ASPECTS OF BONE QUALITY IN THE BROILER CHICKEN

By

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Thesis submitted for the degree of Doctor of Philosophy

in the

Faculty of Veterinary Medicine, University of Glasgow

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ACKNOWLEDGEMENTS

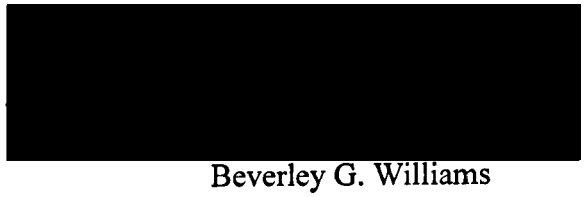
My biggest thank you must go to Dr Colin Farquharson and Dave Waddington, whose support and encouragement kept me going, and whose advice kept me straight. Colin's refrain of "keep smiling" sometimes resulted in more of a manic grin, but it always helped, and I also appreciated Dave's patience and humour while I attempted to grasp the fine art of statistics. Thanks also to Professor Sally Solomon, whose faith in our team, and timely support and advice was much appreciated. Drs Colin Whitehead, Murdo Macleod, and Jim McNab gave me advice on poultry nutrition, which was gratefully received, and Dr Barry Thorp arranged the initial funding, and introduced me to the mysteries of bone biology, for which I thank him. Thanks also to Ross Breeders Ltd and John Thompson and Sons Ltd, who not only provided funding, but also support and encouragement. I would also like to acknowledge MAFF for additional project funding, and British Poultry Science for funding my visit to the Workshop on Metabolic Disorders in Poultry in Turkey, 1999.

This project would have been impossible without the advice and help I received from many of the staff in the Department of Integrative Biology at the Roslin Institute. Special thanks go to Heather McCormack and Lynn Mcteir for their help and expertise with syringe, scalpel and mixing bowl, to Elaine Seawright for help with the plasma assays, to Bob Fleming for instructing me in the art of image analysis and the illicit fun of breaking bones, and to everyone who cheerfully mucked in on highly organised sampling days. Thanks also to Dr Sandra Wilson, Laura Dick, Louise Dunn, and Katrina Kivlin for help and advice on histology, to Dr Bob Scougal for discussions on the analysis of diets for phytate, and to the poultry staff for their help and co-operation. A big thank you goes to the members of the Roslin Institute Graphics Department, and especially Norrie Russell for all his hard work preparing the plates for this thesis. Special thanks must also go to the staff of Roslin Nutrition, Doug Currie, Anne Knox, and the boys in the feedmill for their hard work getting the diets right, and Audrey Laurie and Etta Leadbetter for advice and help with the mineral analysis. I would also like to thank Drs Yves Nys and John Williams and the staff at INRA Nouzilly for their advice and help with the osteocalcin assay, and their kind hospitality, and Drs Alan Bailey and Nick Avery at the University of Bristol for performing the pyridinoline analysis, and explaining to me how they did it.

Finally, I would like to say a big thank you to my family, Caroline Channing, Kay Farmer, and especially Colin Miller, for their total support, and welcome distractions during the course of this project, and for being there throughout the problems life threw at me along the way.

DECLARATION

I hereby declare that the work presented in this thesis was carried out by me personally, with the exceptions of the pyridinoline analysis which was performed by Dr Nick Avery at the University of Bristol, the cryosectioning and ALP and TRAP reactions, which were performed by Dr Colin Farquharson and Elaine Seawright, and Figures 1.1 and 1.2, which were adapted from diagrams published by Banks (1986).



Beverley G. Williams

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DEDICATION

I wish to dedicate this thesis to my family and my late Dad and Pop, they would have been proud.

SUMMARY

The high incidence of bone disorders causing lameness has been a cause of concern to the Broiler Industry for a number of years. Although some attention has been paid to bone mineral content, research into skeletal health has been predominantly concerned with the epiphyseal growth plates rather than the shaft of the long bones. The work contained in this thesis explores the development of the tibiotarsus shaft and the quality of cortical bone in the modern broiler, in relation to genetic background, growth rate and dietary mineral content. This was achieved through a series of experiments predominantly using a modern selected strain of broiler and an older, unselected control strain, and occasionally utilising current commercial stock. Birds were exposed to a variety of dietary mineral contents, and feeding regimes, and assessments were made of a number of aspects of bone quality, bone turnover, and mineral homeostasis at selected ages.

A number of phases in tibiotarsus development were identified from the embryo through to slaughter age at approximately six weeks; these were seen in all strains of broiler chicken investigated. Resorption and replacement of the embryonic cartilage model of the tibiotarsus was found to begin before day 16 of incubation, and a reduction in cortical thickness was observed between this age and hatch as the marrow cavity was enlarged. During this period, the cortex was observed to develop from a maze like scaffold, to display recognisable Haversian canals with the new bone being rapidly mineralised; by day 6, the cortex displayed a distinctive pattern of Haversian canals which remained consistent throughout much of the birds life-span. From hatch, cortical width began a rapid increase in thickness, which was seen to halt at approximately 18 days of age, cortical thickness remaining approximately constant from this age. The rapid increase in cortical mineral content was also seen to plateaux at approximately 11 days. These changes are discussed in terms of the skeletal function required at the various stages of development. Periosteal solidity was seen to increase, and endosteal solidity to decrease with age over the course of the bird's life-span, and hypotheses are put forward to account for these changes.

Although all the strains investigated appeared to show similar cortex development in the tibiotarsus, there were significant differences in aspects of the quality of the bone produced. Cortical bone of the selected and commercial strains was found to be thinner, less well mineralised and more porous than that of the control strain. Tibiotarsi were also found to be significantly shorter in the modern strains, which may counteract some of the reductions in cortical bone quality, however, the modern strains still had considerably

weaker and more elastic tibiotarsi at a given body weight. None of these differences were detected at the embryonic stage, and they are suggested to develop during rearing. Some evidence was observed of a dietary mineral effect on bone mineral content, however, it is suggested that other factors are also involved. Growth rate appeared to be the main factor controlling the solidity and thickness of the cortex, as evidenced by the improvements in these aspects of bone quality with feed restriction; potential mechanisms are discussed. The results indicate that either growth rate or body weight may be partly responsible for the shortening of the tibiotarsus in modern strain, however, there also appear to be genetic factors. Suggestions on further work needed to find practical ways of improving bone quality in the context of the Broiler Industry are made.

CHAPTER 1
BONE: A LITERATURE REVIEW & AIMS OF THESIS

1.1: INTRODUCTION

Man has bred domestic fowl since 2500BC, and throughout this time different characteristics have been predominant in selection according to usage. The use of quantitative and biometrical methods in poultry breeding have more recently resulted in an unprecedented improvement in the production efficiency of birds reared for meat; approximately 85-90% of the improvement in the growth of broilers has come about through genetic selection for body weight (Sherwood 1977; Havenstein *et al* 1994). There is a limit to what genetics can achieve, however, and the long-term consequences of this selection are unknown. The hyperphagic feeding behaviour of these fast growing birds leads to excessive fat deposition and reproductive problems. In addition, intense selection for growth rate has led to a number of health problems, including skeletal abnormalities (Reddy 1996).

Skeletal problems are an important welfare issue, and they are also recognised as one of the major factors limiting the performance of meat type birds (Day 1990). There are five primary causes of leg problems in broilers: nutritional disorders, infectious diseases, metabolic conditions, conformational problems, and mycotoxins in the feed (Morris 1993). Fast growing broilers that reach market weight at earlier ages are perceived to have more leg problems, and suffer greater losses than slower growing birds (Sullivan 1994). In addition, the severity of leg problems is highly correlated with the body weight of broilers (Morris 1993). In a survey 90% of commercially reared broilers were found to have a detectable gait abnormality, and 4% were affected to the point where they could walk only with great difficulty when strongly motivated. Lame birds may prefer to squat on litter when not forced to move; prolonged contact with litter results in breast blisters, hock burns, and ulcerated feet (Kestin *et al* 1992). Leg problems can therefore reduce growth performance due to reduced feed consumption (Sullivan 1994), and increase the mortality rate due to problems reaching food and water, the number of culls, carcass condemnations due to septicemia-toxemia, and downgrades due to trimming of the breast and legs (Morris 1993).

It is obvious that an improvement in the skeletal health of broilers would be of benefit both to the birds and the producers. Bone is a highly specialised form of connective tissue, and the principle calcified tissue of vertebrates. The most striking difference of bone from other forms of connective tissue is that bone is hard. This results from the deposition within an organic matrix of a complex mineral substance, composed chiefly of calcium, phosphate, carbonate, and citrate. The presence of this substance allows bone to fulfil its three main biological functions: provision of support to the musculature, protection of the internal organs, and storage of a reservoir of calcium for the body. Bone also has cells peculiar to it: osteoblasts, osteocytes, and osteoclasts. The interstitial substance of bone has a fibrillar structure similar to other connective tissue, and is composed of the organic framework (matrix) of bone, the inorganic (mineral) part, and water. The organic matrix has two chief components: collagenous fibres, and non-collagenous proteins.

Most bones have the same hollow cylindrical structure. Except in certain locations; for example the neck of the femur, the patella, and intrascapular joint surfaces; there is a specialised membrane of connective tissue surrounding the outside of a bone known as the periosteum. In young animals, especially in regions of rapid growth, this consists of an outer dense layer of collagenous fibres and fibroblasts, and an inner loose layer of osteoblasts and their precursor cells. In the quiescent adult state, the periosteum serves for the attachment of tendons and ligaments, and carries blood vessels, lymphatics and nerves. The inner layer retains osteogenic potency, and in fractures is activated to produce osteoblasts and new bone. Covering the inner surface of a bone is the endosteum, a thin layer of cells lining the walls of bone marrow cavities and Haversian canals of cortical bone, and covering the surface of trabecullar bone (McLean and Urist 1968).

1.2: THE FUNCTIONS OF BONE

Bone has many different functions: it provides a rigid skeleton, and by the muscle attachments of various bones allows locomotion; it protects vital organs, for example the brain, lungs, and heart; and it plays a crucial role in mineral homeostasis, helping to maintain a constant level of certain essential ions in plasma and tissue fluid (Patt 1976; Patt and Maloney 1975).

1.2.1: Mechanical Support

The mechanical role of the skeleton has been the subject of many studies. In 1638 Galileo made a theoretical assessment of bone structure, and concluded that hollow cylinders are stronger than solid rods (Bell 1959; 1969), whilst Ward in 1892 compared the architectural features of trabecullar bone in the femoral neck to a triangular bracket supporting a street lamp (Murray 1936). Later studies looked at bone as a material - one that has the tensile strength of cast iron, yet is three times lighter, and ten times more flexible (Ascenzi and Bell 1972). These properties are achieved through a structure composed of collagen fibrils with mineral incorporated in the junctions between them, and between fibres. The fibres in bone that resist pulling forces are composed of type I collagen, whilst the solid particles that resist compression are a crystalline form of calcium phosphate. Seventy to 80% of the variance in the ultimate strength of bone is accounted for by density (Smith and Smith 1976); however, this can be offset by changes in the material composition and structural geometry (Einhorn 1992). Variation in the arrangement of fibrils in individual bones allows adaptation for the role of that particular bone. For example the femur is capable of withstanding a greater force longitudinally than transversely, and of compression rather than tension (Reilly and Burstein 1975), which is ideal for its role in load bearing.

1.2.2: Adaptations for Flight

All birds possess a skeleton adapted for flight (Fedducia 1975) which is lighter and more rigid than the equivalent mammalian skeleton. For example, the skeleton of the pigeon accounts for 4.4% of its total body weight, whilst that of the rat accounts for 5.6% (Welty 1962). Similarly, the skeleton of the eagle is less than 7% of its body weight, whilst man's is double this (Hildebrand 1972). Bone density measured in over 20 species of birds and several species of mammals showed that the femur, tibia, ulna and radius are 10-15% less dense in birds than in mammals (Chappel 1978).

In birds, many bones of the skull and postcranial skeleton are hollow, and contain air sacs bounded by an epithelium continuous with that of the respiratory system (Bellairs and Jenkin 1960). Different species are pneumatised to different degrees. In the domestic fowl most cervical vertebrae, the fifth thoracic vertebra, the second and third sternal ribs, the first two vertebral ribs, the lumbosacral mass, pelvic girdle, parts of the sternum and coracoid, and the skull and humerus are all pneumatised (King 1957). The cavity of a pneumatised bone has a relatively greater volume and reduced density compared to the corresponding bone of other species of similar size. It also has a greater circumference but thinner cortex, giving it greater resistance to bending strains. Hence lightening of the skeleton has been achieved without compromising strength (Bellairs and Jenkins 1960).

Rigidity and further strengthening are provided by the fusion and concomitant deletion of some bones (Huxley 1871, Fedduccia 1975). The cranium and pelvic girdle are the most obvious areas of fusion; however, fusion and deletion occur extensively in the appendicular skeleton. In the wing, carpals, metacarpals and phalanges exhibit fusion, whilst the tarsals and metatarsals are fused in the leg (Wilson 1994).

1.2.3: Mineral Homeostasis and Acid-Base Buffering

Calcium, magnesium and phosphorus ions, in appropriate and constant concentrations, are essential for the proper working of many of the most delicate cellular mechanisms throughout the body (Rasmussen 1972). Of these, calcium is probably the most important. It is essential that the concentration of this ion stays constant in plasma, and in extra- and intracellular fluids. Mineral homeostasis is maintained by a complex system of controlling factors acting on the skeleton, absorption from the intestinal tract, and on excretion by the kidneys (Vaughan 1981). The skeleton contains a mineral reservoir that can be drawn on in conjunction with these other mechanisms in times of mineral imbalance. This reservoir contains 89% of the body's total calcium, 35% of its sodium, 80% of its carbonate and citrate, and 60% of its magnesium (Martin *et al* 1987). When the nutritional status is normal, the gut and kidneys are in control of mineral homeostasis. However, when the diet is low in calcium, and possibly also during early morning, calcium is mobilised from the skeleton to maintain plasma calcium concentration (Peacock and Nordin 1973). A drop in plasma calcium results in the secretion of parathyroid hormone, the main targets of which are the kidney and bone. This hormone plays a major role in the regulation of 1,25 dihydroxyvitamin D_3, the active metabolite of vitamin D_3, which increases active calcium uptake from the gut and tubular resorption from the kidneys (Taylor and Dacke 1984), and increases the activation of new bone remodelling units (Breslau 1992). Conversely, calcitonin, which inhibits bone resorption, is thought to combat hypercalcaemia and to protect the skeleton from excessive resorption in times of calcium stress (Taylor and Dacke 1984).

The role of bone remodelling in calcium homeostasis is particularly important during egg laying in birds, antler formation in deer, and in pregnancy and lactation in mammals (Dacke 1979; Parfitt 1981; Miller *et al* 1989). It is important for long term mineral homeostasis as it is virtually unlimited in capacity (Rasmussen 1961, Martin *et al* 1987), and it also allows for a relatively rapid mobilisation, and replacement over a longer period (Parfitt 1981). There is a labile fraction of bone mineral located chiefly, if not exclusively, in the newly formed and incompletely mineralised bone (McLean and Urist 1968). When blood plasma is depleted of calcium there is a rapid movement of this

element from the intercellular fluids to buffer any rapid changes in the Ca^{2+} content of plasma. The intercellular calcium is then replenished from the labile fraction of the bone mineral. As a rough guide, it may be said that the transfer of calcium from intercellular fluid to plasma provides a minute to minute adjustment, from the labile fraction to intercellular fluid an hour to hour adjustment, and from stable bone to blood by hormonal and cellular intervention a day to day adjustment (McLean and Urist 1968). There have been suggestions that different bone sites differ in their sensitivity and response to various levels of calcium stress (Wilson 1994). In large animals, physiological calcium stresses generally result in increased remodelling of cortical bone rather than trabecullar bone, so that a large amount of calcium can be mobilised with the minimum of impact on skeletal integrity (Parfitt 1981; Parfitt 1988). Bone formed by intramembranous ossification, for example the skull, is less commonly involved in the metabolic activities of the body.

Acid-base homeostasis is normally maintained by the excretion of acid at a rate equal to the rate of its production; however, in many pathological states the rate of non-carbonic acid production exceeds its excretion (Bettice 1984). It has long been demonstrated that bone carbonate content can be diminished in acidosis in animals (Goto 1918; Pellegrino and Farber 1960), and both carbonate and calcium have been found to be lost from human bone due to uraemia, the loss proportional to the severity of the condition. Thirty seven percent of carbonate and 5% of Ca in bone is in a labile form elutable with NH_4Cl, and it is this labile carbonate which is partly or completely lost during uraemia (Pellegrino and Biltz 1965). In metabolic acidosis, it has been suggested that the H^+ is neutralised in bone by CO_3^{2-}, thus increasing the HCO_3^- concentration in the extracellular fluid. In contrast, it is thought that in respiratory acidosis, dissolved CO_2 diffuses into bone, where it is hydrated to carbonic acid (H_2CO_3). (Biltz *et al* 1981). A large amount of sodium is lost from bone during acute acidosis, and it is thought that sodium is being exchanged for H^+ (Swan and Pitts 1955). The loss of bone calcium (Barzel and Jowsey 1969) and increase in urinary calcium excretion during chronic metabolic acidosis could also reflect proton buffering by bone (Lemann *et al* 1967). However, artificially induced acidosis in lambs resulted in a prompt increase in urinary calcium excretion, accompanied by a fall in plasma calcium, and an increase in circulating parathyroid hormone (PTH), which acts to increase circulating calcium by promoting bone resorption. It was suggested that the main site of the response to acidosis was in fact the kidney, where there was a failure to reabsorb calcium. The increased PTH levels and subsequent release of calcium from bone tissue were thought to be acting to redress the calcium balance, rather than directly participating in acid buffering (Scott et al 1993).

1.3: THE CONSTITUENTS OF BONE

1.3.1: Cellular Complement

Throughout the extracellular matrix of bone are channels and cavities, the surfaces of which are occupied by cells; these make up 15% of the volume of the bone. There are three main cellular components of bone, each associated with specific functions: osteoblasts with the formation of bone, osteocytes with the maintenance of bone as a living tissue, and osteoclasts with the resorption of bone.

Osteoblasts

Osteoblasts are mononucleate cells characterised by the presence of membrane-bound alkaline phosphatase; the amount present depends on the state of development and functional activity of the cell (McLean and Urist 1968).

In young bone, the surface of the bone where apposition is taking place is covered by active columnar osteoblasts and their precursors, osteoprogenitor cells. Active osteoblasts are 15-20 μm wide (McLean and Urist 1968), with the nucleus positioned away from the bone surface. They are irregular in contour and have many fine protoplasmic processes on the matrix surface. These penetrate unmineralised matrix, known as osteoid, through canaliculi and eventually contact osteocytes (Cameron 1972, Ham and Cormack 1979). These cell to cell junctions resemble the gap junctions associated with facilitated ion transport between cardiac and smooth muscle cells (Holtrop and Weinger 1971). Electron dense particles shown to have high calcium and phosphorus contents are occasionally found within mitochondria. In addition, within osteoblasts there are large extracellular membrane bound particles 100 nm in diameter known as matrix vesicles. The first mineral micro-crystals formed during mineralisation are associated with these, and the inner aspect of their membrane (Ali 1992, Anderson 1989). The surfaces of old bone where bone is not being laid down are covered by a single-cell layer of flattened quiescent osteoblasts (Kimmel and Jee 1977,1978). Resting osteoblasts have fewer intracellular organelles, however, these cells cannot be said to be inactive, and, surprisingly, many more of these cells synthesise DNA than osteoprogenitor cells. They are therefore capable of proliferation (although cell division not been observed), and there is no evidence that they cannot, with appropriate stimulation, perform the same functions as the osteoblasts of younger bone (Kimmel and Jee 1977). In areas of repair the osteoblasts adopt the 'active' morphology and, together with osteoclasts, replace old bone.

It has long been recognised that the main function of the osteoblast is to lay down osteoid (Owen 1963). The osteoblast synthesises procollagen type I, which is extruded and

cleaved to collagen; assembly into fibrils is achieved extracellularly (Prockop *et al* 1979). In addition to the synthesis of collagen, the osteoblast also secretes a number of non-collagenous proteins such as sialoprotein, osteocalcin, and osteonectin (Termine 1990). These, together with the carbohydrate protein complexes (proteoglycans) constitute the osteoid. The osteoblast has also been implicated in the deposition and exchange of calcium and other ions (Talmage and Grubb 1977, Anderson 1978, Matthews *et al* 1978). The synthesis of the inorganic matrix requires nucleation and accretion onto existing mineral crystals. The osteoblast may play at least three roles in the mechanism of calcification: they are the origin of matrix vesicles (Anderson 1978) which create several factors that facilitate crystallisation; they may exert some control on the movement of calcium ions in and out of bone fluid; and they have the capacity to store calcium in their mitochondria, and possibly pass it onto the matrix (Posner 1978a).

Osteoblasts are derived from osteoprogenitor cells, which are themselves derived from the primitive mesenchyme of the perichondrium (Friedenstein 1976, Jotereau and LeDouarin 1978, Owen 1978). The earliest formation of bone in the perichondrium arises from cells that differentiate on the outer edge of the original mesenchymal condensation. Studies have shown that osteogenic connective tissue cells on the periosteal and endosteal surfaces and in the Haversian canals are continuous with the stromal elements of the marrow (Vaughan 1981). TGF-ß is known to promote the proliferation of osteoblast precursors, IGF-1 has been found to stimulate the differentiation of these precursors into osteoblasts (Mundy 1999). Differentiated osteoblasts are affected by calciotropic hormones (parathyroid hormone, vitamin D metabolites), sex steroids (oestrogen), cytokines (interleukins), growth factors (IGF-1, TGF-ß) and thyroid hormones, amongst others (Price and Russell 1992).

Osteocytes

An osteocyte is an osteoblast that has been entombed within the matrix. The formation of osteocytes from functional osteoblasts proceeds by accumulation of collagen fibrils about the periphery of the plasma membrane. In newly forming osteocytes there is a preosseous zone immediately surrounding the cell being buried, with little or no mineral present. The collagen formed is loose in arrangement but in close contact with the entire plasma membrane; lacunae are not initially visible. There is then additional deposition of collagen by osteoblasts above and further reduction in cytoplasm and organelles. This is accompanied by collagen maturation, increased collagen fibril size, and mineralisation (Tonna 1973).

The morphology of the osteocyte varies with the age of the cell. Newly embedded osteocytes reduce in volume by approximately 30% (Marrotti 1976a). This continues as the osteocyte fills the lacuna with new bone, and the smallest osteocytes tend to be farthest from the bone surface (Yeager *et al* 1975). Organelles within the buried cell are reduced in number, but are initially qualitatively similar to those of functional osteoblasts. A further reduction in the number of organelles, and a decrease in the volume of cytoplasm (Ham 1969) accompany the process of cell burial. The significant reduction in the size of the cell eventually makes the lacuna more obvious. Nuclear integrity is retained during the degeneration of all other ultra-structures, however, in highly degenerated osteocytes the plasma membrane, and eventually nuclear integrity is lost (Tonna 1973). Recent evidence has indicated that osteocytes die through the process of apoptosis (programmed cell death); however, the significance of this observation has yet to be fully understood (Noble *et al* 1997).

Both the morphology and distribution of osteocytes vary with the site of the cell. In young woven bone osteocytes are present as closely but irregularly packed cells, almost indistinguishable from osteoblasts, and with relatively few and short cytoplasmic processes. In mature lamellar bone osteocytes are flattened and ovoid, possess numerous fine branching processes, and tend to be evenly spaced and uniformly oriented with respect to the long and radial axes of the lamellar system they occupy (Vaughan 1981).

The function of osteocytes is still very controversial. Some workers suggest that they are concerned only with the maintenance of the bone matrix (Ham and Cormack 1979). No osteocyte is further than 0.1-0.2 mm from a capillary that could serve as a source of nutrients, and those cytoplasmic processes running through canaliculi are bathed in bone fluid, another source of nutrients. The lacuna-canalicular system bathed in bone fluid forms an exchange area of 250 mm^2 per mm^3 (Baud 1968). It also communicates directly with the sub microscopic interfibrillar spaces of the bone matrix, representing an exchange area of 35,000 mm^2 per mm^3. The osteocyte network is therefore well equipped for the maintenance of bone matrix as a tissue. It may also act as a sensory array to detect the need for, and start the process of, bone remodelling (Lanyon 1992).

Other workers suggest osteocytes play an important role in mineral homeostasis by micro dissolution of bone mineral (Bonucci 1990). It has been suggested that there is a mini remodelling cycle involving osteocytes (Baud and Boivin 1978). There are repeated phases of perilacunar osteolysis (resorption) and osteoplasis (apposition) seen during the ageing of an osteocyte. These have been described as morphological aspects of the participation of osteocytes in bone turnover, mineral metabolism, and calcium homeostasis, under hormonal control (Tonna 1973). It has been suggested that there are two types of

bone resorption shown by osteocytes: the periodic removal and replacement of perilacunar bone; and resorption affecting bone beyond the perilacunar region, which occurs in close juxtaposition with osteoclastic resorption (Parfitt 1976, Chambers 1980). In this manner, it is suggested, osteocytes play a role in the release of mineral from bone to blood, and therefore in the homeostatic regulation of calcium concentration in body fluids (McLean and Urist 1968). There is no satisfactory evidence that any of the calciotropic hormones affect osteocytes directly. However, it has been suggested that those osteocytes associated by cytoplasmic processes with surface osteoblasts respond to changing levels of plasma calcium by releasing calcium from the lacuna walls (Talmage and Grubb 1977, Matthews *et al* 1978). Changes in the ultra-structure of such osteocytes have been observed following administration of parathyroid hormone (Krempin *et al* 1978).

Osteoclasts

Osteoclasts are large multinucleate cells, usually found on the surface of bone in close relationship with areas of resorption. During growth and reformation of the trabeculae of spongy bone in rapidly growing animals they are commonly seen enveloping the tip of each spicule of bone undergoing resorption (McLean and Urist 1968). The average number of nuclei is species dependent and also varies considerably within the individual (Kaye 1984). Bone resorption is preceded by the appearance of a large 'clear zone' on the cell surface, surrounding the ruffled border characteristic of an active osteoclast. The ruffled border consists of cytoplasmic folds interdigitating with the bone surface. It provides a large surface area for the extrusion of lytic enzymes and the uptake of degraded material from the resorption site. The clear zone acts as a seal, creating a microenvironment in which pH and enzyme concentrations suitable for osteolysis can be maintained (Vaes 1988). Podosomes within the clear zone mediate adherence of the osteoclast to the bone surface in a manner similar to the interactions between integrins on the cell surface and specific matrix components (Teti *et al* 1992).

It is now generally accepted that the key functional feature that defines a cell as an osteoclast is the ability to form lacuna resorption pits on a bone substrate (Anderson *et al* 1992, Chambers *et al* 1984). Osteoclasts adhere to the matrix through integrins (Parfitt 1984), and create a highly acidic micro-environment within the ruffled border, sealed between the osteoclast and the bone surface (Vaes 1968 a, b), which allows the removal of mineral from the bone. Removal of apatite crystals, leaving a temporary layer of demineralized matrix below the osteoclast (Parfitt 1984), has been attributed to the production of both citric and lactic acids (Vaes 1968a; b). Digestion of the organic matrix constituents then follows, involving lysosomal enzymes (including tartrate resistant acid

phosphatase, or TRAP) transferred from the ruffled border into the sub osteoclastic space (Vaes 1973; Doty and Schofield 1971). Three distinct cysteine proteases are able to both depolymerise fibrillar collagen and to degrade the resulting gelatin. These enzymes are regulated by the differing calcium concentrations that exist within the regions of the ruffled border. Both phases of bone resorption are tightly regulated, and changes in osteoclast acidity result from various enzyme mechanisms within the osteoclast; these include H^+/K^+ ATPases, Na^+/H^+ antiports (Baron *et al* 1985; Hall and Chambers 1990), chloride/bicarbonate exchanges, and carbonic anhydrase (Hall and Kenny 1985).

Osteoclasts form by the fusion of circulating mononuclear cells; themselves derived from the pluripotent haemopoietic stem cell (Walker 1975, Scheven *et al* 1985, Hagenaars *et al* 1989). The nature of the circulating mononuclear precursor remains uncertain, however, cells of the mononuclear phagocytic system, including monocytes and tissue macrophages, appear the most likely candidates for osteoclast precursors as they share a number of functional, cytochemical, and morphological features with osteoclasts. Several recent investigations have shown that a bone-derived stromal cell population is essential for osteoclastic differentiation (Takahashi *et al* 1988, Udagawa *et al* 1990). It is now thought that these cells produce macrophage colony stimulating factor-1 (MCSF-1), which attaches to receptors on the precursor cells, stimulating their differentiation into osteoclasts (Mundy 1999).

Control of the osteoclast population is thought to be by a combination of calcium concentration and prostaglandins. It is also recognised that certain locally produced agents, possibly other lymphokines, and osteoclast stimulating or activating factor, are also involved in bone resorption. Parathyroid hormone is a potent bone resorptive agent, and increases the numbers as well as the activity of osteoclasts (Vaughan 1981). Calcitonin also alters the formation and activity of osteoclasts (Chambers and Magnus 1982), and 1,25 dihydroxyvitamin D_3 increases bone resorption by enlarging the size of the ruffled border and increasing cell activity (Vaughan 1981). Although osteoclasts contain specific receptors for calcitonin, it is generally accepted that 1,25 dihydroxyvitamin D_3 and parathyroid hormone receptors are not present. It has been suggested that modulation of osteoclast function is by the production of osteoclast stimulating factor by the osteoblast; which has receptors for both calciotropic hormones; and that bone resorption can only proceed if osteoblasts are also present (McSheehy and Chambers 1986). However, this theory has been cast into some doubt, since recent evidence now suggests that osteoclasts do contain 1,25 dihydroxyvitamin D_3 (Mee et al 1996), and parathyroid hormone (Agarwala and Gay 1992) receptors.

Recent work (e.g. Kartsogiannis *et al* 1999) has identified a specific membrane-bound molecule produced by the osteoblast that stimulates the differentiation of osteoclast precursors in conjunction with macrophage colony stimulating factor. This has been assigned several names, for example osteoprotegin ligand (OPGL), receptor activator of NF_kB ligand (RANKL), and tumour necrosis factor related activator-induced cytokine (TRANCE). Two receptors have been proposed: RANK, the membrane-bound signalling receptor for RANKL, and a secreted receptor OPG, which is thought to act as a natural decoy to limit the actions of RANKL.

1.3.2: Bone Matrix

Bone matrix comprises a variety of organic molecules. Collagen is the major macromolecule in bone as in other connective tissues, though its structure varies with tissue (Prockop *et al* 1979). A high proportion of mature cortical bone by weight is collagen. There is also approximately 11% non-collagenous organic material present in mature cortical bone (Herring 1972), made up of proteoglycans, glycoproteins, plasma proteins, peptides and lipids. Whenever intense and rapid calcification of bone matrix occurs, increased amounts of non-collagenous proteins are present (Pugliarello *et al* 1973) Many matrix proteins have been postulated to play a role in mineralisation (Boskey 1989); these include chondrocalcin, proteoglycans, osteonectin, osteocalcin, and phosphoproteins. Their role may be to act either as nucleators (Neuman and Neuman 1958) or inhibitors of crystal growth; possibly depending on whether they are attached to collagen or are in solution (Linde *et al* 1989).

Collagen

A collagen molecule is a 300 nm long triple chain helix, containing 1014 amino acid residues per polypeptide chain (α chain). Each a chain has a repeating amino acid triplet sequence, Gly-X-Y, where the X and Y residues are frequently proline and hydroxyproline respectively. The repetitive triplet structure is essential for helical formation; glycine is the only amino acid small enough to be accommodated in the interior of the helix (Robins 1988), and the presence of proline and hydroxyproline in the α chains increases the stability of the triple helix. A variety of other amino acids which occupy the X and Y positions decrease helix stability, but are essential for their assembly into fibrils (Vaughan 1981). There is dramatic genetic polymorphism in collagen, resulting in at least 15 different variations on the molecule. The major forms that appear in bone and cartilage are known as type I and type II collagen, respectively. The collagen I molecule of bone is

composed of two slightly different types of α chains with different amino acid sequences; these are designated α_1(I) (2 chains) and α_2(I) (1 chain) (Robins 1988). Type I and II collagen are synthesised by osteoblasts and chondrocytes as part of longer procollagen molecules containing additional pro-peptides. The procollagen molecules are secreted into the matrix for further processing, and the collagen molecules produced by cleavage of procollagen spontaneously assemble into fibrils. Collagen molecules aggregate so that each molecule is longitudinally displaced by approximately one quarter of its length relative to its nearest neighbour. This leaves a hole between the end of one triple helix and the beginning of the next, and it has been suggested that this hole may serve as a nucleation site for calcium apatite crystal formation (Vaughan 1981).

Newly deposited collagen fibrils are stabilised by cross-links formed by the action of lysyl oxidase on lysine and hydroxylysine residues in the teleopeptide domains of the collagen molecule. These eventually mature to the trivalent structures: pyridinoline, incorporating a hydroxylysine residue, and deoxypyridinoline incorporating a lysine residue (Calvo *et al* 1996, Farquharson *et al* 1989). These cross-links provide the collagen with the tensile strength necessary for its function (Farquharson *et al* 1989). In the last decade, urinary levels of the two forms of pyridinoline cross-links have been the most studied markers of bone resorption (Demers and Kleerekoper 1994). These markers are highly specific: both are absent from skin, except for a small amount in blood vessels (Reiser *et al* 1987), and although they are present in the diet, they are not absorbed, thus diet does not contribute to their urinary levels (Calvo *et al* 1996). In addition, since the formation of these cross-links occurs only as a final stage in the maturation of collagen fibrils, they mark the degradation of mature collagen, not its intermediates (Robins 1995). Deoxypyridinoline is present mainly in bone; pyridinoline has a wider tissue distribution, its concentration being highest in cartilage, however, it also constitutes one of the major cross-links in bone. The ratio of the two in bone is 4:1 pyridinoline to deoxypyridinoline (Eyre *et al* 1988).

Carbohydrate-Protein Complexes

Carbohydrate-protein complexes can be divided into two categories: protein-polysaccharides (proteoglycans), largely found in cartilage; and glycoproteins, largely found in bone matrix. Proteoglycans consist of a protein core, or chain, to which are attached a number of polysaccharide chains (the glycosaminoglycans) containing repeating disaccharide units. The glycosaminoglycans are thought to be responsible for the diverse number of functions attributed to proteoglycan molecules. Glycoproteins differ from proteoglycans in the nature of the carbohydrate groups. These do not have a repeating

disaccharide group, usually contain a relatively small number of monosaccharide residues, and have a wide variety of sugars (Vaughan 1981).

A number of proteoglycans are found in bone matrix (Ruoslahti 1989). During early bone development a large chondroitin sulphate proteoglycan, versican, is found primarily in the loose interstitial mesenchyme; its function may be to capture space destined to become bone (Fisher and Termine 1985). As development proceeds, it is replaced by 2 smaller species: in pericellular areas and lacunae, by biglycan (previously known as PG-I); and distributed throughout the developing bone matrix, by decorin (previously PG-II), thought to regulate collagen fibril growth (Bianco *et al* 1990, Robey *et al* 1992).

In the growth plate proteoglycans perform a structural function in conjunction with collagen. The combination of these two macromolecules is responsible for the elastic yet tough property of the tissue in non calcified regions, and the load bearing properties of the growth plate are largely attributed to the osmotic properties of the proteoglycans confined within the collagen network. These macromolecules are also involved in mineralisation, although the exact nature of this involvement is not yet understood (Howell and Dean 1992).

A number of specific glycoproteins have now been isolated from bone matrix and analysed. These include sialoprotein, osteonectin, osteopontin, and osteocalcin, all of which appear to be synthesised by osteoblasts (Herring 1979), although osteocalcin is the only one thought to be exclusively synthesised by osteoblasts (Beresford *et al* 1984, Corlett *et al* 1990). Glycoproteins have many different functions in bone. Some may have a role in collagen interactions, controlling fibril formation as in tendon (Anderson *et al* 1977), and they are clearly associated with the complex process involved in matrix calcification.

Osteocalcin (also called bone Gla-protein or BGP) is a single-chain, γ-carboxyglutamic-acid-containing protein of low molecular weight, approximately 58000 daltons (Beresford *et al* 1984, Corlett *et al* 1990). Approximately 15% of newly synthesised osteocalcin is released into the circulatory system; the other 85% is incorporated into bone (Parfitt and Kleerekoper 1984), where it binds strongly to the surface of hydroxyapatite crystals, and less strongly to amorphous calcium phosphate (Price *et al* 1977, Ndiaye *et al* 1993). It is the most abundant non-collagenous protein of bone, comprising 15-20% of this protein fraction (Corlett *et al* 1990). Synthesis of osteocalcin is stimulated by the action of 1,25 dihydroxyvitamin D_3 (Markose *et al* 1990), and is dependent on the presence of vitamin K (Price *et al* 1977). However, the regulation of synthesis is multi-factorial, involving vitamins, minerals, and local growth factors (Ndiaye *et al* 1993).

The physiological role of osteocalcin is currently unknown; however, it has been suggested that it may play a part in preparing osteoid for mineralisation (Lian *et al* 1982). Glutamic acid-containing proteins, including osteocalcin, appear coincident with, or just prior to the mineralisation to the embryonic chick bone (Hauschka and Reid 1978), in the foetal rat bone (Allen *et al* 1981), and in ectopic bone induced by implanted de-mineralised bone powder in the rat (Hauschker and Reddi 1980). However, evidence has also been found for the involvement of osteocalcin in the bone resorption process. In vitro, the protein is chemotactic for osteoclasts and their precursors (Malone *et al* 1982, Mundy and Poser 1983). An in vivo resorption model has also shown that bone particles depleted in osteocalcin are poorly resorbed compared with normal bone (Lian *et al* 1984). In addition, Glowacki *et al* (1991) showed that hydroxyapatite crystals prepared with purified osteocalcin were partially resorbed and generated multinucleate cells with osteoclastic characteristics, while those containing collagen, albumin, or apatite alone were not resorbed. They suggested that osteocalcin acts as a matrix signal for the recruitment and differentiation of bone resorbing cells. In spite of the fact that its function remains undefined, serum osteocalcin is now widely used in both clinical and basic research as an indicator of the rate of bone formation activity (Patterson-Buckendahl *et al* 1995). Plasma osteocalcin levels have been found to correlate well with known bone development patterns determined by other measures of bone formation rate, for example the number of chondrocytes in the columnar zone of the growth plate, tetracycline labelling, and ^{45}Ca labelling (Corlett *et al* 1990).

1.3.3: Mineral Content

The inorganic mineral phase of bone constitutes two thirds of the total matrix volume (Green 1994). For vertebrate tissues, it has long been known to be composed principally of calcium and phosphorus; carbonate was subsequently found to be a constituent by Levy (1894). The x-ray structure of bone and tooth mineral was identified by Rosenberry *et al* (1931) as hydroxyapatite. Although hydroxyapatite is now generally accepted as the basic template for mature bone mineral, the exact structure and chemical composition of this mineral are still uncertain. The theoretical fraction of calcium in hydroxyapatite is 40.3%, and of phosphorus, 18.4%. However, the calcium and phosphorus content of hydroxyapatite in human bone vary between 18.5 and 62%, and 7.7 to 27% respectively (Grynpas *et al* 1997, Cohen *et al* 1991).

Hydroxyapatite and The Apatite Family

Hydroxyapatite is a member of a large family of isomorphous substances named apatites (Narasaraju and Phebe 1996). Apatite structure can be represented as $A_{10}(XO_4)_6Z_2$ (Nàray-Szabò 1930), in the case of hydroxyapatite, $Ca_{10}(PO_4)_6(OH)_2$ (Vaughan 1981), giving a molar Ca:P ratio of 1.67 to 1. 'A' can be any of a number of ions, including Ca, Na, Mg, Pb, Mn, Sr, K, Ag, Zn, Cd, Bi, Sc^{3+}, and U^{4+}, whilst 'XO_4' can be PO_4, SiO_4, SO_4, VO_4, AsO_4, CrO_4, or CO_3 groups (Nathan 1984, Posner 1987). The most biologically important substitution for PO_4 is CO_3. 'Z_2' is generally OH, F, or Cl (Liu and Comodi 1993), however carbonate can also substitute for OH (Posner 1987).

A characteristic property of apatites, and therefore hydroxyapatite, is their ability to undergo a series of both isoionic (same ion type) and heteroionic (different ion type) substitutions involving both cations and anions, the criteria being a similarity of charge and sizes between the ions concerned. In addition, a few substitutions involving the replacement of an ion by one of a different charge are possible, with appropriate compensation of the imbalance in charge occurring elsewhere in the lattice (Narasaraju and Phebe 1996).

Composition of Real Bone Mineral

Real bone mineral crystals are not ideal in structure or composition. Many discontinuities are formed as the crystals grow, and others are formed with time, meaning that not all unit cells of a crystal are identical in structure (McLean and Urist 1968). Thus, while mature crystals in bone have an overall structure similar to hydroxyapatite, they should properly be referred to in a general sense as a carbonate apatite containing acid phosphate groups (Rey *et al* 1995). It is this lack of perfection, and the incorporation of foreign, sometimes unstable ions, which gives bone mineral its specific and complex biological properties. Its rate of dissolution, binding capacity, and potential for cellular interactions are altered to make bone mineral crystals metabolically active (Landis 1996).

Bone mineral is also not a single, homogeneous chemical entity. It has been described as consisting mainly of Ca^{2+}, PO_4^{3-}, OH^-, CO_3^{2-}, and citrate, with an inclusion of small amounts of other ions, especially Na^+, Mg^{2+}, and F^-. The dimensions of the citrate ion ($C_6H_5O_7^{3-}$) are too large for substitution into the crystal lattice, and citrate is thought to exist as a separate phase with a special relationship to the surfaces of crystals in bone mineral. In addition, bone mineral is also thought to include amorphous calcium phosphate with a range of molar Ca:P ratios of 1.45-1.55 (McLean and Urist 1968), and labile forms of various ions, including calcium, carbonate, and phosphate. This heterogeneity of bone mineral leads to variations in the molar Ca:P ratio of bone from the 1.67 to 1 predicted by

the hydroxyapatite crystal structure. For example, Bogart and Hastings (1931) found molar ratios of 1.57-1.61 in Bovine bone, consistently lower than the 1.67 molar Ca:P ratio of hydroxyapatite.

Bone Carbonate

Carbonate is the third most abundant ion in bone mineral, and constitutes approximately 5% of the total weight of the bone (Eastoe 1961). The overall carbonate content of bone has been shown to be dependent on the Ca:P ratio of the serum, as influenced by dietary Ca:P ratios and contents (Sobel and Hanok 1948). In addition, when the bone molar Ca:P ratios of 16 different species were examined in relation to carbonate content, a very good correlation between the two was found, suggesting that the differences seen in molar Ca:P ratios are directly attributable to variations in carbonate content (Pellegrino and Biltz 1968).

The structural role of carbonate has been a matter of controversy. This is perhaps compounded by the fact that unless heating of a carbonate apatite is performed in an atmosphere of CO_2, the carbonate ions decompose, leading to the formation of hydroxyl ions (Rey *et al* 1995). In well-crystallised mineral, the position of carbonate appears to be within the crystal lattice itself (Rey *et al* 1991a), where it is the most abundant foreign ion in the hydroxyapatite lattice (Vignoles *et al* 1988). Carbonate can occupy either one of two distinct lattice sites (Bonel 1964). Type A substitution involves the replacement of one OH^- group by CO_3^{2-}, a much larger ion, and the removal of a neighbouring OH^- ion; this resolves both the size and charge differences (Driessens *et al* 1983). Type B substitution involves the replacement of a single PO_4^{3-} group by a CO_3^{2-} group (De Maeyer *et al* 1993; Liu and Comodi 1993). This type of substitution occurs only in biological apatite, and in synthetic hydroxyapatite prepared at high temperatures (Elliot *et al* 1985). The ratio of site A to site B substitution remains constant (0.7-0.9) throughout the ageing of bone mineral in a variety of species (Rey *et al* 1989). The conditions that govern the specific quantitative relationship between the two sites have not yet been established (Rey *et al* 1991b). The presence of carbonate within the lattice induces structural disorders (Cuisinier *et al* 1992), which may subsequently favour biological apatite nucleation; the solubility product of crystals is increased, and for the same pH value, more calcium and phosphate ions are available for further calcification (Cuisinier *et al* 1995).

Eanes and Posner (1970) assumed that most of the carbonate in bone is adsorbed onto the surface of the mineral crystal, and many consider that this readily exchangeable surface carbonate in bone may be needed to help maintain a constant serum pH in conditions such as acidosis (Vaughan 1981). Modern techniques have recently revealed a

third type of carbonate species regularly present in the apatite structure of bone mineral (Rey *et al* 1991b). This species has been shown to be related to the easily soluble domains of crystals (Rey *et al* 1989), and appears to correspond to an unstable (labile) site. It is thought that it is variations in the concentrations of this particular carbonate species that may be important in the role bone mineral plays as a carbonate ion reservoir (Rey *et al* 1991b).

Variation in Mineral Composition with Site and Age

Bone is a biomaterial that is structurally adapted to different functions and loading situations, therefore the exact composition of mineral may vary depending on the type and site of bone (Mellors and Solberg 1966). For example, Urist and Dowell (1967) noted that the chemical composition of the earliest mineral deposits is different in the zone of provisional calcification (cartilage) to the composition in new diaphyseal bone. The molar Ca:P ratio in calcifying cartilage was found to be below 1.6, whilst in bone it was found to be 1.6 or higher (McLean and Urist 1968). It was suggested that the mineral in calcified cartilage is either octacalcium phosphate ($Ca_8H_2(PO_4)_6.5H_2O$) or hydrated tricalcium phosphate ($Ca_9H(PO_4)_6OH$) (Urist and Dowell 1967).

Woodard (1962) found that the molar Ca:P ratio in human bone varied between 1.37-1.71, with the lowest values being found in children and the elderly. The first calcium phosphate mineral formed in bone is suggested to be amorphous in form, and is thought to be comprised of tricalcium phosphate ($Ca_3(PO_4)_2$), with a molar Ca:P ratio of 1.5 to 1. This mineral is apparently predominant in younger bones, and is partially transformed into crystalline hydroxyapatite with age (Narasarju and Phebe 1996); in the rat, the crystallinity of bone mineral levels off at 65% at maturity (Termine 1966).

While stoichiometric hydroxyapatite contains phosphate as PO_4^{3-}, vertebrate bone mineral contains, in addition, HPO_4^{2-}, and $H_2PO_4^{1-}$ (Landis 1996), and the phosphate content has been found to vary both with the age and maturation of the mineral and the bone tissue (Roberts *et al* 1992). It has been recently found that the HPO_4 ions in bone mineral are different from those in other forms of calcium phosphate (Rey *et al* 1995). In general, bone mineral contains approximately 5-10% HPO_4^{2-} (Rey *et al* 1991b).

Combined with the presence of labile, very reactive non-apatitic carbonate, HPO_4 and PO_4 groups, and the significant changes in their concentration with crystal age and maturation, evidence suggests that the biological functions of bone crystals are intimately related to a specific arrangement and environment of their atomic and ionic constituents (Rey *et al* 1995).

Bone Crystal Shape and Surface Chemistry

The qualities of bone crystals satisfy the need of bone mineral to be insoluble enough to be stable, but reactive enough for normal resorption needs. The most distinguishing features of bone apatite are a small crystal size, a lack of chemical perfection, and internal crystalline disorder (Posner 1978b). The crystals are very small, approximately 200 x 30-70 Å, giving an enormous surface area of 100-200 m^2/g of bone. The crystal's impurity follows on from this, since at the time of formation some of its exposed constituent ions can be replaced by other ions (Neuman and Neuman 1958).

Like its chemical composition, the exact size and shape of the hydroxyapatite crystals has long been a matter for discussion. Early and more recent studies have indicated that crystals are either needle-like (*e.g.* Engstrom and Finean 1953, Traub *et al* 1992, Fratzl *et al* 1993) or thin and plate-like (*e.g.* Robinson and Bishop 1950, Arsenault 1988). One of the problems in studying the size and shape of bone crystals is the dense accumulation of crystals, and their intimate association with collagen fibrils. After separation of the crystals from the organic matrix, a more recent study found that crystals from the bone of species as varied as chicken, fish, and bovine exist as small, thin plates. These were found to be curved, bent, and folded on themselves and immediately adjacent crystals, giving the appearance of dense thin lines, and the illusion that thin needle-like crystals were being viewed. Although they found variability in the size and distribution of crystals in the four species, they found little difference in their average dimensions of 16 nm width, by 27 nm length, by approximately 2 nm thick (Kim *et al* 1995). The same method was also used to isolate crystals from calcified cartilage. Again, no needle-like crystals were seen. Crystals were larger in the cartilage when compared with bone of the same species, with an average size of 103 nm long, by 68 nm wide; thickness was not noted in this case (Kim *et al* 1996).

Since the crystals of bone mineral have such a large surface area, half to one third of unit cells are on the surface and possess one or more unshared sides. The nature and structure of bone mineral, and the dynamics of its behaviour, are therefore largely problems in surface chemistry (McLean and Urist 1968). A solid surface, when exposed to a liquid, takes on a very thin field of bound solvent approximately 100 Å thick known as a hydration shell. This is formed due to an electric charge asymmetry at the crystal-solution interface (Green 1994). Due to its large surface area, bone mineral crystal binds a large hydration shell; on a volume basis, every crystal binds a hydration shell 1.9 times its own volume (Neuman and Neuman 1958). There are therefore three zones in a crystal: the interior, the surface, and the hydration shell, all of which present opportunities for the exchange of ions. Exchange in the interior is probably very slow, however, that on the

surface or in the hydration shell can be rapid (Vaughan 1981). It is presumed that any ion will penetrate the hydration shell, however, only specific ions tend to congregate there, for example UO_2^{2+} and citrate^{3+}, those ions which are transferred through the hydration shell to and from the crystal surface, and those which can be substituted into the lattice (Neuman and Neuman 1958).

1.4: BONE TYPES

The proportions of all the various components of bone can vary; as can the size and arrangement of the cells and collagen fibre bundles; producing different types of bone to fulfil different functions.

1.4.1: Bundle and Woven Bone

Bundle bone is made up of regularly arranged coarse fibre bundles of collagen with osteocytes in columns between them. It is found extensively in the skeletons of lower vertebrates, and at the attachment of ligaments and tendons in mammals and birds. Woven bone is very rich in large randomly packed osteocytes, and has coarse, variable sized fibres, highly irregular in orientation. It is highly vascularised and mineralised, and in birds and mammals is mainly a temporary tissue found, for example, after a fracture. It is replaced later by more permanent lamellar bone. Both bundle and woven bone are associated with rapid bone formation. In reproducing female birds a special type of woven bone known as medullary bone is present in the marrow cavity, and forms the main component of the mineral reservoir for laying birds. The loosely organised structure allows constant remodelling as part of the laying cycle (Bloom *et al* 1941).

1.4.2: Lamellar Bone

Lamellar bone is the principle bone type in birds and mammals. The osteocytes are relatively small, uniformly ovoid, and widely spaced in the bone matrix. It is formed slowly in sheets (lamellae) as a lining to vascular channels in existing bone or calcified cartilage, or as a narrow surface layer on existing bone in the adult skeleton (Wilson 1994). In mature bone the collagen fibres in each layer are arranged in helical pattern along the length of the bone. The angle of the helix differs between each layer of bone, and it is this pattern of fibre orientation that is partly responsible for the tensile properties of bone (Banks 1986). Lamellar bone can be further subdivided into trabecular (cancellous) and cortical (compact) bone, the two types of bone structure seen in the mature skeleton.

Trabecular Bone

Trabecular bone occupies the marrow cavity in the form of narrow plates that interconnect and cris-cross the cavity through the marrow, or in female birds the medullary bone. The network of fine interlacing partitions encloses cavities containing either red or fatty marrow, and provides great structural strength. Trabeculae are composed of fine lamellae of bone with their osteocytes, and the occasional fragment of calcified cartilage in young growing bone. They are covered by the endosteum, a key source for osteoblasts (Pritchard 1972). Trabecular bone is found in the vertebrae, in most flat bones, and in the metaphyses of long bones. Remodelling takes place on the surface, and is more active than in cortical bone (Vaughan 1981).

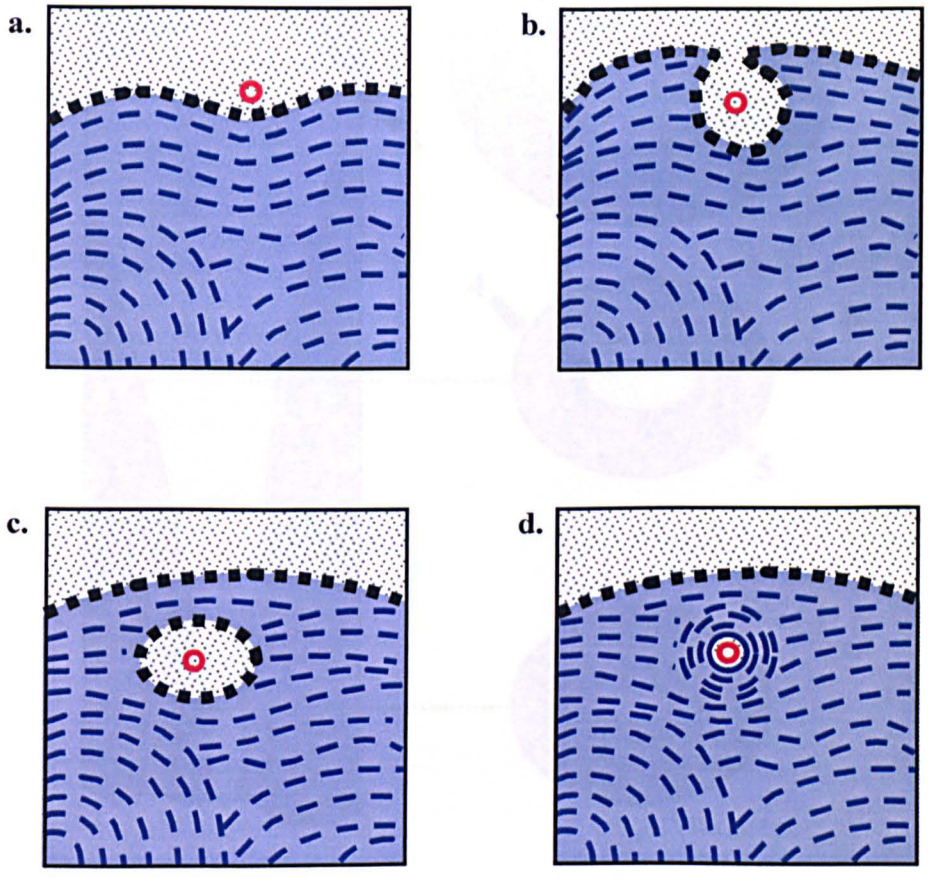
Cortical Bone

Cortical bone is found mainly in the shafts of long bones and surrounds the marrow cavities. The pattern of structure and mineralisation varies with age, and is very variable within a small area (Vaughan 1981). Cortical bone may be formed in three ways: as primary solid bone on exposed periosteal or endosteal surfaces of existing cortices (found throughout birds and mammals where new bone is formed), as cylinders of new bone (1° osteons), or as 2° osteons as a result of cortical bone remodelling (Wilson 1994). Osteons surround Haversian canals containing blood vessels, with their long axis mainly parallel to the long axis of the bone. Haversian canals are lined by flat osteoprogenitor cells or inactive osteoblasts to form the endosteum for new bone formation. They are surrounded by layers of lamellar bone containing rings of osteocytes, regularly arranged and oriented around canals within the osteons. Canaliculi extend radially and anastomose with those of neighbouring osteocytes (Pritchard 1972).

