at all doses in both sham and oophx rats ($P<0.05$) but remained significantly elevated throughout the experiment (Table 3.1).

3.3.2 Urine Volume and 24 hour urine creatinine excretion.

Urine volume and 24 hour urine creatinine excretion were not affected by oophorectomy (Appendix A.1, Appendix A.2). DHT administration did not significantly affect urine volume or 24 hour urine creatinine excretion in either group and remained unchanged throughout the duration of the experiment (Appendix A.1, Appendix A.2). The effect of oophorectomy and DHT treatment on the urine biochemical variables did not differ when they were expressed as a ratio to creatinine (mmol/mmol) or as an excretion (L/day), therefore all urine biochemical measurements are expressed as a ratio to creatinine to correct for dilutional and bladder emptying errors.

Table 3.1 Serum dihydrotestosterone levels (pmol/L) measured in control and oophorectomised rats.

Time (Weeks)	Control				Oophx			
	Vehicle	40mg	80mg	160mg	Vehicle	40mg	80mg	160mg
2	-	2143 (423)^{ac} n=6	2253 (289)^{ac} n=6	5060 (1241)^{abc} n=6	-	1338 (334)^{ac} n=5	2394 (602)^{ac} n=6	5772 (1073)^{abc} n=6
5	-	1307 (251)^{a} n=6	1483 (244)^{a} n=7	4355 (1118)^{ab} n=5	-	1355 (575)^{a} n=6	1864 (530)^{a} n=5	4372 (482)^{ab} n=6
8	231 (38) n=8	1335 (237)^{a} n=6	1369 (234)^{a} n=6	3922 (626)^{ab} n=6	158 (21) n=5	1011(210)^{a} n=6	1882 (695)^{a} n=6	3570 (488)^{ab} n=6

DHT dose is mg per kg body weight. Values are Mean (SE). $^{a}P<0.05$ versus vehicle within operation group, $^{b}P<0.05$ versus 40mg and 80mg DHT, $^{c}P<0.05$ versus weeks 8 within dosage group.

3.3.3 Bone Biochemical Markers

3.3.3.1 Effect of Oophorectomy

Serum alkaline phosphatase and osteocalcin were raised as a result of oophorectomy in rats receiving vehicle ($P<0.001$) (Figure 3.1, Figure 3.2). In addition, oophorectomy increased deoxypyridinoline/creatinine and urine hydroxyproline/creatinine in rats receiving vehicle ($P<0.001$, $P<0.01$, respectively) (Table 3.2, Figure 3.3).

3.3.3.2 Effect of DHT Treatment in Sham and Oophorectomised Rats

Alkaline phosphatase was elevated following DHT administration independent of dose ($P<0.001$) in both sham and oophx rats. This effect was time dependent ($P<0.001$) with maximal stimulation occurring between weeks 2 to 8 after commencing treatment (Figure 3.1). Serum alkaline phosphatase did not differ between sham and oophx rats following DHT treatment (Figure 3.1). Osteocalcin was unaffected by DHT treatment in either operation group (Figure 3.2). There was no difference in serum osteocalcin levels between the sham and oophx rats following DHT treatment due to an insignificant rise in the sham rats and an insignificant fall in the oophx rats (Figure 3.2).

Urine deoxypyridinoline/creatinine and hydroxyproline/creatinine were unaffected by DHT administration in either group (Table 3.2, Figure 3.3). Urine deoxypyridinoline/creatinine increased throughout the duration of the experiment in both sham and oophx rats ($P<0.001$) (Table 3.2). Although not statistically significant, DHT treatment resulted in an increase in both urine deoxypyridinoline/creatinine and hydroxyproline/creatinine in sham rats such that no statistical difference was observed between DHT-treated sham and oophx rats (Table 3.2, Figure 3.3).

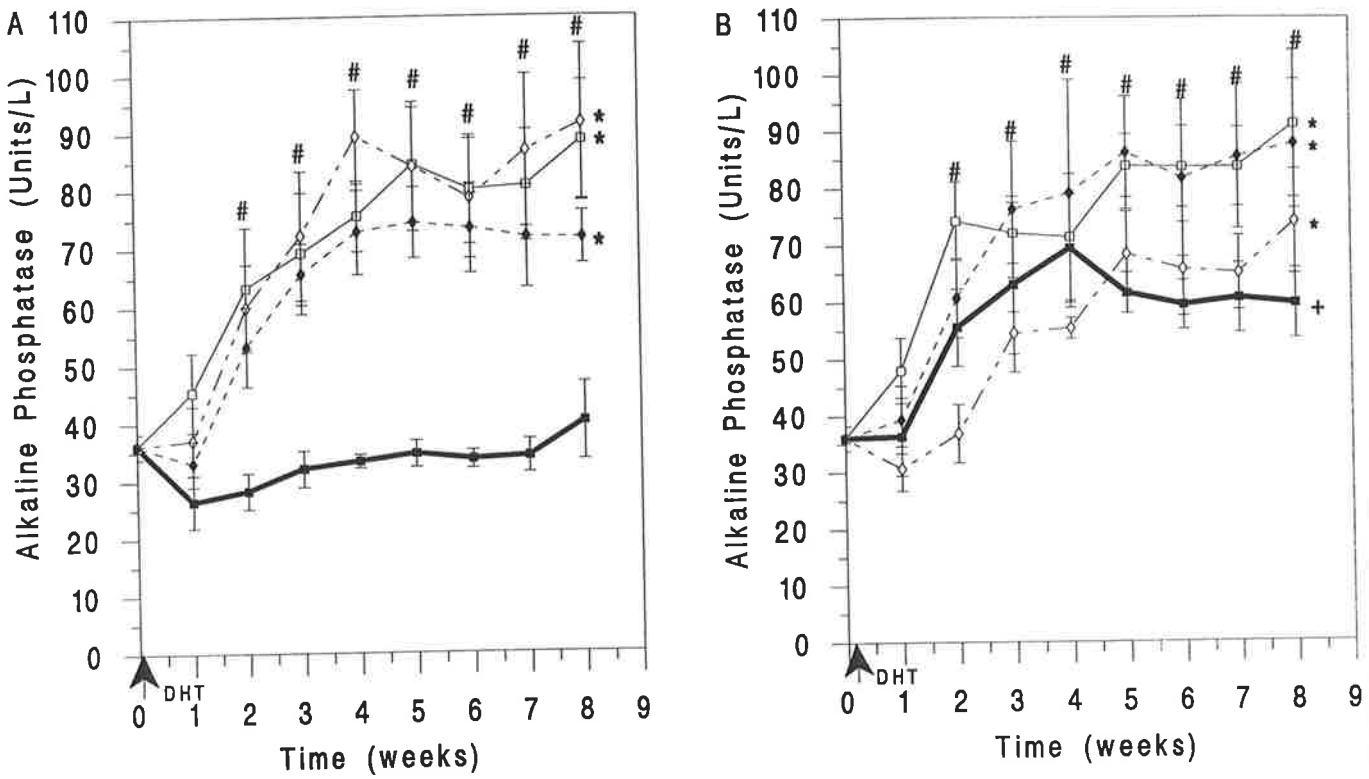


Figure 3.1 Serum alkaline phosphatase (units/L) in sham (a) and oophx (b) rats following DHT administration at the time of operation. Values are Mean ± SE, n=6. (■) Vehicle, (□) 40mg/kg bw DHT, (◊) 80mg/kg bw DHT, (◆) 160mg/kg bw DHT. $^{+}P<0.001$ versus sham (a) within dosage group, $*P<0.001$ versus vehicle within operation group, $^{\#}P<0.001$ versus week 1.

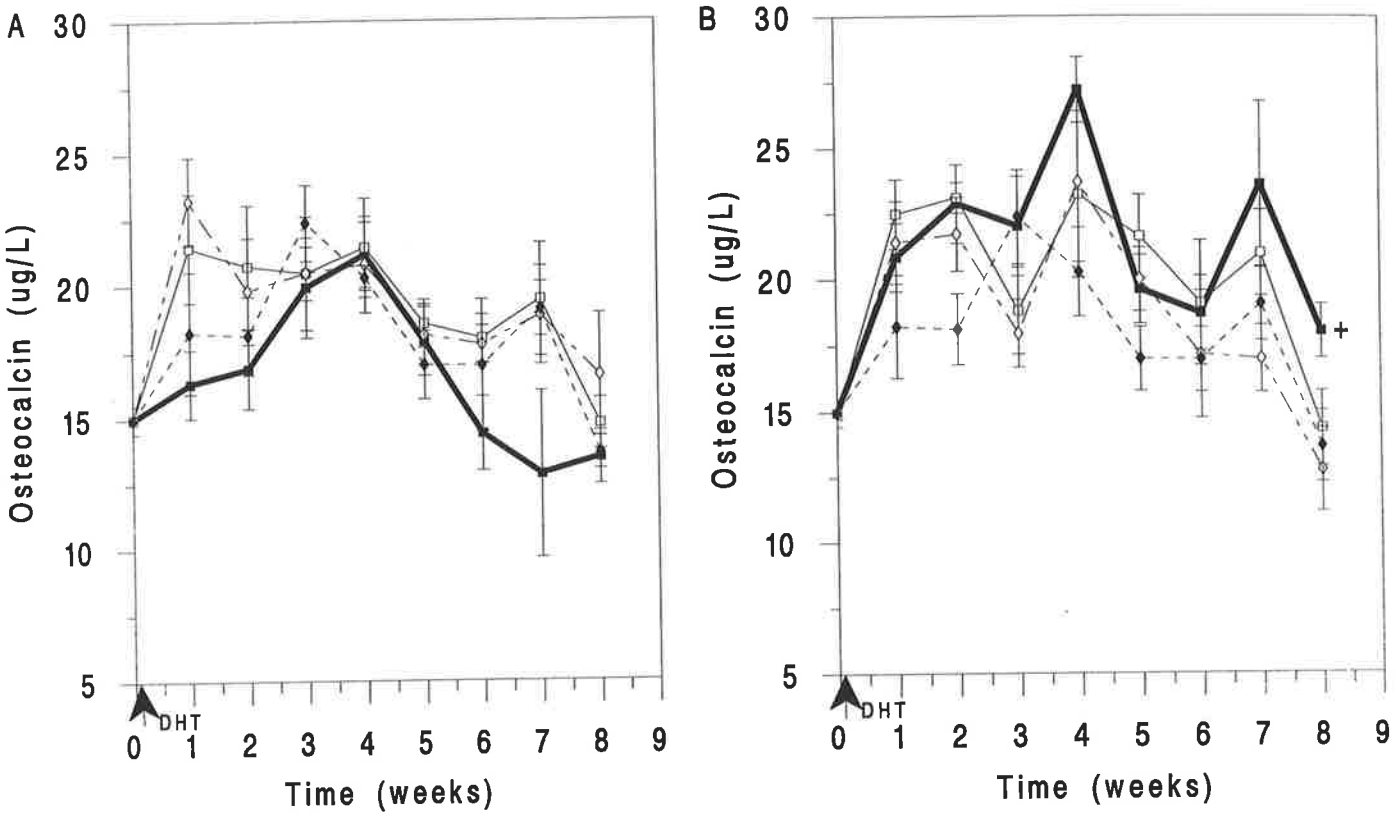


Figure 3.2 Serum osteocalcin (μg/L) in sham (a) and oophx (b) rats following DHT administration at the time of operation. Values are Mean ± SE, n=6. (■) Vehicle, (□) 40mg/kg bw DHT, (◊) 80mg/kg bw DHT, (◆) 160mg/kg bw DHT. $^{+}P<0.001$ versus sham (a) within dosage group.

Table 3.2 Urine deoxypyridinoline/creatinine (nmol/mmol) in sham and oophorectomised rats following DHT treatment.

Time (Weeks)	Control				Oophx			
	Vehicle	40mg	80mg	160mg	Vehicle	40mg	80mg	160mg
1	24.2 (3.3)[b] n=6	35.5 (4.3)[b] n=6	21.1 (0.5)[b] n=5	20.7 (1.5)[b] n=4	18.2 (1.7) n=3	23.1 (2.9) n=3	26.8 (4.4) n=2	27.7 (5.0) n=5
8	31.1 (3.9) n=8	59.7 (11.1) n=7	49.1 (13.9) n=6	51.7 (8.4) n=6	49.6 (4.6)[a] n=7	49.0 (11.9) n=9	97.0 (25.3) n=6	43.2 (7.6) n=7

DHT dose is mg per kg body weight. Values are mean (SE). [a]$P<0.001$ versus sham within dosage group, [b]$P<0.001$ versus week 8 within dosage group.

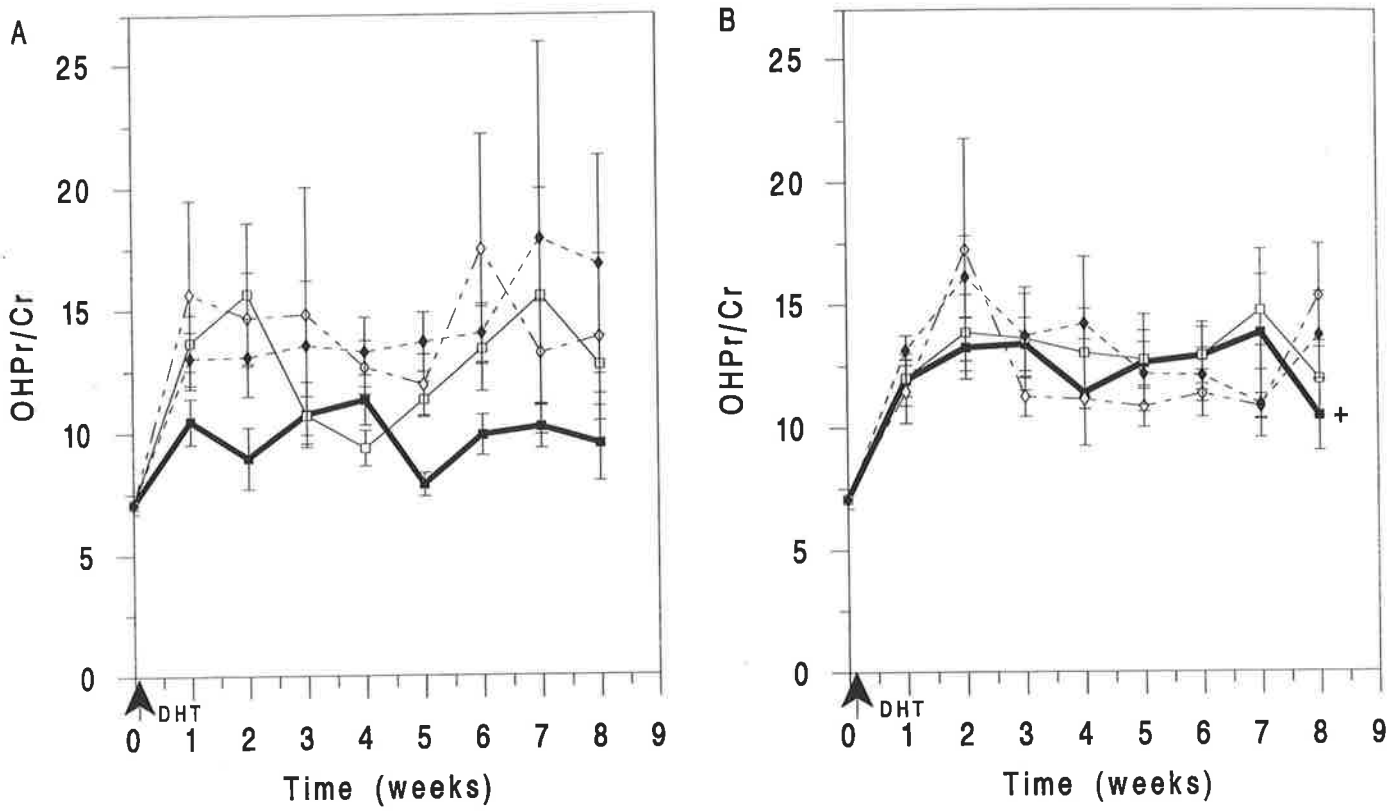


Figure 3.3 Urine hydroxyproline/creatinine (μmol/mmol) in sham (a) and oophx (b) rats following DHT administration at the time of operation. Values are Mean ± SE, n=6. (■) Vehicle, (□) 40mg/kg bw DHT, (◊) 80mg/kg bw DHT, (◆) 160mg/kg bw DHT. $^{+}P<0.01$ versus sham (a) within dosage group.

3.3.4 Parathyroid Hormone and 1,25 Dihydroxyvitamin D_3

Parathyroid hormone and 1,25 Dihydroxyvitamin D_3 were unaffected by oophorectomy in rats receiving vehicle and DHT (Table 3.3) and were unaffected by DHT administration in either sham or oophx rats (Table 3.3).

3.3.5 Urine Calcium

3.3.5.1 Effect of Oophorectomy

Urine calcium/creatinine and TmCa were unaffected by oophorectomy in rats receiving vehicle and DHT (Figure 3.4, Figure 3.5).

3.3.5.2 Effect of DHT Treatment in Sham and Oophorectomised Rats

DHT administration decreased urine calcium with doses of 80mg and 160mg/kg bw ($P<0.001$) in both sham and oophx rats. This effect was time dependent and occurred at 2 weeks following commencement of DHT treatment ($P<0.001$) (Figure 3.4). TmCa was increased following DHT administration at doses of 80mg and 160mg/kg bw ($P<0.05$) (Figure 3.5) and this effect was time dependent ($P<0.005$) with maximal responses occurring at weeks 6 to 8, following DHT administration.

Table 3.3 Serum parathyroid hormone (pmol/L) and 1,25 dihydroxyvitamin D_3 (pmol/L) levels in sham and oophorectomised rats at 8 weeks following DHT administration.

Variable	Operation	Vehicle	40mg DHT	80mg DHT	160mg DHT
Parathyroid Hormone (pmol/L)	Sham	4.4 (0.1) n=2	-	-	4.0 (2.5) n=6
	Oophx	3.8 (1.2) n=2	-	-	3.9 (2.5) n=6
1,25 Dihydroxyvitamin D_3 (pmol/L)	Sham	77.0 (10.5) n=3	60.7 (6.9) n=3	71.7 (10.4) n=3	79.3 (1.5) n=3
	Oophx	64.0 (7.0) n=2	65.0 (2.3) n=3	72.3 (7.6) n=3	82.0 (6.7) n=3

DHT dose is mg per kg body weight. Values are Mean (SE) of week 8.

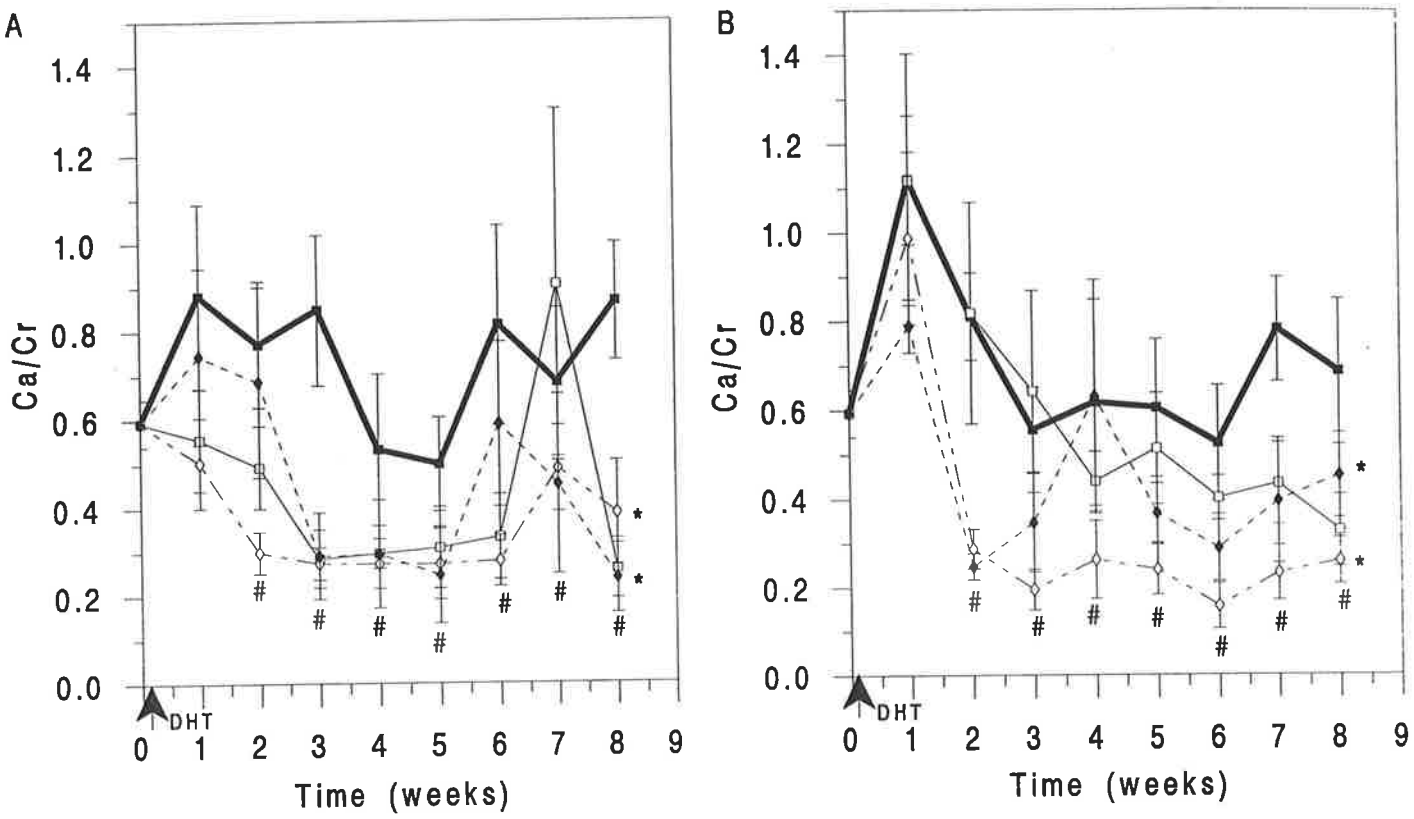


Figure 3.4 Urine calcium/creatinine (mmol/mmol) in sham (a) and oophx (b) rats following DHT administration at the time of operation. Values are Mean ± SE, n=6. (■) Vehicle, (□) 40mg/kg bw DHT, (◊) 80mg/kg bw DHT, (◆) 160mg/kg bw DHT. *P<0.001 versus vehicle within operation group, $^{\#}P$<0.001 versus week 1.

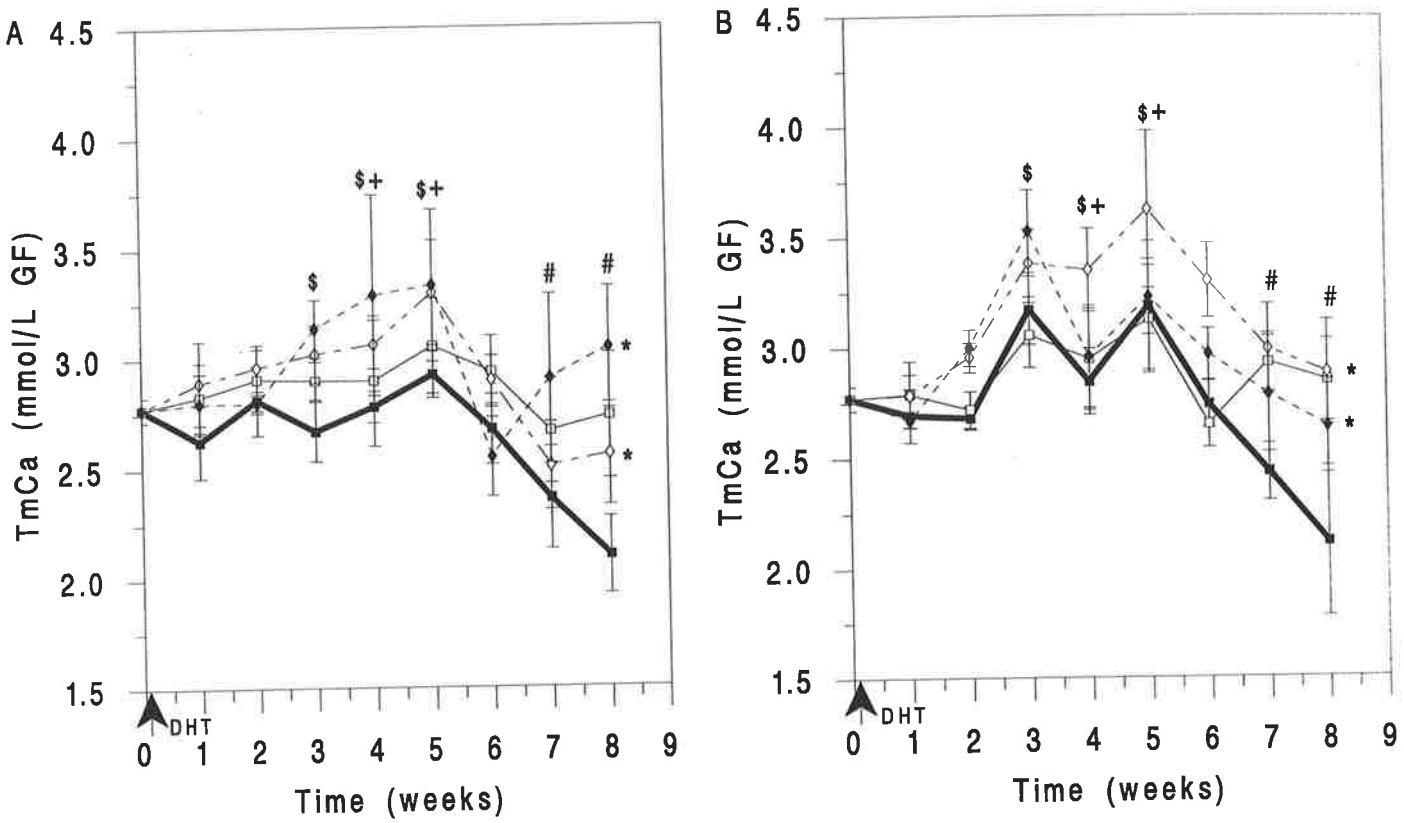


Figure 3.5 Tubular reabsorption of calcium (mmol/L GF) in sham (a) and oophx (b) rats following DHT administration at the time of operation. Values are Mean ± SE, n=6. (■) Vehicle, (□) 40mg/kg bw DHT, (◊) 80mg/kg bw DHT, (◆) 160mg/kg bw DHT. *$P<0.05$ versus vehicle within operation group, $^{\#}P<0.005$ versus weeks 7 and 8 within dosage group, $^{\$}P<0.005$ versus week 1, $^{+}P<0.005$ versus week 6.

3.3.6 Calcium Fractions

3.3.6.1 Effect of oophorectomy

Ionised calcium (Figure 3.6), total serum calcium (Table 3.4), ultrafiltrable calcium (Table 3.5) and complexed calcium (Table 3.6) were unaffected by operation in rats receiving vehicle. Protein bound calcium was decreased in oophorectomised rats receiving vehicle ($P<0.05$) (Table 3.7).

3.3.6.2 Effect of DHT Treatment in Sham and Oophorectomised Rats

Ionised calcium was significantly decreased with doses of 80mg and 160mg/kg bw DHT ($P<0.05$), with maximal suppression occurring at weeks 6 to 8 following commencement of DHT administration ($P<0.05$)(Figure 3.6). Total serum calcium was unaffected by DHT treatment in sham or oophx rats but decreased throughout the duration of the experiment ($P<0.001$)(Table 3.4). Although not significant, DHT treatment resulted in a greater decrease in total serum calcium in sham rats than in oophx rats resulting in a significantly higher level of total serum calcium in oophx rats compared to sham rats ($P<0.01$) only in DHT-treated rats (Table 3.4). Ultrafiltrable calcium, complexed calcium and protein bound calcium were unaffected by DHT administration but decreased throughout the duration of the experiment ($P<0.005$, $P<0.025$, $P<0.005$ respectively) in sham and oophx rats (Table 3.5, Table 3.6, Table 3.7).

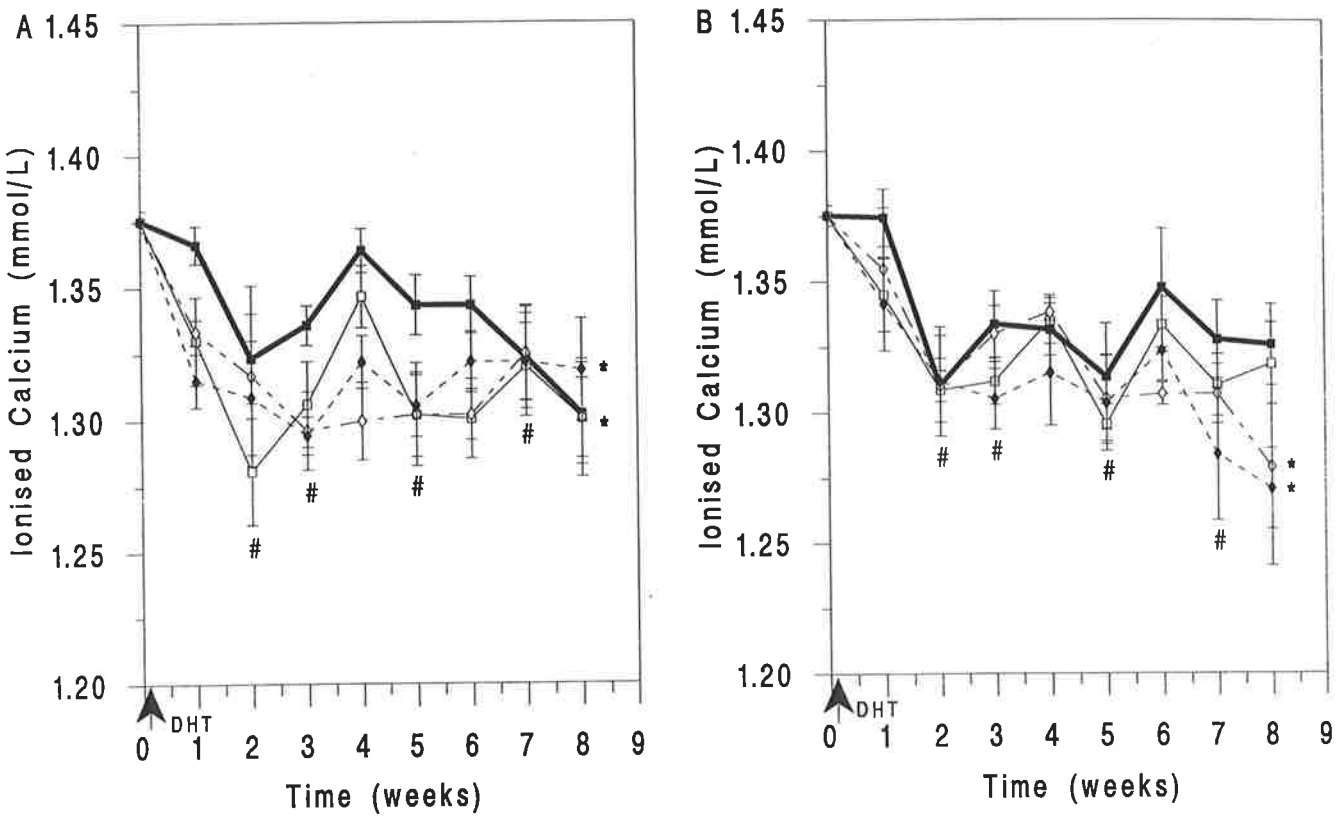


Figure 3.6 Serum ionised calcium (mmol/L) in sham (a) and oophx (b) rats following DHT administration at the time of operation. Values are Mean ± SE, n=6. (■) Vehicle, (□) 40mg/kg bw DHT, (◊) 80mg/kg bw DHT, (◆) 160mg/kg bw DHT. *P<0.05 versus vehicle within operation group, #P<0.05 versus week 1.

Table 3.4 Total serum calcium (mmol/L) in sham and oophorectomised rats following DHT treatment.

Time (Weeks)	Control				Oophx			
	Vehicle	40mg	80mg	160mg	Vehicle	40mg	80mg	160mg
1	2.66 (0.03)^{bc} n=6	2.68 (0.06)^{bc} n=6	2.69 (0.10)^{bc} n=6	2.71 (0.05)^{bc} n=6	2.76 (0.05)^{bc} n=6	2.75 (0.09)^{abc} n=6	2.73 (0.07)^{abc} n=6	2.56 (0.05)^{abc} n=6
2	2.83 (0.07)^{b} n=6	2.64 (0.07)^{b} n=6	2.61 (0.02)^{b} n=6	2.62 (0.03)^{b} n=6	2.66 (0.09)^{b} n=6	2.58 (0.05)^{ab} n=6	2.49 (0.03)^{ab} n=6	2.51 (0.05)^{ab} n=6
3	2.68 (0.08)^{bc} n=5	2.52 (0.02)^{bc} n=5	2.62 (0.08)^{bc} n=5	2.63 (0.04)^{bc} n=5	2.95 (0.07)^{bc} n=4	2.77 (0.06)^{abc} n=6	2.66 (0.05)^{abc} n=6	2.82 (0.13)^{abc} n=6
4	2.67 (0.02)^{bc} n=4	2.54 (0.22)^{bc} n=4	2.61 (0.05)^{bc} n=4	2.57 (0.06)^{bc} n=4	2.75 (0.04)^{bc} n=3	2.72 (0.09)^{abc} n=3	2.75 (0.02)^{abc} n=3	2.75 (0.01)^{abc} n=3
5	2.69 (0.09)^{bc} n=6	2.49 (0.06)^{bc} n=6	2.62 (0.07)^{bc} n=6	2.56 (0.13)^{bc} n=6	2.80 (0.09)^{bc} n=6	2.77 (0.11)^{abc} n=6	2.79 (0.11)^{abc} n=6	2.70 (0.08)^{abc} n=6
6	2.67 (0.09)^{b} n=6	2.46 (0.07)^{b} n=6	2.40 (0.10)^{b} n=6	2.29 (0.12)^{b} n=6	2.61 (0.13)^{b} n=5	2.41 (0.08)^{ab} n=6	2.49 (0.07)^{ab} n=6	2.56 (0.09)^{ab} n=6
7	2.42 (0.14) n=6	2.50 (0.12) n=6	2.32 (0.13) n=6	2.42 (0.09) n=6	2.48 (0.10) n=5	2.64 (0.10)^{a} n=6	2.45 (0.09)^{a} n=6	2.43 (0.10)^{a} n=6
8	2.16 (0.18) n=6	2.62 (0.19) n=6	2.34 (0.19) n=6	2.41 (0.15) n=6	2.08 (0.28) n=5	2.45 (0.07)^{a} n=6	2.38 (0.12)^{a} n=6	2.36 (0.13)^{a} n=6

DHT dose is mg per kg body weight. Values are Mean (SE). $^{a}P<0.01$ versus sham within dosage group, $^{b}P<0.001$ versus week 8 within operation and dosage group, $^{c}P<0.001$ versus weeks 6 and 7 within operation and dosage group.

Table 3.5 Serum ultrafiltrable calcium (mmol/L) in sham and oophorectomised rats following DHT administration.

Time (Weeks)	Control				Oophx			
	Vehicle	40mg	80mg	160mg	Vehicle	40mg	80mg	160mg
1	1.85 (0.03)^{abc} n=6	1.88 (0.04)^{abc} n=6	1.90 (0.05)^{abc} n=6	1.92 (0.03)^{abc} n=6	1.91 (0.02)^{abc} n=6	1.95 (0.06)^{abc} n=6	1.93 (0.05)^{abc} n=6	1.82 (0.04)^{abc} n=6
2	1.92 (0.04)^{bc} n=6	1.85 (0.03)^{bc} n=6	1.83 (0.02)^{bc} n=6	1.86 (0.03)^{bc} n=6	1.86 (0.04)^{bc} n=6	1.83 (0.02)^{bc} n=6	1.76 (0.02)^{bc} n=6	1.77 (0.04)^{bc} n=6
3	1.88 (0.06)^{abc} n=5	1.76 (0.01)^{abc} n=5	1.85 (0.06)^{abc} n=5	1.84 (0.03)^{abc} n=5	1.67 (0.42)^{abc} n=5	1.95 (0.04)^{abc} n=6	1.86 (0.03)^{abc} n=6	2.02 (0.10)^{abc} n=6
4	1.87 (0.04)^{abc} n=4	1.77 (0.15)^{abc} n=4	1.82 (0.02)^{abc} n=4	1.76 (0.06)^{abc} n=4	1.92 (0.03)^{abc} n=3	1.91 (0.07)^{abc} n=3	1.97 (0.05)^{abc} n=3	1.95 (0.03)^{abc} n=3
5	1.86 (0.06)^{abc} n=6	1.75 (0.04)^{abc} n=6	1.85 (0.05)^{abc} n=6	1.78 (0.09)^{abc} n=6	2.00 (0.09)^{abc} n=6	1.94 (0.09)^{abc} n=6	1.96 (0.08)^{abc} n=6	1.91 (0.06)^{abc} n=6
6	1.82 (0.06) n=6	1.73 (0.05) n=6	1.70 (0.06) n=6	1.61 (0.09) n=6	1.52 (0.31) n=6	1.67 (0.05) n=6	1.73 (0.05) n=6	1.73 (0.07) n=6
7	1.65 (0.10) n=6	1.74 (0.09) n=6	1.62 (0.09) n=6	1.70 (0.10) n=6	1.43 (0.29) n=6	1.85 (0.07) n=6	1.71 (0.06) n=6	1.69 (0.07) n=6
8	1.51 (0.13) n=6	1.59 (0.13) n=6	1.64 (0.12) n=8	1.70 (0.02) n-=6	1.21 (0.29) n=6	1.70 (0.05) n=6	1.67 (0.09) n=6	1.65 (0.09) n=6

DHT dose is mg per kg body weight. Values are Mean (SE). $^{a}P<0.005$ versus week 6, $^{b}P<0.005$ versus week 7, $^{c}P<0.005$ versus week 8.

Table 3.6 Serum complexed calcium (mmol/L) in sham and oophorectomised rats following DHT administration.

Time (Weeks)	Control				Oophx			
	Vehicle	40mg	80mg	160mg	Vehicle	40mg	80mg	160mg
1	0.44 (0.01)^{abc} n=6	0.45 (0.01)^{abc} n=6	0.46 (0.02)^{abc} n=6	0.47 (0.01)^{abc} n=6	0.46 (0.01)^{abc} n=6	0.46 (0.01)^{abc} n=6	0.46 (0.01)^{abc} n=6	0.44 (0.01)^{abc} n=6
2	0.45 (0.01)^{c} n=6	0.45 (0.01)^{c} n=6	0.45 (0.01)^{c} n=6	0.45 (0.01)^{c} n=6	0.46 (0.01)^{c} n=6	0.45 (0.01)^{c} n=6	0.43 (0.01)^{c} n=6	0.43 (0.01)^{c} n=6
3	0.45 (0.02)^{abc} n=5	0.43 (0.01)^{abc} n=5	0.45 (0.02)^{abc} n=5	0.45 (0.01)^{abc} n=5	0.41 (0.10)^{abc} n=5	0.48 (0.01)^{abc} n=6	0.46 (0.01)^{abc} n=6	0.51 (0.03)^{abc} n=6
4	0.44 (0.01)^{abc} n=4	0.44 (0.04)^{abc} n=4	0.45 (0.01)^{abc} n=4	0.34 (0.12)^{abc} n=4	0.48 (0.01)^{abc} n=3	0.48 (0.02)^{abc} n=3	0.49 (0.02)^{abc} n=3	0.50 (0.01)^{abc} n=3
5	0.45 (0.02)^{abc} n=6	0.44 (0.01)^{abc} n=6	0.46 (0.01)^{abc} n=6	0.45 (0.02)^{abc} n=6	0.50 (0.03)^{abc} n=6	0.48 (0.02)^{abc} n=6	0.49 (0.03)^{abc} n=6	0.48 (0.02)^{abc} n=6
6	0.44 (0.01) n=6	0.43 (0.01) n=6	0.43 (0.02) n=6	0.41 (0.02) n=6	0.37 (0.08) n=6	0.42 (0.01) n=6	0.43 (0.01) n=6	0.29 (0.09) n=6
7	0.38 (0.03) n=6	0.41 (0.03) n=6	0.38 (0.03) n=6	0.42 (0.02) n=6	0.34 (0.07) n=6	0.46 (0.02) n=6	0.42 (0.01) n=6	0.42 (0.02) n=6
8	0.35 (0.03) n=6	0.38 (0.03) n=6	0.40 (0.03) n=6	0.41 (0.02) n=6	0.29 (0.07) n=6	0.35 (0.07) n=6	0.40 (0.02) n=6	0.40 (0.02) n=6

DHT dose is mg per kg body weight. Values are Mean (SE). $^{a}P<0.005$ versus week 6, $^{b}P<0.005$ versus week 7, $^{c}P<0.005$ versus week 8.

Table 3.7 Serum protein bound calcium (mmol/L) in sham and oophorectomised rats following DHT treatment.

Time (Weeks)	Control				Oophx			
	Vehicle	40mg	80mg	160mg	Vehicle	40mg	80mg	160mg
1	0.81 (0.01)[a] n=6	0.79 (0.03)[a] n=6	0.79 (0.05)[a] n=6	0.80 (0.02)[a] n=6	0.84 (0.03)[a] n=6	0.80 (0.03)[a] n=6	0.80 (0.03)[a] n=6	0.74 (0.02)[a] n=6
2	0.90 (0.03)[a] n=6	0.79 (0.03)[a] n=6	0.77 (0.02)[a] n=6	0.76 (0.01)[a] n=6	0.79 (0.04)[a] n=6	0.75 (0.02)[a] n=6	0.72 (0.02)[a] n=6	0.73 (0.02)[a] n=6
3	0.80 (0.03)[a] n=5	0.76 (0.01)[a] n=5	0.77 (0.02)[a] n=5	0.79 (0.01)[a] n=5	0.68 (0.18)[a] n=5	0.82 (0.02)[a] n=6	0.80 (0.02)[a] n=6	0.81 (0.03)[a] n=6
4	0.80 (0.05)[a] n=4	0.78 (0.07)[a] n=4	0.78 (0.04)[a] n=4	0.81 (0.04)[a] n=4	0.83 (0.02)[a] n=3	0.82 (0.02)[a] n=3	0.78 (0.02)[a] n=3	0.80 (0.03)[a] n=3
5	0.82 (0.03)[a] n=6	0.74 (0.02)[a] n=6	0.77 (0.03)[a] n=6	0.78 (0.04)[a] n=6	0.80 (0.03)[a] n=6	0.82 (0.03)[a] n=6	0.83 (0.02)[a] n=6	0.79 (0.03)[a] n=6
6	0.84 (0.04) n=6	0.73 (0.02) n=6	0.70 (0.04) n=6	0.68 (0.03) n=6	0.66 (0.14) n=6	0.73 (0.03) n=6	0.76 (0.02) n=6	0.83 (0.05) n=6
7	0.77 (0.04) n=6	0.76 (0.04) n=6	0.71 (0.04) n=6	0.73 (0.02) n=6	0.64 (0.13) n=6	0.79 (0.03) n=6	0.74 (0.03) n=6	0.74 (0.03) n=6
8	0.66 (0.05) n=6	0.67 (0.06) n=6	0.70 (0.07) n=6	0.72 (0.05) n=6	0.52 (0.13) n=6	0.75 (0.03) n=6	0.71 (0.03) n=6	0.70 (0.04) n=6

DHT dose is mg per kg body weight. Values are Mean (SE). [a]$P<0.05$ versus week 8.

3.3.7 Phosphate

3.3.7.1 Effect of Oophorectomy

Urine phosphate/creatinine was significantly decreased following oophorectomy in animals receiving vehicle alone and in animals receiving DHT treatment ($P<0.05$) (Figure 3.7). TmP and serum phosphate were increased as a result of oophorectomy in rats receiving vehicle alone and in rats treated with DHT ($P<0.005$) (Figure 3.8, Figure 3.9).

3.3.7.2 Effect of DHT Treatment in Sham and Oophorectomised rats

DHT treatment had no effect on urine phosphate/creatinine in sham or oophx rats (Figure 3.7). TmP (Figure 3.8) and serum phosphate (Figure 3.9) however, were elevated following DHT treatment in sham rats only ($P<0.001$) and this effect was not time dependent but was dose dependent with significant stimulation occurring at 80mg and 160mg/kg bw DHT.

3.3.8 Serum Creatinine and Body Weight

3.3.8.1 Effect of Oophorectomy

Serum creatinine was unaffected by oophorectomy (Figure 3.10). Body weight was increased in oophorectomised rats receiving vehicle ($P<0.01$) (Figure 3.11).

3.3.8.2 Effect of DHT Treatment in Sham and Oophorectomised rats

Serum creatinine decreased following DHT treatment in both sham and oophx rats ($P<0.05$) and this effect was time dependent with maximal suppression occurring by 5 weeks following DHT administration ($P<0.05$) and dose dependent with maximal suppression occurring with 160mg/kg bw DHT (Figure 3.10). The effect of DHT to decrease serum creatinine was greater in the oophx rats and thus following DHT treatment

the oophx rats had significantly lower serum creatinine levels compared to sham rats (P<0.05) (Figure 3.10). Body weight increased over time (P<0.005) however it was unaffected by DHT (Figure 3.11). Although not significant, DHT increased body weight in sham-operated rats such that there was no significant difference in body weight between sham and oophx rats treated with DHT treatment (Figure 3.11). No relationship was observed between body weight and serum creatinine in either sham (r^2=0.015) or oophx (r^2=0.005) rats (data not presented).

3.3.9 Serum Alanine Aminotransferase, Albumin and Total Protein

3.3.9.1 Effect of Oophorectomy

Serum alanine aminotransferase (Table 3.8), albumin (Table 3.9) and total protein (Table 3.10) were unaffected by oophx in rats receiving vehicle.

3.3.9.2 Effect of DHT Treatment in Sham and Oophorectomised Rats

Serum alanine aminotransferase and albumin were unaffected by DHT treatment in sham and oophx rats (Table 3.8, Table 3.9). Serum alanine aminotransferase decreased throughout the duration of the experiment in both sham and oophx rats (P<0.01) (Table 3.8), however albumin levels remained unchanged throughout the duration of the experiment (Table 3.9). DHT administration decreased serum total protein in both sham and oophx rats (P<0.01) and this effect was not dose dependent but was time dependent with minimal levels occurring at weeks 1-3 (P<0.05) (Table 3.10).

3.3.10 Serum Electrolytes

Serum electrolytes are presented in Appendix A.

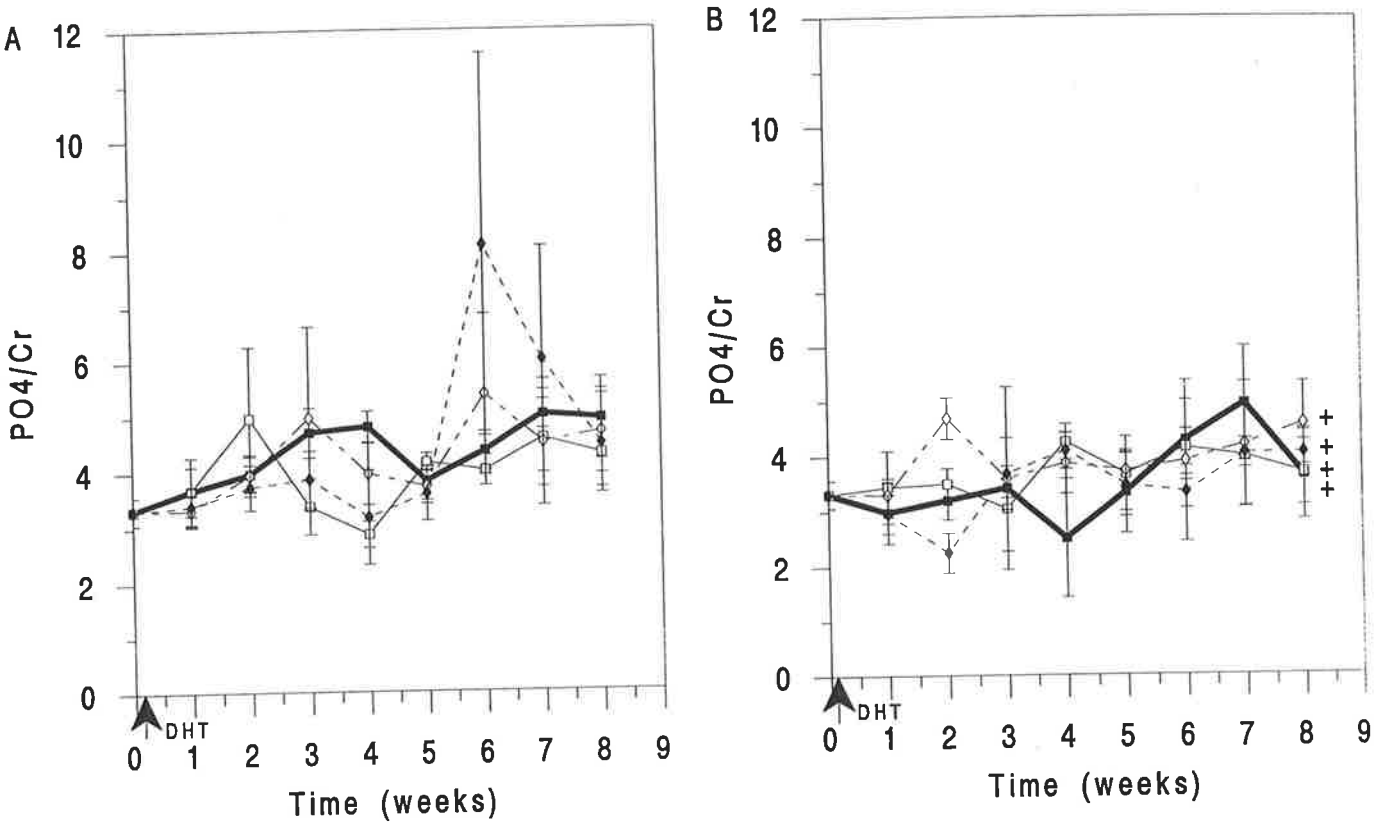


Figure 3.7 Urine phosphate/creatinine (mmol/mmol) in sham (a) and oophx (b) rats following DHT administration at the time of operation. Values are Mean ± SE, n=6. (■) Vehicle, (□) 40mg/kg bw DHT, (◊) 80mg/kg bw DHT, (◆) 160mg/kg bw DHT. $^{+}P<0.05$ versus sham (a) within dosage group.