Primary osteonal systems tend to have several blood and nerve vessels in their Haversian canals, and are small with no definite boundary between individual osteons (Pritchard 1972). They are formed by differential osteoblast activity at the periosteal surface of a bone. A longitudinal depression is formed at the periosteal surface along the length of the bone, and this is then covered over by more new bone, trapping blood vessels. The resulting hollow cylinder is then filled in by the formation of a few concentric laminae of bone by osteoblasts (Banks 1986; Figure 1.1). Secondary osteonal systems are formed by the removal of bone from the surface of a pre-existing Haversian canal by osteoclasts, resulting in an expansion of its original limits. Each system is discrete, being bound by the cement, or reversal line, which is the limit at which resorption ceased, and the surface upon which lamellae of new bone were laid down by osteoblasts (Banks 1986).

Figure 1.1:

A diagram of primary osteon development around a blood vessel at the periosteal surface



a. A longitudinal depression develops at the periosteal surface

b. Differential osteoblast activity results in this depression becoming covered in bone

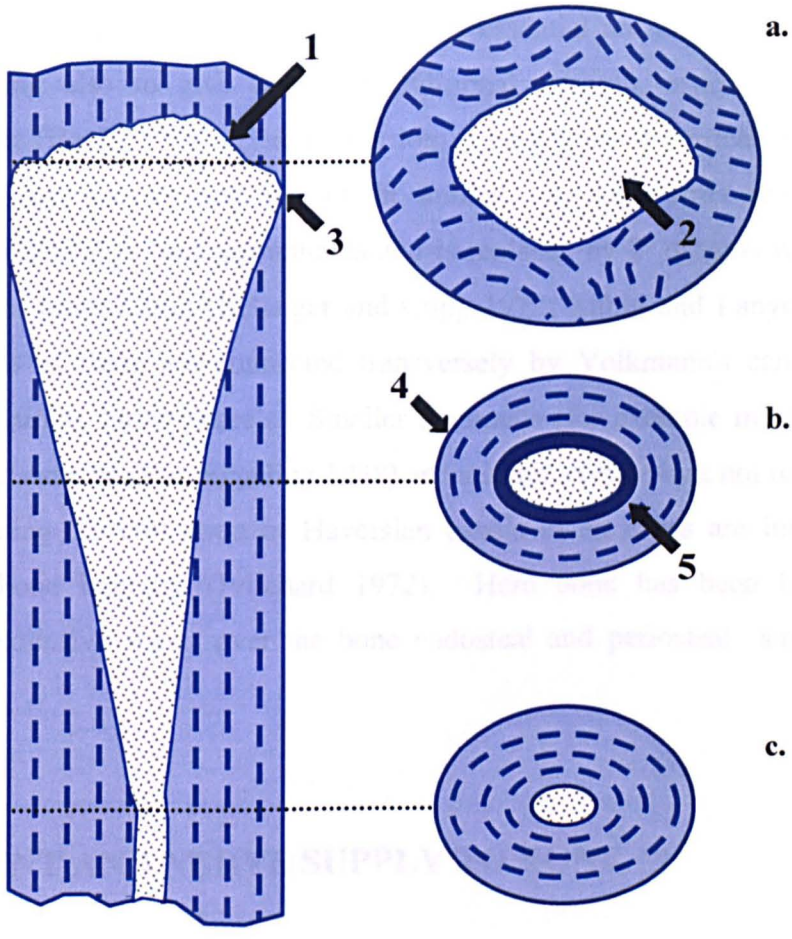
c. Blood vessels and periosteal elements become trapped within the enclosed space.

d. Appositional bone growth within the enclosed space results in the formation of a primary osteon.

Redrawn and modified from Banks (1986).

Figure 1.2:

A diagram of osteonal bone remodelling



a. The osteoclasts of the cutting cone (arrow 1) move through the bone, removing tissue longitudinally and radially to form the resorption space (arrow 2).

b. Once the resorption space reaches the radial reversal line (arrows 3&4), resorption in this direction ceases. Osteoblasts begin laying lamellae of new bone that are subsequently mineralised. Osteons undergoing this process have a seam of osteoid present (arrow 5).

c. Eventually a mature osteon is formed, and osteoblastic activity ceases.

Redrawn and modified from Banks (1986).

Each osteon is also surrounded by interstitial bone lamellae consisting of the remains of remodelled osteons or the blind ends of other osteon branches (Pritchard 1972). The larger Haversian canals often have an irregular edge and are known as resorption cavities. In such cavities removal of mineral and matrix may be occurring on one part of the surface, while osteoid is laid down on another (Vaughan 1981). Resorption begins at a point in a Haversian canal due to some activating stimulus. The osteoclasts then proceed along the canal as a cutting cone, with osteoblasts following behind to form new bone (Banks 1986; Figure 1.2).

The presence of osteons in cortical bone is essential not only to carry blood and nerve vessels, but also to give the bone additional strength, especially towards the periosteal surface (Banks 1986). The proportion of each type of cortical bone, however, depends on the age, species, and size of the animal. In large birds and mammals 1° osteonal bone is typical of younger animals and is replaced by 2° osteons with increasing age as a result of remodelling (Belanger and Copp 1972; Rubin and Lanyon 1988). The Haversian canals of these are connected transversely by Volkmann's canals (Pritchard 1972), also containing blood vessels. Smaller mammals (for example mice, rats) do not normally have 2° osteons (Von Eggeling 1938) as their thin cortices are not remodelled.

Surrounding the collection of Haversian canals in all cases are inner and outer circumference bone lamellae (Pritchard 1972). Here bone has been laid down by osteoblasts in extensive sheets over the bone endosteal and periosteal surfaces (Banks 1986).

1.5: NUTRIENT AND NERVE SUPPLY TO BONE

1.5.1: Nervous Supply

The periosteum contains abundant myelinated and non-myelinated nerve fibres ending in networks on the surface of the bone tissue. There are some myelinated and non-myelinated nerve fibres accompanying blood vessels in Haversian canals, and myelinated nerves are numerous in the marrow; terminating in the endosteum as delicate fibrils running along the blood vessels (McLean and Urist 1968).

1.5.2: Vascular supply

The blood supply of a long bone comes from three sources: the nutrient artery, the periosteal arteries, and the epiphyseal arteries. The nutrient artery perforates the bone

obliquely through the shaft and divides into ascending and descending branches. Each branch arborises to reach the endosteum, the metaphyses, and the epiphyseal plates.
The periosteum has a rich blood supply consisting of small arteries and veins forming a continuous vascular sheath that encloses the bone. The number of periosteal vessels in the centre of the shaft is relatively few, but adds up to hundreds at the metaphyseal ends of the bone. The periosteal blood supply is greatly increased when the medullary arterial supply is damaged. The medullary blood supply may increase if the periosteal route is damaged; this represents the collateral route.

The epiphyses are supplied by 1-3 main epiphyseal arteries, each coursing towards the centre and then branching outwards to supply a specific area of the spongiosa and articular surfaces. The avian epiphyseal plate is supplied by blood vessels from 3 sources; perforating epiphyseal, perforating metaphyseal, and circumferential epiphyseal arteries (McLean and Urist 1968). In contrast, the mammalian growth plate is not traversed by blood vessels. Instead, capillary tufts supply nutrients from the epiphyseal side, while metaphyseal capillary loops appear to be involved in the ingrowth of blood vessels and the mineralisation of matrix produced by hypertrophied chondrocytes (Trueta 1997).

Veins in bone are thin walled. The centrally placed nutrient vein or sinus in the human tibia accompanies the nutrient artery. Venous drainage of the epiphysis is by veins that parallel the arterial supply. Drainage of the metaphysis is through a series of longitudinally arranged sinusoids and into the metaphyseal veins (McLean and Urist 1968).

1.5.3: Bone Fluid

Bone fluid is continuous throughout the Haversian canals, on the endosteal and periosteal surfaces, in the canaliculi, and in osteocyte lacunae (Vaughan 1981). The surface area of bone is extensive when Haversian systems and Volkmann's canals, the lining of marrow cavities, and the external bone surface are all taken into account. In a human vertebral body of 40 cm^3, the trabecular surface area is of the order of 1000 cm^2, compared with a periosteal area of 80 cm^2 (Dunnill *et al* 1967). This provides a massive area for the exchange of nutrient between bone and bone fluid. The passage of fluid through cortical bone is in the same net direction as the blood moves, mainly from the endosteal to the periosteal surface (Owen *et al* 1977). It is speculated that the composition of bone fluid may vary with site; however, this has never been demonstrated as it has not yet proved possible to obtain samples. Although the character of the barrier between them is unknown, it is accepted that there are probably important differences between bone fluid and extravascular tissue fluid (Vaughan 1981).

1.6: DEVELOPMENT AND GROWTH OF BONE

In early foetal life, the skeleton begins as a condensation of mesenchyme cells. Its development involves the commitment of cells within this mass to chondrogenic and osteogenic lineages, the positioning of cells with similar differentiation potential at appropriate sites within the mesenchymal mass, and their subsequent differentiation. Bones can initially develop by two methods: intramembranous and endochondral ossification. The bones of the skull are formed by intramembranous ossification, whilst all the long bones in the skeleton are formed by endochondral ossification. Until adult stature is reached, further growth in the length of the long bones occurs by endochondral means, and growth in diameter by apposition (Vaughan 1981).

1.6.1: Intramembranous Ossification

In intramembranous ossification, the mesenchymal mass is converted directly to bone. Condensations of cells with an osteoblastic potential form within the mesenchymal mass. The increased cellular interaction is immediately followed by differentiation into osteoblasts, forming centres of ossification. These secrete osteoid, and in doing so some are surrounded by matrix and become osteocytes (Thomson *et al* 1989). Other osteoblasts continue to secrete osteoid and surround ingrowing capillaries that will bring in the haemopoietic cells of the future marrow. Spicules of bone are found radiating out from centres of ossification as a result of matrix formation by the osteoblasts lying on the surface of the condensation (Vaughan 1981). There are usually two such centres of ossification in each bone, surrounded by proliferating cells which increase the number of active osteoblasts. Each is expanded by peripheral bone formation, thickened by the addition of perpendicular elements, and cross-linked by the formation of secondary trabeculae. The spaces enclosed by the resulting spongy bone become filled with vascular tissue and marrow (Loveridge *et al* 1992).

1.6.2: Endochondral Ossification

In the case of endochondral ossification, bone is formed indirectly, via an intermediate cartilage stage. Cells with a chondrogenic potential accumulate within an area of the mesenchyme condensation of the limb bud, differentiate, and then proliferate to form a model of the future bone. These cells surround themselves with an organic matrix largely composed of glycoproteins peculiar to cartilage (Vaughan 1981). Cartilage models of most of the bones of the skeleton are formed during embryonic life in this manner.

At a certain stage of embryonic life dependant on the species, the hyaline cartilage undergoes degenerative changes; and the cells enlarge, hypertrophy, and accumulate glycogen, glycolytic enzymes, and alkaline phosphatase. The changes in cartilage cells begin at a relatively small number of foci originating from the mesenchyme forming the perichondrium; these are destined to become ossification centres for the shafts of the bones. At the time that the foci of hypertrophic cells are seen in the cartilage model, or shortly thereafter, calcification of the cartilage matrix occurs. Replacement of the embryonic cartilage with bone within the model is guided by a network of calcified cartilage matrix (McLean and Urist 1968).

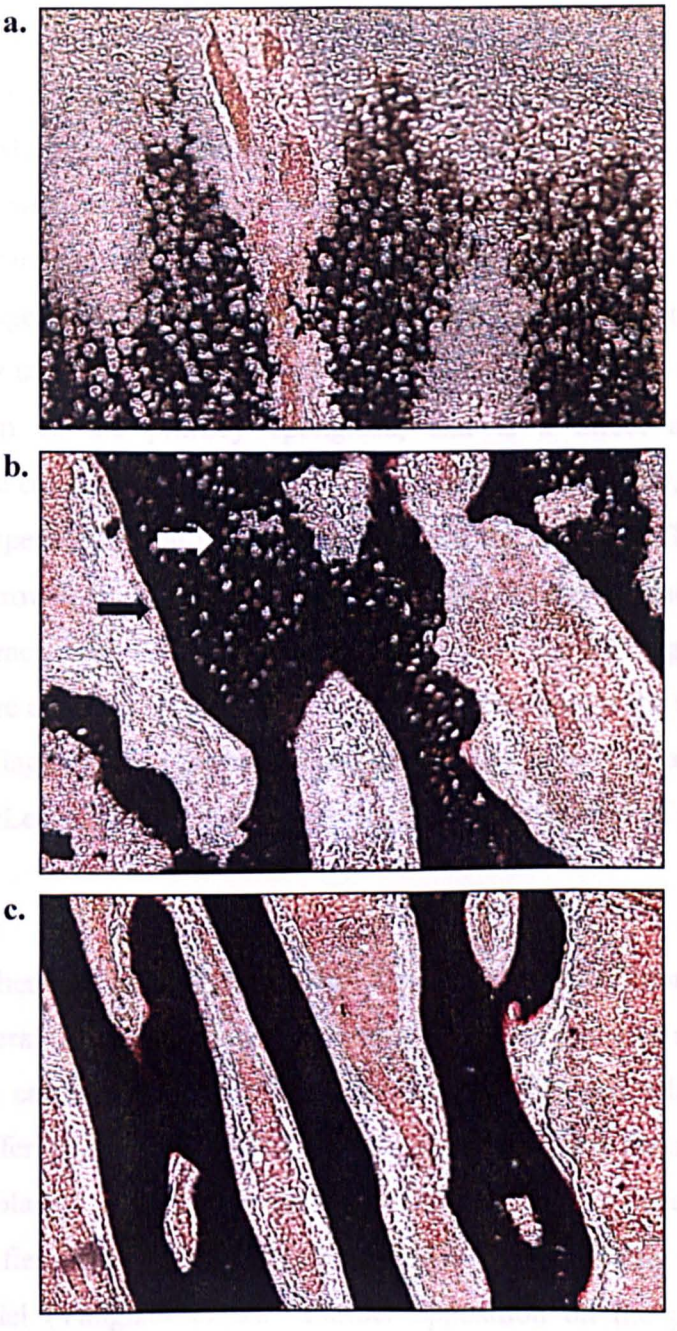
As the cells undergo these changes, there is ingrowth of capillaries through the perichondrium and periosteal bone. These penetrate the area of hypertrophic cartilage cells and carry with them stromal cells from the inner layers of the perichondrium that will form the marrow stroma (Jotereau and LeDouarin 1978). The vessels arborise, and ultimately; together with their stromal cuff derived from the perichondrium; replace the degenerating cartilage cells (Vaughan 1981). The original vascular endothelium forms the largely sinusoidal endothelium of the marrow (Weiss 1976; Jotereau and LeDourain 1978). Blood cells in the capillaries leak out either through gaps in the endothelium, or through the endothelial cells themselves (Weiss 1973), to form the cells of the haemopoietic marrow; the precursors of erythrocytes, lymphocytes, megakaryocytes, granulocytes, and monocytes of peripheral blood (Vaughan 1981). From the monocyte, the macrophage and the osteoclast differentiate (Owen 1978). The widespread replacement of cartilage is slowed only as it approaches that portion of the model destined to form the epiphysis and the epiphyseal cartilage (McLean and Urist 1968).

1.6.3: The Epiphyseal Growth Plate

The epiphyseal growth plate is a portion of the embryonic cartilage model that persists through adolescence, proliferates, and participates in the growth in length of the long bones (McLean and Urist 1968). The epiphyseal growth plate of both avians and mammals comprises both chondrocytes and matrix components. The chondrocytes show a series of well-defined maturational stages (Brighton 1978). These are distinguished by changes in proliferation rate, morphology, and the synthesis of extracellular matrix proteins (Hunziker *et al* 1987) and endogenous growth mediators (Loveridge *et al* 1990). As a result, the growth plate can be separated into distinct zones consisting of resting, proliferating, maturing, and hypertrophic chondrocytes. The growth plate stays approximately constant in thickness, being replaced on the diaphyseal front by bone, while new chondrocytes arise from the germinal zone on the epiphyseal face (McLean and Urist 1968).

Plate 1.1:

Growth plate mineral development
(Von Kossa staining)



a. Mineralised matrix of hypertrophic chondrocytes below the proliferation zone.

b. New mineralised bone (black arrow) is deposited on the cartilage scaffold (white arrow) as the chondrocytes mature.

c. The cartilage is completely replaced giving rise to spongy bone.

A bone increases in length due to two distinct processes. The first involves the generation of new chondrocytes by cellular proliferation in the upper zones of the growth plate. The second occurs after the cells have left the proliferating zone; the chondrocytes pass into the maturing and hypertrophic zones, where they differentiate and are characterised by the presence of alkaline phosphatase activity (Fell and Robinson 1929) and collagen type X (Schmid and Linsenmeyer 1985). The precise role of these proteins is uncertain; however, it has been proposed that they are involved in mineralisation of the growth plate cartilage (De Bernard and Vittur 1990). As the cells near the diaphyseal face of the plate, they become larger, develop vacuoles in their cytoplasm, their nuclei swell and lose most of their chromatin (McLean and Urist 1968). They also lose their transverse walls, whilst their longitudinal walls calcify. The mineralised cartilage is eventually invaded by the vascular system carrying osteogenic cells. Blood borne osteoclasts resorb some of the mineralised cartilage, leaving spicules of it as a scaffold for the deposition of osteoid by the osteoblasts; this is subsequently calcified (Vaughan 1981; Plate 1.1). This area of intense activity is known as the primary spongiosa, and is a direct and unreconstructed continuation of the calcified cartilage matrix (McLean and Urist 1968), and it is the process of chondrocyte hypertrophy that is considered to have the greatest influence in controlling the rate of bone growth (Farnam and Wilsman 1987). The development of the secondary spongiosa is characterised by greater reconstruction of the cartilage scaffold, and the appearance of large amounts of new bone. At a time dependant on the species and the bone involved, the cartilage forming the epiphyseal ends of the bone is invaded and replaced by forming bone (McLean and Urist 1968); this results in the cessation of growth.

1.6.4: Apposition

Apposition is where bone matrix is laid down on existing bone surfaces and is subsequently mineralised. It begins on the periosteum of long bones at approximately the same time as the cartilage model is being penetrated. The inner layer of cells of the perichondrium differentiates and can be shown to contain alkaline phosphatase, the enzyme marker for osteoblasts. These differentiated cells lay down a layer of osteoid which immediately calcifies, forming a 'periosteal collar' of bone directly in contact with the cartilaginous model (Vaughan 1981). Further apposition on the periosteal surface is accompanied by resorption from the endosteal surface, thus the original shape of the long bone is maintained, but the three dimensional geometry of the bone is increased in scale (Lacroix 1971). In early foetal life of long gestating species, resorption and apposition also begin in those bones formed by intramembranous ossification. This results in spongy or cancellous bone occupying the centre of the bone mass, and a layer of compact bone

forming on each surface by the continuous addition of new sheets of bone by active osteoclasts. Apposition also occurs in Haversian systems (Vaughan 1981).

1.6.5: Control of Bone Growth and Development

Genetic factors are important in determining the shape of any bone (Fell 1956); however, there are many hormones and growth factors that affect skeletal growth in different and subtle ways. Both local and systemic factors are known to be involved, however, the precise actions of individual growth factors and hormones is not yet fully understood.

The growth spurt at puberty is dependent on the sex hormones, testosterone and oestrogen (Short 1980), and on growth hormone (Aynsley-Green *et al* 1976). In addition, sex differences have been reported for bone development in poultry: in females the tibiotarsi are narrower from hatching, lighter in weight from the age of 26 days, and the length and volume are smaller by 42 days when compared with males. However, the percentage dry matter in this bone is higher in females from hatching, and from 12 days old tibiotarsi in females tend to be less porous with a higher ash content, mineralisation proceeds faster in females until 26 days of age, after which it proceeded faster in male birds. Stiffness in the tibial diaphysis is similar in both males and females, and although the growth of cortical bone is very different between the sexes, in females the narrowness of the bone diaphysis is counter balanced by modifications in the composition of the matrix and in the porosity of the cortex. The tibiotarsi of both sexes ultimately have equal biomechanical characteristics (Rose *et al* 1997).

Within the epiphyseal growth plate it has been suggested that the events of matrix synthesis, cell maturation and degeneration, and mineralisation are regulated by metabolic limitations imposed by a low oxygen partial pressure (Bennet 1994; Brand 1983). Although the epiphyseal plate has its own blood supply, from an anatomical point of view the supply is limited (Arsenault 1987; Howlet *et al* 1984). However, chondrocyte oxygenation was examined in a recent experiment using a hypoxia-sensing drug, and it was found that none of the cells are severely hypoxic. It is now thought that the chondrocyte regulates its own fate by the process of programmed cell death (apoptosis). However, an apparent oxygen gradient was found between the proliferating chondrocytes (highest oxygen tension) and the hypertrophic chondrocytes (lowest oxygen tension), and a role for oxygen gradients in the differentiation of cells in the growth plate cannot be ruled out (Shapiro *et al* 1997). Peptide growth factors are now known to be central to chondrocyte differentiation in the growth plate. For example, ß(TGF-ß) is known to regulate chondrocyte differentiation, and TGF-ß3 has been localised in the prehypertrophic and hypertrophic chondrocytes of the chick growth plate (Thorp *et al* 1995). Other major

regulators of endochronal ossification are found predominantly within the more mature chondrocytes of the growth plate, for example basic fibroblast growth factor (bFGF), insulin-like growth factor-I (IGF-I), epidermal growth factor (EGF), and transforming growth factor α (TGF-α; Farquharson and Jeffries in press, Ren *et al* 1997). In addition, parathyroid hormone-related peptide (PTHrP) has been found to act as a negative regulator of growth plate chondrocyte differentiation (Vortkamp *et al* 1996), while differentiation is stimulated by 1,25 dihydroxyvitamin D_3 (Farquharson *et al* 1993).

1.6.6: Disorders Of The Growth Plate In Broilers

Rickets

Rickets occurs in the growth plates of long bones and is attributed to a deficiency in vitamin D_3, calcium or phosphorus (Nonindez 1928). Calcium to phosphorus ratios in the diet are central to the development of rickets, and a high dietary phosphorus content can have the same effect as a low calcium content (Thorp 1994). Although rickets is precipitated by nutritional deficiencies, there is also a genetic component; strains particularly prone to the disorder can be produced by selecting for susceptibility (Austic *et al* 1977). In all forms of rickets there is a thickened growth plate (Nonindez 1928), and a failure of mineralisation which leads to flexibility in the long bones and lameness. The disorder is usually seen in rapidly growing young chicks (Wise and Nott 1975).

Although there are three main nutritional influences on rickets, there are two categories of the disorder. In hypocalcaemic rickets, the proximal epiphyseal growth plate of the long bone contains a much-thickened zone of proliferation, which has disorganised and poorly oriented chondrocytes (Randall and Reece 1996). This is the main identifying characteristic of this disorder, and is thought to be due to a delay in the initiation of maturation changes in the oldest chondrocytes in the zone (Mankin and Lippello 1969, Bisaz *et al* 1975), although the cause of this has not been identified. There are also other histological characteristics associated with this disorder. The pre-hypertrophic zone may be poorly vascularised by metaphyseal vessels (Randall and Reece 1996), and like the chondrocytes of the proliferation zone are also small and irregular (Jande and Dickson 1980). The bony trabeculae are often distorted, and have a thick layer of unmineralised osteoid on their surface (Randall and Reece 1996). The symptoms of hypocalcaemic rickets can be induced by feeding a diet deficient in vitamin D (Jande and Dickson 1980), and addition of this vitamin to the diet results in normalisation of the growth plate (Belanger and Migicovsky 1958, 1960). These alterations do not necessarily imply a direct effect of vitamin D on bone; they may result from the consequent disturbance to calcium

homeostasis (Jande and Dickson 1980), since intestinal calcium transport is stimulated by 1,25-dihydroxyvitamin D_3, the active metabolite of vitamin D_3 (Norman 1978).

In hypophosphataemic rickets, the zone of proliferation is normal, however, the hypertrophic zone is greatly thickened, although well vascularised by metaphyseal vessels (Randall 1991). The zone is devoid of any mineral at the proximal end, and poorly mineralised at the distal end (Jande and Dickson 1980), although some new bone may be mineralised (Randall and Reece 1996). It has been suggested that the histological changes are caused by a physico-chemical mechanism involving inhibition of the crystallisation of calcium phosphate (Bisaz *et al* 1975), although no exact mechanism has been specified. The disorder can be caused by either phosphorus deficiency or calcium excesses (Randall 1991). In the latter case, birds have been shown to have slightly elevated plasma Ca levels, and marked hypophosphataemia (Jande and Dickson 1980).

Tibial Dyschondroplasia

Dyschondroplasia is another disorder of the growth plate of long bones. Histologically it is identified as an accumulation of avascular cartilage consisting mainly of pre-hypertrophic chondrocytes (Poulos *et al* 1978, Hargest *et al* 1985), and is also characterised by smaller ovoid lacunae and more matrix than is present in normal hypertrophic cartilage (Thorp 1994). At the distal end of the abnormal cartilage, there may be distended blood vessels that have failed to penetrate the cartilage (Randall and Reece 1996). The lesions may be avascular due to an inability of the metaphyseal vessels to penetrate the accumulated cartilage (Thorp and Duff 1988), or through some fault in the blood vessels themselves (Riddell 1975a). In some cases lesions contain areas of necrosis due to oxygen depletion with increasing distance from the vascular supply (Hargest *et al* 1985). The larger the dyschondroplasic mass, the more likely there is to be necrosis (Randall and Reece 1996). Ultrastructural studies have shown that there is sufficient Ca and P present in the matrix to allow mineralisation, yet this does not take place. In addition, incomplete chondrocyte hypertrophy has been observed in affected growth plates (Hargest *et al* 1985). Thus the primary fault is now thought to be impaired chondrocyte differentiation resulting in a matrix of incorrect composition for mineralisation (Thorp 1994), which cannot become vascularised (Poulos 1978).

Dyschondroplasia occurs in most long bones, however in broilers it is most common in the tibiotarsus, the fastest growing long bone in the chick (Farquarson and Jeffries in press), and is known as tibial dyschondroplasia (Siller 1970). It is most frequently found in the proximal growth plate, which is the end of the bone which produces new bone at the greatest rate (Farnum and Wilsman 1993). The disorder causes economic

losses through both increased mortality and poor performance. It has not been linked with heavier slaughter weight, however, it has been shown to be associated with larger breast weights and longer tibiotarsi (Yalcin 1996). Strains with a high incidence of TD have slightly faster-growing long bones than those with a low incidence (Riddell 1975b), and the disorder tends to occur early in development when growth is proceeding most rapidly (Lilburn 1994, Marks 1979).

A wide variety of factors have been shown to influence the incidence and severity of dyschondroplasia. The incidence varies markedly in response to genetic selection (Sheridan *et al* 1978). It can also be reduced by restricted or meal feeding to reduce growth rate during the second or third week (Lilburn *et al* 1989, Edwards and Sorenson 1987). TD can be induced by alterations in dietary Ca:P ratios (Riddell and Pass 1987, Rennie *et al* 1993), chloride and magnesium levels (Luo *et al* 1992), and acid base balance (McCaskey *et al* 1982), and by mycotoxins (Walser *et al* 1982). Levels of dietary calcium and phosphorus have an effect on both the incidence and severity of TD. Raising calcium levels (Edwards and Veltmann 1983) or increasing the Ca:P ratio (Riddell and Pass 1987) reduce both the incidence and severity of TD. Low Ca and High P diets have been reproducibly used to induce TD (Edwards and Veltmann 1983, Rennie *et al* 1993). In addition, evidence continues to be collected indicating that high phosphorus levels in diets borderline in calcium can significantly increase the incidence of TD (Halley *et al* 1987 a, b).

Supplementary vitamin D_3 metabolites in the diet have been found to be useful in reducing the incidence of TD. Edwards (1989) found that a dramatic reduction in incidence was obtained by supplementing low calcium diets (0.45-0.95%) with dietary 1,25 dihydroxyvitamin D_3 at 10μg/kg diet, and Rennie *et al* (1993) found that with 0.75% calcium, 2.5 μg/kg reduced, and 5μg/kg prevented TD. Further studies by Edwards revealed that all of the vitamin D_3 metabolites with the 1-OH group were effective in preventing the development of TD and stimulating bone calcification, whilst those metabolites without the 1-OH group were not (Edwards 1989, 1990, 1992). In particular, 1,25-dihydroxyvitamin D_3 is thought to stimulate chondrocyte differentiation, the inhibition of which is thought to be a major factor in the aetiology of TD (Farquharson *et al* 1993). Unfortunately, high doses of this metabolite result in hypercalcaemia and growth depression, particularly when combined with higher dietary calcium content. For a diet containing 1% calcium, the optimum concentration effective in reducing TD without resulting in hypercalcaemia has been found to be 3.5μg/kg (Rennie *et al* 1995). However, ascorbic acid supplements have been found to act synergistically with 2μg/kg 1,25

hydroxyvitamin D_3 to prevent TD, although the vitamin is not effective on its own (Rennie and Whitehead 1996). Ascorbic acid is required as a co-factor for prolyl and lysyl hydroxylase in the synthesis of stable triple-helical collagen. Its synergistic effect may, therefore, be due to its involvement in the growth plate matrix composition, which, along with 1,25 dihydroxyvitamin D_3, promotes chondrocyte differentiation. In addition, ascorbic acid is also thought to stimulate the effect of 1α-hydroxylase, the enzyme that converts 25- to 1,25 dihydroxyvitamin D_3 (Farquharson *et al* 1993). Finally, although Edwards found only those vitamin D metabolites with the 1-OH group to be useful, Rennie and Whitehead (1996) have found that supplementation with 25-dihydroxyvitamin D_3 at 75 μg/kg also reduces the incidence of TD. In contrast to 1,25 dihydroxyvitamin D_3, this metabolite is non-toxic and inexpensive, and has potential as a practical method of combatting this disorder.

1.7: BONE MINERALISATION

The mineralisation of bone is a complex process that is not yet fully understood. Initial micro crystal formation first occurs in the cartilage template, and bone is subsequently laid down on spicules of calcified cartilage. During the mineralisation of cartilage, in order to form the first micro crystal of hydroxyapatite the thermodynamic equilibrium must be overcome, and the energy barrier crossed. The mineralisation process in bone, however, is generally concerned with accretion and nucleation onto existing crystals in the calcified cartilage template, and then with the growth and proliferation of crystals in the osteoid (Ali 1992).

Mineralisation is brought about by a series of complex physico-chemical and biochemical processes that act in concert to facilitate the deposition of a solid phase in specific areas of an organic matrix. In the first stage of mineralisation, soluble constituent ions such as calcium and phosphorus must be complexed to form an insoluble compound, and therefore form small solid particles. The second stage involves these particles growing and reorganising to form the first crystals of hydroxyapatite, and the third stage involves an increase in the number and size of crystals to saturate the matrix of the tissue. A final stage, where required, involves further solid phase changes and transformations, sometimes entailing dissolution and re-crystallisation, before stable, organised, and structurally sound bone mineral is formed (Glimcher 1976; Posner 1969). There are, therefore, numerous factors that are involved in the mineralisation process. The supply and transformation of the constituent ions to the growing crystals, and the structure and composition of those

crystals are fundamental to mineralisation with respect to nucleation, growth, and development in association with the organic matrix components (Landis 1996).

There appear to be two principle patterns of mineral deposition. The first is that associated with small extra-cellular, membrane-bound vesicles. This results in a radial-shaped spherulitic deposit of numerous, disorganised, individual crystallites of various lengths about a central point (Christofferson and Landis 1991). This type of deposition has been observed in calcifying cartilage at the growth plate (Anderson 1969; Bonucci 1971), and occasionally in the initial stages of intramembranous (Bernard and Pease 1969) and appositional bone formation (Bonucci 1971). The second pattern seen is a much more regular and highly organised appearance of crystals extra-cellularly, with a periodic distribution of approximately 64-70nm. The crystals are associated with collagen fibrils in an organised manner, with their long axis generally parallel to the fibrils (Christofferson and Landis 1991). This pattern of mineralisation is common during intramembranous and appositional bone development (Bernard and Pease 1969).

1.7.1: Growth Plate Mineralisation: Matrix Vesicles

These extracellular vesicles are generally circular in cross section with a diameter of 40-400 nm, and are bound by a trilaminar membrane. They are thought to arise by budding from the cell membranes of chondrocytes and osteoblasts (Anderson 1995), and contain a variety of enzymes, most notably alkaline phosphatase. The association of these vesicles with mineralisation is based on observations that they occur in regions of tissue that are mineralising, and that they appear to contain crystals of calcium phosphate (Anderson 1969; Bonnuci 1971). Several factors are created by the matrix vesicles that facilitate crystallisation. The alkaline phosphatase present cleaves a phosphate-containing substrate to release PO_4^{3-}, thus increasing local concentrations of both calcium and phosphate ions to a level where spontaneous precipitation occurs. In addition, there are other phosphatases present, such as ATPases and another isolated from chick chondrocytes, 3XIIA (Houston *et al* 1999), which may also be involved in the mineralisation process. Furthermore, the vesicles may provide for the exclusion of compounds that inhibit crystal formation; pyrophosphates, glycoproteins, and some proteoglycans have been postulated (Anderson 1989).

Within the epiphyseal growth plate clusters of matrix vesicles are seen in the longitudinal septum in the lower zone of proliferation, however, no crystals are associated with them at this stage. In the maturation zone matrix vesicles can be seen with needle-like crystals within them (Ali 1992). Earlier observations of these crystals came into question due to potential artefacts induced during tissue preparation. However, this mineral phase

has also been observed in vesicles prepared by anhydrous means to avoid possible sources of inorganic artefacts (Landis and Glimcher 1982).

The formation of the hydroxyapatite crystals occurs in association with the inner membrane of the vesicles, which eventually ruptures giving rise to the spherulitic growth of crystals (Christofferson and Landis 1991). The membranes of matrix vesicles have a characteristic composition (Wuthier 1975), which led to speculation concerning the role in mineral nucleation and growth of the acidic phospholipids and phophatidyl serine present in the membrane (Eanes 1989). Vesicles are thought to induce primary mineralisation by accumulating calcium and phosphorus ions on the charged nucleating regions of the phospholipids on their inner membrane surface (Holing *et al* 1995).

With the proliferation and growth of the crystals within the matrix vesicles, the membrane is disrupted, and crystals are found protruding from the vesicles. These crystals can now grow by accretion, and at this stage the rapid proliferation and growth of hydroxyapatite crystals may become independent of the matrix vesicles and more dependent on calcium and phosphorus availability. Some organic components of the matrix; such as proteoglycans, chondrocalcin, and collagen type X may also be involved at this second stage in controlling the progress of further calcification. In the next stage of mineralisation at the growth plate, the crystal clusters and mineral nodules grow and coalesce to calcify the whole longitudinal septum, and at this stage crystals are seen associated with collagen fibrils. As the matrix becomes saturated with mineral, the crystals appear to align themselves between long collagen fibrils in the direction of the long axis of the fibres (Ali 1992), and the second pattern of mineralisation becomes predominant.

It is currently uncertain as to whether matrix vesicles are the initial sites of mineral deposition. There is evidence that this is the case in calcifying turkey leg tendon (Landis 1986), however, the situation in other vertebrate tissue is less clear. Some immunocytochemical studies have shown that matrix vesicles are not the sites of initial mineral deposition in calcifying cartilage (Poole *et al* 1986), the tissue in which they are most commonly observed. It is also unclear whether in systems in which deposition is initially within matrix vesicles, any relationship exists between mineralisation within the vesicles and that occurring later in collagen. Once the crystals have been extruded, they could affect external mineralisation, but it is thought that this effect would be in the immediate vicinity of the vesicles. However, in the case of bone, rapid and massive mineralisation clearly occurs at sites spatially distinct from local sites of vesicle mineral deposition. No satisfactory model has been proposed to date which can explain what effect vesicles could have on this, and it has been suggested that they have only a minor and indirect role in mineral deposition as a whole (Christofferson and Landis 1991).

1.7.2: Osteon Mineralisation: The Organic Matrix

As osteoid is laid down in an active Haversian system it becomes mineralised, except in pathological conditions such as rickets or osteomalacia (Vaughan 1981). Bone mineralisation occurs predominantly extracellularly within an organic matrix supersaturated with calcium and phosphate (Christofferson and Landis 1991). When mineral is first observed, it appears at spatially distinct sites in the collagen matrix (Landis *et al* 1977). Despite the recognition of the importance of this type of deposition, accounts of how it occurs are still inconsistent. However, it is generally accepted that collagen is laid down prior to mineralisation, that it is the principle organic matrix constituent in which mineral is deposited, and that the mechanism of calcification relies on a very close association between the organic matrix and an inorganic phase or phases (Christofferson and Landis 1991).

Crystal Nucleation In The Matrix

Mineral crystals in collagen fibrils occur initially in the hole zone. One of the main controversies in extracellular mineralisation is that while estimates of crystal size are in the range of 20-50 nm long, 10-40 nm wide, and 3-5 nm thick (Weiner and Price 1986), estimates of the size of the collagen hole zones are in the range of 35-40 nm long, with a diameter of only 1.5-3 nm (Miller 1984). It is therefore difficult to understand how even a single crystal can be accommodated. However, some workers have shown that crystals 30-80 nm long in the growth plate cartilage and cortical bone of the rat are actually composed of microcrystals 12-17 nm long and approximately 5 nm wide and thick (Arsenault and Grynpas 1988, Arsenault and Hunziker 1988). Accommodation of such microcrystals and their aggregates is conceivable, although little is known about their growth (Christofferson and Landis 1991). There is also evidence that the alignment of collagen hole regions is such that continuous channels or grooves are created, traversing many molecules and fibrils (Katz *et al* 1989, Weiner and Traub 1989).

It is generally considered that the central dark line, which is a lattice line situated in the central portion of hydroxyapatite crystallites in several calcifying tissues, is the nucleation site of crystallites (Nakahara and Kakei 1989). During the formation of the initial line there appears to be a conversion of a non-apatitic precursor to the apatitic form, however, the conversion mechanism has been a controversial issue for many years (Kakei *et al* 1997). It has been reported that magnesium and carbonate ions exist as major impurities in crystallites, and that the central portion of hydroxyapatite crystals is rich in both ions (Quint *et al* 1980). Magnesium ions strongly disturb the conversion from non-apatitic to apatitic mineral (LeGeros 1981); the alteration of that inhibitory effect is

necessary for mineralisation (Kakei *et al* 1997). Contrary to this, carbonate ions generated by carbonic anhydrase activity may act as a trigger for initiating biological crystal growth (Kakei and Nakahara 1996). It has been suggested that the nucleating step takes place simultaneously with carbonate incorporation, which might inactivate the inhibitory magnesium ion in the initial non-apatitic mineral, supporting the development of apatite crystal (Kakei *et al* 1997). Casciani *et al* (1979) claimed that huntite ($Mg_3Ca(CO_3)_4$) develops prior to the development of hydroxyapatite crystals in calcifying tissues, which would be one way of removing the inhibitory Mg ion. The presence of a magnesium-carbonate compound has since been confirmed in 6-day old rat calvaria, although the form of the compound was not identified. It is suggested that an initial lattice line comprised of calcium and phosphate groups is decorated by the magnesium carbonate compound, and thus transformed into the central dark line (Kakei *et al* 1997).

Numerous components of the organic matrix of bone have been suggested to feature in the initiation of mineralisation. A current candidate is bone sialoprotein, a sialophosphoprotein expressed only in mineralised tissues (Sodek *et al* 1992). Recent studies have shown that this protein can nucleate hydroxyapatite in a steady-state agarose gel system (Hunter and Goldberg 1993). This activity is not affected by dephosphorylation of the protein (Hunter and Goldberg 1994), but appears to be associated with regions of contiguous glutamic acid residues in a helical conformation (Goldberg *et al* 1996). However, a better understanding of nucleating sites in mineralised tissues awaits consensus on the identity of the proteins involved. This requires a correlation of the properties of a potential nucleator protein in vitro, and its relationship to mineral crystals in vivo (Hunter 1996).

Crystal Growth

After nucleation, the initial growth of apatite crystals is rapid, occurring in a matter of minutes or less (Eanes and Posner 1965; Christofferson and Landis 1991); approximately 70% of the mineralisation within an osteon is achieved within days, yet complete mineralisation requires at least 6 weeks (Frost 1963). The formation and growth of biominerals is a complex process. At the chemical level, it involves the control of supersaturation and ion activities, which lead to nucleation and growth of crystals (Simkiss and Wilbur 1989). It is generally agreed that much of the process depends on interactions occurring at the solid-solution interface (Mann 1988).

Whenever crystals grow in an aqueous medium, two processes occur consecutively: transport of ions from the bulk of the solution to the solution adjacent to the crystal surface, and a surface process whereby ions are incorporated into the crystal. The rate of growth

can never be faster than the transport-controlled rate, however, this is usually faster than the surface process, and therefore, generally bulk diffusion plays no important role. However, the maturation and growth of highly mineralised bone crystals could be controlled by the transport mechanism in addition to the surface mechanism, since the supply pathways become narrower as mineralisation progresses (Christofferson and Landis 1991). From its initial seeding, through growth, maturation, and dissolution, the hydroxyapatite crystal must interact with the water in the bone matrix. For the crystal to grow, ions must diffuse in from circulation via the extracellular fluid. When bone mineral is laid down, it displaces an equal volume of matrix water. The water spaces between the crystals and collagen fibres are reduced until ions can no-longer diffuse at appreciable rates (Neuman and Neuman 1958). Bone mineralisation should therefore continue maximally only up to a critical water content; in fact, bone never becomes 100% mineralised, calcification usually stops at 80-85% of theoretically complete mineralisation (Robinson 1964).

1.7.3: Hydroxyapatite Precursors

The newly deposited mineral in osteons is more reactive with bone-seeking radio-isotopes, tetracycline, alizarin and porphyrins than more mature mineral (McLean and Urist 1968). The mineral component from new bone also has a lower calcium to phosphate ratio, a higher HPO_4^{2-} content, a higher content of tightly bound water, and a lower degree of crystallinity than that from more mature tissue (Glimcher 1976). Theories of bone mineral formation and development fall into two broad categories (Glimcher *et al* 1981). The continuum view suggests that a single apatite phase with variable chemical composition is present in bone. This apatite gradually becomes more crystalline, and its composition, and Ca:P ratio, is presumed to approach that of hydroxyapatite as the mineral ages (Glimcher 1976), mainly by the addition of Ca^{2+} (McLean and Urist 1968). Alternatively, the two phase view suggests the presence of a distinct precursor phase with a Ca:P ratio lower than the 1.67:1 of hydroxyapatite. This precursor is thought to be converted to hydroxyapatite, or to become mixed with hydroxyapatite as the bone mineral matures (Glimcher *et al* 1981). The two most prominent precursor-phase hypotheses are those involving amorphous calcium phosphate (Harper and Posner 1966), and octacalcium phosphate (Brown 1966).

The amorphous calcium phosphate theory, which is currently the more popular hypothesis, is based on the low x-ray diffracted intensity of bone mineral compared to the intensity of pure crystalline hydroxyapatite (Glimcher *et al* 1981). Amorphous calcium phosphate has apparently been found at sites immediately preceding the calcification front

(Robinson and Watson 1955). However, it has also been found distributed throughout the bone (Harper and Posner 1966). Although its occurrence decreases with age as bone formation is reduced, approximately 40% of the mineral of adult human, cow, and rat femurs is still thought to be non-crystalline. Like the bone apatite crystals, amorphous calcium phosphate is not thought to have a rigidly defined chemical composition, its molar Ca:P ratio varying between 1.44-1.55 to one (Vaughan 1981), however its basis is thought to be tricalcium phosphate (McLean and Urist 1968). Once formed, the amorphous salt is thought to become the controlling source of ions for the precipitation of apatite crystals (Eanes *et al* 1973). However, subsequent to the original work done on amorphous calcium phosphate, the hydroxyapatite used as a standard was adjusted to account for the effects of crystal size and carbonate content on diffracted x-ray intensity; this led to lower apparent bone contents of the precursor. In addition, Legros *et al* (1987) found that the mineral of rat and bovine bone, subjected to a number of techniques, displayed a constant proportion of calcium ions. They found that with age, the content of HPO_4^- was reduced, while the carbonate content increased, which would account for the increase in Ca:P ratio observed with age. It has been proposed that there are many other factors that might also reduce the x-ray diffraction intensity of a mineral, and that these might in themselves account for the reduced intensity seen in bone mineral. It has therefore been suggested that there is no need for an amorphous calcium phosphate theory (Glimcher *et al* 1981).

It is also still unclear what the initial form of the crystalline mineral phase is, or how many intermediates there are between the proposed amorphous calcium phosphate and the mature hydroxyapatite crystals, however octacalcium phosphate and brushite ($CaHPO_4.2H_2O$) have both been implicated (Roufoss *et al* 1979, Kakei *et al* 1997). Octacalcium phosphate ($Ca_8H_2(PO_4)_6.5H_2O$) has a molar Ca:P ratio of 1.33, it has calcium phosphate-rich layer with a configuration resembling hydroxyapatite, and alternating with these, water-rich layers (Brown and Chow 1976). This mineral gives an x-ray powder diffraction pattern very similar to hydroxyapatite (Glimcher *et al* 1981), and it has been found in dental calculus (Saxton 1968). Brown (1966) suggested that it may be the initial solid deposit in bone; however, although it was observed in bone by Münzenberg and Gebhardt (1973), extensive studies by other workers have failed to find any. Brushite ($CaHPO_4.2H_2O$) has been found in developing chick bone. Tissue from the mid-diaphysis of 17-day chick embryos was subjected to density fractionation, whereby the denser fractions are expected to correspond to more mature tissue. Brushite was the only phase detectable by diffraction methods in the lowest density fractions, and as density (and age of tissue) increased, hydroxyapatite became more predominant. In the densest fractions, only hydroxyapatite was detected. It therefore appears that, in chicks at least, brushite is the

initial solid phase, and that there is no evidence of any phase other than hydroxyapatite being deposited as mineralisation progresses beyond its early stages (Glimcher *et al* 1981).

1.8: BONE REMODELLING

The term remodelling is used where old lamellar bone is replaced by new bone, with little or no change in the mass or shape of the whole bone (Frost 1973). The constant remodelling of cortical bone accounts for the varying degrees of mineralisation found around Haversian canals; it is a process that goes on continuously throughout life, although the rate and degree at which it occurs are highly variable (Vaughan 1981). However, it has been estimated that at any given time in an adult 3.5% of the total skeleton is being remodelled (Rasmussen 1968), and that the whole adult skeleton is replenished every ten years.

Remodelling is necessary for the repair of micro damage to bones incurred through normal loading (Frost 1986); however, it is also increased in response to increased loading (Hert *et al* 1972, Bouvier and Hylander 1981). In addition to the maintenance of the skeleton's mechanical competence, bone remodelling is necessary to maintain mineral homeostasis (Lacroix 1971, Frost 1973, Parfitt *et al* 1983).

1.8.1: Mechanism of Bone Remodelling

Remodelling is carried out by an organised group of bone cells collectively referred to as bone remodelling units. Their actions can be separated into four phases: activation, resorption, reversal, and formation (Baron 1977, Frost 1986). Activation is the process that changes a resting bone surface into a resorbing surface. It involves the recruitment and subsequent fusion of osteoclast precursors, and the penetration of the layer of bone lining cells. In the adult human, a new bone remodelling unit is activated every ten seconds. Resorption is then carried out by the newly recruited osteoclasts, which create Howships lacunae in trabecullar bone, and 'cutting cones' in compact bone (Parfitt *et al* 1983). The next stage is the reversal from bone resorption to formation, which results in a balancing, or coupling, of the two processes. The mechanism of this coupling is not understood, however, osteoblasts are attracted to resorption sites; possibly as a consequence of the release of growth factors such as TGF-ß from the resorbed matrix; and bone formation occurs (Price and Russell 1992). The uncoupling of these two processes so that the rates are no longer equal results in bone pathologies such as osteoporosis.

Bone formation occurs in two stages. Osteoblasts lay down layers of osteoid in areas previously excavated by osteoclasts; these lamellae are later slowly mineralised after a time lag of several days, the exact length of which is species-specific. There is always a thin layer of uncalcified preosseous tissue during the formation of bone, even in animals with an optimum intake of minerals. This layer is no longer present after the apposition of bone is complete (McLean and Urist 1968). It has been suggested that during the time lag between osteoid being lain down and mineralised, biochemical changes occur in the matrix to make it receptive to mineralisation (Anderson 1989). Primary mineralisation is relatively rapid, and within a few days 75% of the final mineral level is achieved (Amprino and Engstrom 1952). Secondary mineralisation is slower, and has been reported as taking from 18 weeks in the dog (McLean and Urist 1968) to as long as a year (Amprino and Engstrom 1952) to reach the final level of mineralisation. Once the Haversian canal reaches its final diameter, apposition stops and the osteons metabolic activity is at a minimum (McLean and Urist 1968).

1.8.2: Control of Bone Remodelling

Why resorption or apposition should occur in any osteon at any point in time is not known, many remain quiescent for long periods (Vaughan 1981). Various factors are known to alter formation and resorption rates; however, the specific control of bone cells is poorly understood. Controlling factors are thought to be produced locally within the bone under the influence of local and systemic factors and environmental factors such as loading on the bone (Rawlinson *et al* 1991).

Although genetic factors determine the gross configuration of a bone, it is mechanical influences, such as cardiovascular action, gravity, muscle tone, voluntary muscle activity, and the impact between the skeleton and the environment, which modify and develop the fine detail of bone structure (Murray 1936). It has been suggested that control of remodelling is largely dependent on the indirect effect produced when mechanical energy is transduced to electrical energy by the bone matrix and vasculature. A deforming force produces stress that is converted to a proportional electrical command signal; this signal tells the bone cells when, how, and in what orientation to function to adjust the mechanical properties of the bone to the need (Vaughan 1981). More recently, however, experiments have shown that bone cells respond to loading by producing almost immediately prostacyclin in the case of osteocytes and osteoblasts, and prostaglandin E_2 in the case of osteocytes (Rawlinson *et al* 1991). This results in increased bone cell metabolism; indicated by elevated glucose 6-phosphate dehydrogenase activity; followed by RNA production (Pead *et al* 1988). As osteocytes are not concerned with matrix

synthesis, the RNA may be coding for a cytokine or growth factor, such as TGF-ß or IGF-I. It is therefore possible that growth factor production may be able to control certain aspects of bone remodelling in relation to loading (Lanyon 1992).

Factors circulating in the blood stream are also known to affect bone remodelling. For example, parathyroid hormone is known to cause bone resorption by increasing the cyclic AMP within osteoblasts, and utilises secondary messengers by activation of the phosphoinositide pathway. This results in the production of local cytokines and growth factors which act directly on osteoclasts. 1,25 dihydroxyvitamin D_3, the active metabolite of vitamin D_3, tends to increase bone resorption. Receptors for this vitamin are present on osteoblasts and osteocytes (Boivin *et al* 1987), and are up-regulated by oestradiol, glucocorticoids, parathyroid hormone, and 1,25 vitamin D_3 itself (Goff *et al* 1990, Hirst and Feldman 1982). Calcitonin, which is produced by the c cells of the thyroid, inhibits resorption by interaction with its osteoclast receptors (Chambers and Magnus 1982).

Local factors affecting bone turnover include the cytokine IL-6; a potent bone resorptive agent synthesised by many cells including cultured osteoblast-like cells when stimulated by IL-1. Another local factor is TGF-ß, a large gene family including the bone morphogenic proteins. These are important in both formation and resorption, and some bone morphogenic proteins can induce bone formation in other tissues. TGF-ß is mitotic for osteoprogenitor cells and osteoblasts, and can increase bone formation both by increasing the activity of individual osteoblasts, and by raising the numbers of osteoblasts (Centrella *et al* 1986). Two other local factors, osteoclast stimulating/activating factor and prostaglandins, particularly prostaglandin-PGE_2, are also recognised as taking part in the process of bone remodelling (Horton *et al* 1972). Osteoclast stimulating/activating factor is a protein-containing macromolecule (Strumpf *et al* 1978). It appears to be produced by lymphocytes activated by prostaglandins of the E series, which are in turn synthesised and released by macrophages (Yoneda and Mundy 1979). All the classical prostaglandins and their metabolites so far studied are stimulators of bone resorption in vitro (Seyberth *et al* 1978); however, those of the E series are the most potent. Their effect on resorption is similar to that of parathyroid hormone, and both are inhibited by calcitonin. However the prostaglandins merely stimulate the activity of osteoclasts, whilst parathyroid hormone also increases their numbers, and prostaglandins are more sensitive to the inhibiting effect of cortisol (Vaughan 1981).

1.8.3: Osteopenia and Osteoporosis

Pathology can occur in bone that has previously been laid down with no apparent problems. Any reduction in the amount of structural bone is termed an osteopenia, and

there are a variety of types and causes. Bone loss can be permanent or temporary, and a bone remodelling disorder may or may not be involved. For example, bone loss can occur due to a reduction in muscle strength and activity. Normal activities would not cause fracture, and the remodelling process is considered normal since bone strength and mass is adequate for the needs of the reduced mechanical usage (Frost 1997). Transient, or naturally reversible (Jaworski 1976) osteopenias can occur after a severe injury. This is partly due to disuse, and partly due to the regional acceleratory phenomenon (Frost 1997). Infection, injury and some tumours can accelerate all normally ongoing tissue processes, which usually hastens healing and improves local resistance to infection (Frost 1986, 1995). When the injury heals and normal mechanical usage return, most of the lost bone is replaced with no special treatment (Frost 1997).

True osteoporosis is said to be a naturally irreversible osteopenia accompanied by such increased bone fragility that the subject's usual physical activities cause bone pain and/or spontaneous fracture. There may be increased bone microdamage, and the remodelling process is considered impaired since the bone strength and mass is not adequate for the needs of normal mechanical usage (Frost 1997). This can be through deficient bone formation, enhanced bone resorption, or a combination of the two. Osteoporosis can occur with age and with the hormonal changes associated with the menopause in humans (Aguado *et al* 1997). It can also occur due to poor mineral availability in the diet at a critical stage of development (Chan *et al* 1984, Thorp 1994), or declining availability or intake of minerals with age, when bone mass is increased (Heaney 1996).

Osteoporosis is generally clinically assessed in terms of bone mass within key areas of the skeleton, and attention is most often directed towards cancellous rather than cortical bone. However, the risk of fracture in the human femoral neck increases 13-fold between the ages of 60 and 80 years (Bell *et al* 1999), whilst De Laet *et al* (1997) have estimated that the decline in bone mass between these ages would contribute only to a doubling of the fracture risk. Therefore changes in other factors such as the bone structure, its distribution, and its quality are also likely to be important (Kanis and McCloskey 1996). Although comparatively few studies have looked at cortical bone as opposed to cancellous bone with respect to osteoporosis, cortical bone loss has been observed in humans with increasing age, associated with increasing Haversian canal size and canal number (Thompson 1980).