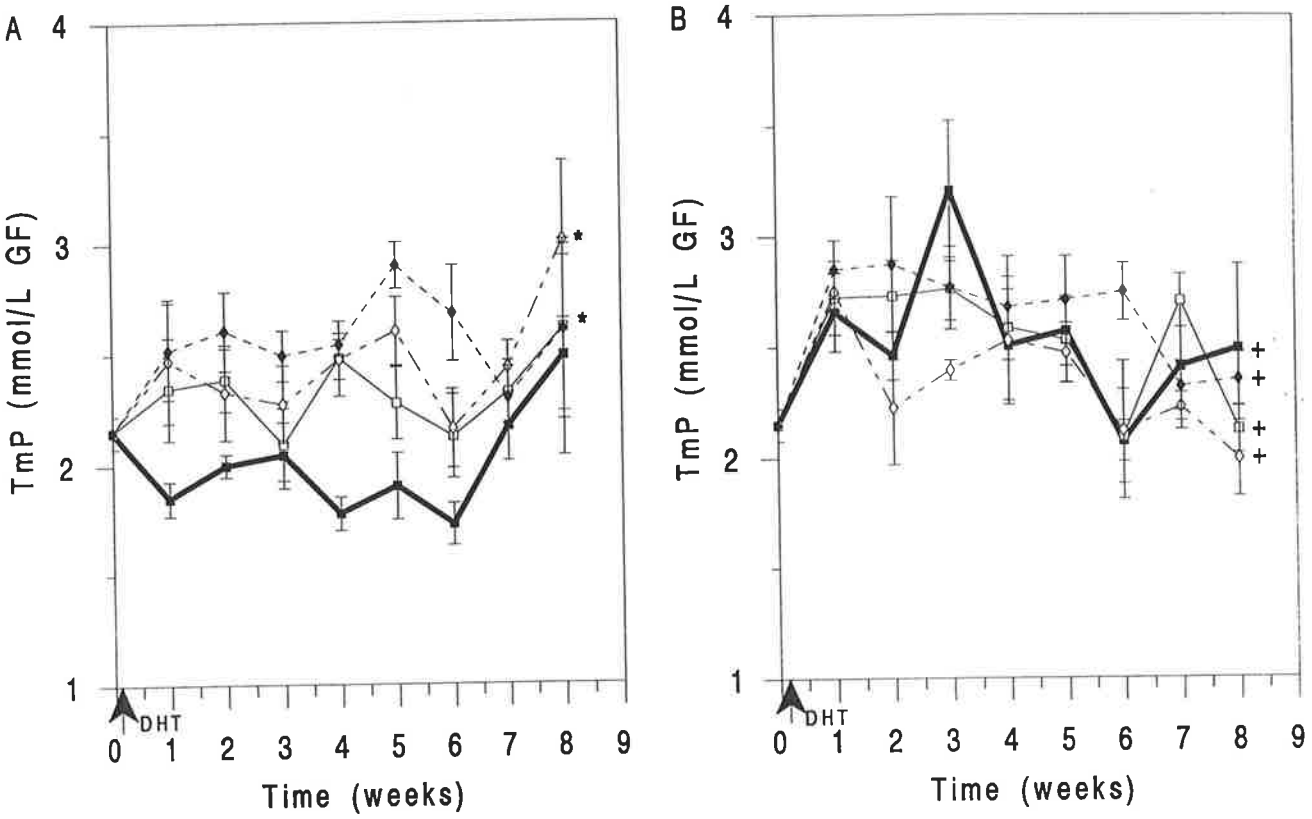


Figure 3.8 Tubular reabsorption of phosphate (mmol/L GF) in sham (a) and oophx (b) rats following DHT administration at the time of operation. Values are Mean ± SE, n=6. (■) Vehicle, (□) 40mg/kg bw DHT, (◊) 80mg/kg bw DHT, (◆) 160mg/kg bw DHT. $^{+}P<0.005$ versus sham (a) within dosage group, $*P<0.001$ versus vehicle within operation group.

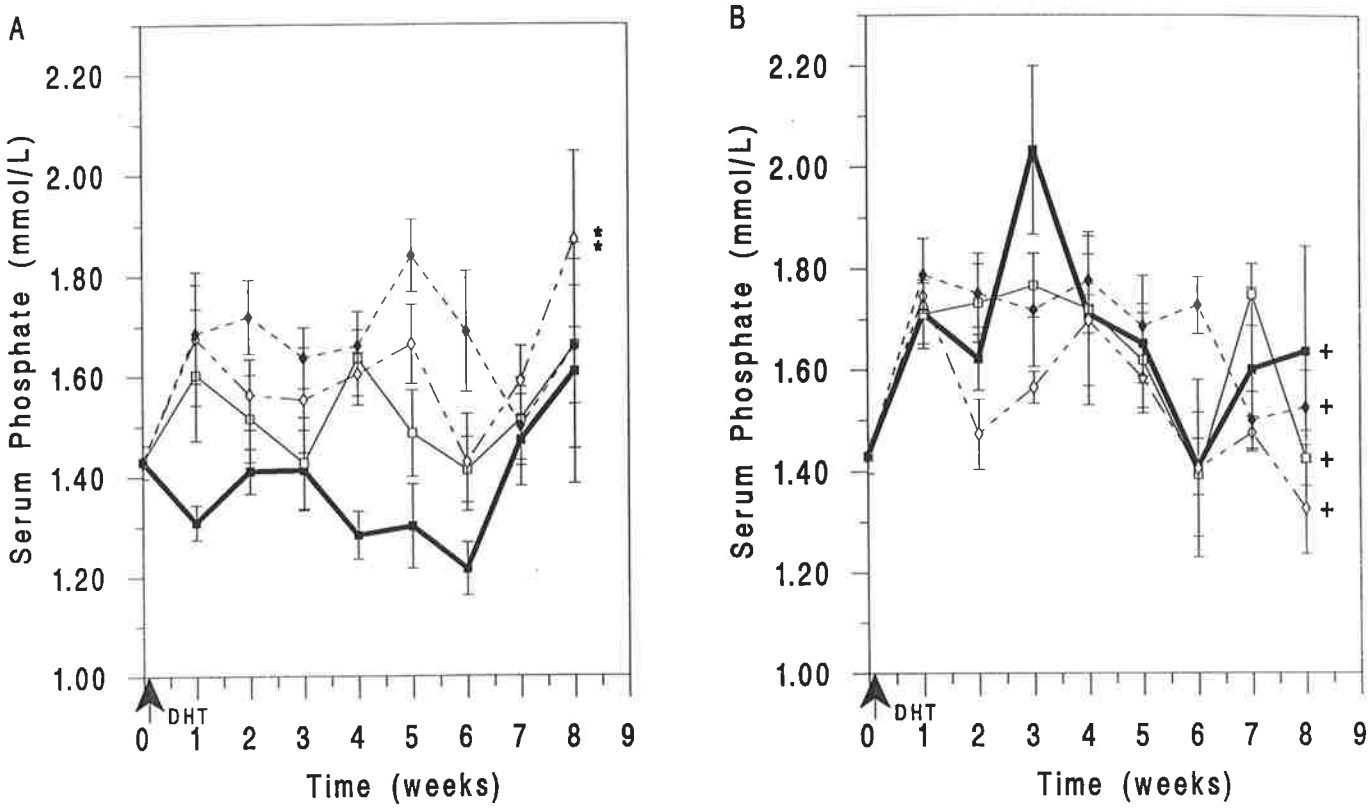


Figure 3.9 Serum phosphate (mmol/L) in sham (a) and oophx (b) rats following DHT administration at the time of operation. Values are Mean ± SE, n=6. (■) Vehicle, (□) 40mg/kg bw DHT, (◊) 80mg/kg bw DHT, (◆) 160mg/kg bw DHT. $^{+}P<0.005$ versus sham (a) within dosage group, $*P<0.001$ versus vehicle within operation group.

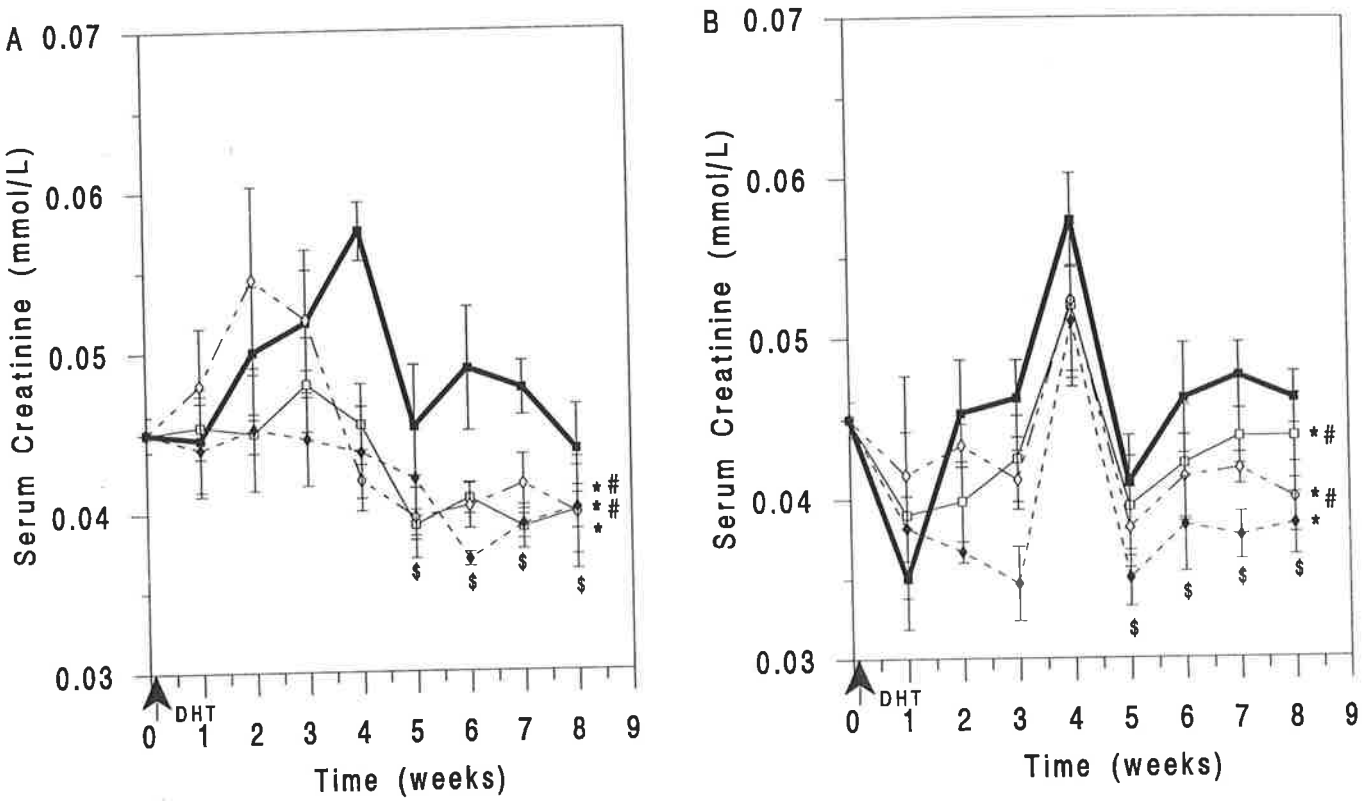


Figure 3.10 Serum creatinine (mmol/L) in sham (a) and oophx (b) rats following DHT administration at the time of operation. Values are Mean ± SE, n=6. (■) Vehicle, (□) 40mg/kg bw DHT, (◊) 80mg/kg bw DHT, (◆) 160mg/kg bw DHT. *P<0.05 versus vehicle within operation group, #P<0.05 versus 160mg/kg bw DHT within operation group, $$P$<0.05 versus week 1.

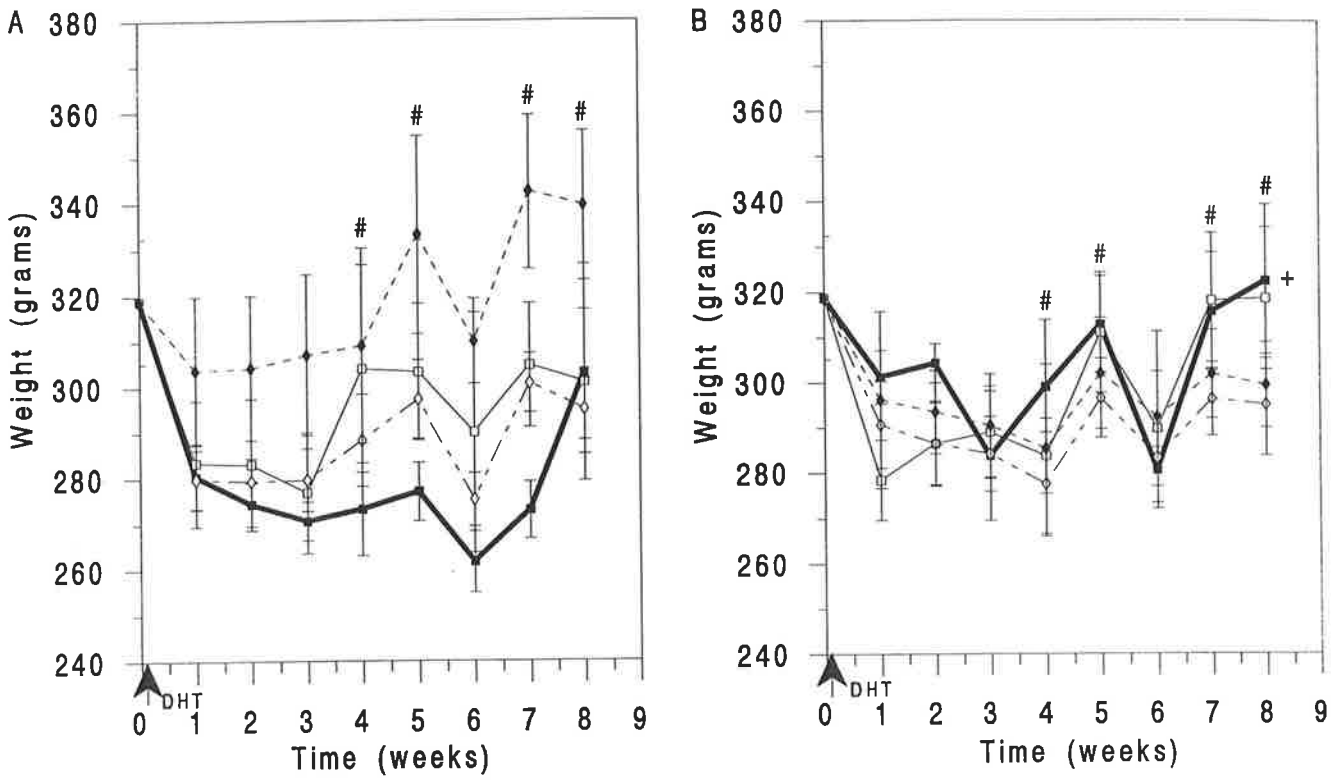


Figure 3.11 Body weight (grams) in sham (a) and oophx (b) rats following DHT administration at the time of operation. Values are Mean ± SE, n=6. (■) Vehicle, (□) 40mg/kg bw DHT, (◊) 80mg/kg bw DHT, (◆) 160mg/kg bw DHT. $^{+}P<0.01$ versus sham (a) within dosage group, $^{\#}P<0.005$ versus weeks 1, 2 and 3.

Table 3.8 Serum alanine aminotransferase (Units/L) in sham and oophorectomised rats following DHT treatment.

Time (Weeks)	Control				Oophx			
	Vehicle	40mg	80mg	160mg	Vehicle	40mg	80mg	160mg
0	22.02 (4.4) n=8	-	-	-	-	-	-	-
2	24.8 (8.7) n=4	18.0 (8.9) n=5	14.7 (6.4) n=6	11.9 (5.2) n=6	26.0 (7.3) n=6	30.4 (7.8) n=6	23.4 (7.4) n=6	30.7 (6.5) n=6
8	11.1 (3.5) n=5	13.1 (8.1) n=5	18.8 (6.6) n=6	10.3 (4.0) n=5	11.0 (4.8) n=5	16.8 (7.7) n=5	5.8 (3) n=5	9.2 (4.5) n=5

DHT dose is mg per kg body weight. Values are Mean (SE). Week 0 is immediately prior to DHT administration.

Table 3.9 Serum albumin (mmol/L) in sham and oophorectomised rats following DHT treatment.

Time (Weeks)	Control				Oophx			
	Vehicle	40mg	80mg	160mg	Vehicle	40mg	80mg	160mg
1	33.2 (1.5) n=6	32.7 (0.8) n=6	33.0 (0.73) n=6	32.2 (0.6) n=6	35.3 (1.2) n=6	33.2 (0.8) n=6	32.3 (0.9) n=6	31.8 (1.2) n=6
2	36.2 (1.4) n=6	34.5 (0.8) n=6	33.3 (1.0) n=6	33.0 (0.6) n=6	34.0 (1.4) n=6	33.2 (1.0) n=6	31.8 (0.5) n=6	34.8 (0.7) n=6
3	34.4 (1.0) n=5	34.8 (0.4) n=5	34.0 (1.0) n=5	34.0 (0.6) n=5	29.0 (6.1) n=4	34.7 (0.6) n=6	35.3 (0.7) n=6	34.0 (1.4) n=6
4	34.3 (4.8) n=4	35.0 (0.7) n=4	34.0 (1.7) n=4	35.5 (1.2) n=4	34.0 (1.5) n=3	33.0 (0.6) n=3	29.0(2.3) n=3	30.7 (4.1) n=3
5	35.2 (1.1) n=6	33.3 (0.8) n=6	33.7 (1.0) n=6	34.8 (0.6) n=6	31.2 (2.5) n=6	34.5 (0.8) n=6	34.2 (0.9) n=6	32.3 (2.5) n=6
6	35.3 (1.0) n=6	34.0 (0.6) n=6	33.3 (0.8) n=6	34.8 (0.7) n=6	33.8 (0.4) n=5	34.0 (0.7) n=6	34.7 (0.8) n=6	34.7 (0.6) n=6
7	35.7 (1.4) n=6	35.8 (0.5) n=6	34.2 (0.8) n=6	35.2 (0.9) n=6	35.2 (0.6) n=5	34.0 (0.5) n=6	35.3 (0.9) n=6	36.2 (0.3) n=6
8	33.3 (0.8) n=6	32.2 (0.9) n=6	33.0 (1.5) n=6	33.3 (0.7) n=6	32.8 (0.7) n=5	34.5 (1.1) n=6	34.0 (1.0) n=6	34.8 (1.1) n=6

DHT dose is mg per kg body weight. Values are Mean (SE).

Table 3.10 Serum total protein (mmol/L) in sham and oophorectomised rats following DHT treatment.

Time (Weeks)	Control				Oophx			
	Vehicle	40mg	80mg	160mg	Vehicle	40mg	80mg	160mg
1	67.7 (1.6) n=6	63.7 (2.1)[a] n=6	62.2 (3.1)[a] n=6	63.7 (1.6)[a] n=6	64.8 (1.8) n=6	59.7 (1.5)[a] n=6	62.0 (1.4)[a] n=6	61.7 (2.2)[a] n=6
2	69.8 (3.0) n=6	63.0 (2.5)[a] n=6	63.7 (1.7)[a] n=6	60.7 (1.2)[a] n=6	63.0 (2.2) n=6	61.2 (1.1)[a] n=6	60.3 (1.8)[a] n=6	58.7 (0.8)[a] n=6
3	61.4 (1.2) n=5	63.8 (1.5)[a] n=5	61.4 (1.5)[a] n=5	63.8 (0.9)[a] n=5	67.5 (1.7) n=4	60.7 (1.1)[a] n=6	62.7 (2.1)[a] n=6	57.7 (0.8)[a] n=6
4	64.3 (6.8)[d] n=4	66.0 (0.8)[ad] n=4	64.3 (4.7)[ad] n=4	62.5 (2.6)[ad] n=4	66.0 (1.5)[d] n=3	67.0 (1.5)[ad] n=3	64.0 (2.0)[ad] n=3	66.3 (1.8)[ad] n=3
5	66.0 (1.3) n=6	64.0 (0.9)[a] n=6	62.8 (1.8)[a] n=6	66.3 (2.1)[a] n=6	62.5 (1.5) n=6	62.7 (2.0)[a] n=6	64.67 (1.2)[a] n=6	64.3 (1.2)[a] n=6
6	71.2 (2.0)[bcd] n=6	63.2 (1.1)[abcd] n=6	61.0 (1.3)[abcd] n=6	63.5 (1.5)[abcd] n=6	66.8 (2.1)[bcd] n=5	67.3 (1.7)[abcd] n=6	66. 8 (1.6)[abcd] n=6	66.2 (1.3)[abcd] n=6
7	68.8 (1.5)[d] n=6	62.8 (1.6)[ad] n=6	63.3 (1.8)[ad] n=6	63.3 (2.1)[ad] n=6	67.4 (1.0)[d] n=5	65.2 (1.7)[ad] n=6	64.2 (1.7)[ad] n=6	65.2 (1.6)[ad] n=6
8	66.2 (1.5) n=6	65.0 (1.2)[a] n=6	64.0 (2.4)[a] n=6	62.8 (1.7)[a] n=6	65.4 (2.0) n=5	62.3 (1.2)[a] n=6	63.0 (1.9)[a] n=6	61.7 (1.8)[a] n=6

DHT dose is mg per kg body weight. Values are Mean (SE). [a]$P<0.01$ versus vehicle within operation group, [b]$P<0.05$ versus week 1, [c]$P<0.05$ versus week 2, [d]$P<0.05$ versus week 3.

3.4 DISCUSSION

The serum DHT concentrations achieved with silastic tubing implants in this study were comparable to those achieved by Chen and coworkers (1994). Although the serum concentrations of DHT were decreased at week 8, these decreased levels remained considerably elevated at all doses compared to the vehicle implants. The decrease in serum DHT levels in oophx rats compared to sham rats in the vehicle treated groups, although not statistically significant was most likely due to the absence of ovarian tissue in these rats which contributes to the circulating level of androgens (Fearnley et al 1978). It is surprising that this difference was not of a larger magnitude, since approximately half of the secreted androgens are produced by the ovaries and the remaining proportion is produced by the adrenal glands with this contribution from the adrenal glands decreasing with age (Mauvais-Jarvis et al 1981). This observation however, may have reached statistical significance with a larger number of rats.

An increase of serum alkaline phosphatase was observed in both sham and oophx rats following DHT treatment. The response was rapid with stimulation evident after 2 weeks and was sustained throughout the duration of the experiment. No changes were detected in serum albumin levels, a protein synthesised by the liver, or alanine aminotransferase, a liver enzyme, suggesting that the effect of DHT is on the production of alkaline phosphatase in bone, rather than liver alkaline phosphatase. This effect is consistent with the induction of alkaline phosphatase by DHT in isolated osteoblast cells invitro, which was blocked by hydroxyflutamide, an androgen receptor antagonist, confirming this activity is mediated by the androgen receptor (Kasperk et al 1989). Furthermore, the

synthetic androgen nandrolone decanoate has been demonstrated to have an anabolic effect on longitudinal and periosteal bone growth in young oophx rats, resulting in an increase in cortical width, femoral length and calcium content (Schot et al 1993). The resultant levels of alkaline phosphatase in both sham and oophx rats were comparable, suggesting that the doses of DHT used in the present study provided maximal stimulation of osteoblasts independent of ovarian status.

In contrast, DHT treatment did not effect serum osteocalcin levels in sham-operated rats or oophx rats. Treatment with nandrolone decanoate to young oophx rats has been demonstrated to decrease serum osteocalcin levels (Schot et al 1993), however such data is difficult to interpret as nandrolone decanoate can be metabolised to estrogen and serum osteocalcin levels decrease following estrogen treatment in oophx rats (Sims et al 1996b). In the present study therefore, a differential effect of DHT on the osteoblast products alkaline phosphatase and osteocalcin has been observed. It is proposed that alkaline phosphatase and osteocalcin synthesis reflect different aspects of osteoblastic activity (Owen et al 1990), and therefore this discordant effect of DHT on their serum levels may indicate that androgens act at a specific stage of osteoblast maturation. These data are consistent with a model that DHT stimulates osteoblasts at the matrix maturation stage of development when alkaline phosphatase is synthesised without stimulating the osteoblasts at the stage of mineralisation when osteocalcin is synthesised.

DHT administration had no effect on urine deoxypyridinoline excretion and urine hydroxyproline in either sham or oophx rats. Such data indicates that androgens do not directly effect bone resorption, independent of estrogen status. Similarly, treatment with

nandrolone decanoate in osteoporotic, post-menopausal women has been shown to have no effect on urine hydroxyproline (Need et al 1987). In contrast, Johanssen and colleagues (1989) proposed that an increase in bone mineral content observed in postmenopausal women following nandrolone decanoate treatment was due to a decrease in bone resorption, as biochemical markers of bone formation were unchanged during the treatment. No markers of bone resorption were measured in this experiment however, and calcium supplements which have anti-resorptive actions were also administered. Contrary to this proposal, DHT has no effect on rat osteoclast cultures or osteoclast-osteoblast co-cultures (Tobias and Chambers 1991). Treatment of young and old oophx rats with nandrolone decanoate administered immediately following operation results in a decrease in serum osteocalcin levels (Schot et al 1993), which was attributed to an overall anti-resorptive effect. The biochemical markers measured in the present study however, better reflect the changes in bone resorption following DHT treatment than osteocalcin, an osteoblast specific product. Hydroxyproline is produced from the breakdown of collagen and deoxypyridinoline is a specific breakdown product derived from bone collagen.

Urine hydroxyproline, urine deoxypyridinoline, alkaline phosphatase and osteocalcin were increased as a result of oophorectomy compared to ovary-intact rats receiving vehicle alone. This is consistent with increased bone turnover associated with the bone loss observed following oophorectomy and is in agreement with previous findings (Ismail et al 1988, Kalu 1991a, Morris et al 1992, Sims et al 1996a). It is interesting to note that DHT abolished the effect of oophorectomy on bone turnover, as the resultant levels of all bone biochemical variables measured following DHT administration were not significantly different between sham and oophx rats. The major factors contributing to this effect were

the greater increase of alkaline phosphatase and the slight but not significant increase in serum osteocalcin, deoxypyridinoline and hydroxyproline excretion in sham rats compared with oophx rats. These results provide further evidence for an interaction between DHT and estrogen which has previously been reported in the oophx rat model (Coxam et al 1996) and requires additional investigation.

Urine calcium excretion and the tubular reabsorption of calcium at the kidney was unaffected by oophorectomy, which is in agreement with previous findings (Yamazaki and Yamaguchi 1989, Morris et al 1992). In a recent study however, urine calcium was shown to be elevated in oophx rats (Morris et al 1995). Morris and colleagues (1995) investigated the effects of oophorectomy for 130 days post-operation and thus the duration of the current study may have been too short to observe these changes in urine calcium following oophorectomy.

Urine calcium excretion was significantly decreased with DHT administration in both sham and oophx rats. Nandrolone decanoate administration also decreases urine calcium excretion in post-menopausal women (Riggs et al 1972, Chestnut et al 1983, Need et al 1987) and in young oophx rats (Schot et al 1993). The decrease in urine calcium observed in the present study was associated with an increase in the tubular reabsorption of calcium in the kidney suggesting a direct action of DHT on the kidney in agreement with the findings of Need and colleagues (1987) in postmenopausal women. Ionised calcium was significantly decreased in sham and oophx rats administered DHT. This may reflect increased calcium incorporation into bone with the increased bone formation, indicated by the rise in alkaline phosphatase observed following DHT treatment. The ability of DHT to

conserve calcium at the level of the kidney contributes to the availability of calcium for incorporation into bone, and subsequent increase in bone volume.

Parathyroid hormone and 1,25 dihydroxyvitamin D_3 were unaffected by oophorectomy which is consistent with previous studies (Turner et al 1989, Kalu et al 1989) and were unchanged following DHT treatment in sham and oophx rats. These data indicate that androgens do not affect calcium homeostasis at the level of the calciotropic hormone, parathyroid hormone or 1,25 dihydroxyvitamin D_3 mediated intestinal calcium absorption. In contrast, radiocalcium absorption increases following nandrolone decanoate treatment in postmenopausal women (Need et al 1987), however serum levels of 1,25 dihydroxyvitamin D_3 were not measured. Johanssen and colleges (1989) demonstrated no change in vitamin D metabolites following nandrolone decanoate treatment combined with calcium supplements in postmenopausal women. Since nandrolone decanoate is metabolised to estrogen the observed changes in calcium absorption following treatment may be due to an estrogenic effect on the gut to increase intestinal calcium absorption (Civitelli et al 1988) and not as a result of androgenic activity. A rise in parathyroid hormone following DHT treatment may have been expected as serum ionised calcium levels were decreased following DHT treatment. Changes in ionised calcium of a larger magnitude than observed in the current study however, are required to evoke a change in serum parathyroid hormone levels in the rat (unpublished observation, Mason and Morris 1994).

Oophorectomy decreased urine phosphate excretion and increased serum phosphate levels which was associated with an increase in the tubular reabsorption of phosphate in the kidney. These data are consistent with previous findings in young (Yamazaki and

Yamaguchi 1989) and mature rats (Morris et al 1992) and estrogen replacement therapy decreases serum phosphate and the tubular reabsorption of phosphate in postmenopausal women (Selby et al 1985). DHT treatment had no effect on urine phosphate excretion in either sham or oophx rats. Serum phosphate was increased following DHT administration in sham-operated rats only which was associated with an increase in the tubular reabsorption of phosphate. Such data suggest that there is an interaction of estrogens and androgens on the renal handling of phosphate to increase serum levels of phosphate and the tubular reabsorption of phosphate in the kidney without altering the excretion of phosphate in the urine. This is the first time the effect of androgens on phosphate homeostasis in sham rats has been identified.

Body weight was increased following oophorectomy which has been associated with a partial protective effect against the development of osteopenia, proposed to occur by stimulation of bone formation by mechanical stress in weight bearing bones (Wronski et al 1987). It is interesting to note that there was no difference in body weight between the sham and oophx rats following DHT treatment due to a non significant increase in sham rats. DHT treatment decreased serum creatinine in all groups. This is in contrast to previous human studies which have demonstrated an increase in serum creatinine following androgen treatment and is attributed to an increase in muscle mass, consistent with weight gain (Chestnut et al 1983, Erdstieck et al 1994). In the present study however, no correlation was observed between serum creatinine and weight in any group which suggests that the rise in alkaline phosphatase was due to a direct effect of DHT on bone cells and not solely due to increases in mechanical stress on the skeleton and subsequent stimulation of bone formation.

Serum albumin, total calcium and total protein were unaffected by oophx which is in contrast to previous findings where albumin was decreased following oophorectomy (Morris et al 1992). DHT treatment had no effect on the serum levels of albumin or total calcium which is consistent with findings in post menopausal women following nandrolone decanoate treatment (Riggs et al 1972). In the present study, the ultrafiltrable calcium and complexed calcium components of total serum calcium were unaffected by DHT treatment while protein bound calcium was decreased.

In summary, this study has demonstrated that even at high doses DHT exerts a stimulatory effect on alkaline phosphatase levels with a decrease in ionised calcium levels consistent with stimulation of bone formation. The action of DHT is most likely due to a direct effect on bone cell activity rather than by stimulation of muscle tissue with an indirect effect on bone, as serum creatinine levels were unchanged and the increase in body weight in sham rats was not significant. DHT had no effect on bone resorption as indicated by urine hydroxyproline and urine deoxypyridinoline excretion in the estrogen sufficient or estrogen-deficient rats. The primary action of DHT when administered immediately following operation was to increase bone formation with no effect on bone resorption. In addition, DHT increased the renal tubular reabsorption of calcium suggesting a direct effect on the kidney. Since DHT is not metabolised to estrogen each of these actions must be mediated by the androgen receptor.

CHAPTER 4

EFFECT OF DHT ON BONE BIOCHEMICAL MARKERS IN OSTEOPENIC OOPHORECTOMISED RATS

4.1 INTRODUCTION

Oophorectomised rats with established osteopenia have been used as a model of post-menopausal bone loss that more closely represents the human menopausal state. In osteopenic oophorectomised rats, the initial phase of increased bone resorption and bone loss following ovarian hormone deficiency has passed and the trabecular bone volume is stabilised at a lower level to that before oophorectomy (Wronski et al 1988b).

DHT treatment in osteopenic oophx rats results in a net gain in bone volume rather than the prevention of further bone loss (Tobias et al 1994). The accrual of bone is due to an increase in bone formation rate with increased surface extent of flurochrome labels resulting in both increased trabecular thickness and number in the tibiae (Tobias et al 1994) and stimulation of periosteal bone formation in the femur (Coxam et al 1996). At high doses of DHT, the extent of osteoclast surface and number is reduced (Tobias et al 1994).

Although the effect of androgens on bone turnover in osteopenic oophorectomised rats is well documented by histomorphometric techniques, few studies have investigated the effects of androgens on the biochemical markers of bone turnover. The following study

demonstrates significant biochemical changes with DHT treatment providing further information on the activities of androgens on bone and calcium metabolism.

4.2 EXPERIMENTAL PROTOCOL

4.2.1 Experimental Procedure

24 animals were randomly divided into 4 groups and all animals were oophorectomised under halothane anaesthesia (Chapter 2.3.2). At 15 weeks post-operation the rats were administered either vehicle (silastic tubing), 40mg, 80mg or 160mg/kg/body weight dihydrotestosterone as described in Chapter 3.2.1. 24 hour fasting blood (Chapter 2.3.4) and urine samples (Chapter 2.3.5) were collected every 2 weeks for 14 weeks.

4.2.2 Biochemical Analyses

Urine volumes were recorded. Urine was analysed for hydroxyproline (Chapter 2.4.2), free deoxypyridinoline (Chapter 2.4.3), creatinine (Chapter 2.4.1), calcium and phosphate (Chapter 2.4.4). Serum was analysed for DHT (Chapter 2.4.9), osteocalcin (Chapter 2.4.7), alkaline phosphatase (Chapter 2.4.13), alanine aminotransferase (Chapter 2.4.12), ionised calcium (Chapter 2.4.5), sodium, potassium, chloride, bicarbonate (Chapter 2.4.6), calcium, phosphate, creatinine (Chapter 2.4.16), albumin (Chapter 2.4.14), and total protein (Chapter 2.4.15). Urinary hydroxyproline, calcium, phosphate and creatinine excretion were determined in 24 hour fasting urine specimens (Chapter 2.5). TmCa and TmP were calculated as described in Chapter 2.5.

4.2.3 Statistical analyses

Statistical analyses for bone biochemical variables were performed on corrected data (Chapter 2.5). The effect of DHT administration, time and interactions of these variables were determined using multiple comparisons of mean values (Chapter 2.6.2). The data were further analysed using a Tukey's post hoc test (Chapter 2.6.3). Regression analysis was used to determine the relationship between weight and serum creatinine (Chapter 2.6.4).

4.3 RESULTS

4.3.1 Serum Dihydrotestosterone

Serum DHT levels correlated with dose ($P<0.001$) (Table 4.1) and the levels at 29 weeks post-implantation did not significantly differ from those measured at 8 weeks post-implantation reported in Chapter 3.3.1.

Table 4.1 Serum DHT (pmol/L) in osteopenic oophorectomised rats following DHT administration at 15 weeks post-oophorectomy.

Time (Weeks)	Dose Dihydrotestosterone (mg/kg body weight)			
	Vehicle	40	80	160
17	-	1338 (334)[ac] n=5	2394 (602)[ac] n=6	5772 (1073)[abc] n=6
23	158 (21) n=5	1011 (210)[a] n=6	1882 (695)[a] n=5	3750 (488)[ab] n=6
29	172 (24) n=5	1022 (148)[a] n=6	1352 (186)[a] n=6	2580 (451)[ab] n=6

Values are Mean (SE). Time is weeks post-oophorectomy. DHT was administered following collection of blood and urine samples at 15 weeks post-oophorectomy. Values at weeks 17 and 23 post-oophorectomy (2 and 8 weeks post-treatment) were taken from Chapter 3, Table 3.1. [a]$P<0.001$ versus vehicle, [b]$P<0.001$ versus 40mg/kg bw and 80mg/kg bw DHT, [c]$P<0.001$ versus weeks 23 and 29.

4.3.2 Urine Volume and 24 hour urine creatinine excretion

Urine volume and 24 hour urine creatinine excretion were unaffected by DHT administration and remained unchanged throughout the duration of the experiment (Appendix B.1, Appendix B.2). The urine biochemical analyses therefore were expressed as a ratio to creatinine.

4.3.3 Bone Biochemical Markers

Serum alkaline phosphatase was increased as a result of DHT administration at doses of 80mg and 160mg/kg body weight DHT ($P<0.05$) and this effect was time dependent with maximal serum levels not occurring until 14 weeks after commencing treatment (29 weeks post-operation) ($P<0.001$) (Figure 4.1). Serum osteocalcin was decreased following DHT treatment ($P<0.01$) and this effect was independent of dose but was time dependent with maximal suppression occurring at 14 weeks after commencement of treatment (29 weeks post-oophorectomy) ($P<0.01$) (Figure 4.2).

Urine deoxypyridinoline/creatinine was decreased by DHT treatment ($P<0.001$) and this effect was dose dependent with maximal suppression occurring at 160mg/kg bw DHT (Table 4.2). Urine hydroxyproline/creatinine was unaffected by DHT treatment but was elevated at 14 weeks post-treatment (29 weeks post-oophorectomy) ($P<0.05$)(Table 4.3).

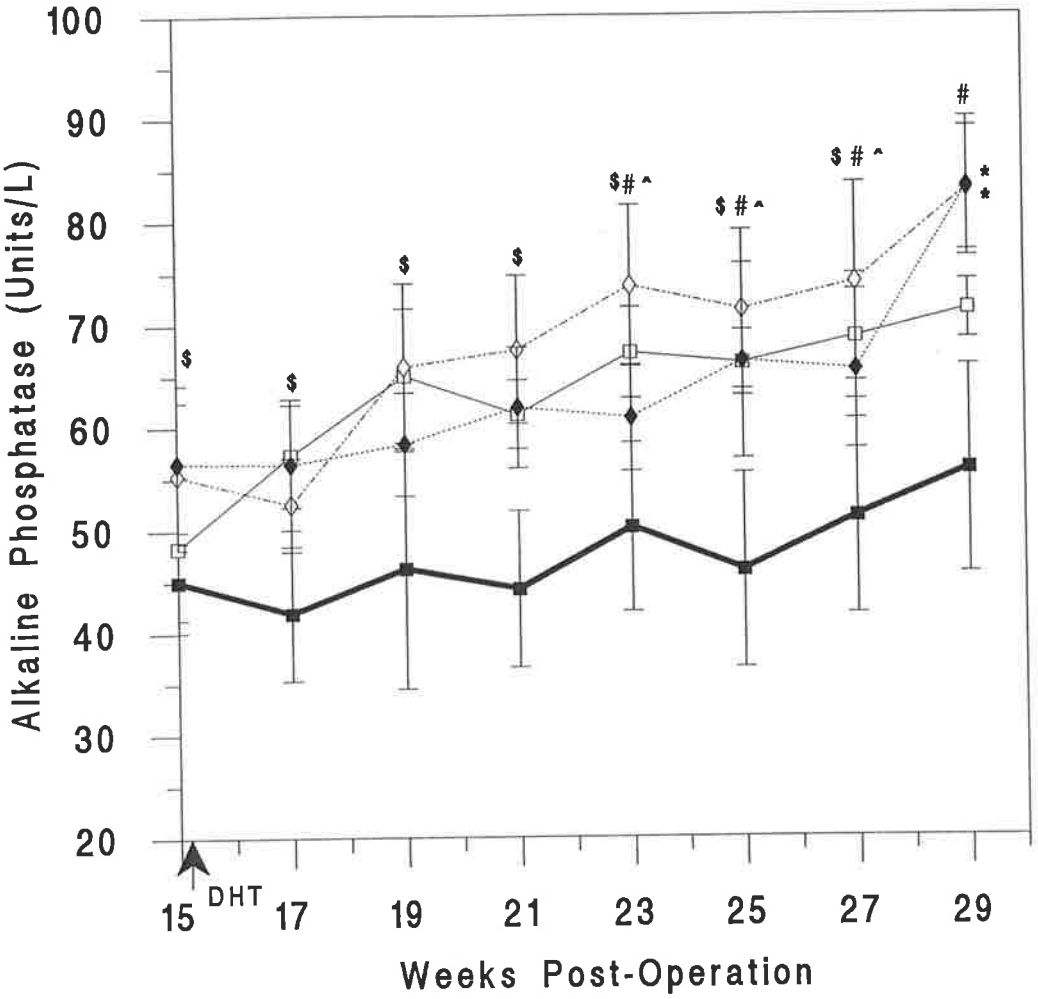


Figure 4.1 Serum alkaline phosphatase (units/L) in osteopenic oophx rats administered DHT at 15 weeks post-oophorectomy. Values are Mean ± SE, n=6. (■) Vehicle, (□) 40mg/kg bw DHT, (◊) 80mg/kg bw DHT, (◆) 160mg/kg bw DHT. *P<0.05 versus vehicle, #P<0.001 versus week 15, $$P$<0.001 versus week 29, ^$P$<0.001 versus week 17.

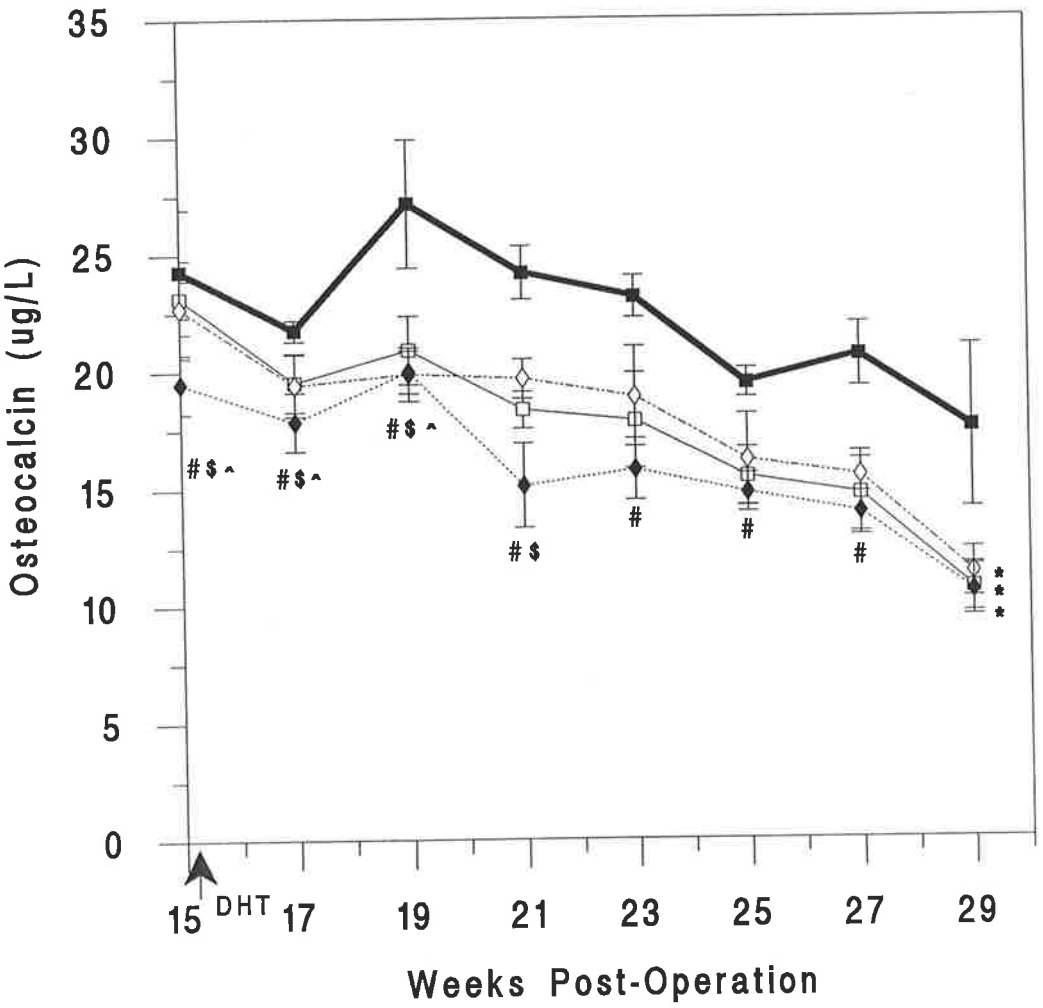


Figure 4.2 Serum osteocalcin (μg/L) in osteopenic oophx rats administered DHT at 15 weeks post-oophorectomy. Values are Mean ± SE, n=6. (■) Vehicle, (□) 40mg/kg bw DHT, (◊) 80mg/kg bw DHT, (◆) 160mg/kg bw DHT. *P<0.01 versus vehicle, $^{\#}P$<0.01 versus week 29, $^{\$}P$<0.01 versus week 27, $^{\wedge}P$<0.01 versus week 25.

Table 4.2 Urine deoxypyridinoline/creatinine (nmol/mmol) in osteopenic oophorectomised rats following DHT administration at 15 weeks post-oophorectomy.

Time (Weeks)	Dose Dihydrotestosterone (mg/kg body weight)			
	Vehicle	40	80	160
15	48.9 (5.8)[a] n=5	60.9 (4.7)[a] n=5	48.9 (6.9) n=6	29.4 (1.9) n=5
29	44.5 (2.0)[ab] n=5	30.5 (3.6)[ab] n=6	25.6 (2.9)[b] n=6	24.5 (3.1)[b] n=5

Values are Mean (SE). Time is weeks post-oophorectomy. DHT was administered following collection of blood and urine samples at 15 weeks post-oophorectomy. [a]$P<0.05$ versus 160mg DHT/kg bw, [b]$P<0.001$ versus week 15.

Table 4.3 Urine hydroxyproline/creatinine (μmol/L) in osteopenic oophorectomised rats following DHT administration at 15 weeks post-oophorectomy.

Time (Weeks)	Dose Dihydrotestosterone (mg/kg body weight)			
	Vehicle	40	80	160
15	10.4 (0.9) n=5	8.8 (0.6) n=6	8.8 (1.0) n=6	7.3 (0.8) n=6
17	10.4 (0.4) n=5	10.4 (0.7) n=6	10.9 (0.4) n=6	10.6 (1.2) n=5
19	10.5 (0.8) n=5	10.2 (0.4) n=6	10.2 (0.6) n=6	12.5 (1.3) n=6
21	10.5 (0.8) n=5	11.5 (0.5) n=6	11.7 (0.7) n=6	12.4 (1.0) n=6
23	10.1 (0.6) n=5	9.7 (0.8) n=6	10.2 (0.8) n=6	13.3 (1.3) n=6
25	10.5 (0.9) n=5	9.6 (0.5) n=5	10.0 (0.7) n=6	10.6 (0.7) n=6
27	11.0 (1.0) n=5	10.0 (0.9) n=5	10.0 (1.0) n=5	11.1 (0.9) n=6
29	11.1 (0.6)[a] n=3	9.6 (1.5)[a] n=5	12.1 (0.8)[a] n=6	15.4 (1.2)[a] n=6

Values are Mean (SE). Time is weeks post-oophorectomy. DHT was administered following collection of blood and urine samples at 15 weeks post-oophorectomy. [a]$P<0.05$ versus week 15.

4.3.4 Urine Calcium

The fall in urine calcium/creatinine following DHT treatment did not reach statistical significance (P=0.13) (Figure 4.3) but decreased throughout the duration of the experiment in all groups (P<0.05). TmCa was elevated by DHT treatment (P<0.05) (Figure 4.4) and this was dose dependent with maximal stimulation occurring with 80mg and 160mg/kg body weight DHT and was time dependent with maximal stimulation occurring between 6 and 12 weeks following treatment (21 and 27 weeks post-oophorectomy) (P<0.05).

4.3.5 Calcium Fractions

Serum ionised calcium, total serum calcium, ultrafiltrable calcium, complexed calcium and protein bound calcium were unaffected by DHT administration (Table 4.4, Table 4.5, Table 4.6, Table 4.7, Table 4.8). Serum ionised calcium was increased at 12 weeks following commencement of DHT treatment (27 weeks post oophorectomy) (P<0.005)(Table 4.4). Total serum calcium decreased throughout the duration of the experiment (P<0.001)(Table 4.5). Ultrafiltrable calcium, complexed calcium and protein bound calcium were unchanged throughout the duration of the experiment (Table 4.6, Table 4.7, Table 4.8).

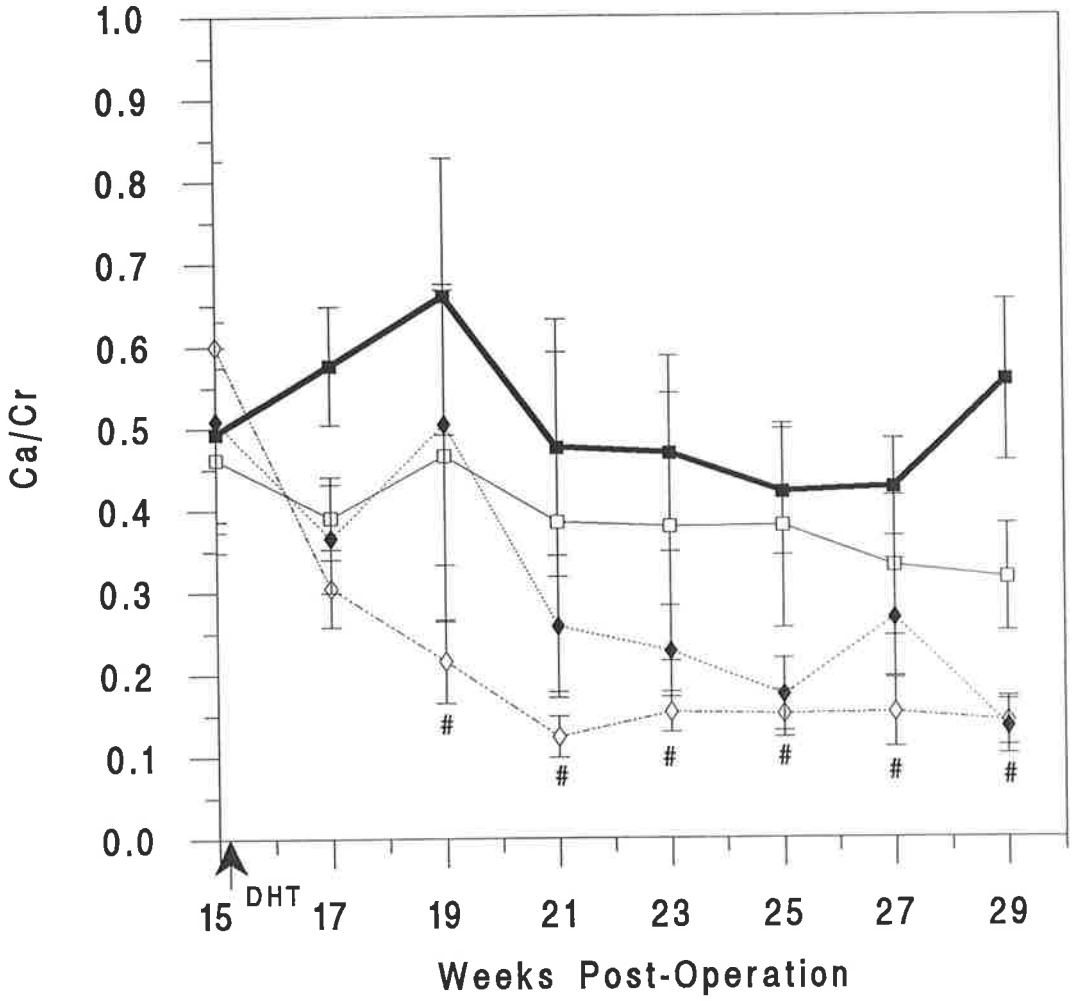


Figure 4.3 Urine calcium/creatinine (mmol/mmol) in osteopenic oophx rats administered DHT at 15 weeks post-oophorectomy. Values are Mean ± SE, n=6. (■) Vehicle, (□) 40mg/kg bw DHT, (◊) 80mg/kg bw DHT, (◆) 160mg/kg bw DHT. $^{\#}P<0.05$ versus week 15.

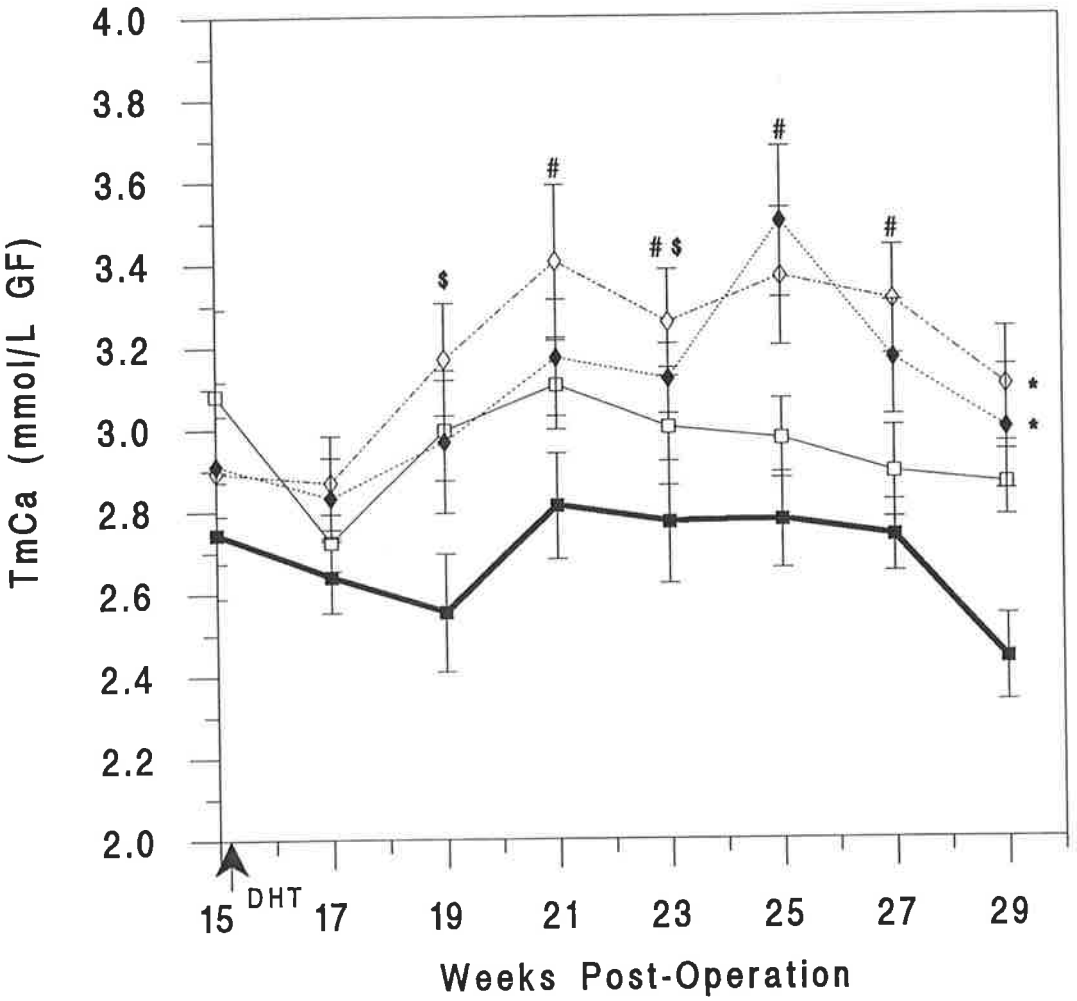


Figure 4.4 Tubular reabsorption of calcium (TmCa) (mmol/L GF) in osteopenic oophx rats administered DHT at 15 weeks post-oophorectomy. Values are Mean ± SE, n=6. (■) Vehicle, (□) 40mg/kg bw DHT, (◊) 80mg/kg bw DHT, (◆) 160mg/kg bw DHT. *P<0.05 versus vehicle, $^{\#}P$<0.05 versus week 17, $^{\$}P$<0.05 versus week 29.

Table 4.4 Serum ionised calcium (mmol/L) in osteopenic oophorectomised rats following DHT administration at 15 weeks post-oophorectomy.

Time (Weeks)	Dose Dihydrotestosterone (mg/kg body weight)			
	Vehicle	40	80	160
15	1.33 (0.02)[a] n=5	1.33 (0.01)[a] n=6	1.35 (0.02)[a] n=6	1.34 (0.03)[a] n=6
17	1.36 (0.02)[a] n=5	1.36 (0.02)[a] n=6	1.36 (0.01)[a] n=6	1.36 (0.01)[a] n=6
19	1.33 (0.02)[a] n=5	1.33 (0.01)[a] n=6	1.33 (0.01)[a] n=6	1.32 (0.01)[a] n=6
21	1.34 (0.01)[a] n=5	1.34 (0.01)[a] n=6	1.34 (0.01)[a] n=6	1.35 (0.01)[a] n=6
23	1.34 (0.01)[a] n=5	1.34 (0.01)[a] n=6	1.33 (0.01)[a] n=6	1.34 (0.01)[a] n=6
25	1.37 (0.02)[a] n=5	1.37 (0.01)[a] n=6	1.35 (0.01)[a] n=6	1.34 (0.01)[a] n=6
27	1.42 (0.01) n=5	1.41 (0.01) n=6	1.41 (0.04) n=6	1.39 (0.01) n=6
29	1.29 (0.03)[a] n=5	1.30 (0.02)[a] n=6	1.38 (0.03)[a] n=6	1.39 (0.05)[a] n=6

Values are Mean (SE). Time is weeks post-oophorectomy. DHT was administered following collection of blood and urine samples at 15 weeks post-oophorectomy.

[a] $P<0.005$ versus week 27.

Table 4.5 Serum total calcium (mmol/L) in osteopenic oophorectomised rats following DHT administration at 15 weeks post-oophorectomy.

Time (Weeks)	Dose Dihydrotestosterone (mg/kg body weight)			
	Vehicle	40	80	160
15	2.53 (0.07)[a] n=5	2.56 (0.04)[a] n=6	2.62 (0.04)[a] n=6	2.64 (0.06)[a] n=6
17	2.54 (0.03)[ab] n=5	2.39 (0.02)[ab] n=6	2.45 (0.04)[ab] n=6	2.43 (0.02)[ab] n=6
19	2.44 (0.04)[ab] n=5	2.54 (0.02)[ab] n=6	2.51 (0.04)[ab] n=6	2.48 (0.04)[ab] n=6
21	2.50 (0.03)[a] n=5	2.57 (0.05)[a] n=6	2.48 (0.05)[a] n=6	2.51 (0.03)[a] n=6
23	2.50 (0.05)[ab] n=5	2.47 (0.04)[ab] n=6	2.46 (0.02)[ab] n=6	2.49 (0.06)[ab] n=6
25	2.47 (0.04)[a] n=5	2.55 (0.03)[a] n=5	2.46 (0.03)[a] n=6	2.56 (0.02)[a] n=6
27	2.49 (0.05)[ab] n=5	2.43 (0.02)[ab] n=6	2.51 (0.02)[ab] n=6	2.52 (0.04)[ab] n=6
29	2.27 (0.02)[b] n=5	2.37 (0.04)[b] n=6	2.30 (0.06)[b] n=6	2.27 (0.08)[b] n=6

Values are Mean (SE). Time is weeks post-oophorectomy. DHT was administered following collection of blood and urine samples at 15 weeks post-oophorectomy. [a]$P<0.001$ versus week 29, [b]$P<0.001$ versus week 15.

Table 4.6 Serum ultrafiltrable calcium (mmol/L) in osteopenic oophorectomised rats following DHT administration at 15 weeks post-oophorectomy.

Time (Weeks)	Dose Dihydrotestosterone (mg/kg body weight)			
	Vehicle	40	80	160
15	1.76 (0.05) n=5	1.90 (0.11) n=6	1.80 (0.03) n=6	1.82 (0.04) n=6
17	1.76 (0.02) n=5	1.68 (0.05) n=6	1.72 (0.03) n=6	1.71 (0.01) n=6
19	1.71 (0.04) n=5	1.79 (0.01) n=6	1.76 (0.02) n=6	1.75 (0.03) n=6
21	1.76 (0.04) n=5	1.78 (0.04) n=6	1.73 (0.03) n=6	1.75 (0.02) n=6
23	1.75 (0.03) n=5	1.74 (0.02) n=6	1.72 (0.01) n=6	1.75 (0.04) n=6
25	1.73 (0.03) n=5	1.48 (0.30) n=6	1.74 (0.03) n=6	1.79 (0.01) n=6
27	1.73 (0.04) n=5	1.70 (0.02) n=6	1.73 (0.01) n=6	1.75 (0.03) n=6
29	1.61 (0.02) n=5	1.70 (0.02) n=6	1.64 (0.04) n=6	1.63 (0.05) n=6

Values are Mean (SE). Time is weeks post-oophorectomy. DHT was administered following collection of blood and urine samples at 15 weeks post-oophorectomy.

Table 4.7 Serum complexed calcium (mmol/L) in osteopenic oophorectomised rats following DHT administration at 15 weeks post-oophorectomy.

Time (Weeks)	Dose Dihydrotestosterone (mg/kg body weight)			
	Vehicle	**40**	**80**	**160**
15	0.43 (0.02) n=5	0.46 (0.03) n=6	0.43 (0.01) n=6	0.44 (0.01) n=6
17	0.42 (0.01) n=5	0.41 (0.01) n=6	0.42 (0.01) n=6	0.42 (0.01) n=6
19	0.41 (0.02) n=5	0.44 (0.01) n=6	0.43 (0.01) n=6	0.42 (0.02) n=6
21	0.40 (0.01) n=5	0.36 (0.07) n=6	0.41 (0.01) n=6	0.42 (0.01) n=6
23	0.41 (0.01) n=5	0.42 (0.01) n=6	0.42 (0.01) n=6	0.43 (0.01) n=6
25	0.40 (0.02) n=5	0.36 (0.07) n=6	0.42 (0.01) n=6	0.43 (0.01) n=6
27	0.40 (0.01) n=5	0.41 (0.01) n=6	0.42 (0.01) n=6	0.42 (0.01) n=6
29	0.38 (0.01) n=5	0.42 (0.01) n=6	0.40 (0.01) n=6	0.40 (0.01) n=6

Values are Mean (SE). Time is weeks post-oophorectomy. DHT was administered following collection of blood and urine samples at 15 weeks post-oophorectomy.

Table 4.8 Serum protein bound calcium (mmol/L) in osteopenic oophorectomised rats following DHT administration at 15 weeks post-oophorectomy.

Time (Weeks)	Dose Dihydrotestosterone (mg/kg body weight)			
	Vehicle	**40**	**80**	**160**
15	0.77 (0.02) n=5	0.66 (0.13) n=6	0.81 (0.01) n=6	0.82 (0.02) n=6
17	0.78 (0.02) n=5	0.70 (0.03) n=6	0.72 (0.01) n=6	0.72 (0.02) n=6
19	0.73 (0.01) n=5	0.75 (0.01) n=6	0.75 (0.02) n=6	0.74 (0.02) n=6
21	0.74 (0.03) n=5	0.79 (0.04) n=6	0.75 (0.02) n=6	0.76 (0.01) n=6
23	0.75 (0.02) n=5	0.73 (0.02) n=6	0.74 (0.01) n=6	0.75 (0.01) n=6
25	0.74 (0.01) n=5	0.65 (0.30) n=6	0.72 (0.01) n=6	0.78 (0.01) n=6
27	0.76 (0.01) n=5	0.74 (0.02) n=6	0.78 (0.01) n=6	0.77 (0.01) n=6
29	0.66 (0.01) n=5	0.67 (0.02) n=6	0.66 (0.03) n=6	0.65 (0.04) n=6

Values are Mean (SE). Time is weeks post-oophorectomy. DHT was administered following collection of blood and urine samples at 15 weeks post-oophorectomy.

4.3.6 Serum Creatinine and Body Weight

Body weight was unaffected by DHT administration and remained unchanged throughout the experiment (Figure 4.5). Serum creatinine was decreased following DHT treatment ($P<0.001$) and this effect was not dose dependent but was time dependent ($P<0.05$) with maximal suppression occurring by 2 weeks following DHT treatment (17 weeks post-oophorectomy) (Figure 4.6). There was no correlation between weight and serum creatinine ($r^2=0.001$)(Data not shown).

4.3.7 Serum Alanine Aminotransferase, Albumin and Total Protein

Alanine aminotransferase, albumin and total protein were unaffected by DHT administration (Table 4.9, Table 4.10, Table 4.11). Alanine aminotransferase remained unchanged throughout the duration of the experiment (Table 4.9). Albumin ($P<0.01$) and total protein ($P<0.01$) decreased during the experiment (Table 4.10, Table 4.11).

4.3.8 Serum Electrolytes

Serum electrolytes are presented in Appendix B.

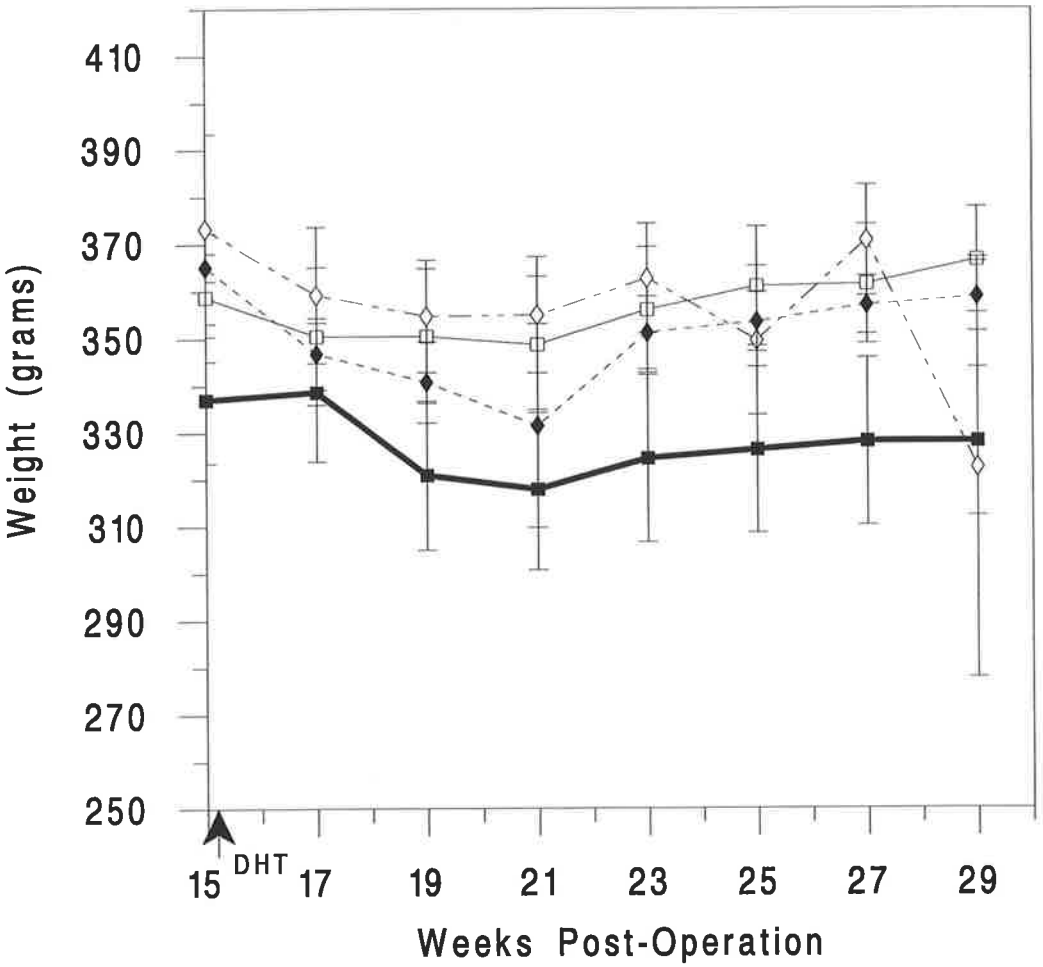


Figure 4.5 Body weight (grams) in osteopenic oophx rats administered DHT at 15 weeks post-oophorectomy. Values are Mean ± SE, n=6. (■) Vehicle, (□) 40mg/kg bw DHT, (◊) 80mg/kg bw DHT, (◆) 160mg/kg bw DHT.

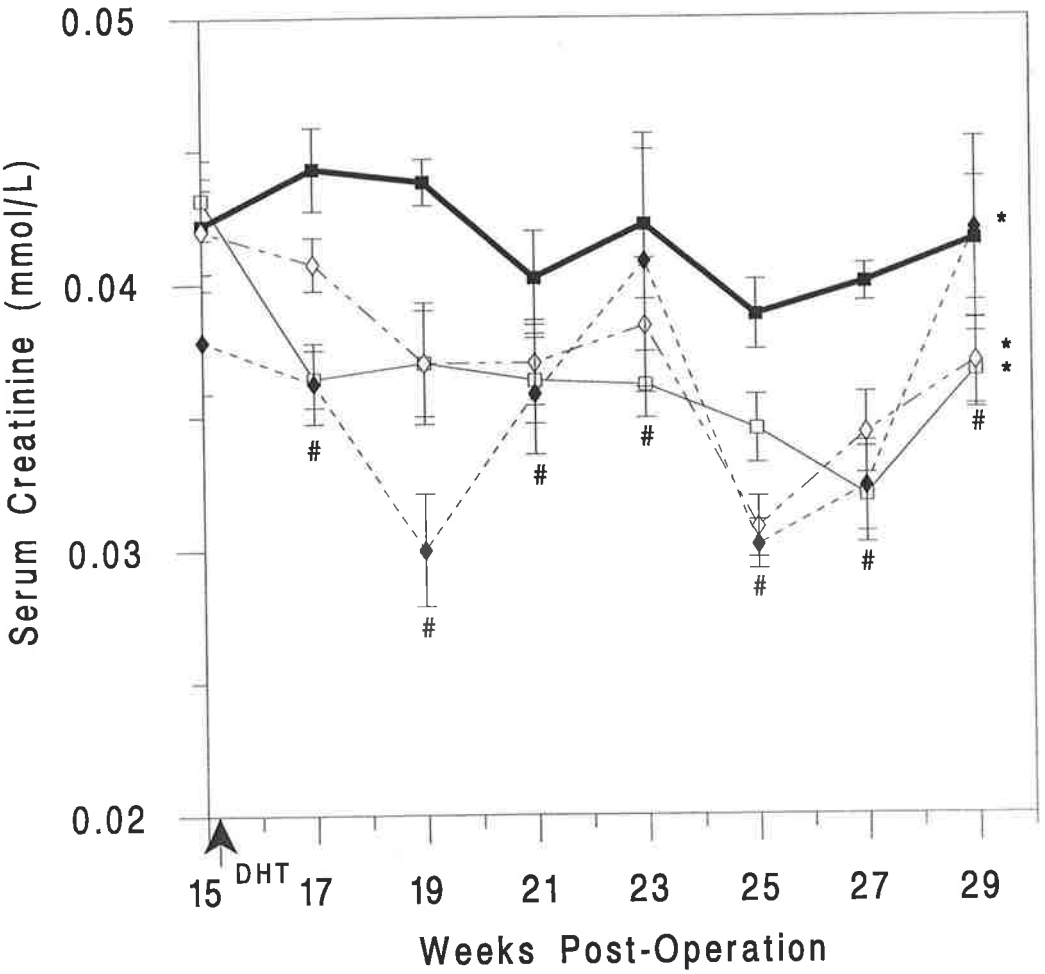


Figure 4.6 Serum creatinine (mmol/L) in osteopenic oophx rats administered DHT at 15 weeks post-oophorectomy. Values are Mean ± SE, n=6. (■) Vehicle, (□) 40mg/kg bw DHT, (◊) 80mg/kg bw DHT, (◆) 160mg/kg bw DHT. *P<0.001 versus vehicle, $^{\#}P$<0.05 versus week 15.

Table 4.9 Serum alanine aminotransferase (units/L) in osteopenic oophorectomised rats following DHT administration at 15 weeks post-oophorectomy.

Time (Weeks)	Dose Dihydrotestosterone (mg/kg body weight)			
	Vehicle	40	80	160
15	-	11.9 n=1	21.2 (6.6) n=4	26.5 (7.8) n=3
29	15.7 (1.6) n=5	19.9 (1.7) n=6	17.9 (1.5) n=6	19.6 (1.0) n=4

Values are Mean (SE). Time is weeks post-oophorectomy. DHT was administered following collection of blood and urine samples at 15 weeks post-oophorectomy.

Table 4.10 Serum albumin (mmol/L) in osteopenic oophorectomised rats following DHT administration at 15 weeks post-oophorectomy.

Time (Weeks)	Dose Dihydrotestosterone (mg/kg body weight)			
	Vehicle	40	80	160
15	34.6 (1.0)[a] n=5	35.2 (0.8)[a] n=5	35.8 (0.2)[a] n=6	35.8 (1.0)[a] n=6
17	34.4 (0.8)[ab] n=5	34.5 (0.4)[ab] n=6	33.7 (0.5)[ab] n=6	35.2 (0.9)[ab] n=6
19	33.8 (1.4)[ab] n=5	34.0 (0.6)[ab] n=6	34.8 (1.0)[ab] n=6	34.7 (1.0)[ab] n=6
21	33.4 (1.5)[ab] n=5	33.7 (1.0)[ab] n=6	34.7 (0.9)[ab] n=6	35.8 (0.4)[ab] n=6
23	33.2 (1.1)[a] n=5	34.2 (0.6)[a] n=6	36.3 (1.0)[a] n=6	35.3 (0.6)[a] n=6
25	33.2 (1.1)[a] n=5	36.0 (1.0)[a] n=6	32.8 (2.2)[a] n=6	36.5 (1.4)[a] n=6
27	35.6 (0.1)[a] n=5	36.3 (0.3)[a] n=6	37.5 (0.9)[a] n=6	37.0 (1.3)[a] n=6
29	32.2 (0.7) n=5	31.3 (0.9) n=6	32.2 (0.9) n=6	31.8 (1.0) n=6

Values are Mean (SE). Time is weeks post-oophorectomy. DHT was administered following collection of blood and urine samples at 15 weeks post-oophorectomy. [a]$P<0.01$ versus week 29, [b]$P<0.01$ versus week 27.

Table 4.11 Serum total protein (mmol/L) in osteopenic oophorectomised rats following DHT administration at 15 weeks post-oophorectomy.

Time (Weeks)	Dose Dihydrotestosterone (mg/kg body weight)			
	Vehicle	40	80	160
15	66.2 (1.1)[b] n=5	64.8 (1.5)[b] n=6	66.8 (1.7)[b] n=6	67.2 (1.6)[b] n=6
17	65.8 (1.2)[ab] n=5	60.7 (2.1)[ab] n=6	62.2 (1.1)[ab] n=6	61.3 (1.6)[ab] n=6
19	64.2 (1.0)[a] n=5	61.8 (1.8)[a] n=6	62.2 (1.3)[a] n=6	60.5 (1.1)[a] n=6
21	61.0 (3.8)[a] n=5	62.2 (1.2)[a] n=6	63.3 (1.5)[a] n=6	61.7 (1.1)[a] n=6
23	64.0 (0.6)[ab] n=5	60.8 (2.3)[ab] n=6	62.3 (1.1)[ab] n=6	61.2 (0.7)[ab] n=6
25	63.2 (1.1)[ab] n=5	61.8 (1.3)[ab] n=5	62.2 (1.0)[ab] n=6	61.7 (1.3)[ab] n=6
27	63.4 (1.0)[ab] n=5	62.3 (1.1)[ab] n=6	64.5 (1.2)[ab] n=6	61.2 (0.7)[ab] n=6
29	60.1 (2.2) n=5	58.7 (1.8) n=6	58.7 (1.8) n=6	58.5 (2.5) n=6

Values are Mean (SE). Time is weeks post-oophorectomy. DHT was administered following collection of blood and urine samples at 15 weeks post-oophorectomy. [a]$P<0.01$ versus week 15 within dosage group, [b]$P<0.01$ versus week 29 within dosage group.

4.4 DISCUSSION

The serum DHT concentrations achieved with silastic tubing implants were comparable to those achieved previously (Chapter 3.3.1). The serum DHT levels measured at 14 weeks post-implantation did not differ significantly from those measured at 8 weeks post-implantation (Chapter 3.3.1) at any dose. These data suggest that the use of silastic tubing implants to administer DHT at the dosages used in the present study can adequately maintain the serum DHT concentrations up to 14 weeks post-implantation.

Serum alkaline phosphatase was increased following DHT treatment in osteopenic oophx rats, which is consistent with the increase in alkaline phosphatase observed in sham and oophx rats following DHT administered at the time of operation (Chapter 3.3.3). Since no changes were detected in the liver protein albumin or the liver enzyme alanine aminotransferase following DHT treatment, it is suggestive that the effect of DHT is to stimulate bone alkaline phosphatase rather than the liver isozyme. The increase in alkaline phosphatase following DHT treatment in the osteopenic oophx rats was not as rapid as that observed in the sham and oophx rats administered DHT at the time of operation (Chapter 3.3.3) as maximal levels did not occur until 14 weeks post-treatment. Furthermore, the anabolic effect of DHT on serum alkaline phosphatase was not more prominent in the osteopenic oophx rats as proposed (Chapter 1.8.2). The effect of DHT to increase alkaline phosphatase in osteopenic rats is consistent with the stimulation of bone formation rate resulting in increased trabecular thickness and number in the tibiae (Tobias et al 1994). The effect of DHT to stimulate trabecular thickness is stronger than the stimulatory effect on trabecular number, with trabecular thickness being restored to that of control animals. It

appears therefore, that androgens act to increase cancellous bone volume by enlarging existing trabeculae and by protecting existing trabeculae from dissolution rather than stimulating their formation (Tobias et al 1994). Furthermore, DHT treatment in osteopenic oophx rats increases the mean specific mass in the mid diaphysis (Schot et al 1993), stimulates endocortical bone formation in the tibiae (Tobias et al 1994) and periosteal bone formation in the femur (Coxam et al 1996), suggesting that DHT has an anabolic effect on cortical as well as cancellous bone.

In contrast, osteocalcin was decreased following DHT treatment with maximal suppression not occurring until 14 weeks following treatment. Since alkaline phosphatase and osteocalcin are produced at different stages of osteoblast development (Owen et al 1990), this discordancy may reflect a differential effect of DHT on the different stages of bone formation. It has been proposed that alkaline phosphatase is correlated to the extent of active bone forming surface as indicated by the extent of double flurochrome-labelled surface and mineralising surface, and osteocalcin is correlated to the rate of active mineralisation as indicated by mineral apposition rate (Sims et al 1996a). The findings of the present study therefore, suggest that androgens act to increase the amount of bone surface actively synthesising new bone matrix as indicated by increased alkaline phosphatase and to inhibit the rate of mineralisation of the newly formed bone matrix as indicated by osteocalcin. Supportive evidence for this theory is an increase in double labelled surface area and mineralising surface with no effect on mineral apposition rate following DHT treatment in the femur of osteopenic oophorectomised rats (Coxam et al 1996). The effect of DHT to decrease osteocalcin synthesis may be due to the inability of

the mineralisation process to meet the increased demand by the greater extent of newly formed bone matrix induced by DHT treatment.

The effects of DHT on biochemical markers of bone resorption were similar to those observed on serum osteocalcin levels. Urine deoxypyridinoline was significantly reduced in osteopenic rats when DHT was administered 15 weeks post-oophorectomy, while urine hydroxyproline excretion was unaffected. Since deoxypyridinoline is considered to be a more specific marker of bone resorption (Robins et al 1991) it may better reflect such changes compared to hydroxyproline. Consistent with the present finding, the administration of high doses of DHT to osteopenic oophx rats, comparable to those used in the present study, decreases bone resorbing surfaces which is associated with a decrease in osteoclast surface and number (Tobias et al 1994). The suppression of bone resorption may contribute to the increase in bone volume observed following DHT treatment (Tobias et al 1994). In addition, nandrolone decanoate, a synthetic androgen decreases osteocalcin in young and old rats when administered immediately following operation (Schot et al 1993), which was attributed to an overall anti-resorptive effect.

The differences between the effects of DHT on the biochemical variables when DHT was administered either at the time of operation (Chapter 3) or 15 weeks post-oophorectomy, possibly reflects an interaction between DHT and the rate of bone turnover at the time of treatment. In osteopenic rats, bone turnover rate as measured by osteoclast and osteoblast surface is not significantly different from that of sham rats in the cancellous bone of the tibiae at 120 days post-operation (Coxam et al 1996, Wronski et al 1989a). In addition, the extent of gene activation in osteoblast cells decreases with age in the rat (Liang et al 1992).

When DHT was administered at the time of operation (Chapter 3) the effects on bone resorption may have been masked due to the increased number and activity of bone cells associated with the elevated bone turnover rate following estrogen deficiency (Wronski et al 1989a). In contrast, the osteopenic rats had a lower number of active bone cells at the time of treatment since trabecular bone volume and bone turnover were stabilised (Wronski et al 1988b) and the osteopenic rats were 15 weeks older than those used in the previous study (Chapter 3). Thus the effect of DHT to decrease bone resorption was detectable. Serum osteocalcin was also decreased by DHT treatment in the osteopenic oophx rats further supporting this theory. The effects of DHT on the biochemical markers of bone turnover measured in the present study were not as rapid as the changes observed in the rats administered DHT immediately following oophorectomy (Chapter 3) which may also be due to decreased bone cell activity at the time of treatment.

DHT treatment decreased urine calcium excretion, although not significantly, which was associated with an increase in the tubular reabsorption of calcium by the kidney. These effects are consistent with the findings in sham and oophx rats following DHT treatment at the time of operation (Chapter 3) and are consistent with androgen treatment in post-menopausal women (Need et al 1987) and in young and old rats (Schot et al 1993). The conservation of calcium at the level of the kidney following DHT treatment was not accompanied by a decrease in serum ionised calcium or protein-bound calcium as observed with DHT treatment immediately following oophx (Chapter 3). Furthermore, the rise in alkaline phosphatase in the osteopenic oophx rats did not reach maximal stimulation until 14 weeks post-implantation, thus any calcium requirement for incorporation into bone therefore may not have been as great as in the sham and oophx rats administered DHT at

the time of operation where alkaline phosphatase was raised immediately following DHT administration.

Body weight and serum creatinine were unaffected by DHT treatment and were not correlated suggesting that the action of DHT to increase bone formation, as indicated by alkaline phosphatase, is through a direct effect on bone and not only through mechanical stress by increases in muscle mass.

This study demonstrates that the increase in bone formation observed following DHT administration to osteopenic oophx rats in previous studies (Tobias et al 1994, Coxam et al 1996, Schot et al 1993) is associated with an increase in serum alkaline phosphatase. In addition, DHT treatment suppressed urine deoxypyridinoline excretion and serum osteocalcin levels indicating an anti-resorptive action in osteopenic oophx rats. This action however, appears to be relatively weak since it was not detectable when DHT was administered immediately following either sham or oophorectomy operations (Chapter 3). It was also demonstrated that DHT has a differential effect on osteoblast products, suggesting that DHT acts to increase osteoblast bone formation at the level of matrix development and maturation and to suppress mineralisation in osteopenic oophx rats. Furthermore, DHT has a direct effect on the kidney to conserve calcium. In conclusion, the action of DHT on bone is dependent on the rate of bone turnover at the time of treatment.

CHAPTER 5

EFFECT OF OOPHORECTOMY AND DHT TREATMENT ON mRNA LEVELS OF OSTEOBLAST AND OSTEOCLAST GENES

5.1 INTRODUCTION

The rapid phase of bone loss following oophorectomy is accompanied by an increase in the rate of bone resorption and formation which is associated with increases in mRNA levels of the osteoblast genes c-*myc*, type 1α collagen and osteocalcin (Salih et al 1993, Ikeda et al 1993) and the osteoclast genes carbonic anhydrase II and tartrate-resistant acid phosphatase (Zheng et al 1994). Following this initial, rapid phase of bone loss, the rate of bone turnover decreases as osteopenia develops (Wronski et al 1988b). It is likely that the mRNA levels of osteoblast and osteoclast genes decrease with the decline of bone cell activity.

The administration of androgens has an anabolic effect on bone including nandrolone decanoate treatment in oophx rats which increases longitudinal and periosteal bone growth (Schot et al 1993) and DHT treatment in osteopenic oophx increases trabecular bone volume and bone mineral density (Tobias et al 1994, Coxam et al 1996). The increase in bone formation rate following DHT treatment in estrogen-sufficient and estrogen-deficient rats is associated with an increase in serum alkaline phosphatase (Chapter 3, Chapter 4). In addition, high doses of DHT administered to osteopenic oophx rats suppresses osteoclast

bone resorption as indicated by decreased osteoclast surface and number (Tobias et al 1994) and by decreased urine deoxypyridinoline excretion (Chapter 4). The observed changes in bone cell metabolism following DHT administration in sham, oophx and osteopenic oophx rats however, has not been established at the level of osteoblast and osteoclast gene expression. The following chapter therefore investigates the effect of oophorectomy and DHT administration to sham and oophx rats immediately following operation and to osteopenic oophx rats on the mRNA levels of the osteoblast genes expressed during osteoblast development and the osteoclast gene calcitonin receptor.

5.2 EXPERIMENTAL PROTOCOL

5.2.1 Experimental Procedure

The experimental procedures for experiment A and experiment B are as described in Chapter 3.2.1 and Chapter 4.2.1 respectively. A group of 6 rats in both experiments A and B were sacrificed at the beginning of the experiments prior to operation to provide baseline mRNA levels. At the end of the experiments the rats were exsanguinated by cardiac puncture under halothane anaesthesia and sacrificed by cervical dislocation. One femur and both tibia from each rat were excised as described in Chapter 2.7.3.1.

5.2.2 Analysis of mRNA levels

Total RNA was extracted from the bones as described in Chapter 2.7.3.2. The mRNA levels for the osteoblast genes c-*fos*, type 1α collagen, alkaline phosphatase, osteopontin, osteocalcin, and the osteoclast genes tartrate-resistant acid phosphatase and carbonic

anhydrase were determined by Northern blot analysis as described in Chapter 2.7.4. The mRNA levels for the osteoclast gene calcitonin receptor were amplified by reverse transcriptase polymerase chain reaction (RT-PCR) (Chapter 2.7.6) and these PCR products were semi-quantified by Southern blot analysis as described in Chapter 2.7.6.3.

5.2.3 Statistical Analyses

All mRNA levels analysed were corrected for glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mRNA levels as described in Chapter 2.7.4.6. The mRNA levels of the osteoblast genes determined in experiment A were calculated as described in Chapter 2.7.4.6, however the mRNA levels determined in experiment B were expressed as a fold increase relative to baseline rats. Since no dose effect of DHT administration was observed, the mRNA levels of the osteoblast genes obtained for each of the three doses of DHT (ie 40mg, 80mg and 160mg/kg body weight) were pooled for each group of rats. Statistical analyses were performed as described in Chapter 2.7.4.6.

5.3 RESULTS

The signals obtained from the ImageQuant program (Chapter 2.7.4.4) for the mRNA levels of the osteoclast and osteoblast genes from each Northern blot and Southern blot analyses are presented in Appendix C.1.

5.3.1 Experiment A

The mRNA levels for tartrate-resistant acid phosphatase and carbonic anhydrase were undetectable by northern blot analysis in any group in either experiments A or B.

5.3.1.1 Effect of Oophorectomy

The mRNA levels of c-*fos* were unaffected by oophorectomy (Figure 5.1). Type 1α collagen and osteocalcin were increased by 30 fold, and 10 fold respectively as a result of oophorectomy ($P<0.05$) (Figure 5.2, Figure 5.5). The mRNA levels of alkaline phosphatase and osteopontin were also increased as a result of oophorectomy by 30 fold and 7 fold respectively but did not reach statistical significance (Figure 5.3, Figure 5.4). Calcitonin receptor mRNA levels were increased by 2.5 fold following oophorectomy (Figure 5.6).

5.3.1.2 Effect of DHT Treatment in Sham and Oophorectomised Rats

c-*fos* mRNA levels were unaffected by DHT administration in either sham or oophx rats (Figure 5.1). DHT administration in sham rats resulted in a 7 fold increase in type 1α collagen mRNA levels ($P<0.05$). In contrast, DHT administration to oophx rats resulted in a suppression of mRNA levels by 20 fold ($P<0.05$) (Fig 5.2).

Alkaline phosphatase mRNA levels were not significantly affected by DHT administration in either sham or oophx rats (Fig 5.3). DHT administration resulted in an increase of 4 fold in osteopontin mRNA levels in sham rats but did not reach statistical significance while mRNA levels in oophx rats were unaffected (Fig 5.4).

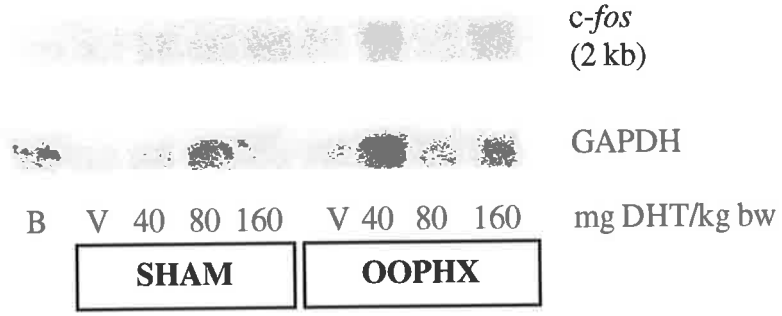
Osteocalcin mRNA levels were increased by DHT treatment in sham rats of 4 fold ($P<0.05$), while in contrast DHT administration suppressed osteocalcin mRNA levels in oophx rats by 6 fold ($P<0.05$)(Fig 5.5).

Calcitonin receptor mRNA levels were increased by 1.5 fold in sham rats following treatment with 160mg/kg bw DHT. In contrast, calcitonin receptor mRNA levels were suppressed by 2.5 fold and 2 fold in oophx rats administered 40mg and 80mg/kg body weight DHT respectively (Figure 5.6).

Figure 5.1 **A:** Representation of Northern Blot analysis for c-*fos* mRNA and GAPDH mRNA. B-Baseline, V-Vehicle. **B:** c-*fos* mRNA levels corrected for GAPDH and expressed as a fold increase relative to Sham + Vehicle in sham and oophorectomised rats administered DHT from the time of operation. Values are Mean ± SE. Dose of DHT is mg/kg body weight. DHT doses 40mg, 80mg and 160mg/kg body weight have been pooled for both sham and oophx rats.

DHT Administered Immediately Following Operation

A



B

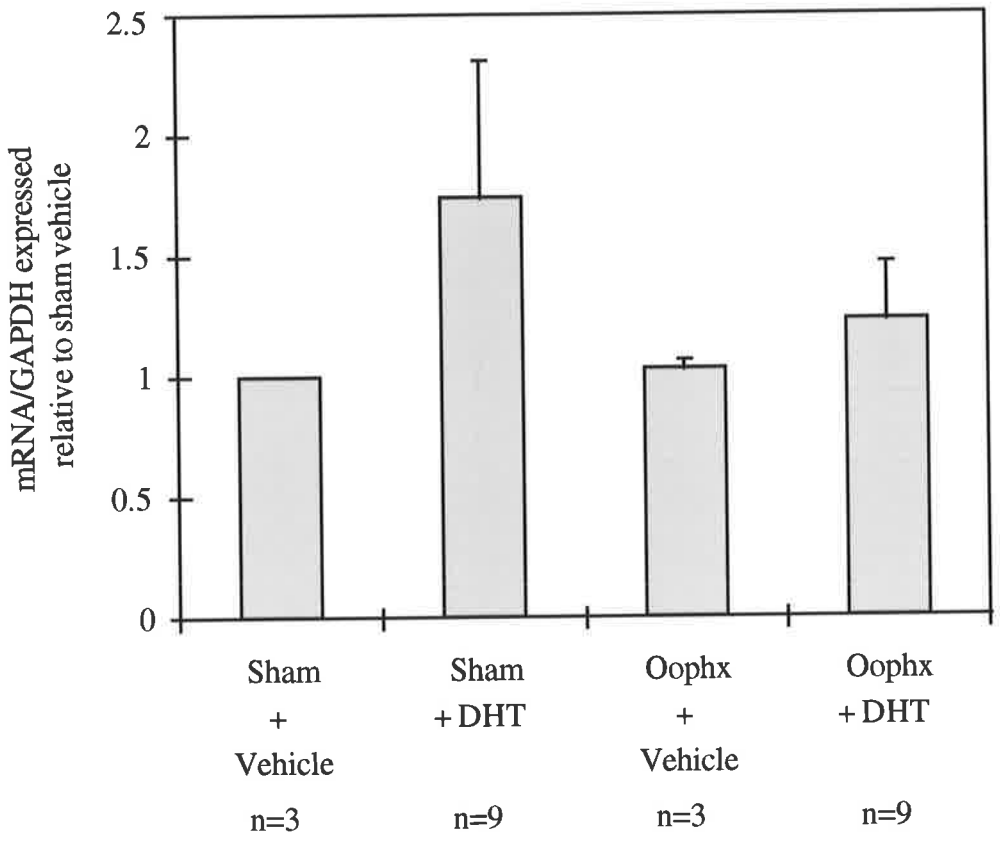
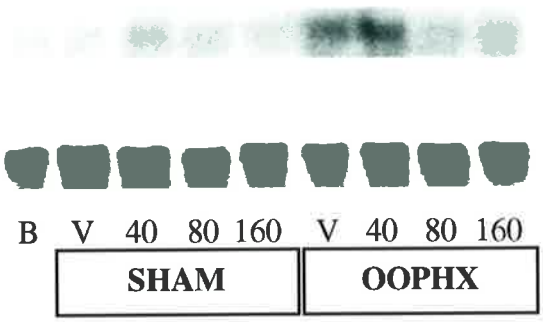


Figure 5.2 A: Representation of Northern Blot analysis for type 1α collagen mRNA and GAPDH mRNA. B-Baseline, V-Vehicle. **B:** Type 1α Collagen mRNA levels corrected for GAPDH and expressed as a fold increase relative to Sham + Vehicle in sham and oophorectomised rats administered DHT from the time of operation. Values are Mean ± SE. Dose of DHT is mg/kg body weight. DHT doses 40mg, 80mg and 160mg/kg body weight have been pooled for both sham and oophx rats. $^{*}P<0.05$ versus Sham + Vehicle, $^{\#}P<0.05$ versus Oophx + Vehicle.

DHT Administered Immediately Following Operation

A



B

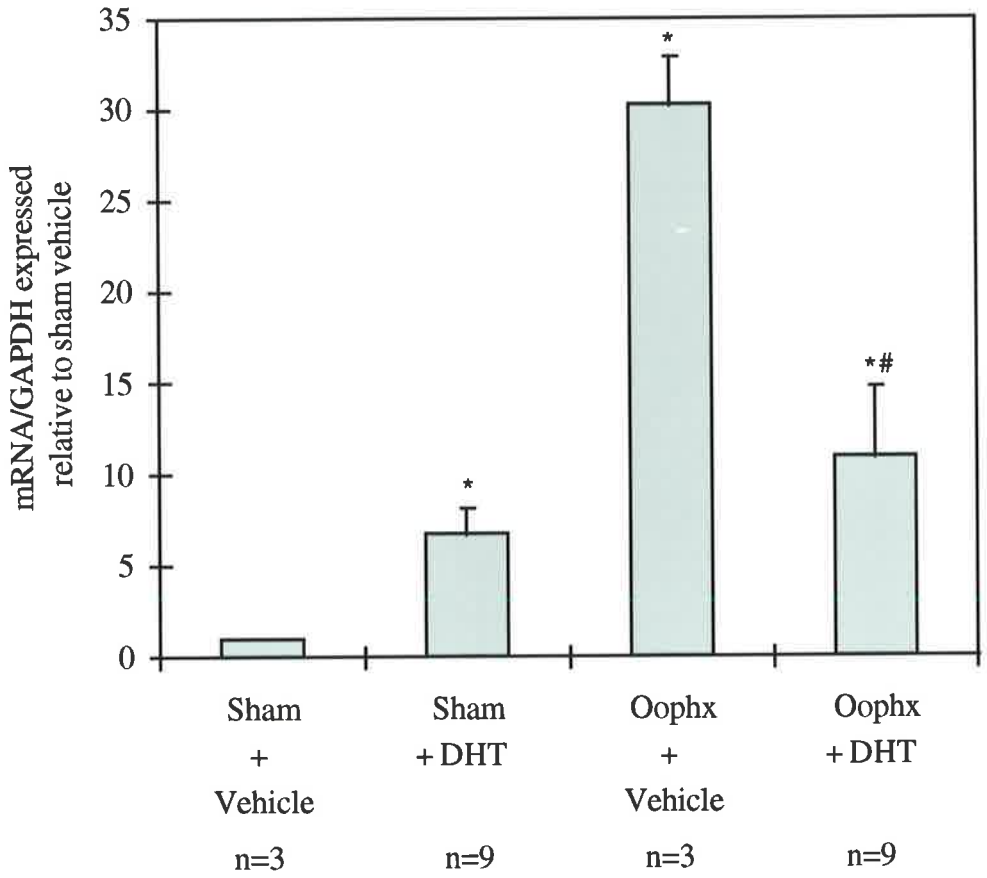
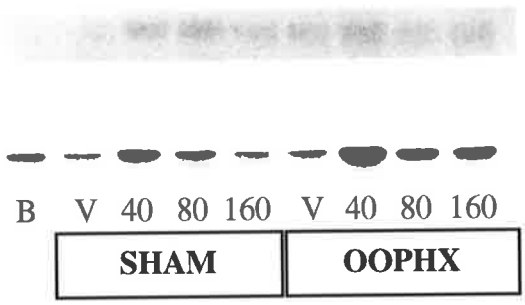


Figure 5.3 A: Representation of Northern Blot analysis for alkaline phosphatase mRNA and GAPDH mRNA. B-Baseline, V-Vehicle. **B:** Alkaline Phosphatase mRNA levels corrected for GAPDH and expressed as a fold increase relative to Sham + Vehicle in sham and oophorectomised rats administered DHT from the time of operation. Values are Mean ± SE. Dose of DHT is mg/kg body weight. DHT doses 40mg, 80mg and 160mg/kg body weight have been pooled for both sham and oophx rats.

DHT Administered Immediately Following Operation

A



B

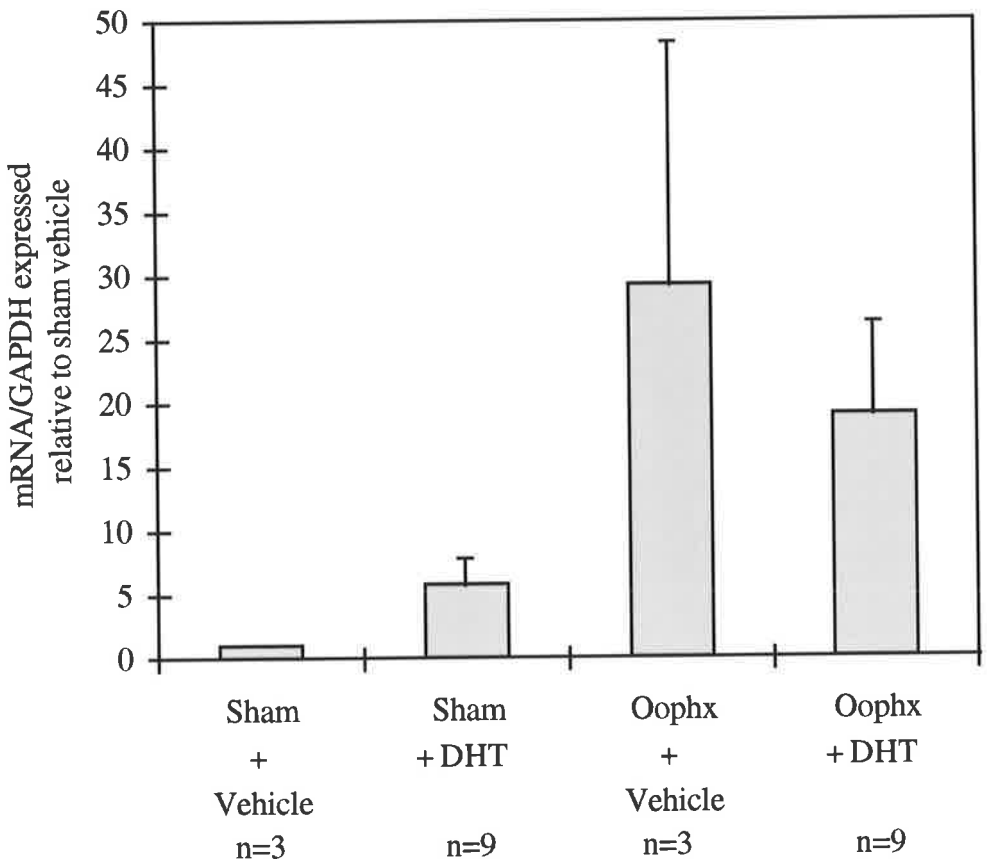
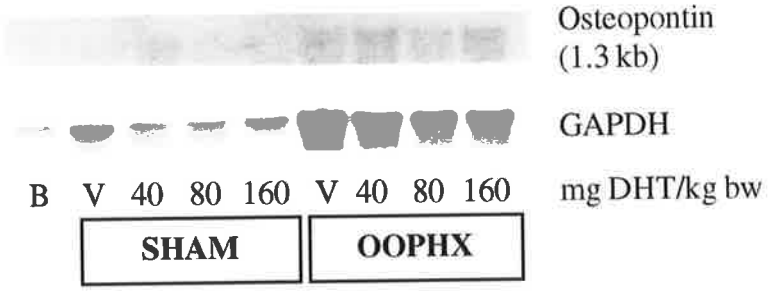


Figure 5.4 **A:** Representation of Northern Blot analysis for osteopontin mRNA and GAPDH mRNA. B-Baseline, V-Vehicle. **B:** Osteopontin mRNA levels corrected for GAPDH and expressed as a fold increase relative to Sham + Vehicle in sham and oophorectomised rats administered DHT from the time of operation. Values are Mean ± SE. Dose of DHT is mg/kg body weight. DHT doses 40mg, 80mg and 160mg/kg body weight have been pooled for both sham and oophx rats.