Both cortical porosity, and the density of Haversian canals have been shown to have implications for bone strength (Barth *et al* 1992; Squillante and Williams 1993). For example, the elastic modulus of bovine cortical bone has been found to decrease as a power

(-0.55) of increasing porosity (Schaffler and Burr 1988). In addition, osteoporitic human femur shafts have been found to be less strong and stiff than control bone, both parameters being related to increased porosity (Dickenson *et al* 1981). Porosity has also been investigated in respect to loading and fracture risk. The calcaneus of sheep, which has cortices that are predominantly in compression or tension, demonstrates marked differences in porosity associated with these loading environments; the cortex in tension had higher porosity than that in compression (Lanyon 1973). It has also been shown that the compression cortex has a greater density of osteons (Skedros *et al* 1994), implying a greater rate of bone turnover. Reduced mechanical loading, or its reduced perception (Lanyon 1996), is a major mechanism for the development of bone loss and osteoporosis. A lack of a porosity gradient between cortex sites under compression and tension, and an increase in porosity in sites under compression were found in human females with a femoral neck fracture. This suggests that the fracture subjects experienced different strain environments, or that the ability to sense such strains was impaired (Bell *et al* 1999). Recent evidence has suggested that osteocyte viability decreases with age in the femoral head (Dunstan *et al* 1993); as these cells are thought to act as mechanosensors (Mullender and Huiskes 1997), this may lead indirectly to structural failure.

Concerning poultry, laying hens are the most common victims of osteoporosis. Exercise (Riddell 1989) and environmental factors such as cage design (Rowland and Harms 1970) have been shown by some to result in small improvements in the breaking strength of bones from hens. However, laying hens are an example of the importance of mineral requirements in the development of osteoporosis. The calcium requirements of hens during growth, pre-lay, and lay vary markedly (Roland 1986), yet despite considerable variation in the age of birds at the onset of lay, all members of a flock are changed from grower, to pre-lay, to layer diets simultaneously. These changes in diet give marked alterations in the amount of calcium, phosphorus, and possibly vitamins available to the birds (Thorp 1994), and the requirements of some birds will be incorrectly met at a critical point in their skeletal development (Roland and Rao 1992). This may be an important cause of the individual variation seen in bone volume within a flock of layers (Thorp 1994).

Among some European poultry meat producers there is also growing concern about excessive bone fragility in broilers resulting in bone fractures during carcass processing. Thirty-five day old broilers from a control population considered to have good skeletal health had reasonable quality tibiotarsus cortical bone as indicated by the cortical thickness and low porosity. However, samples from commercial populations of broilers showed marked individual variation, ranging from samples showing extensive cortical porosity, to

examples in which the quality was equal to that of the controls. Those birds with the most porous cortical bone also had the lowest %ash content, and it was suggested that they may be prone to fractures, and account for those seen during carcass processing. The causes of the variability in cortical bone porosity and %ash are uncertain, but it was suggested to be due to varying mineral uptake by individual birds, possibly caused by enteric or other factors (Thorp and Waddington 1997). However, there may also be a similar effect to that suggested by Roland and Rao (1992) for layers: there may be some variation in growth rate and stage of skeletal development between birds of a flock, yet all are swapped between starter, grower, and finisher diets simultaneously. If at any point sufficient mineral is not provided in the diet for the bone production required, it is possible that an attempt is made to obtain it by reduced formation or increased resorption at other less essential sites of bone, resulting in osteopenia.

1.9: AIMS OF THESIS

For a number of years there have been concerns about the welfare of broiler chickens due to the unacceptably high incidence of bone disorders which can cause lameness. This has promoted the funding of a large volume of research into skeletal health, by Research Councils such as BBSRC, MAFF and by the industry itself. Much of the research to date has focussed on infectious diseases, nutritional disorders such as rickets, and metabolic disorders such as TD, which cause large economic losses to the industry each year. However, despite a large body of information on a variety of causes of leg problems, poultry farmers are still concerned about the skeletal quality of modern broilers, and in particular, the number of fractures seen in birds at slaughter.

Although some attention has been paid to bone mineral content, research into skeletal health has been predominantly concerned with growth plate cartilage at the proximal and distal ends of the long bones. There has been relatively little work done on the compact bone of the shaft of the long bones, which is, after all, the main load bearing tissue, and the area where many of the observed fractures occur. The work contained in this thesis aims to further the understanding of long bone development in the modern meat-type chicken. In particular, it aims to expand knowledge of several aspects of cortical bone quality in the diaphysis of the tibiotarsus, the fastest growing and major weight-bearing long bone. Of primary interest is whether cortical bone quality has been compromised in modern strains of broilers, and if so, why, and can the problem be rectified. The aspects of quality investigated include cortical bone thickness, porosity, mineral content and mineral

composition, and on a more general note, growth plate pathology, bone turnover, and bone breaking strength and stiffness. This work also aims to examine the effect of dietary mineral content on these aspects of bone quality, whether current mineral inclusion rates are adequate for the modern, rapidly growing broiler, and if possible, to identify optimum dietary Ca and P contents for the modern strain. In addition, it is also intended to investigate what effect the rapid growth rate and heavy weight of the modern strain has on bone quality.

CHAPTER 2
EXPERIMENTAL METHODS

2.1: ASSESSMENT OF BLOOD MINERAL STATUS

2.1.1: Ionised Calcium Measurement

One to 2 ml of blood were taken from the wing vein and transferred to a 2 ml heparin tube, which was stored on ice until measurement (usually within 1 hour). Samples were shaken, and circulating ionised Ca was measured using a Ciba-corning 634Ca^{++}/pH analyser.

2.1.2: Plasma Total Calcium

Blood samples were centrifuged at 2000 g and 1°C for 10 minutes, and plasma samples were transferred to Eppendorfs for storage at –20°C until the assay was performed. Total Ca was measured using a commercial kit (Wako Chemicals GmbH, Germany), which is based on the o-cresolphthalein complexon (OCPC) colour development method. OCPC combines with alkaline earth metals to assume a purplish red colour, and the 8-hydroxyquinoline in the colour reagent affords colour development of calcium specifically.

Standards and Samples

Two stock standards were supplied with the kit. The first contained 20 mg/dl Ca, and was used as the top standard. The second contained 10 mg/dl Ca, and this was used as the second standard. Three further standards containing 5, 2.5 and 1.25 mg/dl Ca were produced by a series of 2x dilutions of the second standard with distilled water. Four microlitres of each standard were pipetted into 2 adjacent wells on a 96 well plate. Four microlitres of distilled water were pipetted into two adjacent wells as duplicate blanks, and 4 µl of each plasma sample (neat) were also pipetted into duplicate wells in the plate.

Assay Procedure

Two hundred microlitres of the buffer solution provided (0.2% boric acid, 5.4% monoethanolamine) were pipetted into each well, followed by 20 µl of the colour reagent (1% 8-hydroxyquinoline, 0.04% o-cresolphthalein complexon, 0.7% hydrochloric acid). The plate was left to incubate at room temperature for 15 minutes before the absorbance of the standards and samples was measured in a Dynatech MR500 plate reader at a wavelength of 560 nm; colour intensity was directly proportional to total Ca content.

2.1.3: Plasma Inorganic Phosphorus

Samples were prepared and stored in the same manner as for total Ca measurement. Plasma inorganic P was measured using a commercial kit (Wako Chemicals GmbH, Germany), which is based on the molybdenum-blue method. When the colour reagents are combined and added to plasma samples, inorganic P in the samples combines with molybdate in the colour reagent to form phosphomolybdic acid. This is then reduced by ferrous sulphate to form molybdenum-blue.

Standards and Samples

The stock standard supplied with the kit contained 10 mg/dl inorganic P. This was used as the highest standard, and a series of 2x dilutions were made with distilled water to produce 3 more standards containing 5, 2.5, and 1.25 mg/dl P. Five microlitres of each standard were pipetted into 2 adjacent wells on a 96 well plate, and duplicate wells with 5 µl of distilled water were used as blanks. Five microlitres of each plasma sample (neat) were also pipetted into duplicate wells in the plate.

Assay Procedure

Colour reagent A and B supplied with the kit (highly purified preparations of molybdate and ferrous sulphate) were mixed in equal measures, and 125 µl of the resulting solution were pipetted into each well in the plate. The plate was left to incubate at room temperature for 15 minutes, and then read in a Dynatech MR500 plate-reader at a wavelength of 690 nm; colour intensity was directly proportional to inorganic P content.

2.1.4: 1,25-dihydroxyvitamin D

Circulating levels of 1,25-dihydroxyvitamin D, the active metabolite of vitamin D involved in Ca homeostasis, were measured using a commercially available kit (IDS, Tyne and Wear, UK). This is a competitive binding radioimmunoassay, using 1,25 dihydroxyvitamin D radioactively labelled with ^{125}Iodine to bind excess antibody not bound by the native 1,25 dihydroxyvitamin D.

Standards and Samples

To prepare each sample, 350 µl of plasma were transferred to a tube, and 35 µl of the delipidation reagent provided (dextran sulphate and magnesium chloride) were added. The tubes were then vortexed, and centrifuged at 2000 g for 30 minutes. Six standards were provided containing 0, 7, 15, 54, 170, and 488 pmol/l of 1,25-dihydroxyvitamin D. These were provided in lyophilised form, and approximately 15 minutes before use 1 ml of

distilled water was added to each bottle to reconstitute them. Also provided were 2 bottles containing lyophilised human serum as quality controls, which were prepared in the same manner as the standards.

Immunoextraction of Samples

This stage purified the 1,25 dihydroxyvitamin D present in the plasma samples. The immunocapsules provided contained monoclonal antibody to 1,25 dihydroxyvitamin D linked to solid particles. The capsules were vortexed, and allowed to stand for 5 minutes. The caps were removed from the capsules, and 100 µl of the delipidated samples were added to each of 2 immunocapsules, the capsules were then rotated end-over-end for 3 hours at room temperature. After mixing, the capsules were allowed to settle for 5 minutes. The caps were then removed, the bottom stopper broken off, and each capsule was placed over a tube, and centrifuged at 1000 g for 1 minute to aid the removal of unbound constituents of the sample. Still over the tubes, 500 µl of the wash solution (phosphate buffered saline with tween-20) were added to each capsule, and centrifuged as above; this step was repeated a second time.

After the capsules had been washed, they were transferred to fresh tubes, and 150 µl of the elution reagent provided were added to each capsule and allowed to soak in for 2 minutes. The tubes with their capsules were again centrifuged at 1000 g for 1 minute, and this step was repeated a further 2 times, giving a total eluted volume of 450 µl. The tubes containing the elutes were dried under a flow of nitrogen. The residues in each tube were then reconstituted in 100 µl of assay buffer (BSA-phosphate buffer) and vortexed.

Assay Procedure

One hundred microlitres of each standard were placed in appropriately labelled duplicate tubes, and the 100 µl samples were assembled. Two hundred microlitres of the primary antibody provided (sheep anti-1,25 dihydroxyvitamin D) were added to each tube. Non-specific binding (NSB) tubes were prepared using 300 µl of assay buffer, and nothing further was added to these tubes at this stage. The function of the NSB tubes was to demonstrate the amount of ^{125}I-1,25 dihydroxyvitamin D that binds to proteins other than the primary antibody. All tubes were vortexed, and incubated at 4°C overnight (16-24 hours).

The next day, 200 µl of ^{125}I-1,25 dihydroxyvitamin D were added to each tube, including duplicate total count tubes, used to measure the counts per minute given by this amount of label. The tubes were then incubated at room temperature for 2 hours.

After incubation, immunoprecipitation of the bound ^{125}I-1,25 dihydroxyvitamin D was performed by adding 100 µl of anti-sheep IgG antibody provided to each tube, except the total count tubes. The tubes were vortexed, and allowed to incubate at room temperature for 30 minutes. Four millilitres of the wash concentrate solution provided (phosphate buffered saline containing tween to aid decanting) was added to each tube except the total count tubes, and all tubes were centrifuged at 2000 g for 20 minutes. Finally, all but the total count tubes were decanted and allowed to drain, before counting in a gamma counter for 1 minute. A computer programme was then used to convert the counts per minute of each sample into pmol/l of native 1,25 dihydroxyvitamin D; concentration was inversely proportional to the number of counts per minute.

2.2: ASHING AND DETERMINATION OF MINERAL CONTENT

2.2.1: Bone Ash, Ca, P and Mg Determination

Bone Sample Preparation and Ashing

Bones destined to be subject to ashing analysis (tibiotarsi or humeri) were dissected from the appropriate culled birds, cleaned, and stored at -20°C until required. Their length was measured, and 5 equal segments were marked off along the bones. The proximal head segment was removed, and the shaft segments cut using either a hacksaw, or a hand-held motorised circular blade.

Bone samples were oil extracted in petroleum spirit using a Tecator Soxtec System HT 1043 Extraction Unit. Samples were wrapped in filter paper labelled with the sample identification number, placed in Whatman single thickness cellulose extraction thimbles (diameter 26 mm, length 60 mm), and immersed in boiling petroleum spirit for 1 hour (birds aged under 25 days) or 1.5 hours (birds aged 32-45 days). They were then suspended above the petroleum spirit and rinsed in the falling condensed vapour for the same amount of time. Samples were subsequently placed into labelled beakers (age under 14 days 50 ml, 100 ml for samples from older birds) and into an oven at 100°C for drying. Samples were generally left in the oven for 4 hours, although those from birds under 11 days old could be removed after 2-3 hours.

The samples were then allowed to cool in a dessicator. The beakers and the dry, fat free bone samples were weighed separately, and the samples were placed in a muffle furnace at 550°C for a minimum of 16 hours. After this time, they were removed from the furnace and allowed to cool in a dessicator before weighing the ash and beakers together.

Percentage ash (total bone mineral content) was then calculated as a percentage of the dried, fat-free weight of the sample.

Ash Sample preparation

After weighing, 5 ml (50 ml beaker) or 10 ml (100 ml beaker) of 6 N HCl was pipetted into each beaker. The beakers were placed on a hot plate at a temperature between 105-200°C to allow complete digestion of the ash. Samples were removed from the hotplate once they had boiled dry, 5 or 10 ml of distilled water (or 6 N HCl, which was found to work better) was pipetted into each beaker, and they were briefly warmed on the hot plate to reconstitute the mineral.

The sample solutions were then filtered through 9 cm No 1 Whatman filter paper into an appropriately sized flask (16 day embryo 25 ml, age 1-16 days 50 ml, age 18-45 100 ml). The beakers and filter paper were rinsed 3 times with distilled water, and the samples were diluted up to volume using distilled water.

Individual Mineral Determination (P, Ca, Mg)

The P contents of the solutions were measured by colorimetry using a Technicon Traacs 800 automated system. The sample solution was mixed with molybdovanadate reagent, and the resulting yellow-coloured solution was measured at a wavelength of 420 nm. Standard solutions contained 0%, 0.5%, 1%, 1.5%, 2% and 2.5% P, and the solutions in the flasks were diluted further if necessary to bring the sample on range. The system then calculated % P content of the original dry, fat free bone sample.

To measure Ca or Mg content, the samples were diluted a further 100 times, and analysed using a Varian AA-875 series atomic absorption spectrophotometer with a hollow cathode lamp (Cathodeon Ltd) specific for Ca and Mg analysis. For Ca analysis 0.1 ml of the solution used for P determination was added to 8.9 ml distilled water, and 1 ml of Lanthanum Chloride (this prevents the presence of other ions interfering with the Ca analysis). Absorption readings were taken at a wavelength of 422.7 nm. Standard solutions contained 0, 1, 2, 3, 4 and 5 Ca ppm, and sample absorption readings were converted to Ca ppm. To measure Mg, 0.1ml of the solution used for P determination was added to 9.9ml distilled water, and absorption readings were taken at a wavelength of 285.2nm. Standard solutions contained 0, 0.1, 0.2, 0.3, 0.4 and 0.5 ppm Mg, and sample absorption readings were converted to Mg ppm.

The % Ca and % Mg contents of the dry, fat free bone samples were calculated by:

[(flask volume / bone weight) x further dilutions x ppm] / 10,000.

The molar Ca:P ratio was calculated by:

$$[(\%Ca \times 10{,}000) / 40.08] / [(\%P \times 10{,}000) / 30.975]$$

2.2.2: Eggshell Ca, P and Mg Determination

Approximately 1 cm square samples were cut from the wide end of the eggshell using a dentist's drill with a small circular saw fitted. The samples were briefly soaked in distilled water to enable easy removal of the eggshell membranes, and were then dried at 100°C for 4 hours, and ashed as above. Digestion, reconstitution and the determination of P, Ca and Mg was the same as for bone ash samples. All samples were diluted up to 50 ml for P and Mg analysis, with a further 2-times dilution made before Ca analysis.

2.2.3: Diet Ca, P, Mg and Zn Determination

Feed samples were not dried or oil extracted. Samples were ground in a Retsch grinder (Germany) with a 1 mm mesh, and mixed in a food mixer. Duplicate 2 g sub-samples were placed in labelled 100 ml beakers and ashed in a muffle furnace at 550°C. Percentage ash was not calculated. The digestion, and reconstitution were the same as for the bone samples, as were the methods for measuring P, Ca, and Mg. All ash samples were diluted up to 100 ml.

Zinc content was measured by atomic absorption spectrophotometry using the same method as for Ca and Mg, however the lamp was exchanged for one specific for Zn analysis, and absorption was read at a wavelength of 213.9 nm. The standards contained 0, 0.25, 0.5, 0.75, 1, and 1.25 Zn ppm, and % Zn was calculated in the same manner as % Ca and % Mg.

2.3: BONE COLLAGEN (HYDROXYPROLINE) ASSAY

Hydroxyproline is an amino acid that occurs frequently in the polypeptide chains comprising the triple helix of collagen, and serves to increase the stability of the helix. This assay uses chloramine-T to oxidise hydroxyproline to a compound which reacts with dimethylamino-benzaldehyde to form a chromophore (Stegeman and Stalder 1967).

2.3.1: Sample Hydrolysation

Small (20-55mg) samples of cortical bone were placed in tightly capped, labelled extraction tubes, and 0.5 ml of 6 M HCl was added to each tube. The tubes were placed in an oven at 107°C for a minimum of 18 hours. At the end of this period, the tubes were

removed from the oven and allowed to cool, before transferring the samples to small vials. Each extraction tube was rinsed with 1 ml of distilled water, which was then added to the relevant vial. Samples were vacuum dried overnight (longer if necessary) with NaOH pellets in a dish below them to neutralise the evaporating acid. Once dry, the samples were removed from the vacuum drier and reconstituted in 0.5 ml of distilled water. They were then transferred to Eppendorfs and centrifuged at 6500 rpm for 1 minute to pellet non-soluble material, and 300-400 µl of the supernatant from each sample were transferred to fresh Eppendorfs, and stored at -20°C prior to their use in the assay.

2.3.2: Standards and Samples

A range of standards was produced from a stock hydroxyproline solution of 500 nmoles/ml. The stock was diluted from 0.4 ml to 4 ml with distilled water to give a top standard of 50 nmoles/ml, or 6.55µg/ml (1 mole = 131 g). Serial dilutions were performed to produce a range of standards with concentrations of 0.2, 0.4, 0.8, 1.6, 3.3, and 6.7 µg/ml

To assess what sample dilution was required to bring the sample within the range of the assay sensitivity, two samples were taken at random and used to produce a range of dilutions from 1:50 to 1:16000. Each of these dilutions was used in the assay procedure (see below), and for 14-day-old broilers, a 1:400 dilution was found to be suitable.

2.3.3: Assay Procedure

(After Creemers et al 1997.) Stock buffer was prepared, containing 0.24 M citric acid, 0.88 M sodium acetate (anhydrous), 0.88 M sodium acetate trihydrate, 0.21 M acetic acid, 0.85 M sodium hydroxide, and was adjusted to pH 6.1. Both the chloramine-T reagent (10 ml: 0.282 g chloramine-T, 1 ml n-propanol, 1 ml de-mineralised water, and 8 ml stock buffer) and the DMBA reagent (20 ml: 10 g dimethylaminobenzaldehyde, 6.25 ml n-propanol, and 13.75 ml perchloric acid) were prepared fresh before use. Sixty microlitres of the standards and samples were dispensed in duplicate into 96-well microplates. To each well, 20 µl of assay buffer (n-propanol, de-mineralised water, and stock buffer in a ratio of 3:2:10) was added , followed by 40 µl of the chloramine-T reagent. The microplate was left to incubate at room temperature for 15 minutes before 80 µl of the DMBA reagent was added to each well. The plate was then parafilmed to prevent evaporation, before being placed in a gently shaking water bath at 60°C so that the water just touched its underside. After 20 minutes, the microplate was removed from the bath and placed on ice to cool for 5 minutes before being read at a wavelength of 560 nm. Results are presented as mg hydroxyproline per g bone.

2.4: HISTOLOGY PROCESSING

2.4.1: Wax Sections

Fixing

At the time of dissection, pieces of bone tissue were trimmed to the required size, placed in a labelled cassette, and then into buffered neutral formalin (BNF: formaldehyde 500ml, distilled water 4500ml, sodium dihydrogen orthophosphate 20g, di-sodium hydrogen orthophosphate (anhydrous) 32.5g). The tissue was left in BNF for 7 days for fixation.

Decalcifying

After fixation, the cassettes were transferred to Goodings and Stewarts solution for decalcification (formic acid 5ml, formaldehyde 5ml, distilled water 100ml). Samples from birds up to 4 days old were left overnight; samples from birds 6-18 days old were left for 2 days; for birds 25 days old or more, tissue was left in the solution for one week.

Samples were checked for total decalcification at the end of these periods by x-raying at 35 kVp for 15 seconds (Faxitron 804, Livingston Electronics Ltd., Watford, Herts.). If any residual mineral was identified (as a white area on the x-ray) the samples were returned to Gooding and Stewarts solution. If the samples were clear, the tissue was washed in tap water overnight to remove the formic acid, and placed back in BNF until further processing.

Further Processing

Further tissue processing was done by an automatic processor (Shandon Hypercenter, Shandon Scientific Ltd., Runcorn, Cheshire). This machine ran a 23-hour cycle during which the bone samples were passed through BNF (ambient temperature), 70% ethanol (ambient temperature), 2 washes of 96% ethanol (35°C), 2 washes of 99% ethanol (35°C), 1 wash of a mix of 99% ethanol and CNP (35°C), 3 washes of CNP (35°C, 40°C, and 45°C), and 2 wax baths (53°C and 60°C). This ultimately achieved the replacement of water in the tissue samples with wax, via water-alcohol, alcohol-CNP, and CNP-wax mixing and replacement. Once the cycle was finished, the samples were held in the 60°C wax bath until they were removed.

Embedding and Cutting

Once processed, the tissue was embedded in paraffin wax with a melting point of 60°C using appropriate sized plastic moulds. The wax blocks were allowed to cool, and then

excess wax was trimmed away in 10 μm slices on the microtome to leave the sample surface exposed for cutting.

Once trimmed, blocks were cooled at -20°C for 30 minutes before sections of 4-5 μm thickness were cut. The sections were transferred to a water bath at 40-50°C and left to soften for 5-10 minutes before being transferred to a poly-l-lysine-coated microscope slide. The slides were then placed in an oven at 50°C overnight to ensure a good attachment of the section to the slide.

2.4.2: Frozen Sections

(After Chayen and Bitensky 1991.)

Preparation of Freezing Bath

A large glass jar with a lid was inserted into polystyrene, and filled 1/3 full with absolute alcohol. Dry ice chips were added until a saturated solution was obtained; the mixture became viscous, and stopped bubbling when more dry ice was added. Hexane was poured into a small beaker, which was placed into the alcohol / dry ice mix. The lid was screwed down, and the bath was left for 20 minutes to reach optimum temperature (-70°C).

Preparation of PVA

Polyvinyl alcohol (PVA) aids the cutting of frozen mineralised tissue. A 5% solution was prepared by gradually adding 5 g of PVA to 100 ml of warm water on a heated magnetic stirrer (hand hot). The solution was left stirring at a low heat for 30-50 minutes, and then allowed to cool.

Freezing and Cutting the Tissue

One at a time, bone tissue samples were dipped into PVA, and then dropped into the hexane. After 30 s, they were retrieved using forceps pre-cooled in dry-ice, placed in pre-cooled self sealing bags containing a piece of tissue paper to absorb any remaining hexane, and stored at -80°C until use. Using optimal cutting temperature compound to attach the samples to the chucks, sections 10 μm in thickness were cut at –30°C (Brights model OT cryostat), and picked up on poly-l-lysine coated microscope slides. The sections were then air died at room temperature.

2.4.3: Plastic Sections

Preparation of the Polymethylmethacrylate

Equal volumes of methylmethacrylate and 5% sodium hydroxide were added to a separating funnel and shaken vigorously, and the lower NaOH, water, and oxidised phenolate-containing fraction was discarded into the fume-hood sink. This process was performed 3 times, and a similar process was performed once with distilled water. The resulting 'washed' (purified) methylmethacrylate solution was then frozen, and passed through a vacuum filter twice to remove all the water (drying). The polymethymethacrylate for embedding purposes was then prepared by dissolving 40 g of 40% polymethylmethacrylate in 100 ml of the washed and dried methylmethacrylate. This was done on rollers at room temperature over a 2-3 day period.

Fixing and Defatting

Tissue samples were fixed in 70% alcohol for 10 days, then transferred to glass universal vials on rollers for further processing at room temperature. Samples were placed in defatting solution (chloroform: xylene: ethanol at 4:4:1) for 3 days, and then transferred to 100% ethanol for 3 days.

Embedding and Cutting

Samples were transferred to a 50:50 solution of ethanol and methylmethacrylate for 3 days, and then through 3 changes of methylmethacrylate for 3 days each, all at room temperature on rollers. They were then impregnated in polymethylmethacrylate at 4°C for 2 weeks, before being transferred to plastic moulds and submerged in polymethylmethacrylate, which was then polymerised at 35°C for 48 hours. If necessary, the polymethylmethacrylate was topped up, and the samples were returned to the 35°C oven for a further 48 hours. This process was continued until the hardened plastic had ceased to shrink, and then the samples were transferred to a 45°C oven for a further 48 hours for final hardening. Ten-micrometre-thick sections of the tissue were then cut using a Leica SM 2500E polycut.

2.5: GROWTH PLATE PATHOLOGY ASSESSMENT

2.5.1: Wax Sections

Histological Staining

Wax growth plate sections, containing epiphyseal and metaphyseal tissue, were cleared of paraffin wax by immersion in xylene for 5 minutes, then hydrated by washing in 99% then 96% ethanol, followed by water. They were stained with haematoxylin for 5 minutes, washed in tap water, then counterstained in eosin for 2 minutes. Finally, they were dehydrated by washing back up the ethanols, and cleared in xylene again before a coverslip was attached. Nuclei stained blue, cytoplasm pink.

Assessment Methods

Growth plates were assessed for the presence or absence of the pathologies described below (Thorp 1994); severity was not recorded. Normal growth plates displayed chondrocytes in the proliferation zone in regular stacks, and metaphyseal blood vessels that extended to, or close to, the proliferation zone. Growth plates were said to display hypocalcaemic rickets when a thickened and disorganised proliferation zone was observed. They were considered to be displaying tibial dyschondroplasia if there was a thickened and avascularaised transitional zone forming a cartilage lesion.

The incidence of hypophosphataemic rickets was generally not scored from de-mineralised wax sections. However, the majority of normal growth plates were observed to have a hypertrophic zone approximately 3 times the size of the proliferation zone, while hypophosphotaemic rickets is known to result in extended metaphyseal vessels, and a thickened hypertrophic zone. Growth plate samples in one experiment (Chapter 4) were therefore marked positive if the combined thickness of the transition and hypertrophic zone was more than 3 times the thickness of the proliferation zone, with numerous extended metaphyseal vessels, and there was no evidence of TD to account for this. However, this was found to be an arbitrary and potentially unreliable measurement, and hypophosphataemic rickets was not scored from demineralised wax sections in subsequent work.

2.5.2: Un-demineralised Frozen/ Plastic/Wax Sections

Histological Staining

Von Kossa and Haemotoxylin stains were utilised on un-demineralised growth plate sections containing epiphyseal and metaphyseal tissue. The slides were soaked in water, then placed in haemotoxylin for 3 minutes to stain nuclei blue. They were rinsed in running water until the water ran clear, then placed in a 5% solution of silver nitrate for 30 minutes to stain calcified tissue black. Slides were washed 3 times in distilled water, then placed in sodium thiosulphate for 5 minutes to fix the silver nitrate stain. They were washed in distilled water, placed in eosin for 1 minute to stain cytoplasm pink, and briefly rinsed. Sections were dehydrated in 96% and 99% alcohol, and cleared in xylene before coverslips were mounted with DPX.

Assessment Methods

Growth plates were assessed for health status using the criteria described previously, except in the case of hypophosphataemic rickets. Additional evidence of normality was the presence of well-mineralised matrix in the hypertrophic zone. Growth plates displaying hypocalcaemic rickets had little or no black staining in the hypertrophic zone, while those displaying TD had no black staining within the site of the lesion (Plate 2.1). The primary reason for using this method was the reliable identification of growth plates displaying hypophosphataemic rickets. Samples were marked as positive for this disorder if cells in the proliferation zone displayed a normal columnar distribution (i.e. no evidence of hypocalcaemic rickets) and there were numerous metaphyseal vessels extending to the proliferation zone (i.e. no evidence of TD), yet the hypertrophic zone was poorly mineralised.

2.6: BONE SOLIDITY ASSESSMENT

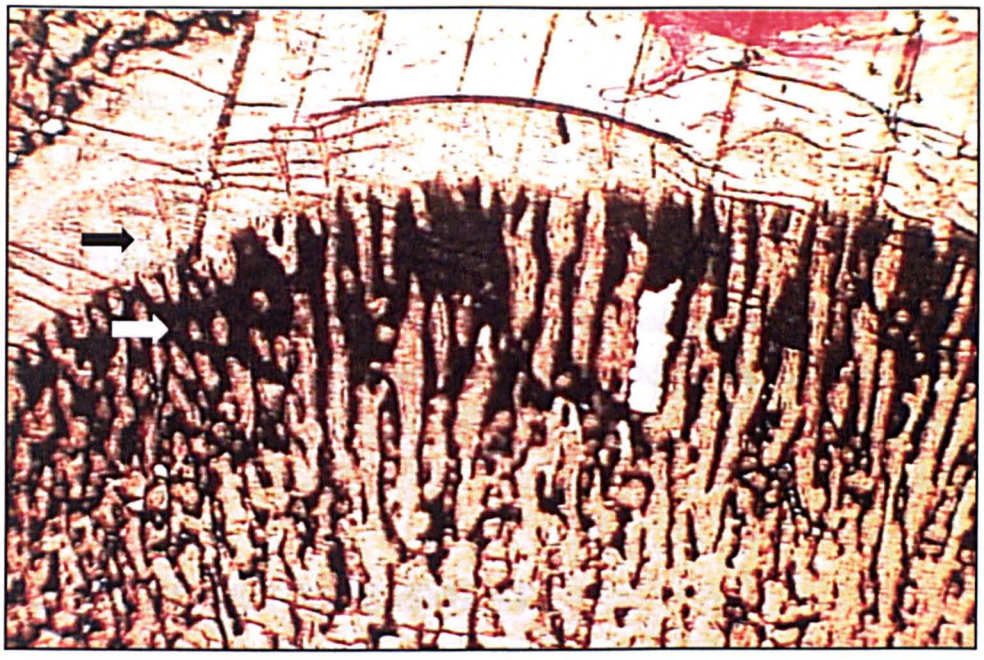
2.6.1: Histological Staining

Wax-embedded cross sections of mid diaphysis were de-paraffinised by immersion in xylene for 5 minutes, and then hydrated in 99% then 96% ethanol, and then water. They were stained in 1% Toluidine Blue in 50% isopropanol for 15 minutes at room temperature, then placed in isopropanol for 1 minute. Sections were then blotted dry, before clearing in xylene and attaching a coverslip with DPX.

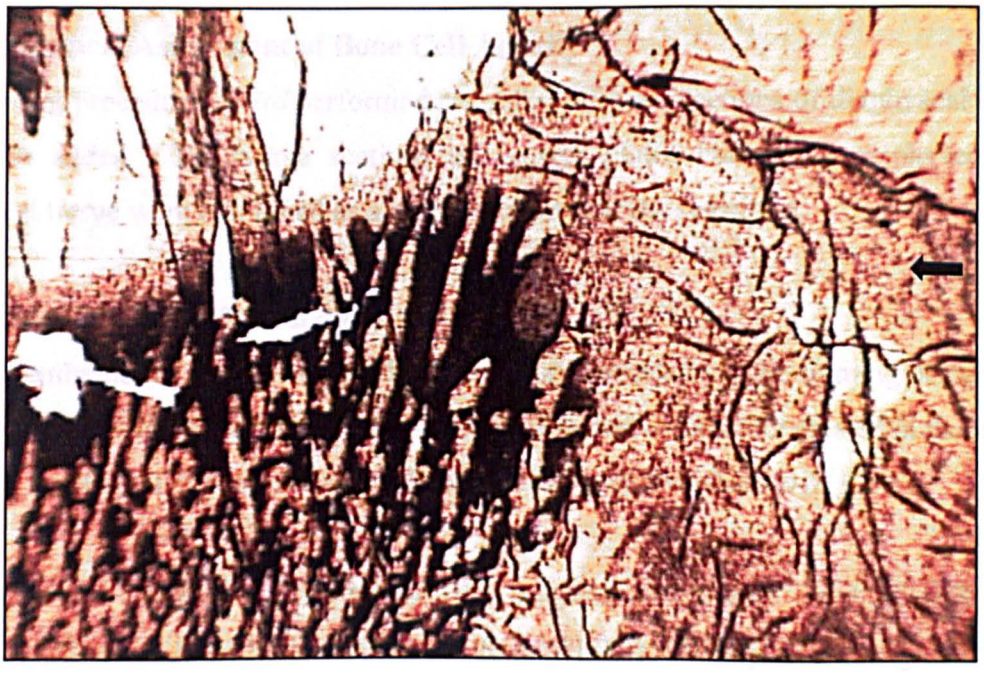
Plate 2.1:

Comparison of a normal growth plate with one displaying tibial dyschondroplasia (Von Kossa staining)

a.



b.



a. Normal growth plate showing the unmineralised proliferation zone (black arrow) and the mineralised hypertrophic zone (white arrow)

b. Growth plate exhibiting the unmineralised hypertrophic lesion characteristic of tibial dychondroplasia (arrow).

2.6.2: Image Analysis

Sections were analysed using 'NIH image' (version 1.59, a public domain image analysis software package) to measure the proportion of the field of view at x20 magnification which contained cortical bone (bone solidity, the inverse of porosity). Measurements were made across the total cortical width in samples from chicks up to 4 days old, and for endosteal (inner surface) and periosteal (outer surface) areas in older birds.

Each field of view was captured in duplicate onto a Power-Mac computer running NIH Image, and one of each pair converted to a binary (black and white) picture by 'density slicing', where the operator entered what levels of light and dark in the image corresponded to bone. The binary picture was then edited, using the duplicate image in its original form as a reference, to ensure that the black areas were representative of the areas of bone. The software then measured what percentage of the edited picture was black, i.e. bone. Four fields of view on each of 3 sections were analysed in this manner for each bone area for each bird, and these values were averaged to give a single value per bone area per bird.

2.7: BONE TURNOVER ASSESSMENT

2.7.1: Histological Assessment of Bone Cell Activity

The following procedures were performed on unfixed frozen sections of diaphyseal cross-sections on slides. Transverse sections of growth plates containing epiphyseal and metaphyseal tissue were employed as a control to monitor the reactions.

Alkaline Phosphatase (ALP) Reaction

ALP is a membrane-bound enzyme produced in large amounts by bone-forming osteoblasts. It is thought to be involved in the mineralisation process, and the amount present in these cells depends on the state of differentiation and functional activity (Bradbeer *et al* 1992).

Procedure: Two hundred microlitres of 10% magnesium chloride ($MgCl_26H_2O$) were added to 50 ml of 0.1 M sodium barbitone buffer (pH 9.4). Twenty five milligrams of α-napthyl phosphate (0.5 mg/ml) were then added to the solution, which was placed in a 37°C oven to warm for 20-30 minutes. On removing the solution from the oven, 50 mg fast blue salt (1 mg/ml) were added just before use, the solution was stirred quickly, and transferred to a coplan jar containing the sections. The slides were incubated in the solution at 37°C for 2 minutes, then the reaction was halted by pouring out the blue

solution, and adding 50 ml of ice-cold 0.1 M acetic acid. The slides were washed twice with distilled water, and a cover slip was mounted with aquamount (BDH, Poole, Dorset). The presence of ALP results in a blue-black reaction product; a higher occurrence of cells (osteoblasts) on the bone surfaces containing this product implies a higher occurrence of active bone formation (Zanelli and Loveridge 1990).

Tartrate Resistant Acid Phosphatase (TRAP) Reaction

Acid phosphatase (AP) is a lysosomal enzyme characteristic of active macrophages, giant cells, and osteoclasts. Unlike other forms of AP, osteoclastic AP is resistant to the inhibitory effect of tartrate, hence the inclusion of tartrate in the reagent enables the specific identification of active osteoclasts (Minkin 1982).

Procedure: 50 ml of citrate buffer were warmed to 37°C, and then 25 mg napthol-ASBI-phosphate (0.5 mg/ml) and 115 mg of 10 mM potassium sodium tartrate (2.3 mg/ml) were added and dissolved in it. The slides were incubated in this solution for 2 minutes at 37°C, then rinsed with distilled water, followed by 50 mM sodium fluoride. The sections were 'post coupled' in 50 ml of 0.1 M acetate buffer (pH 6.2) containing 50 mg of Fast Garnet (1 mg/ml), then rinsed in distilled water before a cover slip was mounted using aquamount. The presence of TRAP results in a purple reaction product. Multinucleate cells containing this product are actively resorbing osteoclasts; a purple haze at the bone surface may imply the recent presence of active osteoclasts that had left the site at the time of tissue sampling (Zanelli and Loveridge 1990).

2.7.2: Fluorescent (Calcein) Assessment of Bone Formation Rate

Calcein (fluoresceindi-(methylimino diacetic acid) sodium salt) is a fluorescent indicator that is taken up by bone in preference to calcium. Injection of a specified dose of calcein is used to mark the mineralisation front around the time of injection; a ring excitable by UV-radiation is produced corresponding to that new bone being actively mineralised around the periosteal and/or osteonal surface. By giving a bird two injections of such a fluorescent marker a known number of days apart, it is possible to calculate the rate at which new bone has been laid down (Compston 1998).

Solution preparation and Injection

Syringes containing solutions of calcein in sterile phosphate-buffered saline (PBS) were prepared in advance for intra peritoneal injections in the abdomen. Using previously obtained mean body weights of the lightest broiler strain involved at the appropriate ages

as a guideline, solutions were prepared to give birds 20 mg calcein per kg of body weight, to be delivered in 0.5ml of solution per bird. Birds of other strains which were heavier at a given age therefore received a dose below 20 mg/kg, however, the dose received was adequate for the purpose of producing detectable rings in most cases, and this approach prevented potentially toxic doses of calcein being given to lighter birds. For each gram of calcein to be added to the solution, 2 g of sodium bicarbonate was first dissolved in the appropriate amount of PBS with the aid of a magnetic stirrer. The calcein was then added to the solution, left to stir for 10 minutes, and the resulting solution was then stored in the dark at 4°C until use. Two injections were given a known number of days apart before the animal was culled.

Bone Sectioning

Mid-diaphyseal segments were taken from the injected birds, and stored in 70% alcohol in the dark. Due to the small size of the segments, one end of each sample was embedded in a block of LR White resin (Agar Scientific Ltd, Essex, UK), leaving the other end free for cutting. The samples were first de-hydrated in two changes of 100% alcohol for a day each to aid binding to the resin. Each sample was pushed through a hole in a piece of card labelled with the sample number. The card was then laid across the mouth of an Eppendorf filled with LR White, so that one end of the sample was submerged in the resin, and the other end was free. The lids on the Eppendorfs had been removed previously to enable the cards to balance easily, and they were slightly over-filled with resin to allow for shrinkage. The samples were then placed in an oven at 60°C overnight (18-24 hours) to polymerise.

Once the bone segments were securely mounted, three sections 100-200 μm thick were cut from each sample on a diamond saw (Microslice2, Metals Research Ltd, Cambridge, UK), using the resin-filled Eppendorf to clamp the sample in position. These sections were stored in labelled cassettes in 70% alcohol, again in the dark, until use.

Assessment of Bone Formation

Sections were temporarily mounted in UV-free aqueous mountant (Raymond Lamb, London, UK) on microscope slides with a well, and coverslips were added. They were then examined under a microscope using a Leica I3 fluorescent filter cube with an excitation wavelength of 470±20 nm bandpass. If two rings were present, the distance between their outer edges was measured at the four 'compass-points' of each section (12 measurements per sample) using the NIH image analysis system. These measurements were then used to calculate the thickness of bone laid down per day between injections.

2.7.3: Circulating Levels of Markers

Osteocalcin

This is a protein exclusively synthesised by bone-forming osteoblasts, and most of that produced is incorporated into the newly forming bone matrix. However, approximately 15% of the protein produced is released into the circulatory system, and although the physiological function of osteocalcin is still unclear, the concentration of this circulating osteocalcin has been found to reflect the rate of bone formation. The following method (after Vandershueren *et al* 1990) is a competitive binding radioimmunoassay using osteocalcin radioactively labelled with ^{125}Iodine to bind excess antibody not bound to native osteocalcin.

Standards and Samples: A series of concentrations of unlabelled osteocalcin were made up to produce a standard curve of counts per minute versus protein concentration. The source solution of osteocalcin was at a concentration of 1.5 mg/ml PBS-HCl solution. An aliquot of this was diluted in diluent (fraction V bovine serum albumin in PBS at 1 g/l; 0.5 M, pH 7.4) to produce a stock standard of 1500 ng/ml. Serial dilutions were then made with diluent to produce 10 standards containing osteocalcin at concentrations of 0.7, 1.4, 2.7, 5.5, 10.9, 21.9, 43.8, 87.5, 175.0, and 350.0 ng/ml. One hundred microlitres of each standard were pipetted into tubes in triplicate, the zero standards consisting of 100 µl of diluent. Plasma samples were diluted 1:10 in diluent to reduce non-specific binding of the labelled osteocalcin, and 100 µl of each diluted sample were transferred to tubes in triplicate. Samples of previously analysed chicken plasma, diluted 1:10 as above, were used as quality controls by comparing the current results with previous analyses.

Assay Procedure: Five hundred microlitres of osteocalcin antibody (1 part serum to 12500 parts diluent) were added to each tube. Non-specific binding tubes containing 600 µl of diluent were prepared to assess the binding of ^{125}I-osteocalcin to proteins other than osteocalcin antibody. The tubes were mixed well, and incubated at 4°C overnight.

The following day, in order to assess the current radiation emission from the labelled osteocalcin, 10 µl of the stock ^{125}I-osteocalcin were pipetted into two tubes, and these were placed in a gamma count reader (PackardBell). From the reading, the number of counts emitted per minute (cpm) from 1 µl of stock (cpm_{stock}) was calculated. In order to place enough ^{125}I-osteocalcin to give approximately 20,000 cpm into each of the triplicate total count tubes, ($20{,}000/cpm_{stock}$) µl of stock, mixed in 100 µl of diluent, was

added to each tube, and to each sample, standard, and NSB tube. The tubes were then mixed, and incubated at 4°C overnight.

On the third day, the bound ^{125}I-osteocalcin was immuno-precipitated by adding 100 µl of diluted sheep-anti-rabbit antibody (1 part serum to 20 parts diluent) to all tubes except the Total Counts Tubes, mixing, and incubating at 4°C for 2 hours. Following this, 1 ml of 4% polyethylene glycol (which improves precipitation) containing 0.1% Tween 20 was pipetted into each tube, and the tubes were mixed, and left standing at room temperature for a further 30 minutes. At the end of this period, the tubes (except the Total Counts) were centrifuged at 3500 rpm for 30 minutes at 4°C, after which the liquid was decanted off leaving the radioactively labelled precipitate in the tube to be read. Each tube was counted for 1 minute, and the data was transferred to a PC with a specially designed program (Williams, INRA Nouzilly) for calculation of the quantity of native osteocalcin in each 100 µl plasma sample. Results were expressed as ng osteocalcin per ml plasma.

Pyridinoline Cross-Links

Plasma samples were sent to the University of Bristol for pyridinoline cross-link analysis using a method after Bailey *et al* (1995). These cross-links provide the stability in mature collagen, and are released only as a product of collagen degradation. They are not present in skin, and are present in blood vessels only in small amounts. Since the majority of the remaining collagen in the body is found in the bone and cartilage of the skeletal system, circulating levels of these cross links are largely attributed to bone or cartilage resorption in the skeleton.

Assay Procedure: Five hundred microlitres of plasma were added to an equal volume of concentrated HCl and made up to 2 ml with 6 M HCl. Samples were then hydrolysed at 112°C in a sealed container for 24 hours, prior to lyophillisation and subsequent rehydration in 100 µl of an aqueous solution of 1% trifluoroacetic acid (TFA). Aliquots of all the samples were analysed after 0.2 µm filtration by direct injection onto a 0.46 x 10 cm Shandon HyperCarb S column eluted at 1 ml/min using a linear gradient from 0-12% tetrahydrofuran in water, both containing 0.5% TFA. Detection and quantitation of pyridinoline was by means of its natural fluorescence at 295nm excitation and 405nm emission when compared to standard material previously quantified by amino acid analysis, and content was expressed as pMol pyridinoline per 30 µl plasma. Further aliquots of selected samples (approximately 1 in 5) were run as duplicates after pre-chromatography using CF1 cellulose columns (Sims and Bailey 1992) in order to

concentrate the pyridinoline, remove contaminants and to confirm accurate and reliable quantitation within the whole plasma hydrolysates.

2.8: BONE RADIODENSITY AND DIMENSION MEASUREMENT

2.8.1: X-Ray Procedure

Tibiotarsi were x-rayed alongside an aluminium wedge with steps of known thickness at 35 KVP for 15 seconds (Faxitron 804; Plate 2.2).

2.8.2: Dimension Measurement

Using a light box, bone length was measured from the x-ray with a ruler, and 5 equal segments were marked off to one side of the image along the length of the bone image. A black and white camera was used to capture an image of a ruler onto a Power-Mac computer running 'NIH Image'. A line was then drawn between two points on the ruler, and the user entered values for the distance measured, and the aspect ratio for the camera in use in order to set the scale of pixels per mm for further measurements in mm. An image of the bone x-ray was then captured, and the mid-shaft section of the bone was selected. The total and marrow widths of this section were measured automatically at approximately 30 equally spaced points along its length and average total and marrow width of the mid-section were calculated for each bone.

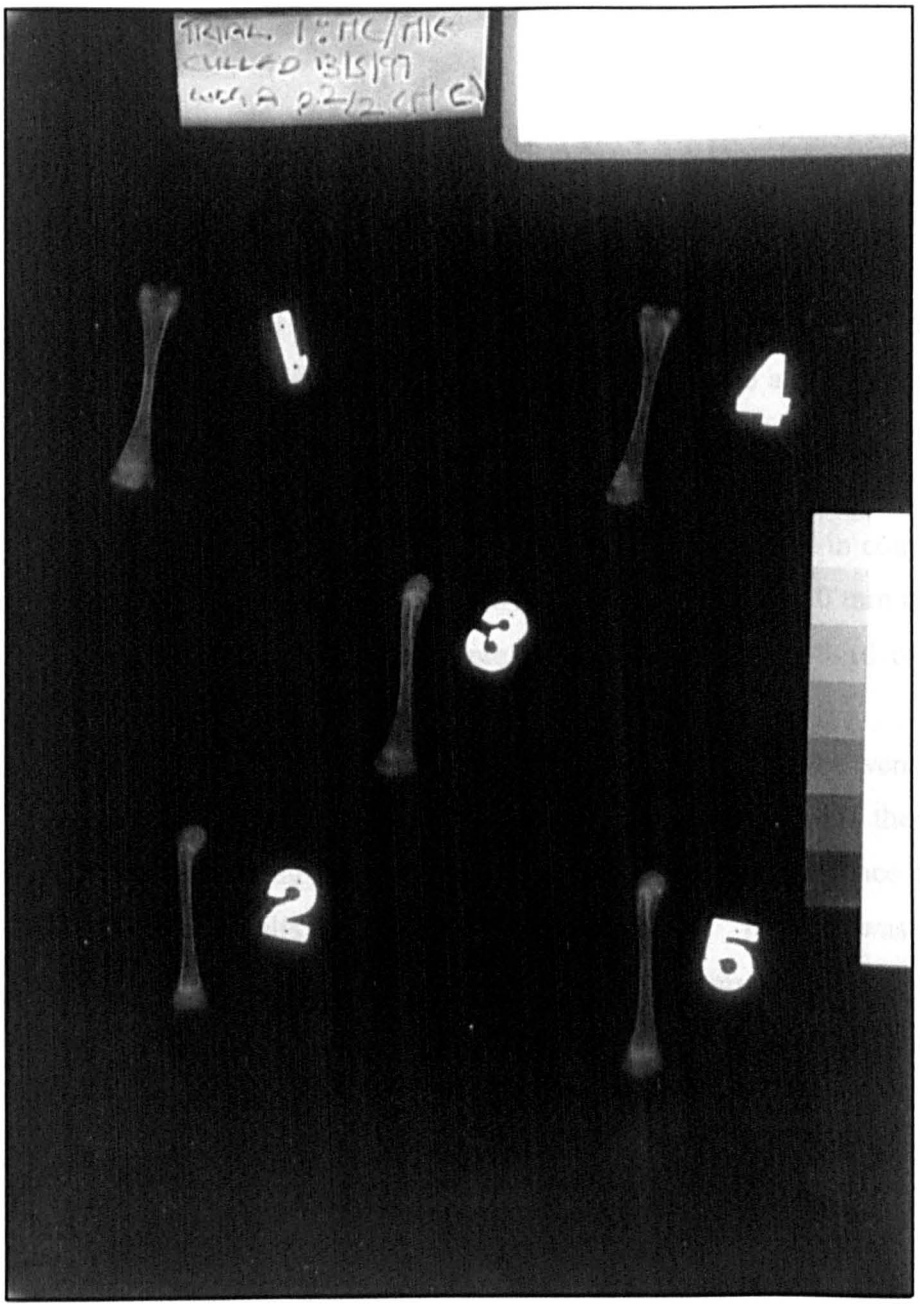
2.8.3: Radiodensity Measurement

(After Fleming *et al* 2000.) The light box was turned to maximum brightness and allowed to warm up for a minimum of 30 minutes before analysis began to ensure that no changes occured in the light level emitted. All parts of the light box other than that immediately under the camera were covered to prevent glare from other parts of the x-ray affecting measurements of the selected image. Room blinds were drawn to prevent changes in lighting due to variations in the weather, and artificial lighting level was kept constant.

For each film, the aperture on the camera was adjusted so that the brightest points on the bones on the film were just below saturation point (when differences in brightness can no longer be identified; saturated pixels show up red in the live NIH image). After this adjustment, an image of each of the steps on the aluminium step wedge x-ray was captured in turn on the computer, starting with the darkest square (no aluminium). A representative region within each square was selected, and its average pixel value (brightness) on the greyscale was measured.

Plate 2.2:

An example of the x-ray layout for bone radiodensity and dimension analysis



This procedure was performed on all un-saturated squares, and any steps not being measured were masked to prevent glare from them affecting the current measurement. These pixel values and the equivalent known aluminium step thickness' were paired, and a 3rd degree polynomial curve was fitted to calibrate future radiodensity measurements from this film in mmAl equivalent. For each bone x-ray on the film, all but one segment of bone were masked, and an image of the required segment was captured, and its radiodensity measured in mmAl; i.e. the segment was said to have a radiodensity equivalent to that of aluminium at a particular thickness. Segment radiodensity values could then be compared with the ash analysis of the equivalent shaft section.

2.9: BONE STRENGTH AND STIFFNESS ANALYSIS

Bones were first cleaned of all meat and periosteal membrane, taking care not to damage them in the process. Mechanical properties were then measured using an LRX materials tester (Lloyds Instruments, UK) with a load cell value of 2500 N, and a cell calibration of 105.5%. The materials tester was under remote control of a PC running the software package 'Nexygen 2.2' via the software package 'LrLrxConsole' (both Lloyds Instruments, UK). The compress to rupture test within Nexygen was used in combination with a 3-point bend jig on the materials tester (two curved lower rests of 10 mm diameter, with a 30 mm fixed span, on which the sample was balanced, and a third of similar dimensions applying a downward force central to them).

Bone length and width were measured using calipers, and the values were entered into the computer, along with the batch and sample references. Samples were then placed on the jig, always with the distal end to the left, with the flat distal surface pointing upwards, to ensure that the results were as consistent as possible. The test was then set running, the bone broken, and Nexygen calculated the maximum load applied to the sample before it ruptured, and stiffness (the slope of the tangent to the steepest part of the load versus extension graph).

CHAPTER 3
SKELETAL HEALTH AND DEVELOPMENT IN THE MEAT-TYPE CHICKEN

3.1: INTRODUCTION

A variety of health and reproductive problems occur in broilers (Reddy 1996), and some of the most common health problems observed are skeletal disorders (Kestin *et al* 1992). Skeletal problems compromise the birds' welfare. They also reduce growth, increase mortality, increase carcass downgrading due to lesions, and are recognised as one of the four major factors adversely affecting the performance of broilers (Day 1990).

It is generally recognised that selection pressure for muscle growth in the broiler has resulted in increased demands being placed on skeletal integrity, and over the past decade meat-type chicken lines have been selected for improved skeletal quality in addition to growth performance. However, this process is made difficult by the numerous specific causes of leg problems. These include a variety of nutritional disorders, infectious diseases, metabolic conditions, conformational problems, and toxins (Riddell *et al* 1983; Thorp 1994). In addition, increased porosity and a Ca:P ratio greater than expected have been found in the bones of some modern broilers at 35 days of age, and it is possible that both may contribute to bone weakness (Thorp and Waddington 1997). The growth performance of the modern broiler has changed considerably over recent years; however, the diets provided have changed little in terms of mineral content. Thorp and Waddington (1997) postulated that the observed porosity associated with lowered bone mineral contents could be due to an inadequate mineral supply (primarily Ca and P) in the diets currently used commercially. If the supply of one or both of these minerals is inadequate, it is possible that normal circulating levels of both are maintained by increased bone resorption, or by reduced bone formation within the osteons of cortical bone (Stevens and Lowe 1992); both would result in greater porosity.

3.2: AIMS

The main aim of the present study was to ascertain how the tibiotarsi of modern meat-type chickens develop under a fast-growth rearing regime typical of commercial conditions, and

to compare their development with that of tibiotarsi from a slower growing genetic precursor. Changes over 6 weeks were observed for measures of bird growth and body conformation, and bone radiodensity, dimensions, porosity and mineral content, expanding on the results of Thorp and Waddington (1997). A preliminary investigation into possible links between weight, conformational changes, growth rate, and bone growth and mineralisation was also made.