DHT Administered Immediately Following Operation

A



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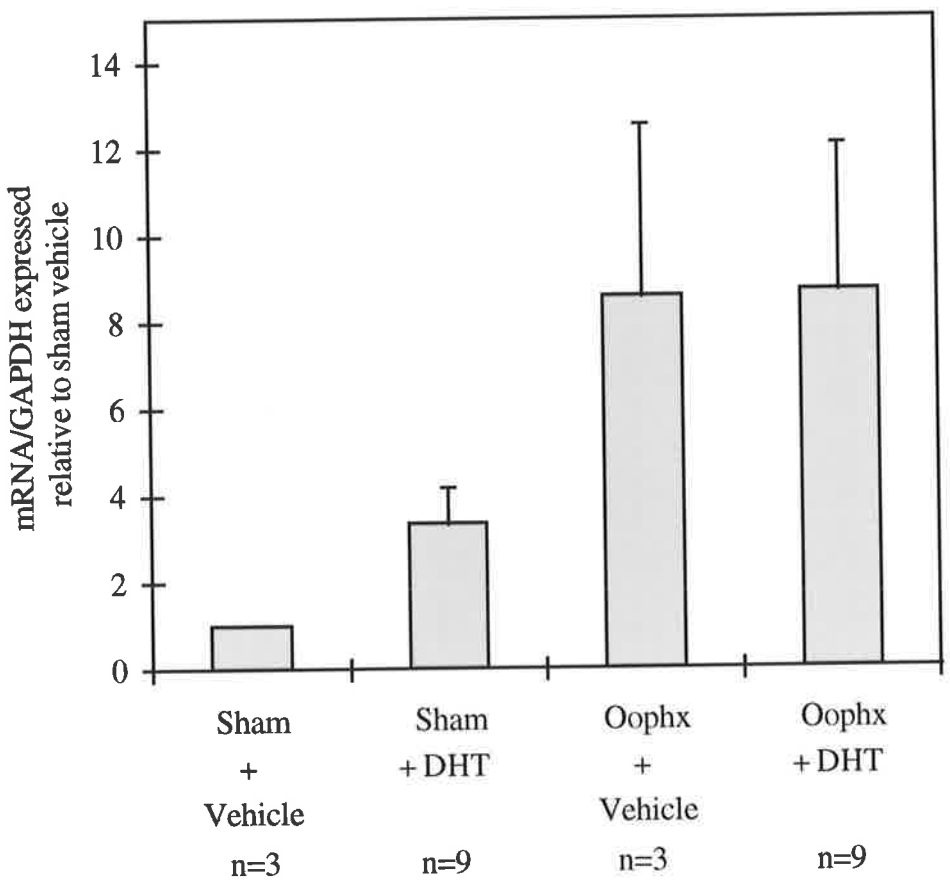
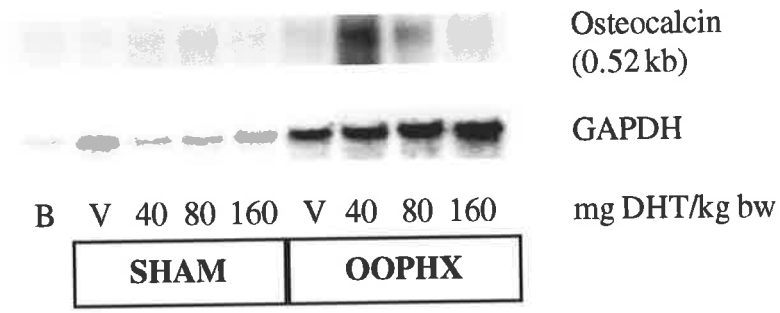


Figure 5.5 A: Representation of Northern Blot analysis for osteocalcin mRNA and GAPDH mRNA. B-Baseline, V-Vehicle. **B:** Osteocalcin mRNA levels corrected for GAPDH and expressed as a fold increase relative to Sham + Vehicle in sham and oophorectomised rats administered DHT from the time of operation. Values are Mean ± SE. Dose of DHT is mg/kg body weight. DHT doses 40mg, 80mg and 160mg/kg body weight have been pooled for both sham and oophx rats. $^{*}P<0.05$ versus Sham + Vehicle, $^{\#}P<0.05$ versus Oophx + Vehicle.

DHT Administered Immediately Following Operation

A



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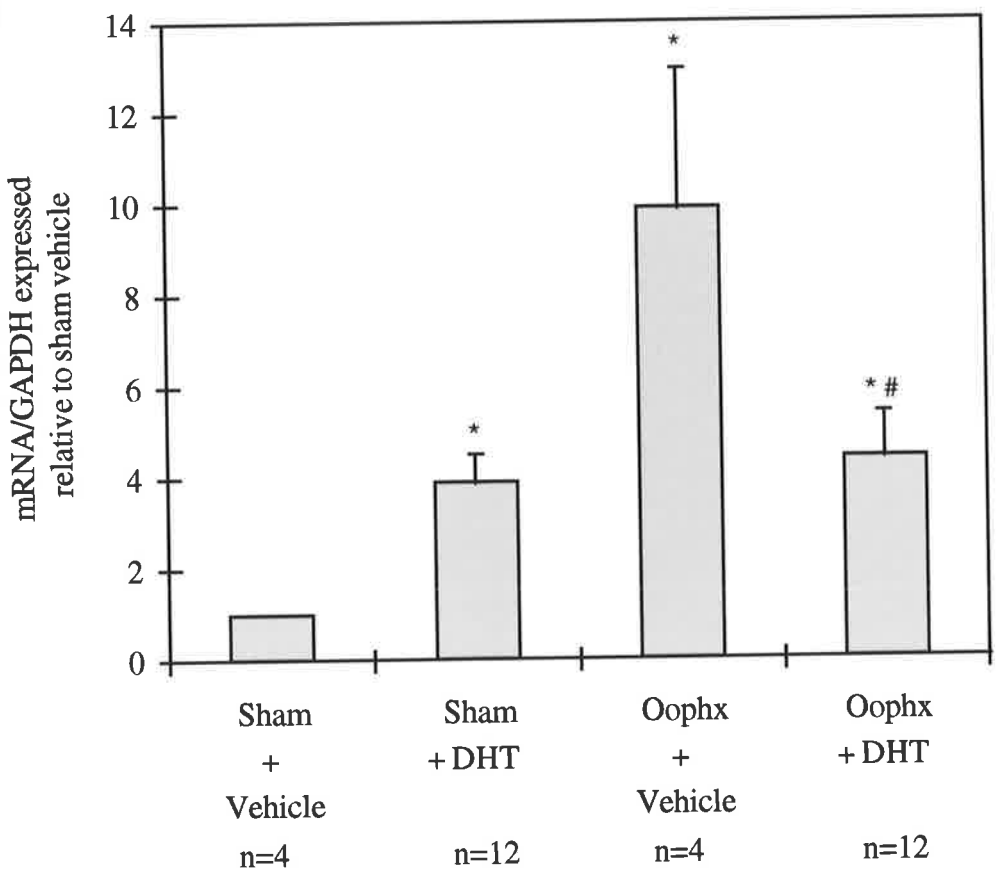
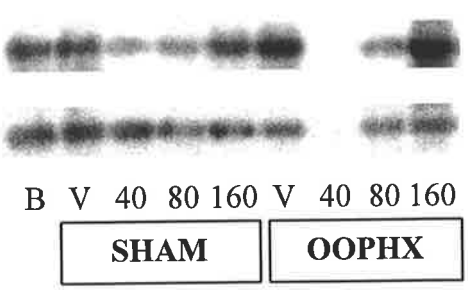


Figure 5.6 **A:** Representation of Southern blot analysis of RT-PCR products for calcitonin receptor (CTR) mRNA and GAPDH mRNA. B-Baseline, V-Vehicle, S-Sham-operated, O-Oophorectomised. **B:** Calcitonin receptor mRNA levels are corrected for GAPDH and expressed as a fold increase relative to sham + vehicle in sham and oophx rats administered DHT from the time of operation. Bars represent mean values, (◆) data points contributing to mean (n=2). Dose of DHT is mg/kg body weight.

DHT Administered Immediately Following Operation

A

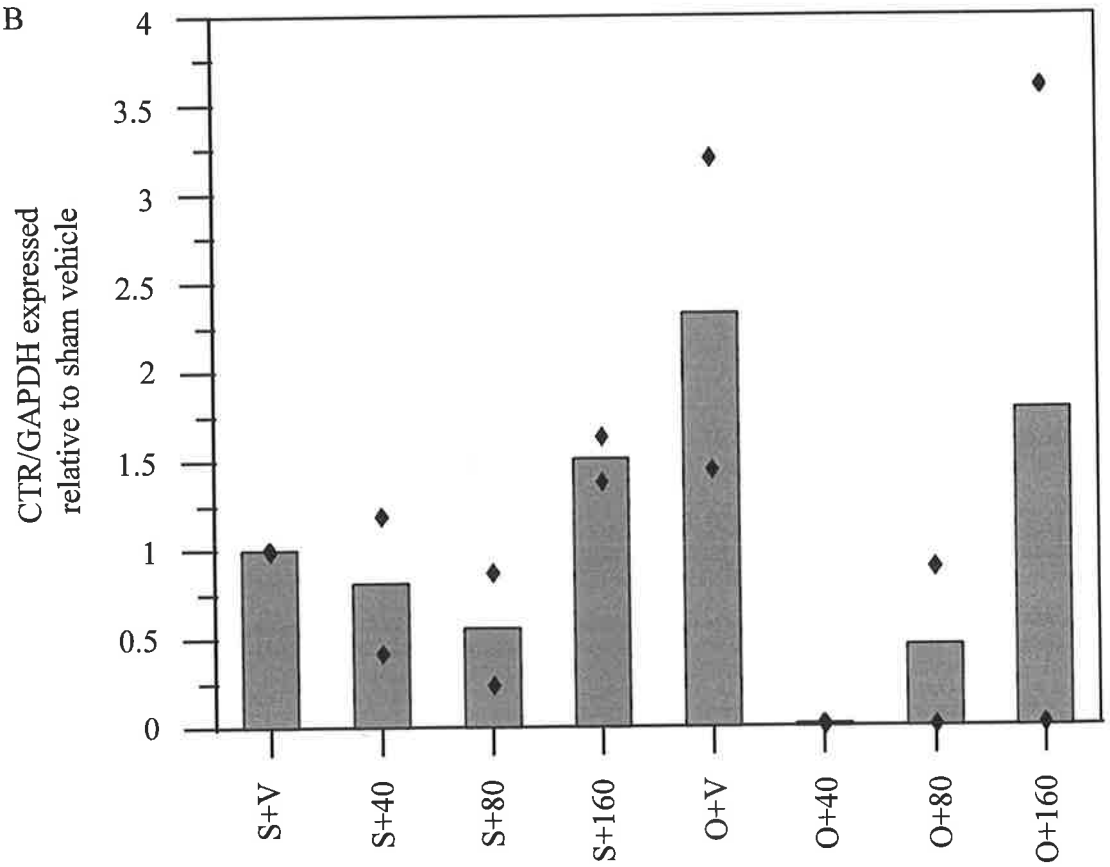


Calcitonin Receptor (437 bp)

GAPDH

mg DHT/kg bw

B



5.3.2 Experiment B

In order to determine the effect of osteopenia in oophx rats on the mRNA levels of the osteoblast and osteoclast genes, the mRNA levels in experiment B were compared to the mRNA levels of the sham-operated rats determined in experiment A. To allow for direct comparisons to be made between the mRNA levels determined in these two experiments, the mRNA levels of the sham-operated rats were expressed as a fold increase relative to the mRNA levels measured in the baseline group of rats in experiment A.

c-*fos* mRNA levels were undetectable in osteopenic oophx rats receiving vehicle or DHT (Data not presented).

5.3.2.1 Effect of Osteopenia in Oophorectomised Rats

The mRNA levels of osteocalcin ($P<0.01$) and calcitonin receptor were increased in osteopenic oophx rats by 1.4 fold and 1.8 fold respectively, compared to sham rats (Figure 5.10, Figure 5.11). Type 1α collagen and alkaline phosphatase mRNA levels were increased in osteopenic oophx rats by 6 fold and 2.5 fold respectively, however these did not reach statistical significance (Figure 5.7, Figure 5.8). Osteopontin mRNA levels were unchanged in osteopenic oophx rats (Figure 5.9).

5.3.2.2 Effect of DHT Treatment in Osteopenic Oophorectomised Rats

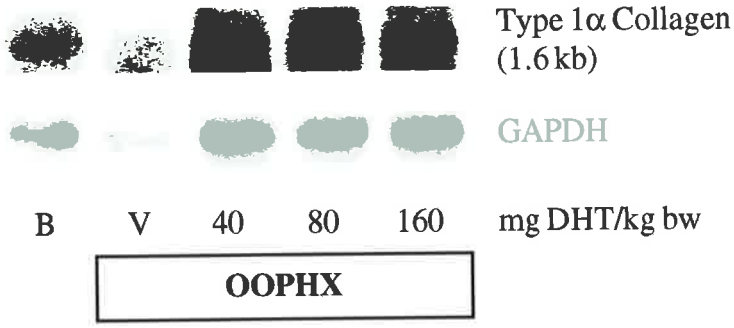
Type 1α collagen and alkaline phosphatase mRNA levels were suppressed in osteopenic oophx rats following DHT treatment by 4.5 fold ($P<0.01$) and 1.2 fold ($P<0.05$) respectively (Figure 5.7, Figure 5.8). Osteopontin mRNA levels were increased by 3.2 fold following DHT treatment, however this was not significant (Figure 5.9). Osteocalcin

mRNA levels were unaffected by DHT treatment (Figure 5.10). Calcitonin receptor mRNA levels were increased following DHT treatment and this was dose dependent with maximal stimulation of 5 fold occurring at 160mg/kg bw DHT (Figure 5.11).

Figure 5.7 **A:** Representation of Northern Blot analysis for type 1α collagen mRNA and GAPDH mRNA. B-Baseline, V-Vehicle. **B:** Type 1α collagen mRNA levels corrected for GAPDH and expressed as a fold increase relative to Baseline in sham and osteopenic oophorectomised rats. DHT was administered at 15 weeks post-oophorectomy. Values are Mean ± SE. Dose of DHT is mg/kg body weight. DHT doses 40mg, 80mg and 160mg/kg body weight have been pooled. *P<0.01 versus Oophx + Vehicle.

DHT Administered to Osteopenic Oophorectomised Rats

A



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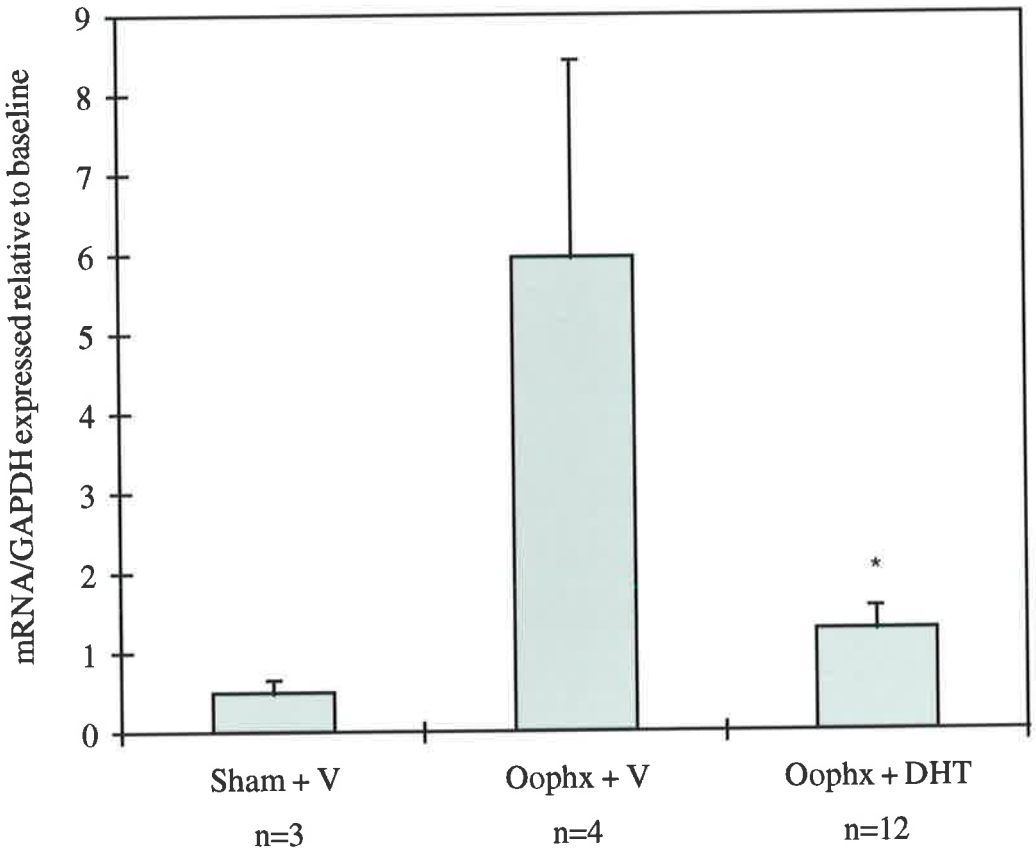
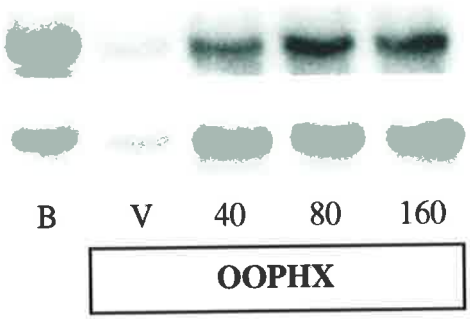


Figure 5.8 **A:** Representation of Northern Blot analysis for alkaline phosphatase mRNA and GAPDH mRNA. B-Baseline, V-Vehicle. **B:** Alkaline phosphatase mRNA levels corrected for GAPDH and expressed as a fold increase relative to Baseline in sham and osteopenic oophorectomised rats. DHT was administered at 15 weeks post-oophorectomy. Values are Mean ± SE. Dose of DHT is mg/kg body weight. DHT doses 40mg, 80mg and 160mg/kg body weight have been pooled. *$P<0.05$ versus Oophx + Vehicle.

DHT Administered to Osteopenic Oophorectomised Rats

A



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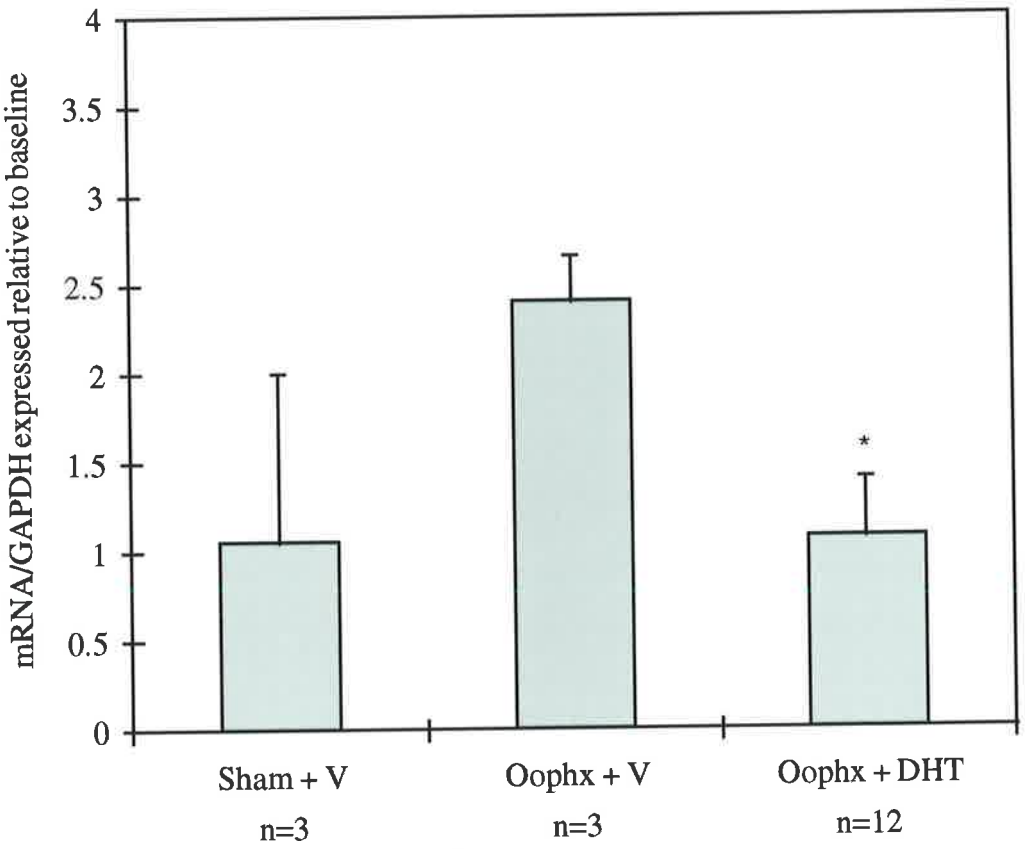
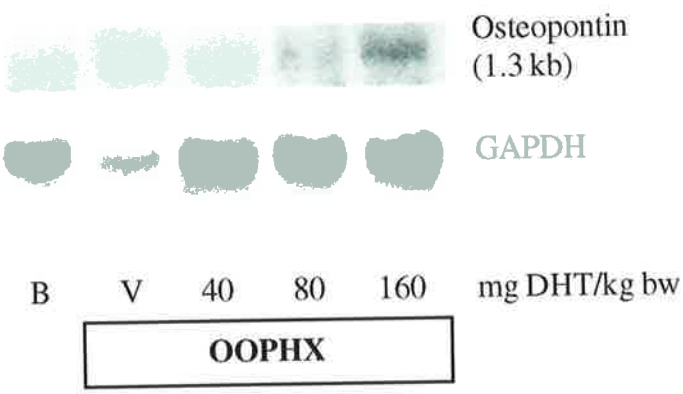


Figure 5.9 **A:** Representation of Northern Blot analysis for osteopontin mRNA and GAPDH mRNA. B-Baseline, V-Vehicle. **B:** Osteopontin mRNA levels corrected for GAPDH and expressed as a fold increase relative to Baseline in sham and osteopenic oophorectomised rats. DHT was administered at 15 weeks post-oophorectomy. Values are Mean ± SE. Dose of DHT is mg/kg body weight. DHT doses 40mg, 80mg and 160mg/kg body weight have been pooled.

DHT Administered to Osteopenic Oophorectomised Rats

A



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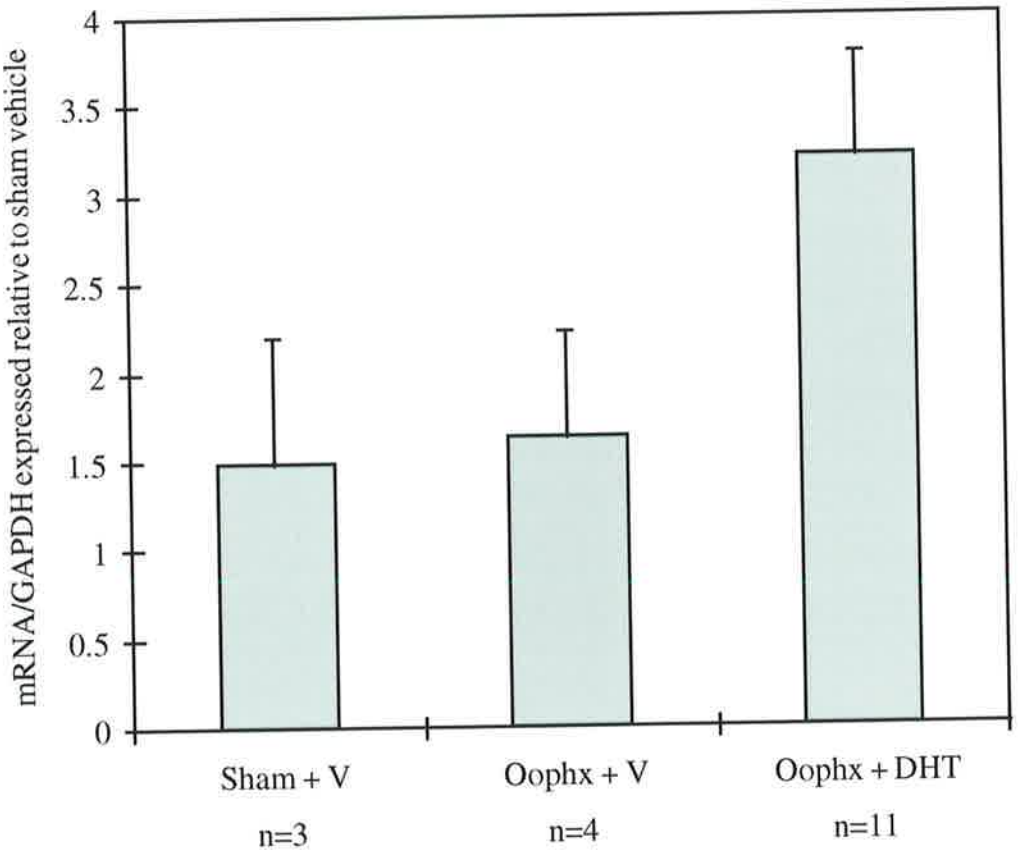
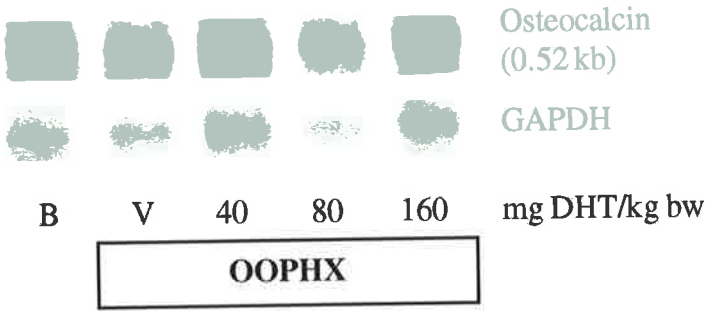


Figure 5.10 **A:** Representation of Northern Blot analysis for osteocalcin mRNA and GAPDH mRNA. B-Baseline, V-Vehicle. **B:** Osteocalcin mRNA levels corrected for GAPDH and expressed as a fold increase relative to Baseline in sham and osteopenic oophorectomised rats. DHT was administered at 15 weeks post-oophorectomy. Values are Mean ± SE. Dose of DHT is mg/kg body weight. DHT doses 40mg, 80mg and 160mg/kg body weight have been pooled. $^{*}P<0.01$ versus Sham + Vehicle, $^{\#}P<0.05$ versus Sham + Vehicle.

DHT Administered to Osteopenic Oophorectomised Rats

A



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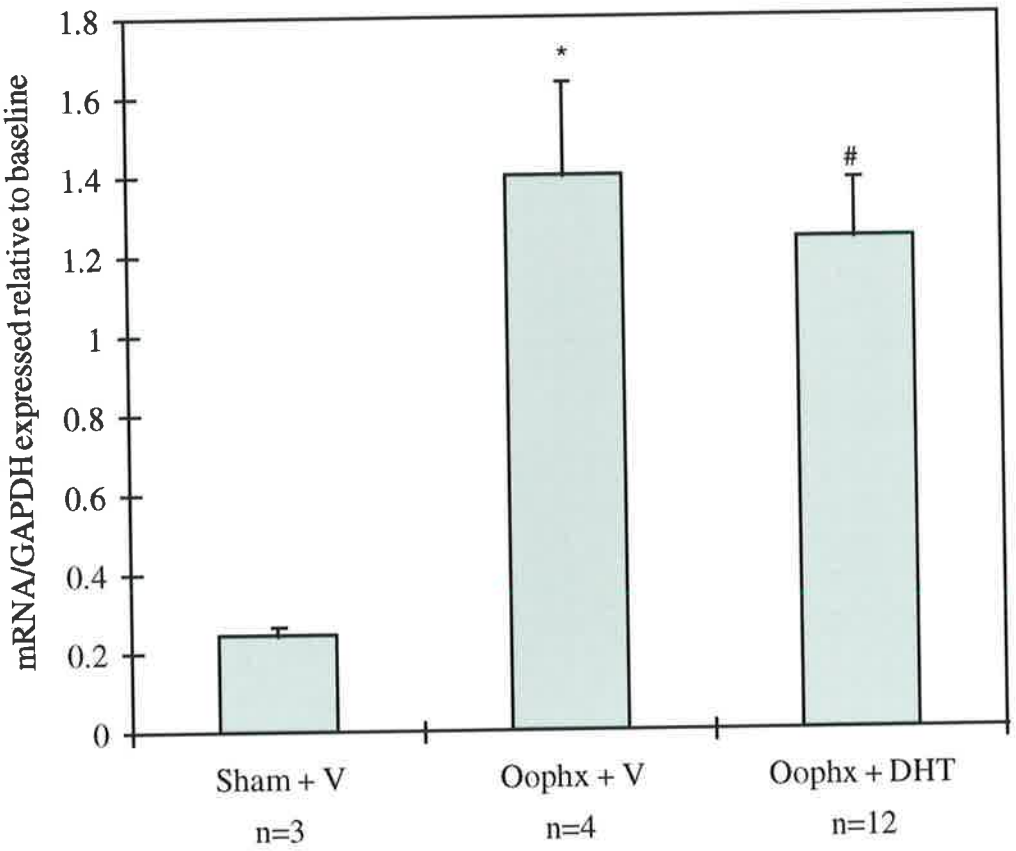
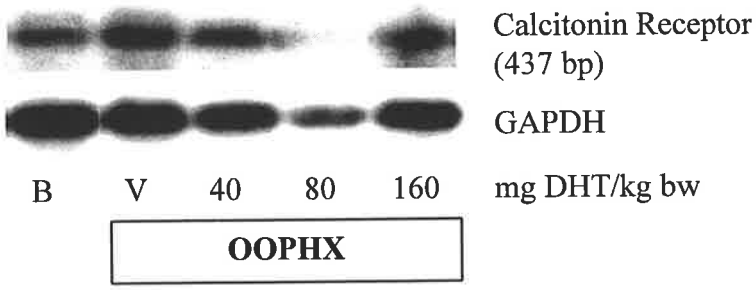


Figure 5.11 **A:** Representation of Southern Blot analysis of RT-PCR products for calcitonin receptor (CTR) mRNA and GAPDH mRNA. B-Baseline, V-Vehicle. **B:** Calcitonin receptor mRNA levels corrected for GAPDH and expressed as a fold increase relative to Baseline in sham and osteopenic oophorectomised rats. DHT was administered at 15 weeks post-oophorectomy. Bars represent mean values, (◆) data points contributing to mean (n=2). Dose of DHT is mg/kg body weight.

DHT Administered to Osteopenic Oophorectomised Rats

A



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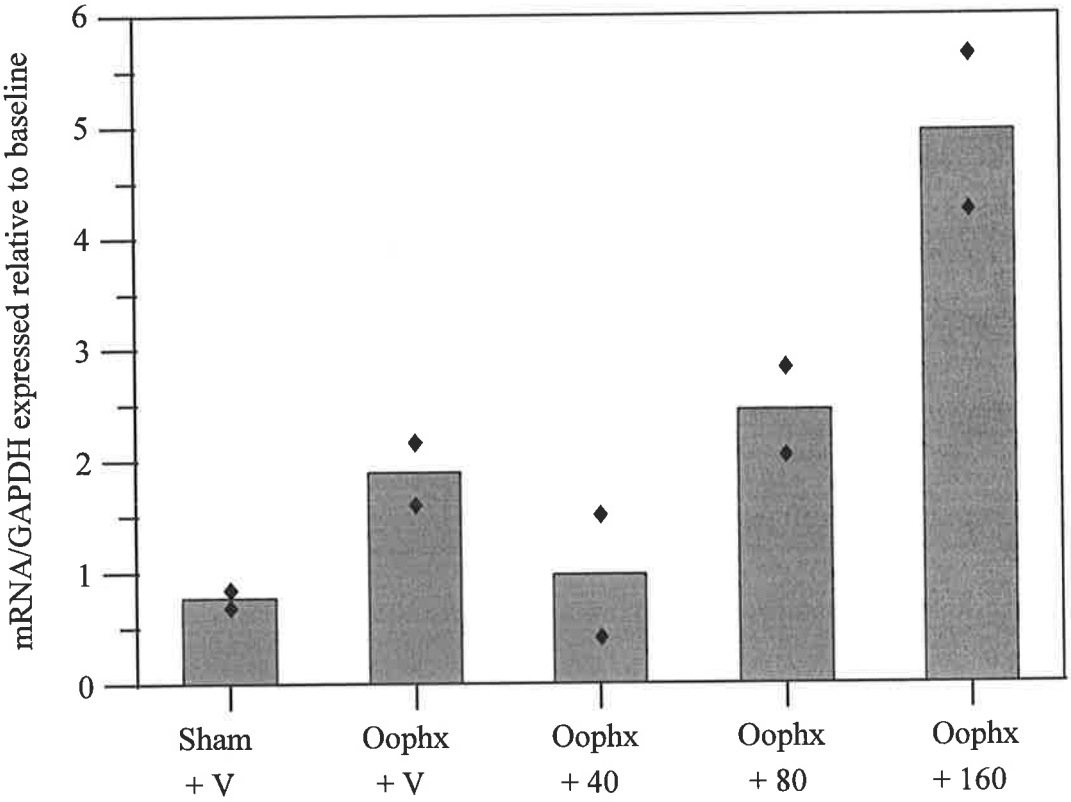


5.4 DISCUSSION

Oophorectomy increased the mRNA levels of the osteoblast genes type 1α collagen, osteopontin, osteocalcin and although not achieving statistical significance, the mRNA levels of alkaline phosphatase. Calcitonin receptor mRNA levels were increased in oophx rats also. Calcitonin receptor is a specific receptor located on osteoclasts and reflects osteoclast activity as decreased calcitonin receptor mRNA levels are associated with a decrease in bone resorption activity (Wada et al 1996). These data therefore, indicate that osteoblast and osteoclast gene expression are elevated following oophorectomy, consistent with the increase in bone resorption and bone formation observed as this time. The present findings confirm the increase in type 1α collagen and osteocalcin mRNA levels previously reported following oophorectomy (Salih et al 1993, Ikeda et al 1993) and are consistent with the increase in the biochemical markers of bone resorption and bone formation (Chapter 3). This is the first time that the effect of oophorectomy on all of the genes expressed during osteoblast development and the osteoclast gene calcitonin receptor has been identified.

c-*fos* mRNA levels were unaffected by oophorectomy which in contrast to previous findings where c-*myc* mRNA levels, a gene expressed concurrently with the expression of c-*fos* during proliferation of the osteoblast (Owen et al 1990) are elevated following oophorectomy (Salih et al 1993). The elevation of c-*myc* mRNA levels however was detected at 2 weeks post-oophorectomy while the mRNA levels in the present study were measured at 8 weeks following oophorectomy. This may have been too late to detect the oophorectomy induced stimulation of osteoblast development at the early stages of

proliferation when c-*fos* is expressed. c-*fos* expression is detected at 12 days following fracture of the tibiae in the rat during a period of bone formation supporting this theory, while expression of c-*fos* persists for 28 days following fracture, it then decreases to a low level (Ohta et al 1991). Furthermore, the mRNAs for c-*fos* and c-*myc* have half-life values as short as 15 minutes and are unstable allowing for their rapid disappearance after induction (Brawerman 1987).

The oophorectomy-induced stimulation of bone formation which follows the increased osteoclastic bone resorption (Dempster et al 1995, Sims et al 1996a) leads to the stimulation of osteoblasts at all stages of development. Based on the model of osteoblast development proposed by Owen and colleagues (1990) estrogen deficiency stimulates osteoblast bone formation at the level of late proliferation, matrix development and maturation and mineralisation as indicated by the increase in the mRNA levels of type 1α collagen, alkaline phosphatase, osteopontin and osteocalcin. The stimulation of osteoblast development in estrogen deficiency may occur at the early stage of proliferation when c-*fos* is expressed, which results in a cascade of stimulation of the proceeding stages of osteoblast development. The failure to detect an increase in c-*fos* mRNA levels in the present study however is probably due to its time course.

The mRNA levels for the osteoclast genes carbonic anhydrase and tartrate-resistant acid phosphatase were undetectable by northern blot analysis in the present study. In contrast, Zheng and colleagues (1994) demonstrated marked increases in these genes following oophorectomy in the rat by northern blot analysis. The failure to detect changes in the mRNA levels of these osteoclast genes following oophorectomy may be due to our

collection procedure of the bones prior to extraction of total RNA. Zheng and colleagues (1994) extracted total RNA from whole tibiae where the epiphyses, growth plate and bone marrow were present, while in the present study the epiphyses including the growth plate and bone marrow were removed prior to total RNA extraction. Perhaps the bone marrow contains a large proportion of osteoclasts in addition to the bone matrix and were removed by the collection procedure of the bones used in the present study. The osteoclast gene calcitonin receptor was however detected in the present study following amplification by reverse transcriptase polymerase chain reaction.

Following the development of osteopenia at 29 weeks post-oophorectomy the mRNA levels of the osteoblast and osteoclast genes were not stimulated to the same degree as the mRNA levels measured at 8 weeks post-oophorectomy (experiment A). In the osteopenic oophx rats the mRNA levels of osteocalcin was significantly elevated by approximately 1.4 fold compared to the control rats and calcitonin receptor mRNA levels were elevated by 1.8 fold. Type 1α collagen and alkaline phosphatase mRNA levels however, although elevated were not statistically significant and osteopontin mRNA levels were unaffected in the osteopenic oophx rats. In contrast, at 8 weeks post-oophorectomy, all of the osteoblast genes were significantly elevated by 3 to 30 fold compared with control rats and the osteoclast gene calcitonin receptor was elevated by 2.5 fold. These differences in the magnitude of stimulation of mRNA levels are perhaps due to the differences in the rate of bone turnover between the two time points following oophorectomy. The marked increase in the mRNA levels of the osteoblast and osteoclast genes observed at 8 weeks post-oophorectomy is consistent with the rapid phase of bone loss occurring during the first 3 months following oophorectomy (Wronski et al 1989a). In contrast, at 29 weeks post-oophorectomy, the bone turnover rate does not differ

significantly from sham rats (Coxam et al 1996, Wronski et al 1989a) which in the present study is consistent with the slight, non significant increases in the mRNA levels of the osteoblast and osteoclast genes in the osteopenic oophx rats. In addition, the mRNA levels of c-*fos* were not detectable in the osteopenic oophx rats at 29 weeks post-oophorectomy which is not surprising as at 8 weeks post-oophorectomy when bone turnover is high, c-*fos* was detectable only at low levels.

DHT treatment in estrogen sufficient rats increased the mRNA levels of the osteoblast genes type 1α collagen, alkaline phosphatase, osteopontin and osteocalcin but had no effect on c-*fos* mRNA levels. The increase in alkaline phosphatase and osteopontin mRNA levels in the estrogen sufficient rats however did not reach significance possibly due to the individual variation observed in these mRNA levels. It is interesting to note that serum levels of alkaline phosphatase also vary greatly between individual rats. The action of DHT treatment to increase the mRNA levels of type 1α collagen, alkaline phosphatase, osteopontin and osteocalcin suggests that androgens in the presence of ovarian hormones stimulate all stages of osteoblast differentiation. Consistent with these findings is the increase in the mRNA levels of type 1α(I) procollagen in osteoblast-like cells observed following treatment with testosterone and DHT (Benz et al 1991) and the increase in the number of differentiated bone cells as indicated by positive staining for alkaline phosphatase following DHT treatment (Kasperk et al 1989). DHT treatment had no effect on the mRNA levels of c-*fos* which is consistent with the previous findings of Bodine and colleagues (1995) who demonstrated that treatment of human osteoblast-like cells with DHT, testosterone or androstenedione for 30 minutes and 24 hours invitro had no effect on the mRNA levels of c-*fos* or c-*jun*. These data suggest that androgens do not stimulate

proliferation of osteoblast cells. In contrast however, DHT has been shown to stimulate the proliferation of mouse calvarial cells (Kasperk et al 1989), rat bone cells (Somjen et al 1991) and MC3T3-E1 osteoblast-like cells (Masuyama et al 1992) in vitro. In the present study however, any effect of DHT treatment on c-*fos* mRNA levels may not have been detectable at 8 weeks post-oophorectomy as c-*fos* mRNA is unstable and can decay rapidly following induction (Brawerman 1987). The data from the present study are consistent with a model of androgens in estrogen sufficient rats acting to stimulate the differentiation of the osteoblast at all stages of development as indicated by the mRNA levels of type 1α collagen, alkaline phosphatase, osteopontin and osteocalcin. The effect of DHT on osteoblast proliferation as indicated by c-*fos* however, is inconclusive and requires further investigation at shorter time points following DHT treatment.

DHT treatment in estrogen sufficient rats increased the mRNA levels of the osteoclast gene calcitonin receptor and this effect was dose dependent with maximal stimulation occurring at the highest dose of DHT. In contrast, DHT and testosterone have no effect on osteoclasts in vitro (Tobias et al 1991, Caputo et al 1976). Interestingly, no dose effects of DHT were observed on the mRNA levels of the osteoblast genes and therefore perhaps osteoblasts are more responsive to lower circulating levels of androgens than osteoclasts in estrogen sufficient rats. The increase in the mRNA levels of calcitonin receptor may be a response to the increase in the mRNA levels of the osteoblastic genes due to the tight coupling between bone formation and bone resorption, however this theory requires further investigation.

DHT treatment in oophorectomised rats immediately following operation suppressed the mRNA levels of type 1α collagen and osteocalcin. Immediately following oophorectomy, bone cell activity is high and thus it appears that androgens can suppress the oophorectomy-induced increase in type 1α collagen and osteocalcin. DHT however had no effect on the mRNA levels of c-*fos*, alkaline phosphatase or osteopontin. These data are consistent with a model of androgens acting to suppress osteoblast differentiation at the stage of early differentiation as indicated by type 1α collagen and at the stage of mineralisation as indicated by osteocalcin in oophorectomised rats immediately following operation. The significant suppression of osteocalcin mRNA levels following DHT treatment however, may be associated with increased bone formation as it has been recently proposed, based on studies of the osteocalcin knockout mouse, that osteocalcin functions to limit bone formation without impairing bone resorption or mineralisation (Ducy et al 1996). In these osteocalcin-negative mice there was an increase in cortical thickness, bone mineral matrix and cancellous bone volume associated with an increase in cortical and cancellous bone formation rates and double-labelled fluorescence surfaces. If osteocalcin does in fact limit bone formation, then the action of androgens to suppress the mRNA levels of osteocalcin may also be associated with an increase in bone formation rate and bone volume. Certainly stimulation of bone formation has been reported with DHT treatment in osteopenic oophx rats (Tobias et al 1994, Coxam et al 1996) and with nandrolone decanoate treatment, the latter being associated with suppression of serum osteocalcin levels (Schot et al 1993).

In contrast, the mRNA levels of alkaline phosphatase and osteopontin were unaffected by DHT treatment in oophx rats. Furthermore, DHT significantly suppressed calcitonin

receptor mRNA at the lowest doses of 40mg and 80mg/kg body weight indicating that androgens can have an inhibitory action on osteoclast bone resorption in oophx rats immediately following operation when bone turnover rate is high.

It is of interest that the action of DHT to increase the mRNA levels of osteocalcin in estrogen-sufficient rats and to suppress the mRNA levels in estrogen-deficient rats is reflected in changes in the serum osteocalcin levels following DHT treatment. No effect of oophorectomy was observed on the serum levels of osteocalcin in rats receiving DHT due to a small non significant rise in osteocalcin in sham rats and a small non significant decrease in oophx rats. In contrast, alkaline phosphatase mRNA levels were only increased in sham rats following DHT treatment while serum alkaline phosphatase levels were elevated in both sham and oophx rats. Calcitonin receptor mRNA levels were decreased at 40mg/kg body weight DHT which was not reflected in the biochemical markers of bone resorption urine hydroxyproline and urine deoxypyridinoline. It is obviously difficult to make direct comparisons between serum levels of biochemical bone markers and the gene expression.

DHT administration to osteopenic oophx rats suppressed type 1α collagen and alkaline phosphatase mRNA levels and had no effect on the mRNA levels of osteopontin and osteocalcin. The mRNA levels of type 1α collagen were also suppressed in DHT-treated oophx rats immediately following oophx however alkaline phosphatase mRNA levels were unaffected. The actions of androgens on osteoblast gene expression therefore, may be dependent on the rate of bone turnover at the time of administration. Immediately following operation the level of bone turnover is high but this declines with time being

similar to ovary-intact rats after 15 weeks post-operation. These data suggest that androgens inhibit osteoblast development in osteopenic oophx rats at an early stage of differentiation when type 1α collagen and alkaline phosphatase are expressed but have no effect at later stages of osteoblast differentiation when osteopontin and osteocalcin are expressed. In contrast to the suppression of calcitonin receptor in the first experiment when DHT was administered at the time of operation, DHT administration to osteopenic oophx rats increased the mRNA levels of the osteoclast gene calcitonin receptor, indicating that androgens stimulate bone resorption when bone turnover rate is low. Interestingly, the changes in the mRNA levels of the osteoblast and osteoclast genes following DHT treatment in osteopenic oophx rats were not reflected in the biochemical markers of bone turnover measured.

The changes in the mRNA levels following DHT treatment of all the osteoblast genes and the osteoclast gene in estrogen-sufficient and estrogen-deficient rats were lower in magnitude than the effects of oophorectomy alone. Furthermore, the small changes in sham and oophx rats following DHT treatment were not observed in all the biochemical markers of bone turnover. In contrast, the predominant effect of oophorectomy was observed in both changes in gene expression and biochemical markers of bone turnover. These data indicate, not surprisingly that estrogen is the predominant steroid sex hormone regulator of bone cell gene expression in female rats. Furthermore, the mechanism by which androgens increased the mRNA levels of the osteoblast and osteoclast genes in the estrogen sufficient rats requires further investigation. The increase in mRNA levels following androgen treatment could be due to either an increase in the actual levels of mRNA or by an increase in the stability of the mRNA, since the rate of decay of some

mRNA species can be altered in response to physiological signals such as hormone induction in animals (Brawerman 1987).

This study has demonstrated for the first time that at 8 weeks post-oophorectomy the mRNA levels of all of the genes expressed during osteoblast development and the osteoclast gene, calcitonin receptor are increased. Furthermore, in osteopenic oophx rats at 29 weeks post-oophorectomy these genes are not stimulated to the same degree as observed at 8 weeks post-oophorectomy. The effects of DHT on osteoblast and osteoclast gene expression differ in estrogen sufficient and estrogen deficient rats and therefore the action of androgens on osteoblast and osteoclast gene expression appears to be estrogen dependent. In estrogen sufficient rats DHT increases osteoblast and osteoclast gene expression while in oophorectomised rats DHT administered at the time of operation inhibits osteoblast differentiation at the level of type 1α collagen and osteocalcin and inhibits the osteoclast gene calcitonin receptor. Furthermore, in osteopenic oophx rats DHT treatment inhibits osteoblast differentiation at the level of type 1α collagen and alkaline phosphatase while stimulating calcitonin receptor mRNA levels. These data suggest that there is an interaction of estrogens and androgens on the mRNA levels of the osteoblast and osteoclast genes, which requires further investigation.

CHAPTER 6

EFFECTS OF ESTRADIOL TREATMENT ALONE AND IN COMBINATION WITH DHT ON BONE CELL METABOLISM IN OSTEOPENIC OOPHORECTOMISED RATS

6.1 INTRODUCTION

The increase in trabecular bone volume following androgen treatment in osteopenic oophorectomised rats is associated with an increase in bone formation rate (Tobias et al 1994, Coxam et al 1996) and DHT treatment at high doses inhibits bone resorption (Tobias et al 1994). In contrast, estrogen treatment suppresses bone resorption in post-menopausal women (Lafferty et al 1964, Lindsay et al 1976, Riggs et al 1972) and in oophorectomised rats whether administered from the time of oophorectomy (Wronski et al 1988, Kalu et al 1991b, Frolik et al 1996) or at 13 weeks post-oophorectomy (Abe et al 1993) but does not restore the bone lost prior to treatment (Abe et al 1993).

Since androgens and estrogens have contrasting effects on bone there is a potential therapeutic use for combination treatment of these agents to prevent bone loss associated with the menopause, while partially restoring the bone lost prior to treatment. In post-menopausal osteoporotic women, the addition of nandrolone decanoate to hormone replacement therapy (HRT) for 3 years results in an increase in lumbar bone mineral content which is not observed with HRT alone (Erdstieck et al 1994). Furthermore,

combined androgen therapy with HRT can reverse the inhibitory effects of estrogen on the markers of bone formation without altering the inhibitory effects of estrogen on bone resorption (Raisz et al 1996). Similarly, in osteopenic oophorectomised rats, combined estrogen and androgen treatment increased bone mineral density in the femur to a greater level than with estrogen treatment alone (Coxam et al 1996).

The following chapter investigates the effects of DHT and estradiol treatment alone and in combination in osteopenic oophorectomised rats to identify changes in the mRNA levels of the osteoclast and osteoblast genes and the biochemical markers of bone cell metabolism.

6.2 EXPERIMENTAL PROTOCOL

6.2.1 Experimental Procedure

40 animals were randomly divided into 4 groups and pre-operative fasting blood and urine samples were collected to provide baseline biochemistry. The rats were oophorectomised (Chapter 2.3.2) and at 15 weeks post-oophorectomy the rats were administered either vehicle (silastic tubing) or 80mg/kg body weight dihydrotestosterone as described in Chapter 3.2.1. 24 hour fasting blood (Chapter 2.3.4) and urine samples (Chapter 2.3.5) were collected at 2 and 4 weeks. At 19 weeks post-oophorectomy (4 weeks post DHT treatment) the rats were administered either vehicle (silastic tubing) or 20mg/kg bw estrogen by silastic implants of length 1cm as described in Chapter 2.3.3. A 24 hour fasting blood (Chapter 2.3.4) and urine sample (Chapter 2.3.5) was collected 1 week following estradiol administration. The treatment groups therefore are as follows; Group 1,

vehicle + vehicle; Group 2, vehicle + estradiol; Group 3, DHT + vehicle; Group 4, DHT + estradiol. On completion of the experiment the rats were exsanguinated by cardiac puncture under halothane anaesthesia and sacrificed by cervical dislocation. One femur and both tibia from each rat were excised as described in Chapter 2.7.3.1.

6.2.2 Biochemical Analyses

Urine volumes were recorded. Urine was analysed for hydroxyproline (Chapter 2.4.2), free deoxypyridinoline (Chapter 2.4.3), creatinine (Chapter 2.4.1) and calcium (Chapter 2.4.4). Serum was analysed for estradiol (Chapter 2.4.10), osteocalcin (Chapter 2.4.7), alkaline phosphatase (Chapter 2.4.13), sodium, potassium, chloride, bicarbonate (Chapter 2.4.6), calcium, creatinine (Chapter 2.4.16), albumin (Chapter 2.4.14) and total protein (Chapter 2.4.15). Urinary hydroxyproline, calcium and creatinine excretion were determined in 24 hour fasting urine specimens and expressed as their ratio to creatinine (Chapter 2.5). Ionised calcium and TmCa were calculated as described in Chapter 2.5.

6.2.3 Analysis of mRNA Levels

Total RNA was extracted from the bones collected as described in Chapter 2.7.3.2. The mRNA levels for the osteoblast genes c-*fos*, type 1α collagen, alkaline phosphatase, osteopontin and osteocalcin were determined by Northern blot analysis as described in Chapter 2.7.4. The mRNA levels for the osteoclast gene calcitonin receptor were amplified by reverse transcriptase polymerase chain reaction (RT-PCR) and these RT-PCR products were semi quantified by Southern blot analysis as described in Chapter 2.7.6.

6.2.4 Statistical Analyses

The biochemical variables varied at 15 weeks post-oophorectomy between the groups and therefore statistical analyses were performed on the difference between the experimental values (17, 19 and 20 weeks post-oophorectomy) and the value measured at 15 weeks post-oophorectomy in order to correct for individual variation. The effect of DHT administration at 2 and 4 weeks post-treatment (17 and 19 weeks post-oophorectomy) were analysed as described in Chapter 2.6.1. The effect of DHT, estradiol and an interaction between DHT and estradiol treatment at 5 weeks post-treatment DHT, 1 week post-treatment estradiol (20 weeks post-oophorectomy) was analysed as described in Chapter 2.6.5. All mRNA levels were normalised for glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mRNA levels as described in Chapter 2.7.4.6. The mRNA levels of the osteoblast and osteoclast genes were expressed as a fold increase relative to oophorectomised rats receiving vehicle only (Chapter 2.7.4.6). Statistical analyses were performed as described in Chapter 2.7.4.6.

6.3 RESULTS

The data presented for each biochemical variable is the change from 15 weeks post-oophorectomy (Chapter 6.2.4). The uncorrected data for each biochemical variable is presented in Appendices D.1 - D.18.

6.3.1 Biochemical Analyses

6.3.1.1 Serum Estradiol

Serum estradiol levels were elevated following estradiol administration ($P<0.001$) and were unaffected by DHT administration (Table 6.1). The estradiol levels achieved were supraphysiological being well in excess of the normal range for intact rats (86-258 pmol/L (Sims et al 1996)).

6.3.1.2 Urine Volume and 24 Hour Creatinine Excretion

Urine volume was significantly decreased by DHT administration at 17 weeks post-oophorectomy ($P<0.05$) but was unaffected at 19 and 20 weeks post-oophorectomy (Appendix D.1). In contrast, 24 hour creatinine excretion was unaffected by DHT treatment at any time point (Appendix D.2). The urine biochemical analyses therefore were expressed as a ratio to creatinine.

6.3.1.3 Bone Biochemical Markers

Serum alkaline phosphatase was increased as a result of DHT administration at all time points ($P<0.01$ week 17, $P<0.001$ weeks 19 and 20 post-oophorectomy) (Figure 6.1). Serum osteocalcin was decreased following DHT treatment at all time points ($P<0.005$ weeks 17 and 19, $P<0.05$ week 20 post-oophorectomy) (Figure 6.2). Serum alkaline phosphatase and osteocalcin were unaffected by estradiol treatment at 20 weeks post-oophorectomy (Figure 6.1, Figure 6.2). There was an interaction of combined DHT and estradiol treatment on the serum levels of alkaline phosphatase such that the serum levels of alkaline phosphatase in the combined DHT and estradiol treatment group were elevated compared to rats receiving DHT alone ($P<0.01$) (Figure 6.1).

Urine deoxypyridinoline/creatinine was suppressed following DHT treatment at 20 weeks post-oophorectomy ($P<0.05$) but was unaffected by estradiol treatment (Table 6.2). Urine hydroxyproline/creatinine was unaffected by DHT treatment but was increased following 1 week of estradiol treatment at 20 weeks post-oophorectomy ($P<0.05$) (Table 6.3).

6.3.1.4 Urine Calcium

Urine calcium/creatinine was decreased at all time points following DHT administration ($P<0.001$ weeks 17 and 19, $P<0.005$ week 20 post-oophorectomy) (Figure 6.3). TmCa was elevated by DHT treatment at all time points of the experiment ($P<0.001$ weeks 17 and 19, $P<0.05$ week 20 post-oophorectomy) (Figure 6.4). Urine calcium/creatinine and TmCa were unaffected by estradiol treatment at 20 weeks post-oophorectomy and no interactions of combined DHT and estradiol treatment were observed (Figure 6.3, Figure 6.4).

6.3.1.5 Calcium Fractions

Serum ionised calcium (calculated) ($P<0.05$), total serum calcium ($P<0.005$), ultrafiltrable calcium ($P<0.05$) and protein bound calcium ($P<0.001$) were decreased following DHT administration at 20 weeks post-oophorectomy but were unaffected by estradiol treatment (Table 6.4, Table 6.5, Table 6.6, Table 6.7). Serum complexed calcium was unaffected by DHT or estradiol treatment at any time point (Table 6.8).

Table 6.1 Serum estradiol (pmol/L) in osteopenic oophorectomised rats following DHT administration at 15 weeks post-oophorectomy and estradiol administration at 19 weeks post-oophorectomy.

Time (Weeks)	Vehicle + Vehicle	Vehicle + Estradiol	DHT + Vehicle	DHT + Estradiol
20	75 (14) n=8	2626 (444)[a] n=10	114 (40) n=9	3538 (340)[a] n=10

Values are Mean (SE). Time is weeks post-oophorectomy. DHT (80mg/kg bw) was administered at 15 weeks post-oophorectomy and Estradiol (20mg/kg bw) was administered at 19 weeks post-oophorectomy. [a]$P<0.001$ versus Vehicle + Vehicle and versus DHT + Vehicle.

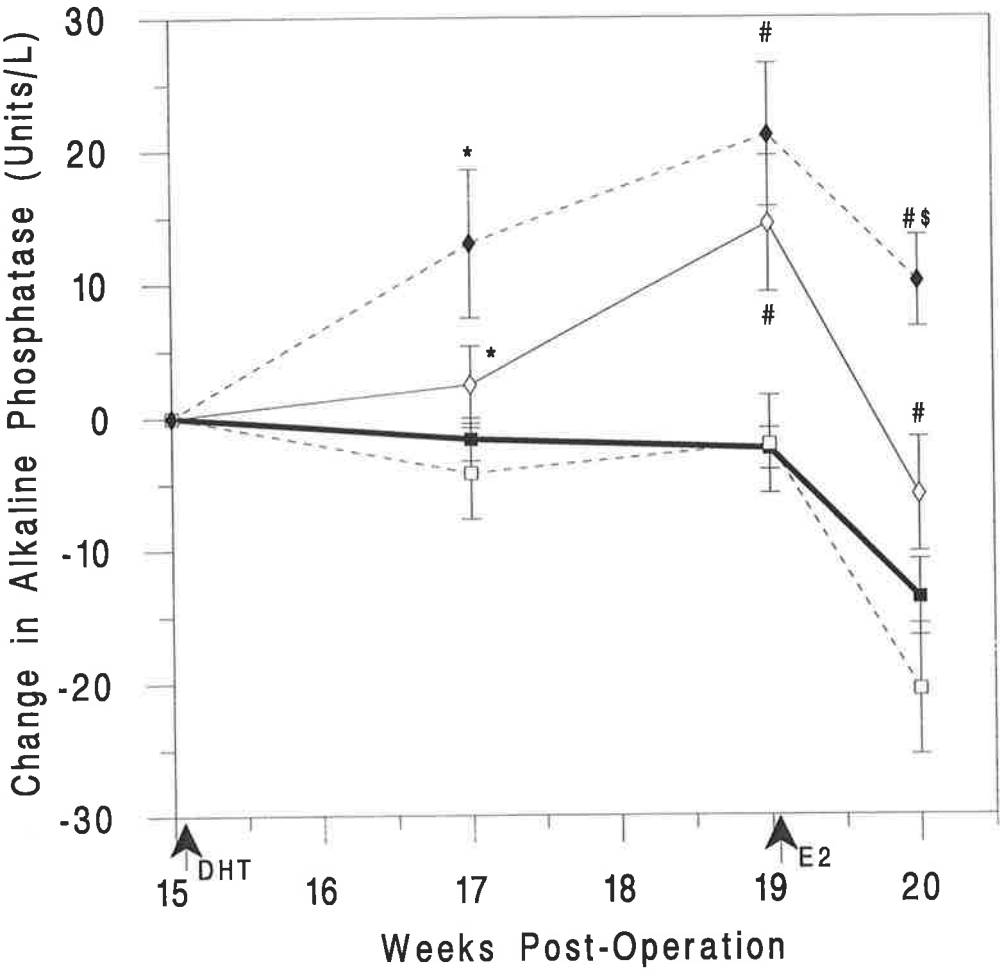


Figure 6.1 Change in serum alkaline phosphatase (units/L) from 15 weeks post-oophorectomy in osteopenic oophx rats following DHT (80mg/kg body weight) administration at 15 following oophorectomy and estradiol (20mg/kg body weight) administration at 19 weeks following oophorectomy. Values are Mean ± SE, n=9. (■) Vehicle + Vehicle, (□) Vehicle + Estradiol, (◊) DHT + Vehicle, (◆) DHT + Estradiol. *$P<0.01$ versus Vehicle + Vehicle and versus Vehicle + Estradiol, $^{\#}P<0.001$ versus Vehicle + Vehicle and versus Vehicle + Estradiol, $^{\$}P<0.01$ versus DHT + Vehicle.

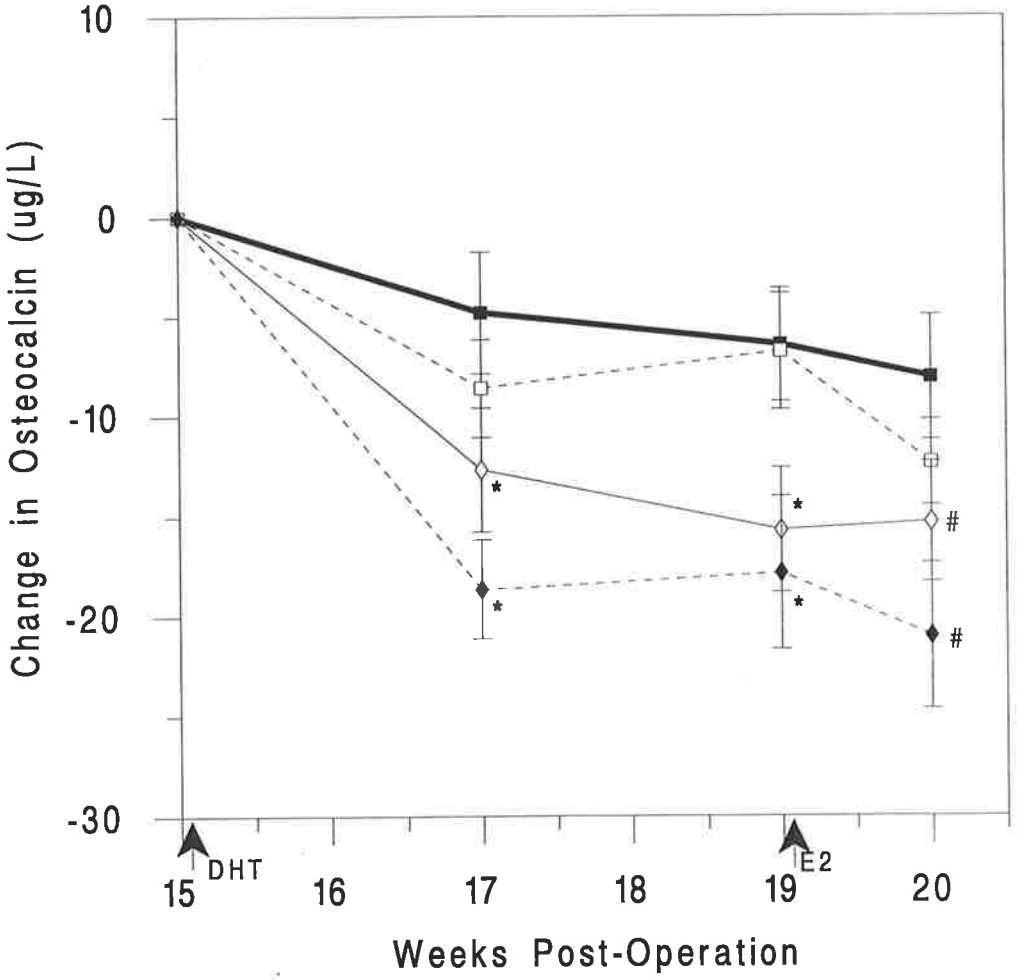


Figure 6.2 Change in serum osteocalcin (μg/L) from 15 weeks post-oophorectomy in osteopenic oophx rats following DHT (80mg/kg body weight) administration at 15 following oophorectomy and estradiol (20mg/kg body weight) administration at 19 weeks following oophorectomy. Values are Mean ± SE, n=9. (■) Vehicle + Vehicle, (□) Vehicle + Estradiol, (◊) DHT + Vehicle, (◆) DHT + Estradiol. *P<0.005 versus Vehicle + Vehicle and versus Vehicle + Estradiol, #P<0.05 versus Vehicle + Vehicle and versus Vehicle + Estradiol.

Table 6.2 Urine deoxypyridinoline/creatinine (nmol/mmol) in osteopenic oophorectomised rats following DHT administration at 15 weeks post-oophorectomy and estradiol administration at 19 weeks post-oophorectomy.

Time (Weeks)	Vehicle + Vehicle	Vehicle + Estradiol	DHT + Vehicle	DHT + Estradiol
20	37.9 (2.1) n=9	36.9 (3.5) n=10	28.8 (2.4)[a] n=10	26.4 (4.2)[a] n=10

Values are Mean (SE). Time is weeks post-oophorectomy. DHT (80mg/kg bw) was administered at 15 weeks post-oophorectomy and Estradiol (20mg/kg bw) was administered at 19 weeks post-oophorectomy. [a]$P<0.05$ versus Vehicle + Vehicle and versus Vehicle + Estradiol.

Table 6.3 Change in urine hydroxyproline/creatinine (μmol/mmol) from 15 weeks post-oophorectomy in osteopenic oophorectomised rats following DHT administration at 15 weeks post-oophorectomy and estradiol administration at 19 weeks post-oophorectomy.

Time (Weeks)	Vehicle + Vehicle	Vehicle + Estradiol	DHT + Vehicle	DHT + Estradiol
17	0.91 (0.24) n=9	0.91 (0.57) n=9	0.06 (2.61) n=9	1.25 (1.09) n=10
19	1.09 (0.49) n=9	0.25 (0.35) n=9	-0.67 (2.66) n=9	1.59 (1.02) n=10
20	-0.25 (0.63) n=9	1.2 (0.45)[a] n=9	-1.4 (1.78) n=9	3.42 (2.61)[a] n=5

Values are Mean (SE). Values are corrected for 15 weeks post-oophorectomy. Time is weeks post-oophorectomy. DHT (80mg/kg bw) was administered at 15 weeks post-oophorectomy and Estradiol (20mg/kg bw) was administered at 19 weeks post-oophorectomy. [a]$P<0.005$ versus Vehicle + Vehicle and versus DHT + Vehicle.

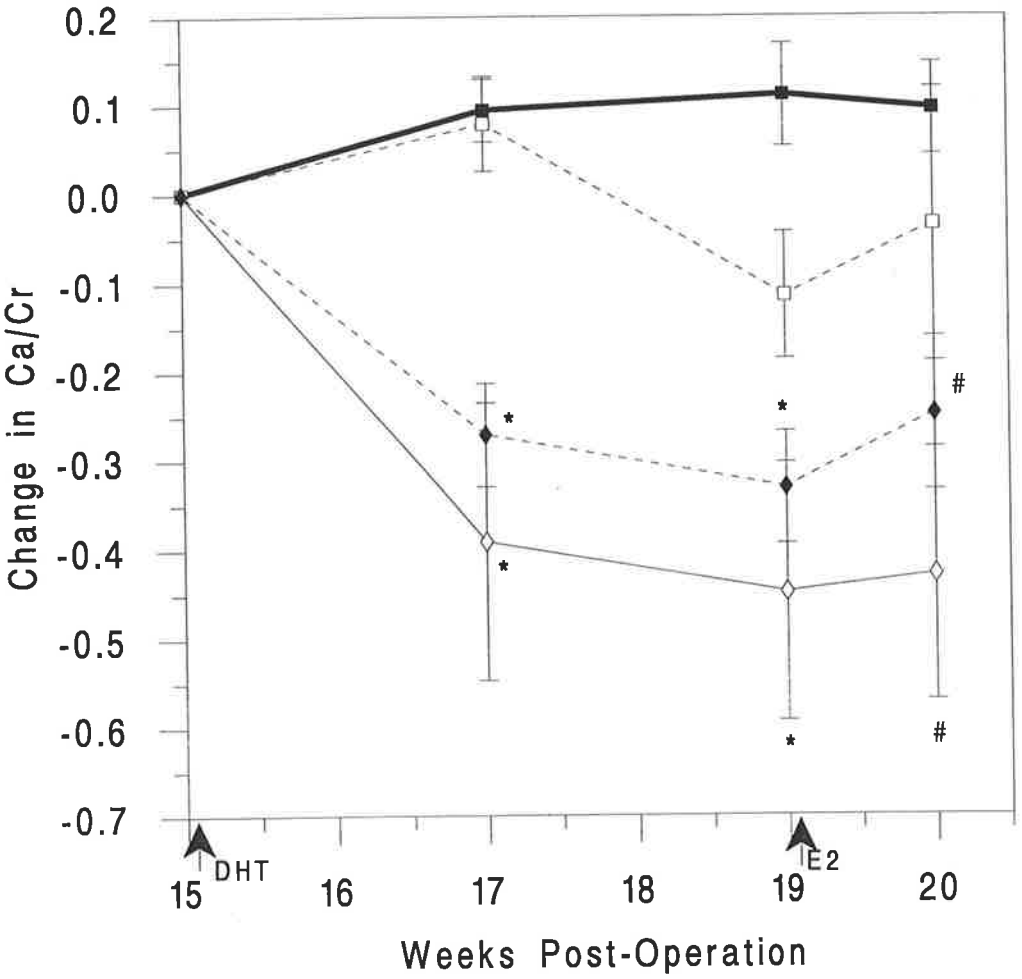


Figure 6.3 Change in urine calcium/creatinine (mmol/mmol) from 15 weeks post-oophorectomy in osteopenic oophx rats following DHT (80mg/kg body weight) administration at 15 following oophorectomy and estradiol (20mg/kg body weight) administration at 19 weeks following oophorectomy. Values are Mean ± SE, n=9. (■) Vehicle + Vehicle, (□) Vehicle + Estradiol, (◊) DHT + Vehicle, (◆) DHT + Estradiol. *P<0.001 versus Vehicle + Vehicle and versus Vehicle + Estradiol, $^{\#}P$<0.005 versus Vehicle + Vehicle and versus Vehicle + Estradiol.

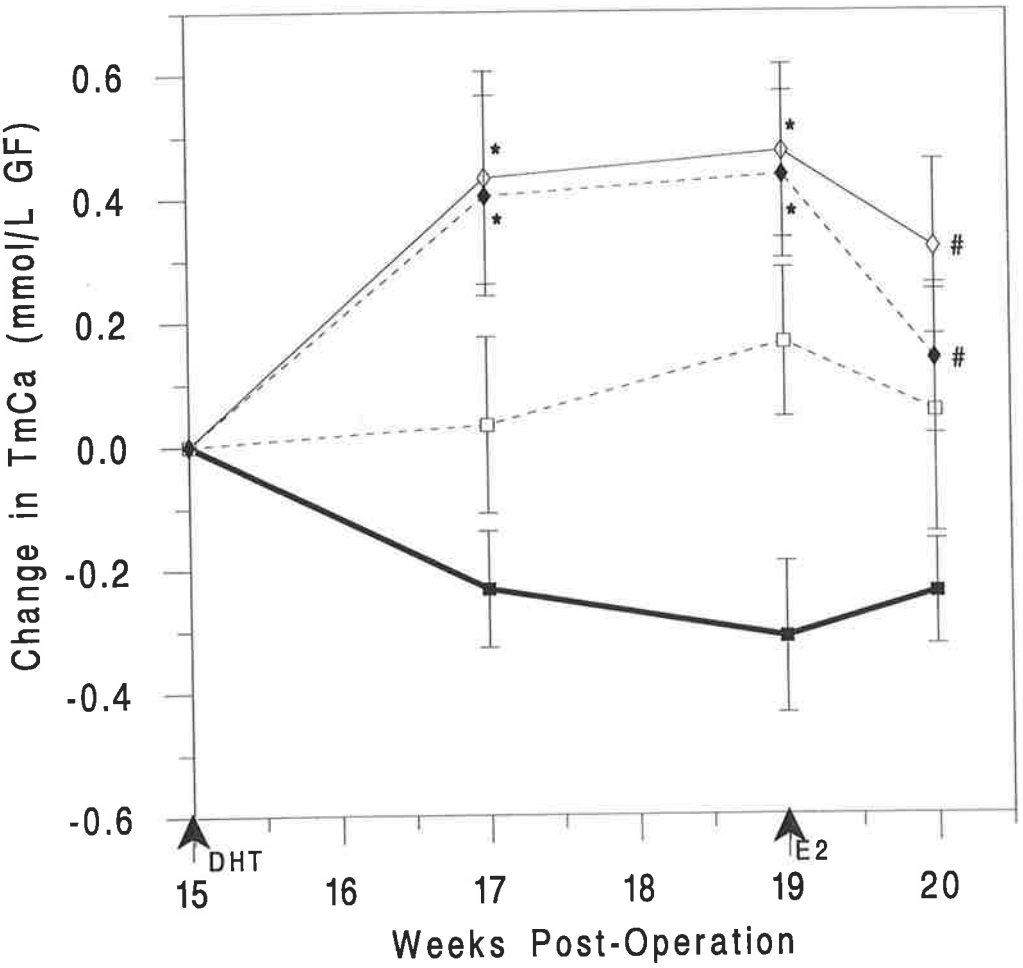


Figure 6.4 Change in the tubular reabsorption of calcium (TmCa) (mmol/L GF) from 15 weeks post-oophorectomy in osteopenic oophx rats following DHT (80mg/kg body weight) administration at 15 following oophorectomy and estradiol (20mg/kg body weight) administration at 19 weeks following oophorectomy. Values are Mean ± SE, n=9. (■) Vehicle + Vehicle, (□) Vehicle + Estradiol, (◊) DHT + Vehicle, (◆) DHT + Estradiol. *P<0.001 versus Vehicle + Vehicle and versus Vehicle + Estradiol, $^{\#}P$<0.05 versus Vehicle + Vehicle and versus Vehicle + Estradiol.

Table 6.4 Change in serum ionised calcium (calculated) (mmol/L) from 15 weeks post-oophorectomy in osteopenic oophorectomised rats following DHT administration at 15 weeks post-oophorectomy and estradiol administration at 19 weeks post-oophorectomy.

Time (Weeks)	Vehicle + Vehicle	Vehicle + Estradiol	DHT + Vehicle	DHT + Estradiol
17	-0.02 (0.03) n=9	0.02 (0.04) n=9	-0.01 (0.02) n=9	-0.03 (0.03) n=9
19	-0.07 (0.04) n=9	0.02 (0.03) n=9	-0.07 (0.04) n=9	-0.06 (0.03) n=9
20	-0.03 (0.02) n=9	-0.01 (0.04) n=9	-0.10 (0.03)[a] n=9	-0.08 (0.03)[a] n=9

Values are Mean (SE). Values are corrected for 15 weeks post-oophorectomy. Time is weeks post-oophorectomy. DHT (80mg/kg bw) was administered at 15 weeks post-oophorectomy and Estradiol (20mg/kg bw) was administered at 19 weeks post-oophorectomy. [a]$P<0.05$ versus Vehicle + Vehicle and versus Vehicle + Estradiol.

Table 6.5 Change in total serum calcium (mmol/L) from 15 weeks post-oophorectomy in osteopenic oophorectomised rats following DHT administration at 15 weeks post-oophorectomy and estradiol administration at 19 weeks post-oophorectomy.

Time (Weeks)	Vehicle + Vehicle	Vehicle + Estradiol	DHT + Vehicle	DHT + Estradiol
17	-0.10 (0.06) n=9	-0.23 (0.08) n=9	-0.07 (0.06) n=9	-0.11 (0.07) n=9
19	-0.20 (0.08) n=9	-0.33 (0.07) n=9	-0.19 (0.09) n=9	-0.16 (0.07) n=9
20	-0.14 (0.05) n=9	-0.07 (0.08) n=9	-0.32 (0.06)[a] n=9	-0.29 (0.06)[a] n=9

Values are Mean (SE). Values are corrected for 15 weeks post-oophorectomy. Time is weeks post-oophorectomy. DHT (80mg/kg bw) was administered at 15 weeks post-oophorectomy and Estradiol (20mg/kg bw) was administered at 19 weeks post-oophorectomy. [a]$P<0.005$ versus Vehicle + Vehicle and versus Vehicle + Estradiol.

Table 6.6 Change in ultrafiltrable calcium (mmol/L) from 15 weeks post-oophorectomy in osteopenic oophorectomised rats following DHT administration at 15 weeks post-oophorectomy and estradiol administration at 19 weeks post-oophorectomy.

Time (Weeks)	Vehicle + Vehicle	Vehicle + Estradiol	DHT + Vehicle	DHT + Estradiol
17	-0.04 (0.04) n=9	0.03 (0.06) n=9	0.01 (0.02) n=9	-0.01 (0.04) n=9
19	-0.11 (0.05) n=9	0.01 (0.05) n=9	-0.08 (0.05) n=9	-0.07 (0.05) n=9
20	-0.06 (0.03) n=9	-0.01 (0.05) n=9	-0.14 (0.04)[a] n=9	-0.12 (0.04)[a] n=9

Values are Mean (SE). Values are corrected for 15 weeks post-oophorectomy. Time is weeks post-oophorectomy. DHT (80mg/kg bw) was administered at 15 weeks post-oophorectomy and Estradiol (20mg/kg bw) was administered at 19 weeks post-oophorectomy. [a]$P<0.05$ versus Vehicle + Vehicle and versus Vehicle + Estradiol.

Table 6.7 Change in serum protein bound calcium (mmol/L) from 15 weeks post-oophorectomy in osteopenic oophorectomised rats following DHT administration at 15 weeks post-oophorectomy and estradiol administration at 19 weeks post-oophorectomy.

Time (Weeks)	Vehicle + Vehicle	Vehicle + Estradiol	DHT + Vehicle	DHT + Estradiol
17	-0.07 (0.02) n=9	-0.05 (0.03) n=9	-0.08 (0.04) n=9	-0.12 (0.03) n=9
19	-0.10 (0.03) n=9	-0.04 (0.03) n=9	-0.11 (0.04) n=9	-0.09 (0.03) n=9
20	-0.08 (0.03) n=9	-0.06 (0.03) n=9	-0.18 (0.03)[a] n=9	-0.17 (0.02)[a] n=9

Values are Mean (SE). Values are corrected for 15 weeks post-oophorectomy. Time is weeks post-oophorectomy. DHT (80mg/kg bw) was administered at 15 weeks post-oophorectomy and Estradiol (20mg/kg bw) was administered at 19 weeks post-oophorectomy. [a]$P<0.001$ versus Vehicle + Vehicle and versus Vehicle + Estradiol.

Table 6.8 Change in serum complexed calcium (mmol/L) from 15 weeks post-oophorectomy in osteopenic oophorectomised rats following DHT administration at 15 weeks post-oophorectomy and estradiol administration at 19 weeks post-oophorectomy.

Time (Weeks)	Vehicle + Vehicle	Vehicle + Estradiol	DHT + Vehicle	DHT + Estradiol
17	-0.02 (0.01) n=9	0.01 (0.01) n=9	0.03 (0.01) n=9	0.02 (0.02) n=9
19	-0.03 (0.01) n=9	-0.01 (0.01) n=9	-0.02 (0.01) n=9	-0.01 (0.01) n=9
20	0.03 (0.01) n=9	-0.01 (0.02) n=9	-0.04 (0.01) n=9	-0.04 (0.02) n=9

Values are Mean (SE). Values are corrected for 15 weeks post-oophorectomy. Time is weeks post-oophorectomy. DHT (80mg/kg bw) was administered at 15 weeks post-oophorectomy and Estradiol (20mg/kg bw) was administered at 19 weeks post-oophorectomy.

6.3.1.6 Serum Creatinine and Body Weight

Serum creatinine was unaffected by DHT or estradiol treatment at any time point (Figure 6.5). Body weight was increased following DHT treatment at all time points ($P<0.01$ week 17, $P<0.005$ week 19, $P<0.001$ week 20 post-oophorectomy) but was unaffected by estradiol treatment (Figure 6.6). There was no correlation between serum creatinine and body weight ($r^2= 0.018$) (Data not shown).

6.3.1.7 Serum Albumin and Total Protein

Serum albumin was decreased by DHT treatment at 20 weeks post-oophorectomy ($P<0.001$) but was unaffected by estradiol treatment (Table 6.9). Serum total protein was not affected by either DHT or estradiol treatment (Table 6.10).

6.3.1.8 Serum Electrolytes

Serum electrolytes are presented in Appendices D.20 to D.29.

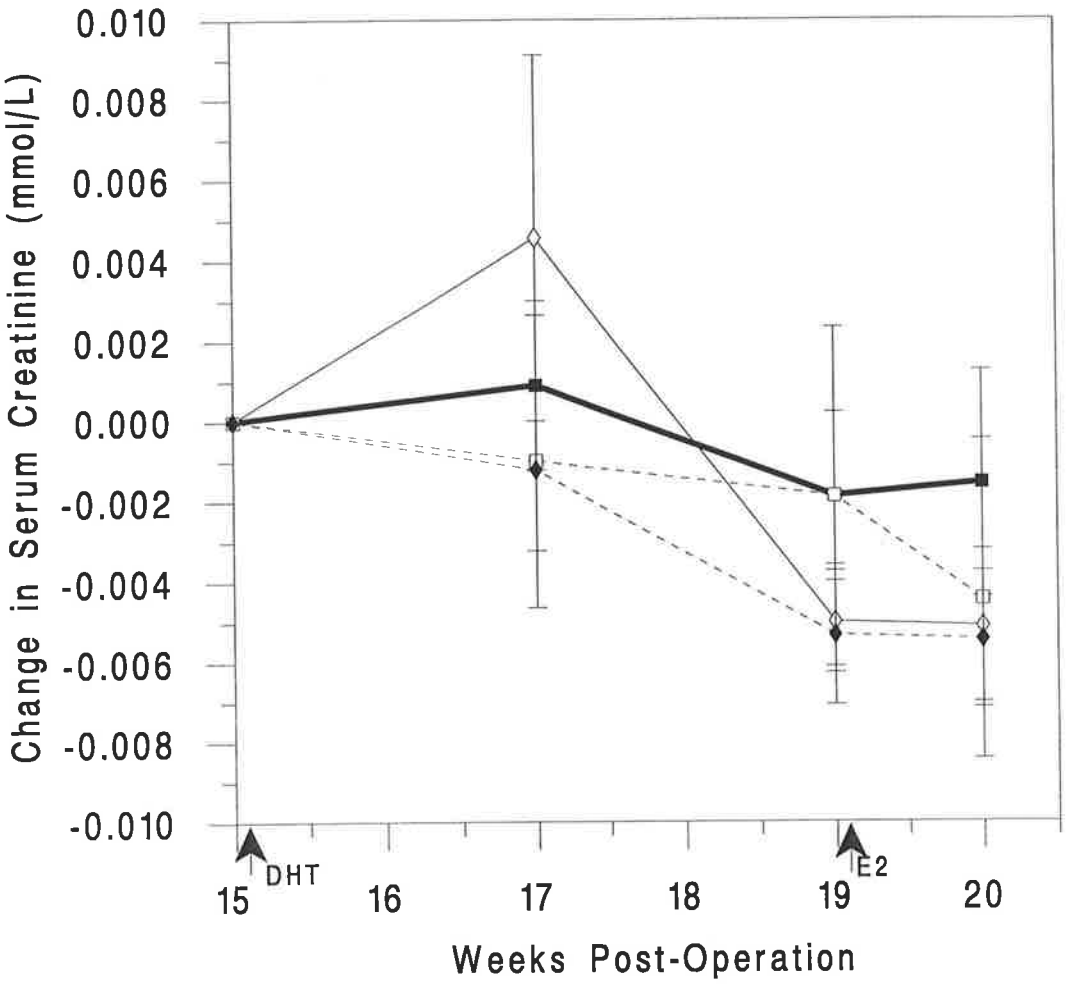


Figure 6.5 Change in serum creatinine (mmol/L) from 15 weeks post-oophorectomy in osteopenic oophx rats following DHT (80mg/kg body weight) administration at 15 following oophorectomy and estradiol (20mg/kg body weight) administration at 19 weeks following oophorectomy. Values are Mean ± SE, n=9. (■) Vehicle + Vehicle, (□) Vehicle + Estradiol, (◊) DHT + Vehicle, (◆) DHT + Estradiol.

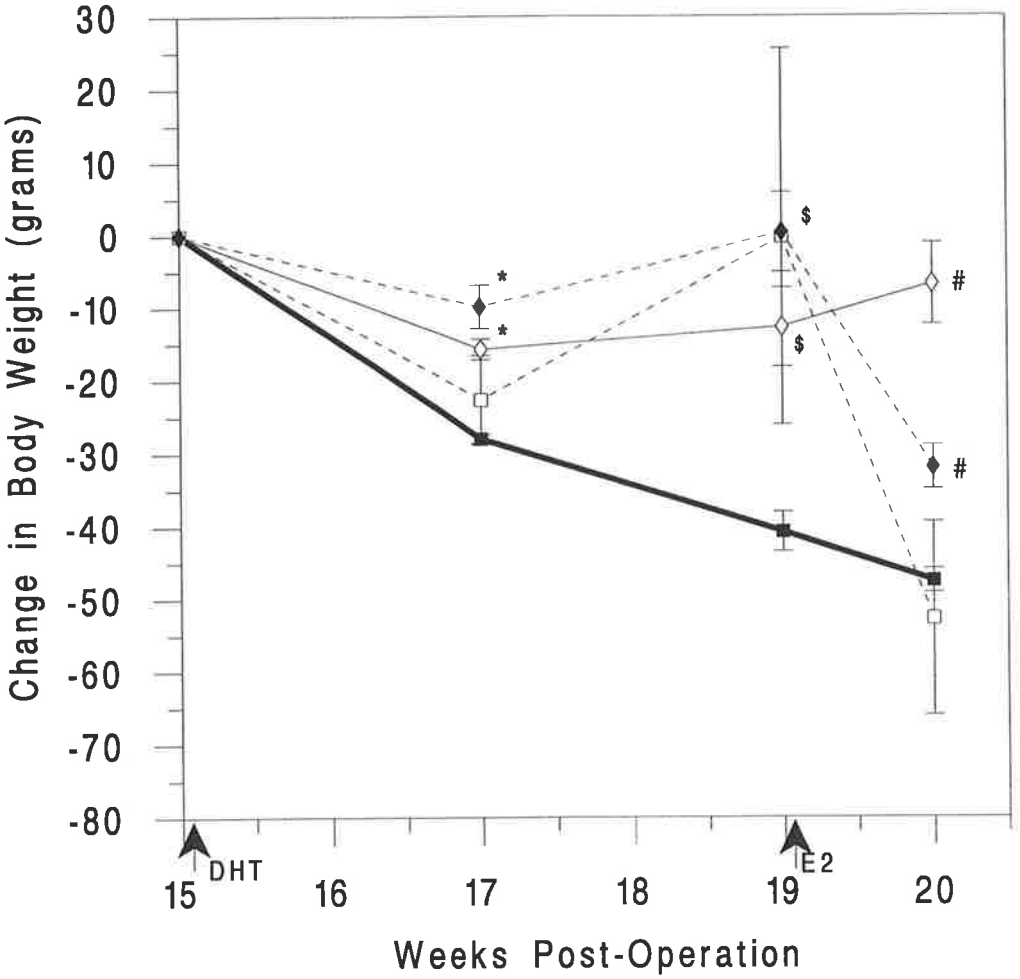


Figure 6.6 Change in body weight (grams) from 15 weeks post-oophorectomy in osteopenic oophx rats following DHT (80mg/kg body weight) administration at 15 following oophorectomy and estradiol (20mg/kg body weight) administration at 19 weeks following oophorectomy. Values are Mean ± SE, n=9. (■) Vehicle + Vehicle, (□) Vehicle + Estradiol, (◊) DHT + Vehicle, (◆) DHT + Estradiol. $^{*}P<0.01$ versus Vehicle + Vehicle and versus Vehicle + Estradiol, $^{\$}P<0.005$ versus Vehicle + Vehicle and versus Vehicle + Estradiol, $^{\#}P<0.001$ versus Vehicle + Vehicle and versus Vehicle + Estradiol.

Table 6.9 Change in serum albumin (mmol/L) from 15 weeks post-oophorectomy in osteopenic oophorectomised rats following DHT administration at 15 weeks post-oophorectomy and estradiol administration at 19 weeks post-oophorectomy.

Time (Weeks)	Vehicle + Vehicle	Vehicle + Estradiol	DHT + Vehicle	DHT + Estradiol
17	-3.9 (0.6) n=9	-2.8 (1.3) n=9	-5.3 (1.8) n=9	-4.2 (0.9) n=9
19	-3.7 (0.7) n=9	-2.6 (1.0) n=9	-3.6 (1.5) n=9	-2.78 (0.9) n=9
20	-3.2 (0.9) n=9	-1.2 (1.6) n=9	-6.6 (1.4)[a] n=9	-5.7 (0.9)[a] n=9

Values are Mean (SE). Values are corrected for 15 weeks post-oophorectomy. Time is weeks post-oophorectomy. DHT (80mg/kg bw) was administered at 15 weeks post-oophorectomy and Estradiol (20mg/kg bw) was administered at 19 weeks post-oophorectomy. [a]$P<0.001$ versus Vehicle + Vehicle and versus Vehicle + Estradiol.

Table 6.10 Change in serum total protein (mmol/L) from 15 weeks post-oophorectomy in osteopenic oophorectomised rats following DHT administration at 15 weeks post-oophorectomy and estradiol administration at 19 weeks post-oophorectomy.

Time (Weeks)	Vehicle + Vehicle	Vehicle + Estradiol	DHT + Vehicle	DHT + Estradiol
17	-2.6 (1.4) n=9	-5.0 (1.6) n=9	-2.9 (2.3) n=9	-7.4 (2.2) n=9
19	-3.6 (1.8) n=9	-4.3 (2.1) n=9	-5.1 (2.9) n=9	-4.4 (2.5) n=9
20	-5.0 (2.3) n=9	-6.2 (2.7) n=9	-9.3 (2.9) n=9	-10.2 (2.5) n=9

Values are Mean (SE). Values are corrected for 15 weeks post-oophorectomy. Time is weeks post-oophorectomy. DHT (80mg/kg bw) was administered at 15 weeks post-oophorectomy and Estradiol (20mg/kg bw) was administered at 19 weeks post-oophorectomy.

6.3.2 mRNA Levels of Osteoblast and Osteoclast Genes

The signals obtained from the ImageQuant program (Chapter 2.7.4.4) for the mRNA levels of the osteoclast and osteoblast genes from each Northern blot and Southern blot analyses are presented in Appendix D.19.

6.3.2.1 Effect of DHT Treatment

The mRNA levels of type 1α collagen, alkaline phosphatase, osteopontin, osteocalcin and calcitonin receptor were unaffected by DHT treatment (Figure 6.7, Figure 6.8, Figure 6.9, Figure 6.10).

6.3.2.2 Effect of Estradiol Treatment

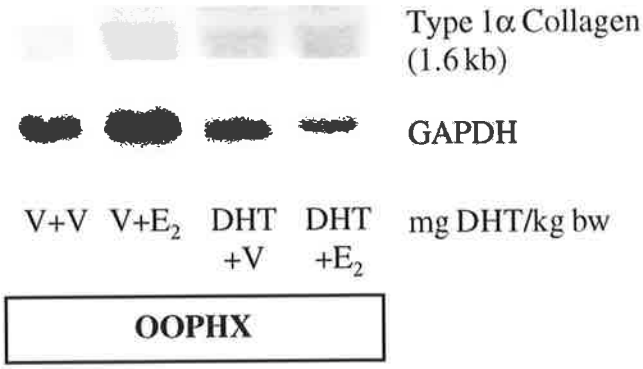
Estradiol treatment increased the mRNA levels of type 1α collagen ($P<0.05$) by 2 fold (Figure 6.7). The mRNA levels of alkaline phosphatase were also increased by 2.8 fold following estradiol treatment, although this did not reach significance (Figure 6.8). The mRNA levels of osteopontin, osteocalcin and calcitonin receptor were unaffected by estradiol administration (Figure 6.9, Figure 6.10, Figure 6.11).

6.3.2.3 Effect of Combined DHT and Estradiol treatment

Alkaline phosphatase mRNA levels were increased by 2.2 fold following combined DHT and estradiol treatment ($P<0.001$) (Figure 6.8). In contrast, osteocalcin and calcitonin receptor mRNA levels were decreased by 0.4 fold ($P<0.05$) and 0.3 fold respectively by combined DHT and estradiol treatment (Figure 6.10, Figure 6.11). Type 1α collagen and osteopontin mRNA levels were unaffected by combined treatment (Figure 6.7, Figure 6.9).

Figure 6.7 A: Representation of Northern Blot analysis for type 1α collagen mRNA and GAPDH mRNA. **B:** Type 1α collagen receptor mRNA levels corrected for GAPDH and expressed as a fold increase relative to vehicle in osteopenic oophorectomised rats. DHT (80mg/kg body weight) was administered at 15 weeks post-oophorectomy and Estradiol (20mg/kg bw) was administered at 19 weeks post-oophorectomy. Values are Mean ± SE. *P<0.05 versus Vehicle + Vehicle.

A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GAPDH
V+V
V+E2
DHT +V
DHT +E2
mg DHT/kg bw
OOPHX

B

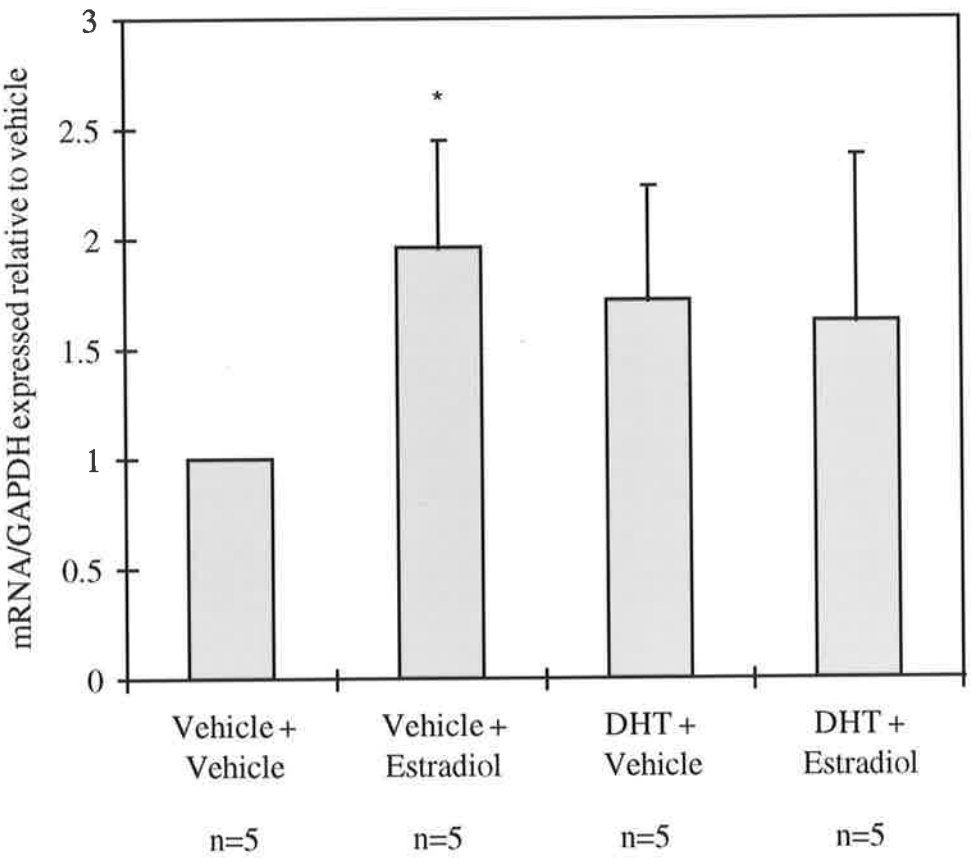
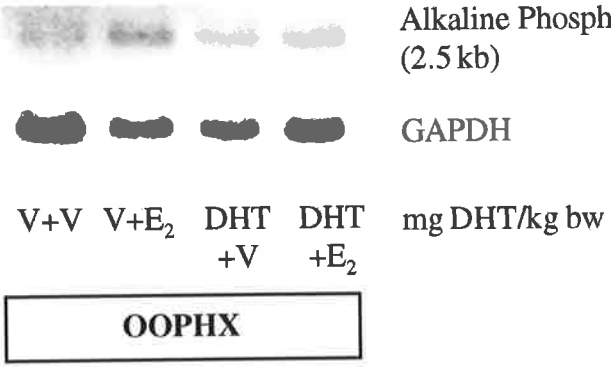

mRNA/GAPDH expressed relative to vehicle
3
2.5
2
1.5
1
0.5
0
*
Vehicle + Vehicle
Vehicle + Estradiol
DHT + Vehicle
DHT + Estradiol
n=5
n=5
n=5
n=5

Figure 6.8 A: Representation of Northern Blot analysis for alkaline phosphatase mRNA and GAPDH mRNA. **B:** Alkaline phosphatase receptor mRNA levels corrected for GAPDH and expressed as a fold increase relative to vehicle in osteopenic oophorectomised rats. DHT (80mg/kg body weight) was administered at 15 weeks post-oophorectomy and Estradiol (20mg/kg body weight) was administered at 19 weeks post-oophorectomy. Values are Mean ± SE. *P<0.001 versus Vehicle + Vehicle.

A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(2.5 kb)
GAPDH
V+V
V+E2
DHT +V
DHT +E2
mg DHT/kg bw
OOPHX

B

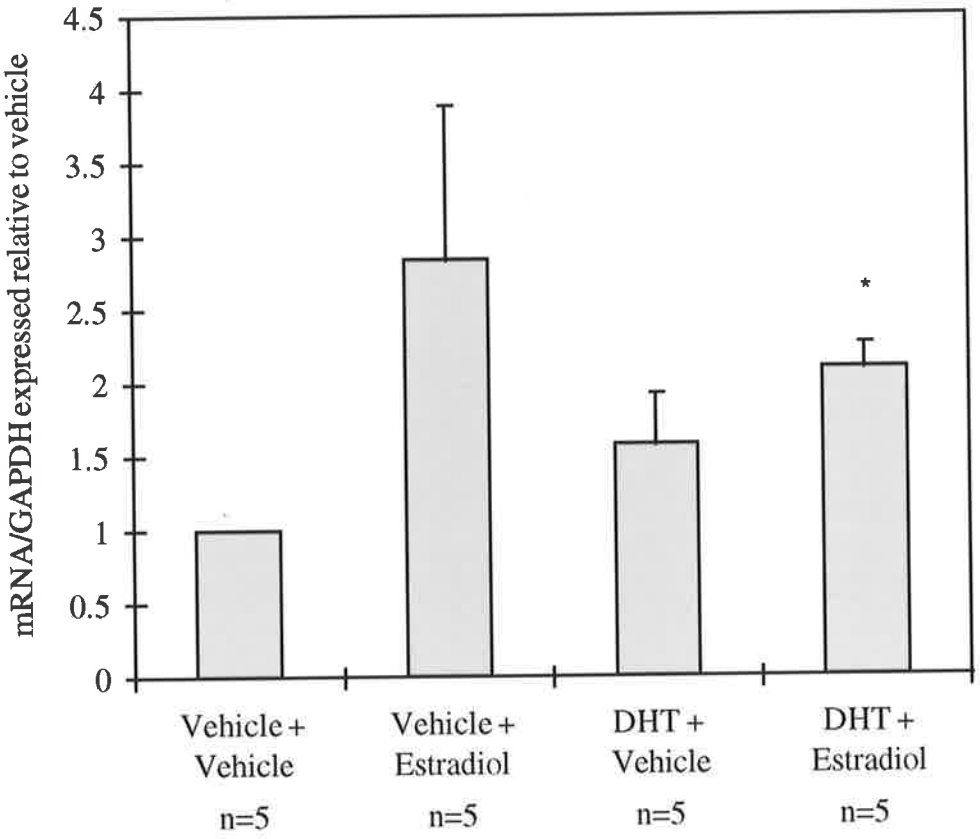
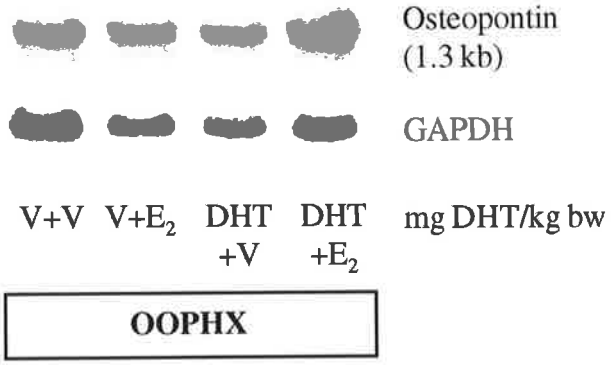

mRNA/GAPDH expressed relative to vehicle
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
*
Vehicle + Vehicle
n=5
Vehicle + Estradiol
n=5
DHT + Vehicle
n=5
DHT + Estradiol
n=5

Figure 6.9 **A:** Representation of Northern Blot analysis for osteopontin mRNA and GAPDH mRNA. **B:** Osteopontin mRNA levels corrected for GAPDH and expressed as a fold increase relative to vehicle in osteopenic oophorectomised rats. DHT (80mg/kg body weight) was administered at 15 weeks post-oophorectomy and Estradiol (20mg/kg body weight) was administered at 19 weeks post-oophorectomy. Values are Mean ± SE.

A


Osteopontin
(1.3 kb)
GAPDH
V+V
V+E2
DHT +V
DHT +E2
mg DHT/kg bw
OOPHX

B

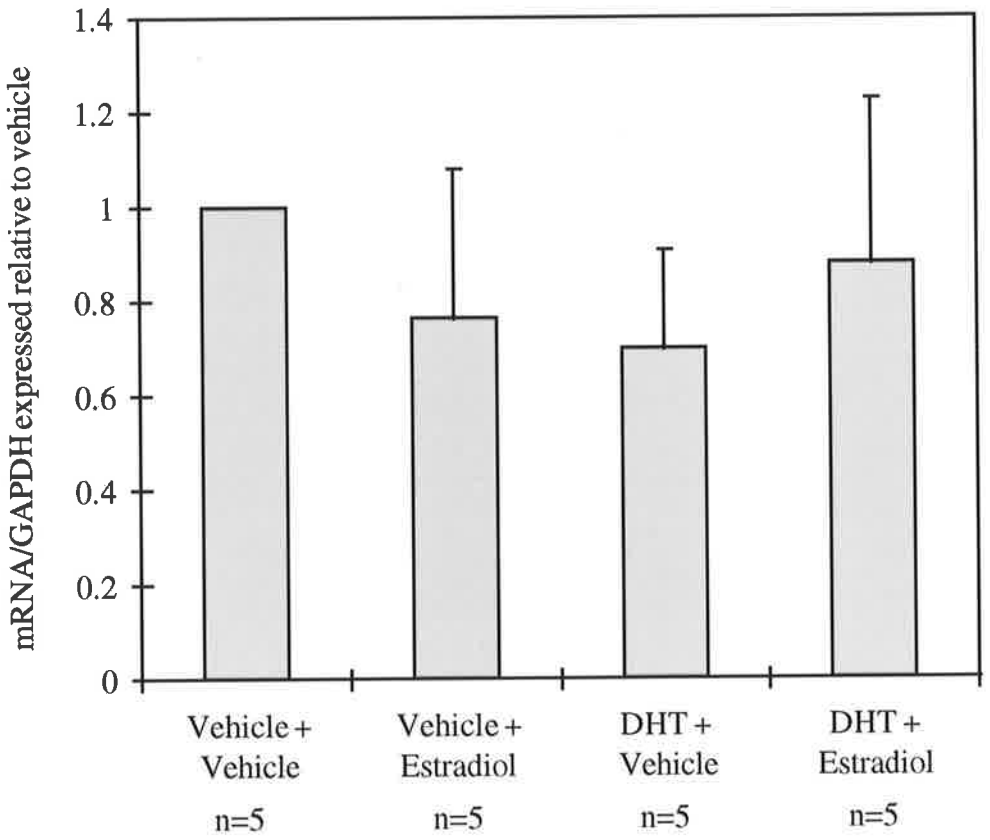
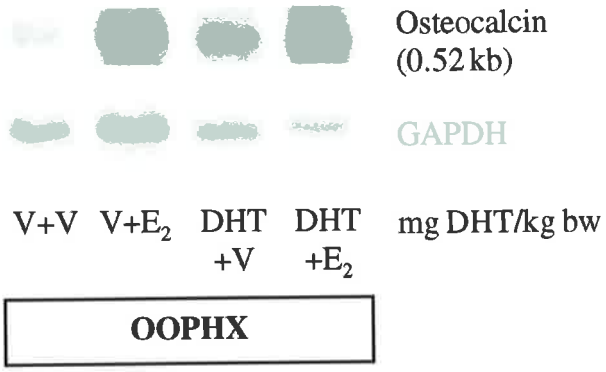

mRNA/GAPDH expressed relative to vehicle
1.4
1.2
1
0.8
0.6
0.4
0.2
0
Vehicle + Vehicle
n=5
Vehicle + Estradiol
n=5
DHT + Vehicle
n=5
DHT + Estradiol
n=5

Figure 6.10 A: Representation of Northern Blot analysis for osteocalcin mRNA and GAPDH mRNA. **B:** Osteocalcin mRNA levels corrected for GAPDH and expressed as a fold increase relative to vehicle in osteopenic oophorectomised rats. DHT (80mg/kg bw) was administered at 15 weeks post-oophorectomy and Estradiol (20mg/kg bw) was administered at 19 weeks post-oophorectomy. Values are Mean ± SE. $^{*}P<0.05$ versus Vehicle + Vehicle, $^{\#}P<0.05$ versus DHT + Vehicle.