3.3: MATERIALS AND METHODS

3.3.1: Birds and Housing

Male birds from two lines of Ross birds were used: a slow-growing control strain (MK) that has not been selected for growth performance since 1972, and a fast-growing strain selected for growth, food conversion, and skeletal quality (MCX). Unfortunately, both strains were not available at the same time, and 110 selected birds were housed at a Ross pedigree farm (farm 1), while 110 control and 55 selected birds were later housed at a different Ross farm (farm 2). The selected birds at the second farm were controls for any differences between the two farms, although environmental and nutritional conditions were expected to be the same. The birds were reared from day old to 6 weeks under standard conditions at the farms. They were fed the starter and grower diets normally used by Ross Breeders (Ca 9.5 g/kg, available P 5.0 g/kg, crumb; and Ca 9.0 g/kg, available P 4.8 g/kg, pelleted respectively). They were housed in a floor pen with wood shavings, and a space allowance of 0.81 square feet per bird. A minimum of 4 Maco pan feeders and Rainbow bell drinkers was provided. The temperature was maintained at 29°C on day one, gradually lowered to 25°C at day 21, and then lowered to 19°C at day 35. Light was provided for 21 hours per day, starting at 21 lux, and reducing to 16 lux by day 21.

3.3.2: Sampling Procedure

All the birds were wing-banded on day one, and individually weighed every week. Any birds that became lame were excluded from the study. Ten selected birds at farm 1, and 10 control and 5 selected birds at farm 2 were randomly chosen and culled by cervical dislocation at 4, 11, 18, 25, 32, and 39 days of age. The final total body weight was obtained for each bird, both tibiotarsi were dissected out, and the breast, thigh, and calf muscles were dissected out and weighed. Samples of feed were taken from each batch delivered to the farms during the experiment for elemental analysis.

3.3.3: Sample Processing

(Chapter 2.) The right tibiotarsus was x-rayed, and its length was measured from the x-ray using a ruler. The x-ray was then used to measure radiodensity, and total and marrow width of the mid shaft section by image analysis. After x-raying, the diaphyseal shaft was divided into three sections (proximal, mid, and distal) for ashing, and the measurement of ash, total P and Ca content as a proportion of the dry fat free sample. The feed samples were ashed, and the trace elements copper and zinc were measured in addition to Ca and P. Duplicate samples were analysed for each batch, and the mean was calculated for each element. A portion of mid-diaphyseal bone from the left tibiotarsus was histologically processed, and used to assess solidity by image analysis. Samples of the proximal growth plate were also taken for histological processing and pathology assessment.

3.3.4: Derived Measurements

Instantaneous growth rate was calculated for the last week of life using the equation:

$$IGR = [ln(weight_2) - ln(weight_1)] / [day_2 - day_1]$$

(Brody 1945). For those birds which were culled before two weekly weights had been taken, the weekly weight taken before culling and the final total body weight were used. The proportion of each muscle type was calculated as a percentage of total body weight, and the molar Ca:P ratio was calculated from measurements of bone Ca and P content by:

$$molar\ Ca{:}P\ ratio = [\%Ca / 40.08] \times [30.975 / \%P].$$

Mean cortical thickness was calculated from measurements of total and marrow width, and the tibiotarsus area moment of inertia (MI, an estimate of resistance to bending) was calculated after Biewener (1992), assuming the tibiotarsus to be a circular tube, as:

$$MI = T \times (\pi / 2^6) \text{ where } T = (total\ width)^4 - (marrow\ width)^4$$

.

3.3.5: Statistical Analysis

Residual maximum likelihood (REML) analysis (equivalent to ANOVA) for unbalanced split plot designs (Patterson and Thomson 1971) was performed on all the measurements. Farm and bird (where applicable) were fitted as random factors to account for any differences due to variations in farm conditions or individual birds. The effects of age, strain, shaft section (where applicable), and their interactions were explored before final simplified models were determined for each variable. Effects were assessed using Wald tests, a generalisation of the Student's t-test, which has a chi-squared distribution for large data sets. The effects of body weight, IGR, and the proportion of various muscles on skeletal development were investigated, allowing for age and strain, by using these measures as covariates in REML analyses. The residuals of significant covariates were

plotted against those of the measure affected to assess plausibility; those relationships which appeared to rely on a few select data points were rejected. Possible strain differences in the slopes of the allometric relationships of MI, tibiotarsus length and width to body weight were examined by linear regression of logarithmically transformed data. Two-tailed T-tests were performed on molar Ca:P ratio data to determine whether observed values differed from the expected value of 1.67 (Pellegrino and Biltz 1968). The significance levels for the T-tests were adjusted by a Bonferroni correction to allow for the 12 simultaneous tests.

3.4: RESULTS

3.4.1: Feed Quality

The first batch of feed at each farm was of a starter diet, all other samples were of grower-finisher diet. Contents of each mineral varied with the batch, including between batches of the same grower-finisher diet; amounts of Ca and copper were most variable (Table 3.1). The contents of copper did not become low enough to cause deficiency in the mineral; however, the ratio of Ca to total P varied widely between batches of feed: 1.04-1.5 at farm 1, and 0.93-1.39 at farm 2 for the grower-finisher feed.

3.4.2: Growth and Weight Variables

Final Growth Rate

Final instantaneous growth rate was inversely related to the age of the bird culled, and was generally higher in the selected birds than the control birds except at 18 days (age by strain interaction: Wald statistic (W) 9.9, degrees of freedom (df) 4, P=0.05; see Figure 3.1).

Figure 3.1: Changes in mean instantaneous relative growth rate with age, together with standard errors

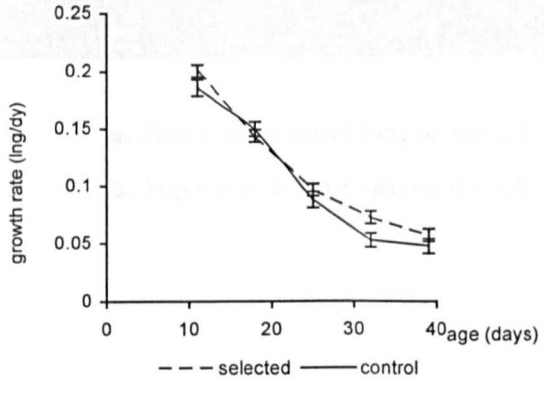
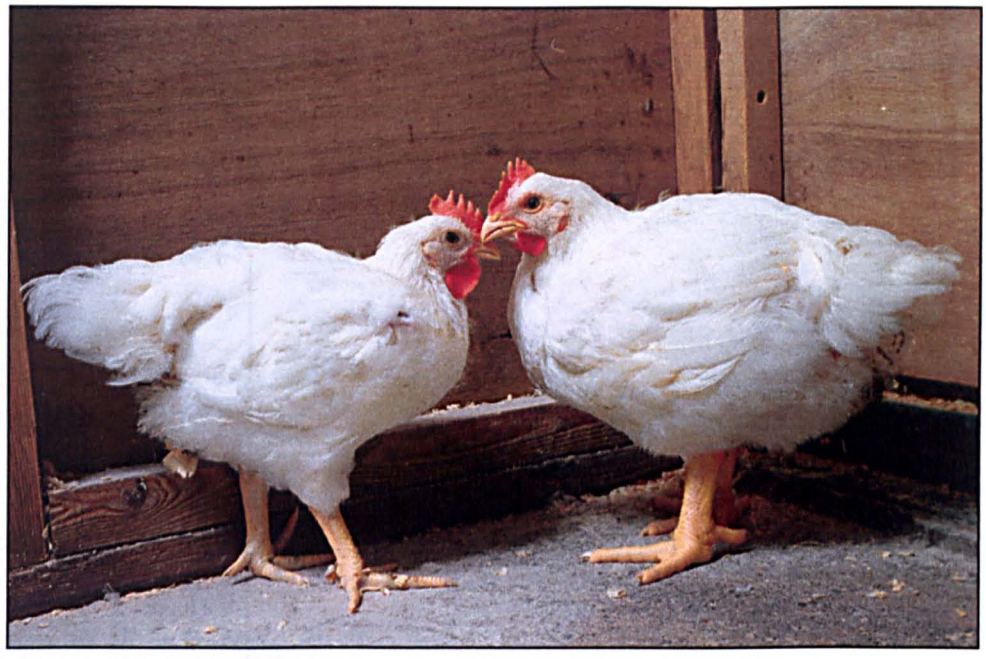


Plate 3.1:

Comparing the body size of selected and control birds at age 40 days

a.



b.

a. Side view, control bird on the left.

b. Top view, control bird on the left.

Table 3.1. Mineral contents of feed batches

	Farm 1					Farm 2			
feed type	Ca (g/kg)	P (g/kg)	Cu/ppm	Zn/ppm	feed type	Ca (g/kg)	P (g/kg)	Cu/ppm	Zn/ppm
starter	9.9	7.5	0.57	0.66	starter	9.9	7.1	0.40	0.59
grower-finisher	7.9	7.6	0.49	0.61	grower-finisher	6.3	6.8	0.37	0.51
grower-finisher	9.6	6.4	0.47	0.62	grower-finisher	8.5	7.2	0.42	0.66
grower-finisher	7.8	6.4	0.28	0.54	grower-finisher	8.8	7.1	0.44	0.65
grower-finisher	8.9	7.4	0.40	0.62	grower-finisher	6.8	7.4	0.39	0.56
					grower-finisher	6.3	7.6	0.44	0.67

Total Body Weight At Cull

Examination of the residual plots from analysis of the raw data showed that variance was not constant over the treatment groups, and the data were therefore transformed by taking natural logs. Body weight at cull increased with the age of the bird, was always higher in the selected birds, and increased at a much greater rate in the selected birds (age by strain interaction: W 575.4, df 5, $P<0.001$; Figure 3.2a, Plate 3.1)

Percentage Breast Muscle

This was much higher in the selected birds than the control birds throughout their life spans. It increased with age in both strains, however, this increase was much larger in the selected birds (age by strain interaction: W 87.9, df 5, $P<0.001$; Figure 3.2b).

Percentage Thigh and Calf Muscle

The proportion of thigh and calf muscle increased with age in a similar manner for both strains (age effect: thigh W 60.4, calf W 139.6, df 5, $P<0.001$), however there was a consistently greater proportion in the selected strain (strain effect: thigh W 28.5, df 1, $P<0.001$; calf W 5.0, df 1, $P<0.05$ Figures 3.2c and d).

3.4.3: Growth Plate Pathology

Examples of transverse sections through growth plates showing evidence of various pathologies can be found in Plate 3.2. Overall levels of pathology were similar in both strains, as were levels of TD (Table 3.2). The incidence of pathology was quite small, involving only up to 3 birds per strain at any one age, hence no statistical analyses were performed on the data. There was no pathology seen at 4 days in either strain, and beyond 4 days, there appeared to be no observable pattern of pathology occurring with age.

Table 3.2: The incidence of growth plate pathologies

age	normal		hypocalcaemic		TD		unidentified	
	selected	control	selected	control	selected	control	selected	control
4	15	10	0	0	0	0	0	0
11	14	7	0	0	1	0	1	0
18	12	10	1	0	2	0	0	0
25	12	5	0	1	2	0	1	1
32	13	6	0	0	1	3	1	0
39	9	5	0	1	3	2	1	0
totals	75	43	1	2	9	5	4	1
percents	84%	84%	1%	4%	10%	10%	5%	2%

Figure 3.2: Changes in means with age, together with standard errors: a. final weight at cull; b. percentage breast muscle; c. percentage thigh muscle; d. percentage calf muscle.

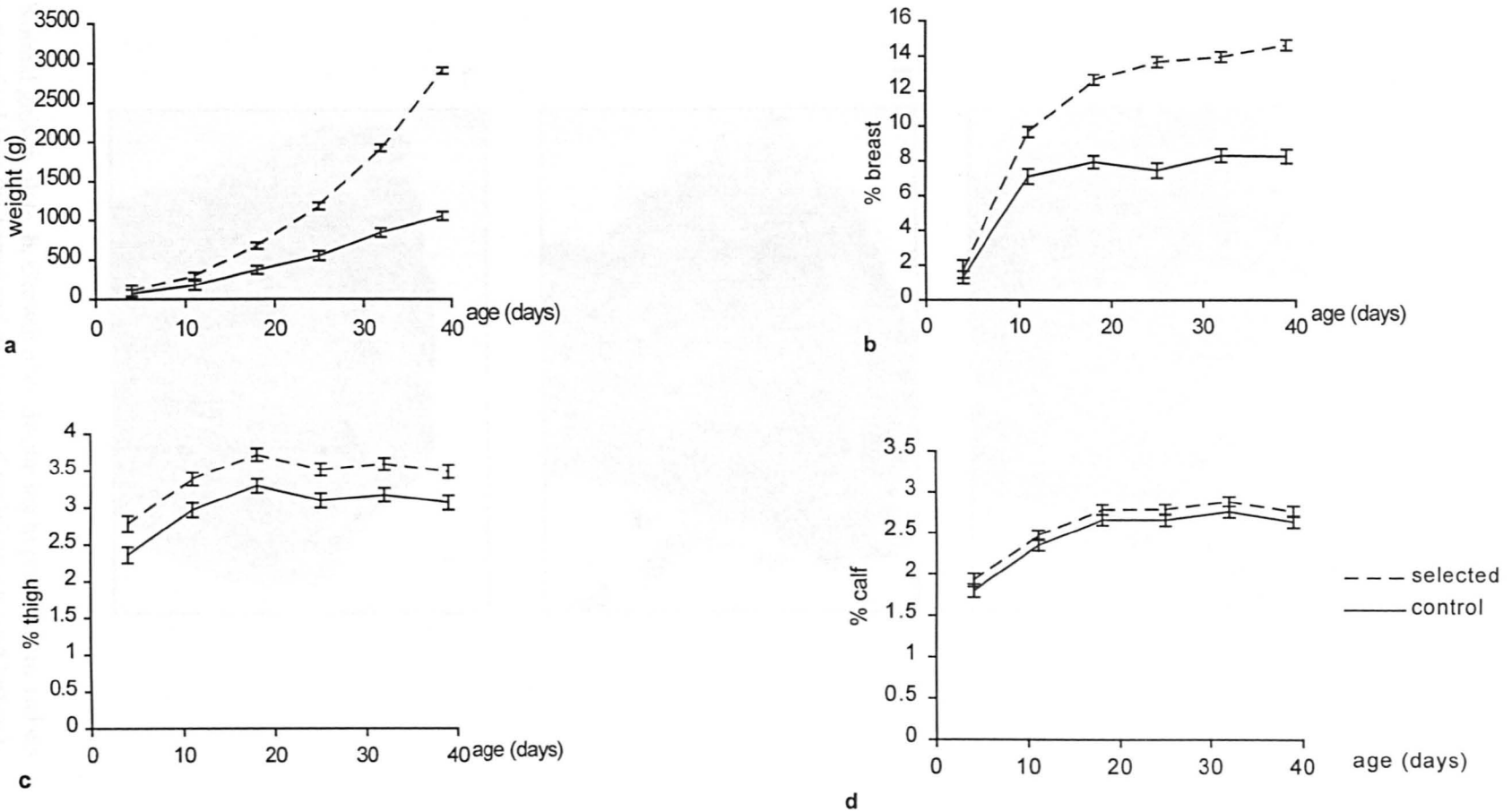
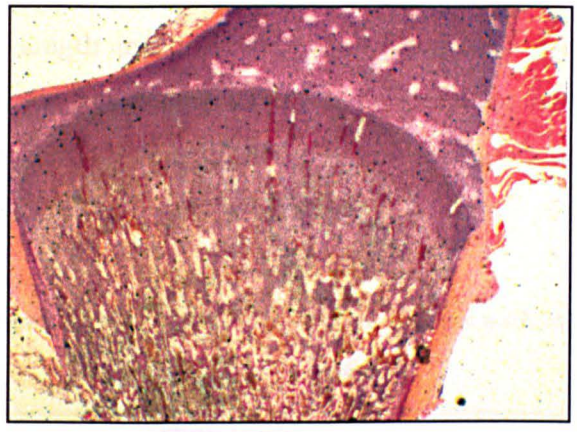


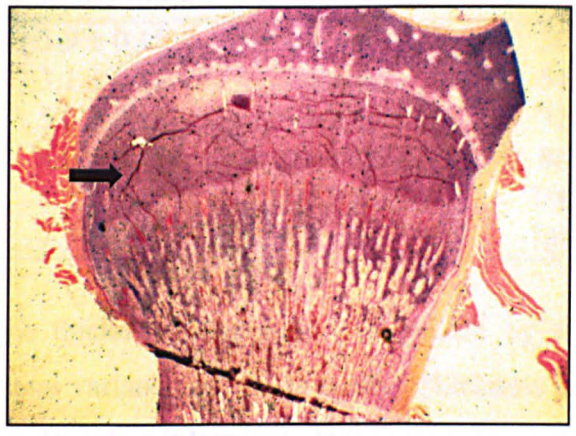
Plate. 3.2:

Growth plate sections displaying selected pathologies

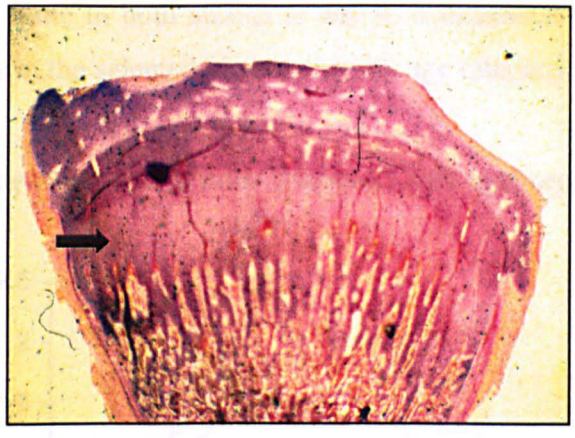
a.



b.



c.



a. Normal growth plate. **b.** Growth plate displaying hypocalcaemic rickets. Characterised by a thickened and disorganised proliferation zone (arrow). **c.** Growth plate displaying tibial dychondroplasia. Characterised by an unmineralised lesion in the pre-hypertrophic zone (arrow).

3.4.4: Age and Strain Effects on Bone

Tibiotarsus Dimensions

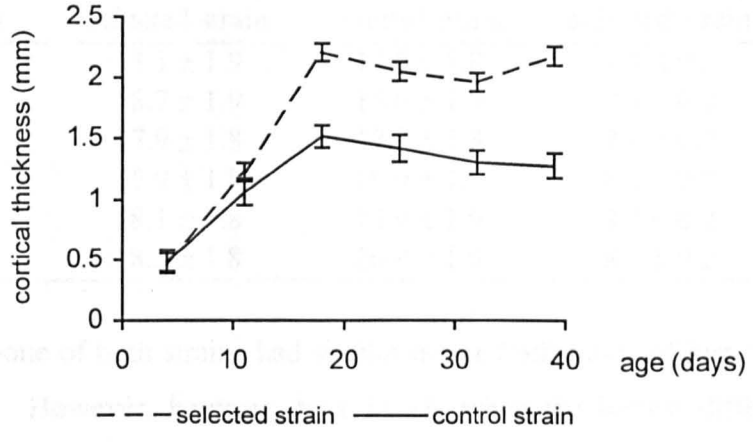
Tibiotarsi increased in length with age in both strains, and the rate of increase was approximately constant. Those of the selected birds were of a similar length to those of control birds at day 4 (Table 3.3), however, they grew more rapidly in the selected strain. This difference in length increased with age (strain by age interaction: W 92.8, df 5, $P<0.001$). Tibiotarsus width also increased with age in both strains; again, width was similar in both strains at day four (Table 3.3), and increased more rapidly over the 6 weeks in the selected strain (strain by age interaction: W 44.5, df 5, $P<0.001$).

Table 3.3. Changes in mean tibiotarsus dimensions with age, together with standard errors

	width (mm)		length (mm)	
age (days)	selected strain	control strain	selected strain	control strain
4	1.9 ± 0.15	1.6 ± 0.18	36 ± 1.0	34 ± 1.2
11	3.1 ± 0.15	2.6 ± 0.21	54 ± 1.0	48 ± 1.4
18	5.0 ± 0.15	3.8 ± 0.18	72 ± 1.0	63 ± 1.2
25	6.0 ± 0.15	4.7 ± 0.21	86 ± 1.0	76 ± 1.4
32	6.7 ± 0.15	5.1 ± 0.19	100 ± 1.0	89 ± 1.3
39	7.7 ± 0.16	5.3 ± 0.20	116 ± 1.0	94 ± 1.3

Cortical bone thickness data were transformed by taking natural logs to give approximately constant variance over treatments. Thickness increased rapidly with age in both strains until day 18, when it levelled off (Figure 3.3). Although cortical thickness was approximately the same in both strains at day 4, it increased more rapidly and reached a much greater value in the selected birds (strain by age interaction: W 41.9, df 5, $P<0.001$).

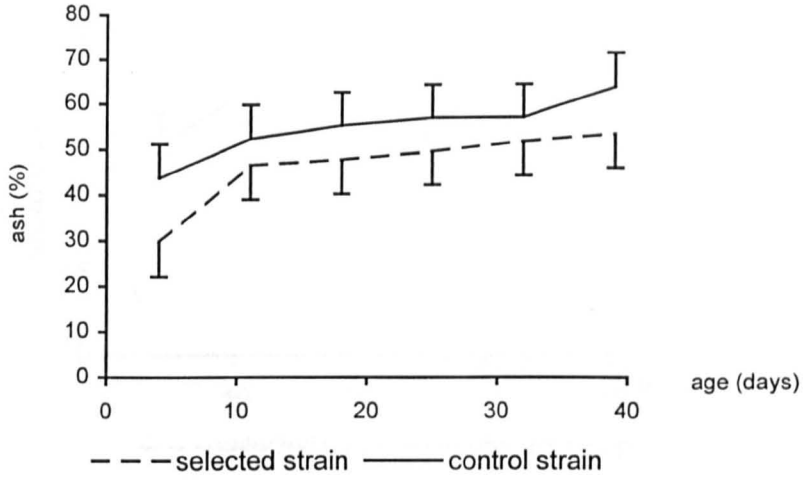
Figure 3.3: Changes in cortical bone thickness with age, together with standard errors



Mineral Content

There was a rapid increase in the mean ash content of the tibiotarsus shaft between days 4-11, after which the increase continued in a less pronounced manner (Figure 3.4). At all ages, the control birds had higher ash content than the selected birds, with the largest differences at 4 and 39 days (strain by age interaction: W 50.3, df 6, P<0.001).

Figure 3.4: Changes in mean cortical bone mineral content with age, together with standard errors



Mean shaft Ca content of both strains was higher at day 39 than at day 4 (Table 3.4). However, Ca content fluctuated differently in each strain over the 6 weeks, resulting in a strain by age interaction (W 195.9, df 5, P<0.001). Phosphorus content also tended to increase with age (Table 3.4), but again, fluctuations in P contents over the 6 weeks resulted in a strain by age interaction (W 82.7, df 5, P<0.001).

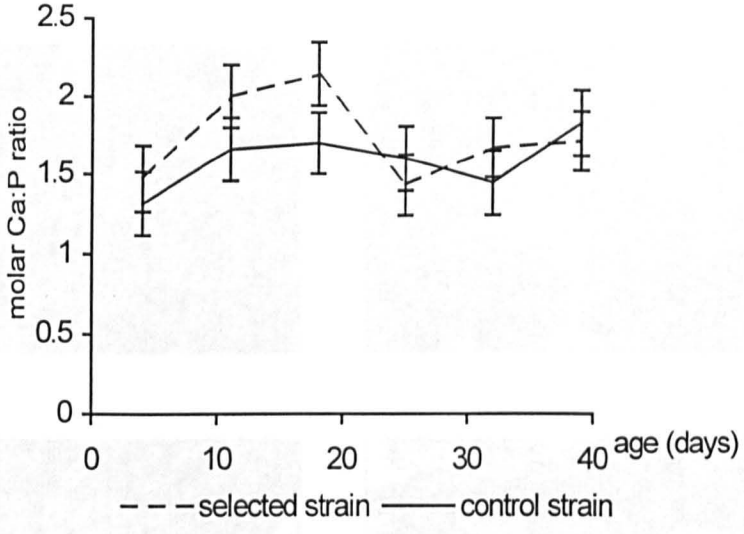
Table 3.4. Changes in mean cortical bone Ca and P content with age, together with standard errors

	Calcium (%)		Phosphorus (%)	
age (days)	selected strain	control strain	selected strain	control strain
4	13.1 ± 1.9	12.0 ± 1.8	7.1 ± 0.2	7.1 ± 0.3
11	18.7 ± 1.9	15.6 ± 1.9	7.7 ± 0.2	7.5 ± 0.3
18	17.9 ± 1.8	17.3 ± 1.8	7.0 ± 0.2	8.0 ± 0.3
25	15.9 ± 1.8	19.9 ± 1.9	8.8 ± 0.2	9.5 ± 0.3
32	18.1 ± 1.8	13.9 ± 1.9	8.4 ± 0.2	7.3 ± 0.3
39	18.5 ± 1.8	26.9 ± 1.9	8.4 ± 0.2	11.4 ± 0.3

The bone of both strains had similar molar Ca:P ratios of just over 1.7 by 39 days (Figure 3.5). However, between days 11-18, when the largest difference between the strains was observed, the ratio of the selected strain averaged over the 3 diaphyseal

sections reached values which were significantly higher than 1.67 (days 11 and 18: t 5.52 and 6.79 respectively; df 123, P<0.01). The control strain showed a ratio significantly lower than 1.67 at day 4 (t -3.39, df 123, P<0.01); however, it kept much closer to the predicted ratio at other ages (strain by age interaction: W 26.6, df 5, P<0.001).

Figure 3.5: Changes in mean molar Ca:P ratio of cortical bone with age, together with standard errors



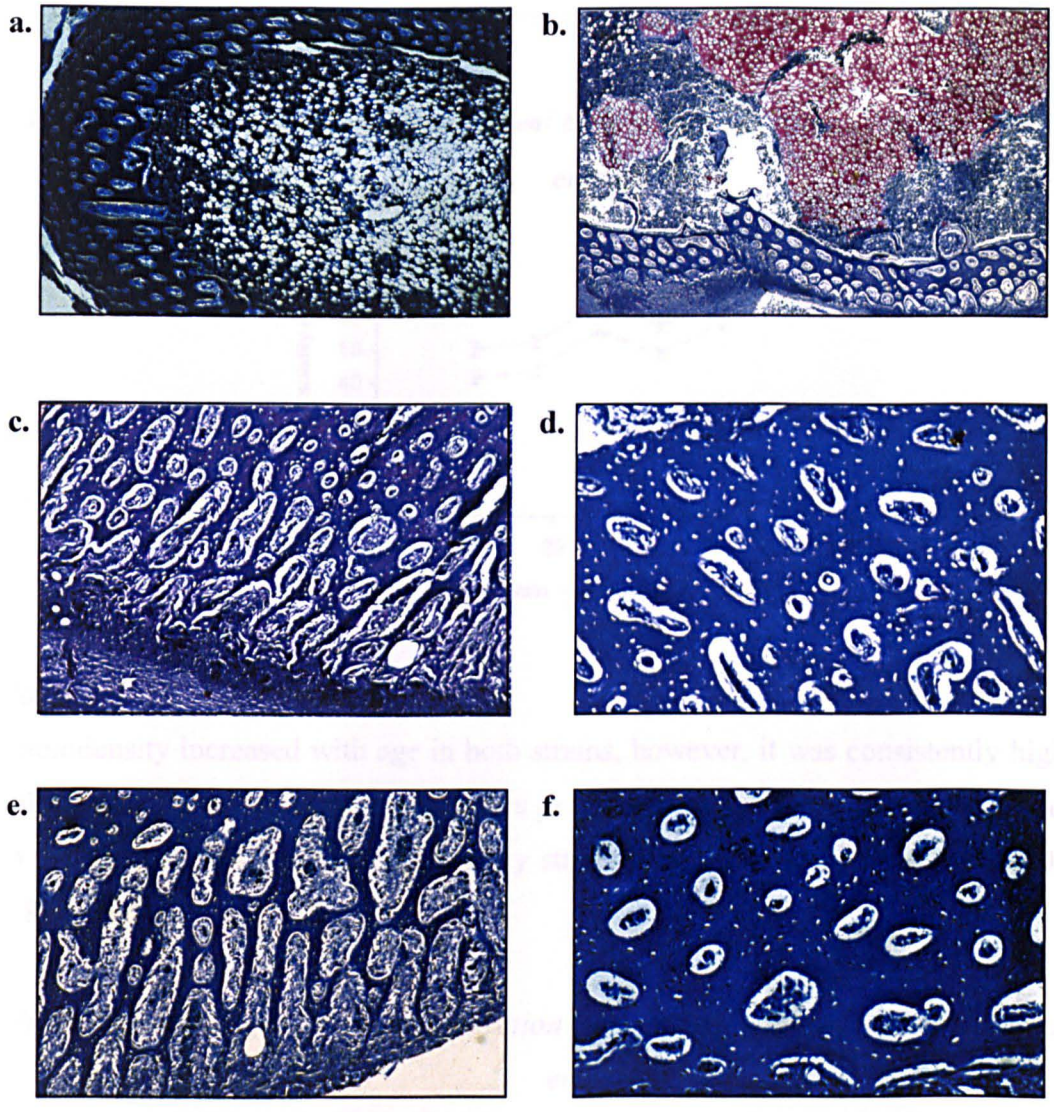
Solidity

Bone solidity across the cortical width of 4 day old birds was significantly greater in the control strain (0.68) than the selected strain (0.62; strain effect: W 6.2, df 1, P<0.05). Endosteal bone became more porous from 11 days onwards in both strains (age effect: W 12.2, df 4, P<0.05; Figure 3.6); each week on average there was 1.1% less bone present within a given cross-sectional area. Bone was more porous in the selected birds (strain effect: W 5.5, df 1, P<0.05). This difference was consistent with age, and the selected strain had a mean of 5% less endosteal bone than the control strain.

Similarly, periosteal bone was more porous in the selected birds (strain effect: W 27.5, df 1, P<0.001), and the difference was consistent with age. However, this difference was greater than for endosteal bone; the selected strain had a mean of 15% less bone than the control strain. In addition, periosteal bone solidity increased with age in both strains (age effect: W 72.5, df 4, P<0.001; Figure 3.7); on average an extra 3.7% bone tissue was present within a given area each week (Plate 3.3).

Plate. 3.3:

Cross sections of the mid-diaphysis comparing periosteal cortical bone solidity of the selected and control strains at different ages



a. Selected strain at 4 days. **b.** Control strain at 4 days. **c.** Selected strain at 25 days. **d.** Control strain at 25 days. **e.** Selected strain at 39 days. **f.** Control strain at 39 days.

Figure 3.6: Changes in mean endosteal bone solidity with age, together with standard errors

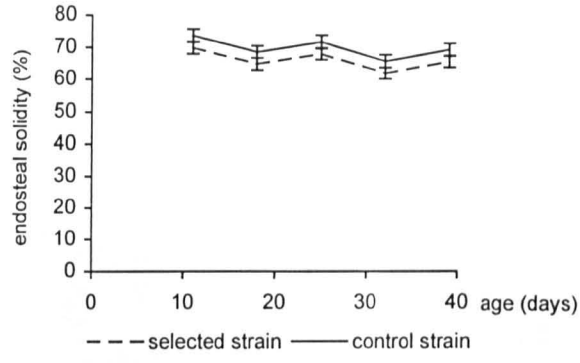
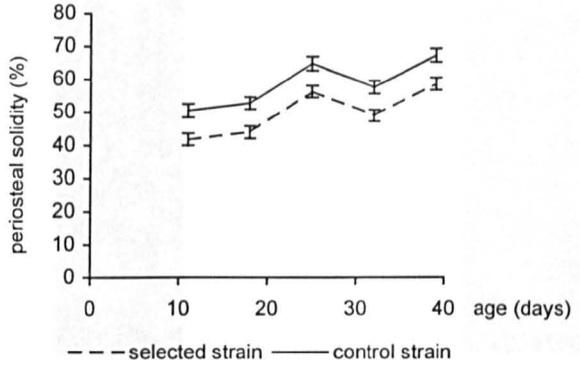


Figure 3.7: Changes in mean periosteal bone solidity with age, together with standard errors



Radiodensity

Radiodensity increased with age in both strains, however, it was consistently higher in the selected strain. It levelled off at 25 days in the control strain, but continued to increase up to 39 days in the selected strain (age by strain interaction: W 456, df 5, P<0.001; Figure 3.8, Plate 3.4).

Figure 3.8: Changes in mean mid-section radiodensity with age, together with standard errors

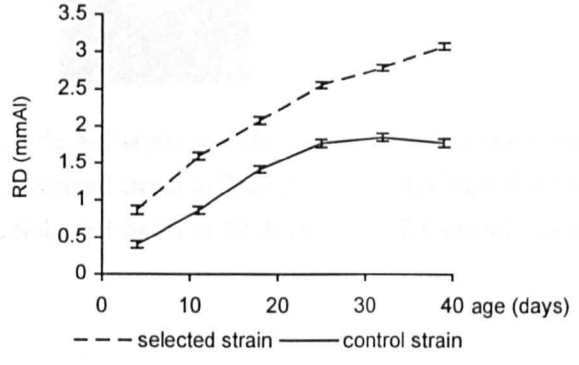
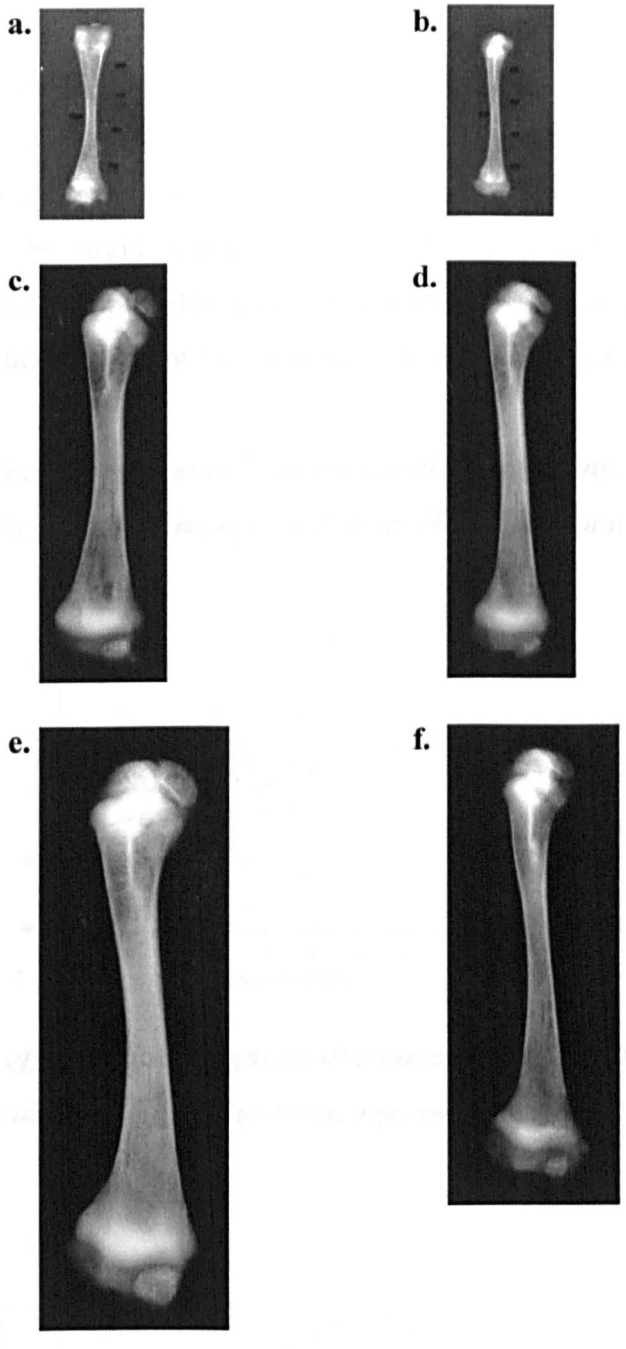


Plate 3.4:

Comparison of bone x-rays between selected and control strains at different ages



a. Selected strain at 4 days
b. Control strain at 4 days
c. Selected strain at 25days
d. Control strain at 25 days
e. Selected strain at 39 days
f. Control strain at 39 days

3.4.5: Effects of Body Growth, Weight and Conformation on Bone

There were no covariate effects on bone mineralisation. Only one covariate effect was observed on solidity; the solidity of endosteal bone tended to be greater in birds with a larger proportion of breast muscle (slope 1.7, se 0.7, df 1, $P<0.05$, Figure 3.9). Covariate analysis also showed that, on average, tibiotarsi were wider in heavier birds allowing for strain and age (slope 0.0029, se 0.0003, df 1, $P<0.001$; Figure 3.10). On average, tibiotarsi were also longer and cortical bone was thicker within strain by age combinations in birds with a greater IGR (slope 98, se 24, and slope 8.3, se 1.9 respectively; df 1, $P<0.001$), and heavier total weight (slope 0.015, se 0.002, and slope 0.00068, se 0.00018 respectively; df 1, $P<0.001$; Figure 3.11). Allometric comparisons suggested that tibiotarsus length relative to body weight increased more quickly in control birds (slope 0.35) than in selected birds (slope 0.33; ln(weight) by strain interaction t 2.71 df 124 $P<0.01$; see Figure 3.12a). However, both strains had similar tibiotarsus width increases with body weight (t 1.3, df 124; data not shown), and also similar increases in MI (t 0.43, df 123; Figure 3.12b).

Figure 3.9: Linear effect of breast muscle on endosteal bone solidity. Plotted values are residuals obtained by fitting strain, age, and farm. (Far right point reduces slope.)

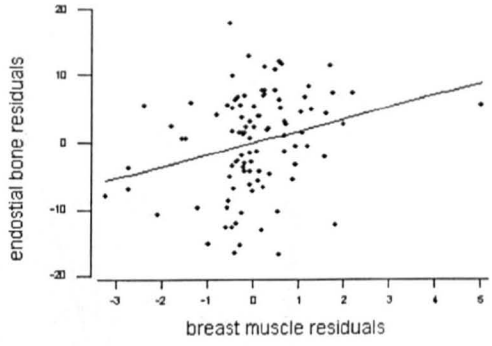


Figure 3.10: Linear effect of bird weight on tibiotarsus width. Plotted values are the residuals obtained by fitting strain, age, and farm to the data.

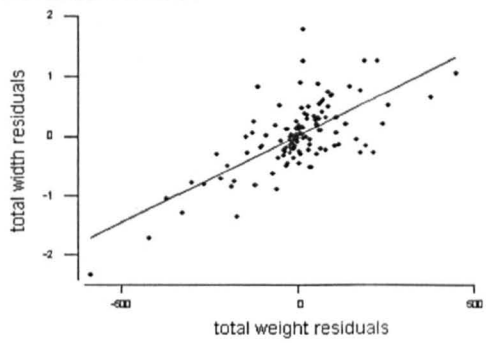


Figure 3.11: Linear effects of a. growth rate on tibiotarsus length, b. growth rate on cortical thickness, c. bird weight on tibiotarsus length, d. bird weight on cortical thickness

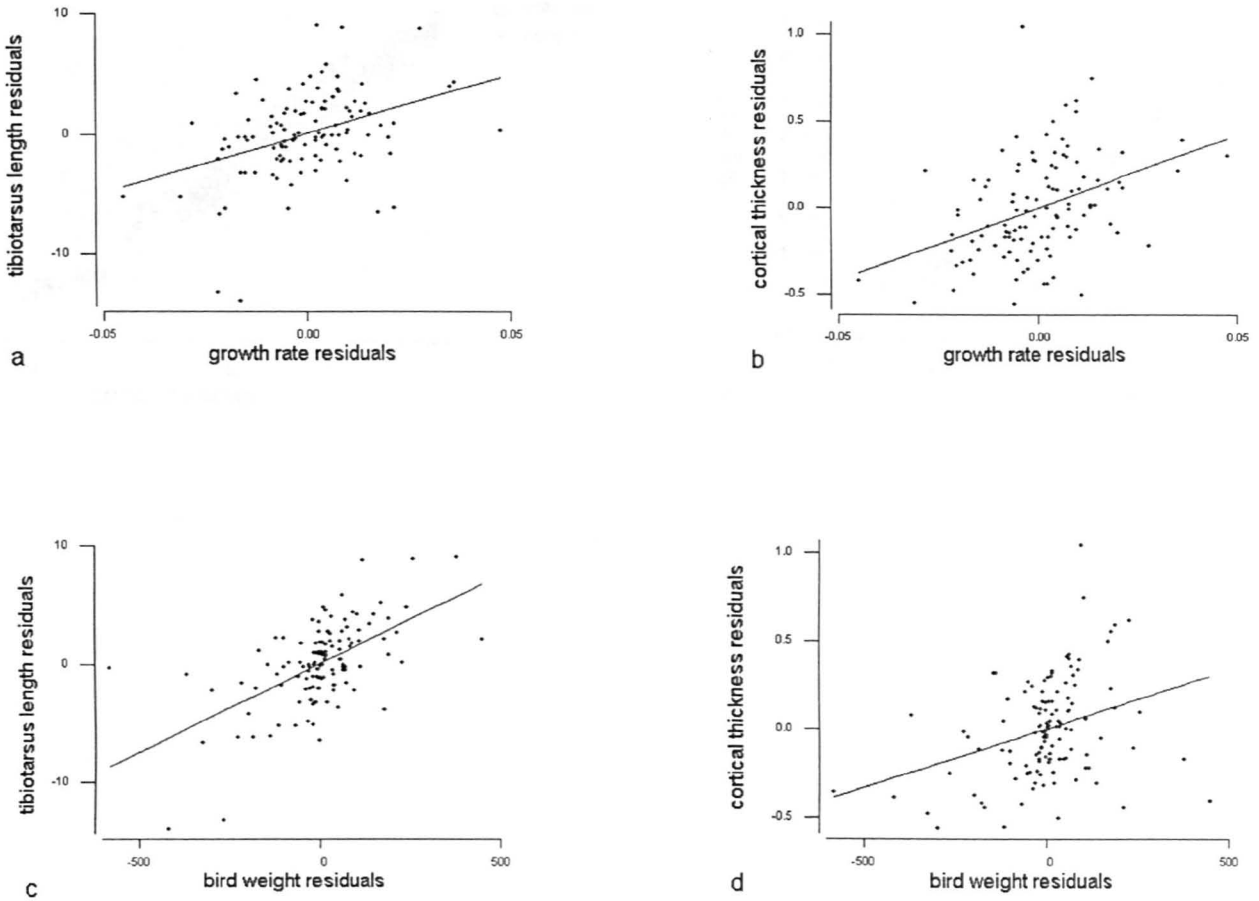
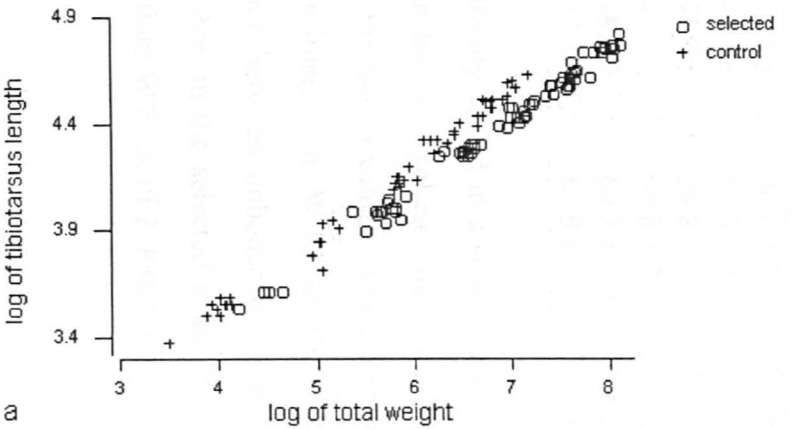
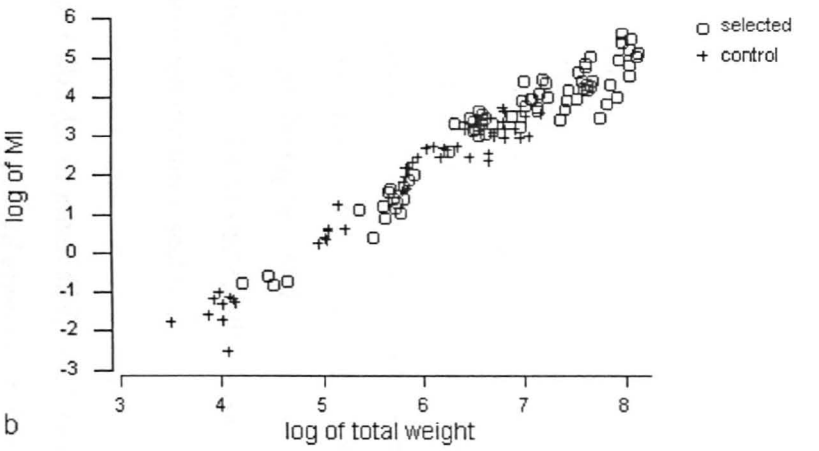


Figure 3.12: The allometric relationship of tibiotarsus dimensions to weight: a. length, b. MI





3.4.6: Shaft Section Effects

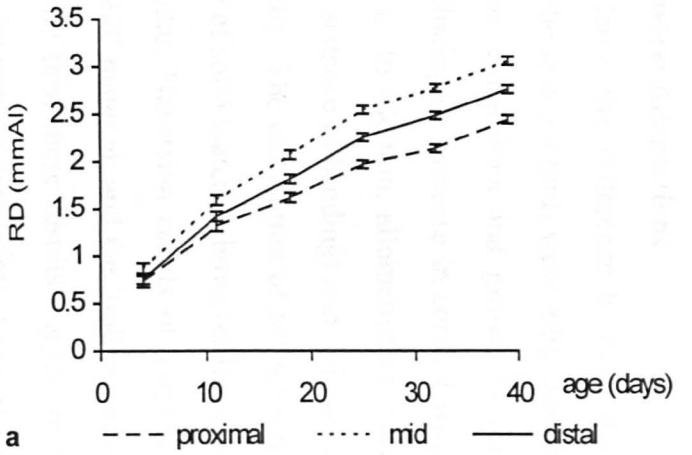
Ash content was similar for all diaphyseal sections at day 4, and changes in mineral content with age in all sections followed a similar pattern to that in Figure 3.4. However, the values reached from day 11 onwards varied with section, leading to a section by age interaction (W 36.3, df 10, $P<0.001$; Table 3.5). The mid-section showed the greatest ash content, followed by the distal section and then the proximal section, which had the lowest level of overall mineralisation. Calcium content also varied between sections (W 128.9, df 2, $P<0.001$); the mid section had the highest content of 190 mg/g, followed by the distal section (171 mg/g) and then the proximal section (154 mg/g). These differences were consistent over ages. There was a section by age interaction for P content due to varying fluctuations in content over the 6 weeks (W 19.9, df 10, $P<0.05$; Table 3.5). Overall, the mid-section showed the highest bone P content, followed by the distal section and then the proximal section with the lowest levels. There were no significant differences in the bone Ca:P ratios between the three sections.

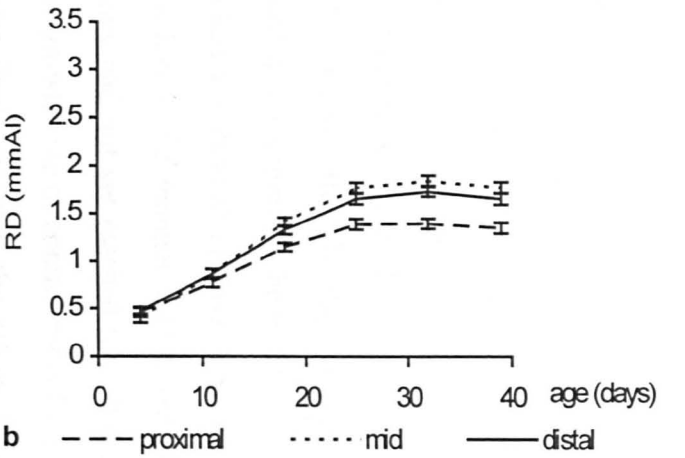
Table 3.5: Changes in mean shaft section mineral content with age together with standard errors (strain effect accounted for)

	Ash (%)			Phosphorus (%)		
age (days)	Proximal	Mid	Distal	Proximal	Mid	Distal
4	36.5 ± 7.5	36.0 ± 7.5	37.9 ± 7.5	65 ± 2.7	7.8 ± 0.3	6.9 ± 0.3
11	44.7 ± 7.5	54.5 ± 7.5	49.4 ± 7.5	71 ± 2 9	8.9 ± 0.3	6.9 ± 0.3
18	45.9 ± 7.5	56.8 ± 7.5	52.1 ± 7.5	68 ± 2.7	8.2 ± 0.3	7.7 ± 0.3
25	47.3 ± 7.5	59.8 ± 7.5	52.5 ± 7.5	83 ± 3.1	10.2 ± 0.3	8.9 ± 0.3
32	48.1 ± 7.5	60.2 ± 7.5	54.5 ± 7.5	67 ± 2.8	8.7 ± 0.3	8.2 ± 0.3
39	52.5 ± 7.5	63.9 ± 7.5	59.5 ± 7.5	87 ± 3.0	11.1 ± 0.3	9.6 ± 0.3

Radiodensity varied in a similar manner to ash between the shaft sections, in that the mid section had the highest values, followed by the distal values, with the proximal section having the lowest values. The difference between the sections increased with age (age by section interaction: W 88, df 10, $P<0.001$; Figure 3.13). However, there was a larger difference between radiodensity in the proximal versus the distal section of the control strain than in the selected strain from approximately 18 days of age (strain by section interaction: W 25.3, df 2, $P<0.001$).

Figure 3.13: Changes in mean cortical bone radiodensity with age, together with standard errors: a. selected strain; b. control strain





3.5: DISCUSSION

3.5.1: Growth and Weight Variables

In general the results from the analysis of these variables held no surprises; they confirmed the differences between the strains which were expected: selected birds grew faster, reached heavier weights, and had a greater proportion of all muscle types measured (Figures 3.1 & 3.2). The selected birds have been specifically bred over numerous generations for increased growth rate, a large market weight, and a large proportion of breast muscle; whereas the control strain has not been subjected to such selection pressures since 1972. Percentages of thigh and calf muscle initially increased, and then remained approximately constant after 18 days in both strains of birds, whilst percentage breast muscle showed a rapid increase between 4-11 days in both strains. In control birds, this increase reached a plateau at 11 days, however, the amount of breast muscle continued to increase in a less rapid manner throughout the rest of the life-span of selected birds, suggesting they had not yet reached their full potential. In contrast, the lower values for instantaneous growth rate of control birds from day 25 onwards suggest that the birds may be nearer their final size than the selected strain birds. However, no birds reached a sufficient age to confirm this.

3.5.2: Allometric Adaptations

Due to the large size difference between the 2 strains, the longer and wider tibiotarsi observed in the selected birds were fully expected (Table 3.3). Expectations of strong links between bone dimensions and growth performance were also borne out by covariate analysis, including an increase in cortical width with fast growth and heaviness (Figures 3.10 & 3.11). In addition, allometric analysis revealed that both strains had similar MI (estimated resistance to bending) and tibiotarsus width values at a given total body weight (Figure 3.12b). The calculation of MI involves the assumption that the bone is a hollow circular tube of solid material, however its structure is much more complex; the bone is not exactly circular, Haversian canals of varying sizes and densities run the length of the assumed 'solid' material, and the 'hollow' tube contains both bone marrow and trabecular bone. Despite this, these results suggest that the selected strain successfully produced tibiotarsi of the correct dimensions to resist bending at a given weight. In addition, at a particular weight, bones from selected birds were shorter than those from controls (Figure 3.12a), reducing the effect of leverage on the bending moment on the tibiotarsus in the selected strain (Biewener 1992). This may be an accidental but advantageous result of the selection process for muscle growth. Alternatively, it could be due to an inability to

produce growth in tibiotarsus length at the same rate as muscle growth, perhaps due to limits in the supply of appropriate proteins and/or minerals for growth, or to a genetic limit on the rate of the process itself. A similar observation has been made previously in a comparison between a relaxed strain and a modern broiler. The difference was reduced by lowering growth rate in the modern strain by feed restriction (Sandra Corr 1999), suggesting that a fast growth rate does have some adverse effect on longitudinal growth of bones.

Covariate analysis also showed that denser endosteal bone occurred in those birds within a strain with a greater proportion of breast muscle (Figure 3.9); this is possibly a method of adding strength to the bone under these altered conditions of load distribution. However, although the selected strain had a much larger proportion of breast muscle, it had more porous endosteal bone. This was probably due to the effect of other factors on bone porosity such as the more rapid increase in tibiotarsus diameter, and the interaction of nutritional and growth effects perhaps resulting in greater requirements for Ca and P.

3.5.3: Similarities in Development

Overall, the development of the tibiotarsus in the 2 strains followed very similar patterns. The rapid increase in cortical bone width between days 4-18 in both strains (Figure 3.3) implies a period of intensive bone formation to provide adequate skeletal support for the growing bird. It is possible that by day 18, the optimum cortical thickness required to support the bird was already present; the increase in the total width of the tibiotarsus throughout a birds life may have been adequate in providing additional support during further growth. This is supported by the fact that MI increased linearly with increased body weight despite the plateau in cortical width.

Within the period of cortical bone growth, there was an initial rapid mineralisation of bone between days 4-11 in both strains (Figure 3.4). This pattern of development is similar to that observed by LeTerrier and Nys (1992) in alternative fast and slow growing broiler strains. There is little need for strong, well-mineralised bones whilst in the egg, and the amount of mineral available may reflect this. Once hatched, mobile, and growing rapidly, the need for a strong supporting skeleton becomes increasingly paramount, and a higher availability of minerals in the feed may allow this rapid mineralisation.

There was a more gradual change over time in the porosity of bone. Periosteal bone became more solid with age in both strains (Figure 3.7). Bone is constantly added by apposition at the periosteal surface to increase tibiotarsus width (Baron 1996). Growth rate is reduced as the birds become older (Figure 3.1), and the need for rapid expansion in tibiotarsus width is reduced. More resources may therefore be directed towards filling in

the newer osteons, and it is possible that this allows a natural increase in periosteal solidity with age. Whatever the mechanism, this new bone became more solid, and therefore more structurally sound, in line with support requirements as the birds became heavier. Conversely, endosteal bone became more porous and trabecular in morphology with age in both strains (Figure 3.6). There is increased osteoclastic activity at the endosteal surface of long bones to constantly increase the size of the medullary cavity (Baron 1996), and it is possible that increased Haversian resorption in this area was augmenting this process.

3.5.4: Differences in Bone Quality

Although there was little incidence of growth plate pathology in either strain (Table 3.2), ash content was consistently lower in the selected birds (Figure 3.4). This complements the results of LeTerrier and Nys (1992), who also found a slow growing broiler strain to have higher bone mineral content than a fast growing strain. In the case of the present study, this could indicate that the feed given to the birds, containing current recommended levels of Ca and P, had an inadequate mineral content for the modern strain. Alternatively, this strain may have been unable to utilise the mineral provided quickly enough, perhaps due to limitations in mineral uptake or bone calcification rates. If either of the above hypotheses are true, the results also suggests that the bone mineralisation process is more sensitive to inadequacies in mineral supply than development at the growth plate; however further investigation is needed to confirm this.