A


Osteocalcin
(0.52 kb)
GAPDH
V+V
V+E2
DHT +V
DHT +E2
mg DHT/kg bw
OOPHX

B

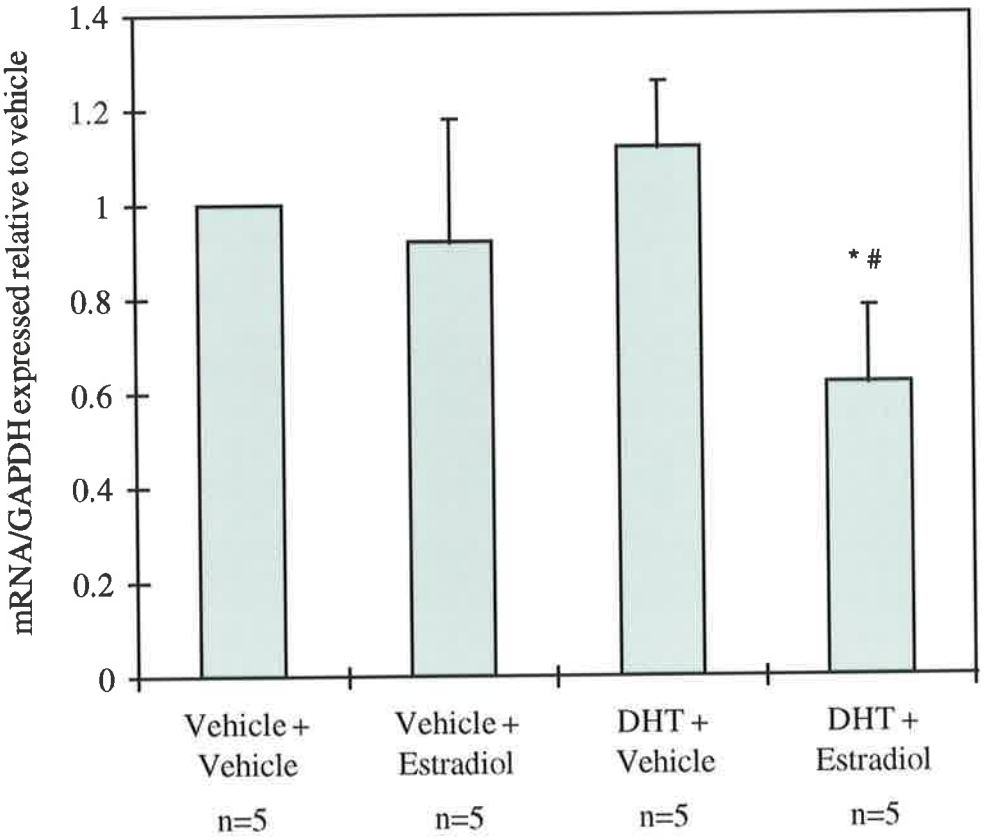
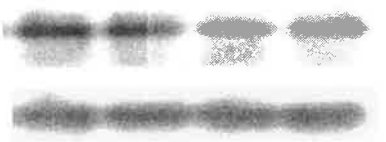

mRNA/GAPDH expressed relative to vehicle
1.4
1.2
1
0.8
0.6
0.4
0.2
0
* #
Vehicle + Vehicle
Vehicle + Estradiol
DHT + Vehicle
DHT + Estradiol
n=5
n=5
n=5
n=5

Figure 6.11 **A:** Representation of Southern Blot analysis of RT-PCR products for calcitonin receptor (CTR) mRNA and GAPDH mRNA. **B:** Calcitonin receptor mRNA levels corrected for GAPDH and expressed as a fold increase relative to vehicle in osteopenic oophorectomised rats. DHT (80mg/kg body weight) was administered at 15 weeks post-oophorectomy and Estradiol (20mg/kg body weight) was administered at 19 weeks post-oophorectomy. Bars represent mean values, (◆) data points contributing to mean (n=2).

A



Calcitonin Receptor (437 bp)

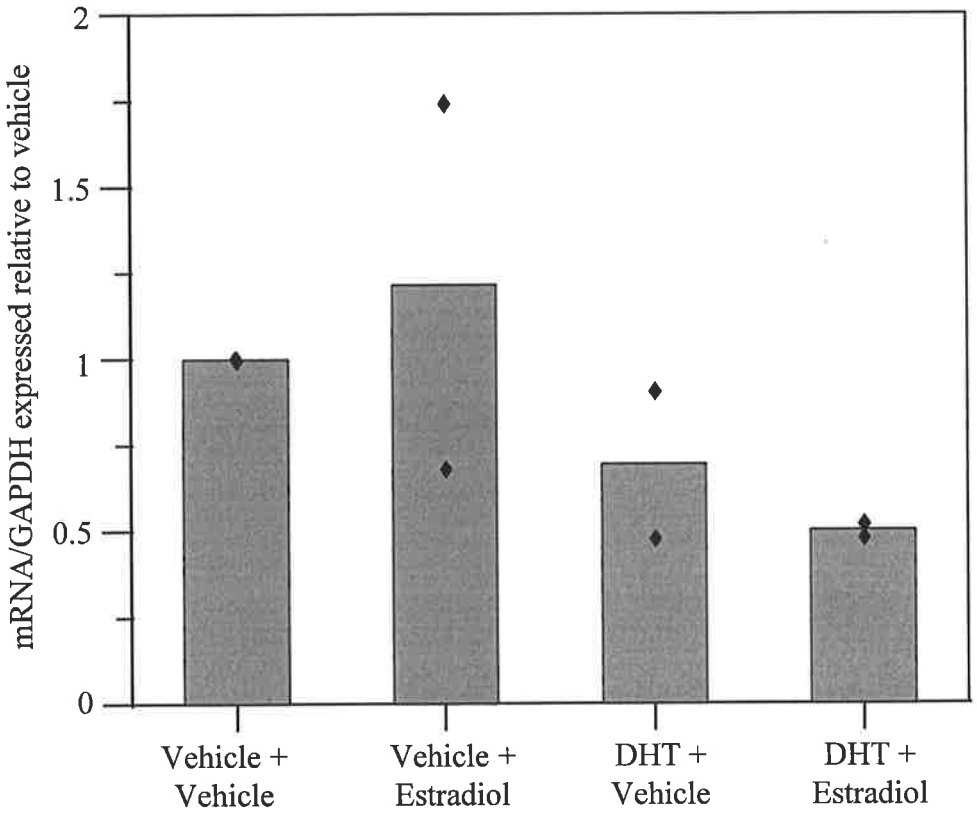
GAPDH

V+V V+E_2 DHT +V DHT +E_2 -ve

mg DHT/kg bw

OOPHX

B



6.4 DISCUSSION

In the present study, at 7 days following treatment with 20mg/kg body weight estradiol administered by silastic tubing implants, the serum levels of estradiol observed were almost identical to the serum levels of estradiol observed at 2 hours following a subcutaneous injection of 20μg/kg body weight estradiol (Sims et al 1996b). An advantage of using silastic implants as a means of administering estradiol, is that supraphysiological levels of estradiol are maintained at a constant level, while in comparison at 24 hours following subcutaneous injection of estradiol, the circulating levels of estradiol fall to below that of control animals (Sims et al 1996b).

DHT treatment increased serum alkaline phosphatase levels in osteopenic oophx rats which suggests an increase in bone formation at the level of matrix development and maturation and is consistent with findings reported in Chapter 4. Estradiol treatment had no effect on the serum levels of alkaline phosphatase at 7 days post-treatment. In contrast, previous studies have demonstrated a suppression in alkaline phosphatase from 6 days post-treatment (Sims et al 1996b). It is of interest however, that the levels of alkaline phosphatase in the rats receiving a combined treatment of DHT and estradiol were elevated in comparison to rats receiving DHT treatment alone. These data suggest that DHT and estradiol act synergistically to stimulate bone formation at the level of matrix development and maturation as indicated by alkaline phosphatase. It has been proposed that alkaline phosphatase is correlated to the extent of active bone forming surface as indicated by the extent of double-labelled flurochrome surface (Sims et al 1996a). Consistent with this theory is the increased double-labelled flurochrome surface observed in the tibiae of

osteopenic oophx rats following combined androgen and estrogen treatment (Coxam et al 1996).

Urine deoxypyridinoline and serum osteocalcin were decreased following DHT treatment which is associated with an anti-resorptive action of DHT and possibly an inhibitory effect of DHT on mineralisation in osteopenic oophx rats, which is consistent with the effects of DHT reported previously (Chapter 4). If in fact osteocalcin is an inhibitor of bone formation, as suggested by the development of the osteocalcin-deficient mouse (Ducy et al 1996), the suppression of serum osteocalcin following DHT treatment may suggest a stimulation of bone formation, however this requires further investigation.

It is well established that estradiol is a potent inhibitor of bone resorption as indicated by histomorphometric techniques (Wronski et al 1988) and by measurement of bone biochemical variables (Frolik et al 1996, Kalu et al 1991b, Sims et al 1996b). In contrast, urine deoxypyridinoline and osteocalcin were unaffected by estradiol following one week of treatment in the present study. Decreases in urine pyridinoline and urine hydroxyproline have been observed following estradiol administration in oophx rats by 6 and 21 days post-treatment respectively (Sims et al 1996b, Frolik et al 1996). Furthermore, previous studies have identified transient increases in osteocalcin at 6 and 9 days post-treatment (Sims et al 1996b) followed by a suppression from 14 days post-treatment (Sims et al 1996b, Frolik et al 1996). The failure to detect these changes following estradiol treatment in the present study may be due to the method of estradiol administration. The serum levels of estradiol achieved in the present study by silastic implants, although comparable to levels achieved in a previous study (Sims et al 1996b), may not have been achieved as rapidly in

comparison to administration by daily subcutaneous injection. 7 days of treatment with estradiol in the present study therefore may not be of sufficient duration to observe significant changes in the biochemical markers of bone cell metabolism.

Urinary calcium excretion was decreased following DHT treatment which was associated with an increase in the tubular reabsorption of calcium at the kidney. These data are consistent with the effects of DHT in osteopenic oophx rats reported previously (Chapter 4) indicating that androgens have a direct effect on the kidney to conserve calcium. Treatment with estradiol over this time period however, had no effect on the renal handling of calcium. Furthermore, the combined treatment of DHT and estradiol had no additional effect of the renal handling of calcium compared with DHT treatment alone, suggesting that the effect of androgens predominate at the level of the kidney. Alternatively, 7 days of estradiol treatment was perhaps not of sufficient duration to observe these expected changes as estrogen therapy in post-menopausal women stimulates the tubular reabsorption of calcium at the kidney resulting in decreased excretion of calcium in the urine (Lindsay 1988, Adami et al 1992).

Ionised calcium was decreased following 5 weeks of DHT treatment (20 weeks post-oophorectomy) which may reflect the increased incorporation of calcium into bone as a result of increased bone formation. Since bone formation, as indicated by serum alkaline phosphatase, was elevated from 2 weeks post-treatment, this suggests the increased requirement for calcium incorporation into bone was not reflected in the ionised calcium until 5 weeks post-treatment. In addition, DHT treatment decreased the serum levels of ultrafiltrable calcium, total calcium and protein bound calcium which was accompanied by

a decrease in albumin levels. These changes in the serum calcium fractions have not been observed previously following DHT treatment in osteopenic oophx rats (Chapter 4) and were not affected by estradiol treatment.

DHT treatment increased body weight in the osteopenic oophx rats throughout the experiment. This increase was not correlated with serum creatinine, suggesting that the weight gain was not due to increased muscle production by DHT but may be due to increased production of fat tissue. The increase in body weight by DHT treatment may act to provide a partial protective effect on the skeleton (Wronksi et al 1987). Estradiol treatment in the present study had no effect on body weight, although estradiol has been demonstrated to reverse the increase in body weight associated with oophorectomy (Wronski et al 1988, Kalu et al 1991b, Shen et al 1992, Turner et al 1993). These data indicate that the effect of DHT to increase body weight is predominant in the presence of estrogen.

DHT administration had no effect on the mRNA levels of the osteoblast and osteoclast genes following 5 weeks of treatment in osteopenic oophx rats. In contrast, at 14 weeks post-treatment, DHT suppressed the mRNA levels of the osteoblast genes type 1α collagen and alkaline phosphatase and increased the mRNA levels of the osteoclast gene, calcitonin receptor (Chapter 5). Presumably this time period was not of sufficient duration to observe significant changes in the mRNA levels of these genes.

Treatment of osteopenic oophx rats with estradiol increased the mRNA levels of type 1α collagen which suggests that estradiol stimulates the osteoblast at the stage of late

proliferation and early differentiation (Owen et al 1990). Supportive evidence for this theory is that estradiol treatment has been demonstrated to stimulate gene transcription of type 1α collagen in mature osteoblasts in culture (Ernst et al 1989). Furthermore, estradiol treatment of pure rat osteoblast-like cells in culture increases their proliferation (Ernst et al 1988) while estradiol increases creatinine kinase activity and [^{3}H]thymidine incorporation into DNA in rat epiphyseal cartilage cells (Somjen et al 1989). In contrast, estradiol inhibits the proliferation of osteoblast-like UMR106-06 cells (Gray et al 1987). UMR106-06 cells however are of immature phenotype and therefore the effect of estradiol on osteoblast proliferation may be dependent on the maturity of the osteoblast at the time of treatment.

Estradiol had no effect on the mRNA levels of alkaline phosphatase or osteopontin which are expressed during the matrix development and maturation stage of osteoblast development (Owen et al 1990). Since estradiol treatment in oophx rats suppresses the extent of double-labelled surface (Shen et al 1992, Turner et al 1993, Coxam et al 1996, Sims et al 1996b), a decrease in the mRNA levels of alkaline phosphatase and osteopontin may have been expected following estradiol treatment as the extent of double-labelled surface has been shown to correlate with serum alkaline phosphatase levels (Sims et al 1996a).

Osteocalcin mRNA levels were unaffected by estradiol administration in osteopenic oophx rats which suggests that estrogens do not effect the process of mineralisation, the stage of osteoblast development during which is it proposed osteocalcin is expressed (Owen et al 1990). Shen and colleagues (1992) demonstrated that mineral apposition rate, which has

been previously identified to be related to serum osteocalcin levels (Sims et al 1996b) was unaffected following estradiol treatment in oophx rats. In contrast however, a number of studies have identified an inhibitory effect of estradiol on mineral apposition rate in oophx rats (Turner et al 1993, Coxam et al 1996, Sims et al 1996b). Since the duration of treatment with estradiol in the present study was shorter in comparison to previous studies, this may have contributed to the differences observed. The present study clearly demonstrates that estradiol increases the mRNA levels of type 1α collagen following 7 days of treatment, however the role of estradiol on the proliferation and maturation of osteoblasts remains controversial and requires further investigation.

Estradiol suppresses osteoclastic bone resorption as indicated by decreased osteoclast surface and osteoclast number in oophx rats (Turner et al 1993, Shen et al 1992, Wronski et al 1988). In the present study however, the mRNA levels of the osteoclast gene calcitonin receptor were unaffected by estradiol treatment after 7 days. Furthermore, administration of estradiol shortly before sacrifice in oophx rats suppresses the mRNA levels of the osteoclast genes carbonic anhydrase and tartrate-resistant acid phosphatase in oophx rats (Zheng et al 1994). It is difficult however to make direct comparisons with this study as estradiol was administered as a bolus dose of 20μg of estradiol and the significant suppression of the mRNA levels of carbonic anhydrase II and tartrate-resistant acid phosphatase were only observed at 18 hours post-treatment (Zheng et al 1994).

Although DHT treatment and estradiol treatment alone had no effect on the mRNA levels of alkaline phosphatase, osteocalcin and calcitonin receptor, the combined treatment of DHT and estradiol in osteopenic oophx rats increased the mRNA levels of alkaline

phosphatase while suppressing the mRNA levels of calcitonin receptor and osteocalcin. These data suggest that DHT in combination with estradiol can inhibit bone resorption, as indicated by the mRNA levels of calcitonin receptor, while stimulating bone formation at the stage of matrix development and maturation as indicated by alkaline phosphatase mRNA levels. In post-menopausal women, the addition of an oral androgen to estrogen therapy can overcome the suppressive effects of estrogen on bone formation as indicated by increased lumbar bone mineral content (Erdstieck et al 1994) and by biochemical markers of bone metabolism (Raisz et al 1996). Furthermore, the combination of androgen and estrogen treatment in oophx rats has been demonstrated to have a beneficial effect on bone density which was reflected in an increase in mineral apposition rate and bone formation rate (Coxam et al 1996). Interestingly, it appears that DHT was less able to overcome the suppressive effects of estrogen on bone formation in trabecular bone than in cortical bone. Although osteocalcin mRNA levels in the present study were significantly suppressed following combined DHT and estradiol treatment, this however may not reflect a suppression of bone formation at the level of mineralisation. The mutation of the osteocalcin gene in rats decreases bone resorption with no effect on mineralisation, suggesting that in fact, osteocalcin inhibits bone formation and may act as a termination signal for the bone formation process (Ducy et al 1996).

This study confirms that DHT administration to osteopenic oophx rats increases bone formation as indicated by alkaline phosphatase and inhibits bone resorption as indicated by urine deoxypyridinoline. Furthermore, DHT treatment suppresses osteocalcin synthesis and conserves calcium at the level of the kidney in osteopenic oophx rats. In contrast, treatment with estradiol for 7 days had no effect on the biochemical markers of bone

turnover. Combined DHT and estradiol treatment increased the serum levels of alkaline phosphatase to a greater extent than observed in rats treated with DHT alone, suggesting a synergistic action of estrogen and androgens on bone formation at the level of matrix development and maturation. Estradiol treatment increased the mRNA levels of type 1α collagen suggesting that estrogens stimulate osteoblast proliferation. Furthermore, the results from this study provide insight to the action of combined of DHT and estradiol treatment at the level of gene expression. Combined DHT and estradiol treatment increased alkaline phosphatase mRNA levels while suppressing osteocalcin and calcitonin receptor mRNA levels. These data suggest that combined androgen and estrogen treatment stimulate osteoblast development at the level of matrix development and maturation and suppress osteoclastic bone resorption. The inhibition of osteocalcin mRNA levels following combined treatment may reflect a suppression of mineralisation (Owen et al 1990) or an increase in bone formation (Ducy et al 1996). A study of longer duration than the present study is required to determine whether the effects of combined DHT and estradiol treatment on the biochemical variables and bone cell gene expression are maintained.

CHAPTER 7

SUMMARY AND CONCLUSIONS

Utilising the model of gene expression during the three stages of osteoblast development proposed by Owen and colleagues (1990) can provide insight into the mechanism by which androgens and estrogens affect the process of bone formation. Furthermore, it may provide some insight into the coupling between the processes of bone resorption and bone formation.

7.1 The Effect of Oophorectomy on Bone Cell Metabolism

The present study demonstrates for the first time that osteoblast and osteoclast gene expression were markedly elevated at 8 weeks post-oophorectomy. Oophorectomy increased the mRNA levels of the osteoblast genes type 1α collagen, expressed during late proliferation, alkaline phosphatase and osteopontin, expressed during matrix development and maturation and osteocalcin expressed during mineralisation (Figure 7.1). Associated with these changes in gene expression following oophorectomy was an increase in the biochemical markers of bone resorption, urine deoxypyridinoline and hydroxyproline and the biochemical markers of bone formation, serum alkaline phosphatase and osteocalcin which is consistent with the increased bone turnover and subsequent bone loss observed

following oophorectomy (Ismail et al 1988, Kalu 1991a, Morris et al 1992, Sims et al 1996a).

The oophorectomy-induced stimulation of bone formation associated with the increased osteoclastic bone resorption as indicated by increased calcitonin receptor mRNA levels, resulted in the complete stimulation of the osteoblast at all stages of differentiation (Figure 7.1). The stimulation of osteoblast development in estrogen deficiency probably originates at the level of proliferation when c-*fos* is expressed, as the mRNA levels of c-*myc*, a gene expressed concurrently with the expression of c-*fos* is elevated following oophorectomy (Salih et al 1993). Since c-*myc* increased soon after oophorectomy and c-*fos* and c-*myc* have short half-lives, the increase in c-*fos* mRNA levels following estrogen deficiency may have already occurred by 8 weeks post-oophorectomy in the present study. These data are consistent with a model whereby estrogen deficiency stimulates osteoblast development by stimulating osteoclasts and bone resorption. This process in turn stimulates osteoblasts at the level of proliferation which subsequently results in a cascade of stimulation of osteoblast differentiation which is reflected in the increase in the mRNA levels of the genes expressed at each of these stages.

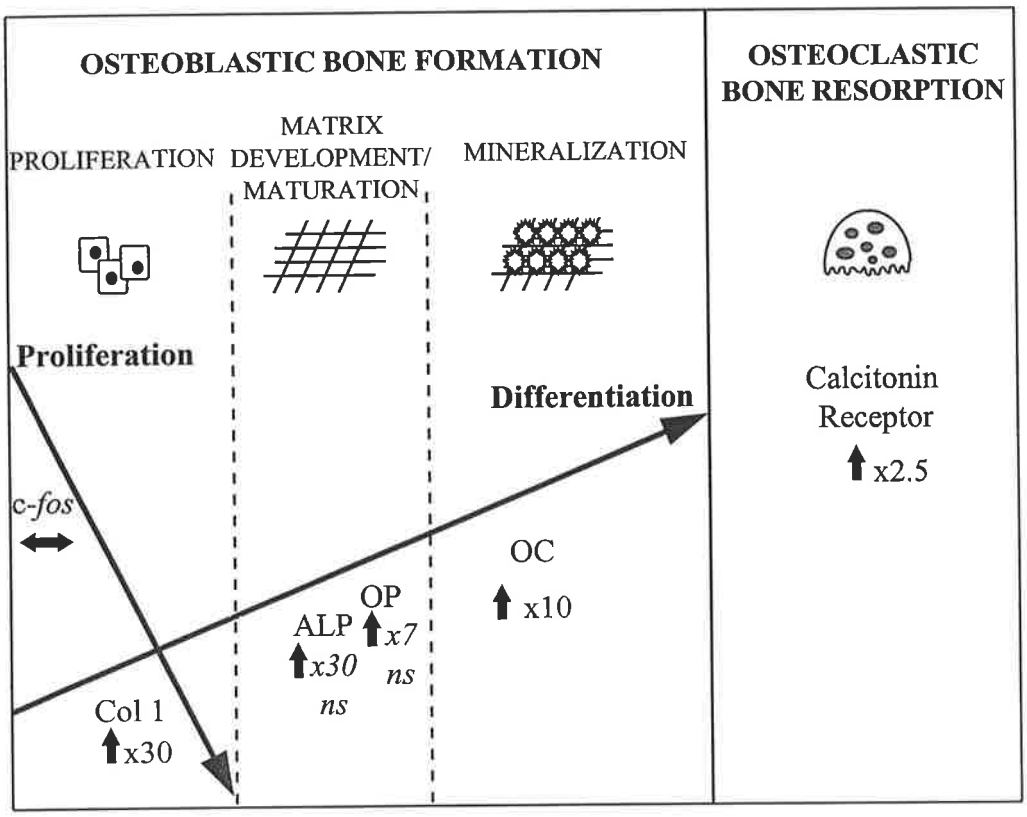


Figure 7.1 Osteoblast and osteoclast gene expression in oophorectomised rats at 8 weeks post-operation. The proposed model of gene expression during osteoblast development is from Owen and colleagues 1990. (↔) no change, (↑) increase and (↓) decrease. 'x' refers to fold increase relative to sham rats receiving vehicle, '*ns*' refers to changes in mRNA levels that did not reach statistical significance.

7.2 The Development of Osteopenia and Bone Cell Metabolism

The mRNA levels of the osteoblast and osteoclast genes remained elevated at 29 weeks post-oophorectomy in osteopenic rats but not to the same degree as observed at 8 weeks post-oophorectomy (Figure 7.2). The marked stimulation of the osteoblast and osteoclast genes at 8 weeks post-oophorectomy probably reflects the rapid phase of bone loss that occurs in the first 3 months following oophorectomy in the rat (Wronski et al 1989a) while the bone turnover rate at 29 weeks post-oophorectomy has stabilised these activities to similar levels observed in ovary-intact rats. This was is reflected in the slight increase in the mRNA levels of the osteoblast and osteoclast genes observed in the osteopenic oophx rats (Figure 7.2). Furthermore, the mRNA levels of c-*fos* were undetectable in the osteopenic oophx rats, an observation which is consistent with the stabilised rate of bone turnover, as c-*fos* mRNA levels in oophx rats at 8 weeks post-operation, were only detected at low levels when bone turnover is high.

7.3 DHT Administration to Estrogen Sufficient Rats

The administration of DHT to estrogen sufficient rats resulted in the complete stimulation of osteoblast differentiation (Figure 7.3). The increase in the mRNA levels of type 1α collagen, alkaline phosphatase, osteopontin and osteocalcin indicates that DHT stimulates osteoblast development at the stage of late proliferation, matrix development and maturation and mineralisation. The results from the present study do not indicate whether the stimulation of osteoblast development by DHT originates at the level of proliferation,

as no changes were observed in the mRNA levels of c-*fos* following DHT treatment. At 8 weeks post-treatment however the mRNA levels of c-*fos* may have degraded following induction as the mRNA of c-*fos* has a short half-life of no more than 15 minutes (Brawerman 1987). Since there are numerous studies which suggest that androgens stimulate proliferation of mouse (Kasperk et al 1989), rat (Somjen et al 1991) osteoblasts and osteoblast-like cells (Masuyama et al 1992) while few suggest that androgens have no effect (Bodine et al 1995), the stimulation of osteoblast bone formation by androgens most likely originates at the level of proliferation.

Since the processes of bone formation and bone resorption are tightly coupled, the increase in osteoblast bone formation may be the result or the source of an increase in bone resorption as suggested by calcitonin mRNA levels. Since androgens have been demonstrated to increase bone formation in post-menopausal women (Riggs et al 1972, Johanssen et al 1989, Need et al 1987) and in rats (Tobias et al 1994, Schot et al 1993, Coxam et al 1996) it appears likely therefore that the increase in bone resorption is a result of the DHT-stimulated osteoblast activity. Alternatively, if the primary action of DHT was to increase bone resorption in the estrogen sufficient rats, this increase in bone resorption increases bone formation at all stages of osteoblast development.

The increase in the mRNA levels of the osteoblast genes alkaline phosphatase and osteocalcin and the osteoclast gene calcitonin receptor in estrogen sufficient rats were reflected in the serum levels of these variables. Following DHT administration serum alkaline phosphatase was markedly increased and although not statistically significant, serum osteocalcin and urine deoxypyridinoline were also increased. The increase in bone

formation as indicated by serum alkaline phosphatase following DHT treatment was associated with a decrease in the serum levels of ionised calcium which probably reflects the increased incorporation of calcium into bone. In addition to the marked effects of DHT on bone cell metabolism, DHT had a direct effect on the kidney to conserve calcium which was reflected in a decrease in the excretion of calcium in the urine.

7.4 DHT Administration to Oophorectomised Rats

DHT treatment in oophx rats immediately following operation suppressed the oophorectomy-induced stimulation of calcitonin receptor, type 1α collagen and osteocalcin mRNA levels (Figure 7.4). These data are consistent with a model of androgens at high doses acting to inhibit bone resorption and to inhibit osteoblast bone formation at the stage of late proliferation and mineralisation. The mechanism of action of DHT in the estrogen-deficient rat when the rate of bone turnover is high, may be to inhibit bone resorption which due to the tight coupling between the processes bone resorption and formation, inhibits osteoblast bone formation at the stages of late proliferation and mineralisation. Alternatively, the primary action of DHT may be to inhibit bone formation at these stages which in turn inhibits bone resorption.

The suppression of osteocalcin mRNA levels may not necessarily reflect an inhibition of bone formation at the level of mineralisation. In osteocalcin knockout mice, cortical thickness, bone mineral matrix and bone volume were increased, suggesting that under normal conditions osteocalcin acts to limit bone formation without impairing bone

resorption or mineralisation (Ducy et al 1996). Perhaps osteocalcin acts as a termination signal for the completion of the bone formation process. In the present study therefore, the action of DHT to inhibit osteocalcin mRNA levels in the oophx rat when administered immediately following operation may actually reflect an increase in bone formation and bone volume. Supportive evidence for this theory is the increase in trabecular bone volume in osteopenic oophx rats observed following treatment with DHT (Tobias et al 1994, Coxam et al 1996) and nandrolone decanoate (Schot et al 1993). The positive effect of nandrolone decanoate on bone volume was associated with a suppression of serum osteocalcin levels and was attributed to an overall anti-resorptive effect (Schot et al 1993) which is consistent with the small non-significant decrease in the serum levels of osteocalcin and the suppression of calcitonin receptor mRNA levels observed following DHT treatment in the present study.

DHT treatment had no effect on the mRNA levels of alkaline phosphatase or osteopontin and these genes remained stimulated as a result of oophorectomy (Figure 7.4). These data indicate that DHT has no effect on osteoblast bone formation at the level of matrix development in estrogen deficient rats with a high rate of bone turnover.

The serum levels of alkaline phosphatase were increased following DHT administration in oophx rats indicating a stimulation of bone formation, however this was not reflected in the mRNA levels of this gene which remained unchanged following treatment. The absence of an increase in the mRNA levels of alkaline phosphatase associated with the stimulation of the serum levels of this enzyme may be due to a number of factors. The DHT-induced increase in serum alkaline phosphatase was of a smaller magnitude in the oophx rats

compared to the sham rats, in which an increase in the mRNA levels of alkaline phosphatase was also observed. As well, DHT could stabilise alkaline phosphatase mRNA such that more protein is translated for each transcription event. Furthermore, perhaps the changes due to DHT treatment were obscured in the oophx rats as estrogen deficiency had the most marked effect on the mRNA levels of the osteoblast and osteoclast genes.

Associated with the increase in bone formation as indicated by serum alkaline phosphatase following DHT treatment was a decrease in the serum ionised calcium levels which perhaps reflects the increased requirement for calcium incorporation into bone. The suppression of calcitonin receptor mRNA was also reflected in the biochemical markers of bone resorption urine deoxypyridinoline and hydroxyproline which were unaffected. It is difficult however to make direct comparisons between the serum and urine levels of the biochemical bone markers and the mRNA levels of these genes.

The positive effect of DHT on the kidney to conserve calcium was also observed in the estrogen deficient rats resulting in decreased urine calcium excretion.

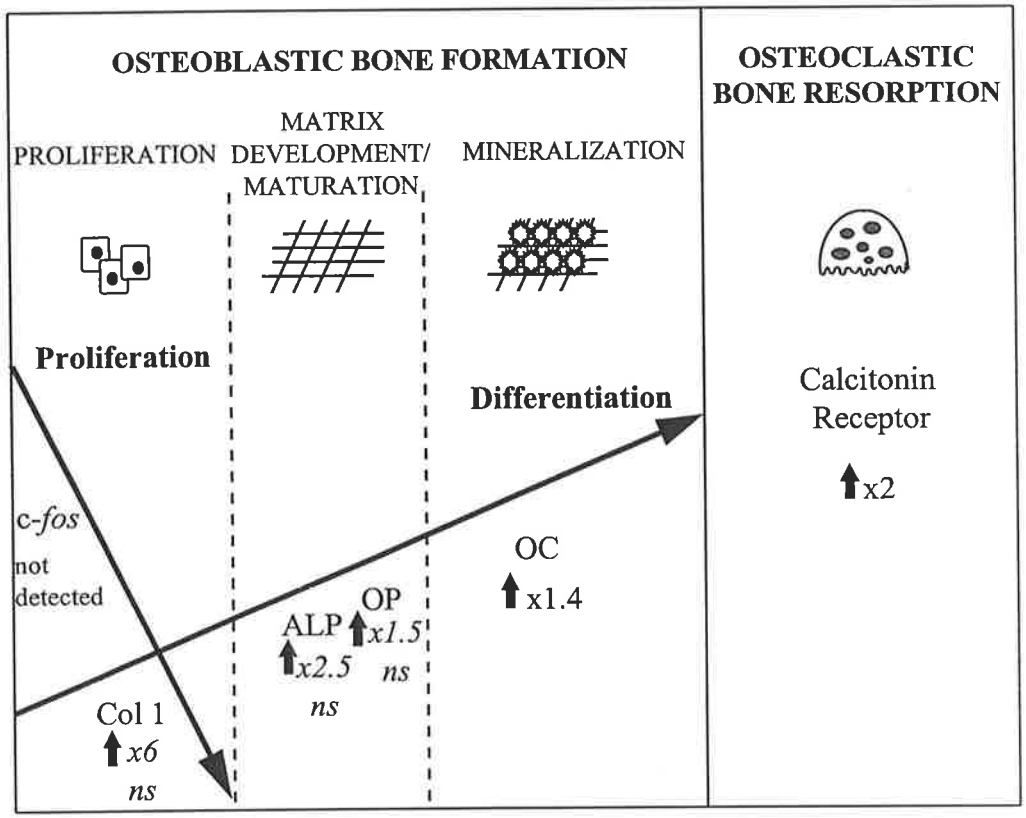


Figure 7.2 Osteoblast and osteoclast gene expression in osteopenic oophorectomised rats at 29 weeks post-operation. The proposed model of gene expression during osteoblast development is from Owen and colleagues 1990. (↔) no change, (↑) increase and (↓) decrease. 'x' refers to fold increase relative to sham rats receiving vehicle, '*ns*' refers to changes in mRNA levels that did not reach statistical significance.

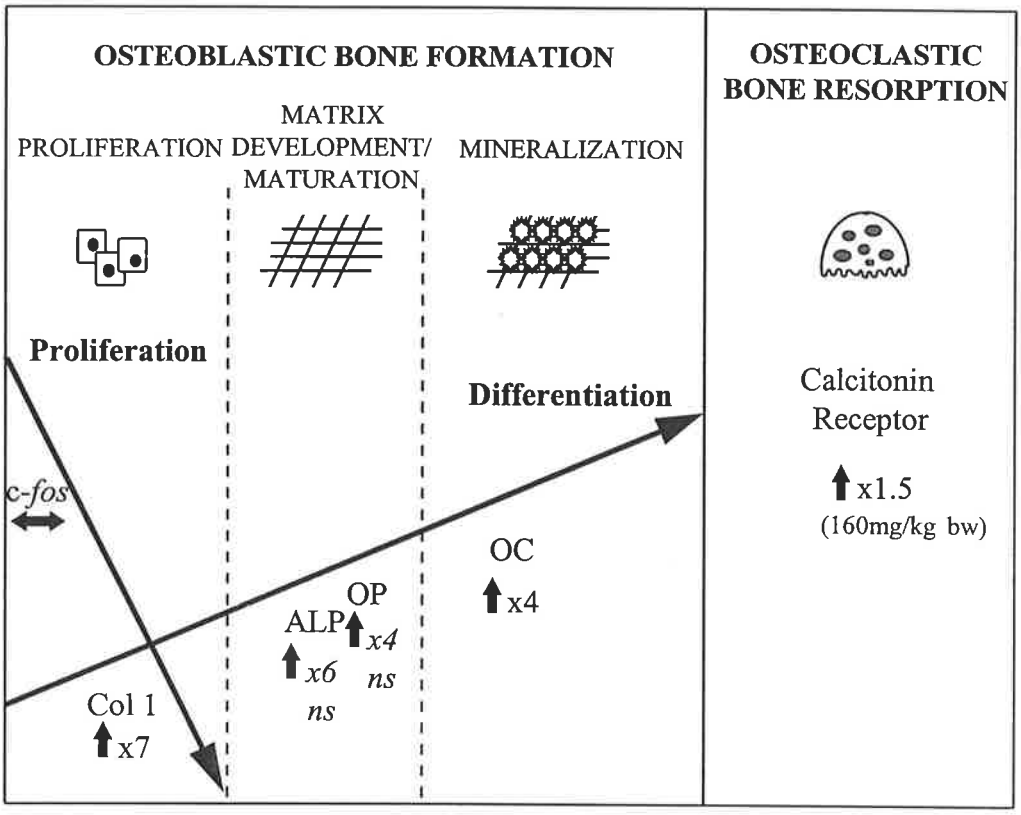


Figure 7.3 Osteoblast and osteoclast gene expression following DHT treatment in sham-operated rats at 8 weeks post-operation. DHT was administered at the time of operation. The proposed model of gene expression during osteoblast development is from Owen and colleagues 1990. (↔) no change, (↑) increase and (↓) decrease. 'x' refers to fold increase relative to sham rats receiving vehicle, '*ns*' refers to changes in mRNA levels that did not reach statistical significance.

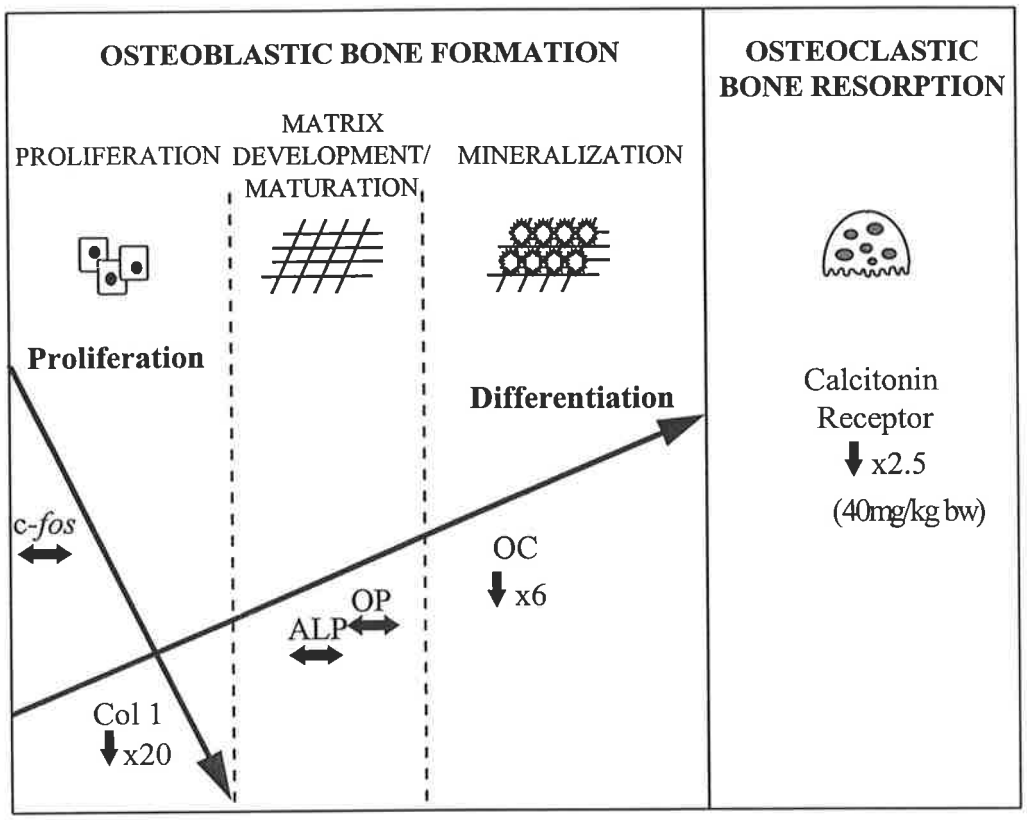


Figure 7.4 Osteoblast and osteoclast gene expression following DHT treatment in oophorectomised rats at 8 weeks post-operation. DHT was administered at the time of operation. The proposed model of gene expression during osteoblast development is from Owen and colleagues 1990. (↔) no change, (↑) increase and (↓) decrease. 'x' refers to fold increase relative to sham rats receiving vehicle.

7.5 DHT Administration to Osteopenic Oophorectomised Rats

DHT treatment in the osteopenic oophx rats resulted in a suppression of osteoblast bone formation at the stage of late proliferation, as indicated by type 1α collagen mRNA levels and at the early stages of matrix development and maturation as indicated by alkaline phosphatase mRNA levels (Figure 7.5). The administration of DHT had no effect on the later stages of osteoblast differentiation when osteopontin and osteocalcin are expressed. The effect of DHT on bone formation in estrogen deficient rats therefore may be dependent on the rate of bone turnover at the time of treatment as the rate of bone turnover in osteopenic oophx rats does not differ significantly from control rats while bone turnover at 8 weeks post-oophorectomy is high. It is of interest to note that in the osteopenic oophx rats, DHT treatment increased the serum levels of alkaline phosphatase and decreased the serum levels of osteocalcin which were not detected at the mRNA level.

Calcitonin mRNA levels was increased following DHT treatment at the highest dose (Figure 7.5) suggesting an increase in bone resorption. In contrast, DHT at high doses has been demonstrated to suppress osteoclastic bone resorption in osteopenic oophx rats as identified by histomorphometric techniques (Tobias et al 1994) and by measurement of the biochemical markers of bone turnover (Chapter 4). Perhaps the increase in calcitonin receptor mRNA levels actually reflects a suppression of bone resorption as the hormone calcitonin is a potent inhibitor of bone resorption. Since these rats have circulating calcitonin, an increase in the mRNA levels of its receptor may indicate an increase in the number of receptors available to bind calcitonin and elicit an inhibitory action on osteoclastic bone resorption.

Type 1α collagen mRNA levels were suppressed in oophorectomised rats treated with DHT immediately following oophorectomy when calcitonin receptor mRNA levels were suppressed (Figure 7.4) and in osteopenic oophx rats treated with DHT at 15 weeks post-oophorectomy when calcitonin receptor mRNA levels were stimulated (Figure 7.5). These data suggest that perhaps expression of type 1α collagen is not coupled to bone resorption as mRNA levels are not correlated with changes in bone resorption activity.

DHT treatment in osteopenic oophx rats had a positive effect on the kidney to increase the tubular reabsorption of calcium and to decrease the loss of calcium in the urine. DHT therefore has a direct positive effect on the kidney independent of estrogen status and the rate of bone turnover.

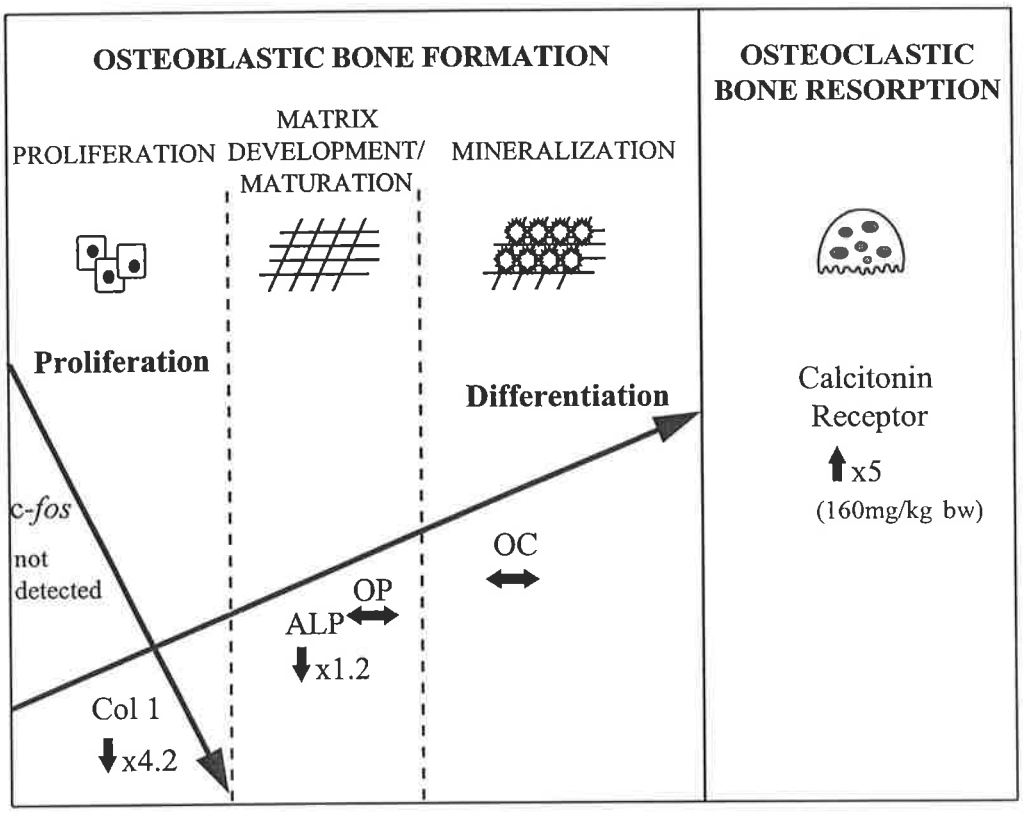


Figure 7.5 Osteoblast and osteoclast gene expression following DHT treatment in osteopenic oophorectomised rats at 29 weeks post-operation. DHT was administered at 15 weeks post-oophorectomy. The proposed model of gene expression during osteoblast development is from Owen and colleagues 1990. (↔) no change, (↑) increase and (↓) decrease. 'x' refers to fold increase relative to sham rats receiving vehicle.

7.6 Estrogen Treatment to Osteopenic Oophorectomised Rats

Estradiol replacement to osteopenic oophx rats for one week surprisingly had no effect on bone resorption as indicated by calcitonin receptor mRNA levels (Figure 7.6) and urine deoxypyridinoline and hydroxyproline excretion. In contrast, estrogen treatment in oophx rats has been demonstrated to inhibit bone resorption within 6 days (Sims et al 1996b) as identified by urine hydroxyproline excretion and to suppress carbonic anhydrase II and tartrate-resistant acid phosphatase mRNA levels at 18 hours post-treatment (Zheng et al 1994). In the present study, differences in the method of estradiol administration employed and the time course of treatment may account for these differences.

Estrogen treatment increased the mRNA levels of type 1α collagen (Figure 7.6) suggesting that estrogens stimulate proliferation of osteoblasts which is consistent with previous findings (Ernst et al 1989). Estradiol treatment however had no effect on matrix development and maturation as indicated by alkaline phosphatase and osteopontin mRNA levels or mineralisation as indicated by osteocalcin mRNA levels (Figure 7.6). The serum measurements of alkaline phosphatase and osteocalcin were also unaffected by estrogen treatment. The mRNA levels of alkaline phosphatase although not statistically significant were elevated following estradiol treatment. Estradiol has been previously demonstrated to increase alkaline phosphatase activity per cell number in osteoblast-like cells (Gray et al 1987) suggesting that estrogens may also increase bone matrix production.

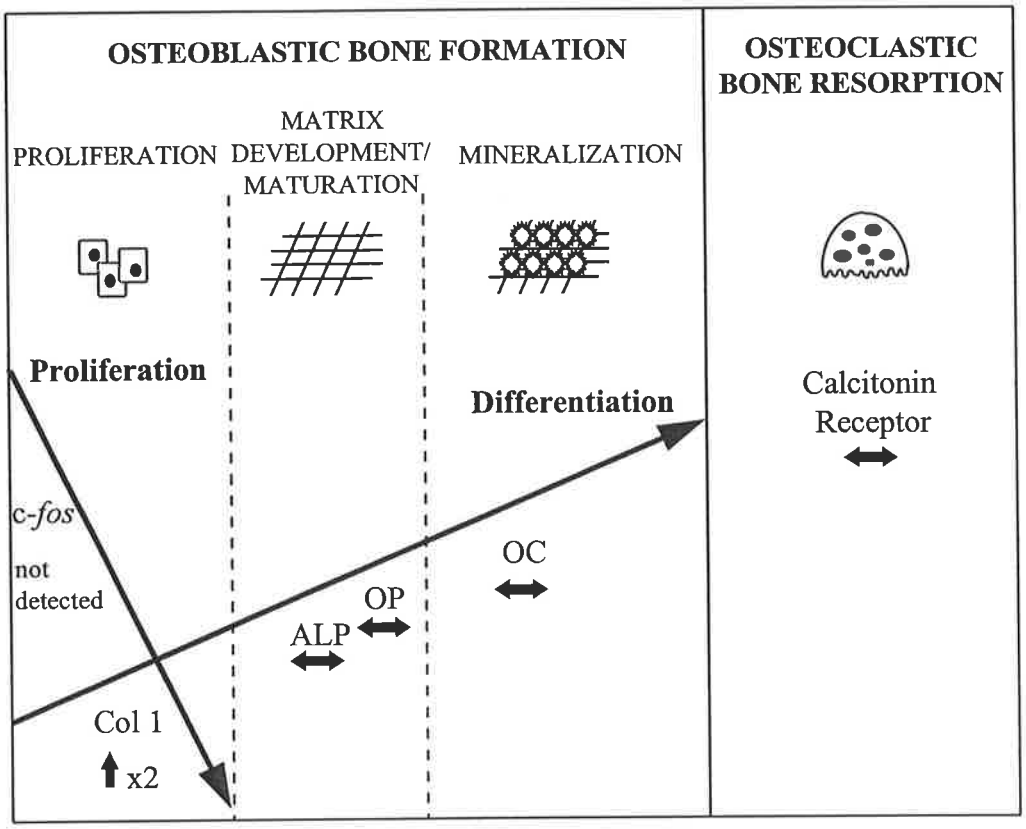


Figure 7.6 Osteoblast and osteoclast gene expression following estradiol treatment in osteopenic oophorectomised rats at 20 weeks post-operation. Estradiol was administered at 19 weeks post-oophorectomy. The proposed model of gene expression during osteoblast development is from Owen and colleagues 1990. (↔) no change, (↑) increase and (↓) decrease. 'x' refers to fold increase relative to sham rats receiving vehicle.

7.7 Combined DHT and Estrogen Treatment in Osteopenic Oophorectomised Rats

Combination treatment of DHT and estrogen resulted in a suppression of bone resorption as indicated by calcitonin receptor mRNA levels and an increase in bone formation at the stage of matrix development and maturation as indicated by alkaline phosphatase mRNA levels (Figure 7.7). These data provide further supportive evidence to the theory that androgens are able to overcome the suppressive effects of estrogens on bone formation. It has been demonstrated previously that in postmenopausal women (Erdstieck et al 1994) and in rats (Coxam et al 1996) that bone mineral content is increased following combined estrogen and androgen treatment compared to estrogen treatment alone. The combined treatment of DHT and estradiol to increase alkaline phosphatase mRNA levels was also reflected in the serum level of this variable. Estradiol treatment acted synergistically with DHT to stimulate serum levels of alkaline phosphatase to a greater degree than observed with DHT treatment alone. Increases in double-labelled fluorochorme surfaces have been observed following combined treatment of estradiol and DHT (Coxam et al 1996), a finding consistent with the increased alkaline phosphatase observed in the present study as it has been proposed that alkaline phosphatase best reflects the extent of active bone surface in oophx rats (Sims et al 1996a).