LeBlanc *et al* (1985), have previously compared fast and slow growing strains of turkey, and found the tibiotarsus cortical width to be similar in both strains at six weeks, despite considerable differences in body weight. In contrast, the cortical width achieved by the selected strain of broiler in the present study (Figure 3.3) was greater than that of the slow strain from 11 days onwards. The fast growing turkey strain used by LeBlanc *et al* was notorious for leg problems, and it was suggested that the inadequate cortical thickness observed in the strain might contribute to these. As mentioned previously, the fast growing strain in the present study has been selected for skeletal health in addition to growth performance. The MI data from this study suggest that these birds are capable of producing bones of the correct dimensions to support the body weight at any stage of development, and the greater cortical thickness is an essential component of this.

However, whilst the cortical width achieved by the selected strain was greater than that of the slow strain, all areas of cortical bone were consistently more porous in the selected birds (Figures 3.6 & 3.7). In adults of numerous species there is constant resorption and formation (remodelling) of bone around the Haversian canals of individual

osteons. This maintains the skeleton's mechanical competence and allows its role in mineral homeostasis (Frost 1986, Parfitt *et al* 1983). In many cases, increased porosity can be due to an imbalance in this remodelling mechanism (Frost 1997). Whether the increased porosity observed in our selected strain of broilers is due to increased resorption or reduced formation within osteons is unclear at this point, however, there was no evidence observed of secondary osteon formation in this study. This would imply that, in chickens within the age range used here, the bulk of bone turnover is achieved by the simultaneous expansion in tibiotarsus width, and growth in the marrow cavity, rather than by active cutting cones.

Total bone width increases more rapidly in the selected strain than in the control strain, while the cortical width stays approximately constant after 18 days. The growth of the marrow cavity, therefore, occurs more rapidly in the selected strain. It is possible that the increased endosteal porosity is due to a mechanism whereby the increased resorption of primary osteons in this area augments the more rapid growth of the marrow cavity. Increasing the total bone width of the tibiotarsus during weight gain is a priority. The greater difference in porosity between the strains seen in the periosteal area (15% difference compared with 5% in the endosteal area) would, therefore, seem to suggest that the bigger problem in the modern strain was in producing sufficient bone matrix, or in obtaining sufficient mineral for bone formation at this site. Primary osteons are initially formed at the periosteal surface as a result of differential osteoblast activity. Longitudinal depressions develop at the surface, which are then covered over, trapping blood vessels and periosteal elements, and these hollow canals are then filled with concentric lamellae to form the osteon (Banks 1986). It is possible that the total width of the tibiotarsus is expanding so rapidly that this process cannot keep up. This could be due to the proposed limited supplies of bone matrix or mineral being preferentially directed to periosteal appositional growth, rather than apposition to fill in the new osteons. There is insufficient evidence at this point to come to any firm conclusions on the mechanism for this increased porosity in the selected strain.

It has been suggested that, in broilers, the cause of the increased porosity might be due to an inadequate mineral supply in the feed given to modern strains (Thorp and Waddington 1997). The accompanying low ash content seen in this study is consistent with mineral deficiency as a cause, however, again, the greater porosity could be due to either an inadequate mineral supply, or a problem utilising the mineral provided quickly enough due to the rapid growth rate. Alternatively, the greater porosity observed in the selected strain might simply be part of the phenotype of this strain. For example, LeTerrier *et al* (1998) reduced the growth rate of a fast growing broiler strain by energy restriction,

and observed no reduction in the porosity of cortical bone despite a reduction in bird growth. Further investigation into the exact cause of the increased porosity observed in the selected strain will be performed in the future. Whatever the mechanism, although estimates of MI at a given body weight in the selected strain were similar to the control strain, the high porosity and low mineralisation of cortical bone in the selected strain is likely to reduce the actual breaking strength of the tibiotarsus.

Radiodensity has been used in the past as an indirect measure of bone mineral content in the clinical diagnosis of osteoporosis and fracture risk (Mack *et al* 1939; Vose 1969). The method has generally been replaced over the last 20 years by dual-energy x-ray absorptiometry, however modern image analysis techniques have now returned it to the status of a viable diagnostic tool (Hagiwara *et al* 1998). The selected strain radiodensity increased throughout the life-span of the birds, while that of the control strain appeared to reach a plateau at 25 days of age (Figure 3.8). This could imply that the control birds had reached some developmental end-point at this stage, which the selected strain was still trying to achieve at day 39. However, the results also demonstrated bone from the selected strain to have consistently greater radiodensity than that from the control strain, despite having consistently lower mineral content. The measure is likely to be influenced by a number of factors in addition to mineral content. The porosity and dimensions of a bone will both influence how much tissue the x-rays must pass through before they reach the film, whilst the mineral content, and perhaps the exact form of the mineral, must affect how easily the x-rays pass through a given depth of tissue. The selected strain had bone which was more porous and less well mineralised, yet it was more radiodense; this can only be due to the difference in bone dimensions between the two strains. Some attempts were made to standardise radiodensity for bone dimensions (Appendix 1), however, the results were inconclusive.

Bone Ca and P content fluctuated over time (Table 3.4), however, the changes bore no resemblance to those in the Ca and P content of the feed samples (Table 3.1) and are therefore unlikely to be an artefact of poor batch consistency. In addition, the results of tibiotarsus shaft section effects on mineralisation (Table 3.5) confirmed the expected pattern: the mid-section of the shaft consists of the most mature bone and showed the highest ash, Ca, and P contents. Longitudinal bone growth proceeds faster at the proximal growth plate than the distal one (Farnum and Wilsman 1993), and bone near the proximal end of the bone shaft is therefore likely to be newer and less mineralised than that at the distal end. Again, this was supported by the results: proximal sections demonstrated lower ash, Ca and P contents than distal sections. This difference in mineral content of the various regions of a bone may influence the choice of where to take a sample for ashing,

and should be considered when comparing results between studies using different regions of bone.

The pattern of mineral distribution between the sections was repeated by the radiodensity results: the mid section was the most radio-dense, followed by the distal section, whilst the proximal section was the least radio-dense (Figure 3.13). This implies that the measure does partly reflect mineral content. In addition, radiodensity was the only measure where section differences were influenced by strain. The difference between the proximal section and the other two sections was much greater in the control strain than in the selected strain. However, the reason for this is uncertain due to the difficulties of interpreting what radiodensity is measuring.

Although by no means pure in form, mature bone mineral is generally accepted to have a structure close to that of the calcium phosphate, hydroxyapatite, which has a molar Ca:P ratio of 1.67 to 1 (Pellegrino and Biltz 1968, Legros *et al* 1987). In the control strain the fluctuations of bone Ca and P content were similar for both minerals, and therefore resulted in a molar Ca:P ratio that stayed consistently close to, or below, the ideal 1.67 ratio (Figure 3.5). A low ratio is expected at a young age since there is likely to be a greater proportion of bone mineral precursors of hydroxyapatite present in the young skeleton (Glimcher *et al* 1981); the molar Ca:P ratios of these have been suggested to vary from 1.33-1.55 (McClean and Urist 1968) and will therefore bring the overall ratio down. The control strain ratios were therefore assumed to represent normal development of bone mineral. The fluctuations of Ca and P in the selected strain differed from each other, resulting in a significantly increased molar Ca:P ratio during the period of rapid bone formation discussed earlier. Bone mineral at any stage is not a pure substance; numerous substitutions and deletions in the crystal lattice maintain chemical reactivity, allowing bone to fulfil its role as a mineral reservoir (Landis 1996); this can also alter the molar Ca:P ratio of the mineral. However, few of the possible substitutions involve the replacement of a phosphate group (Narasaraju and Phebe 1996), and Woodard (1962) found that the molar Ca:P ratio of human bone samples ranged from 1.37-1.71. The significantly high ratios observed here, therefore, appear abnormal, and may also affect bone strength through alterations in the crystal structure of bone mineral. Selected birds went on to reach a similar Ca:P ratio to control birds by day 25, and the reason for the high ratio observed between days 11-18 is uncertain at present. It is possible, however, that the increased Ca:P ratio is yet another indicator of mineral deficiency, in this case of P in the selected birds during this critical period of bone development.

3.5.5: Conclusions

Under commercial conditions, the selected strain showed many similarities to the control strain in the development of the tibiotarsus. There was a continuous increase in tibiotarsus length and width, a rapid increase in cortical thickness between 4-18 days, and a rapid increase in bone mineral content between 4-11 days. The selected strain also produced tibiotarsi with an estimated resistance to bending similar to that observed in the control strain, and apparently produced bone of the correct dimensions to support the greater weights attained by these birds. However, the quality of bone in the selected strain was relatively poor in terms of porosity and mineral content and is likely to have a lower effective breaking strength. In addition, there was a transient increase in the bone molar Ca:P ratio between 11-18 days, during the period of rapid cortical expansion; this may also affect strength. The results indicate that the differences observed between the strains could either be due to a problem with the supply of Ca and P in the diet, or due to growth rate or genetic effects on the uptake or utilisation of mineral for bone formation. Further research is needed to elucidate the cause of the differences in bone development between the two strains observed in this trail.

CHAPTER 4

DIETARY Ca AND P REQUIREMENTS AND BONE QUALITY DURING THE STARTER PERIOD IN THE MODERN BROILER

4.1: INTRODUCTION

Broiler growth has changed considerably over recent years as a result of genetic selection for meat production; however, poultry diets have changed little in terms of mineral content. Ca and P are essential for bone formation, and numerous skeletal pathologies are associated with deficiencies in these minerals. In a previous study (chapter 3, Williams *et al* 2000), bone development in a fast growing selected strain was compared with that in a slower growing control strain. Both strains demonstrated similar periods of rapid bone formation (age 4-18 days) and mineralisation (age 4-11 days). However, bone ash content was consistently lower and cortical porosity higher in the selected strain, and between the ages of 11-18 days the mean molar Ca:P ratio of cortical bone in the selected strain reached 2.15 to 1. This deviation was not shown by the control strain, which consistently displayed molar ratios close to the predicted 1.67 to 1 (Pellegrino and Biltz 1968, Legros *et al* 1987). Variation in the molar Ca:P ratio of bone is likely to involve alterations in the bone mineral crystal structure, and it has been suggested that abnormal ratios could result in weakened bone (Thorp and Waddington 1997). Nutrient requirements change with age (NRC 1994), and possibly with the growth profile of the bird; for example, genetic variation in Ca metabolism has been reported in chickens selected for aspects of body fatness (Shafey *et al* 1990a). In the previous experiment, the modern strain grew faster and became heavier than its genetic precursor; the size of its bones, and possibly mineral requirements, reflected this. The observed low ash and high porosity implied a general deficiency in Ca and/or P, while it was proposed that the transient high Ca:P ratio in cortical bone may reflect a specific dietary inadequacy for P during this critical period of bone development.

4.2: AIMS

The present experiment was designed to investigate whether modern broilers might be subject to a dietary mineral deficiency by determining the responses in skeletal health and Ca and P metabolism of 2-week-old broilers to differing dietary Ca and available P (avP) contents. It was also intended to identify optimum dietary Ca and P contents for skeletal health at this age. Since there is some controversy as to whether feeding mineral contents optimum for bone formation impairs growth performance (Shafey 1993, Huyghebaert 1996), the effects of diet on body weight and conformation were also investigated.

4.3: MATERIALS AND METHODS

4.3.1: Birds, Housing and Diet Treatments

Day-old male chicks of a selected strain (Ross MCsib) genetically identical to the MCX strain used previously (Chapter 4, Williams *et al* 2000) were housed in 32 pens ($1m^2$) containing 15 birds each. As shown in Plate 4.1, each pen had a bell drinker, a heat lamp, woodshavings as a floor covering, and a food tray or hopper (trays were replaced by hoppers at 7 days). Birds were given 23 hours of light for the first 4 days, after which the time was gradually reduced to 16 hours of light by day 13.

The birds were fed on diets with a variety of Ca and avP contents in a modified 4 by 4 factorial design (Figure 4.5), with 2 pens of birds per treatment. It was initially considered that the dietary Ca:P ratio of a diet containing 1.3% Ca and 0.3% avP was too extreme for healthy growth, whilst the almost 1:1 Ca:P ratio of a diet containing 0.7% Ca and 0.6% avP was considered to be difficult to formulate and unlikely to give healthy growth (Whitehead pers. comm.). These treatments were therefore replaced by two very high avP diets (0.8%). All the diets were analysed for Ca and total P content prior to feeding. A satisfactory method of assessing non-phytate P was not identified (Appendix 2); figures quoted are therefore expected avP as per the feed formulation (Single-Mix, New Century Software, Format International Ltd, Woking, Surrey, UK), which was based on book values (NRC 1994) for the ingredients. The diets contained approximately 0.1% more calcium than formulated for, suggesting that there was more Ca present in the basal diet than expected. The mean difference between expected and observed Ca content was calculated over all 16 diets, and this mean difference was added to the expected Ca content

for each diet. This gave corrected dietary Ca content values that also minimised the effect of feed sampling/analysis error on the overall experimental analysis.

4.3.2: Sampling Procedure

Birds were individually weighed on day 13, and the five birds closest to the median weight in each pen were marked on the head with a non-toxic dye. These birds were then blood-sampled on day 14, and culled on day 15. Carcasses were weighed, and breast muscle and tibiotarsi were dissected out and weighed.

4.3.3: Sample Processing

(Chapter 2.) Blood Ca^{2+} concentration was measured using a Cibia-Corning 634 Ca^{++}/pH analyser, and plasma total Ca and P_i concentrations were determined by colorimetric methods using commercially available kits (Wako Chemicals GmbH, Neuss, Germany). The concentrations of markers of bone formation (osteocalcin) and resorption (pyridinoline), were measured by radioimmunoassay and HPLC respectively. Due to the expense of the assay, the concentration of 1,25-dihydroxyvitamin D_3, the active metabolite of vitamin D_3, was measured in samples from a subset of diet treatments using a commercially available kit (IDS, Boldon, UK). Cortical thickness was calculated from measurements of x-ray images. Breaking strength (maximum load before rupture) of the tibiotarsi was measured during a 3-point bending test using a material tester. Proximal tibiotarsus growth plates were assessed for rickets and tibial dyschondroplasia (TD) by histological examination of wax embedded sections. A segment of bone was taken from the proximal third of each shaft for ashing and measurement of mineral (%ash), Ca (%Ca) and P (%P) contents by atomic absorption spectrophotometry and colorimetry respectively. A sample of mid-diaphyseal bone was assayed for hydroxyproline, a measure of collagen content.

4.3.4: Statistical Analysis

Final growth rate was calculated as previously (between days 13 and 15), as was the proportion of breast muscle relative to the final total weight of the bird, and the molar Ca:P ratio for bone (Chapter 3). For all variables, ANOVA models based on quadratic response surfaces in dietary Ca, avP and their product were used in Genstat to attempt to identify an optimum diet. When the fitted values from such models are contour plotted in Minitab, they should describe a response surface rising smoothly to a single peak or ridge, in this case corresponding to the optimum dietary Ca and avP content or ratio. In the absence of

such an optimum, linear responses with Ca and avP (both together and separately) were also examined. The proportions of birds with particular growth plate pathologies were analysed as binomial variates on the logistic scale.

4.4: RESULTS

4.4.1: Optimum Diet

Initially, the raw data were contour plotted against Ca and avP; an example of such a plot is shown in Figure 4.1. The mineral combinations at which maximum values were achieved were examined for all the measures. Although the observed maxima of some bone quality and growth measures did coincide, it was apparent that the optimum diet varied with the measure investigated. In addition, the relationships between measures and diet were complex; often more than one Ca/P combination gave a maximum value for a measure.

Using the fitted values from quadratic models to produce contour plots for each measure also failed to clarify the relationships. Again, for many individual variables no clear optimum was identified, and in many cases, any maxima found were along the diagonal perimeter to the bottom right of the plot. These were rejected since the contour lines along this edge are more an artefact of the way in which Minitab forms closed contours than a true representation of any effect. Similarly the response surfaces also failed to identify a single optimum diet for all measures of bone and/or body growth. However, such statistical analysis did reveal a number of relationships between parameters measured and dietary mineral content; these results follow.

Figure 4.1: Example of a contour plot: raw final weight data (g)

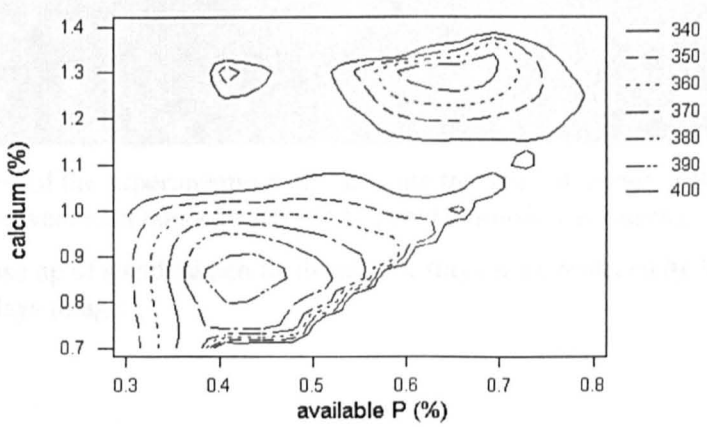
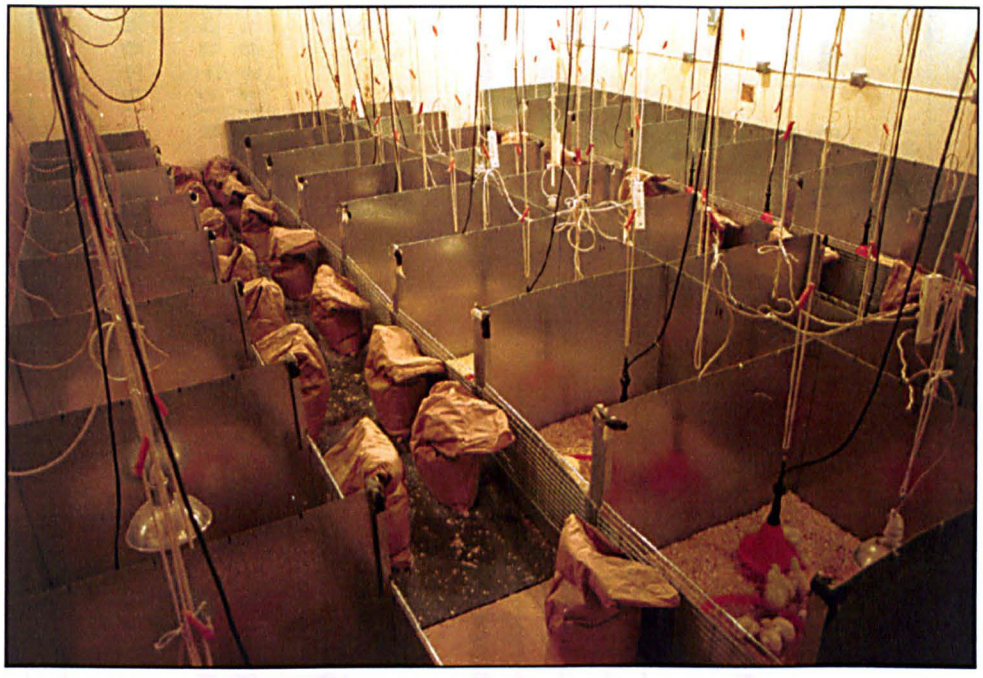


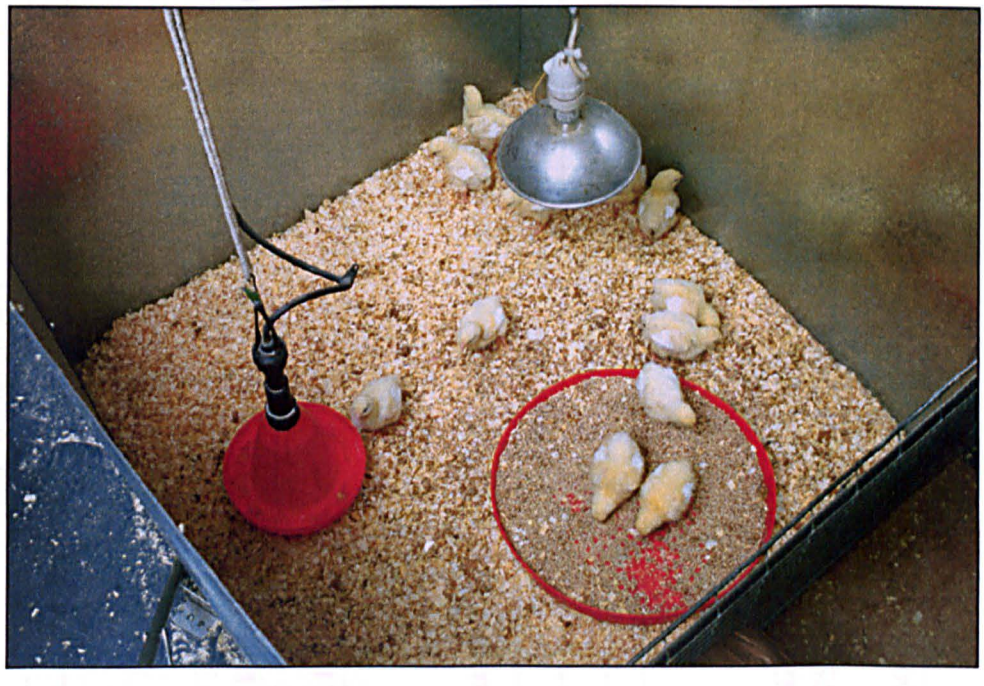
Plate 4.1:

Layout of experimental pens

a.



b.



a. View of the experimental room showing the general layout. Relevant feed bags were kept outside each pen to avoid confusion at feeding.

b. Close up of standard pen facilities. The trays were replaced by hoppers at 7 days of age.

Table 4.1: Means and standard errors of final weight and growth rate achieved with different dietary Ca and avP contents

Ca content (%)	final body weight (g)					growth rate (ln[g]/day)				
	available P content (%)					available P content (%)				
	0.3	0.4	0.5	0.6	0.8	0.3	0.4	0.5	0.6	0.8
1.4					323 ± 14					0.12 ± 0.010
1.3		356 ± 7	336 ± 7	371 ± 27			0.17 ± 0.008	0.15 ± 0.003	0.16 ± 0.020	
1.2					336 ± 13					0.15 ± 0.004
1.1	321 ± 3	323 ± 18	337 ± 3	337 ± 27		0.14 ± 0.007	0.16 ± 0.010	0.16 ± 0.002	0.17 ± 0.009	
0.9	346 ± 10	389 ± 24	380 ± 14	364 ± 2		0.16 ± 0.008	0.17 ± 0.003	0.15 ± 0.005	0.14 ± 0.030	
0.75			369 ± 7					0.16 ± 0.005		
0.7	342 ± 8	386 ± 15				0.16 ± 0.004	0.14 ± 0.022			

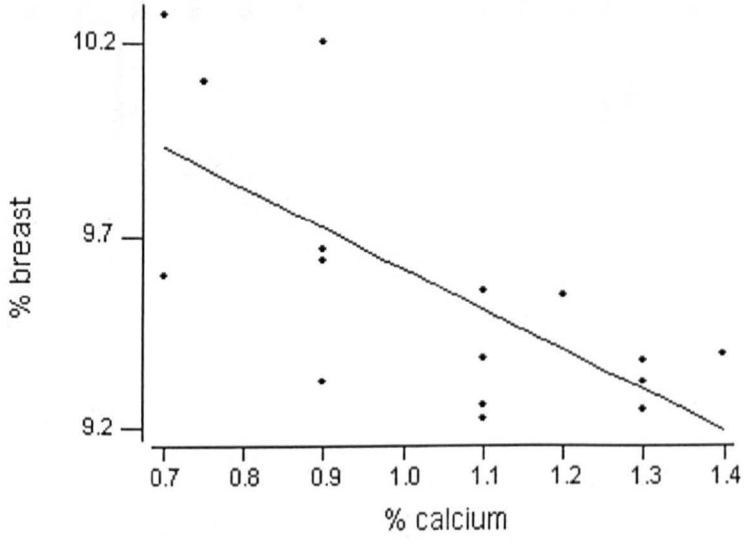
Table 4.2: Means and standard errors of pyridinoline and total Ca concentrations in plasma achieved with different dietary Ca and avP contents

Ca content (%)	pyridinoline cross-links (pMol/30µl)					total plasma Ca (mg/dl)				
	available P content (%)					available P content (%)				
	0.3	0.4	0.5	0.6	0.8	0.3	0.4	0.5	0.6	0.8
1.4					9.5 ± 0.04					1.77 ± 0.100
1.3		10.0 ± 0.10	10.2 ± 0.29	13.5 ± 0.78			2.11 ± 0.141	1.75 ± 0.358	1.64 ± 0.124	
1.2					11.1 ± 1.29					1.37 ± 0.169
1.1	13.3 ± 1.82	12.1 ± 1.71	12.4 ± 0.08	13.0 ± 1.54		1.87 ± 0.154	1.45 ± 0.078	1.94 ± 0.010	1.64 ± 0.087	
0.9	10.3 ± 0.75	10.7 ± 0.12	11.3 ± 1.11	9.9 ± 1.40		1.57 ± 0.230	1.66 ± 0.225	1.80 ± 0.553	1.80 ± 0.151	
0.75			12.8 ±0.10							
0.7	11.3 ± 0.09	11.1 ± 1.28				1.73 ± 0.144	1.79 ± 0.067	1.72 ± 0.101		

4.4.2: Growth Performance

The response of final body weight to diet was too variable to be summarised by a quadratic response surface, and no model was found which accounted for more than 20% of the variation in the final growth rate data. There was no observable regular pattern to final growth rate with dietary mineral content, however, the highest final weights tended to occur at intermediate P contents (0.4-0.6 % avP), and over a wide range of Ca content (0.7–1.1 % Ca; Table 4.1). The proportion of breast muscle, however, was significantly reduced by high dietary Ca in a linear manner (t –4.02, df 15, $P<0.01$, r^2 47.7%; Figure 4.2).

Figure 4.2: Effect of dietary calcium content on the proportion of breast muscle



4.4.3: Blood and Plasma Measures

Concentrations of plasma pyridinoline cross-links and total Ca (Table 4.2) were apparently unaffected by diet. There was a tendency for birds on low Ca or high avP diets to have higher plasma levels of 1,25-dihydroxyvitamin D_3. (Table 4.3), however this effect was not statistically significant.

Assessing both relationships as linear, birds given higher dietary avP showed greater concentrations of plasma P_i (t 2.68, df 15, $P<0.05$, r^2 41.0%) and lower concentrations of Ca^{2+}(t –5.64, df 15, $P<0.001$, r^2 75.5%; Figure 4.3). Plasma osteocalcin concentrations were higher in birds given high avP diets (t 3.28, df 15, $P<0.01$), and lower at higher dietary Ca content (t –2.55, df 15, $P<0.05$, r^2 41.6% for model Ca + P; Figure 4.4).

Figure 4.3: Effect of dietary available phosphorus on plasma a. inorganic P, b. ionised Ca

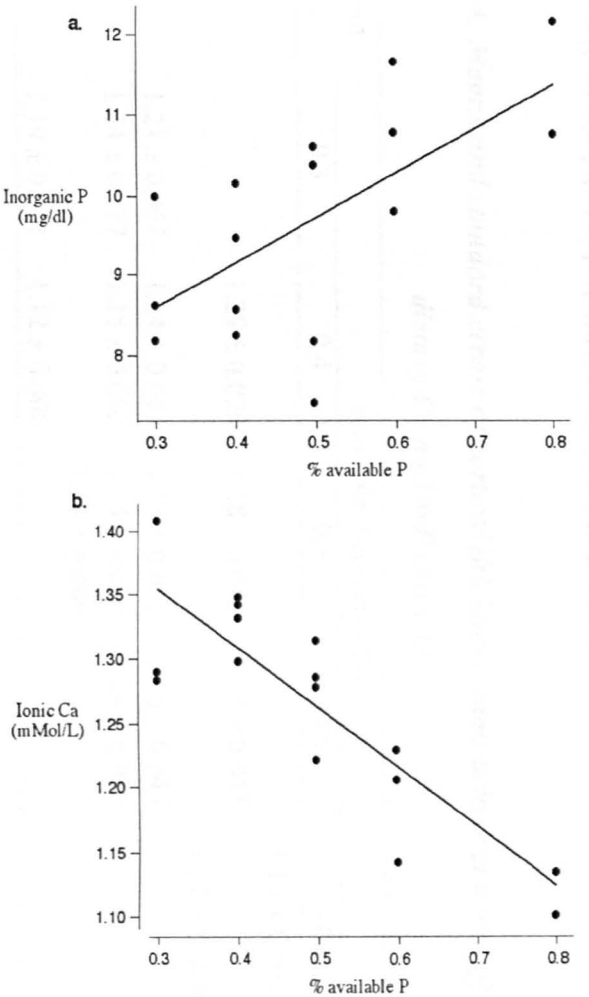


Figure 4.4: Effect on plasma osteocalcin levels of a. dietary calcium, b. dietary available p

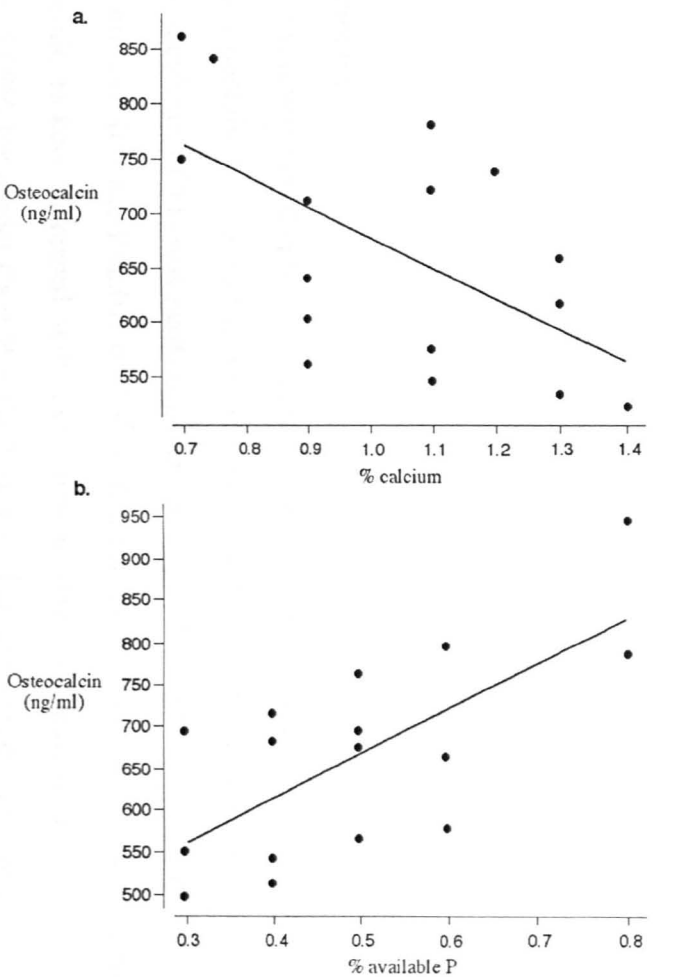


Table 4.3: Means and standard errors of 1,25-dihydroxyvitamin D_3 concentrations (pMol/l) in plasma achieved with different dietary Ca and avP contents

Ca content	available P content (%)				
(%)	0.3	0.4	0.5	0.6	0.8
1.3			133 ± 79		
1.2					203 ± 22
1.1	151 ± 3	158 ± 56	138 ± 3	192 ± 89	
0.9			284 ± 30		
0.75			220 ± 82		

4.4.4: Pathology

While there was no overall optimum diet identified, growth plate assessment indicated that diets should contain 1.1-1.3 % Ca and 0.3-0.6 % avP for normal development (Figure 4.5a). The incidence of TD was markedly increased by reducing Ca (t –3.93, P<0.001) and by increasing avP (t 2.82, P<0.01; df 2 r^2 45.6% for model Ca + P). The incidence of hypocalcaemic rickets increased with avP content along a band of dietary Ca (df 4, P<0.001, r^2 57.9% for model Ca + P + Ca.P +Ca^2). The proportion of birds with normal growth plates therefore increased with dietary Ca (t 3.80, P<0.001) and at lower avP contents (t –5.37, P<0.001; df 2, r^2 46.7% for model Ca + P; Figure 4.5). Hypophosphataemic rickets occurred too rarely for analysis to be performed.

4.4.5: Bone Dimensions

Cortical thickness in the tibiotarsus shaft was apparently unaffected by dietary mineral content (Table 4.4). Tibiotarsus width decreased in a linear manner with increasing dietary Ca content (t –4.056, df 15, P<0.001, r^2 48.5%; Figure 4.6).

Table 4.4: Means and standard errors of cortical thickness (mm) achieved with different dietary Ca and avP contents

Ca content	available P content (%)				
(%)	0.3	0.4	0.5	0.6	0.8
1.4					1.16 ± 0.032
1.3		1.29 ± 0.028	1.18 ± 0.045	1.08 ± 0.043	
1.2					1.17 ± 0.070
1.1	1.21 ± 0.067	1.24 ± 0.003	1.13 ± 0.065	1.20 ± 0.043	
0.9	1.04 ± 0.027	1.35 ± 0.002	1.14 ± 0.032	1.16 ± 0.031	
0.75			1.18 ± 0.005		
0.7	1.19 ± 0.001	1.12 ± 0.006			

Figure 4.5: Diet treatments and predominant growth plate pathologies: a. observed data, b. predicted incidence of hypocalcaemic rickets, c. predicted incidence of TD, d. predicted incidence of normal growth plates.

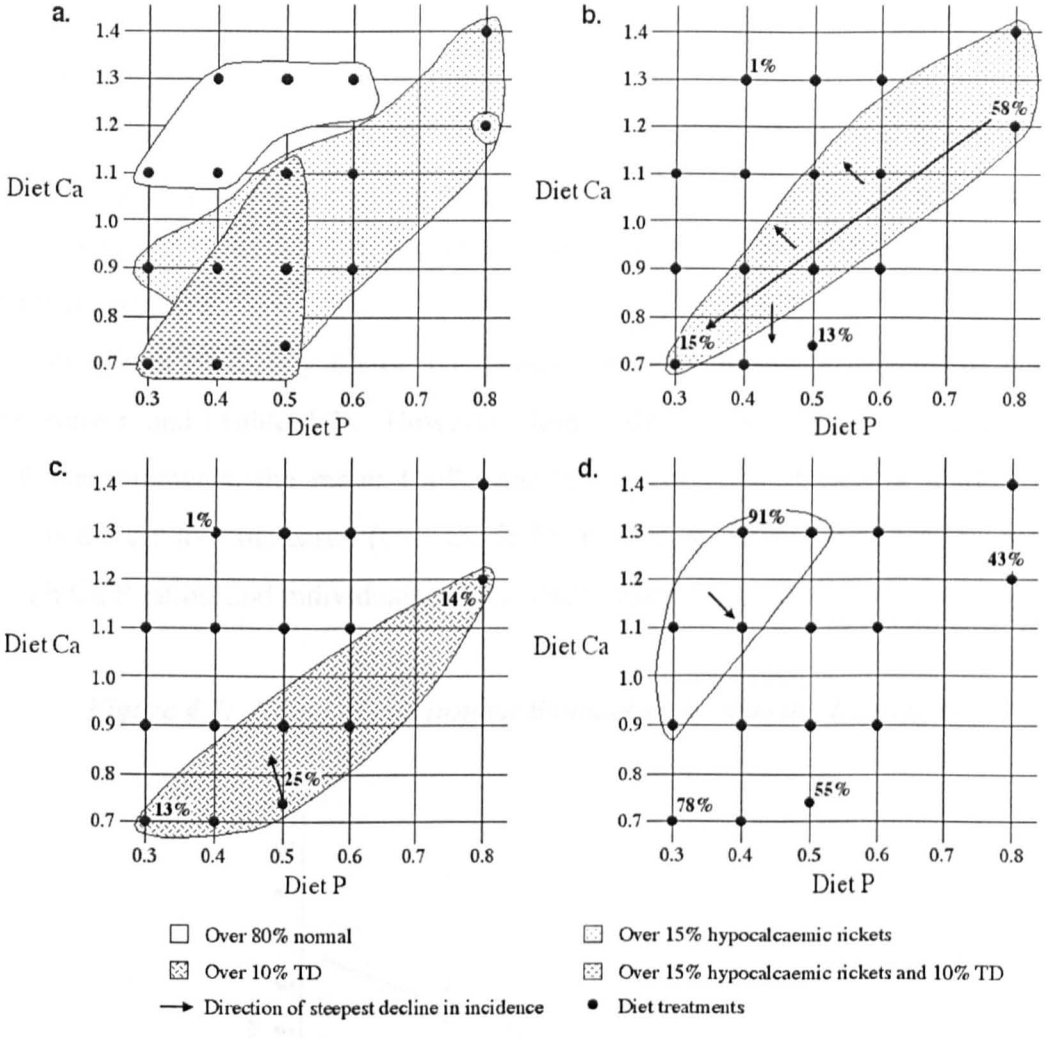
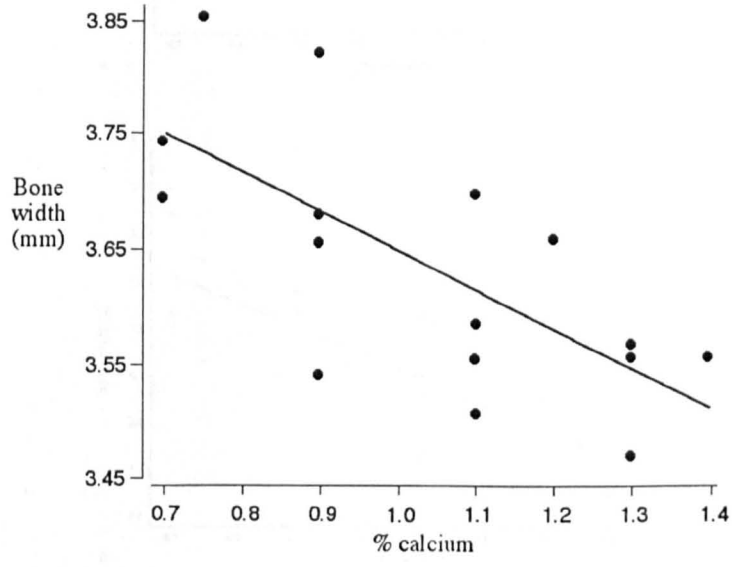


Figure 4.6: The effect of dietary calcium on tibiotarsus width



Tibiotarsus length and weight were also smaller in birds fed on high Ca diets, although these relationships were weaker, and only found in the presence of a P term in the model with a positive, but non-significant effect on both length and weight (t –2.293, df 15, $P<0.05$, r^2 22.1%, and t –3.767, df 15, $P<0.01$, r^2 30.6% respectively for model Ca+P; Figure 4.7).

4.4.6: Bone Quality

The relationships of bone % Ca and % P (Table 4.5), and % ash and radiodensity (Table 4.6) with dietary Ca or avP content were again too complex to be summarised by quadratic response surfaces. However, % ash tended to be highest in birds fed diets with 0.9–1.3 % Ca and 0.4-0.6 % avP

No effects of dietary Ca or avP content on bone breaking strength or collagen content were found (Table 4.7). However, despite the complex dietary effects on other mineral measurements, the molar Ca:P ratio in cortical bone showed a marked linear decline as dietary avP increased (t –2.43, df 15, $P<0.05$, r^2 32.5%; figure 4.8). All diets gave high Ca:P ratios, and individuals values ranged from 1.82-3.89.

Figure 4.7: Effect of calcium on tibiotarsus a. length, b. weight

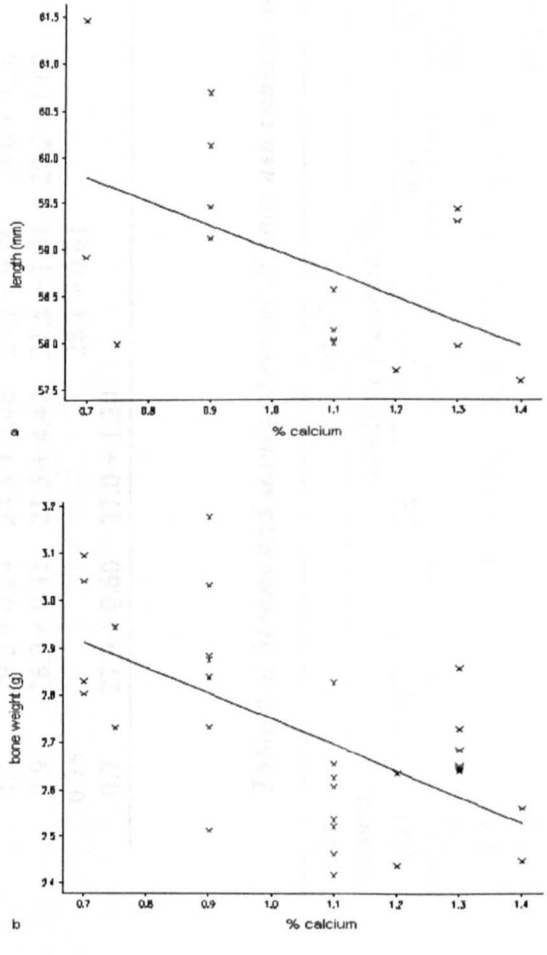


Table 4.5: Means and standard errors of bone Ca and P content achieved with different dietary Ca and avP contents

Ca content (%)	calcium (%)					phosphorus (%)				
	available P content (%)					available P content (%)				
	0.3	0.4	0.5	0.6	0.8	0.3	0.4	0.5	0.6	0.8
1.4					24.9 + 0.02					7.5 + 0.03
1.3		26.5 + 0.61	25.3 + 0.58	25.2 + 1.37			7.9 + 0.38	7.2 + 0.53	6.7 + 0.08	
1.2					29.3 + 0.49					9.1 + 1.03
1.1	29.2 + 0.24	24.5 + 0.48	25.9 + 0.86	24.6 + 0.56		7.7 + 0.53	6.8 + 0.52	7.1 + 0.93	7.7 + 0.37	
0.9	26.9 + 0.31	21.5 + 4.47	25.8 + 1.01	25.2 + 1.67		7.4 + 0.49	5.6 + 0.94	7.3 + 0.51	7.7 + 0.60	
0.75			28.1 + 0.61					6.9 + 0.19		
0.7	27.9 + 0.60	27.0 + 1.20				7.4 + 0.79	7.4 + 1.17			

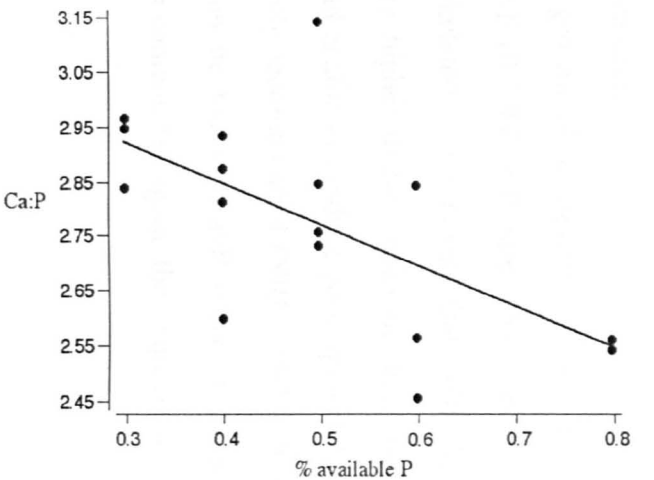
Table 4.6: Means and standard errors of bone ash content and radiodensity achieved with different dietary Ca and avP contents

Ca content (%)	ash (%)					radiodensity (mmAl)				
	available P content (%)					available P content (%)				
	0.3	0.4	0.5	0.6	0.8	0.3	0.4	0.5	0.6	0.8
1.4					48.2 ± 0.18					1.06 ± 0.011
1.3		55.2 ± 1.52	50.1 ± 1.19	53.1 ± 1.38			1.13 ± 0.020	1.12 ± 0.102	1.09 ± 0.050	
1.2					49.1 ± 0.41					1.10 ± 0.067
1.1	50.5 ± 0.47	50.8 ± 4.39	48.2 ± 0.84	51.8 ± 2.19		1.08 ± 0.005	1.09 ± 0.099	1.10 ± 0.042	1.09 ± 0.067	
0.9	50.2 ± 3.21	52.8 ± 0.47	50.8 ± 0.56	47.8 ± 0.01		0.96 ± 0.042	1.13 ± 0.006	1.07 ± 0.078	1.07 ± 0.008	
0.75			50.7 ± 0.11					1.16 ± 0.021		
0.7	46.9 ± 1.54	49.4 ± 0.66				0.98 ± 0.056	1.04 ± 0.044			

Table 4.7: Means and standard errors of bone breaking strength and collagen content achieved with different dietary Ca and avP contents

Ca content (%)	breaking strength (N)					hydroxyproline content (mg/g)				
	available P content (%)					available P content (%)				
	0.3	0.4	0.5	0.6	0.8	0.3	0.4	0.5	0.6	0.8
1.4					49.0 ± 0.21					30.4 ± 2.43
1.3		61.9 ± 1.21	55.0 ± 9.30	58.0 ± 3.19			36.2 ± 5.42	34.9 ± 2.12	29.8 ± 0.67	
1.2					57.1 ± 6.35					29.3 ± 0.26
1.1	62.6 ±1.92	56.1 ± 6.63	52.5 ± 0.58	54.4 ± 3.45		31.3 ± 0.25	30.7 ± 2.19	32.7 ± 2.27	31.7 ± 13.69	
0.9	60.3 ± 5.88	66.2 ± 7.15	60.8 ± 6.02	52.4 ± 0.69		35.1 ± 1.21	36.6 ± 0.40	32.4 ± 2.55	34.06 ± 3.26	
0.75			61.1 ± 7.13					31.7 ± 1.75		
0.7	60.8 ± 2.18	56.1 ± 2.54				25.5 ± 5.73	36.7 ± 0.39			

Figure 4.8: Relationship of bone molar Ca:P ratio to dietary available P content



4.5: DISCUSSION

4.5.1: Optimum Diet

No single optimum diet was found for overall bone quality, or growth performance. Where optima were suggested for individual measures by mathematical modelling, they were often around the edge of the diet treatment grid, and were therefore considered unreliable due to the manner in which Minitab gives closed contours. It is possible that our range of dietary mineral contents was not large enough for the initial purpose of this experiment. The potential high avP-low Ca diet missing from the bottom right of the treatment grid would have been particularly useful since many of the suspect optima were along that edge. Of those few measures for which the quadratic response surface appeared more likely to be a valid representation of dietary effect, the dietary mineral contents at which maximum values were achieved varied between different measures.

However, pathology assessment of the growth plate clearly indicated that diets should be high in Ca (1.1-1.3%) and low in avP (0.3-0.6%; Figure 4.5) to give normal cartilage development, essential for a healthy skeleton. This means that, with the diets provided, the selected strain appears to require higher dietary calcium than the 1.0% currently recommended for the starter period, and is able to produce good quality bone on diets with avP contents below the 0.45% currently recommended (NRC 1994). It would have been interesting to see how low diets can be made in avP before growth plate problems increase in incidence at high dietary Ca content, but again, the range of diets used here was too constricting for this purpose.

4.5.2: Bird Growth

Dietary mineral content appeared to have no effect on growth rate or body weight, although there was a slight tendency for lower body weights to be achieved at the very highest Ca contents (Table 4.1). There was a definite reduction in the proportion of breast muscle produced by birds fed on a high Ca diet, however (Figure 4.2). Arguments have been made in the past that Ca retards muscle growth only at very high contents, and that this is due to the mineral being toxic at such high contents (Whitehead pers. comm.). Since the Ca contents used in this experiment were in no way toxic, the reason for its present effect on breast muscle is uncertain, however, it may cause some conflict for producers of birds for breast meat.

Cortical bone thickness was apparently unaffected by dietary mineral content (Table 4.3), which might, to some extent, explain the lack of dietary effect observed on

bone strength (Table 4.6). Total bone width had a linear relationship with dietary Ca, and was smaller in birds fed on high calcium diets. Both bone length and bone weight also had a linear relationship with Ca, and, similarly, values were smaller in birds fed diets high in Ca. However, the Ca effect on length and weight was only significant when avP was included in the statistical model; higher avP tended to give higher values of length and weight in a linear manner, but the effect was not significant. The observed dietary Ca effects are consistent with the trend observed for lower bodyweight at high Ca content, since lighter birds generally have smaller and lighter bones. The bone length and bone weight results involving the non-significant avP effect are more tenuous, however. While not having a detectable effect itself, it is possible that allowing for the avP content of the diets may have reduced the variation of the data from the Ca lines shown in Figure 4.7, thus enhancing an otherwise non-significant dietary Ca effect. However, the biological mechanism by which this might occur is unknown at present, and it is not certain that this is a true effect. In addition, more definite changes in bodyweight with diet are likely to be needed in order to account for the changes seen in tibiotarsus dimensions and weight, implying that other factors are probably involved. In particular, bone weight could also be affected by bone porosity and mineralisation in addition to size; however, dietary Ca and avP content would be expected to have the opposite effect on bone weight if it was affected greatly by these factors. The reason for these results is therefore uncertain at present.

4.5.3: Calcium and Phosphorous Metabolism

Maintenance of normal plasma Ca concentration depends on the action of 3 hormones: parathyroid hormone (PTH), 1,25-dihydroxyvitamin D_3, and calcitonin (Taylor and Dacke 1984). In the present experiment, 1,25-dihydroxyvitamin D_3 was changed little, and total plasma Ca concentrations were apparently unaffected by dietary Ca or avP content (Tables 4.2 and 4.3), whilst Ca^{2+} concentration was smaller in birds fed on diets with higher avP (Figure 4.3b). Excess dietary Ca or P may reduce the availability of the other by the formation of insoluble calcium phosphate in the intestine (Shafey *et al* 1990a); however, plasma concentrations of total Ca unaltered by dietary mineral content suggest that such problems were not encountered here. The lower Ca^{2+} concentrations seen in birds fed high avP diets do not necessarily contradict this. Ca circulates in 3 fractions: the biologically important ionised fraction, the protein-bound fraction, and that fraction complexed to anions, including phosphate (Bushinsky and Monk 1998). Plasma P_i concentration was greater in birds given the diets with higher avP content (Figure 4.3a), and it is conceivable

that at least some of this extra P_i was in the form of calcium phosphate in the plasma, thereby lowering Ca^{2+}.

The production of 1,25-dihydroxyvitamin D_3 would be expected to increase under conditions producing low Ca^{2+} concentration by the action of increased circulating PTH on renal 25(OH)D_3-1-hydroxylase (Taylor and Dacke 1984), however, this was not seen here (Table 4.3). At present there are no estimates of 'normal' Ca^{2+} concentration for the broiler strain used, and it is possible that low dietary avP is actually resulting in high Ca^{2+} concentrations. Calcitonin may become an important Ca regulator under such conditions, and it would, therefore, be of interest to measure circulating concentrations of this hormone.

4.5.4: Bone Turnover and Quality

Bone resorption was unaffected by dietary Ca and avP content in this study (Table 4.2). However, the concentration of plasma osteocalcin, the marker for bone formation (Power and Fottrell 1991), was greater in birds on diets higher in avP and lower in Ca (Figure 4.4). Although this is perhaps surprising in view of the pathology results, it agrees with the results of Corlett and Care (1988), and with the observation that low dietary Ca produced birds with wider tibiotarsi. The higher osteocalcin concentration may be a consequence of the low Ca^{2+} concentration in birds fed these diets. 1,25-dihydroxyvitamin D_3 is known to stimulate osteocalcin production by osteoblasts (Gundberg *et al* 1983), and whilst osteocalcin is a marker for bone formation, it has recently been implicated in the recruitment and differentiation of bone-resorbing cells (Lian and Marks 1990). However, as concentrations of 1,25-dihydroxyvitamin D_3 were largely unaffected by diet, these relationships require further investigation. An alternative explanation of the osteocalcin results may be that the higher levels reflect increased bone formation *per se*, rather than increased bone turnover for mineral homeostasis. This would explain the lack of any large dietary effect on both 1,25-dihydroxyvitamin D_3 and pyridinoline cross-links, while not ruling out concurrent mineral homeostasis mechanisms involving bone.

The incidence of TD increased on the lower Ca and higher avP diets, supporting other observations (Rennie *et al* 1993) that the incidence of TD is influenced by dietary Ca:P ratio (Figure 4.5). The incidence of hypocalcaemic rickets also increased at higher avP contents along a band of increasing dietary Ca, upholding the view of Thorp (1994) who suggested that the balance of Ca and P in the diet was important (Figure 4.5). The increased incidence of both disorders in birds given higher dietary avP is associated with lower circulating Ca^{2+}, strongly suggesting that hypocalcaemia was occurring on these

diets. Diagnosis of hypophosphataemic rickets from decalcified sections was generally found to be unreliable. The occasional cases which were assessed with any confidence as having this condition were too few in number to show any obvious pattern of occurrence. Alternative methods of tissue preparation were attempted in a subsequent experiment (Chapter 6) that did not involve decalcification.

There was no dietary effect on bone collagen (hydroxyproline) content, and there appeared to be no simple dietary effect on bone ash content, although there was a tendency for lower ash values to occur at the lowest Ca contents and highest avP contents (Table 4.5). The largest bone ash contents did tend to occur at higher dietary Ca contents than the highest final bodyweight values (Table 4.1), however, the results from this study gave little evidence of a conflict between bone mineralisation and final body weight or growth rate within the range of dietary Ca and P contents presented here. It is possible that the weak trends observed for lower bone ash contents and higher body weights on the lowest Ca contents act in concert to produce the observed increases in bone width and circulating osteocalcin concentrations. As discussed previously, bone widths are larger in heavier birds. If the same birds had lower bone ash content, which would be expected to have a weakening effect (Boskey *et al* 1999), bone width might be increased more than expected on the basis of weight alone in order to compensate bone strength. The weak dietary effects on body weight and bone ash could therefore be combining to give a stronger, but indirect, effect on bone width.

Despite complex effects on bone Ca and P content, a relatively simple dietary effect was seen on the molar Ca:P ratio – higher dietary avP give smaller cortical bone Ca:P ratios. This was probably caused by the lower circulating Ca^{2+} and greater P_i concentration produced by the higher avP diets, however, it is difficult to prove a relationship between instantaneous blood measures and cumulative bone measures. All diets gave bone molar Ca:P ratios higher than the expected 1.67 to 1, values ranging from 1.82-3.89 to 1. This was surprising since avP contents in some diets were almost double those currently recommended. In addition, although increasing avP in the diet gave ratios closer to the expected 1.67 to 1, it also resulted in poor growth plate development and would not, therefore, be desirable. The reason for the high ratios is at present unclear, but it appears unlikely to be due to a P deficiency.

In addition, a Ca:P ratio of up to 2.97 to 1 does not seem to preclude relatively good quality bone. One of the main concerns about bone quality is the likelihood of bone fractures in birds of market age (6 weeks). Variation in the molar Ca:P ratio of bone is likely to involve alterations in the bone mineral crystal structure, and it has been suggested

that abnormal ratios could result in weakened bone (Thorp and Waddington 1997). Our results show tibiotarsus breaking strength was unaffected by dietary Ca and avP content at 2 weeks of age, despite great variation in molar Ca:P ratios occurring in the tibiotarsus. It is likely that the structure and distribution of bone, and the proportion of collagen and mineral in it (which remained unchanged with diet in this investigation) are far more important to bone strength than the exact form of the mineral crystal. However, it is possible that a dietary effect on bone strength may only become apparent later in the birds' development; this will be investigated at a later date.