The mRNA levels of type 1α collagen were unaffected by combined DHT and estradiol treatment (Figure 7.7). This is perhaps not surprising since DHT treatment alone exerted an inhibitory action on type 1α collagen mRNA levels while estradiol treatment alone

exerted a stimulatory effect on type 1α collagen mRNA levels, resulting in no detectable effect when these treatments were combined.

Osteopontin mRNA levels were unaffected by combined DHT and estradiol treatment (Figure 7.7). It appears from the results of this study that osteopontin is not regulated by either estradiol or DHT treatment alone or in combination in estrogen deficient rats as the mRNA levels of this gene were not affected by any treatment in either oophx or osteopenic oophx rats.

In oophx rats receiving combined DHT and estradiol treatment, the action of DHT on bone cell metabolism as indicated by the biochemical markers appeared to predominate over the actions of estradiol. This is probably a reflection of the protocol employed in the present study as DHT was administered 4 weeks prior to the addition of estradiol and perhaps a longer duration of estradiol was required to observe changes in the biochemical markers of bone cell metabolism and calcium homeostasis.

It is of interest to note that in each of the following groups, estrogen deficient rats, sham and oophx rats treated with DHT immediately following operation, osteopenic oophx rats administered estradiol and osteopenic oophx rats treated with combined DHT and estradiol, the effects on the mRNA levels of the osteocalcin and calcitonin receptor genes were the same, with the only discordant effect being observed in osteopenic oophx rats treated with DHT. Perhaps the process of bone resorption is coupled to the process of bone formation at the stage of bone mineralisation when osteocalcin is expressed, however to date little is

known about the process which couples bone resorption and bone formation to either support or dismiss this speculation.

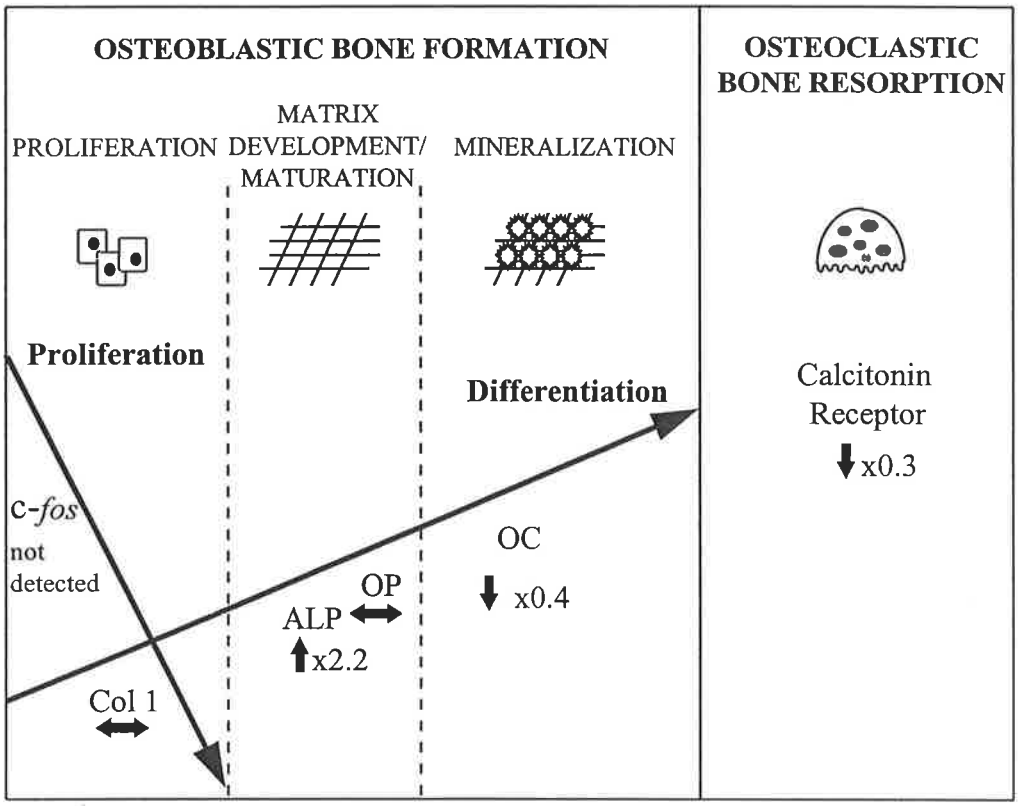


Figure 7.7 Osteoblast and osteoclast gene expression following combined DHT and estradiol treatment in osteopenic oophorectomised rats at 20 weeks post-operation. DHT was administered at 15 weeks post-oophorectomy and estradiol was administered at 19 weeks post-oophorectomy. The proposed model of gene expression during osteoblast development is from Owen and colleagues 1990. (↔) no change, (↑) increase and (↓) decrease. 'x' refers to fold increase relative to sham rats receiving vehicle.

In summary, this study has identified a number of actions of DHT alone and in combination with estradiol on bone cell metabolism as indicated by bone biochemical markers and osteoblast gene expression. Interestingly, the administration of DHT to oophx rats immediately following oophorectomy abolished the effects of estrogen-deficiency on the biochemical markers of bone cell metabolism. The action of DHT alone and in combination with estradiol on the mRNA levels of the osteoblast and osteoclast genes in estrogen-sufficient and estrogen-deficient rats following treatment were much smaller in magnitude compared to the effect of oophorectomy alone. Estrogen therefore, not surprisingly is the predominant sex steroid hormone regulator of bone cell gene expression in oophx rats. Furthermore, the data from this study indicates that there is an interaction of estrogens on the mRNA levels of osteoblast and osteoclast genes.

The data from this study suggests that the increase in bone density previously identified following androgen treatment in postmenopausal women (Need et al 1987, Johanssen et al 1989) and in osteopenic oophx rats (Tobias et al 1994, Coxam et al 1996) is associated with an increase in serum alkaline phosphatase, and adds further support for the potential therapeutic use of androgens to partially restore the bone loss following the menopause. The measurement of alkaline phosphatase therefore, is perhaps a better indication of the change in bone formation following androgen treatment than osteocalcin. The effects of combined DHT and estradiol treatment on bone cell metabolism identified in the present study, in addition to the previous findings of Raisz and colleagues (1996) in postmenopausal women and Coxam and colleagues (1996) in osteopenic oophx rats, provides further evidence for the additional benefit of combined androgen and estrogen

treatment in postmenopausal women than either treatment alone, acting to inhibit bone resorption while stimulating bone formation.

The findings of the present study also raises a few central questions which provide direction for further investigations into the mechanisms by which androgens and estrogens effect bone cell metabolism. It has to be determined whether the suppression of the serum levels and mRNA levels of osteocalcin by DHT observed in the present study, correlate with increased bone mineral density as suggested by the recent development of the osteocalcin knockout mouse (Ducy et al 1996). Further investigations are required to confirm the relationship between serum alkaline phosphatase levels and extent of double-labelled flurochrome surface and osteocalcin and mineral apposition rate as proposed by Sims and colleagues (1996a).

In conclusion, the findings of this study supports the first hypothesis that oophorectomy results in an increase in the mRNA levels of the osteoclast and osteoblast genes. In support of the second hypothesis, DHT treatment increased the mRNA levels of the osteoblast genes but this was only observed in estrogen-sufficient rats and was accompanied by an increase in calcitonin receptor mRNA levels. Contrary to the second hypothesis, DHT treatment actually suppressed the oophx-induced increase in type 1α collagen and osteocalcin mRNA levels in oophx rats and type 1α collagen and alkaline phosphatase mRNA levels in osteopenic oophx rats. In support of the second hypothesis, the anti-catabolic effect of DHT on calcitonin receptor mRNA levels was observed in oophx rats only when DHT was administered immediately following operation. The anabolic effect of DHT on bone cell metabolism as indicated by serum alkaline phosphatase levels was not

more prominent in osteopenic oophx rats as proposed. The third hypothesis that DHT in combination with estrogen could overcome the suppressive effects of estrogen treatment is supported by the present findings. This was reflected by an increase in serum alkaline phosphatase and alkaline phosphatase mRNA levels with a suppression of osteocalcin mRNA and calcitonin receptor mRNA. The findings of the present study have provided insight into the mechanisms by which estrogens and androgens effect bone cell metabolism in the rat.

Appendix A

Serum Electrolytes

Effect of Oophorectomy

Serum sodium and anion gap were unaffected by oophorectomy (Appendix A.3, Appendix A.7). Serum chloride was significantly decreased in oophorectomised rats receiving vehicle alone ($P<0.001$) and in those treated with DHT ($P<0.0005$) (Appendix A.5). Serum potassium and bicarbonate were significantly increased following oophorectomy in rats receiving vehicle alone ($P<0.05$) (Appendix A.4, Appendix A.6).

Effect of DHT Treatment in Sham and Oophorectomised Rats

Serum sodium, chloride and bicarbonate were unaffected by DHT treatment in either sham or oophx rats (Appendix A.3, Appendix A.5, Appendix A.6). Serum potassium was increased following DHT treatment in sham rats only ($P<0.05$) and this effect was not time dependent but was dose dependent with maximal stimulation occurring at 80mg and 160mg/kg bw DHT. As a result there was no significant difference in serum potassium between sham and oophx rats following DHT treatment. Anion gap was significantly increased following DHT treatment in both sham and oophx rats ($P<0.05$) and this effect was dose dependent with maximal stimulation occurring at 40 and 160mg/kg bw DHT and was time dependent with maximal levels occurring between 5 and 6 weeks in sham rats and 4 weeks in oophx rats ($P<0.001$) (Appendix A.7). The increase in anion gap following DHT treatment was due to the increase in serum potassium observed. Serum sodium increased between weeks 4 to 6 in both sham and oophx rats ($P<0.001$). In contrast, serum bicarbonate was decreased at week 6 ($P<0.025$)

(Appendix A.6). Serum bicarbonate levels did not differ between sham and oophx rats following DHT treatment due to a greater rise, although not significant, in sham rats than in oophx rats (Appendix A.6).

Appendix A.1 Urine volume (mLs/day) in sham and oophorectomised rats following DHT administration.

Time (Weeks)	Control				Oophx			
	Vehicle	40mg	80mg	160mg	Vehicle	40mg	80mg	160mg
1	7.7 (1.4) n=4	6.8 (0.6) n=6	5.5 (2.0) n=4	6.6 (1.4) n=6	7.4 (1.0) n=5	8.7 (1.0) n=4	7.6 (0.7) n=5	6.7 (1.0) n=6
2	7.9 (0.9) n=4	5.7 (1.0) n=5	7.5 (1.3) n=5	5.5 (1.5) n=6	6.3 (1.3) n=5	7.8 (1.0) n=5	7.4 (1.0) n=6	6.3 (0.8) n=6
3	7.7 (1.2) n=5	6.6 (1.5) n=5	4.4 (2.0) n=4	5.2 (0.9) n=4	6.9 (1.0) n=5	6.2 (2.1) n=2	7.6 (2.2) n=4	4.2 (1.8) n=6
4	8.7 (0.5) n=3	2.9 (0.8) n=4	6.3 (0.5) n=2	5.2 (2.3) n=3	7.0 (1.4) n=3	10.4 n=1	2.3 n=1	5.1 (1.7) n=3
5	7.9 (1.3) n=4	7.4 (0.6) n=5	4.7 (1.0) n=6	5.7 (1.2) n=4	6.7 (1.9) n=5	7.6 (1.4) n=2	7.1 (1.3) n=6	4.5 (1.4) n=5
6	7.9 (0.6) n=4	6.6 (1.4) n=5	5.7 (1.2) n=6	7.6 (0.9) n=5	6.9 (1.4) n=5	8.0 (1.2) n=4	8.5 (0.5) n=6	6.1 (1.2) n=6
7	8.2 (1.0) n=4	5.9 (1.4) n=5	6.0 (1.8) n=5	6.8 (0.9) n=5	7.4 (1.4) n=4	6.8 (0.7) n=5	8.8 (0.7) n=5	4.8 (1.6) n=4
8	7.2 (0.7) n=3	5.8 (0.4) n=4	5.7 (1.1) n=5	7.3 (1.2) n=5	7.5 (1.0) n=4	7.9 (1.3) n=5	7.4 (1.1) n=4	5.6 (1.2) n=6

DHT dose is mg per kg body weight. Values are Mean (SE).

Appendix A.2 Urine 24 hour creatinine excretion (mmol/day) in sham and oophorectomised rats following DHT administration.

Time (Weeks)	Control				Oophx			
	Vehicle	40mg	80mg	160mg	Vehicle	40mg	80mg	160mg
1	0.072 (0.006) n=4	0.052 (0.007) n=6	0.053 (0.019) n=4	0.067 (0.010) n=6	0.079 (0.007) n=5	0.070 (0.004) n=4	0.066 (0.009) n=5	0.073 (0.018) n=6
2	0.080 (0.007) n=4	0.048 (0.009) n=5	0.054 (0.009) n=5	0.050 (0.007) n=6	0.060 (0.003) n=5	0.062 (0.006) n=5	0.059 (0.012) n=6	0.073 (0.008) n=6
3	0.053 (0.012) n=3	0.057 (0.004) n=5	0.054 (0.012) n=4	0.070 (0.015) n=4	0.056 (0.006) n=5	0.086 (0.011) n=2	0.064 (0.014) n=4	0.036 (0.011) n=6
4	0.040 (0.006) n=3	0.050 (0.011) n=4	0.069 (0.003) n=2	0.062 (0.014) n=3	0.070 (0.012) n=3	0.041 n=1	0.047 n=1	0.054 (0.007) n=3
5	0.067 (0.010) n=4	0.062 (0.007) n=5	0.049 (0.006) n=6	0.064 (0.006) n=4	0.054 (0.013) n=5	0.062 (0.006) n=5	0.056 (0.011) n=6	0.050 (0.010) n=6
6	0.069 (0.007) n=4	0.044 (0.005) n=5	0.050 (0.001) n=6	0.065 (0.014) n=5	0.067 (0.010) n=5	0.057 (0.007) n=4	0.053 (0.009) n=6	0.061 (0.006) n=6
7	0.040 (0.007) n=4	0.050 (0.010) n=5	0.045 (0.013) n=5	0.064 (0.014) n=5	0.050 (0.016) n=4	0.057 (0.011) n=5	0.057 (0.012) n=5	0.055 (0.002) n=4
8	0.068 (0.005) n=3	0.050 (0.007) n=4	0.053 (0.014) n=5	0.064 (0.009) n=5	0.071 (0.003) n=4	0.073 (0.004) n=5	0.049 (0.007) n=4	0.061 (0.010) n=6

DHT dose is mg per kg body weight. Values are Mean (SE).

Appendix A.3 Serum sodium (mmol/L) in sham and oophorectomised rats following DHT treatment.

Time (Weeks)	Control				Oophx			
	Vehicle	40mg	80mg	160mg	Vehicle	40mg	80mg	160mg
1	138.2 (0.3)[a] n=6	138.5 (0.7)[a] n=6	138.5 (0.7)[a] n=6	139.4 (1.1)[a] n=6	137.5 (1.0)[a] n=6	136.6 (1.0)[a] n=6	136.8 (0.7)[a] n=6	136.6 (0.7)[a] n=6
2	136.7 (0.8)[a] n=6	138.5 (0.8)[a] n=6	138.7 (0.4)[a] n=6	137.3 (0.5)[a] n=6	136.9 (0.8)[a] n=6	137.3 (0.3)[a] n=6	136.9 (0.6)[a] n=6	136.6 (1.5)[a] n=6
3	136.2 (0.6)[a] n=5	137.2 (0.6)[a] n=5	137.7 (0.3)[a] n=5	137.7 (0.6)[a] n=5	138.2 (0.5)[a] n=5	137.7 (0.6)[a] n=6	138.7 (0.5)[a] n=6	139.0 (0.9)[a] n=6
4	138.9 (0.5) n=4	139.2 (0.6) n=4	137.6 (1.7) n=4	139.4 (1.5) n=3	137.8 (0.6) n=3	138.5 (0.4) n=3	138.7 (1.0) n=3	139.9 (0.6) n=3
5	138.6 (1.0)[b] n=6	139.2 (1.1)[b] n=6	138.7 (0.8)[b] n=6	139.9 (0.6)[b] n=6	136.8 (1.1)[b] n=6	136.4 (0.6)[b] n=6	138.6 (0.8)[b] n=6	138.1 (0.6)[b] n=6
6	138.5 (0.7)[b] n=6	138.3 (0.7)[b] n=6	138.2 (0.4)[b] n=6	137.7 (0.8)[b] n=6	138.7 (0.7)[b] n=5	138.7 (1.1)[b] n=6	138.4 (1.0)[b] n=6	137.1 (1.7)[b] n=4
7	136.5 (0.7)[a] n=6	137.5 (0.7)[a] n=6	137.0 (0.7)[a] n=6	137.5 (0.4)[a] n=6	136.6 (0.9)[a] n=5	136.5 (0.7)[a] n=6	137.4 (0.2)[a] n=6	137.6 (1.2)[a] n=6
8	137.6 (0.2)[a] n=6	138.3 (0.2)[a] n=6	137.2 (0.6)[a] n=6	138.0 (0.6)[a] n=6	135.9 (1.1)[a] n=5	136.0 (1.0)[a] n=6	137.3 (0.7)[a] n=6	138.1 (0.4)[a] n=6

DHT dose is mg per kg body weight. Values are Mean (SE). [a]$P<0.001$ versus week 4 within dosage group, [b]$P<0.001$ versus week 7 within operation and dosage group.

Appendix A.4 Serum postassium (mmol/L) in sham and oophorectomised rats following DHT treatment.

Time	Control				Oophx			
(Weeks)	Vehicle	40mg	80mg	160mg	Vehicle	40mg	80mg	160mg
1	4.80 (0.19) n=6	5.64 (0.48) n=6	5.73 (0.55)[b] n=6	5.59 (0.35)[b] n=6	5.37 (0.20)[a] n=6	5.44 (0.24) n=6	5.59 (0.21) n=6	5.85 (0.35) n=6
2	5.72 (0.57) n=6	5.92 (0.48) n=6	6.14 (0.41)[b] n=6	6.14 (0.57)[b] n=6	6.07 (0.44)[a] n=6	5.70 (0.26) n=6	5.33 (0.45) n=6	5.77 (0.44) n=6
3	5.37 (0.29) n=5	5.60 (0.18) n=5	5.35 (0.14)[b] n=5	6.00 (0.37)[b] n=5	5.54 (0.43)[a] n=5	5.44 (0.17) n=6	5.58 (0.24) n=6	5.68 (0.24) n=6
4	4.70 (0.22) n=4	5.12 (0.21) n=4	5.00 (0.33)[b] n=4	5.88 (0.16)[b] n=3	5.44 (0.08)[a] n=3	5.45 (0.27) n=3	5.54 (0.20) n=3	5.50 (0.20) n=3
5	5.05 (0.30) n=6	5.23 (0.11) n=6	5.61 (0.16)[b] n=6	5.21 (0.15)[b] n=6	2.89 (0.32)[a] n=6	5.88 (0.37) n=6	5.50 (0.26) n=6	5.85 (0.31) n=6
6	5.46 (0.42) n=6	6.36 (0.18) n=5	6.21 (0.24)[b] n=6	5.53 (0.25)[b] n=5	4.84 (0.22)[a] n=5	5.24 (0.19) n=5	4.98 (0.13) n=5	5.39 (0.26) n=3
7	5.12 (0.23) n=6	5.14 (0.30) n=6	5.08 (0.21)[b] n=6	6.07 (0.53)[b] n=6	5.65 (0.33)[a] n=5	5.72 (0.13) n=6	5.83 (0.22) n=6	6.01 (0.18) n=6
8	4.93 (0.26) n=6	5.43 (0.30) n=6	5.86 (0.30)[b] n=6	5.43 (0.25)[b] n=6	5.63 (0.38)[a] n=5	5.73 (0.22) n=6	5.24 (0.10) n=6	5.26 (0.16) n=6

DHT dose is mg per kg body weight. Values are Mean (SE). [a]$P<0.05$ versus sham within dosage group, [b]$P<0.05$ versus vehicle within operation group.

Appendix A.5 Serum chloride (mmol/L) in sham and oophorectomised rats following DHT treatment.

Time (Weeks)	Control				Oophx			
	Vehicle	40mg	80mg	160mg	Vehicle	40mg	80mg	160mg
1	104.0 (0.6) n=6	104.7 (0.8) n=6	103.7 (1.5) n=6	104.0 (0.5) n=6	103.3 (0.9)[a] n=6	103.0 (0.5)[b] n=6	103.3 (0.7)[b] n=6	102.5 (0.6)[b] n=6
2	104.0 (0.3) n=6	104.2 (0.9) n=6	104.2 (0.7) n=6	102.8 (0.4) n=6	102.8 (0.4)[a] n=6	102.2 (0.6)[b] n=6	102.3 (0.8)[b] n=6	102.0 (0.3)[b] n=6
3	102.6 (1.2) n=5	102.4 (1.2) n=5	102.6 (1.5) n=5	102.8 (0.5) n=5	102.8 (0.6)[a] n=5	102.7 (0.5)[b] n=6	103.2 (0.4)[b] n=6	103.0 (1.2)[b] n=6
4	104.5 (0.5) n=4	103.0 (0.7) n=4	102.0 (0.9) n=4	102.7 (0.9) n=3	101.7 (0.3)[a] n=3	102.0 (0.1)[b] n=3	102.3 (1.5)[b] n=3	102.7 (0.5)[b] n=3
5	103.7 (0.3) n=6	103.0 (0.5) n=6	103.0 (0.6) n=6	102.5 (0.6) n=6	100.7 (1.2)[a] n=6	101.7 (0.5)[b] n=6	102.0 (0.5)[b] n=6	101.5 (0.3)[b] n=6
6	104.0 (0.5) n=6	103.2 (0.5) n=6	102.8 (0.5) n=6	101.8 (0.5) n=6	102.8 (0.2)[a] n=5	102.7 (0.6)[b] n=6	102.8 (0.3)[b] n=6	102.0 (0.5)[b] n=4
7	104.8 (0.8) n=6	103.7 (0.4) n=6	103.7 (0.7) n=6	101.8 (0.5) n=6	102.6 (0.8)[a] n=5	100.8 (1.0)[b] n=6	102.5 (0.5)[b] n=6	102.0 (0.8)[b] n=6
8	104.0 (0.3) n=6	103.5 (0.6) n=6	103.0 (0.3) n=6	103.5 (0.6) n=6	102.0 (0.9)[a] n=5	101.8 (1.1)[b] n=6	103.3 (1.0)[b] n=6	103.2 (0.3)[b] n=6

DHT dose is mg per kg body weight. Values are Mean (SE). [a]$P<0.001$ versus sham within dosage group, [a]$P<0.0005$ versus sham within dosage group.

Appendix A.6 Serum bicarbonate (mmol/L) in sham and oophorectomised rats following DHT treatment.

Time (Weeks)	Control				Oophx			
	Vehicle	40mg	80mg	160mg	Vehicle	40mg	80mg	160mg
1	29.0 (0.4)[b] n=6	27.7 (0.6)[b] n=6	28.1 (1.0)[b] n=6	28.3 (0.2)[b] n=6	29.7 (0.4)[ab] n=6	29.0 (0.4)[b] n=6	29.5 (0.5)[b] n=6	30.3 (0.4)[b] n=6
2	27.8 (0.6)[b] n=6	27.8 (0.3)[b] n=6	27.6 (0.6)[b] n=6	29.3 (0.5)[b] n=6	28.5 (0.6)[ab] n=6	29.3 (0.6)[b] n=6	28.0 (0.2)[b] n=6	28.5 (0.6)[b] n=6
3	27.3 (1.0) n=5	28.1 (0.7) n=5	28.7 (1.1) n=5	28.1 (1.0) n=5	28.3 (1.0)[a] n=5	28.2 (1.0) n=6	28.1 (0.5) n=6	28.4 (0.5) n=6
4	28.2 (0.6)[b] n=4	28.6 (0.7)[b] n=4	29.0 (1.4)[b] n=4	28.6 (0.6)[b] n=3	28.0 (0.6)[ab] n=3	28.1 (0.6)[b] n=3	28.0 (0.5)[b] n=3	28.4 (0.8)[b] n=3
5	26.7 (1.0) n=6	27.8 (0.3) n=6	28.0 (0.7) n=6	28.3 (0.4) n=6	30.4 (1.0)[a] n=6	28.2 (1.0) n=6	28.4 (0.6) n=6	28.4 (0.5) n=6
6	26.8 (0.3) n=6	27.0 (0.4) n=6	27.3 (0.5) n=6	28.2 (0.8) n=6	27.8 (0.3)[a] n=5	27.1 (0.3) n=6	26.3 (0.5) n=6	27.4 (0.2) n=4
7	27.6 (0.7)[b] n=6	28.3 (0.9)[b] n=6	28.4 (0.9)[b] n=6	29.5 (0.6)[b] n=6	29.0 (0.7)[ab] n=5	29.2 (0.7)[b] n=6	28.4 (0.4)[b] n=6	28.7 (0.7)[b] n=6
8	28.4 (0.5)[b] n=6	29.8 (0.7)[b] n=6	29.0 (1.0)[b] n=6	29.0 (0.8)[b] n=6	28.1 (0.5)[ab] n=5	27.6 (0.5)[b] n=5	28.9 (0.6)[b] n=6	30.9 (0.5)[b] n=6

DHT dose is mg per kg body weight. Values are Mean (SE). [a]$P<0.05$ versus sham within dosage group. [b]$P<0.025$ versus week 6 within operation and dosage group.

Appendix A.7 Serum anion gap (mmol/L) in sham and oophorectomised rats following DHT treatment.

Time (Weeks)	Control				Oophx			
	Vehicle	40mg	80mg	160mg	Vehicle	40mg	80mg	160mg
1	11.2 (0.6)^{bc} n=6	13.0 (0.9)^{abc} n=6	13.6 (1.1)^{bc} n=6	13.9 (1.2)^{abc} n=6	11.1 (0.4)^{f} n=6	11.2 (0.6)^{af} n=6	10.8 (0.5)^{f} n=6	10.9 (0.6)^{af} n=6
2	11.8 (0.8)^{bc} n=6	13.6 (0.6)^{abc} n=6	14.2 (0.5)^{bc} n=6	12.5 (0.8)^{abc} n=6	13.3 (0.8)^{b} n=6	12.7 (0.8)^{ad} n=6	13.1 (0.6)^{d} n=6	13.1 (0.4)^{ad} n=6
3	12.9 (0.5)^{e} n=5	13.2 (0.2)^{ac} n=5	12.9 (0.4)^{e} n=5	13.8 (0.7)^{ac} n=5	13.9 (0.5)^{de} n=5	13.5 (0.6)^{ade} n=6	14.2 (0.6)^{de} n=6	15.1 (0.5)^{ade} n=6
4	12.2 (1.0)^{c} n=4	13.9 (1.0)^{ac} n=4	12.7 (1.8)^{c} n=4	15.2 (1.1)^{ac} n=3	14.8 (0.8)^{de} n=3	15.0 (0.2)^{ade} n=3	15.1 (0.3)^{de} n=3	15.5 (0.2)^{ade} n=3
5	14.5 (1.5) n=6	14.8 (1.1)^{a} n=6	14.5 (0.9) n=6	15.6 (0.7)^{a} n=6	12.9 (0.8)^{de} n=6	13.6 (0.3)^{ade} n=6	15.0 (0.8)^{de} n=6	15.2 (0.9)^{ade} n=6
6	14.4 (0.4) n=6	15.9 (0.8)^{a} n=6	15.5 (0.5) n=6	14.9 (1.0)^{a} n=6	14.1 (0.5)^{de} n=5	15.9 (0.7)^{ade} n=6	16.3 (0.8)^{de} n=6	15.0 (0.6)^{ade} n=4
7	10.4 (0.6)^{bc} n=6	11.9 (0.9)^{abc} n=6	11.3 (0.7)^{bc} n=6	13.4 (0.8)^{abc} n=6	11.9 (1.1)^{d} n=5	13.4 (0.8)^{ad} n=6	13.5 (0.3)^{d} n=6	14.1 (0.4)^{ad} n=6
8	11.3 (0.5)^{bc} n=6	11.6 (1.1)^{abc} n=6	12.2 (0.7)^{bc} n=6	12.4 (0.9)^{abc} n=6	12.7 (0.9) n=5	14.1 (0.6)^{a} n=5	11.6 (0.6) n=6	10.5 (1.0)^{a} n=6

DHT dose is mg per kg body weight. Values are Mean (SE). $^{a}P<0.05$ versus vehicle within operation group, $^{b}P<0.05$ versus week 5 within operation group, $^{c}P<0.05$ versus week 6 within operation group, $^{d}P<0.05$ versus week 1 within operation group, $^{e}P<0.05$ versus week 8 within operation group, $^{f}P<0.05$ versus sham within dosage group.

Appendix A.8 Percentage changes from pre-treatment in bone biochemical variables at 8 weeks post-oophorectomy following DHT administered at the time of operation.

Variable	Operation	Dose DHT (mg/kg body weight)			
		Vehicle	40mg	80mg	160mg
Serum Alkaline Phosphatase (Units/L)	Sham	10.9	145.4	153.7	98.7
	Oophx	65.2	152.8	105.2	143.1
Serum Osteocalcin (μg/L)	Sham	-10.0	-1.5	10.1	-8.9
	Oophx	20.4	-4.3	-14.9	-15.0
Urine deoxypyridinoline/creatinine (nmol/mmol)	Sham	19.9	44.9	136.9	156.2
	Oophx	135.9	81.7	91.9	53.0
Urine Hydroxyproline (μmol/mmol)	Sham	33.8	79.0	94.8	136.8
	Oophx	47.4	68.5	116.3	93.5

Note. Percentage changes for deoxypyridinoline/creatinine (mmol/mmol) are from 1 week post-treatment.

Appendix B

Serum Electrolytes

Serum potassium was increased following DHT treatment at doses of 40mg and 160mg DHT/kg bw ($P<0.005$) and this effect was time dependent with maximal stimulation occurring at 14 weeks following treatment (29 weeks post-oophorectomy) (Appendix B.4). Serum chloride was decreased following DHT treatment at doses of 40mg and 160mg DHT/kg bw ($P<0.01$) and this effect was not time dependent (Appendix B.5). Serum sodium, bicarbonate and anion gap were unaffected by DHT treatment (Appendix B.3, Appendix B.6, Appendix B.7) and serum sodium remained unchanged throughout the duration of the experiment (Appendix B.3). In contrast, serum bicarbonate increased throughout the duration of the experiment ($P<0.005$) with maximal levels occurring by week 10 following commencement of treatment (25 weeks post-oophorectomy)(Appendix B.6) and serum anion gap was decreased at 12 weeks following treatment (27 weeks post-oophorectomy) ($P<0.05$) (Appendix B.7).

Appendix B.1 Urine volume (mLs) in osteopenic oophorectomised rats following DHT administration at 15 weeks post-oophorectomy.

Time (Weeks)	Dose Dihydrotestosterone (mg/kg body weight)			
	Vehicle	**40**	**80**	**160**
15	6.8 (0.7) n=3	6.1 (0.8) n=6	6.3 (1.6) n=6	4.7 (1.8) n=6
17	6.5 (0.3) n=5	5.5 (0.9) n=6	4.5 (0.5) n=6	3.4 (0.8) n=6
19	7.4 (0.5) n=5	10.4 (5.0) n=6	4.3 (0.4) n=6	4.3 (1.6) n=6
21	6.0 (0.9) n=5	5.7 (1.8) n=6	4.2 (1.0) n=6	4.6 (1.6) n=6
23	6.6 (1.0) n=5	7.3 (2.9) n=6	4.6 (0.7) n=6	4.6 (0.9) n=6
25	7.7 (1.2) n=5	5.2 (1.1) n=6	3.4 (0.3) n=6	3.5 (1.1) n=6
27	6.0 (1.4) n=5	3.9 (0.8) n=5	8.2 (3.9) n=6	4.1 (1.1) n=6
29	5.6 (0.7) n=5	3.9 (0.6) n=6	4.8 (1.0) n=6	4.2 (0.8) n=6

Values are Mean (SE). Time is weeks post-oophorectomy. DHT was administered following collection of blood and urine samples at 15 weeks post-oophorectomy.

Appendix B.2 Urine 24 hour creatinine excretion (mmol/day) in osteopenic oophorectomised rats following DHT administration at 15 weeks post-oophorectomy.

Time (Weeks)	Dose Dihydrotestosterone (mg/kg body weight)			
	Vehicle	40	80	160
15	0.058 (0.014) n=3	0.081 (0.007) n=6	0.064 (0.010) n=6	0.070 (0.012) n=6
17	0.079 (0.004) n=5	0.072 (0.006) n=6	0.074 (0.005) n=6	0.059 (0.006) n=6
19	0.085 (0.006) n=5	0.077 (0.008) n=6	0.069 (0.004) n=6	0.062 (0.013) n=6
21	0.066 (0.007) n=5	0.062 (0.005) n=6	0.077 (0.005) n=6	0.069 (0.013) n=6
23	0.076 (0.007) n=5	0.076 (0.011) n=6	0.083 (0.007) n=6	0.055 (0.006) n=6
25	0.082 (0.009) n=5	0.057 (0.014) n=6	0.078 (0.006) n=6	0.060 (0.009) n=6
27	0.059 (0.008) n=5	0.058 (0.004) n=5	0.078 (0.006) n=5	0.062 (0.010) n=6
29	0.067 (0.008) n=5	0.068 (0.005) n=6	0.078 (0.007) n=6	0.067 (0.006) n=6

Values are Mean (SE). Time is weeks post-oophorectomy. DHT was administered following collection of blood and urine samples at 15 weeks post-oophorectomy.

Appendix B.3 Serum sodium levels (mmol/L) in osteopenic oophorectomised rats following DHT administration at 15 weeks post-oophorectomy.

Time (Weeks)	Dose Dihydrotestosterone (mg/kg body weight)			
	Vehicle	40	80	160
15	139.7 (0.5) n=5	138.7 (0.5) n=6	138.3 (0.8) n=6	137.5 (0.5) n=6
17	138.3 (0.2) n=5	137.2 (1.6) n=6	138.0 (0.9) n=6	137.4 (0.9) n=6
19	137.9 (0.6) n=5	137.0 (0.4) n=6	138.5 (0.8) n=6	137.9 (0.8) n=5
21	137.5 (0.5) n=5	136.6 (0.5) n=6	137.8 (0.7) n=6	137.1 (0.5) n=6
23	137.6 (0.6) n=5	137.8 (0.5) n=6	138.4 (0.7) n=6	138.2 (0.5) n=6
25	136.4 (0.6) n=5	137.1 (0.3) n=6	138.3 (0.4) n=6	137.4 (0.7) n=6
27	136.9 (0.3) n=5	137.4 (0.3) n=6	138.1 (0.4) n=6	137.7 (0.4) n=6
29	137.0 (0.5) n=5	137.3 (0.5) n=6	137.9 (0.6) n=6	137.2 (0.7) n=6

Values are Mean (SE). Time is weeks post-oophorectomy. DHT was administered following collection of blood and urine samples at 15 weeks post-oophorectomy.

Appendix B.4 Serum potassium levels (mmol/L) in osteopenic oophorectomised rats following DHT administration at 15 weeks post-oophorectomy.

Time (Weeks)	Dose Dihydrotestosterone (mg/kg body weight)			
	Vehicle	40	80	160
15	4.41 (0.14)[c] n=5	4.28 (0.08)[ac] n=6	4.32 (0.09)[bc] n=6	4.85 (0.26)[ac] n=6
17	4.51 (0.16)[cd] n=5	4.92 (0.19)[acd] n=6	4.92 (0.15)[bcd] n=6	5.31 (0.16)[acd] n=6
19	4.53 (0.13)[c] n=5	4.96 (0.14)[ac] n=6	5.04 (0.15)[bc] n=6	4.93 (0.10)[ac] n=6
21	4.70 (0.19)[cd] n=5	5.35 (0.37)[acd] n=6	4.82 (0.15)[bcd] n=6	4.97 (0.14)[acd] n=6
23	4.58 (0.18)[c] n=5	4.78 (0.10)[ac] n=6	4.77 (0.10)[bc] n=6	5.03 (0.19)[ac] n=6
25	4.66 (0.12)[c] n=5	4.91 (0.09)[ac] n=6	4.45 (0.10)[bc] n=6	5.04 (0.14)[ac] n=6
27	4.82 (0.11)[c] n=5	4.93 (0.08)[ac] n=6	4.91 (0.08)[bc] n=6	4.81 (0.17)[ac] n=6
29	5.87 (0.14) n=5	6.28 (0.14)[a] n=6	6.13 (0.24)[b] n=6	6.84 (0.42)[a] n=6

Values are Mean (SE). Time is weeks post-oophorectomy. DHT was administered following collection of blood and urine samples at 15 weeks post-oophorectomy. [a]$P<0.005$ versus vehicle, [b]$P<0.005$ versus 160mg DHT/kg bw, [c]$P<0.001$ versus week 29, [d]$P<0.001$ versus week 15.

Appendix B.5 Serum chloride levels (mmol/L) in osteopenic oophorectomised rats following DHT administration at 15 weeks post-oophorectomy.

Time (Weeks)	Dose Dihydrotestosterone (mg/kg body weight)			
	Vehicle	40	80	160
15	104.0 (0.6) n=5	102.8 (0.3)[a] n=6	103.2 (0.6) n=6	102.2 (0.6)[a] n=6
17	104.3 (0.8) n=5	101.7 (1.2)[a] n=6	102.8 (0.6) n=6	102.0 (0.5)[a] n=6
19	103.2 (1.2) n=5	101.7 (0.9)[a] n=6	103.2 (1.1) n=6	103.2 (1.9)[a] n=6
21	105.0 (0.6) n=5	102.7 (0.5)[a] n=6	103.3 (0.8) n=6	103.0 (0.5)[a] n=6
23	103.8 (0.7) n=5	102.3 (0.6)[a] n=6	102.7 (1.2) n=6	102.0 (0.3)[a] n=6
25	103.6 (0.8) n=5	102.2 (0.6)[a] n=6	103.0 (0.9) n=6	102.2 (0.5)[a] n=6
27	104.0 (0.6) n=5	102.2 (0.4)[a] n=6	103.2 (0.6) n=6	103.3 (0.4)[a] n=6
29	104.8 (0.2) n=5	102.8 (0.5)[a] n=6	104.2 (0.6) n=6	103.2 (0.3)[a] n=6

Values are Mean (SE). Time is weeks post-oophorectomy. DHT was administered following collection of blood and urine samples at 15 weeks post-oophorectomy. [a]$P<0.01$ versus vehicle.

Appendix B.6 Serum bicarbonate levels (mmol/L) in osteopenic oophorectomised rats following DHT administration at 15 weeks post-oophorectomy.

Time (Weeks)	Dose Dihydrotestosterone (mg/kg body weight)			
	Vehicle	40	80	160
15	27.4 (0.5)[b] n=5	28.2 (0.5)[b] n=6	28.2 (0.4)[b] n=6	27.7 (0.6)[b] n=6
17	26.5 (0.2)[abc] n=5	27.7 (0.4)[abc] n=6	27.8 (0.4)[abc] n=6	26.5 (1.2)[abc] n=6
19	27.6 (0.5)[b] n=5	27.2 (1.1)[b] n=6	28.0 (0.4)[b] n=6	28.7 (0.6)[b] n=6
21	26.8 (0.2)[b] n=5	27.2 (0.2)[b] n=5	28.7 (0.4)[b] n=6	27.8 (0.6)[b] n=6
23	26.4 (0.6)[abc] n=5	27.0 (0.4)[abc] n=6	27.6 (0.9)[abc] n=6	26.8 (0.5)[abc] n=6
25	27.3 (0.7) n=5	28.2 (0.2) n=6	28.9 (0.5) n=6	28.5 (0.5) n=6
27	28.5 (0.6) n=5	29.2 (0.3) n=6	29.5 (0.4) n=6	29.9 (0.2) n=6
29	27.5 (0.6) n=5	28.8 (0.5) n=6	29.2 (0.4) n=6	28.7 (0.5) n=6

Values are Mean (SE). Time is weeks post-oophorectomy. DHT was administered following collection of blood and urine samples at 15 weeks post-oophorectomy. [a]$P<0.005$ versus week 25, [b]$P<0.005$ versus week 27, [c]$P<0.005$ versus week 29.

Appendix B.7 Serum anion gap (mmol/L) in osteopenic oophorectomised rats following DHT administration at 15 weeks post-oophorectomy.

Time (Weeks)	Dose Dihydrotestosterone (mg/kg body weight)			
	Vehicle	40	80	160
15	13.8 (0.8) n=5	13.2 (0.7) n=6	12.4 (0.9) n=6	13.8 (0.7) n=6
17	13.2 (0.6)[a] n=5	14.0 (0.7)[a] n=6	13.6 (1.0)[a] n=6	15.4 (1.2)[a] n=6
19	12.8 (1.4) n=5	14.2 (2.2) n=6	13.5 (1.8) n=6	11.9 (2.0) n=6
21	11.6 (0.5) n=5	13.1 (0.6) n=5	11.8 (0.2) n=6	12.5 (0.6) n=6
23	13.1 (0.5)[ab] n=5	14.5 (0.4)[ab] n=6	14.4 (0.5)[ab] n=6	15.6 (0.6)[ab] n=6
25	11.4 (1.0) n=5	12.8 (0.9) n=6	12.2 (0.8) n=6	13.0 (1.0) n=6
27	10.4 (0.3) n=5	12.2 (0.3) n=6	11.6 (0.4) n=6	10.6 (0.3) n=6
29	11.8 (0.6) n=5	13.2 (0.6) n=6	11.9 (0.5) n=6	13.7 (0.6) n=6

Values are Mean (SE). Time is weeks post-oophorectomy. DHT was administered following collection of blood and urine samples at 15 weeks post-oophorectomy. [a]$P<0.05$ versus week 27, [b]$P<0.05$ versus week 21.

Appendix B.8 Percentage changes from pre-treatment (15 weeks post-oophorectomy) in bone biochemical variables at 29 weeks post-oophorectomy following DHT administration at 15 weeks post-oophorectomy.

Variable	Dose DHT (mg/kg body weight)			
	Vehicle	40mg	80mg	160mg
Serum Alkaline Phosphatase (Units/L)	24.0	47.6	50.0	47.2
Serum Osteocalcin (μg/L)	-27.7	-53.9	-50.2	-46.0
Urine deoxypyridinoline/creatinine (nmol/mmol)	-9.0	-50.0	-47.7	-16.4
Urine Hydroxyproline (μmol/mmol)	7.3	8.9	37.3	110.0

Appendix C.1

Signals obtained from the ImageQuant program for the mRNA levels of the osteoclast and osteoblast genes in sham and oophx rats administered DHT from the time of operation determined by Northern and Southern blot analyses.

	Northern 3		Northern 5		Northern 6	
Group	*c-fos*	GAPDH	*c-fos*	GAPDH	*c-fos*	GAPDH
Baseline	5108	607809	32071	95115	117243	245529
Sham + V	5809	472712	61650	111804	105972	242710
Sham + 40mg	7102	832987	65405	63115	62052	77122
Sham + 80mg	1810	382929	30224	60359	64799	54514
Sham + 160mg	28680	399437	51019	112317	49004	158228
Oophx + V	6691	511837	43311	79324	40376	91403
Oophx + 40	14871	674402	54629	152276	75729	92942
Oophx + 80	13598	472712	88180	343881	48097	123038
Oophx +160mg	13310	607809	106660	442156	93200	261141
	Northern 1		Northern 2		Northern 3	
Group	Type 1a col	GAPDH	Type 1a col	GAPDH	Type 1a col	GAPDH
Baseline	5630	262709	5702	390288	13899	94071
Sham + V	4718	1191262	2994	566833	7692	64102
Sham + 40mg	17213	479012	22402	333977	35045	89821
Sham + 80mg	8668	256587	11307	251246	4977	103891
Sham + 160mg	6103	466807	7865	363363	43430	33257
Oophx + V	35105	332495	46296	247922	40084	11656
Oophx + 40	45098	466603	55849	304975	94877	150131
Oophx + 80	11623	494325	15091	323165	25804	145091
Oophx +160mg	19388	1008255	31570	430604	52181	124744
	Northern 1		Northern 3		Northern 4	
Group	ALP	GAPDH	ALP	GAPDH	ALP	GAPDH
Baseline	3575	262709	573.6	551542	3171	903475
Sham + V	668.4	1191262	1516	495276	566.6	813356
Sham + 40mg	2168	479012	3947	832987	430.1	884272
Sham + 80mg	2898	256587	5104	382929	1015	1245987
Sham + 160mg	1627	466807	3481	399437	2100	406290
Oophx + V	12245	332495	2686	511837	4863	339059
Oophx + 40	12267	466603	4719	674402	11642	302495
Oophx + 80	3166	494325	687.4	472712	5314	441618
Oophx +160mg	15.92	1008255	2689	607809	7494	294769
	Northern 1		Northern 3		Northern 4	
Group	Osteopontin	GAPDH	Osteopontin	GAPDH	Osteopontin	GAPDH
Baseline	10257	19263	1965	607809	1871	903475
Sham + V	16361	41720	4455	472712	1356	813356
Sham + 40mg	18856	6453	5763	832987	6464	884272
Sham + 80mg	20219	9300	3918	382929	2972	1245987
Sham + 160mg	9142	15852	20734	399437	1780	406290
Oophx + V	20357	5375	6422	511837	8374	339059
Oophx + 40	33375	17842	13420	674402	9829	302495
Oophx + 80	23907	15142	9628	472712	7910	441618
Oophx +160mg	38656	49161	14369	607809	15070	294769

	Northern 1		Northern 2		Northern 3	
Group	Osteocalcin	GAPDH	Osteocalcin	GAPDH	Osteocalcin	GAPDH
Baseline	35446	262709	11191	390288	154298	94071
Sham + V	30683	1191262	4038	566833	27749	64102
Sham + 40mg	31176	479012	13252	333977	82884	89821
Sham + 80mg	31772	256587	12835	251246	13842	103891
Sham + 160mg	63997	466807	7628	363363	85372	33257
Oophx + V	115715	332495	19843	247922	70575	11656
Oophx + 40	50652	466603	24760	304975	151272	150131
Oophx + 80	66195	494325	11425	323165	88947	145091
Oophx +160mg	63212	1008255	33559	430604	151009	124744
	Northern 4					
Group	Osteocalcin	GAPDH				
Baseline	53405	35038				
Sham + V	29318	68561				
Sham + 40mg	49896	28944				
Sham + 80mg	67030	35815				
Sham + 160mg	38660	51067				
Oophx + V	53798	133619				
Oophx + 40	291010	168228				
Oophx + 80	176527	197039				
Oophx +160mg	117163	240326				

DHT dose is mg per kg body weight. Northerns 1-4 represent individual Northern blot analyses and Southerns 1 and 2 represent individual Southern blot analyses. Type 1a col - Type 1α collagen, ALP - Alkaline phosphatase, CTR - Calcitonin Receptor.

Appendix C.2

Signals obtained from the ImageQuant program for the mRNA levels of the osteoclast and osteoblast genes in sham and oophx rats administered DHT at 15 weeks post oophorectomy determined by Northern and Southern blot analyses.

	Northern 1		Northern 2	
Group	**Type 1a Col**	**GAPDH**	**Type 1a Col**	**GAPDH**
Baseline	28850	91477	14343	24514
Oophx + V	19796	61950	52405	7817
Oophx + 40	16782	59579	22384	30094
Oophx + 80	10620	161977	25242	32885
Oophx +160mg	24092	321272	29502	46433
Oophx + V	20228	24254	83348	16323
Oophx + 40	20327	81831	46349	30238
Oophx + 80	32113	131741	35502	19846
Oophx +160mg	23872	345698	54043	90442
	Northern 1		**Northern 2**	
Group	**ALP**	**GAPDH**	**ALP**	**GAPDH**
Baseline	11642	805913	35296	1836796
Oophx + V	30641	734568	20164	464925
Oophx + 40	1811	167729	38127	2125072
Oophx + 80	25991	876152	16219	446420
Oophx +160mg	42717	1635127	18281	1946799
Oophx + V	15374	512253	38728	*
Oophx + 40	25580	820943	23479	1945679
Oophx + 80	7153	1105238	32647	812862
Oophx +160mg	16767	2060985	25209	2873041
	Northern 1		**Northern 2**	
Group	**Osteopontin**	**GAPDH**	**Osteopontin**	**GAPDH**
Baseline	21084	130587	6046	289840
Oophx + V	59741	115097	3132	94234
Oophx + 40	*	28107	9026	340887
Oophx + 80	62438	100216	13512	219878
Oophx +160mg	151354	148821	11294	317933
Oophx + V	7860	70375	6598	300768
Oophx + 40	99104	123916	13599	367319
Oophx + 80	105082	111587	9389	316174
Oophx +160mg	108705	179192	25925	475134
	Northern 1		**Northern 2**	
Group	**Osteocalcin**	**GAPDH**	**Osteocalcin**	**GAPDH**
Baseline	87209	130587	73903	289840
Oophx + V	106139	115097	46568	94234
Oophx + 40	41899	28107	77461	340887
Oophx + 80	98666	100216	28958	219878
Oophx +160mg	91582	148821	79880	317933
Oophx + V	41240	70375	125798	300768
Oophx + 40	140432	123916	64338	367319
Oophx + 80	142001	111587	95054	316174
Oophx +160mg	135312	179192	154168	475134

	Southern 1			
Group	**CTR**	**GAPDH**		
Baseline	102718	6783347		
Oophx + V	179148	5429553		
Oophx + 40	13942	2165181		
Oophx + 80	167030	3876068		
Oophx +160mg	165248	2560218		
Oophx + V	112199	4574684		
Oophx + 40	138635	5991253		
Oophx + 80	95405	1113566		
Oophx +160mg	227136	926143		

DHT dose is mg per kg body weight. Northerns 1 and 2 represent individual Northern blot analyses. Type 1a col - Type 1α collagen, ALP - Alkaline phosphatase, CTR - Calcitonin Receptor.

Appendix D.1 Change in urine volume (mLs/day) from 15 weeks post-oophorectomy in osteopenic oophorectomised rats following DHT administration at 15 weeks post-oophorectomy and estradiol administration at 19 weeks post-oophorectomy.

Time (Weeks)	Vehicle + Vehicle	Vehicle + Estradiol	DHT + Vehicle	DHT + Estradiol
17	-0.1 (0.5) n=9	-0.7 (1.0) n=9	-1.7 (0.4)[a] n=9	-2.6 (0.9)[a] n=9
19	-0.8 (0.9) n=9	-0.8 (0.6) n=9	-0.9 (1.1) n=9	-3.0 (0.8) n=9
20	-0.5 (0.5) n=9	-0.2 (0.8) n=9	-1.1 (1.3) n=9	-2.6 (1.1) n=9

Values are Mean (SE). Values are corrected for 15 weeks post-oophorectomy. Time is weeks post-oophorectomy. DHT (80mg/kg bw) was administered at 15 weeks post-oophorectomy and Estradiol (20mg/kg bw) was administered at 19 weeks post-oophorectomy. [a]$P<0.05$ versus Vehicle + Vehicle and versus Vehicle + Estradiol.

Appendix D.2 Change in urine 24 hour creatinine excretion (mmol/day) from 15 weeks post-oophorectomy in osteopenic oophorectomised rats following DHT administration at 15 weeks post-oophorectomy and estradiol administration at 19 weeks post-oophorectomy.

Time (Weeks)	Vehicle + Vehicle	Vehicle + Estradiol	DHT + Vehicle	DHT + Estradiol
17	-0.001 (0.005) n=9	-0.015 (0.015) n=9	-0.030 (0.007) n=9	-0.053 (0.018) n=9
19	-0.011 (0.011) n=9	-0.014 (0.010) n=9	-0.027 (0.017) n=9	-0.062 (0.017) n=9
20	-0.006 (0.006) n=9	-0.005 (0.011) n=9	-0.039 (0.021) n=9	-0.062 (0.021) n=9

Values are Mean (SE). Values are corrected for 15 weeks post-oophorectomy. Time is weeks post-oophorectomy. DHT (80mg/kg bw) was administered at 15 weeks post-oophorectomy and Estradiol (20mg/kg bw) was administered at 19 weeks post-oophorectomy.

Appendix D.3 Urine volume (mLs/day) in osteopenic oophorectomised rats following DHT administration at 15 weeks post-oophorectomy and estradiol administration at 19 weeks post-oophorectomy.

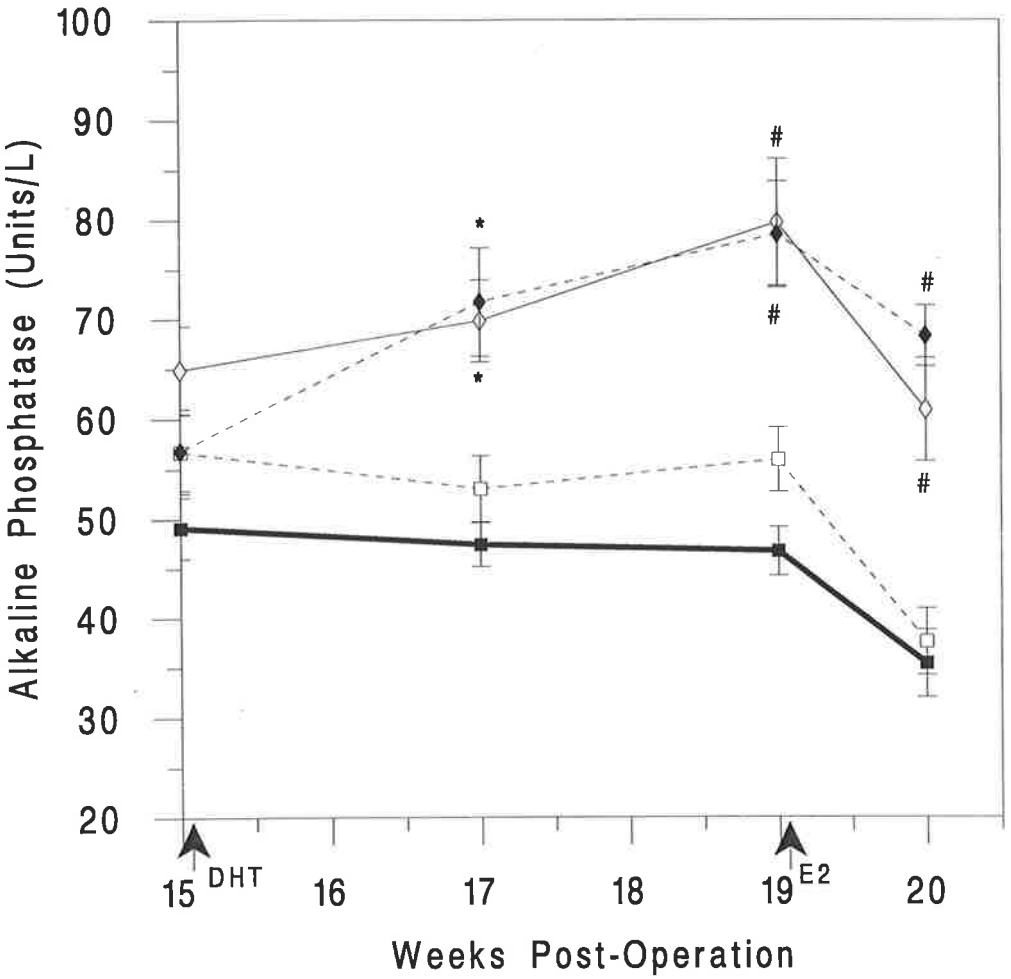
Time (Weeks)	Vehicle + Vehicle	Vehicle + Estradiol	DHT + Vehicle	DHT + Estradiol
15	5.0 (0.9) n=9	5.4 (0.7) n=10	4.5 (0.7) n=10	6.2 (0.8) n=10
17	4.8 (0.6) n=9	4.4 (0.6) n=10	2.8 (0.5)[a] n=10	3.6 (0.4)[a] n=10
19	4.2 (0.4) n=9	4.2 (0.7) n=10	3.3 (0.8) n=10	3.0 (0.5) n=10
20	4.4 (0.7) n=9	5.2 (0.7) n=10	3.2 (0.8) n=10	3.5 (0.7) n=10

Values are Mean (SE). Time is weeks post-oophorectomy. DHT (80mg/kg bw) was administered at 15 weeks post-oophorectomy and Estradiol (20mg/kg bw) was administered at 19 weeks post-oophorectomy. [a]$P<0.05$ versus Vehicle + Vehicle and versus Vehicle + Estradiol.

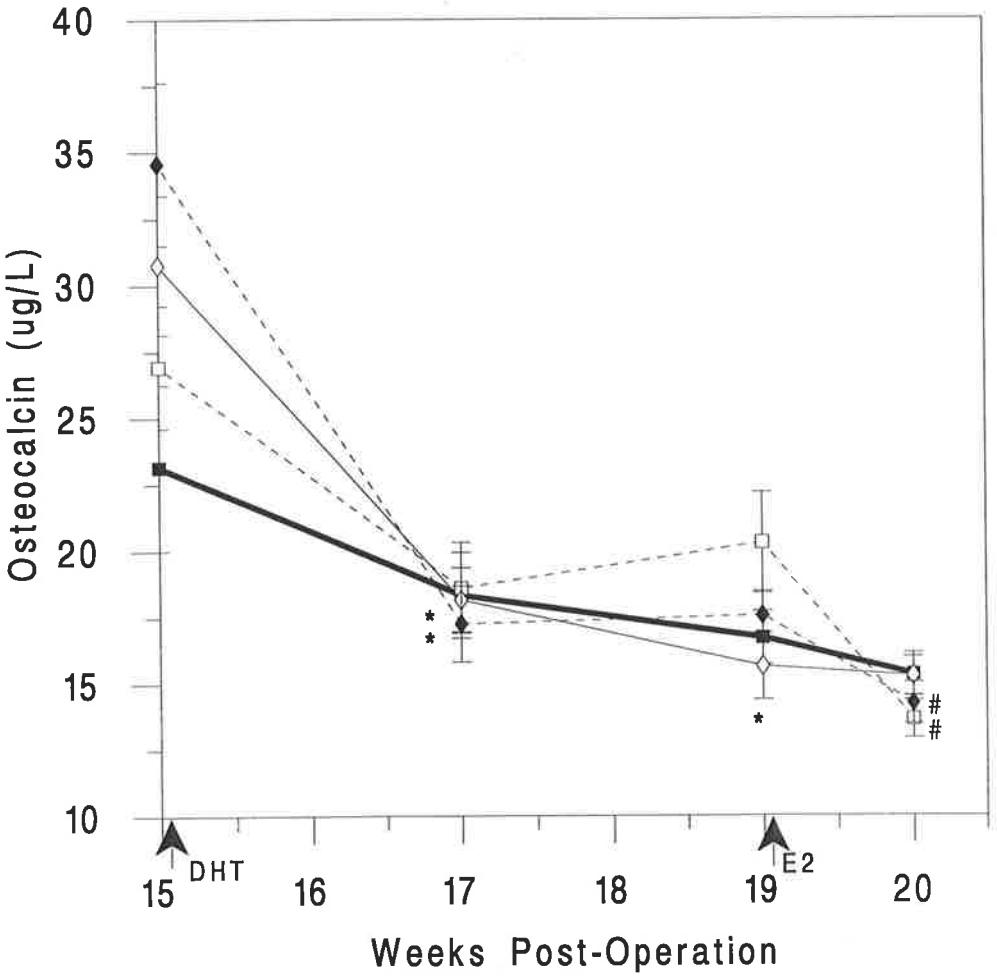
Appendix D.4 Urine 24 hour creatinine excretion (mmol/day) in osteopenic oophorectomised rats following DHT administration at 15 weeks post-oophorectomy and estradiol administration at 19 weeks post-oophorectomy.

Time (Weeks)	Vehicle + Vehicle	Vehicle + Estradiol	DHT + Vehicle	DHT + Estradiol
15	0.061 (0.004) n=9	0.076 (0.007) n=10	0.057 (0.008) n=10	0.074 (0.005) n=10
17	0.056 (0.005) n=9	0.054 (0.005) n=10	0.052 (0.007) n=10	0.070 (0.005) n=10
19	0.049 (0.007) n=9	0.062 (0.007) n=10	0.055 (0.006) n=10	0.061 (0.006) n=10
20	0.051 (0.005) n=9	0.067 (0.008) n=10	0.043 (0.005) n=10	0.049 (0.008) n=10

Values are Mean (SE). Time is weeks post-oophorectomy. DHT (80mg/kg bw) was administered at 15 weeks post-oophorectomy and Estradiol (20mg/kg bw) was administered at 19 weeks post-oophorectomy.



Appendix D.5 Serum alkaline phosphatase (units/L) in osteopenic oophx rats following DHT (80mg/kg body weight) administration at 15 following oophorectomy and estradiol (20mg/kg body weight) administration at 19 weeks following oophorectomy. Values are Mean ± SE, n=10. (■) Vehicle + Vehicle, (□) Vehicle + Estradiol, (◊) DHT + Vehicle, (◆) DHT + Estradiol. *P<0.01 versus Vehicle + Vehicle and versus Vehicle + Estradiol, $^{\#}P$<0.001 versus Vehicle + Vehicle and versus Vehicle + Estradiol $^{\$}P$<0.01 versus DHT + Vehicle.

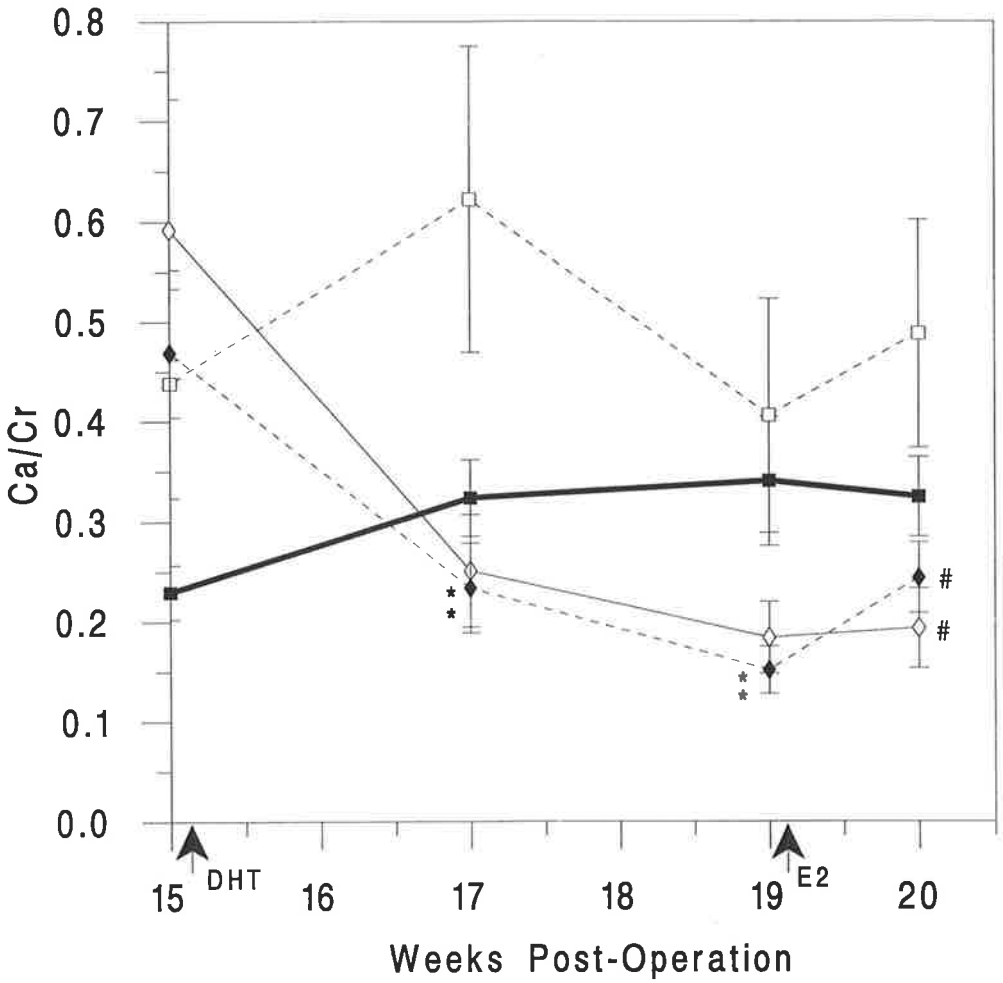


Appendix D.6 Serum osteocalcin (μg/L) in osteopenic oophx rats following DHT (80mg/kg body weight) administration at 15 following oophorectomy and estradiol (20mg/kg body weight) administration at 19 weeks following oophorectomy. Values are Mean ± SE, n=10. (■) Vehicle + Vehicle, (□) Vehicle + Estradiol, (◊) DHT + Vehicle, (◆) DHT + Estradiol. *P<0.005 versus Vehicle + Vehicle and versus Vehicle + Estradiol, #P<0.05 versus Vehicle + Vehicle and versus Vehicle + Estradiol.

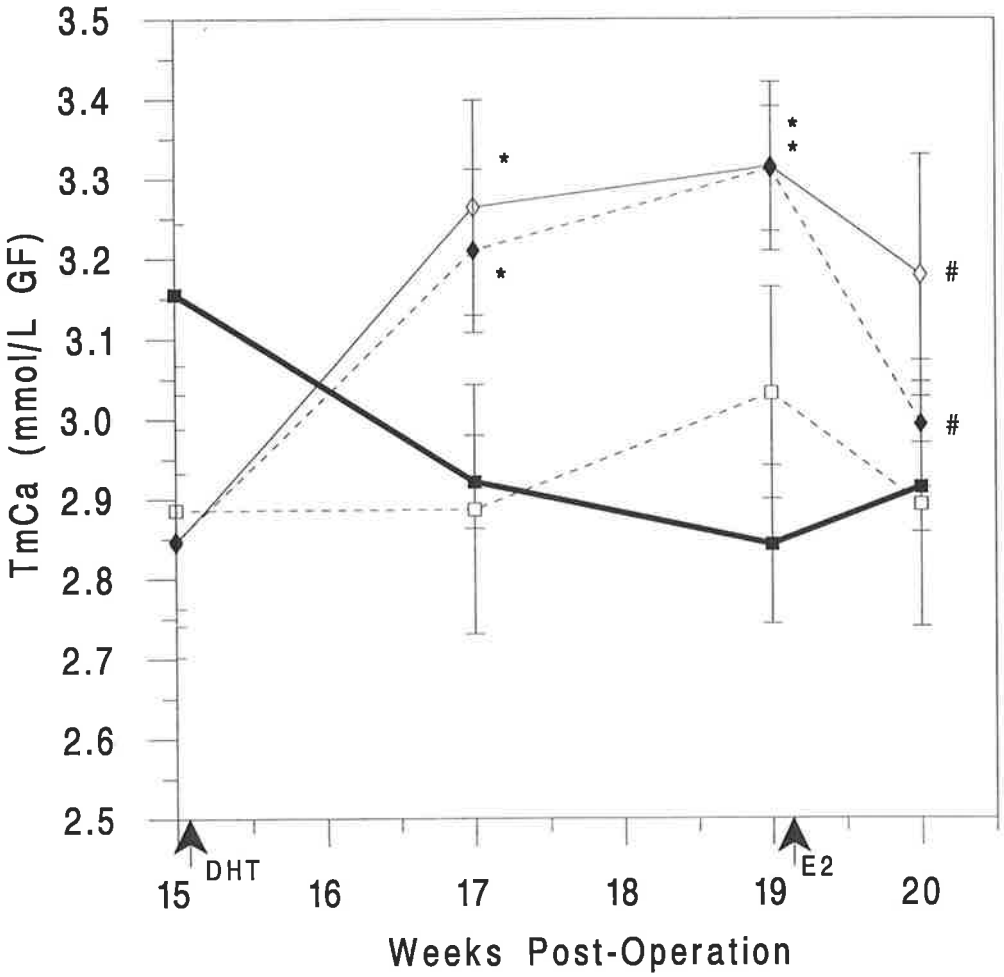
Appendix D.7 Urine hydroxyproline/creatinine (μmol/mmol) in osteopenic oophorectomised rats following DHT administration at 15 weeks post-oophorectomy and estradiol administration at 19 weeks post-oophorectomy.

Time (Weeks)	Vehicle + Vehicle	Vehicle + Estradiol	DHT + Vehicle	DHT + Estradiol
15	8.5 (0.5) n=9	8.7 (0.7) n=10	10.6 (2.1) n=10	9.2 (0.7) n=10
17	9.4 (0.3) n=9	9.2 (0.7) n=10	10.5 (1.1) n=10	10.4 (0.8) n=10
19	9.6 (0.5) n=9	8.4 (0.8) n=10	9.5 (0.9) n=10	10.7 (0.6) n=10
20	8.2 (0.8) n=9	9.6 (0.7)[a] n=10	9.4 (1.1) n=10	10.7 (1.0)[a] n=8

Values are Mean (SE). Time is weeks post-oophorectomy. DHT (80mg/kg bw) was administered at 15 weeks post-oophorectomy and Estradiol (20mg/kg bw) was administered at 19 weeks post-oophorectomy. [a]$P<0.005$ versus Vehicle + Vehicle and versus DHT + Vehicle.



Appendix D.8 Urine calcium/creatinine (mmol/mmol) in osteopenic oophx rats following DHT (80mg/kg body weight) administration at 15 following oophorectomy and estradiol (20mg/kg body weight) administration at 19 weeks following oophorectomy. Values are Mean ± SE, n=10. (■) Vehicle + Vehicle, (□) Vehicle + Estradiol, (◊) DHT + Vehicle, (◆) DHT + Estradiol. *P<0.001 versus Vehicle + Vehicle and versus Vehicle + Estradiol, #P<0.005 versus Vehicle + Vehicle and versus Vehicle + Estradiol.



Appendix D.9 Tubular reabsorption of calcium (TmCa) (mmol/L GF) in osteopenic oophx rats following DHT (80mg/kg body weight) administration at 15 following oophorectomy and estradiol (20mg/kg body weight) administration at 19 weeks following oophorectomy. Values are Mean ± SE, n=10. (■) Vehicle + Vehicle, (□) Vehicle + Estradiol, (◊) DHT + Vehicle, (◆) DHT + Estradiol. *P<0.001 versus Vehicle + Vehicle and versus Vehicle + Estradiol, #P<0.05 versus Vehicle + Vehicle and versus Vehicle + Estradiol.

Appendix D.10 Serum ionised calcium (calculated) (mmol/L) in osteopenic oophorectomised rats following DHT administration at 15 weeks post-oophorectomy and estradiol administration at 19 weeks post-oophorectomy.

Time (Weeks)	Vehicle + Vehicle	Vehicle + Estradiol	DHT + Vehicle	DHT + Estradiol
15	1.36 (0.02) n=9	1.33 (0.03) n=10	1.37 (0.03) n=10	1.36 (0.02) n=10
17	1.34 (0.02) n=9	1.35 (0.01) n=10	1.37 (0.03) n=10	1.34 (0.02) n=10
19	1.29 (0.03) n=9	1.36 (0.02) n=10	1.32 (0.02) n=10	1.31 (0.02) n=10
20	1.33 (0.02) n=9	1.32 (0.02) n=10	1.29 (0.03)[a] n=10	1.29 (0.02)[a] n=10

Values are Mean (SE). Time is weeks post-oophorectomy. DHT (80mg/kg bw) was administered at 15 weeks post-oophorectomy and Estradiol (20mg/kg bw) was administered at 19 weeks post-oophorectomy. [a]$P<0.05$ versus Vehicle + Vehicle and versus Vehicle + Estradiol.

Appendix D.11 Total serum calcium (mmol/L) in osteopenic oophorectomised rats following DHT administration at 15 weeks post-oophorectomy and estradiol administration at 19 weeks post-oophorectomy.

Time (Weeks)	Vehicle + Vehicle	Vehicle + Estradiol	DHT + Vehicle	DHT + Estradiol
15	2.64 (0.05) n=9	2.58 (0.06) n=10	2.65 (0.07) n=10	2.65 (0.04) n=10
17	2.53 (0.04) n=9	2.57 (0.02) n=10	2.61 (0.05) n=10	2.55 (0.03) n=10
19	2.43 (0.05) n=9	2.58 (0.04) n=10	2.49 (0.03) n=10	2.53 (0.04) n=10
20	2.50 (0.03) n=9	2.52 (0.03) n=10	2.38 (0.05)[a] n=10	2.40 (0.04)[a] n=10

Values are Mean (SE). Time is weeks post-oophorectomy. DHT (80mg/kg bw) was administered at 15 weeks post-oophorectomy and Estradiol (20mg/kg bw) was administered at 19 weeks post-oophorectomy. [a]$P<0.005$ versus Vehicle + Vehicle and versus Vehicle + Estradiol.

Appendix D.12 Ultrafiltrable calcium (mmol/L) in osteopenic oophorectomised rats following DHT administration at 15 weeks post-oophorectomy and estradiol administration at 19 weeks post-oophorectomy.

Time (Weeks)	Vehicle + Vehicle	Vehicle + Estradiol	DHT + Vehicle	DHT + Estradiol
15	1.83 (0.03) n=9	1.78 (0.04) n=10	1.83 (0.04) n=10	1.82 (0.03) n=10
17	1.79 (0.03) n=9	1.81 (0.02) n=10	1.86 (0.04) n=10	1.81 (0.02) n=10
19	1.72 (0.03) n=9	1.81 (0.03) n=10	1.77 (0.02) n=10	1.77 (0.03) n=10
20	1.77 (0.03) n=9	1.77 (0.02) n=10	1.72 (0.03)[a] n=10	1.71 (0.02)[a] n=10

Values are Mean (SE). Time is weeks post-oophorectomy. DHT (80mg/kg bw) was administered at 15 weeks post-oophorectomy and Estradiol (20mg/kg bw) was administered at 19 weeks post-oophorectomy. [a]$P<0.05$ versus Vehicle + Vehicle and versus Vehicle + Estradiol.

Appendix D.13 Serum protein bound calcium (mmol/L) in osteopenic oophorectomised rats following DHT administration at 15 weeks post-oophorectomy and estradiol administration at 19 weeks post-oophorectomy.

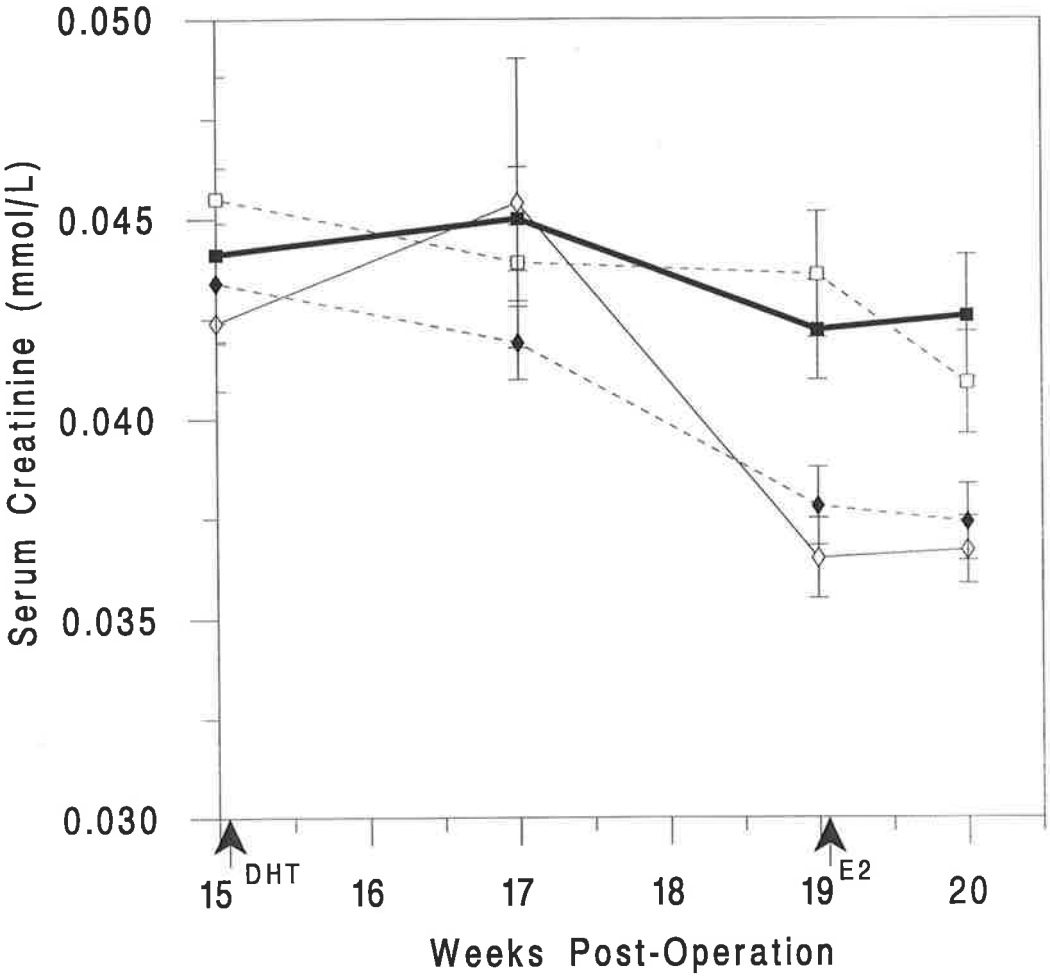
Time (Weeks)	Vehicle + Vehicle	Vehicle + Estradiol	DHT + Vehicle	DHT + Estradiol
15	0.81 (0.02) n=9	0.80 (0.02) n=10	0.82 (0.04) n=10	0.84 (0.02) n=10
17	0.74 (0.01) n=9	0.76 (0.01) n=10	0.75 (0.02) n=10	0.74 (0.01) n=10
19	0.71 (0.02) n=9	0.77 (0.01) n=10	0.72 (0.02) n=10	0.76 (0.02) n=10
20	0.73 (0.01) n=9	0.76 (0.02) n=10	0.66 (0.02)[a] n=10	0.69 (0.01)[a] n=10

Values are Mean (SE). Time is weeks post-oophorectomy. DHT (80mg/kg bw) was administered at 15 weeks post-oophorectomy and Estradiol (20mg/kg bw) was administered at 19 weeks post-oophorectomy. [a]$P<0.001$ versus Vehicle + Vehicle and versus Vehicle + Estradiol.

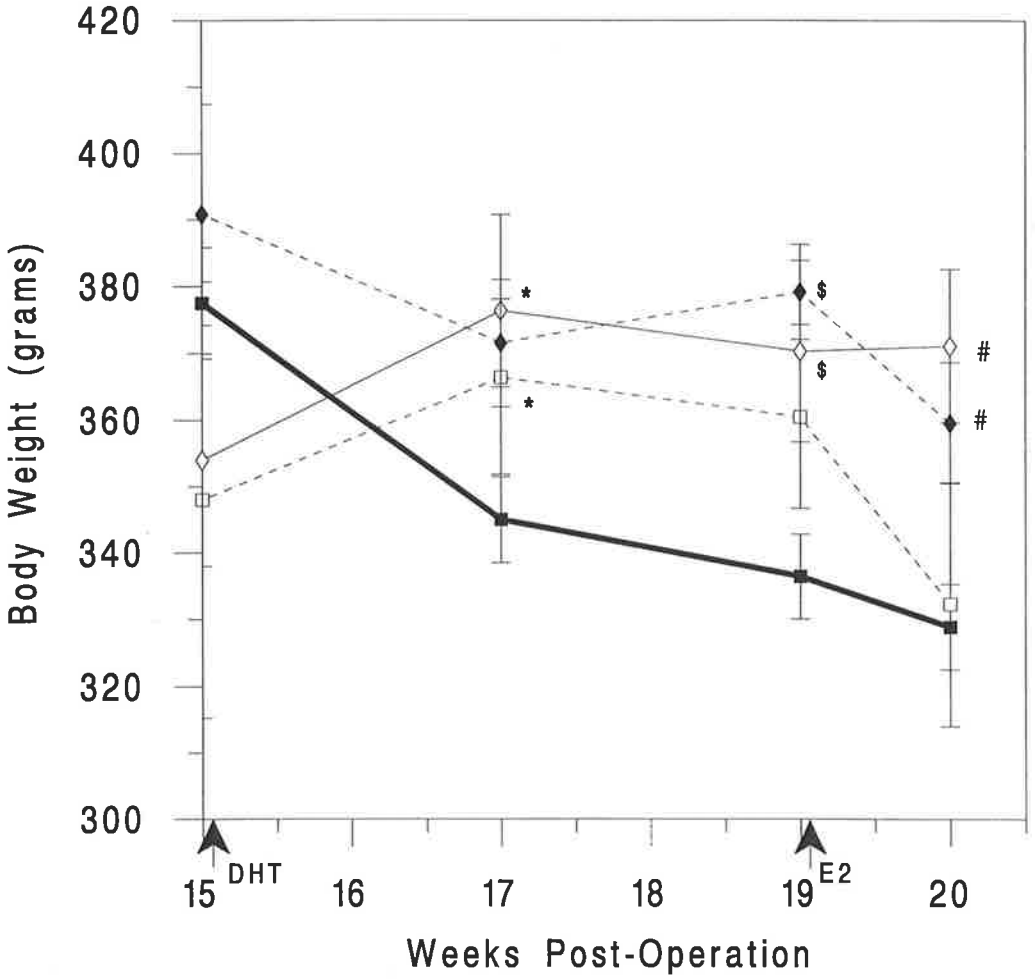
Appendix D.14 Serum complexed calcium (mmol/L) in osteopenic oophorectomised rats following DHT administration at 15 weeks post-oophorectomy and estradiol administration at 19 weeks post-oophorectomy.

Time (Weeks)	Vehicle + Vehicle	Vehicle + Estradiol	DHT + Vehicle	DHT + Estradiol
15	0.47 (0.01) n=9	0.45 (0.01) n=10	0.46 (0.01) n=10	0.46 (0.01) n=10
17	0.45 (0.01) n=9	0.46 (0.01) n=10	0.49 (0.01) n=10	0.48 (0.01) n=10
19	0.43 (0.01) n=9	0.45 (0.01) n=10	0.45 (0.01) n=10	0.46 (0.01) n=10
20	0.44 (0.01) n=9	0.44 (0.01) n=10	0.43 (0.01) n=10	0.43 (0.01) n=10

Values are Mean (SE). Time is weeks post-oophorectomy. DHT (80mg/kg bw) was administered at 15 weeks post-oophorectomy and Estradiol (20mg/kg bw) was administered at 19 weeks post-oophorectomy.



Appendix D.15 Serum creatinine (mmol/L) in osteopenic oophx rats following DHT (80mg/kg body weight) administration at 15 following oophorectomy and estradiol (20mg/kg body weight) administration at 19 weeks following oophorectomy. Values are Mean ± SE, n=10. (■) Vehicle + Vehicle, (□) Vehicle + Estradiol, (◊) DHT + Vehicle, (◆) DHT + Estradiol.



Appendix D.16 Body weight (grams) in osteopenic oophx rats following DHT (80mg/kg body weight) administration at 15 following oophorectomy and estradiol (20mg/kg body weight) administration at 19 weeks following oophorectomy. Values are Mean ± SE, n=10. (■) Vehicle + Vehicle, (□) Vehicle + Estradiol, (◊) DHT + Vehicle, (◆) DHT + Estradiol. *$P<0.01$ versus Vehicle + Vehicle and versus Vehicle + Estradiol, $^{\#}P<0.001$ versus Vehicle + Vehicle and versus Vehicle + Estradiol, $^{\$}P<0.005$ versus Vehicle + Vehicle and versus Vehicle + Estradiol.

Appendix D.17 Serum albumin (mmol/L) in osteopenic oophorectomised rats following DHT administration at 15 weeks post-oophorectomy and estradiol administration at 19 weeks post-oophorectomy.

Time (Weeks)	Vehicle + Vehicle	Vehicle + Estradiol	DHT + Vehicle	DHT + Estradiol
15	35.1 (0.8) n=9	34.4 (1.0) n=10	35.5 (1.5) n=10	36.2 (0.8) n=10
17	31.2 (0.9) n=9	31.6 (0.7) n=10	30.7 (0.5) n=10	32.1 (0.7) n=10
19	31.4 (0.4) n=9	31.8 (0.9) n=10	32.1 (0.6) n=10	33.5 (0.5) n=10
20	31.9 (0.5) n=9	33.7 (1.1) n=10	29.7 (0.5)[a] n=10	31.0 (0.5)[a] n=10

Values are Mean (SE). Time is weeks post-oophorectomy. DHT (80mg/kg bw) was administered at 15 weeks post-oophorectomy and Estradiol (20mg/kg bw) was administered at 19 weeks post-oophorectomy. [a]$P<0.001$ versus Vehicle + Vehicle and versus Vehicle + Estradiol.

Appendix D.18 Serum total protein (mmol/L) in osteopenic oophorectomised rats following DHT administration at 15 weeks post-oophorectomy and estradiol administration at 19 weeks post-oophorectomy.

Time (Weeks)	Vehicle + Vehicle	Vehicle + Estradiol	DHT + Vehicle	DHT + Estradiol
15	68.1 (1.7) n=9	71.4 (1.4) n=10	68.8 (2.4) n=10	71.3 (1.9) n=10
17	65.6 (1.1) n=9	66.6 (0.9) n=10	65.7 (1.3) n=10	64.2 (0.9) n=10
19	64.5 (1.2) n=9	67.3 (1.0) n=10	63.5 (2.2) n=10	67.3 (0.9) n=10
20	63.1 (1.3) n=9	66.1 (2.2) n=10	59.9 (0.8) n=10	62.0 (0.9) n=10

Values are Mean (SE). Time is weeks post-oophorectomy. DHT (80mg/kg bw) was administered at 15 weeks post-oophorectomy and Estradiol (20mg/kg bw) was administered at 19 weeks post-oophorectomy.

Appendix D.19

Signals obtained from the ImageQuant program for the mRNA levels of the osteoclast and osteoblast genes in osteopenic oophx rats administered DHT (80mg/kg bw) at 15 weeks post-oophorectomy and estradiol (20mg/kg bw) at 19 weeks post-oophorectomy determined by Northern and Southern blot analyses.

Northern 4	Type 1a col	GAPDH	Type 1a col	GAPDH
V + V	24498	1809571	3333	526774
	25171	665661	9423	238406
	37551	1527911	6743	525681
V + E2	37039	738810	11448	181964
	13282	483024	975.5	662839
	31835	702325	18960	249619
DHT + V	42239	1355949	19580	840714
	37500	1035740	4387	233133
	37211	475218		
DHT + E2	20974	861211		
	17142	913971		
	Northern 2		Northern 6	
Group	ALP	GAPDH	ALP	GAPDH
V + V	21125	173528	1597	93678
	11482	89712	2959	75777
	20716	186637	11046	96782
V + E2	19243	62961	2706	48350
	10455	129004	5632	139107
	36237	107363	2836	91642
DHT + V	22689	143990	1497	139300
	13262	99683	9024	102258
	25596	97685		
DHT + E2	15850	85102		
	26839	92467		
	29874	134518		
	Northern 2		Northern 6	
Group	Osteopontin	GAPDH	Osteopontin	GAPDH
V + V	64409	173528	215123	93678
	27064	89712	133810	75777
	45067	186637	2347	96782
V + E2	29071	62961	543.4	48350
	43409	129004	118568	139107
	37708	107363	112921	91642
DHT + V	26823	143990	69368	139300
	11575	99683	108691	102258
	35004	97685		
DHT + E2	15894	85102		
	25620	92467		
	71634	134518		

	Northern 1		Northern 5	
Group	**Osteocalcin**	**GAPDH**	**Osteocalcin**	**GAPDH**
V + V	141434	195270	111140	526774
	125416	92284	23616	525681
	239282	264458	129748	662839
V + E2	114434	108675	114264	840714
	99481	59770	126838	238406
	159149	138910	39966	181964
DHT + V	163558	187418	188324	249619
	130824	129146	40020	233133
	158325	124047		
DHT + E2	43141	116514		
	57594	135430		
	188594	189875		
	Southern 1			
Group	**CTR**	**GAPDH**		
V + V	206331	126161		
	185685	125889		
V + E2	240540	84284		
	164829	147267		
DHT + V	154542	104025		
	102250	129992		

DHT dose is mg per kg body weight. Northerns 5 and 6 represent individual Northern blot analyses. E_2- Estradiol, Type 1a col - Type 1α collagen, ALP - Alkaline phosphatase, CTR - Calcitonin Receptor.

Serum electrolytes

Serum potassium ($P<0.001$) and anion gap ($P<0.01$) were increased following DHT treatment at 20 weeks and 17 weeks post-oophorectomy respectively, however were unaffected by estradiol treatment (Appendix D.21, Appendix D.24). In contrast, serum chloride was decreased as a result of DHT treatment only at 17 weeks post oophorectomy ($P<0.05$) but was not affected by estradiol treatment (Appendix D.22). Serum sodium and bicarbonate levels were not affected by DHT or estradiol treatment (Appendix D.20, Appendix D.23). No interactions of combined DHT and estradiol treatment were observed on any of the serum electrolytes.

Uncorrected data is presented in Appendices D.25 to D.29.

Appendix D.20 Change in serum sodium (mmol/L) from 15 weeks post-oophorectomy in osteopenic oophorectomised rats following DHT administration at 15 weeks post-oophorectomy and estradiol administration at 19 weeks post-oophorectomy.

Time (Weeks)	Vehicle + Vehicle	Vehicle + Estradiol	DHT + Vehicle	DHT + Estradiol
17	0.39 (0.9) n=9	-0.21 (0.5) n=9	0.61 (0.6) n=9	0.08 (0.7) n=9
19	0.38 (0.8) n=9	-1.1 (0.7) n=9	-0.86 (0.9) n=9	-0.57 (1.1) n=9
20	1.03 (1.0) n=9	-0.09 (0.8) n=9	-2.3 (0.9) n=9	-0.71 (1.4) n=9

Values are Mean (SE). Values are corrected for 15 weeks post-oophorectomy. Time is weeks post-oophorectomy. DHT (80mg/kg bw) was administered at 15 weeks post-oophorectomy and Estradiol (20mg/kg bw) was administered at 19 weeks post-oophorectomy.

Appendix D.21 Change in serum potassium (mmol/L) from 15 weeks post-oophorectomy in osteopenic oophorectomised rats following DHT administration at 15 weeks post-oophorectomy and estradiol administration at 19 weeks post-oophorectomy.

Time (Weeks)	Vehicle + Vehicle	Vehicle + Estradiol	DHT + Vehicle	DHT + Estradiol
17	0.34 (0.09) n=9	0.29 (0.24) n=9	0.62 (0.31) n=9	0.53 (0.32) n=9
19	0.21 (0.26) n=9	0.47 (0.23) n=9	0.70 (0.13) n=9	0.79 (0.26) n=9
20	0.51 (0.23) n=9	0.22 (0.22) n=9	1.76 (0.35)[a] n=9	1.67 (0.35)[a] n=9

Values are Mean (SE). Values are corrected for 15 weeks post-oophorectomy. Time is weeks post-oophorectomy. DHT (80mg/kg bw) was administered at 15 weeks post-oophorectomy and Estradiol (20mg/kg bw) was administered at 19 weeks post-oophorectomy. [a]$P<0.001$ versus Vehicle + Vehicle and versus Vehicle + Estradiol.

Appendix D.22 Change in serum chloride (mmol/L) from 15 weeks post-oophorectomy in osteopenic oophorectomised rats following DHT administration at 15 weeks post-oophorectomy and estradiol administration at 19 weeks post-oophorectomy.

Time (Weeks)	Vehicle + Vehicle	Vehicle + Estradiol	DHT + Vehicle	DHT + Estradiol
17	1.22 (1.2) n=9	0.11 (0.9) n=9	-1.56 (1.3)[a] n=9	-2.22 (1.4)[a] n=9
19	1.22 (1.0) n=9	0.56 (0.9) n=9	-1.22 (1.3) n=9	-1.22 (1.4) n=9
20	2.79 (1.0) n=9	1.89 (1.0) n=9	-0.56 (1.5) n=9	1.67 (1.9) n=9

Values are Mean (SE). Values are corrected for 15 weeks post-oophorectomy. Time is weeks post-oophorectomy. DHT (80mg/kg bw) was administered at 15 weeks post-oophorectomy and Estradiol (20mg/kg bw) was administered at 19 weeks post-oophorectomy. [a]$P<0.05$ versus Vehicle + Vehicle and versus Vehicle + Estradiol.

Appendix D.23 Change in serum bicarbonate (mmol/L) from 15 weeks post-oophorectomy in osteopenic oophorectomised rats following DHT administration at 15 weeks post-oophorectomy and estradiol administration at 19 weeks post-oophorectomy.

Time (Weeks)	Vehicle + Vehicle	Vehicle + Estradiol	DHT + Vehicle	DHT + Estradiol
17	1.9 (1.3) n=9	4.1 (0.5) n=9	2.5 (0.9) n=9	3.5 (0.7) n=9
19	2.9 (0.8) n=9	3.2 (0.69) n=9	4.0 (0.7) n=9	3.3 (0.6) n=9
20	2.0 (0.7) n=9	3.4 (0.5) n=9	2.6 (0.6) n=9	1.5 (0.7) n=9

Values are Mean (SE). Values are corrected for 15 weeks post-oophorectomy. Time is weeks post-oophorectomy. DHT (80mg/kg bw) was administered at 15 weeks post-oophorectomy and Estradiol (20mg/kg bw) was administered at 19 weeks post-oophorectomy.

Appendix D.24 Change in serum anion gap (mmol/L) from 15 weeks post-oophorectomy in osteopenic oophorectomised rats following DHT administration at 15 weeks post-oophorectomy and estradiol administration at 19 weeks post-oophorectomy.

Time (Weeks)	Vehicle + Vehicle	Vehicle + Estradiol	DHT + Vehicle	DHT + Estradiol
17	-2.6 (1.4) n=9	-4.01 (0.8) n=9	0.34 (1.2)[a] n=9	-0.5 (1.1)[a] n=9
19	-3.5 (1.0) n=9	-4.3 (0.7) n=9	-3.1 (1.3) n=9	-1.9 (0.9) n=9
20	3.4 (0.8) n=9	-3.9 (1.8) n=9	-2.6 (1.1) n=9	-2.1 (1.4) n=9

Values are Mean (SE). Values are corrected for 15 weeks post-oophorectomy. Time is weeks post-oophorectomy. DHT (80mg/kg bw) was administered at 15 weeks post-oophorectomy and Estradiol (20mg/kg bw) was administered at 19 weeks post-oophorectomy. [a]$P<0.01$ versus Vehicle + Vehicle and versus Vehicle + Estradiol.

Appendix D.25 Serum sodium (mmol/L) in osteopenic oophorectomised rats following DHT administration at 15 weeks post-oophorectomy and estradiol administration at 19 weeks post-oophorectomy.

Time (Weeks)	Vehicle + Vehicle	Vehicle + Estradiol	DHT + Vehicle	DHT + Estradiol
15	139.2 (0.8) n=10	140.7 (0.6) n=10	140.5 (0.6) n=10	140.3 (0.9) n=10
17	139.6 (0.5) n=10	140.4 (0.6) n=10	140.8 (0.6) n=10	140.0 (0.5) n=10
19	139.5 (0.6) n=10	139.8 (0.4) n=10	139.7 (0.4) n=10	139.5 (0.4) n=10
20	140.2 (0.3) n=10	140.7 (0.4) n=10	138.2 (0.4) n=10	139.6 (0.5) n=10

Values are Mean (SE). Time is weeks post-oophorectomy. DHT (80mg/kg bw) was administered at 15 weeks post-oophorectomy and Estradiol (20mg/kg bw) was administered at 19 weeks post-oophorectomy.

Appendix D.26 Serum potassium (mmol/L) in osteopenic oophorectomised rats following DHT administration at 15 weeks post-oophorectomy and estradiol administration at 19 weeks post-oophorectomy.

Time (Weeks)	Vehicle + Vehicle	Vehicle + Estradiol	DHT + Vehicle	DHT + Estradiol
15	4.97 (0.26) n=10	4.74 (0.17) n=10	4.78 (0.16) n=10	4.73 (0.20) n=10
17	4.91 (0.16) n=10	5.10 (0.20) n=10	5.38 (0.21) n=10	5.47 (0.15) n=10
19	5.18 (0.30) n=10	5.47 (0.29) n=10	5.37 (0.23) n=10	5.50 (0.23) n=10
20	5.28 (0.12) n=10	5.04 (0.12) n=10	6.31 (0.27)[a] n=10	6.18 (0.33)[a] n=10

Values are Mean (SE). Time is weeks post-oophorectomy. DHT (80mg/kg bw) was administered at 15 weeks post-oophorectomy and Estradiol (20mg/kg bw) was administered at 19 weeks post-oophorectomy. [a]$P<0.001$ versus Vehicle + Vehicle and versus Vehicle + Estradiol.

Appendix D.27 Serum chloride (mmol/L) in osteopenic oophorectomised rats following DHT administration at 15 weeks post-oophorectomy and estradiol administration at 19 weeks post-oophorectomy.

Time (Weeks)	Vehicle + Vehicle	Vehicle + Estradiol	DHT + Vehicle	DHT + Estradiol
15	101.3 (0.9) n=10	103.0 (0.6) n=10	103.4 (0.9) n=10	102.8 (1.2) n=10
17	102.6 (0.7) n=10	102.7 (0.5) n=10	101.5 (0.7)[a] n=10	100.6 (0.3)[a] n=10
19	102.6 (0.6) n=10	103.3 (0.6) n=10	102.0 (0.7) n=10	101.3 (0.5) n=10
20	104.1 (0.5) n=10	104.5 (0.5) n=10	102.6 (0.7) n=10	104.2 (0.9) n=10

Values are Mean (SE). Time is weeks post-oophorectomy. DHT (80mg/kg bw) was administered at 15 weeks post-oophorectomy and Estradiol (20mg/kg bw) was administered at 19 weeks post-oophorectomy. [a]$P<0.05$ versus Vehicle + Vehicle and versus Vehicle + Estradiol.

Appendix D.28 Serum bicarbonate (mmol/L) in osteopenic oophorectomised rats following DHT administration at 15 weeks post-oophorectomy and estradiol administration at 19 weeks post-oophorectomy.

Time (Weeks)	Vehicle + Vehicle	Vehicle + Estradiol	DHT + Vehicle	DHT + Estradiol
15	26.8 (0.6) n=10	27.0 (0.6) n=10	27.9 (0.8) n=10	27.4 (0.8) n=10
17	28.7 (1.1) n=10	30.0 (0.9) n=10	29.2 (1.2) n=10	29.8 (0.9) n=10
19	29.7 (0.6) n=10	29.2 (0.9) n=10	31.5 (0.6) n=10	30.5 (0.6) n=10
20	28.8 (0.6) n=10	29.9 (0.4) n=10	30.2 (0.6) n=10	29.0 (0.8) n=10

Values are Mean (SE). Time is weeks post-oophorectomy. DHT (80mg/kg bw) was administered at 15 weeks post-oophorectomy and Estradiol (20mg/kg bw) was administered at 19 weeks post-oophorectomy.

Appendix D.29 Serum anion gap (mmol/L) in osteopenic oophorectomised rats following DHT administration at 15 weeks post-oophorectomy and estradiol administration at 19 weeks post-oophorectomy.

Time (Weeks)	Vehicle + Vehicle	Vehicle + Estradiol	DHT + Vehicle	DHT + Estradiol
15	17.2 (0.8) n=10	16.7 (0.8) n=10	15.3 (1.1) n=10	16.1 (0.9) n=10
17	14.6 (0.8) n=10	14.0 (0.8) n=10	16.6 (0.7)[a] n=10	16.3 (0.6)[a] n=10
19	13.7 (0.7) n=10	13.9 (1.0) n=10	12.8 (0.5) n=10	14.4 (0.7) n=10
20	13.8 (0.5) n=10	13.5 (1.3) n=10	12.9 (0.3) n=10	13.8 (0.6) n=10

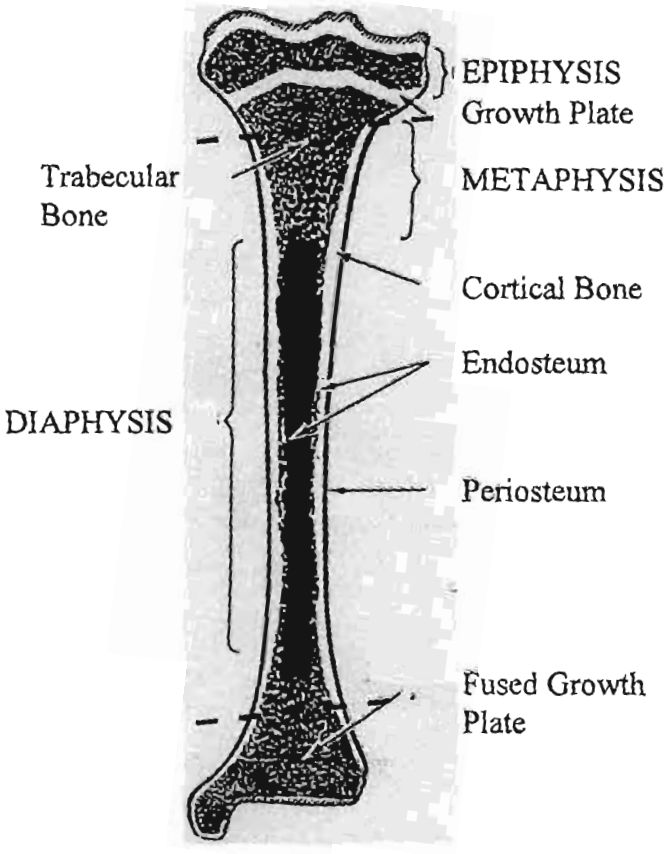
Values are Mean (SE). Time is weeks post-oophorectomy. DHT (80mg/kg bw) was administered at 15 weeks post-oophorectomy and Estradiol (20mg/kg bw) was administered at 19 weeks post-oophorectomy. [a]$P<0.01$ versus Vehicle + Vehicle and versus Vehicle + Estradiol.

Appendix D.30 Percentage changes from pre-treatment (15 weeks post-oophorectomy) in bone biochemical variables at 20 weeks post-oophorectomy following DHT and estradiol administration at 15 and 19 weeks respectively.

Variable	Vehicle + Vehicle	Vehicle + Estradiol	DHT + Vehicle	DHT + Estradiol
Serum Alkaline Phosphatase (Units/L)	-27.8	-33.7	-6.2	20.2
Serum Osteocalcin (µg/L)	-34.9	-49.4	-50.4	-58.8
Urine deoxypyridinoline/creatinine (nmol/mmol)	-22.5	-24.6	-41.2	-46.1
Urine Hydroxyproline (µmol/mmol)	-2.9	10.5	-10.8	29.4

DHT dose is 80mg/kg body weight and Estradiol dose is 20mg/kg body weight.

Appendix E.1



Diagrammatic representation of long bones used for RNA extraction. One femur and both tibiae were excised and the surrounding muscle tissue was removed. The ends of the bones were removed as indicated by the dashed line (---) and the bone cavity was flushed with ice cold normal saline to remove the bone marrow.

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