4.5.5: Conclusions

No single optimum diet was found for overall bone quality, or growth performance. However, pathology assessment of the growth plate indicated that in order to allow normal cartilage development, diets for this strain should be higher in dietary calcium than the 1.0% currently recommended, and that this strain is able to produce good quality bone on diets with available P (avP) contents below current recommendations. The results from this study gave little evidence of a conflict between bone quality (ash content and pathology) and final body weight or growth rate within the range of dietary Ca and P contents used; however, there was a significant reduction in the proportion of breast muscle produced by birds fed on a high Ca diet.

No effect of dietary Ca and avP content on tibiotarsus breaking strength was found at 2 weeks of age; however, it is possible that a dietary effect may only become apparent later in the birds' development. Bone molar Ca:P ratios on all diets were above the expected 1.67 to 1. Increasing avP in the diet gave bone molar Ca:P ratios closer to the expected 1.67 to 1, probably due to the greater inorganic P (P_I) and lower ionised Ca (Ca^{2+}) concentrations seen in the plasma of these birds. However, the incidence of TD and hypocalcaemic rickets also increased on the lower Ca and higher avP diets, and such an approach to normalising bone mineral contents would not, therefore, be desirable. The reason for this result is at present unclear, but a molar Ca:P ratio of up to 2.97 to 1 does not seem to preclude relatively good quality bone.

CHAPTER 5

A COMPARISON OF BONE MINERALISATION AND BREAKING STRENGTH BETWEEN 3 BROILER STRAINS AT 2 AGES

5.1: INTRODUCTION

Chapter 3 (Williams *et al* 2000) looked at how the bones of a pure line selected broiler type chicken develop under a commercial fast growth-rearing regime, and compared their development with that of a slower growing genetic precursor, the control stock. One of the most striking results from this experiment was that the selected strain had a consistently much lower total bone mineral content than the control strain. Another, unexpected, result was that during the first 2-3 weeks of life, the molar Ca:P ratio in the tibiotarsus of the selected strain was considerably higher than the expected 1.67:1 (Pellegrino and Biltz 1968), achieving a ratio of 2.2:1. The control strain had roughly a 1.7:1 ratio throughout its life span, and this was assumed to be the norm.

Originally, it was proposed that there might be a general Ca or P deficiency in the current commercial diets that resulted in the low bone ash content. It was also suggested that the high ratio early in the life of the fast growing birds might be due to problems achieving optimum P levels in the bone during this critical period in tibiotarsus development. However, Chapter 4 identified no convincing dietary effect on bone ash content, and although increased dietary phosphorus did reduce bone Ca:P ratios, none of the diets used gave the 1.67:1 bone molar Ca:P ratio (Williams *et al* in press). Indeed the ratios observed were even higher than in the previous study, ranging from approximately 2.5-3.1 to 1. The results, in fact, suggested that the higher ratios in experiment 2 were due to an increase in Ca uptake into the bone; % P levels in the bone were much the same as in the first experiment (around 6-10%), but bone % Ca was higher (20-29% compared with 15-20%).

Previously, it was suggested that a disruption to the Ca:P ratio of bone mineral might lead to weakness in the bone crystal lattice, and therefore contribute to leg problems (Thorp and Waddington 1997). However, in the second experiment, although the molar Ca:P ratio of cortical bone changed significantly between some of the diets used, there was no dietary mineral content effect on bone breaking strength. In addition, ratios closer to the expected norm were associated with an increased incidence of growth plate

pathologies, and reducing the ratios through dietary means appeared to be detrimental to bone health. It is, therefore, possible that the high ratio observed in the selected strain is the result of an adaptation in the mineralisation process to cope with the much faster bone production rate in this strain. Alternatively, it may simply be a result of a limitation in the rate of the mineralisation process, below the limit of bird growth and bone production potential. A third possibility is that this phenomenon was some unique, possibly genetically based, change in the mineralisation process present only in the selected strain. While confirmation of any of these hypotheses is outside the realm of this experiment, the intention is to investigate bone Ca:P ratios in birds with different genetic backgrounds and growth rates, and in load-bearing and non-load-bearing bones.

5.2: AIMS

From the experiments described above it appears that, other than being less well mineralised, bone mineral development in the selected strain after 25 days is normal (i.e. similar to that of the control strain). However, there is an extreme, and currently unexplained, disruption to the Ca:P ratio of bone in the period before this. The two primary aims of this experiment were to investigate the possibilities that the disruption to the Ca:P ratio seen is either related to growth rate, or peculiar to the Ross MCX strain. This was done by the addition of a third, fast growing, but genetically unrelated broiler strain from Cobb into the experiment. It was also intended to examine whether the occurrence of the aberrant Ca:P ratio was function-related by investigating whether it occurred in a non-load-bearing bone. Finally, it was intended to investigate how bone strength varied between the strains at different ages, and whether bone Ca:P ratio had any apparent effect on this measure at an older age.

5.3: MATERIALS AND METHODS

5.3.1: Birds and Housing

Thirty day-old chicks from 3 strains of birds (selected, Cobb, and control broilers; total 90 birds) were placed in pens at Roslin. The selected strain is a Ross Breeders pure line (MCX) selected for skeletal health in addition to production, and is intended as the basis for future commercial birds. The unrelated strain is Cobb's current commercial broiler, bred purely for production, and is likely to be derived from a four-way cross of different

lines (McKay pers. comm.). The control strain is the MK strain from Ross Breeders which has not been actively selected since 1972, resulting in a markedly lower growth performance to the previous strains. Birds from each strain were split into 2 pens at random, with 15 birds in each pen. All the birds were fed *ad lib* on the same standard commercial type starter feed until 2 weeks of age, when they were transferred on to a standard commercial type grower feed. Feed was provided by the Roslin feedmill, and levels of Ca and P were the same as those specified for the starter and grower diets used on the Ross farms in experiment 1: Ca 9.5 g/kg, available P 5.0 g/kg,; and Ca 9.0 g/kg, available P 4.8 g/kg respectively. Trays were used for feed presentation during week 1, and hoppers replaced these for week 2. Standard wood-shaving floor cover, bell drinkers (*ad lib* water supply) and heaters were deployed in each pen. The lighting regime started with 23hrs light on day one, and switched to 21hrs light on day six.

5.3.2: Sampling Procedure and Processing

Where possible, five male birds from each pen were removed at random for culling on day 14 (ideally 10 from each strain). The remaining birds were switched onto the grower diet. Five of these birds per pen (ideally) were culled on day 35. At culling, the total body weight of each bird was measured, and both tibiotarsi and humeri were dissected out. The left tibiotarsus was used to measure dimensions (length and total width), breaking strength and stiffness, and a segment of the proximal end of the shaft from the right tibiotarsus was subsequently ashed for mineral analysis, along with a segment of the right humerus shaft. The left humerus was put into storage for possible future analysis.

5.3.3: Statistical Analysis

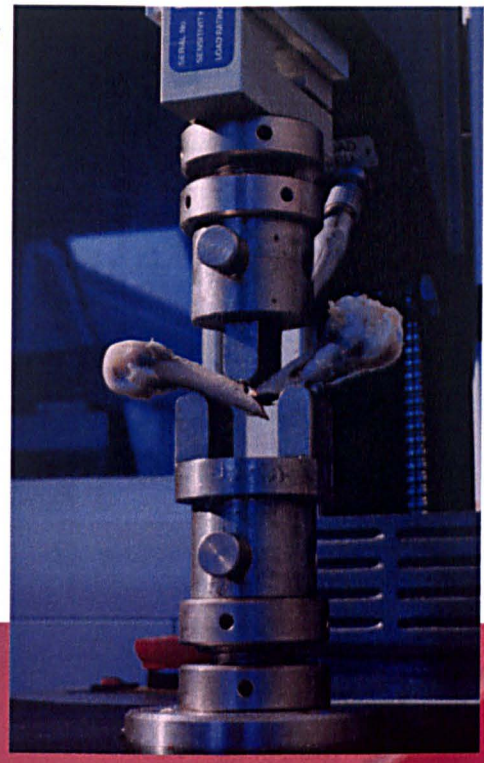
Due to problems with the suppliers, not all the birds were males, and ideal sample numbers were not achieved. Data analysis was, therefore, similar to that performed in Chapter 3: REML analysis was performed on all measurements; 'block' and 'pen' were fitted as random factors to account for any differences due to variations in housing conditions within the hut. The effects of age, strain, and their interactions were explored before final simplified models were determined for each variable. Effects were assessed using Wald tests.

Linear regression analysis was performed on logarithmically transformed allometric data to examine possible differences between strains in the relationships of tibiotarsus length, width, breaking strength and stiffness with weight.

Plate. 5.1:

Testing the mechanical properties of bone

a.



b.

a. Close-up of a bone undergoing 3-point bending.

b. Materials tester and computer link-up.

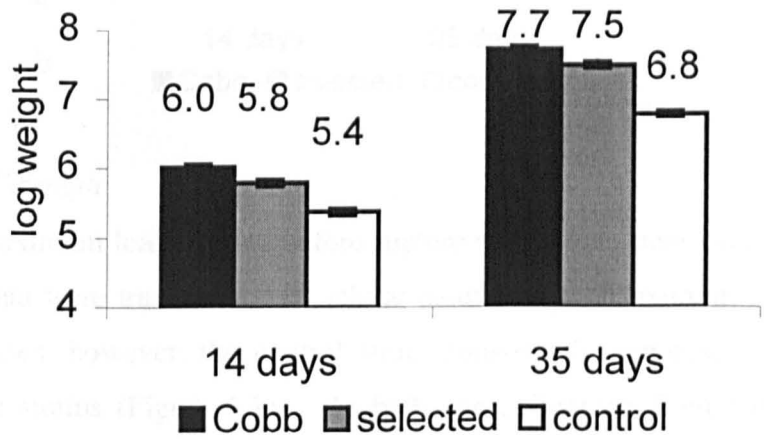
5.4: RESULTS

5.4.1: Age and Strain Effects

Total Body Weight

The variance in body weight was higher for older birds; data were therefore transformed by taking natural logs. Body weight increased between 14 and 35 days in all 3 strains (Figure 5.1). At both ages, the Cobb birds were the heaviest, followed by the selected strain, with the control strain being the lightest; however, this difference between the strains was greater at 35 days than at 14 days (age.strain interaction: W 13.2, df2, P<0.01).

Figure 5.1: Mean body weight along with standard errors

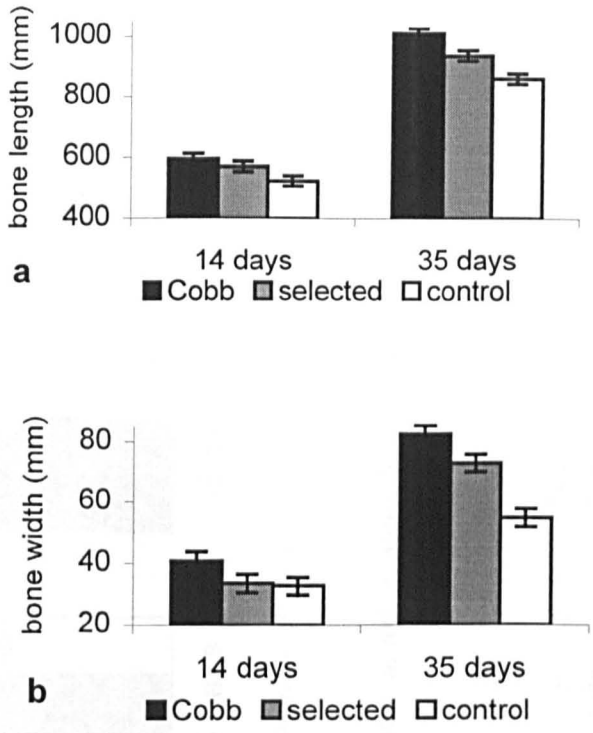


Figures shown are back-transformed means in g.

Tibiotarsus Dimensions

Bone length increased between 14 and 35 days in all three strains (Figure 5.2a). At 35 days, the Cobb birds had the longest bones, followed by the selected line, and then the control strain with the shortest bones. This trend was previously seen at 14 days, however, it was not as marked (age.strain interaction: W 20.2, df 2, P<0.001). Total bone width increased with age in all three strains (Figure 5.2b). At 14 days, bones from the Cobb strain were wider than those from the other two strains. They were also widest at 35 days, followed by the selected strain, with the control strain having the narrowest bones (age.strain interaction: W 27, df 2, P<0.001).

Figure 5.2: Mean tibiotarsus dimensions along with standard errors a. length, b. width

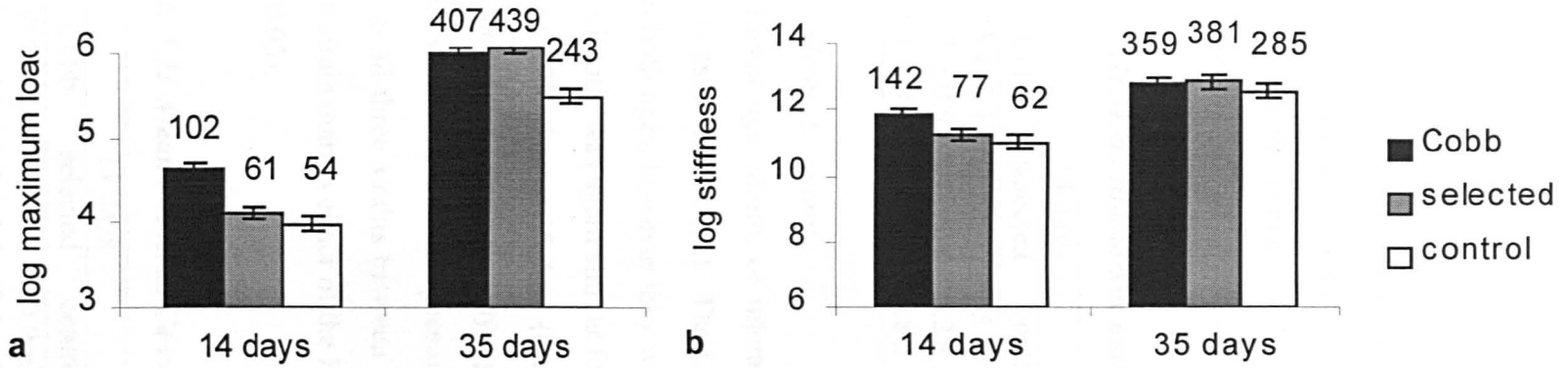


Tibiotarsus Bone Strength

Variance in the maximum load applied before rupture was inconsistent over the treatment groups, and the data were transformed by taking natural logs. Maximum load increased with age in all cases, however, the control strain consistently demonstrated the lowest value of the three strains (Figure 5.3a). At both ages, tibiotarsi from the Cobb strain resisted considerably larger loads than those from the control strain. At 14 days, selected tibiotarsi were similar in strength to the control birds, whilst at 35 days, bones from the selected line resisted similar loads to those from the Cobb strain (age.strain interaction: W 21.5, df 2, $P<0.001$).

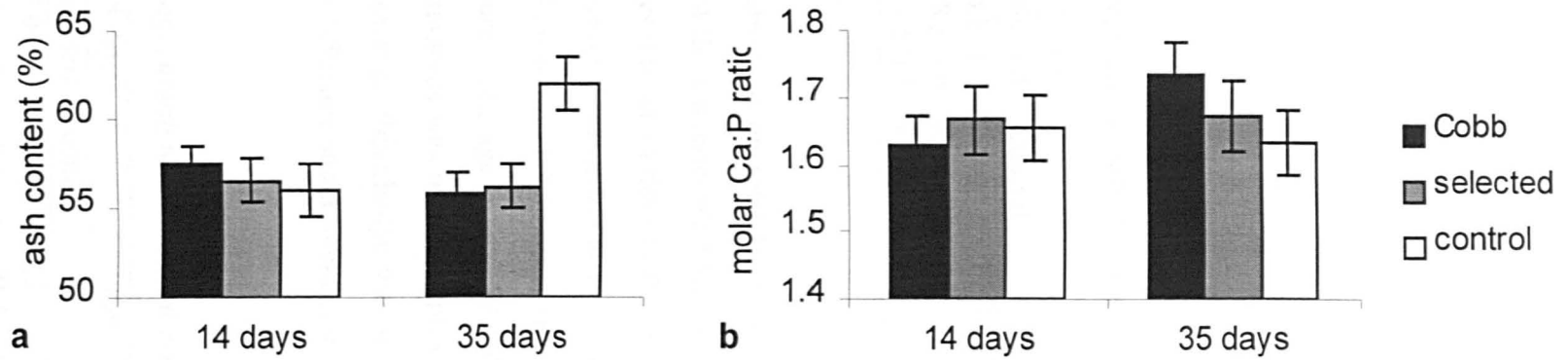
Variance in the stiffness of the tibiotarsi was also inconsistent over the treatment groups, and the data were transformed by taking natural logs. Tibiotarsus stiffness was greater at 35 than at 14 days in all cases (Figure 5.3b), and showed the same pattern as bone strength. Bones from the Cobb strain were much stiffer than those from the control strain at 14 days, and bones from the selected birds were similar in stiffness to those from the control birds at this age. At 35 days, all the strains had bones of similar stiffness (age.strain interaction: W 9.5, df 2, $P<0.01$).

Figure 5.3: Mean tibiotarsus bone strength a. maximum load applied before rupture b. stiffness



Figures shown are back-transformed means, max load N, stiffness 1000 N/m.

Figure 5.4: Mean tibiotarsus mineral contents along with standard errors a. ash content b. molar Ca:P ratio



Tibiotarsus Mineral Content

Total mineral content (% ash) in the tibiotarsus was similar in the 3 strains at 14 days, and in the Cobb and selected strains at 35 days (Figure 5.4a). However, while % ash was unaffected by age in the selected and Cobb strains, in the control strain it increased significantly between 14-35 days (Age.strain interaction: W 7.9, df 2, $P<0.05$). Ca, P and Mg content were similar for all strains at each age, however, overall, mean tibiotarsi contents of Ca and P were significantly higher at 35 days than 14 days, whilst mean Mg content was significantly lower at 35 days (Table 5.1). There was no significant age or strain effect on the molar Ca:P ratio in the tibiotarsus (Figure 5.4b), which closely approximated the 1.67 of hydroxyapatite.

Table 5.1: Mean tibiotarsus mineral content with age, along with standard errors

	14 days			35 days			age effect	
	Cobb	selected	control	Cobb	selected	control	W	$P_{(1)}<$
%Ca	15.4±1.1	17.6±1.2	15.6±1.1	19.0±1.1	18.8±1.2	19.6±1.8	11.3	0.001
%P	7.3 ± 0.5	8.1 ± 0.6	7.2 ± 0.5	8.6 ± 0.5	8.8 ± 0.6	9.3 ± 0.9	7.8	0.01
%Mg	5.1 ± 0.3	5.0 ± 0.3	4.8 ± 0.3	4.3 ± 0.3	4.3 ± 0.3	4.9 ± 0.5	5.6	0.05

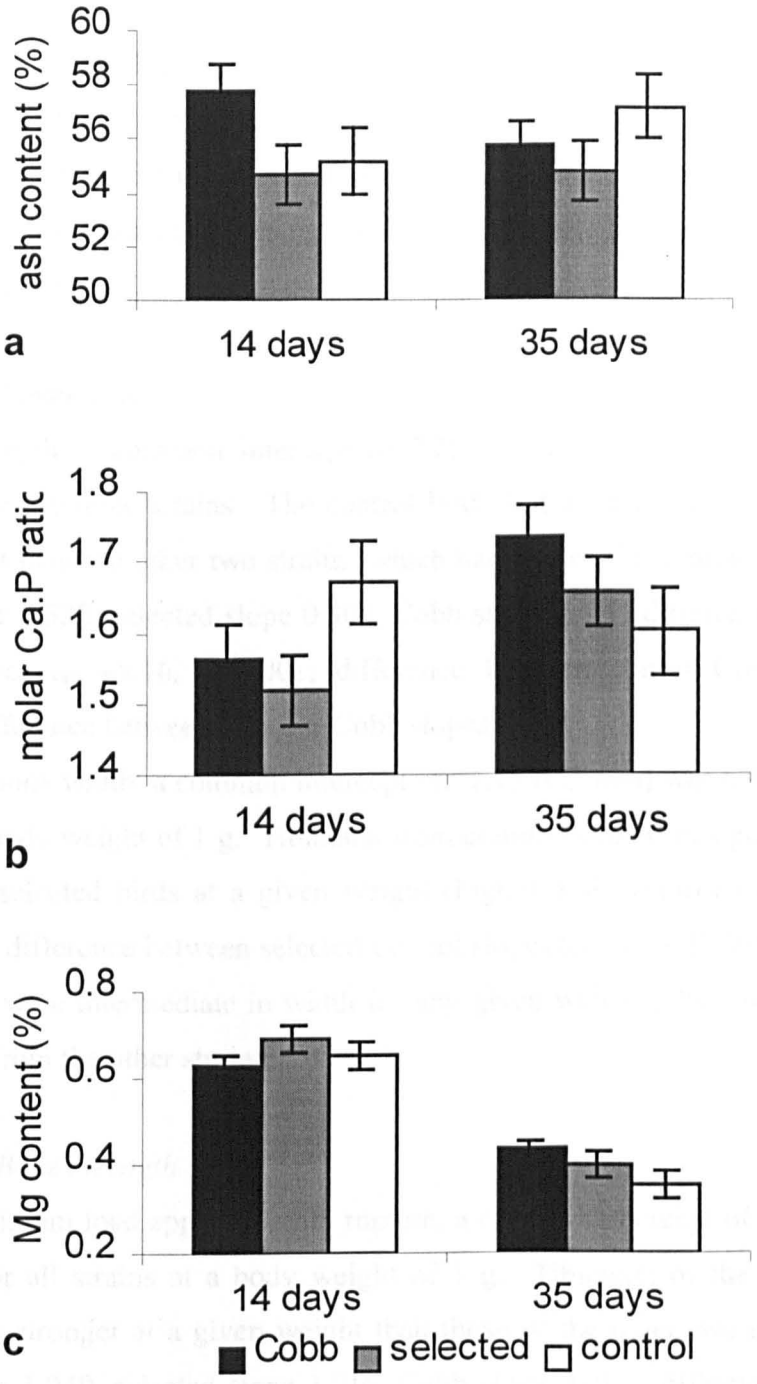
Humerus Mineral Content

No significant age, strain, or interaction effect was observed on total humerus mineral content (% ash; Figure 5.5a). The Ca and P contents of the humerus were similar in all 3 strains at both ages, however they were lower at 35 days than at 14 days (Table 5.2). The molar Ca:P ratio was again similar for the 3 strains, however it was lower at 14 days (mean 1.588, se 0.028) than at 35 days (mean 1.689, se 0.317) when it closely approximated the molar ratio of 1.67 produced by hydroxyapatite (Figure 5.5b; age effect: W 6.4, df 1, $P<0.05$). Variation in the magnesium content of the humerus was more complex; it was reduced in all three strains between 14 and 35 days, however, this change was smaller in the Cobb strain than in either of the Ross strains (Figure 5.5c; age.strain interaction: W 6.2, df 2, $P<0.05$).

Table 5.2: Mean humerus Ca and P content with age, along with standard errors

	14 days			35 days			age effect	
	Cobb	selected	control	Cobb	selected	control	W	$P_{(1)}<$
%Ca	20.1±0.6	19.0±0.6	20.0±0.6	18.4±0.6	17.9±0.7	17.1±0.8	23.7	0.001
%P	9.9 ± 0.2	9.7 ± 0.2	9.3 ± 0.2	8.2 ±0.2	8.6 ±0.2	8.3 ± 0.4	49.4	0.001

Figure 5.5: Mean humerus mineral content along with standard errors a. ash content b. molar Ca:P ratio c. magnesium content



5.4.2: Allometric Adaptations

Since data from only two age groups with body weights relatively far from the origin were available for analysis, it was considered that a regression model could not accurately predict separate intercepts for each strain. An assumption was therefore made for tibiotarsus length, width, and breaking strength, that at the log weight origin (*i.e.* the embryo stage at 1 g), the difference in these parameters would be negligible, allowing us to use a model with a common intercept for all three strains. However, since preliminary analysis did demonstrate differences between the estimated intercepts, the results must be treated with caution.

Tibiotarsus Dimensions

For bone length, a common intercept of 2.25 (9.5 mm) at a body weight of 1g was estimated for the three strains. The control birds had significantly longer tibiotarsi for a given weight than the other two strains, which had bones of a similar length (Figure 5.6a; control slope 0.320, selected slope 0.308, Cobb slope 0.307; difference between selected-control slopes t_{41} –5.16, $P<0.001$; difference between control-Cobb slopes t_{41} 5.64, $P<0.001$; difference between selected-Cobb slopes NS)

For bone width, a common intercept of –1.13 (0.3 mm) was estimated for the three strains at a body weight of 1 g. Tibiotarsi from control birds were significantly wider than those from selected birds at a given weight (Figure 5.6b; control slope 0.425, selected slope 0.412; difference between selected-control slopes t_{41} –2.55, $P<0.05$). Bones from the Cobb strain were intermediate in width for any given weight (slope 0.418; no significant differences from the other strains).

Tibiotarsus Bone Strength

For the maximum load applied before rupture, a common intercept of –1.584 (4.9 N) was estimated for all strains at a body weight of 1 g. Tibiotarsi of the control strain were considerably stronger at a given weight than those of the other two strains (Figure 5.7a; control slope 1.040, selected slope 1.010, Cobb slope 0.013; difference between control-Cobb t_{39} 2.27, $P<0.05$; difference between selected-control t_{39} –2.55, $P<0.05$; difference between selected-Cobb NS). However, the line estimated for the relationship between maximum load and body weight in the Cobb strain does not appear to correspond well with the data points, and the results for this strain should be treated with caution.

There appeared to be two groupings of stiffness measurements within the data-spread at 14 days (Figure 5.7b), and personal observations confirm that bones did tend either towards brittle, or very elastic during the three-point bending test. It was therefore

considered that a model fitting a common intercept, and a single line for each strain may be too simple, and no further analysis was performed on this measure.

Figure 5.6: Changes in the log of tibiotarsus dimensions with the log of body weight a. length b. total width

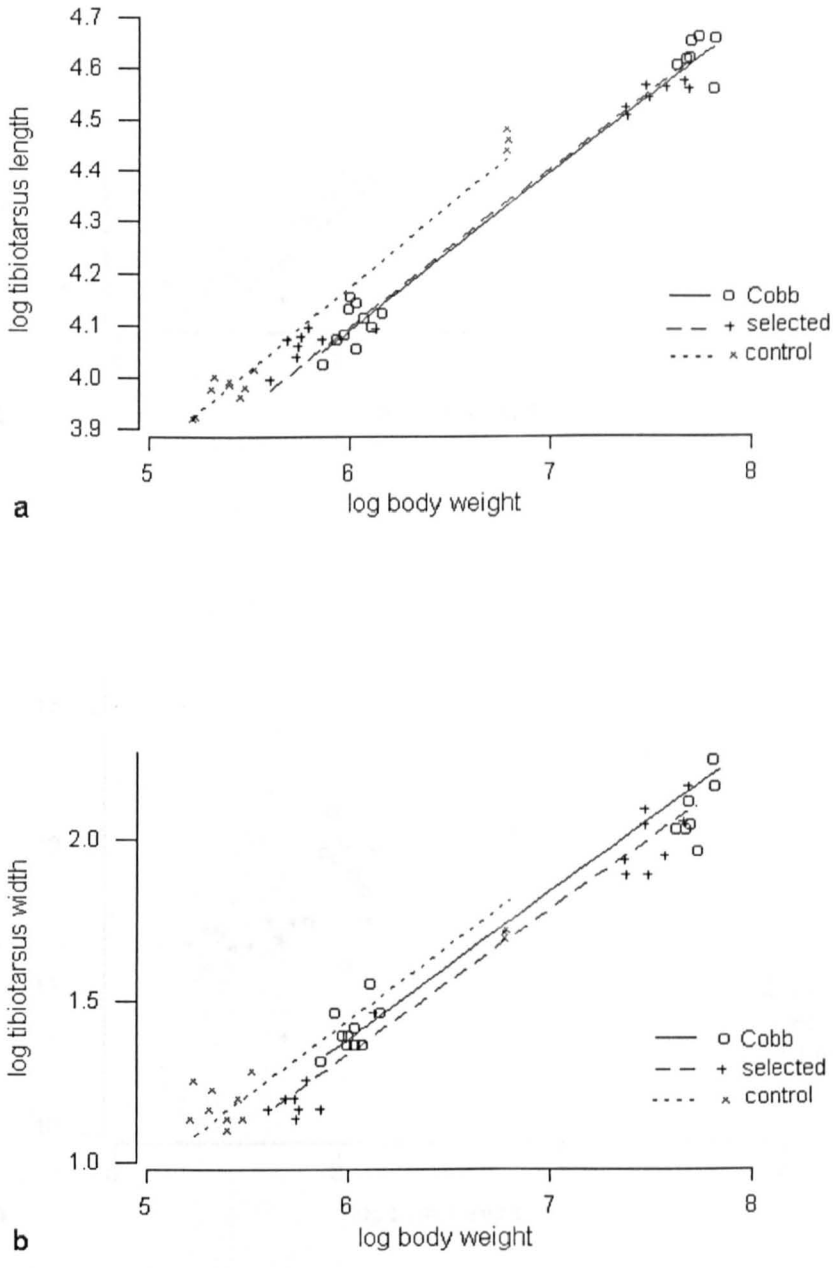
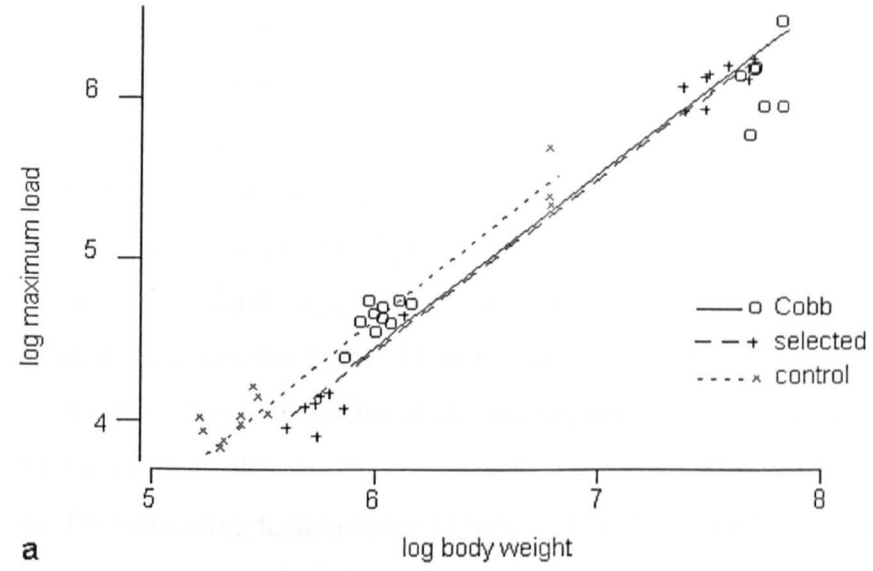
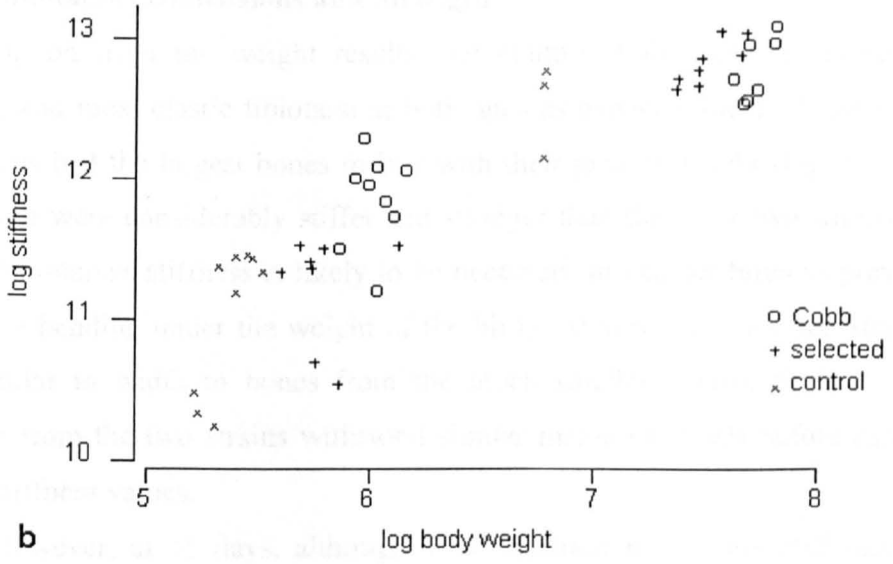


Figure 5.7: Changes in the log of tibiotarsus mechanical properties with the log of body weight, a. maximum load b. stiffness





5.5:DISCUSSION

5.5.1: Body Weight

As expected, the two modern strains were heavier than the control strain at both ages (Figure 5.1). However, contrary to expectations, at 35 days the current commercial strain (Cobb) was heavier than the strain selected for future production. If we compare the mean weight achieved by the selected birds at 35 days in this experiment (1817g) with means achieved at a similar age in a previous experiment (experiment 1, 32 days: 1916g), it would appear that the selected birds were under-performing in this experiment. The reasons for this are uncertain; the lighting regime and diets used were similar in both experiments, thus feed intake and energy consumption should also be similar, and there was no apparent illness in the flock. However, it is likely that the environmental control achieved on the Ross farms in the first study was superior to that achieved at Roslin, where the present experiment was conducted, which may have affected feed intake and/or conversion. Post-brooding temperatures of below 20°C cause birds to eat more in order to keep warm without a corresponding increase in weight, producing a reduction in feed conversion efficiency, while feed intake drops by approximately 5% per degree temperature rise between 32-38°C (Ross Breeders 1996).

5.5.2: Tibiotarsus Dimensions and Strength

Following on from the weight results, the control strain had the shortest, narrowest, weakest, and most elastic tibiotarsi at both ages as expected on the basis of bird weight. Cobb birds had the largest bones in line with their greater weight (Figure 5.2), and at 14 days, these were considerably stiffer and stronger than the other two strains (Figure 5.3). Greater tibiotarsus stiffness is likely to be necessary in heavier birds to prevent the bones constantly bending under the weight of the birds. Bones from selected strain birds were very similar in width to bones from the much smaller control birds at 14 days, and tibiotarsi from the two strains withstood similar maximum loads before rupture, and had similar stiffness values.

However, at 35 days, although selected strain birds were still lighter, and their tibiotarsi were still narrower than those from Cobb birds, they were similar in strength and stiffness to the Cobb tibiotarsi at 35 days. This implies an improvement in some quality of the selected strain bone between 14-35 days which is not seen in the Cobb strain, or perhaps a greater improvement in selected than Cobb birds. In a healthy skeleton, bone mass and architecture is altered to accommodate the load placed upon it. Artificially imposing an intermittent load on a turkey ulna has been found to increase the cross-

sectional area of the bone, primarily by new bone formation at the periosteal surface, including the formation of new primary osteons. In addition, it was found that this increase was proportional to the magnitude of the load placed upon the bone (Rubin and Lanyon 1987). Walking places an intermittent load on the tibiotarsus of the broilers in this study; the heavier the bird, the greater the load, and the larger the cross-sectional area would be expected to be. Although in Chapter 3 the selected strain tibiotarsus cortex was shown to be more porous, it was wider and thicker than that of the MK birds, and for a given weight, the estimated resistance to bending of the tibiotarsus was similar for both strains (Williams *et al* 2000). It is possible that although the bones from the Cobb strain were wider, they did not achieve the correct cortical thickness or solidity for their yet greater weight. However, as neither of these were measured in the present study, further investigation is needed to clarify these results.

5.5.3: Allometric Adaptations

Despite reservations about the quantity and nature of the data available in the present study, the relationships observed between tibiotarsus length and body weight in the selected and control strains are similar to those observed in Chapter 3. For a given weight, bones from the selected strain are shorter than those from the control strain; in addition, the Cobb strain shows a similar response to the selected birds (Figure 5.6a). A force exerts a bending moment on a cylindrical beam that is proportional to both the size of the force, and the length of the beam (Biewener 1992), thus a shorter bone will bend less for a given load. It has previously been suggested (Chapter 3, Williams *et al* in press) that this reduction in bone length in the MCX strain could either be an adaptation to cope with heavier body weights, or simply a co-incidental limitation in the rate of growth plate activity. There is some evidence to support the former hypothesis. When the ulnae of growing rats were subjected to an intermittent loading regime over a period of 12 days, it was found that the loaded ulnae were significantly shorter than the contra-lateral non-loaded bones. It was also found that the size of this effect was dependent on the magnitude of the load applied, and the effect was suggested to be mediated through a reduction in the activity of the growth plate chondrocytes (Mosely *et al* 1997). Bones are constantly altered in shape and mass by remodelling processes to adapt to different loading environments; a role for the growth plates in controlling the growth in bone length for the same reason should perhaps be unsurprising.

For a given weight, tibiotarsi from the control strain were considerably wider than those from selected strain birds, with bones from Cobb birds intermediate in width to these two strains (Figure 5.6b). If the results are real rather than produced by chance, this

suggests that the tibiotarsus bone width achieved in the selected strain is no longer ideal for a particular weight, possibly due to a limitation in the rate of appositional bone growth. However, a previous experiment (Chapter 3, Williams *et al* 2000) demonstrated that control and selected birds produced tibiotarsi with similar potential resistance to bending, estimated on the basis of total and cortical width; at higher weights this was achieved by producing thicker cortical bone. It would therefore appear that in the selected strain, this possible limitation in the rate of increase of total bone width is compensated for by a reduction in the growth of the marrow cavity. The position of the Cobb strain as intermediate to the selected and control strains in tibiotarsus width could be an indication that the reduction in width is occurring within the general broiler selection process, since the selected strain is the most modern. However, Cobb birds are from a completely different genetic background, and their tibiotarsi were not significantly different in width from bones of either the selected or control strains; hence it is impossible to confirm or refute this hypothesis on the basis of this experiment.

The relationship between tibiotarsus bone strength and body weight is less convincing, however, there are indications that bones from the control strain at a given body weight could withstand a considerably larger maximum load before rupturing than the two modern strains (Figure 5.7a). Cortical bone from the selected strain is consistently more porous than that from control birds (Chapter 3, Williams *et al* 2000), and in older birds, both the selected and Cobb strains produce less well mineralised tibiotarsi (Chapter 3 and the present experiment), hence this would appear to be a logical result. However, the data points of the Cobb birds do not appear to fit well with the model used, and the results for this strain should be treated with considerable caution. The data points suggest that Cobb tibiotarsi are able to withstand larger loads than the other strains at smaller weights, but that they lose this ability at greater weights and become weaker than both other strains. This is reflected in the age-strain results (Figure 5.3a), since at 35 days Cobb are heavier than MCX, yet the two strains withstood similar loads before rupture. There are also indications that within strains there are subgroups with two distinct ranges of bone stiffness values, however, there were not enough data points to substantiate this.

5.5.4: Bone Mineralisation

The most startling result of this experiment was a complete lack of any abnormally high tibiotarsus molar Ca:P ratios at 14 days in the selected strain (Figure 5.4b). All three strains demonstrated a range of ratios (1.6-1.75) close to the theoretical ideal of 1.67 at both ages, and there were no age or strain effects observed. This is in contradiction with Chapter 3 (selected strain mean Ca:P: 11 days 2.01, 18 days 2.15), and Chapter 4 (14 days

2.5-3.2 depending on diet treatment). As mentioned previously, the selected strain appeared to be under-performing in this experiment. Since the high ratios observed previously do not appear to be produced by any nutritional problem (Chapter 4, Williams *et al* in press), it is possible that they were due to some imbalance, or adaptation, in the mineralisation process, resulting from the much faster growth rate in the selected strain. The slower growth rate observed in the present study could therefore be one possible explanation for the lack of the high Ca:P ratios. However, this cannot be confirmed at present, and there may be other factors involved.

Total mineral content (% ash) in the tibiotarsus was similar at both ages in the selected and Cobb strains, however, it was considerably higher in the control strain at day 35 (Figure 5.4a). The patterns of mineralisation shown by the control and selected strains, and the difference in bone mineralisation between them is consistent with Chapter 3. However, in Chapter 3, the difference in ash content between the selected and control strains was at least partly accounted for by a similar difference in Ca and P. There was no difference observed between strains in the contents of individual minerals in the tibiotarsus in this study, and it is not certain what mineral was increased in the control strain at 35 days to give the higher total bone mineral content. The skeleton is an important reservoir for a number of minerals in addition to Ca and P, and contains 80% of the body's store of citrate and carbonate (Martin *et al* 1987). It is possible that control tibiotarsi contained more carbonate or citrate ions than those of the other strains. Citric acid metabolism has also been linked to 1,25-dihydroxyvitamin D_3, which increases citrate in serum and bone, and PTH, which decreases bone citrate to increase serum levels (Vaes and Nichols 1961, Svanberg *et al* 1993). Both 1,25-dihydroxyvitamin D_3 and PTH are closely involved in mineral homeostasis and bone turnover (Taylor and Dacke 1984). In addition, although bone mineral crystals are based on the structure of hydroxyapatite, they are not ideal in structure or composition (McLean and Urist 1968). It is this lack of perfection, and the incorporation of foreign, and sometimes unstable, ions into the crystal lattice that gives bone mineral its specific and complex biological properties. The rate of crystal dissolution, the binding capacity, and potential for cellular interactions are altered to make bone crystal more metabolically active (Landis 1996). It is possible that the control birds had achieved a level of mineralisation that allowed further alterations of the bone mineral crystal for this purpose, without compromising mechanical competence. Bones from the selected and Cobb strains were less well mineralised, and perhaps were not capable of increasing the tibiotarsus involvement in mineral homeostasis in such a manner without weakening the bone further.

There was an age effect on individual minerals in the tibiotarsus: % Ca, and % P increased with age in all 3 strains, consistent with Chapter 3, whilst % Mg was reduced with age (Table 5.1). Magnesium ions strongly disturb the conversion of non-apatite mineral deposits to mature hydroxyapatite crystals (LeGeros 1981). It has been reported in both bone fracture calluses and predentine in teeth that Mg and CO_3 ions exist as major impurities in crystallites; the centre of crystals is especially rich in both ions (Quint *et al* 1980). Carbonate ions have been suggested to act as a trigger for biological crystal growth (Kakei and Nakahara 1996), by combining with (and therefore removing) the inhibitory Mg ions to form $MgCO_3$. In rat calvaria, this compound is said to decorate a calcium phosphate core, on which the hydroxyapatite crystal then grows (Kakei *et al* 1997). Should such a process be occurring in the present case, as the crystals grow in size, the effect of the Mg-rich core on the proportion of minerals present in the bone would become less. This could explain the decline in magnesium content as the Ca and P content increased with bone maturity.

The mineralisation of the non-load-bearing humerus demonstrated some differences to that of the tibiotarsus. As in the tibiotarsus, there was no difference in the molar Ca:P ratio between the strains, and at 35 days, all values of the molar Ca:P ratios were within the expected range (Figure 5.5b). However, the ratios averaged over all three strains were significantly lower at 14 days than at 35 days. This could be explained by the presence of a greater proportion of low-Ca:P-ratio hydroxyapatite precursors in the bone mineral (McLean and Urist 1968), and may be an indication that as a non-load bearing bone, the development of bone mineral to mature crystals is under less pressure to occur rapidly.

There were no strain or age effects observed on the total mineral content (% ash) of the humerus, suggesting again that this non-load bearing bone is developing under a different set of constraints to the tibiotarsus, at least in the control strain (Figure 5.5a). In addition, the bone content of Ca, P (Table 5.2) and Mg (Figure 5.5c) were all lower at 35 days than at 14 days in the humerus, the Ca and P contrary to expectations. The reason for this is currently uncertain, however since total mineral content was similar in the two age groups, some other minerals must have replaced the Ca and P. As discussed previously, it is the incorporation of foreign ions into the bone crystallites which makes bone mineral metabolically active, however, too great a disruption in the crystal structure may weaken the lattice, and potentially the bone. Thus it is possible that this type of alteration occurs more in non load-bearing bones, allowing these to fulfil a greater role in mineral homeostasis without compromising the mechanical competence of load bearing bones. This hypothesis is supported by studies on dogs of various breeds and ages. These showed that the bone formation rate (measured by tetracycline labelling) and activation of bone

remodelling units were consistently higher in non-load bearing bones such as vertebrae, the mandible, and ribs, when compared with the shafts of load-bearing long bones (Marrotti 1976b, Nordin *et al* 1977). It is also possible that the humerus is storing more carbonate or citrate ions than the tibiotarsus, perhaps for similar reasons. A comparison of bone mineral compositions in different vertebrates showed an inverse relationship between citrate content, and Ca, Mg, and P content (Biltz and Pellegrino 1969); perhaps a similar relationship occurs when comparing different bones. The role of the humerus as a more metabolically active bone is likely to be even more important in the selected and Cobb strains if their tibiotarsi are not well enough mineralised to fulfil this role as normal.

5.5.5: Conclusions

The abnormally high Ca:P ratio previously observed in the selected strain during the starter period was not seen in this experiment. It was suggested that its occurrence in previous experiments might have been an adaptation / limitation in bone mineralisation rate as a result of the increased rate of bone production and mineralisation observed in the selected strain. This strain appeared to be under-performing in the present study; the reduction in body growth rate is likely to be mirrored by a reduction in bone growth rate, which might account for the absence of the high Ca:P ratios here, however, there may be other factors involved. There appears to be no real reason to doubt the mineral results of any of the experiments, and it is assumed that those factors affecting the Ca:P ratios are biological.

The experiment did demonstrate differences in the bone mineralisation process at load-bearing and non-load-bearing sites in the skeleton. The rate of mineral maturation to hydroxyapatite appeared to be considerably slower in the humerus than in the tibiotarsus. In the tibiotarsus Ca and P content increased with age, while in the humerus, it decreased, despite total mineral content remaining constant. It is not known what these minerals are being replaced by, however, it is possible that the changes are associated with a greater role for the humerus in mineral homeostasis than that of the tibiotarsus.

The results also confirmed that the selected strain had a lower total bone mineral content than control birds, and demonstrated that Cobb birds had a similarly lowered level of mineralisation. However, although mineralisation was similar in Cobb and selected birds, and although bones were generally narrower in the selected than the Cobb strain, there was some suggestion at 35 days that selected birds had stronger and stiffer bones than the Cobb birds at a given weight.

CHAPTER 6

INVESTIGATING THE EFFECT OF DIETARY MINERAL CONTENT ON BODY GROWTH, BONE MINERALISATION AND BREAKING STRENGTH DURING STARTER AND FINISHER PERIODS

6.1: INTRODUCTION

In a previous experiment (Chapter 3, Williams *et al* 2000), it was shown that the fast growing selected strain of broiler (MCX) had significantly lower bone ash content than a slower growing control strain (MK). This was true throughout the birds' life span, up to the final sample at 39 days of age, close to the market age of 42 days. Manipulation of the dietary mineral content in a subsequent experiment (Chapter 4, Williams *et al* submitted) was used to investigate this, and other problems observed in the starter period. However, no effect of dietary manipulation on total bone mineral content was found at this stage of development.

Skeletal problems are recognised as one of the four major factors affecting the performance of broilers (Day 1990), and undermineralised bones at market age could also pose a threat to producer's profits, through fractures during capture, transport, and the slaughter process. For example, Gregory and Wilkins (1990) found that 3% of broilers suffered broken bones during transport, some 18 million birds each year, and some European poultry meat producers are becoming concerned about excessive bone fragility in broilers leading to bone fractures during carcass processing (Thorp and Waddington 1997). This experiment investigates the effect of manipulating dietary mineral content during both starter and finisher periods of growth, on bone quality (mineral content, strength and stiffness) at 16 days, and at an approximate market age of 45 days.

Whilst the ability of broilers to reach market weight at an earlier age is advantageous for the producer, fast growth has been associated with a number of health problems, including ascites, angular bone deformities, tibial dyschondroplasia, and increased mortality. These problems are associated with fast growth during the critical starter phase of development, and restricting growth during this period can reduce their incidence and severity (Julian and Mirsalimi 1992, Classen and Riddell 1989, Lilburn *et al* 1989, Robinson *et al* 1992). Growth restriction can be achieved either by direct quantitative or qualitative feed restriction (e.g. Hester 1994, Walker 1996), or by

employing a variety of lighting schemes to reduce the length of *ad lib* feeding time (e.g. Gordon and Tucker 1998, Hester 1994, Lott *et al* 1996). Depending on the exact regime, in many cases a period of compensatory growth occurs once the birds are switched onto longer hours of light and/or an *ad lib* finisher diet, hence there is no potential loss of profit to the producer (e.g. Hester 1994, Walker 1996). This experiment will investigate whether, in addition to controlling the health problems mentioned, such short-term restricted feeding has any effect on bone mineralisation and mechanical strength.

6.2: AIMS

The present experiment was designed to extend the observations on mineral requirements made in Chapter 4 to approximately market age (45 days). It was also intended to assess what, if any, effect feed restriction had on bone mineralisation and mechanical properties, both during the starter period (16 days) and at 45 days, and to investigate the consequences of feed restriction in the starter period on mineral requirements in the finisher period.

6.3: MATERIALS AND METHODS

6.3.1: Birds, Housing and Diet Treatments

Nine hundred and sixty male selected strain (MCX) chicks were assigned to 24 pens in 2 blocks (40 birds per pen, 12 pens per block). Twenty-three hours of light were given each day, and standard heating, flooring, and water supply were employed. Due to delays in the manufacture of the experimental diets, all pens of birds were fed standard broiler starter diet (1.1% Ca, 0.6% available P) for 3 days, before being switched to one of 12 diet treatments (2 pens per overall treatment). The overall diet treatments consisted of a combination of one of 4 starter treatments with each of 3 finisher treatments (Table 6.1)

6.3.2: Sampling Procedure

Mean pen weights were taken at 16 and 45 days, and feed intake was measured over 2-6 weeks. Eighteen birds per starter treatment (3 per pen) were blood-sampled and culled at 16 days, and both tibiotarsi were dissected out. At 45 days the same was to be done to 12 birds per overall treatment (6 per pen), however, due to time restrictions the number of birds taken was reduced partway through the cull to 9-11 per treatment.

6.3.3: Sample Processing

(Chapter 2.) Ionised and total Ca, and inorganic P were measured from the blood samples. The right tibiotarsi were subjected to 3 point bending to measure breaking strength and stiffness, and were subsequently used for whole bone ashing to measure ash, Ca and P content. The growth plates of the left tibiotarsi were assessed for pathology; 16 day samples were fixed in buffered neutral formalin, and then frozen for cryosectioning, while the 45 day samples were processed through methylmethacrylate. Von Kossa, haemotoxylin and eosin stains were used on the resultant sections. The proximal third of each shaft was taken for cortical bone ashing.

Table 6.1: Experimental design and dietary mineral content

starter diet	mineral content	no. pens per starter	finisher diet	mineral content (all *ad lib*)	no. pens per overall treatment
SH	1.2% Ca 0.5% available P (*ad lib*)	6	FH	1.1% Ca 0.45% available P	2
			FM	0.9% Ca 0.35% available P	2
			FL	0.7% Ca 0.28% available P	2
SM	1.0% Ca 0.4% available P (*ad lib*)	6	FH	1.1% Ca 0.45% available P	2
			FM	0.9% Ca 0.35% available P	2
			FL	0.7% Ca 0.28% available P	2
SMR	1.0% Ca 0.4% available P (17 g/bird/day)	6	FH	1.1% Ca 0.45% available P	2
			FM	0.9% Ca 0.35% available P	2
			FL	0.7% Ca 0.28% available P	2
SL	0.9% Ca 0.3%available P (*ad lib*)	6	FH	1.1% Ca 0.45% available P	2
			FM	0.9% Ca 0.35% available P	2
			FL	0.7% Ca 0.28% available P	2

6.3.4: Derived Measurements

The average bird growth rate from 2-6 weeks was calculated from pen weight data in the same manner as previously described; bone molar Ca:P ratio was also calculated in the same manner as previously (Chapter 3).

Food conversion ratio over the 2-6 week period was calculated by:

total weight of food eaten by pen / total weight gain of pen

Food Conversion Efficiency was calculated by:

total weight gain of pen / total weight of food eaten

6.3.5: Statistical Analysis

16-day data were analysed by ANOVA for starter treatment effect. T-tests were then performed on the means produced by this model to assess whether any differences identified by ANOVA were due to linear mineral content effects (SH versus SL), curved mineral content effects (average of SH and SL versus SM), or feed restriction (SM versus SMR). The log of body weight was added in to the model as a covariate, and logged bone strength and dimension data were re-analysed to investigate whether any apparent starter effects on these measures were actually due to differences in body weight. In the case of cortical bone molar Ca:P ratios, t-tests were also performed on means produced by ANOVA to assess whether ratios deviated from the expected 1.67 to 1. Finally, simple linear regression analysis was used to determine any relationship between Ca:P ratio and bone strength measurements.

Data from day 45 were unbalanced and were therefore subjected to REML analysis for starter and finisher treatment effects, and their interactions. T-tests were performed to assess linear and curved mineral content effects for both starter and finisher treatments, and any effect of feed restriction during the starter period on measures at 45 days. Investigation of the molar Ca:P ratio in cortical bone by t-test and regression analysis was also performed.

6.4: RESULTS

6.4.1: Dietary Effects at 16 Days

Body Growth

The final weight attained at 16 days was affected by starter treatment (Figure 6.1). This was due to the restricted fed group reaching a considerably lower final weight than those fed diet SM *ad lib* (t 9.87, df 19, $P<0.001$); there was no detectable dietary mineral content effect.

Bone Dimensions

Tibiotarsus dimensions were also affected by starter treatment (Figure 6.2). Birds from the restricted fed group had shorter and narrower tibiotarsi than those fed diet SM *ad lib* (t

7.60 and t 8.09 respectively, df 19, P<0.001). There was no dietary mineral content effect. When covariate analysis was performed to allow for body weight, the starter effect on both length and width was no longer present. In both cases, differences in body weight were found to be largely responsible for the differences in dimensions between starter treatments (log weight effect on log length: $F_{1,18}$ 37.62, P<0.001; on log width: $F_{1,18}$ 8.69, P<0.01; Figure 6.3)

Figure 6.1: Mean weight at 16 days, along with standard errors

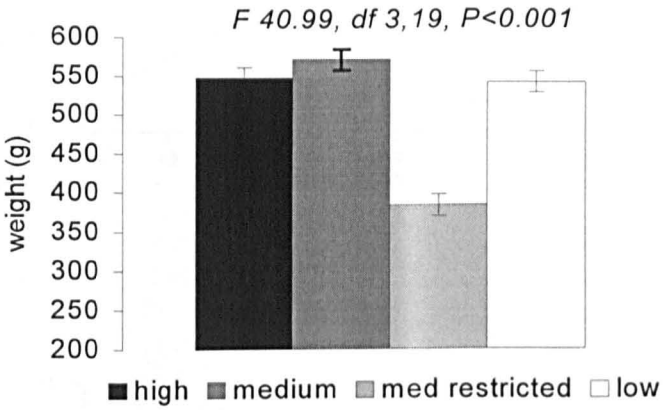


Figure 6.2: Mean tibiotarsus dimensions at 16 days, along with standard errors, a. length, b. width

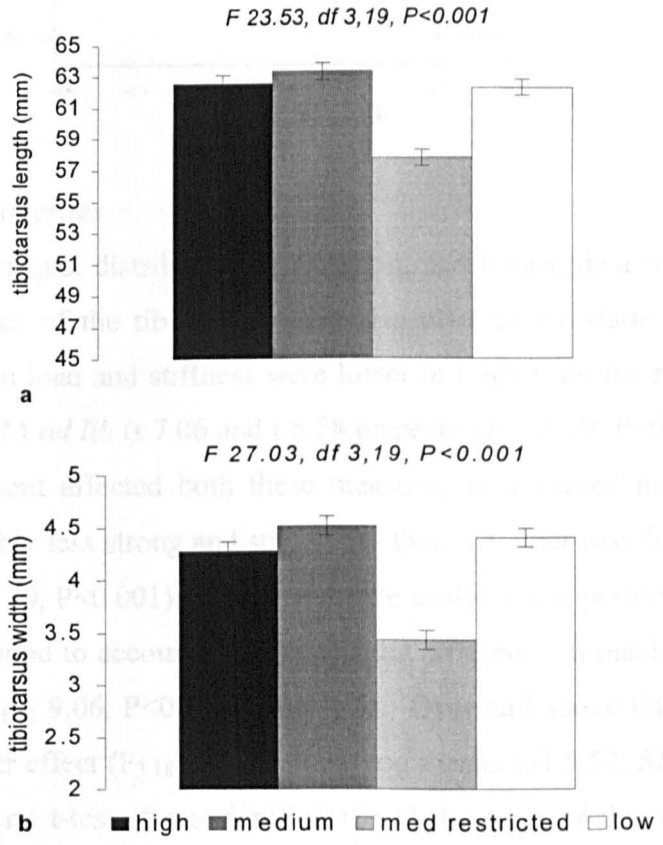
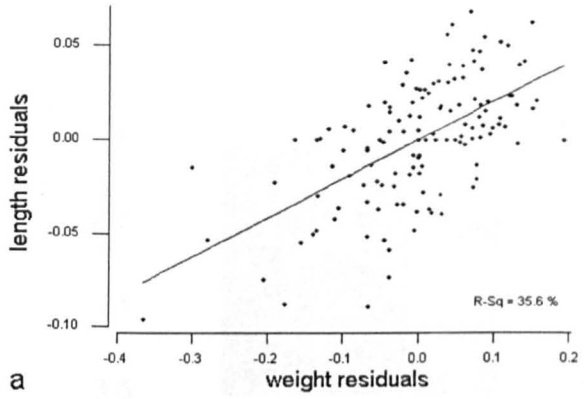
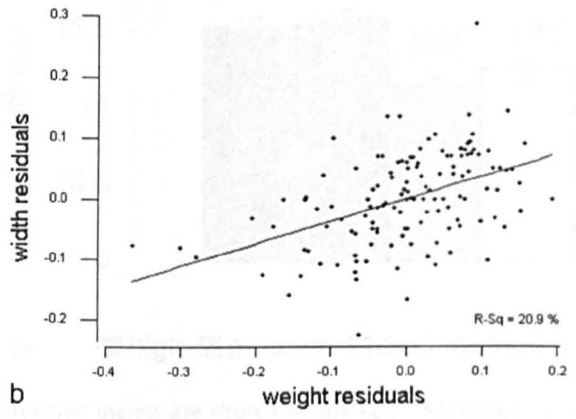


Figure 6.3: Linear effect of log weight on a. log tibiotarsus length, b. log tibiotarsus width. Plotted values are the residuals obtained by fitting starter diets to length, width and weight data separately using ANOVA



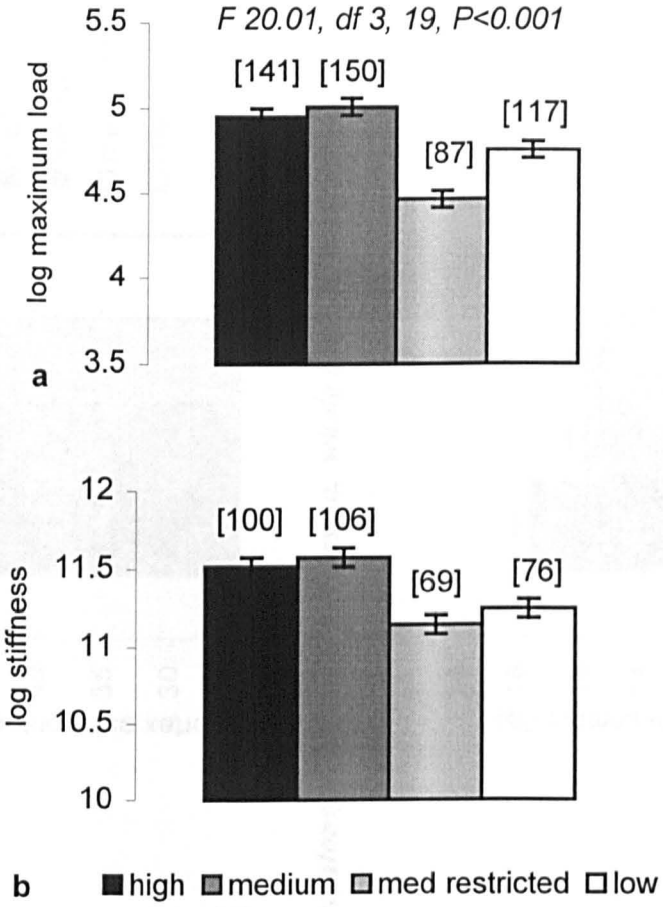


Bone Mechanical Properties

Raw data gave an unequal distribution of variation, and logged data were therefore used. Mechanical properties of the tibiotarsus were also affected by starter treatment (Figure 6.4). Both maximum load and stiffness were lower in birds from the restricted fed group than those fed diet SM *ad lib* (t 7.06 and t 5.28 respectively, df 19, $P<0.001$). In addition, dietary mineral content affected both these measures in a curved manner, with diet L producing considerably less strong and stiff bones than the other two diets (t –2.43 and t –2.78 respectively, df 19, $P<0.001$). When covariate analysis was performed, differences in body weight were found to account for much of the difference in maximum load between starter treatments ($F_{1,18}$ 9.06, $P<0.01$; Figure 6.5). Over and above this, there was also a weaker dietary starter effect ($F_{3,18}$ 3.99, $P<0.05$; log means SH 5.52, SM 5.55, SMR 5.48, SL 5.44), however, no t-tests were significant, and the form of this effect is uncertain.

Body weight was not found to have a detectable effect on bone stiffness, and the starter effects identified previously are likely to be mainly due to diet treatment.

Figure 6.4: Means of mechanical properties of the tibiotarsus at 16 days, along with standard errors, a. maximum load before rupture, b. stiffness



(Back-transformed means are shown in brackets. Maximum load N, Stiffness 1000 N/m)

Figure 6.5: Linear effect of log weight on log tibiotarsus breaking strength. Plotted values are the residuals obtained by fitting starter diets to maximum load and weight data separately using ANOVA

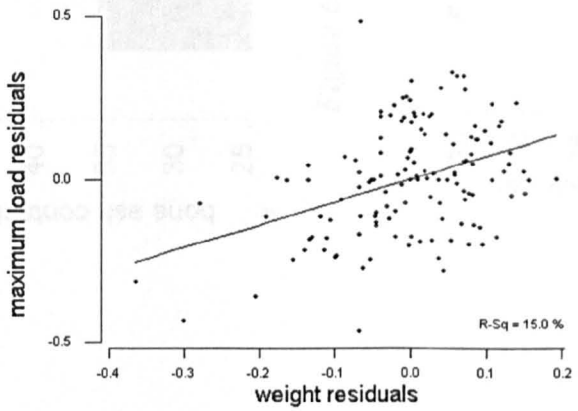


Figure 6.6: Mean total bone mineral content, along with standard errors, a. whole bone, b. cortical bone

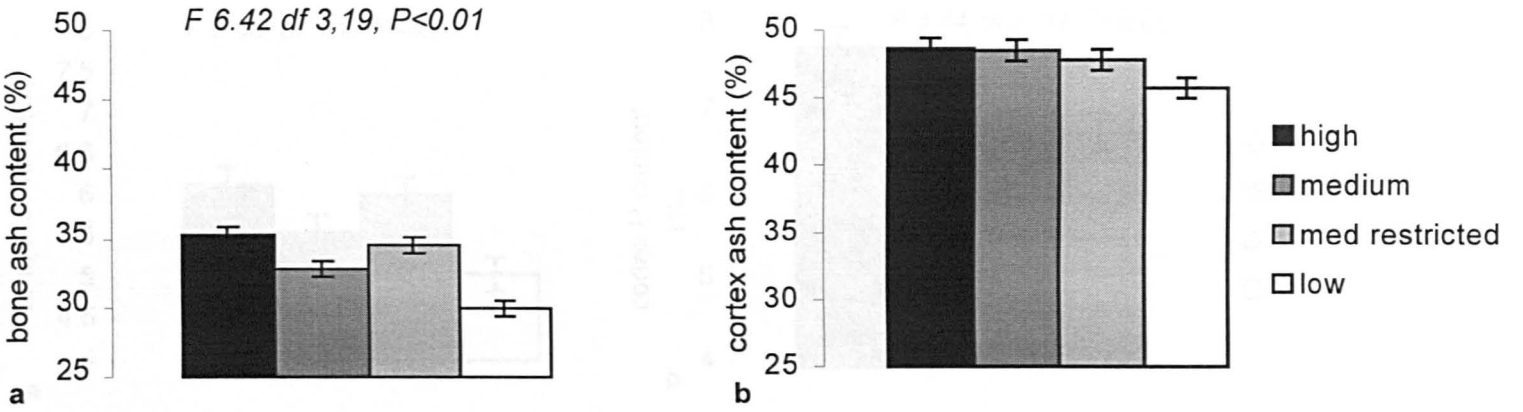


Figure 6.7: Mean calcium content, along with standard errors, a. whole bone, b. cortical bone

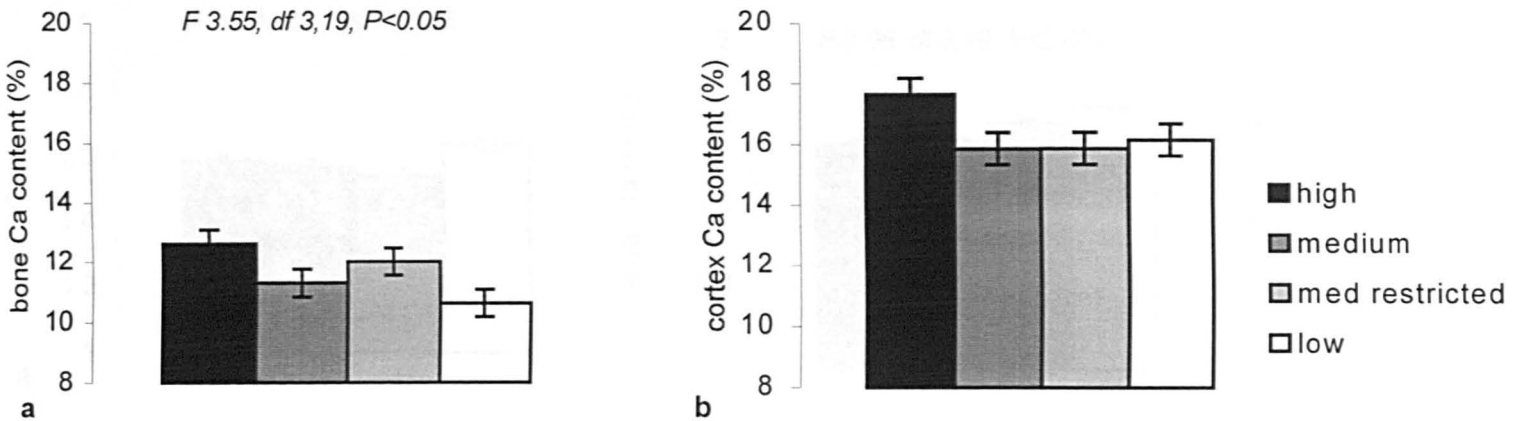


Figure 6.8: Mean phosphorus content, along with standard errors, a. whole bone, b. cortical bone

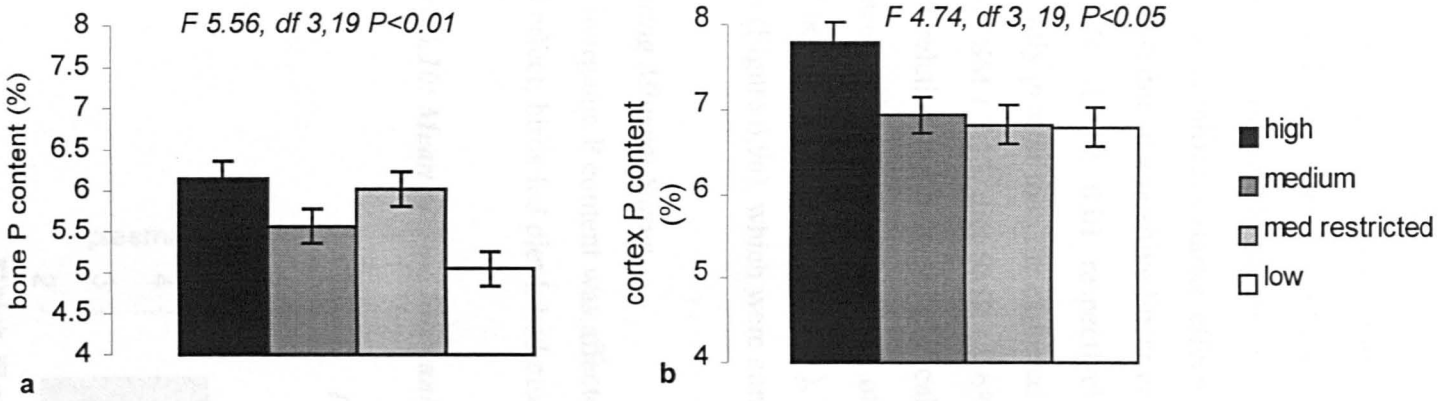
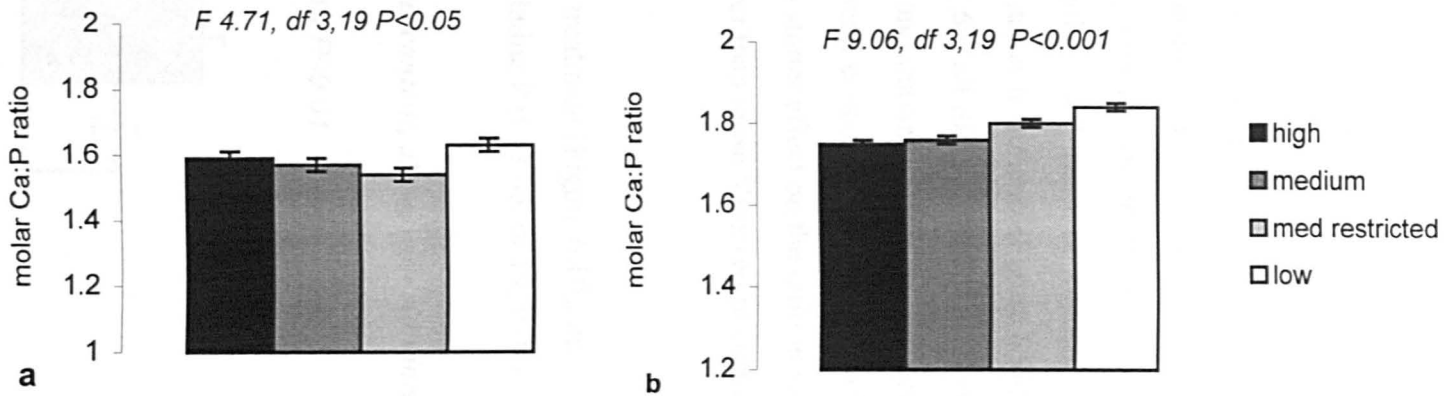


Figure 6.9: Mean molar Ca:P ratio, along with standard errors, a. whole bone, b. cortical bone



Bone Mineral Content

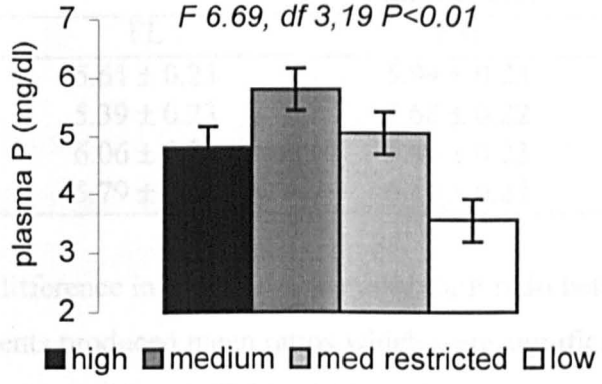
Whole bone mineral content (ash) was affected by starter treatment (Figure 6.6a). This was due to a positive linear dietary mineral content effect (t 4.06, df 19, P< 0.001). ANOVA analysis did not detect an overall starter treatment effect on cortical bone mineral content, however, t-tests showed that there was also a positive linear effect of dietary mineral content in operation at this level, although it was weaker (t 2.68, df 19, P<0.05; Figure 6.6b).

There was a significant starter effect on the Ca content of whole bone (Figure 6.7a), due to a positive linear effect of dietary mineral content (t 3.08, df 19, P<0.01). However, there were no detectable starter effects on the Ca content of cortical bone (Figure 6.7b). There were significant starter effects on the P content of both whole and cortical bone (Figure 6.8) due to a positive linear effect of dietary mineral content (t 3.68, df 19, P<0.01, and t 3.24, df 19, 0.01 respectively). The molar Ca:P ratio of cortical bone was significantly greater than the expected 1.67 for all starter treatments (Figure 6.9b; diet SH t 5.78, diet SM t 7.13, diet SMR t 9.68, diet L t 12.65; all df 19, P<0.001). There was no apparent relationship between cortical Ca:P ratio and maximum load or stiffness. There was a strong, linear negative effect of dietary mineral content on the molar Ca:P ratio of cortical bone (t 4.86, df 19, P<0.001); there was no starter effect on the ratio in whole bone samples (Figure 6.9a), which were consideraly lower than those observed in cortical bone.

Circulating Mineral Status

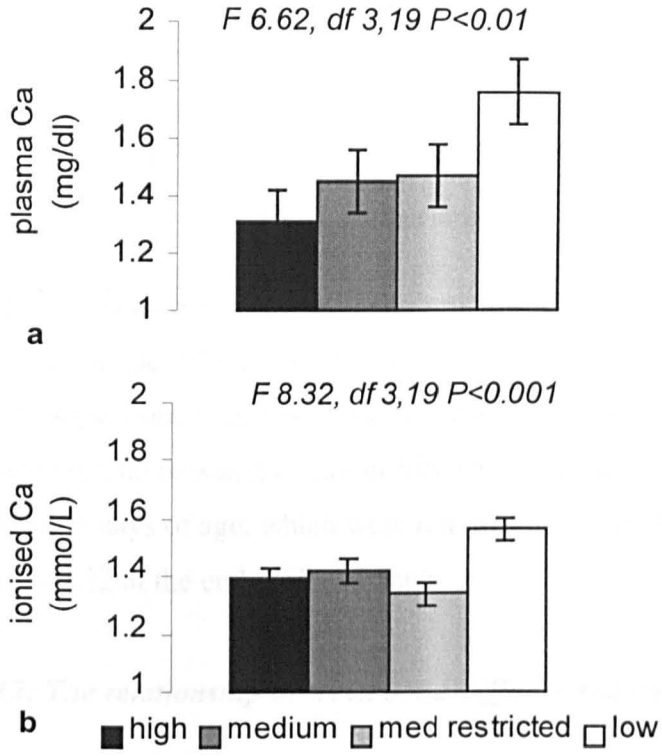
Plasma inorganic P content was affected by starter treatment (Figure 6.10), due to a curved mineral effect; birds fed diet L had much less circulating P (t –3.66, df 19, P<0.01).

Figure 6.10: Mean plasma inorganic phosphorus content, along with standard errors



Both total and ionised circulating Ca were affected by starter treatment (Figure 6.11), this time in a linear manner; higher dietary mineral content resulted in lower circulating Ca (total Ca: t –4.33, df 19, P<0.001; ionised Ca: t –3.64, df 19, P<0.01).

Figure 6.11: Mean circulating calcium content along with standard errors, a. total Ca, b. ionised Ca



6.4.2: Dietary Effects at 45 days

There was only one significant dietary effect at 45 days, a starter – finisher interaction acting on whole-bone P content (W 17.5, df 6, P<0.01; Table 6.2). There appeared to be no consistent pattern, with either starter or finisher diets, and this result probably occurred by chance.

Table 6.2: Predicted mean P content (%) of whole bone, along with standard errors

	finisher diet		
starter diet	FL	FM	FH
SH	5.61 ± 0.23	5.94 ± 0.23	5.64 ± 0.22
SM	5.39 ± 0.23	5.68 ± 0.22	6.03 ± 0.23
SMR	6.06 ± 0.23	5.84 ± 0.23	4.95 ± 0.23
SL	5.79 ± 0.23	6.19 ± 0.23	5.53 ± 0.22

Although there was no difference in cortical bone molar Ca:P ratio between diet treatments at 45 days, some treatments produced mean ratios which were significantly higher than the predicted 1.67, while others did not (Table 6.3). All treatments involving restricted feeding during the starter phase gave ratios close to the expected.

Table 6.3: Predicted mean molar Ca:P ratios for cortical bone, along with standard errors

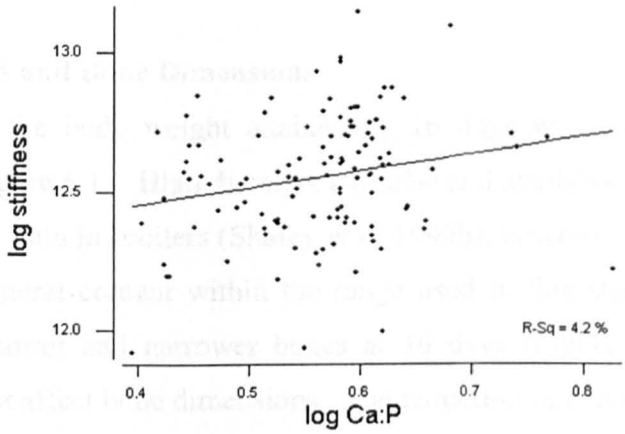
	finisher		
starter	FH	FM	FH
SH	1.83 ± 0.05 $^{**}_{2.85}$	1.73 ± 0.05	1.77 ± 0.05
SM	1.71 ± 0.05	1.77 ± 0.05 $^{*}_{2.02}$	1.78 ± 0.05 $^{*}_{2.06}$
SMR	1.72 ± 0.05	1.71 ± 0.05	1.77 ± 0.05
SML	1.79 ± 0.05 $^{*}_{2.37}$	1.82 ± 0.05 $^{**}_{2.85}$	1.71 ± 0.05

(test of significant difference from 1.67: * P<0.05, ** P<0.01, t values shown in subscript, infinite number of degrees of freedom assumed)

A weak relationship between bone stiffness and cortical Ca:P ratio was found in the 45 day data (logged data used; slope 0.605, se 0.268, t_{117} 2.26, P<0.05; Figure 6.12). This suggests that, on average, bones become more brittle as the molar Ca:P ratio of cortical bone increases, however, there was no relationship with maximum load.

Other data at 45 days of age, which were not affected by diet treatment, can be found in tables 6.6 – 6.22 at the end of this chapter.

Figure 6.12: The relationship between bone stiffness and cortical Ca:P ratio



6.4.3: Growth Plate Pathology

There was only one occurrence of hypocalcaemic rickets (at 16 days). The incidence of TD was also low, 11% at 16 days, and 5 % at 45 days, mainly consisting of very small sub-clinical lesions. The distribution of pathologies between diet treatments can be seen in Tables 6.4 and 6.5.

Table 6.4: Distribution of growth plate pathologies at 16 days

starter diet	normal	hypocalcaemic rickets	tibial dyschondroplasia
SH	14	1	2
SM	13	0	5
SMR	17	0	1
SL	18	0	0

Table 6.5: Distribution of growth plate pathologies at 45 days

starter diet	finisher diet	normal	tibial dyschondroplasia
SH	FH	12	0
	FM	12	0
	FL	10	2
SM	FH	11	1
	FM	12	0
	FL	12	0
SMR	FH	11	1
	FM	11	1
	FL	11	1
SL	FH	12	0
	FM	11	1
	FL	12	0

6.5: DISCUSSION

6.5.1: Growth and Bone Dimensions

As expected, the body weight attained by 16 days was considerably reduced by feed restriction (Figure 6.1). High dietary Ca (2.2%) and available P (1.3%) have been found to reduce weight gain in broilers (Shafey *et al* 1990b), however, body weight was unaffected by dietary mineral content within the range used in this study. Restricted feeding also resulted in shorter and narrower bones at 16 days (Figure 6.2), while dietary mineral content did not affect bone dimensions. The reduction in bone size was found to be largely related to the lower body weights attained on the SMR treatment (Figure 6.3). Since all the birds used in this study were of the same genotype, and were raised in the same pen environment, this result was also expected due to normal body scaling (Swartz and Biewener 1992).

By 45 days, those birds that were feed restricted during the starter period had achieved the expected compensatory growth (Auckland and Morris 1971, Plavnik and Hurwitz 1989), and there was no significant difference in the final weight or bone dimensions between any of the diet treatments. Some workers have found that early feed restriction results in an improvement in feed conversion when *ad lib* feeding is restored, (e.g. Plavnik and Hurwitz 1991), however this was not found to be the case here (Table

6.7). In addition, no treatment effects were found for average pen growth rate over the 16-45 day period (Table 6.9). This is surprising given the difference in weight gained over this period between individual restricted and unrestricted birds It is possible that either the less precise method of calculating growth rate was not sufficient to demonstrate the expected difference, or that data were not collected from enough birds to detect the difference.

6.5.2: Systemic Calcium and Phosphorus Status

Circulating levels of ionised Ca, total Ca and inorganic P at 16 days were not affected by feed restriction, but were affected by dietary mineral content. Both total and ionised Ca reduced linearly with increased dietary mineral content (Figure 6.11), whilst plasma inorganic P content showed a curved positive response, with the lower concentrations resulting from the low mineral starter treatment. The response of ionised Ca is likely to be due to the increased levels of available P in the high mineral diets; ionised Ca has previously been shown to be reduced by high dietary P content (Chapter 4, Shafey *et al* 1990b, Williams *et al* in press). Although in Chapter 4 there was no observed dietary mineral content effect on total plasma Ca, other workers have found that total plasma Ca is also reduced by high dietary P (Gardiner 1973, Shafey *et al* 1990b). Why the present plasma inorganic P content increase with dietary available P content was curved, rather than linear as in the previous experiment, is uncertain. Dietary Ca and P interact during absorption, metabolism, and excretion (Klassing 1998), and it may it may be that the difference in the Ca:P ratios between SH (Ca:P 2.4) and SM (Ca:P 2.5), and SL (Ca:P 3.0) are responsible for the curved effect seen, rather than mineral content *per se.* The SL diet contained less P, and in addition, the higher proportion of Ca in this diet may have been interferring with the uptake of that P present, thus exacerbating the effect of the low mineral content on plasma P concentration. However, no evidence of a dietary Ca effect, or a Ca-P interactive effect on plasma P concentration, was observed in previous work in which a range of dietary Ca and P contents, and a range of dietary Ca:P ratios was utilised (Chapter 4, Williams *et al* in press), and it is not possible to come to any conclusion at present.

6.5.3: Bone Mineral Content

Although restricted feeding has a beneficial effect on some aspects of leg health, for example reducing the incidence of tibial dyschondroplasia (e.g. Riddell et al 1983), it had no effect on any aspect of bone mineral content at any stage of development during this experiment. This confirms observations by other workers that growth restriction, whether

by protein restriction, or energy restriction, does not improve bone ash (Turner and Lilburn 1992, Kirn and Firman 1993, Leterrier *et al* 1998). A fast growth rate, therefore, does not appear to directly impair the efficiency of bone mineralisation in broilers.

Total bone mineral content (ash) at 16 days increased linearly with dietary mineral content in whole-bone samples, and to a lesser extent in cortical bone (Figure 6.6). This agrees with the results of Scheideler *et al* (1995), who showed that bone ash was reduced at 3 weeks in broilers fed a low mineral diet. While both Ca and P content increased in whole bone with higher dietary mineral content, there was no detectable starter treatment effect on cortical bone Ca content. The increase in cortical ash, therefore, appears to be mainly reliant on the increase in cortical P content with higher dietary mineral, while the higher sensitivity of whole bone ash to dietary effect was probably largely mediated by the additional sensitivity of Ca uptake (Figures 6.7, 6.8). Furthermore, cortical bone ash from the proximal diaphysis, while not containing the full mineral complement observed in the mid-diaphysis (Williams *et al* 2000, Chapter 3), still reflects mature bone mineral which has had time to accumulate in a relatively large quantity. Whole bone ash is significantly affected by the inclusion of the growth plates, which contain much less mineral than mature bone. Here, differences in dietary mineral content and balance are likely to have a more pronounced affect on the initiation and process of cartilage mineralisation, and new bone production and mineralisation, than they would on the gradual mineral propagation in more mature bone, rendering whole bone mineral content more sensitive to dietary inflence.

The Ca:P ratio of whole bone was considerably lower than that of cortical bone. Since it has been shown previously that there is no difference in the Ca:P ratio between different segments of the diaphysis (Williams *et al* 2000, Chapter 3), this would imply that the growth plates (cartilage) and metaphyses (new bone and cartilage) had much lower Ca:P ratios than cortical bone. This is consistent with previous work, which showed that calcifying cartilage has a lower Ca:P ratio (below 1.6) than that of mature bone (McClean and Urist 1968), and that newly deposited mineral in bone has an even lower ratio of 1.44-1.55 (Vaughan 1981). It is thought that as bone matures, bone mineral changes from an initial amorphous deposit with a low Ca:P, to a hydroxyapatite-like mature form with a higher Ca:P ratio, by dissolution and crystallisation with additional Ca (McClean and Urist 1968). Taking data from the SM-fed birds as an example, cortical bone contained approximately 23% more P per unit of bone weight than whole bone (influenced by the growth plates), however it contained 41% more Ca. Therefore, Ca does appear to be added to bone mineral during the maturation process, irrespective of dietary treatment.

However, cortical bone Ca:P ratios were again observed to be higher than the 1.67 to 1 expected for hydroxyapatite (Pellegrino and Biltz 1968). The reason for this is still unknown, as is the reason for the differences in the size, or incidence of higher Ca:P ratios between experiments. The hypothesis of a limiting dietary P content as a cause was not supported by the results of a previous experiment (Williams *et al* in press, Chapter 4). Since the ratio was not affected by restricted feeding in the present experiment, it also appears unlikely to be a physiological response to a fast growth rate. Medullary bone in hens, the labile skeletal store of minerals for eggshell production, has been shown to have a higher Ca:P ratio than other types of bone (Tyler 1948). While this may simply reflect the small proportion of P that goes into eggshell, the high ratio may alternatively be an adaptation for fast bone turnover. Similarly, the high ratio seen in the cortical bone of the selected broiler strain may be a genetically mediated adaptation for fast bone production and mineralisation, necessary due to the high growth rate during the starter period in this strain.

Whatever its basis, the bone Ca:P ratio has been shown to be affected by dietary mineral content, both in this experiment, and in previous work (Williams *et al* in press, Chapter 4). In the present experiment higher dietary mineral content resulted in increased P content in cortical bone, while cortical Ca content was unaffected by diet. The molar Ca:P ratio of cortical bone was therefore reduced with higher dietary mineral contents, suggesting that the birds were preferentially incorporating P into cortical bone as more Ca and P became available. In this way, the Ca:P ratio was brought closer to the expected 1.67 as mineral content increased. A reduction in the Ca:P ratio with increased dietary mineral content was also seen to a lesser extent in whole bone samples, despite higher dietary mineral contents also resulting in increased whole bone Ca. The retention of Ca in bone has been shown to be a curvilinear function of Ca intake, and the retention of P to be a linear function of P intake (Shafey *et al* 1990b). This would lead to the reduction in the whole bone Ca:P observed as dietary mineral content increased. The curved Ca response implies an uptake dependant on some requirement, while the linear P response implies a supply-led uptake. It is possible that for the purposes of growth plate function, dietary Ca was limiting, and more Ca was therefore taken up if available; since no dietary effect was seen on cortical bone Ca content, dietary Ca was probably not limiting for the more gradual cortical bone mineralisation. In both types of bone sample, P content increased with dietary mineral content, however, the relationship of whole bone Ca content with dietary mineral reduced the dietary influence on whole bone Ca:P ratio, meaning cortical bone Ca:P ratio was more sensitive to dietary effect.

All differences between diet treatments in bone ash, Ca and P content, and Ca:P ratio disappeared by 45 days. This suggests that whatever problems or programmed responses might be affected by diet during the critical fast-growth starter period, the slower growth rate in the finisher period allows the birds to correct bone mineral content to the strain 'norm'.

6.5.4: Mechanical Properties

Resistance to loading and stiffness of tibiotarsi at 16 days were affected both by restricted feeding (negatively), and mineral content (positively), and diets SL and SMR produced considerably weaker and more elastic bones than the other two starter diets (Figure 6.4). Maximum load before rupture was strongly affected by body weight in addition to starter diet, and the reduction in strength on the SMR treatment was probably due to the much narrower bones produced by these lighter birds. This is a normal effect of body scaling (Swartz and Biewener 1992). However, ANOVA also identified a remaining, though weaker, diet effect on maximum load after weight had been accounted for. T-tests were unable to identify the exact nature of the dietary influence; however, from the pattern of log means obtained from the covariate analysis, it is likely to be the curved positive response to dietary mineral content originally identified. It is possible that there is an optimum dietary mineral content for bone strength versus the cost of mineral supplementation, however, as the effect is so weak it may be difficult to pinpoint such an optimum. Alternatively, the effect may be due to the difference in dietary Ca:P ratio between SH and SM, and the higher ratio of SL. However, no evidence of an effect of dietary Ca or available P content on bone strength was observed in previous work (Chapter 4, Williams *et al* in press), and no firm conclusion can be drawn.

Bone stiffness at 16 days was unrelated to body weight within diet treatments, and the results therefore suggest that it was determined by dietary mineral content and growth rate, not bone size. This lack of a weight effect is perhaps surprising. However, mean area moment of inertia, an estimate of resistance to bending used in a previous study (Chapter 3, Williams *et al* 2000), depends on cortical bone thickness in addition to total bone width (Biewener 1992). It is possible that stiffness is more dependant on the amount and form of mineral present in the bone than on its size; this was supported by the fact that the low mineral starter diet gave more elastic bones. Alternatively, it is possible that there was not enough variation in body weight for any relationship to be detected, however, since weight effects were highly evident on maximum load and bone dimension data, any such undetected relationship is likely to be very weak. Why feed restriction also gave more elastic bones is unknown at present. It is possible that these lighter birds had thinner

cortical bone due to a reduced requirement for stiffness, however, we have no record of this measurement for the present experiment. Again, these differences in maximum load and stiffness between diet treatments disappeared by 45 days.

6.5.5: Conclusions

The results from this study suggest that dietary manipulation may be necessary in the fast-growing starter phase, to improve the health, skeletal quality, and welfare of the birds. At 16 days, low mineral content gave weaker and more elastic bones, probably due to the lower total cortical bone mineral and phosphorus content achieved with lower dietary mineral content. However, within the range of diets used here, manipulation of dietary mineral content did not affect the birds' weight or skeletal quality at market age. The broilers were able to correct any differences in growth performance, circulating Ca and P status, and bone mineralisation and strength due to dietary effects in the starter period, to reach apparently standard values in these measures by market age. In the future, it may be useful to ascertain at what age these standard values are reached. They were also able to achieve this standardisation on a range of finisher diets with varying mineral contents, suggesting that such characteristics are highly robust once the critical starter phase has been passed. Finally, there was no evidence at any stage of development that restricted feeding improved bone mineralisation; any improvement in leg health due to short term feed restriction is likely to be via some other route.

6.6: DATA FROM 45 DAYS NOT GIVEN IN TEXT

6.6.1: Body Growth

Table 6.6: Log of final weight

	finisher					
	1.1%Ca 0.45%avP		0.9%Ca 0.35%avP		0.7%Ca 0.28%avP	
starter	mean	sem	mean	sem	mean	sem
1.2%Ca 0.5%avP	7.98 $_{2925}$	0.04	8.01 $_{2999}$	0.04	8.04 $_{3087}$	0.04
1.0%Ca 0.4%avP	8.02 $_{3053}$	0.04	8.06 $_{3165}$	0.04	8.02 $_{3029}$	0.04
1.0%Ca 0.4%avP (restricted)	7.96 $_{2870}$	0.04	7.96 $_{2870}$	0.04	8.01 $_{3008}$	0.04
0.9%Ca 0.3%avP	8.01 $_{3014}$	0.04	8.02 $_{3047}$	0.04	7.99 $_{2951}$	0.04

(Data logged for analysis, figures in subscript back-transformed means (g))

Table 6.7: Average food conversion efficiency

	finisher					
	1.1%Ca 0.45%avP		0.9%Ca 0.35%avP		0.7%Ca 0.28%avP	
starter	mean	sem	mean	sem	mean	sem
1.2%Ca 0.5%avP	0.491	0.029	0.519	0.029	0.443	0.029
1.0%Ca 0.4%avP	0.582	0.029	0.477	0.029	0.470	0.029
1.0%Ca 0.4%avP (restricted)	0.453	0.029	0.526	0.029	0.536	0.029
0.9%Ca 0.3%avP	0.531	0.029	0.536	0.029	0.534	0.029

Table 6.8: Average food conversion ratio

	finisher					
	1.1%Ca 0.45%avP		0.9%Ca 0.35%avP		0.7%Ca 0.28%avP	
starter	mean	sem	mean	sem	mean	sem
1.2%Ca 0.5%avP	2.05	0.12	1.94	0.12	2.29	0.12
1.0%Ca 0.4%avP	1.72	0.12	2.10	0.12	2.13	0.12
1.0%Ca 0.4%avP (restricted)	2.21	0.12	1.92	0.12	1.87	0.12
0.9%Ca 0.3%avP	1.88	0.12	1.88	0.12	1.88	0.12

Table 6.9: Average growth rate (ln(g)/day)

	finisher					
	1.1%Ca 0.45%avP		**0.9%Ca 0.35%avP**		**0.7%Ca 0.28%avP**	
starter	**mean**	**sem**	**mean**	**sem**	**mean**	**sem**
1.2%Ca 0.5%avP	0.062	0.001	0.061	0.001	0.060	0.001
1.0%Ca 0.4%avP	0.061	0.001	0.061	0.001	0.063	0.001
1.0%Ca 0.4%avP (restricted)	0.061	0.001	0.062	0.001	0.060	0.001
0.9%Ca 0.3%avP	0.076	0.001	0.074	0.001	0.075	0.001

6.6.2: Bone Dimensions

Table 6.10: Tibiotarsus width (mm)

	finisher					
	1.1%Ca 0.45%avP		0.9%Ca 0.35%avP		0.7%Ca 0.28%avP	
starter	mean	sem	mean	sem	mean	sem
1.2%Ca 0.5%avP	8.24	0.27	8.36	0.27	8.05	0.27
1.0%Ca 0.4%avP	7.98	0.27	8.62	0.27	7.95	0.27
1.0%Ca 0.4%avP (restricted)	7.87	0.27	7.99	0.27	8.53	0.27
0.9%Ca 0.3%avP	8.25	0.27	8.10	0.28	8.00	0.27

Table 6.11: Tibiotarsus length (mm)

	finisher					
	1.1%Ca 0.45%avP		0.9%Ca 0.35%avP		0.7%Ca 0.28%avP	
starter	mean	sem	mean	sem	mean	sem
1.2%Ca 0.5%avP	116.1	1.37	115.8	1.30	117.9	1.30
1.0%Ca 0.4%avP	116.6	1.37	117.0	1.30	116.2	1.37
1.0%Ca 0.4%avP (restricted)	114.1	1.37	114.4	1.37	115.3	1.37
0.9%Ca 0.3%avP	114.7	1.37	115.7	1.44	114.7	1.30

6.6.3: Bone Mechanical Properties

Table 6.12: Log of tibiotarsus maximum load

	finisher					
	1.1%Ca 0.45%avP		0.9%Ca 0.35%avP		0.7%Ca 0.28%avP	
starter	mean	sem	mean	sem	mean	sem
1.2%Ca 0.5%avP	5.90 $_{367}$	0.06	5.94 $_{379}$	0.06	5.87 $_{355}$	0.06
1.0%Ca 0.4%avP	5.97 $_{392}$	0.06	5.96 $_{388}$	0.06	5.93 $_{377}$	0.06
1.0%Ca 0.4%avP (restricted)	6.00 $_{404}$	0.06	5.88 $_{359}$	0.06	5.93 $_{377}$	0.06
0.9%Ca 0.3%avP	5.87 $_{353}$	0.06	5.91 $_{367}$	0.06	5.82 $_{337}$	0.06

(Data logged for analysis, figures in subscript back-transformed means (N))

Table 6.13: Log of tibiotarsus stiffness

	finisher					
	1.1%Ca 0.45%avP		0.9%Ca 0.35%avP		0.7%Ca 0.28%avP	
starter	mean	sem	mean	sem	mean	sem
1.2%Ca 0.5%avP	12.64 $_{309}$	0.07	12.61 $_{300}$	0.07	12.39 $_{240}$	0.07
1.0%Ca 0.4%avP	12.63 $_{306}$	0.07	12.54 $_{279}$	0.07	12.63 $_{306}$	0.08
1.0%Ca 0.4%avP (restricted)	12.53 $_{277}$	0.07	12.50 $_{268}$	0.07	12.55 $_{282}$	0.07
0.9%Ca 0.3%avP	12.60 $_{297}$	0.07	12.60 $_{297}$	0.08	12.47 $_{260}$	0.07

(Data logged for analysis, figures in subscript back-transformed means (1000 N/m))

6.6.4: Circulating Mineral Status

Table 6.14: Ionised Ca (mmol/l)

	finisher					
	1.1%Ca 0.45%avP		0.9%Ca 0.35%avP		0.7%Ca 0.28%avP	
starter	mean	sem	mean	sem	mean	sem
1.2%Ca 0.5%avP	1.49	0.04	1.49	0.04	1.51	0.04
1.0%Ca 0.4%avP	1.49	0.04	1.48	0.04	1.49	0.04
1.0%Ca 0.4%avP (restricted)	1.55	0.04	1.44	0.04	1.50	0.04
0.9%Ca 0.3%avP	1.46	0.04	1.50	0.04	1.54	0.04

Table 6.15: Plasma Ca (mg/dl)

	finisher					
	1.1%Ca 0.45%avP		0.9%Ca 0.35%avP		0.7%Ca 0.28%avP	
starter	mean	sem	mean	sem	mean	sem
1.2%Ca 0.5%avP	1.68	0.13	1.67	0.14	1.72	0.13
1.0%Ca 0.4%avP	1.67	0.13	1.74	0.13	1.62	0.13
1.0%Ca 0.4%avP (restricted)	1.54	0.13	1.59	0.13	1.68	0.13
0.9%Ca 0.3%avP	1.51	0.13	1.70	0.13	1.65	0.13

Table 6.16: Plasma P (mg/dl)

	finisher					
	1.1%Ca 0.45%avP		0.9%Ca 0.35%avP		0.7%Ca 0.28%avP	
starter	mean	sem	mean	sem	mean	sem
1.2%Ca 0.5%avP	6.75	0.66	6.68	0.68	6.45	0.66
1.0%Ca 0.4%avP	6.91	0.66	6.41	0.66	7.33	0.66
1.0%Ca 0.4%avP (restricted)	6.37	0.66	6.53	0.67	7.03	0.66
0.9%Ca 0.3%avP	6.47	0.66	6.93	0.66	6.30	0.66

6.6.5: Cortical Bone Mineral Content

Table 6.17: % Ash

	finisher					
	1.1%Ca 0.45%avP		0.9%Ca 0.35%avP		0.7%Ca 0.28%avP	
starter	mean	sem	mean	sem	mean	sem
1.2%Ca 0.5%avP	44.97	2.40	43.91	2.40	41.09	2.41
1.0%Ca 0.4%avP	42.45	2.40	42.64	2.35	43.95	2.40
1.0%Ca 0.4%avP (restricted)	43.71	2.40	41.46	2.40	39.30	2.40
0.9%Ca 0.3%avP	42.51	2.35	42.03	2.40	43.22	2.41

Table 6.18: % Calcium

	finisher					
	1.1%Ca 0.45%avP		0.9%Ca 0.35%avP		0.7%Ca 0.28%avP	
starter	mean	sem	mean	sem	mean	sem
1.2%Ca 0.5%avP	15.68	1.29	14.89	1.29	13.01	1.30
1.0%Ca 0.4%avP	14.70	1.29	15.61	1.26	15.61	1.29
1.0%Ca 0.4%avP (restricted)	16.02	1.29	14.82	1.29	12.55	1.29
0.9%Ca 0.3%avP	15.95	1.26	14.74	1.29	14.83	1.30

6.6.6: Whole Bone Mineral Content

Table 6.19: % Ash

	finisher					
	1.1%Ca 0.45%avP		0.9%Ca 0.35%avP		0.7%Ca 0.28%avP	
starter	mean	sem	mean	sem	mean	sem
1.2%Ca 0.5%avP	36.09	0.66	35.97	0.66	35.26	0.63
1.0%Ca 0.4%avP	35.06	0.66	36.56	0.63	36.44	0.66
1.0%Ca 0.4%avP (restricted)	37.00	0.66	35.67	0.66	35.92	0.66
0.9%Ca 0.3%avP	36.49	0.66	37.24	0.66	35.72	0.63

Table 6.20: % Calcium

	finisher					
	1.1%Ca 0.45%avP		0.9%Ca 0.35%avP		0.7%Ca 0.28%avP	
starter	mean	sem	mean	sem	mean	sem
1.2%Ca 0.5%avP	11.20	0.73	11.10	0.73	10.38	0.70
1.0%Ca 0.4%avP	10.27	0.73	10.23	0.70	11.30	0.73
1.0%Ca 0.4%avP (restricted)	11.17	0.73	10.64	0.73	9.05	0.73
0.9%Ca 0.3%avP	10.58	0.73	11.40	0.73	10.24	0.70

Table 6.21: % Phosphorus

	finisher					
	1.1%Ca 0.45%avP		0.9%Ca 0.35%avP		0.7%Ca 0.28%avP	
starter	mean	sem	mean	sem	mean	sem
1.2%Ca 0.5%avP	5.61	0.23	5.94	0.23	5.64	0.22
1.0%Ca 0.4%avP	5.39	0.23	5.57	0.22	6.03	0.23
1.0%Ca 0.4%avP (restricted)	6.06	0.23	5.84	0.23	4.95	0.23
0.9%Ca 0.3%avP	5.79	0.23	6.19	0.23	5.53	0.22

Table 6.22: Molar Ca:P ratio

	finisher					
	1.1%Ca 0.45%avP		0.9%Ca 0.35%avP		0.7%Ca 0.28%avP	
starter	mean	sem	mean	sem	mean	sem
1.2%Ca 0.5%avP	1.54	0.07	1.44	0.07	1.42	0.07
1.0%Ca 0.4%avP	1.47	0.07	1.43	0.07	1.45	0.07
1.0%Ca 0.4%avP (restricted)	1.43	0.07	1.41	0.07	1.40	0.07
0.9%Ca 0.3%avP	1.41	0.07	1.43	0.07	1.43	0.07

CHAPTER 7

A COMPARISON BETWEEN STRAINS OF EGGSHELL MINERALISATION, AND BONE POROSITY AND MINERAL CONTENT IN EARLY LIFE

7.1: INTRODUCTION

Development of the whole chick, from the initial cell division to hatching, is based entirely on the utilisation of the constituents of the egg (Larbier and LeClercq 1994). The prime role of the egg is as an embryonic chamber, fulfilling the roles of protection and gaseous exchange (Darnell-Middleton 1999), whilst also providing 80% of the chick's Ca needs (Simkiss 1961). All the nutrients necessary for growth are present from the start of embryonic development, and there is no possibility of subsequent supplementation (Larbier and LeClercq 1994).

The minerals required are deposited by the hen into specific portions of the egg at the time of formation. Through the actions of the extra-embryonic membranes, the embryo is then able to acquire the required amount of each mineral at particular points in its development (Richards and Packard 1996). The three main constituents of the egg are the vitellus (yolk), albumen (white), and the eggshell. These are synthesised within different regions of the oviduct; the yolk develops progressively in the ovary, albumen is deposited in the magnum, and eggshell in the uterus (Larbier and LeClercq 1994).

Albumen is essentially water and protein, with small quantities of minerals, water-soluble vitamins, and free glucose. Yolk is an emulsion of protein, lipid and water, containing vitamins and small amounts of glucose and amino acids (Larbier and LeClercq 1994). The majority of the minerals in the egg are present in the yolk. Transport of these to the ovary involves the production of vitellogenin, an oestrogen-induced yolk precursor, in the liver (Richards 1997). This is secreted into the blood during active egg production, where it binds to variety of minerals, which are then actively taken into to the ovary along with the yolk precursor (Shen *et al* 1993). Once in the ovary, the precursor is processed to yield mineral-binding lipovitellin and phosvitin, the latter also itself being an important source of phosphorus (Richards and Packard 1996). The eggshell consists almost entirely of calcium carbonate (90-95%), laid down simultaneously with a protein matrix, which dictates the direction of crystal growth (Larbier and LeClercq 1994). Approximately 80%

or more of the calcium in the hatching chick comes from the eggshell (Simkiss 1961), which contains on average 2.3 g of calcium (Larbier and LeClercq 1994).

Sixty to 75% of the calcium in eggshell is drawn directly from the food, due to an increased production of 1,25 dihydroxyvitamin D_3 by the kidney and the consequent enhancement of calcium uptake in the gut (Nys 1993), along with an increase in digestive tract mobility (Nys and Mongin 1980). However, shell calcification occurs partly, if not mostly, in the dark, and the crop's limited store of food is not enough to supply the calcium requirement during this time (Darnell-Middleton 1999). Transfer of calcium from the blood to the eggshell occurs at a rate of approximately 100-200 mg/h, and if an alternative source of calcium is not available, plasma levels of ionised calcium will be reduced to zero within 7-15 minutes (Etches 1987).

Thirty to forty percent of the calcium in eggshell is therefore rapidly mobilised from medullary bone in the laying hen (Taylor and Dacke 1984). This bone type forms the main component of the mineral reservoir in laying birds (Innoue 1966), and is found only in the marrow cavities of the long bones in reproductively active female birds. The appearance of medullary bone is dependant on the combined influence of oestrogen and testosterone, and coincides with the increase in 1,25-dihydroxyvitamin D_3 (Nys 1993). With its good vascular supply (Bloom *et al* 1958), and loose, woven fibre structure, it is a readily mobilised source of calcium, with re-modelling occurring 10-15 times faster than in cortical bone (Murray and Bloom 1948, Hurwitz 1965). Such is its importance to egg production that in times of calcium stress it is maintained at the expense of cortical bone (Candlish 1971).

Within the eggshell, foetal bone development occurs by the complementary processes of intramembranous and endochondral ossification, which lead to the formation of a complete skeletal system prior to hatching (Gay 1988). Initially, there is no calcium transport from the eggshell, and the first increase in the ash content of the embryo between 1-9 days after setting is due to uptake of a variety of minerals, including some calcium, from the yolk (Johnston and Comar 1955, Tuan and Scott 1977). Accordingly, the calcium content of the yolk tends to fall during the first week of incubation, however, it then begins to rise again (Johnston and Comar 1955). At 10-12 days after setting, utilisation of calcium in the eggshell for skeletal mineralisation begins, and it is thought that there is a flow of calcium from the eggshell through the yolk to the embryo, the yolk acting as a reservoir for excess calcium removed from the eggshell (Tuan and Scott 1977). Increases in embryonic ash and calcium content are parallel after this time (Johnston and Comar 1955), implying that calcium constitutes a major portion of the mineral taken up, and that the destination of such a large amount of calcium is likely to be skeletal. Transport of

calcium from the eggshell reaches a maximum at approximately 19 days after setting (Tuan and Scott 1977), corresponding to the attainment of normal plasma levels of 1,25 dihydroxyvitamin D_3 in the embryo (Kubota *et al* 1981). On day 20, the yolk reservoir is also mobilised, and it has been suggested that it acts as a supplement to the supply of calcium from the eggshell to support the rapid mineralisation of the skeleton occurring on the final day before hatch (Baimbridge and Taylor 1980). At the end of incubation, the more calcium that is present in the embryo, the less there is in the yolk (Romanoff and Faber 1932).

The whole process of egg production and embryonic development is a highly complex one, and a full investigation of this process in relation to final skeletal quality is outside the remit of this project. However, some knowledge of early bone development in the embryo and young chick may be of considerable use. In a previous experiment (Williams *et al* 2000, Chapter 3), cortical bone of a fast-growing selected broiler strain had a consistently lower ash content and was more porous when compared with bone from a slower-growing control strain. The difference in ash was largest at 4 days of age, the youngest age investigated at that time, while the difference in porosity was consistent across age. The current experiment is designed to determine whether these strain differences are present before hatching, or develop later. Specifically, while the movements of minerals, especially calcium, has been well researched over the years, there appears to be little information available on the early development of Haversian canals, which contribute to varying degrees of bone porosity in later life. If differences in ash content and porosity develop after hatching and are not of genetic origin, it may be possible to modify them by changes in chick nutrition. Should the difference be present before hatching, however, this would imply a potential problem at the genetic selection or egg production stage.

The mineral levels in different parts of the egg are variable. The total amount, concentration, and distribution of minerals within the egg depend on the quantity and chemical form of each mineral fed to the laying hen (Stadelman and Pratt 1989). The calcium content of the newly hatched chick has also been found to be influenced by the condition and diet of the bird which laid the egg (Buckner *et al* 1926), which may in turn affect the skeletal health of the chick. The availability of commercial strain eggs from an alternate source allowed a preliminary investigation into whether the diet and husbandry of breeder stock may affect the skeletal health of current commercial broiler chicks in early life.

7.2: AIMS

The main aim of this experiment was to ascertain whether strain differences previously observed in bone ash content and porosity between the selected and control strains develop before or after hatching. An answer to this question might suggest whether the low ash and high porosity seen in the selected strain was a problem of the broiler chicks (genetic or dietary factors), or whether it could be traced back to a problem with the parental stock (genetic, dietary or environmental factors). The early structural development of chicken tibiotarsi, including the development of Haversian canals, was also studied. Finally, 2 groups of eggs from the same commercial strain, produced by breeders on different farms under different husbandry and dietary conditions, were compared to investigate whether they differed in bone ash content and porosity.

7.3: MATERIALS AND METHODS

7.3.1: Eggs, Incubation and Bird Housing

Sixty eggs from the Ross selected (MCY) strain, 45 eggs from the Ross control (MK) strain, and 45 eggs from the Ross 738 commercial strain were provided by a Scottish source. Also supplied were 55 eggs from the commercial strain from an Irish source that rears its broiler breeders under different dietary conditions to the Scottish source (Table 7.1). It is known that eggshell strength (Larbier and LeClercq 1994), incubation time, egg weight, and chick weight at hatch (Suarez *et al* 1997) are all affected by the age of the parent stock, hence the parent flocks were chosen to be of a similar age. The breeder flocks for this experiment were aged 28 weeks (selected and control), 31 weeks (Scottish commercial), and 30 weeks (Irish commercial).

The eggs were stored in a cold room until all the eggs had arrived, and then all 205 eggs were set in a Marcon RS 5000 incubator at 37.5°C, with the four groups of eggs kept separate. At 16 days post-set, the eggs were inspected for fertility by candling (embryos showing dark) and fertile eggs were transferred to a four-tray Marcon SA 700 hatcher at 36.7°C, with the groups of eggs again kept separate. Most chicks hatched 21 days after setting, however, the number of hatchlings were not counted until the following day to allow for stragglers. The chicks were transferred to four pens at 1 day of age (one group of chicks in each), with the standard set-up of woodchip floor covering, feed tray, bell drinker, and heat lamp; the temperature was maintained at 29°C at chick head height. A 23

hours light, 1 hour dark lighting regime was adopted, and all chicks were fed *ad lib* on a standard broiler starter diet containing 1.0% Ca and 5.0% available P.

Table 7.1: Broiler breeder in-lay diets

constituents	Scottish contents (%)	Irish contents (%)
protein	15	14
calcium	2.9	3.1
available phosphorus	0.33	0.35
sodium	0.15	0.16
available metabolisable energy	11.4 MJ/Kg	11.35 MJ/Kg (excluding enzyme effect)
lysine	0.68	0.64
methionine	0.32	0.3
methionine + cystine	0.58	0.58
linoleic acid	1.5	1.04
vitamin E	100 IU	75 g/tonne added
biotin	500 IU	500 mg/tonne added

7.3.2: Sampling Procedure and Processing

After the eggs had been transferred to the hatcher at 16 days post-set, 10 eggs were removed from each tray for sampling. The eggs were broken open and the membranes were removed and discarded from a 1 cm square shell sample that was used for ash, Ca, P, and Mg determination by ash analysis. The embryos were decapitated, sexed by examination of the gonads, and the tibiotarsi were dissected out. The left bone was fixed in buffered neutral formalin for histological preparation and image analysis for total width, cortical width, and cortical bone solidity (measured across the whole cortex). The right bone was used whole for ash analysis.

Samples were taken for ashing from 10 eggshells at random within the four trays after hatching. At 1 and 6 days old, 10 chicks from each group were culled by cervical dislocation, sexed, and the tibiotarsi were dissected out. The left bone was again used for image analysis of cortical bone porosity; in the larger 6-day samples this was measured for endosteal and periosteal areas. The right bone was again used whole for ash analysis.

7.3.3: Statistical Analysis

The data were analysed by ANOVA for unbalanced designs due to the different numbers of males and females. The model age*sex*strain was used, with the alternatively sourced commercial birds being classed as a fourth strain. Eggshell ash data were analysed for strain and sex differences using the 16 day-post-set samples. No sex difference was found in eggshell mineral content at this age, and data from this age was pooled with that from the random eggshell samples taken after hatching to investigate age and strain differences

over the 2 ages. Models were simplified, leaving only those parameters within the main model that were found to significantly affect each variable.

7.4: RESULTS

7.4.1: Hatchability

The number of eggs at each stage of incubation, and the number of infertile or unhatched eggs and dead chicks are shown in Table 7.2. There were very few chick deaths in any group. The eggs of the commercial strain supplied from Ireland had the highest number of infertile and unhatched eggs; however, eggs of the same strain supplied by the Scottish source had the most successful hatch rate. The control strain had a more successful hatch rate than the selected strain.

7.4.2: Eggshell Mineral Content

There were no sex effects on any measure of eggshell mineral content at 16 days post set. No age effect on Mg content was found from the pooled data, however there was a difference between strains ($F_{3,70}$ 5.76, $P<0.001$). Shell samples from Scottish commercial strain eggs contained a similar amount of Mg to samples from control strain eggs. Samples from Irish commercial strain eggs contained more Mg than these 2 strains, and eggshell samples from the selected strain contained the most Mg. There were no strain effects on eggshell ash, Ca or P contents, but all three increased greatly with age ($F_{1,70}$ 8.8, 358.6, and 82.56 respectively, $P<0.001$; Figure 7.2).

7.4.3: Body Weight

The data were log transformed to standardise variances. There was a complex interaction between age, sex and strain effects on body weight ($F_{3,70}$ 3.14, $P<0.05$; Figure 7.3). In day-old chicks of either sex, those from Irish commercial eggs were much heavier than those of the selected or control strains. Female Scottish commercial chicks had a similar weight to the selected and control females, while male chicks has a similar weight to the Irish males at 1 day old. Weight increased with age in all cases; however, the amount by which it increased was dependent on strain and sex. In both sexes, the smallest weight gain was seen in the control strain, followed by the selected strain. In females, the largest gain between 1 and 6 days of age was seen in the Scottish chicks, although the final weight was very similar in commercial chicks from both sources. Conversely, in the male chicks those from the Irish eggs showed the largest weight gain, and were heaviest at 6 days.

Table 7.2: Information on the hatchability of the eggs supplied

strain /source	number of eggs set	number of infertile eggs	number transferred*	number of unhatched eggs	number of dead chicks	number of live chicks	% successfully hatched**
selected	60	9	41	6	1	34	83
control	45	7	28	1	1	26	92
Scottish	45	4	31	2	0	29	94
Irish	55	10	35	12	1	22	63

(* no. of fertile eggs minus the 10 eggs per group taken for sampling when the transfer took place; ** as a percentage of fertile eggs transferred)

Figure 7.1: Comparing eggshell Mg content between strains, along with standard errors

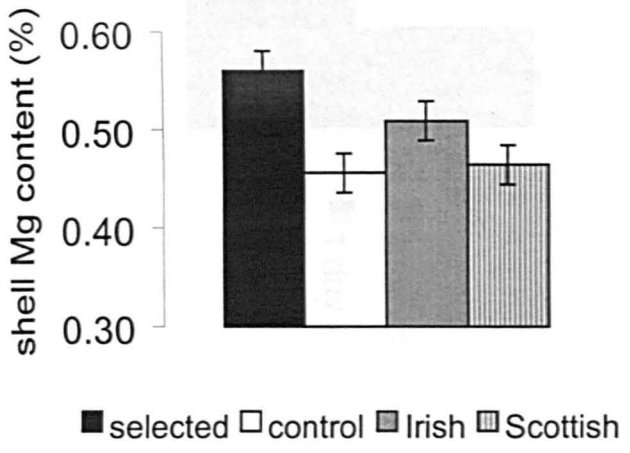


Figure 7.2: Comparing eggshell mineral content between ages, along with standard errors, a. ash, b. Ca, c. P

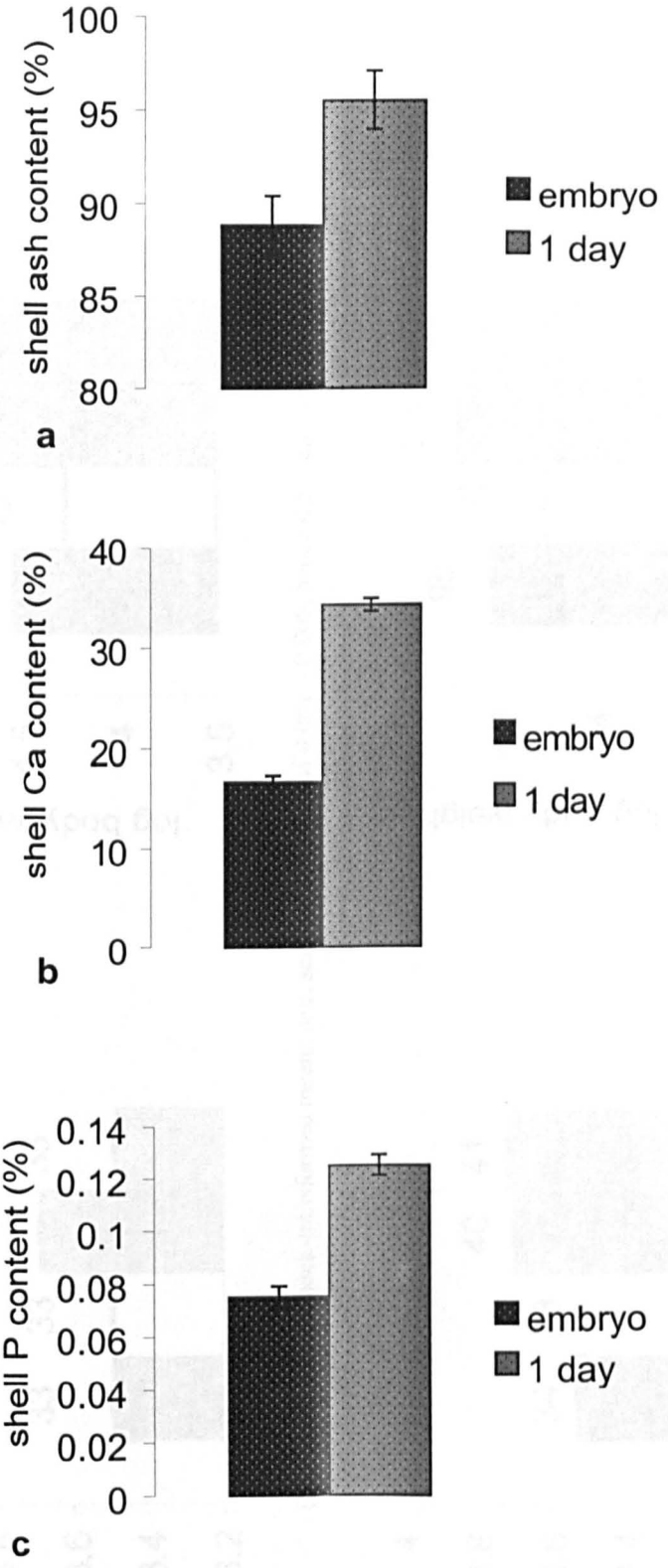
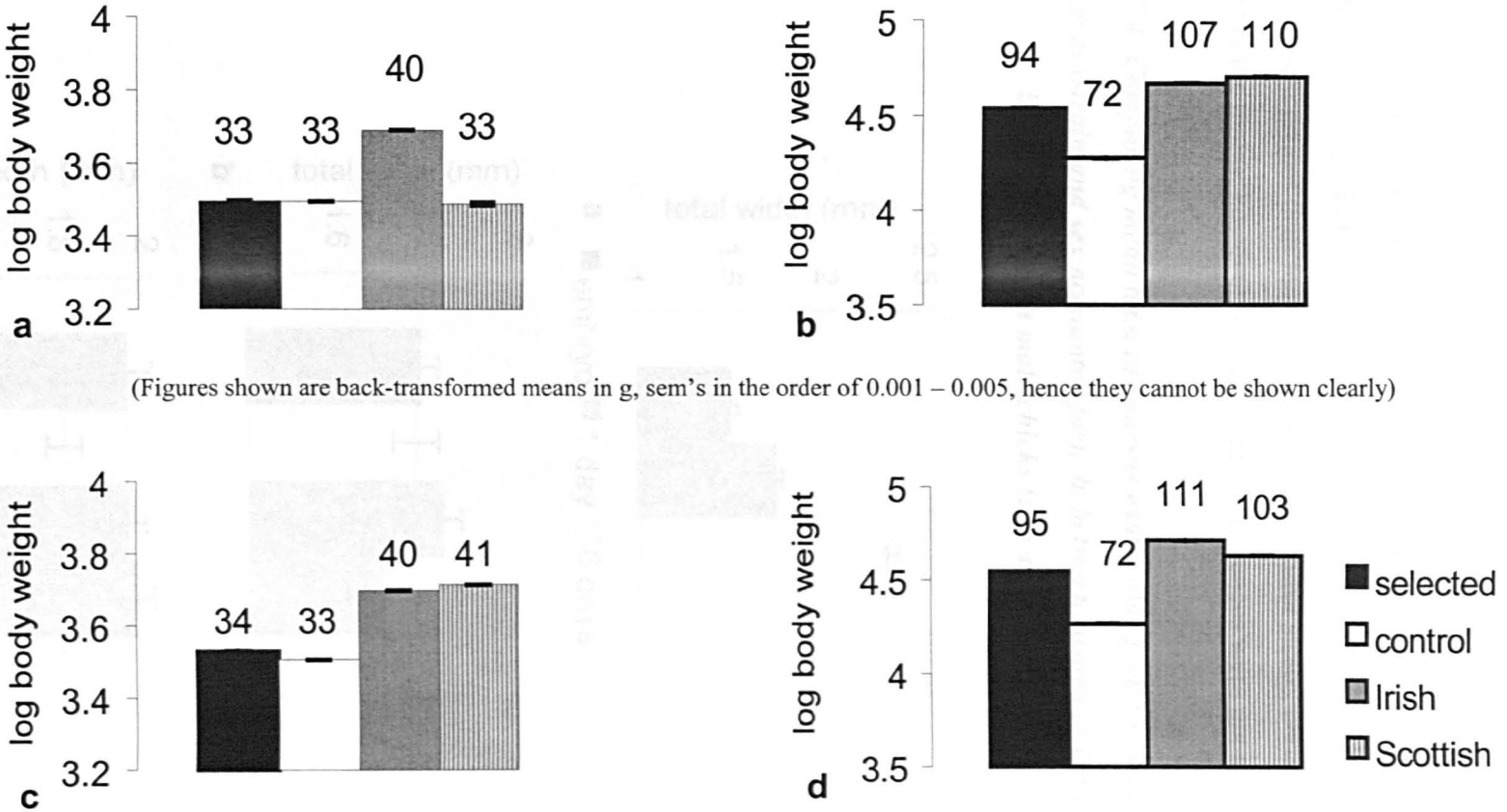


Figure 7.3: Comparing mean body weight between strains, along with standard errors, a. 1 day old females, b. 6 day old females, c. 1 day old males, d. 6 day old males.



7.4.4: Tibiotarsus Dimensions

Total tibiotarsus width increased with age ($F_{2,70}$ 304.21, $P<0.001$; Figure 7.4a). Tibiotarsi from male chicks were wider than those from female chicks in the selected and commercial strains, however, this sexual dimorphism was absent in the control strain ($F_{3,70}$ 3.8, $P<0.05$; Figure 7.4b, c). Cortical thickness increased with age between the embryonic and 6-day samples, however, day-old chicks had thinner cortices than chicks of both other ages ($F_{2,70}$ 76.98, $P<0.001$: Figure 7.5a). There was also an interaction between strain and sex effects on cortical thickness ($F_{3,70}$ 3.36, $P<0.05$: Figure 7.5b, c); male Scottish commercial chicks had slightly thicker cortices than females, while in the control strain, cortical thickness was slightly lower in the males; however, the individual differences were not significant.

Figure 7.4: Comparing mean total tibiotarsus width, along with standard errors, a. between ages (strain and sex accounted for), b. between strains in female chicks, c. between strains in male chicks (age accounted for)

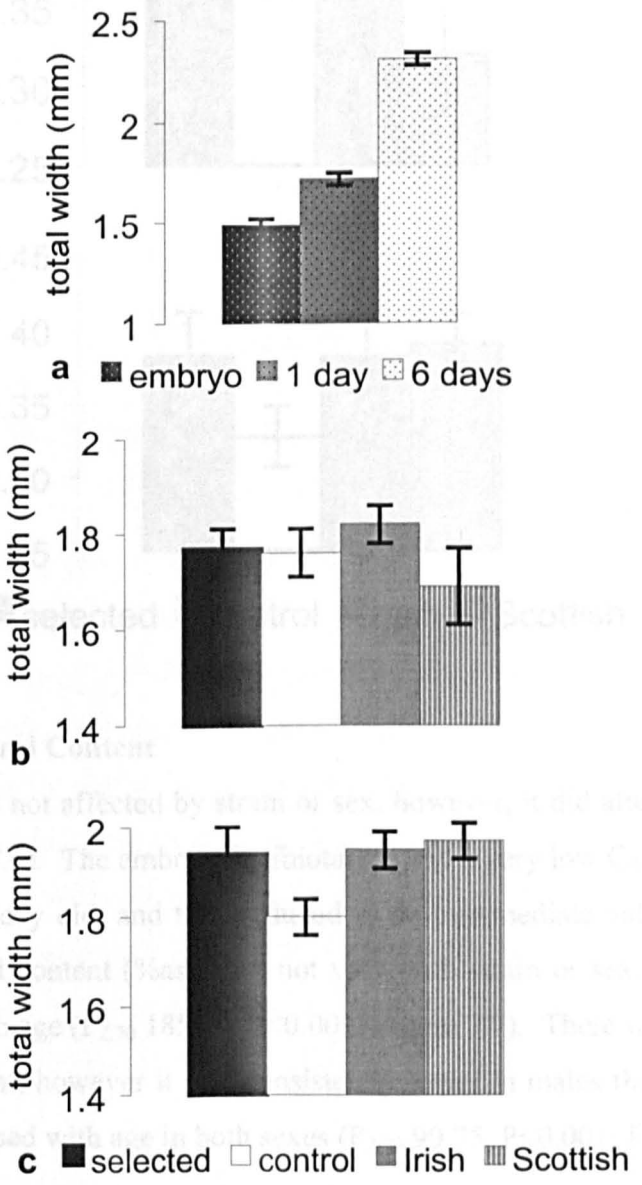
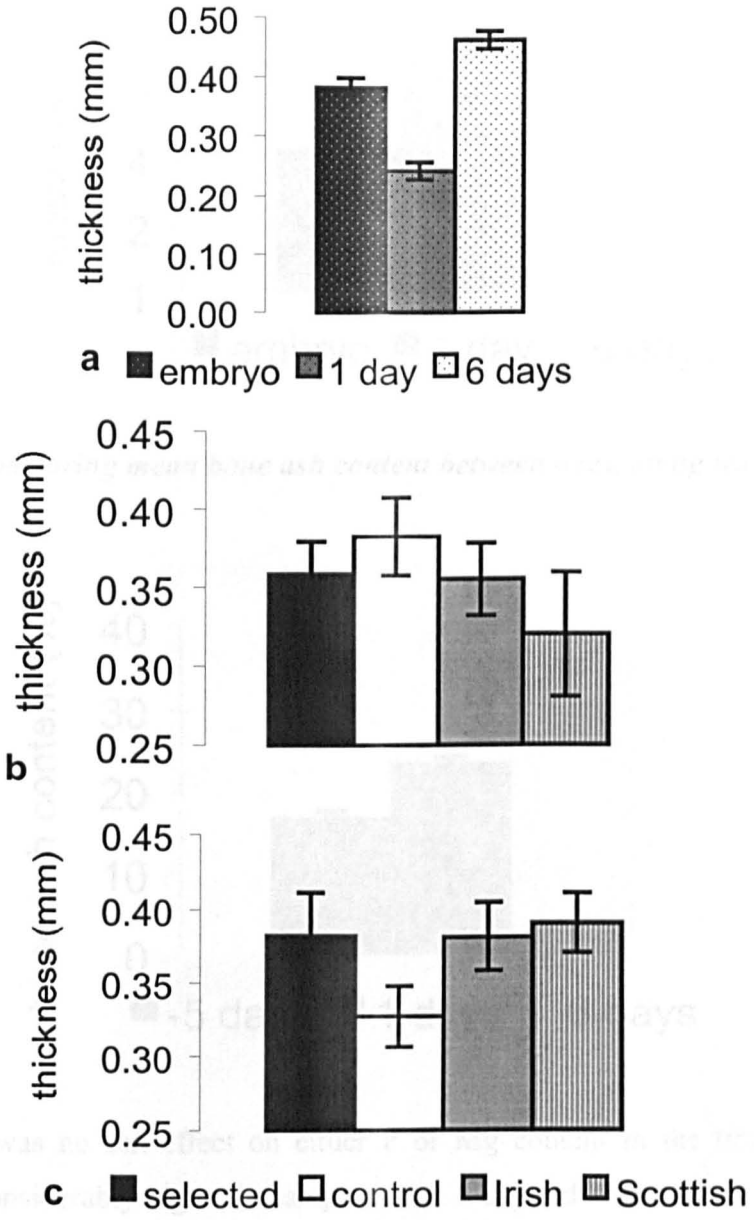


Figure 7.5: Comparing mean cortical width, along with standard errors, a. between ages (strain and sex accounted for), b. between strains in female chicks, c. between strains in male chicks (age accounted for)



7.4.5: Tibiotarsus Mineral Content

The bone Ca:P ratio was not affected by strain or sex, however, it did alter with age ($F_{2,70}$ 36.21, P<0.001; Figure 7.6). The embryonic tibiotarsus had a very low Ca:P ratio; this had risen considerably by 1 day old, and then reduced to an intermediate value by 6 days of age. Total bone mineral content (%ash) did not vary with strain or sex, however, it did increase consistently with age ($F_{2,70}$ 185.79, P<0.001; Figure 7.7). There was also no strain effect on bone Ca content, however it was consistently lower in males than females ($F_{1,70}$ 6.58 P<0.05), and increased with age in both sexes ($F_{2,70}$ 90.75, P<0.001; Figure 7.8).

Figure 7.6: Comparing mean bone molar Ca:P ratio between ages, along with standard errors

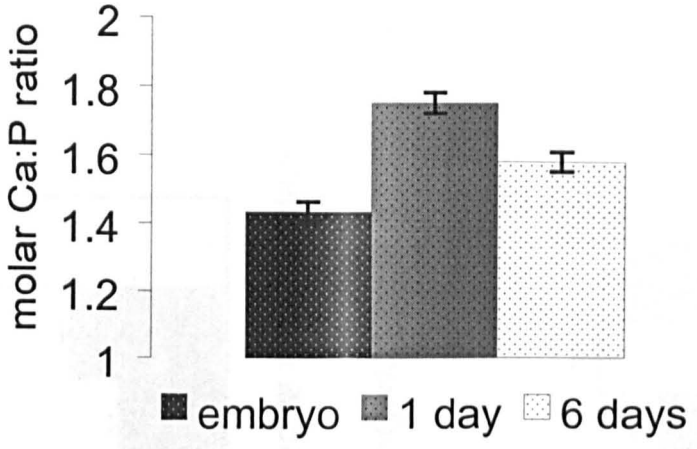
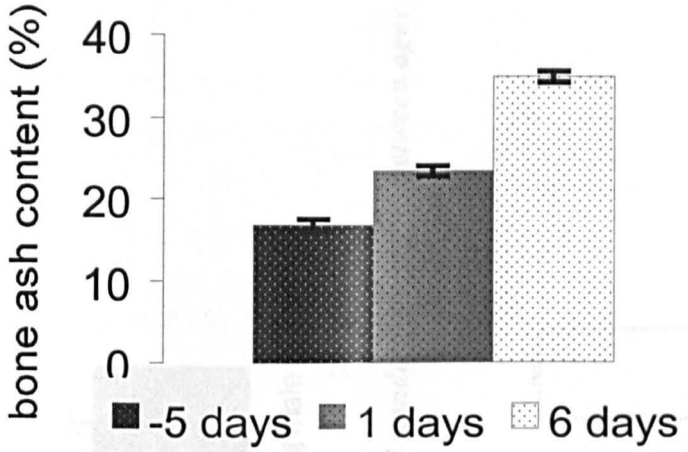


Figure 7.7: Comparing mean bone ash content between ages, along with standard errors



There was no sex effect on either P or Mg content in the tibiotarsus. Bone P content was considerably higher in samples from 6 day old birds than it was in embryonic or day-old samples ($F_{2,70}$ 193.39, $P<0.001$; Figure 7.9). Scottish commercial strain chicks had tibiotarsi with a significantly higher P content than those from the selected strain, with the control strain and Irish chicks having intermediate P contents ($F_{3,70}$ 3.58, $P<0.05$; Figure 7.9). Tibiotarsus Mg content showed a similar pattern; the Scottish chicks had significantly higher Mg contents than the selected strain, with control and Irish chicks having intermediate contents ($F_{3,70}$ 2.94, $P<0.05$; Figure 7.10). Bone Mg content increased overall with age, however samples from day old chicks had considerably less Mg than samples from the other ages ($F_{2,70}$ 126.15, $P<0.001$).

Figure 7.8: Comparing mean tibiotarsus Ca content and standard errors, a. between sex (age accounted for), b. between age (sex accounted for).

(Figures shown are means)

Figure 7.9: Comparing mean tibiotarsus P content and standard errors, a. between ages (strain accounted for), b. between strains (age accounted for).

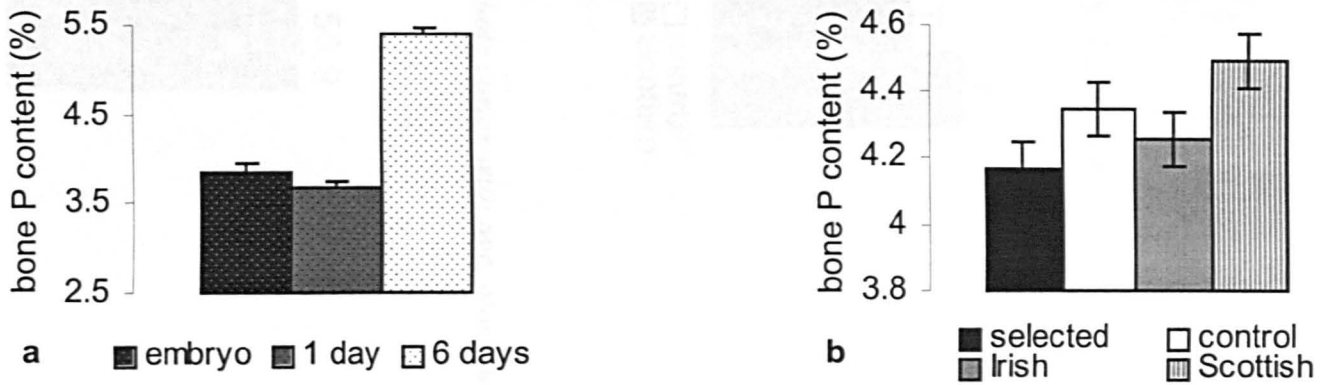


Figure 7.10: Comparing mean tibiotarsus Mg content, along with standard errors, a. between ages (strain accounted for), b. between strains (age accounted for)

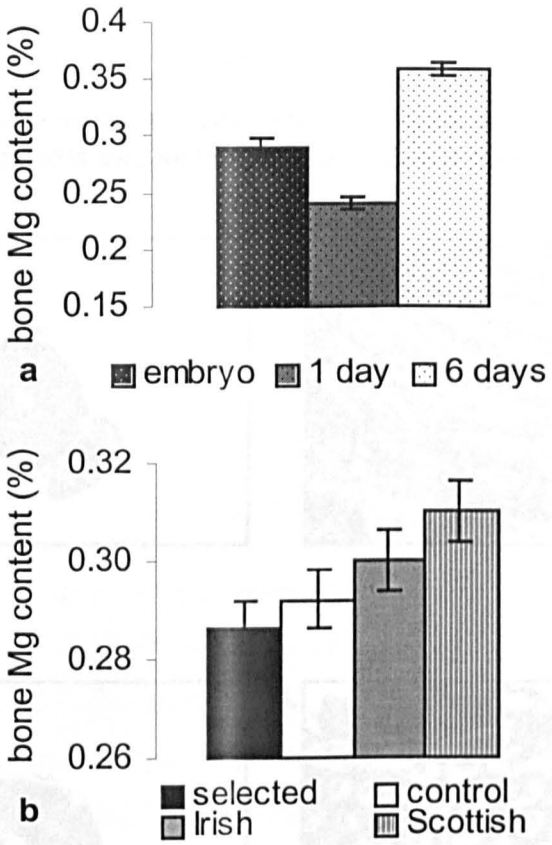
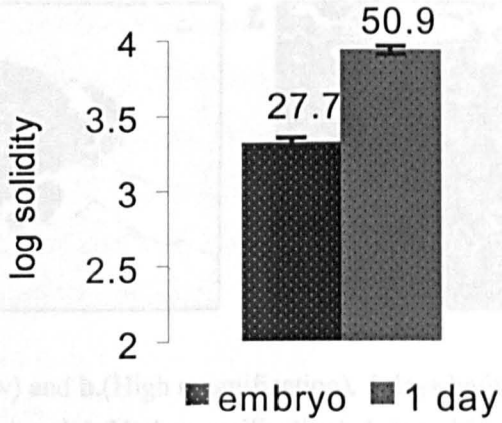


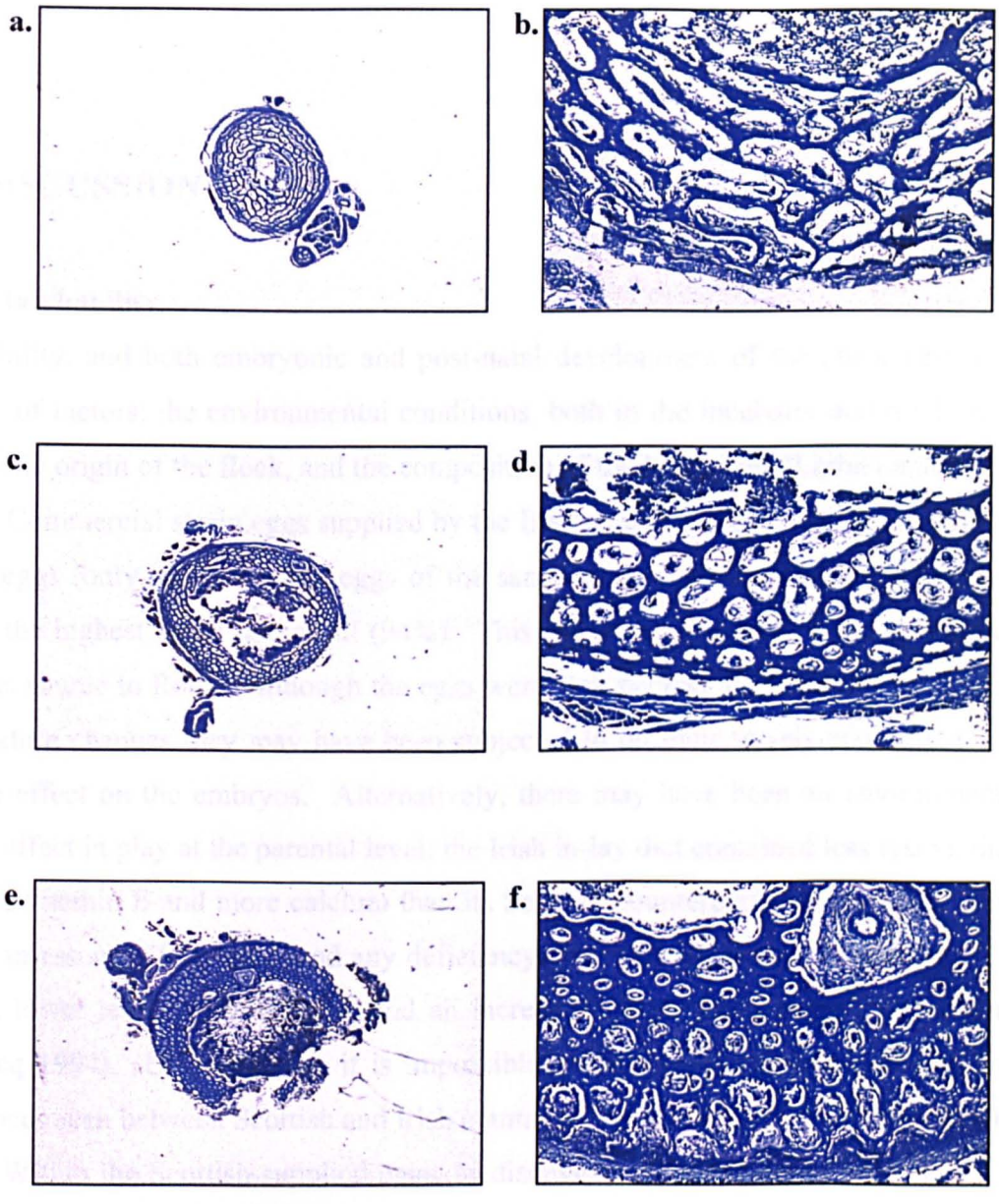
Figure 7.11: Change in mean solidity across the whole cortex with age, along with standard errors



(Figures in brackets are back-transformed means)

Plate 7.1:

Cross sections of mid-diaphyseal bone at different ages in the selected strain showing width and porosity changes (Toluidine Blue staining)



a. (Low) and **b.**(High magnification), 5 days before hatch
c. (Low) and **d.**(High magnification), 1 day old
e. (Low) and **f .**(High magnification), 6 days old

7.4.6: Tibiotarsus Solidity

The data were log transformed to standardise variances. There was no effect of either sex or strain on total cortex solidity in the embryo or at 1 day of age, however, the cortex was considerably more solid in day-old chicks ($F_{1,70}$ 376.51, $P<0.001$; Figure 7.11). At 6 days old, endosteal areas of the cortex continued to become more solid (mean (%) 74.3 ± 1.5), while the periosteal area (mean (%) 48.8 ± 1.3) maintained a similar level of solidity to that seen at 1 day-old across the whole cortex. Neither measure was affected by sex or strain effects.

7.5: DISCUSSION

7.5.1: Hatchability

Hatchability, and both embryonic and post-natal development of the chick depend on a number of factors: the environmental conditions, both in the incubator and the layer unit, the genetic origin of the flock, and the composition of the layer diets (Larbier and LeClercq 1994). Commercial strain eggs supplied by the Irish source had the poorest hatchability of all the eggs (only 63%), despite eggs of the same strain supplied by the Scottish source having the highest hatch rate of all (94%). This can possibly be attributed to the journey from the source to Roslin; although the eggs were well packed, the shaking, vibration, and temperature changes they may have been subjected to on their travels could have had an adverse effect on the embryos. Alternatively, there may have been an environmental or dietary effect in play at the parental level; the Irish in-lay diet contained less lysine, linoleic acid and vitamin E and more calcium than its Scottish counterpart,. In particular, linoleic acid is an essential fatty acid, and any deficiency, even partial, leads to a reduction in egg weight, lower levels of yolk lipid, and an increase in embryonic mortality (Larbier and LeClercq 1994). Unfortunately, it is impossible to distinguish the actual reason for the differences seen between Scottish and Irish commercial strain eggs from the data available.

Within the Scottish-supplied eggs, as discussed, the commercial strain had a good success rate. The control strain hatch rate was similarly high (92%), however, that of the selected strain was considerably lower (83%). This variability between the strains was not unexpected; it is generally accepted within the Ross Breeders company that the selected strain has a relatively poor hatch rate, and supplementary eggs were provided for this experiment precisely for this reason. Genotypical differences have been observed in shell strength (Potts and Washburn 1983, Bowman and Challender 1963), shell thickness (Potts and Washburn 1974), shell weight per unit surface area and egg specific gravity (Hamilton

1978), and egg weight (Marion *et al* 1964). These factors may all affect the development of the embryo, and the success of hatching. The heritability of egg characteristics such as egg weight and density is greater than that for production traits in the laying hen (Besbes *et al* 1992), thus poor egg quality may be a trait inherited as a by-product of selection for increased egg production (Darnell-Middleton 1999). However, further investigation would be needed to confirm the mechanism for this decline in hatch rate in the selected broilers.

7.5.2: Chick Weight

In most cases, there appeared to be little or no difference in chick weight with sex (Figure 7.3); this is in agreement with the results of Bond *et al* (1990) for day-old chicks. Female Scottish commercial day-old chicks were considerably lighter than the equivalent males; however, since this sex difference was not seen in the same genetic strain of birds supplied by the Irish source, the reason for this is currently unknown, and may simply be due to variation in a small sample size. At 1 week of age Bond *et al* (1990) found that male broilers were 14% heavier than females. In the present experiment such a large difference was not seen at 6 days of age. It is possible the strains used in the current experiment had different growth profiles to that used by Bond and his colleagues, and that a sex difference in weight only becomes evident at a later age in the presently used strains.

The biggest effect on body weight was age. The commercial strain was the heaviest at 1 day old, and gained the most weight during the following 5 days, closely followed by the selected strain. The weight increase in the control strain was considerably smaller than that in the other two strains. This was not unexpected; both the commercial and selected strain have been developed for high final body weights, and to reach their full potential, they must grow quickly from the start. Day-old chick weight is likely to be highly related to egg weight, which is known to vary between strains (Marion *et al* 1964), and can therefore be selected for. If we assume that the commercial strain eggs were indeed heavier, this would also appear to rule out linoleic acid deficiency as a cause of the poor hatchability observed in the Irish eggs; the male day-old chicks were of a similar weight to the Scottish males. However, further investigation is needed to confirm the relationship between day-old chick weight and egg weight in these strains, and the effect of dietary variation.

7.5.3: Bone Dimensions

Total tibiotarsus width increased with age, and this increase was not affected by strain or sex (Figure 7.4 a). In the selected and commercial strains, there was a sexual dimorphism in width, with males, on average, having consistently wider bones (Figure 7.4b, c). This

may seem surprising in the absence of any sex effect on body weight, and therefore bone loading, however similar differences in bone width between male and female chickens have been previously documented from hatch (Rose *et al* 1996). The dimorphism in total width was absent in the control strain. The sex difference observed in the modern strains may reflect a developmental adaptation to accommodate the larger difference in body size observed between the sexes at older ages. It is possible that the sexual dimorphism in body weight is much less pronounced in the control strain, making such an early increase in bone dimensions unnecessary, however data are not currently available to confirm this.

The sex by strain interaction observed in cortical width (Figure 7.5b, c) seems weak, and contradictory between strains; it is possible that this significant result occurred by chance. The most interesting effect on cortical width was that of age, and this was again independent of sex or strain. However, rather than a simple increase in thickness with age, the cortex became thinner in day-old chicks than in 16-day embryos, then increased to the highest thickness in 6 day old chicks. A similar finding has been found in the human tibia during the prenatal period of 20-41 weeks; the medullary diameter increased at a greater rate than total diaphyseal diameter, leading to a mild but significant reduction in cortical thickness (Rodriguez *et al* 1992). In the embryo, the tibiotarsus, like all long bones, is initially formed as a solid cartilaginous model. During the development of the embryo, the cartilage undergoes cellular changes characterised by alterations in cell size and matrix deposition. The matrix produced becomes calcified, and is ultimately replaced by bone (McClean and Urist 1968). However, PTH is thought to be secreted by the embryo only after 10 days post set (Narabaitz 1972). In addition, osteoclasts only become recognisable and active at day 12-13 post set (Khan *et al* 1981), and only reach optimum operating efficiency from 19 days post-set (Martini *et al* 1982), when plasma levels of 1,25-dihydroxyvitamin D_3 become normal (Kubota *et al* 1981). Hence the replacement of the cartilaginous model by bone, and the formation of a functional marrow cavity within chick long bones, must occur towards the end of incubation.

Observations in the present experiment suggest that endosteal resorption to produce the marrow cavity had started before 16 days post-set, since diaphyseal cross-sections of the tibiotarsus were already hollow at this age. To create the marrow cavity and increase its size, endosteal resorption apparently occurred at a faster rate than that of periosteal apposition. This resulted in a reduction in cortical thickness, which continued between 16-days post set and hatching. This may be a mechanism to attain an initial optimum marrow cavity size. Bone marrow is the site of red and white blood cell formation, red blood cells (erythrocytes) being vital for O_2 and CO_2 transport in the blood (Alberts *et al* 1983). Pulmonary respiration starts on day 20 of incubation (Baimbridge and Taylor 1980), only a

few hours before hatching; a sufficient supply of erythrocytes is likely to be vital at this point. From previous work discussed in this thesis (Williams *et al* 2000, Chapter 3), it is known that from 4 days old periosteal apposition occurs at a faster rate than endosteal resorption, until an optimum cortical thickness is reached at approximately 18 days, when the two processes become balanced.

7.5.4: Bone Solidity

Bone solidity measured across the whole cortex increased between 16 days post set, and 1 day post-hatch (Figure 7.11). This may have been a compensation mechanism for the reduction in cortical thickness observed between these ages, however, this 'filling in' is more likely to be concurrent with the formation of marrow cavity than a response to it. It was suggested previously that the resorption of the tibiotarsus cartilaginous model in the embryo cannot proceed until at least 12 to 13 days post set. It appears that after this time, massive cartilage resorption occurred, both endosteally and at a number of foci within the cuff destined to become cortical bone. Cross-sections of the tibiotarsus diaphysis from embryos at 16-days post set resembled a circular maze, and were very different from cross-sections of bone from older birds, with the typical radial array of primary osteons and their Haversian canals (Plate 7.1). By 1 day post hatch, this maze had been filled in considerably, and cross sections displayed osteonal systems with large Haversian canals, and a higher solidity value. By the time chicks were 6 days old, the diaphysis was displaying two distinct regions. The endosteal region had small Haversian canals and the highest solidity observed in this experiment. The periosteal region had much larger Haversian canals, and a similar solidity value to that observed at 1 day old across the cortex; this region appeared to consist of newer osteons still actively filling in. This general pattern is continued throughout subsequent bone development in chickens (Williams *et al* 2000, Chapter 3), and has also been described in a heavy, fast growing Turkey strain (LeBlanc *et al* 1986).

Neither strain nor sex effects were detected on bone solidity in the cortex of the tibiotarsus in the embyo or day-old chick. This suggests that the difference previously observed in porosity between the control and selected strain (Williams *et al* 2000, Chapter 3) develops postnataly, which would rule out inadequate diets or environmental conditions in the breeder units as a cause of the high degree of porosity observed in the selected strain. However, in contrast to Chapter 3, in which a difference in porosity was observed between the selected and control strains from 4 days of age, no difference was seen at 6 days in this chapter; the reason for this is uncertain at present. Further work is needed to identify whether the increased porosity in the selected strain is due to a faster growth rate or higher

requirement for dietary mineral (which could be corrected during broiler rearing), or a different body conformation and loading environment, or different genetic programming for bone development (which would have to be addressed through breeding programmes).

7.5.5: Eggshell and Bone Mineral Content

Eggshell total mineral content, and Ca and P content all increased between 16-days post set and hatch (Figure 7.2). The predominant source of P for the developing embryo is the yolk (Richards and Packard 1996), hence it is possible that un-utilised P content in the eggshell might increase proportionally as calcium is removed. However, how total mineral content and Ca content could increase when Ca was being removed for the mineralisation of the embryonic skeleton is unknown. During embryonic development, the bond between the shell and membranes is lysed (Solomon pers. com.), and the inner membranes were much more easily removed from hatched eggshells for analysis than from the eggs containing embryos. It is possible that some portion of the complement of membranes was not successfully removed from the embryo-containing eggs, meaning that shell mineral content measurements were lower than expected at this age. Alternatively, there may have been some degradation of the protein matrix of the eggshell during the latter part of incubation, or after hatching, which might have reduced the organic content of the shell, giving higher mineral contents than expected at hatch. Unfortunately, it is not possible to come to any conclusions at present, and the changes in eggshell mineral content observed with age should be treated with caution until this work can be repeated.

Eggshell magnesium content was apparently not affected by age, however, in the light of the above discussion, this may be debatable. It did, however, vary considerably with strain (Figure 7.1). Eggshells from the selected strain contained the highest amount, followed by the Irish commercial strain, with the Scottish commercial, and control strains having the lowest magnesium content. Conversely, tibiotarsus Mg content was highest in the Scottish commercial strain, and lowest in the selected strain, with the Irish commercial and control strains having an intermediate bone Mg content (Figure 7.10). Magnesium is present as a trace element in diets, and has the ability to substitute for Ca in bone (Posner 1987). It has been suggested that in times of calcium stress, a greater proportion of Mg will be present in the bloodstream, and in hens, a greater proportion will be incorporated into the eggshell (Darnell-Middleton 1999). However, it is unlikely that this is the explanation for the variation observed here. The Scottish commercial, control, and selected eggs were all produced by hens on the same in-lay diet, while the Irish eggs (containing more Mg than the Scottish equivalents) were produced by hens on an in-lay diet with a higher content of Ca than the Scottish diet (Table 7.1).

Mg is known to be deposited primarily in the outer layers of the eggshell (Itoh and Hatano 1964) as magnesium carbonate (Romanoff and Romanoff 1949), and it has been suggested that it may play a role in eggshell strength (Anon 1967). If so, eggshell magnesium may be yet another heritable trait of eggshell quality; however, the difference in shell Mg content between the Irish and Scottish eggs of the same genetic strain suggests that some other factor is involved. The inverse relationship between eggshell and bone Mg content displayed by the 4 groups of chicks implies that there may be varying rates of Mg uptake from the eggshell into the tibiotarsus, resulting in varying amounts of Mg 'left over' in the eggshell. What factor or factors might be responsible for a variation in uptake between strains, or between different groups of the same strain remains to be resolved.

As expected, the total mineral content, and Ca and P content of the tibiotarsus increased with age (Figure 7.7). The ash content for day-old chicks observed in this experiment (23%) is considerably lower than the roughly 40% found by Bond *et al* (1990); the reason for this is uncertain, however it may be due to a difference in bone sampling techniques for ashing between the 2 studies (Williams *et al* 2000, Chapter 3). Whole bone ash values from previous work on birds aged 16 and 45 days ranged from 25-40% (Chapter 6); the results from the current experiment suggest that 60-90% of the final bone mineral content is laid down prior to hatching. No difference in total bone mineral content was detected with sex or strain in the embryo or day-old chick. This suggests that the lower ash content previously observed in the selected strain when compared to the control strains develops postnataly, perhaps for similar reasons as the development of greater porosity. However, the difference previously observed in ash content between the selected and control strain from 4 days onwards (Chapter 3) was not observed in the present experiments; the reason for this remains uncertain.

Ca content of the tibiotarsus was consistently higher in female chicks. This, and the fact that there was no sex effect on any measure of eggshell mineral content, appears to contradict much earlier work, which showed that male chicks utilised more of the eggshell as a source of Ca (Kosin and Munro 1940). However, this early conclusion was based on data which showed that denser eggs produced chicks which showed a greater uptake of calcium from the eggs. Denser eggs might be expected to produce heavier chicks, which would be likely to need a greater quantity of calcium to mineralise a larger skeleton, hence it is possible that the difference observed in calcium utilisation was actually due to a sex difference in weight. No sex difference in weight was observed in 3 of the four groups of chicks in the present experiment, which might also explain the apparent lack of a difference in eggshell Ca utilisation.

P content in the tibiotarsus also varied between strains. The Scottish commercial strain had the highest content, followed by the control strain, then the Irish commercial strain, with the selected strain displaying the lowest bone P content (Figure 7.9). The fact that there was no inverse strain difference in eggshell P content is consistent with the fact that the yolk, rather than the shell, acts as the source of P to the developing chick embryo. However, the reason for the variation in P content between strains is presently unknown. The difference in bone P content between strains was not large enough to produce a variation in bone molar Ca:P ratio between the strains.

The variation of P with age is likely to be responsible for the variation in bone Ca:P ratio seen with age (Figure 7.6). In the bone tissue of young animals, a greater proportion of bone mineral is expected to be in a non-crystalline form with a low (1.45-1.55) Ca:P ratio (McClean and Urist 1968, Narasarju and Phebe 1996). However, in the day-old chicks, a mean ratio of 1.75 was observed. While the bone Ca content of day old chicks was higher than that of the embryos, bone P content was actually slightly lower. These results suggest that during the period between these two ages, calcium uptake into bone was occurring at a much faster rate than P uptake. The reason for this is currently uncertain; however it has been postulated previously (Chapter 4) that transient high Ca:P ratios seen in cortical bone (Williams *et al* 2000, Chapter 3), and the high Ca:P ratio seen in medullary bone (Tyler 1948) may be part of a mechanism allowing rapid mineralisation in times or sites when speed of production and quantity of mineral may be more important than its composition. The high ratios in the current experiment occurred at a time when a large quantity of mineral was being laid down in a relatively short space of time, which would appear to support the proposed hypothesis. P uptake into the tibiotarsus then appeared to increase after hatching, and by 6 days of age, the Ca:P ratio was approximately what was expected.

7.5.6: Conclusions

Late embryonic and early chick tibiotarsus development was broadly similar in all four groups of birds. It appeared to begin with a period of massive cartilage resorption between 12-16 days incubation, to produce a medullary cavity and a cortical framework. The cortex then appeared to be progressively filled in with new bone, and diaphyseal cross-sections displayed a similar pattern of osteons to that of adults by 6 days of age. Sixty to ninety percent of the final tibiotarsus mineral content appeared to be laid down prior to hatching. Endosteal resorption of the medullary cavity appeared to occur at a more rapid rate than periosteal apposition until 1 day old, possibly to ensure than the marrow cavity

was large enough to provide a sufficient supply of erythrocytes for the start of pulmonary respiration just before hatching. Cortical width then began to increase from 1 day old.

There were no strain differences detected in bone ash or Ca contents, or solidity in embryonic or day old chicks; this strongly suggests that the differences in ash content and porosity previously observed between the selected and control strains develops postnataly, although such differences had not appeared by 6 days of age in the current experiment. Further work is needed to determine whether these impairments to bone quality are a consequence of genetic programming, growth rate, body weight and/or dietary effects. There were differences in egg hatchability and chick weight observed between sources in the commercial strain; however, it was impossible to determine the reasons for this from the data available. There were no differences in bone ash content or solidity between the 2 groups of commercial eggs. This suggests that some variation in maternal conditions and diet is possible without affecting skeletal quality in the chick, however further work is needed to confirm this, and to test the range of conditions to which this may apply.

CHAPTER 8

A STUDY ON THE POSSIBLE MECHANISM RESULTING IN POROUS BONES: DIFFERENCES DUE TO STRAIN AND GROWTH RATE

8.1: INTRODUCTION

Previous work showed that cortical bone from a modern selected strain of broiler chicken was consistently more porous and less well mineralised than that from a control strain unselected since 1972 (Chapters 3 and 5, Williams *et al* 2000). The difference in porosity between strains was greater for periosteal bone, the area of appositional bone growth, and in the absence of any evidence of bone remodelling through the formation of secondary osteons, it was hypothesised that the greater porosity could be due either to genetic factors, or to a limiting rate of bone formation within primary osteons. It was suggested that such a limitation may have occurred as a result of the much more rapid expansion in the total width of the tibiotarsus in the selected strain, however, no direct evidence to support this was available. Similarly, it was suggested that the lower mineralisation observed could be due either to a dietary mineral supply inadequate to support the rapid bone expansion, limitations in the rate of mineral uptake or utilisation, or genetic factors.

Both the selected strain and an unrelated commercial broiler were also found to produce considerably shorter bones than the control strain at a given weight, (Chapters 3 and 5, Williams *et al* 2000), suggesting that either there may be a limit to the rate at which bone tissue can be produced at the growth plate, that there is an adaptive shortening response to higher body weight at the growth plate, or that selection processes since 1976 had coincidentally produced heavier, but shorter broilers. This relative shortening in a fast growing broiler strain has also been independantly reported, and was partly reversed by slowing the growth rate by restricted feeding (Corr 1999). This work does not rule out genetic factors, however, it supports the view that either bone production at the growth plate cannot keep pace with the growth rate of modern birds, or that an adaptive response it taking place at higher body weights. If the shortening is due to a limited bone production rate, it is possible that a similar problem occurs within primary osteons of the selected strain, leading to the more porous cortical bone observed. With regard to bone mineralisation, a subsequent feed trail (Chapter 4, Williams *et al* in press) failed to find any convincing evidence that bone mineralisation was seriously impaired in the selected

strain due to dietary constraints. In addition, no differences were observed in the ash content or porosity of embryonic cortical bone from the selected and control strains (Chapter 7). Again, this does not rule out genetic factors, however, the fact that the differences develop during rearing means that growth rate could play a role in both of these aspects of bone quality.

8.2: AIMS

The present experiment seeks to confirm the differences previously observed in bone mineralisation and porosity between modern broiler strains and the control strain, and to investigate the effects of these differences on the mechanical properties of bone. A study will be made of the sites of bone formation and resorption, and of the rate of bone formation, in order to go some way towards elucidating the cause of the increased porosity observed in the selected strain. Information on the circulating mineral status will also be gathered in order to assess whether the poor mineralisation observed in the selected strain might be due to impaired mineral uptake, or a limited rate of utilisation. Manipulation of bird growth rate (and therefore bone growth rate) in the selected strain will be performed to determine the affect of growth rate on the extent of cortical mineralisation and porosity, and on tibiotarsus length. Finally, the opportunity is taken to assess the effectiveness of two non-invasive markers of bone mechanical properties obtained from x-rays, radiodensity for bone strength, and an estimate of resistance to bending based on bone dimensions for stiffness

8.3: MATERIALS AND METHODS

8.3.1: Birds, Housing and Feeding Regimes

Sixty male day-old chicks of the Ross control strain (MK) and the Ross commercial strain (738), and 120 male day-old chicks of the Ross selected strain (MCY) were placed into 12 pens. The pens were divided into 3 blocks, with each block containing one pen each of the commercial and control strains, and two of the selected strain; each pen contained 20 birds. A standard pen set-up of wood-chip floor covering, feeding tray, bell drinker and heat lamp was used. Feed trays were replaced by hoppers after 1 week in *ad lib* fed pens. Lighting started at 23 hours light, 1 hour dark, and switched to 21 hours light and 3 hours dark after 1 week. Temperature was recorded at chick head height with a max-min thermometer;

maximum temperature was maintained between 18-29°C (mean 24°C), minimum temperature ranged from 12-20°C (mean 15°C).

For the first 4 days, all birds were fed *ad lib* on a standard broiler starter diet. From 5 days of age, one pen of selected strain birds in each block was fed at 50% of the *ad lib* consumption rate on a diet which contained twice the Ca and P content, giving a daily Ca and P intake similar to that of the *ad lib* fed birds. Samples of all the diets were analysed before use to check Ca and P content (ash analysis), and gross energy content (bomb calorimetry; Table 8.1). In the absence of previous *ad lib* food intake data on the selected strain, the daily ration was initially based on data from the *ad lib* food intake of the Ross 308 broiler. *Ad lib* food intake in the selected strain was checked on a weekly basis, and the restricted pens' rations for the subsequent week were adjusted where *ad lib* food intake was found to be greater than expected. All other pens continued to be fed *ad lib* on the standard diet. All birds were switched onto finisher diets at 15 days of age. The restricted fed selected birds continued to be fed at 50% of the *ad lib* consumption rate on a finisher diet which again compensated for Ca and P intake. All other birds were fed *ad lib* on a standard finisher diet.

Table 8.1: Composition of the standard ad lib diets, and the compensatory diets for restricted feeding.

diet	total energy (MJ/kg)	Ca (%)		expected available P (%)	total P (%)	
		expected	analysed		expected	analysed
ad lib starter	16.64	1.00	1.05	0.50	0.69	0.62
restricted starter	16.75	2.00	2.01	1.00	1.18	1.06
ad lib finisher	17.87	0.90	0.99	0.45	0.65	0.62
restricted finisher	18.03	1.80	2.01	0.9	1.1	1.07

8.3.2: Sampling Procedure

At 4 days of age, 3 birds from each pen were picked at random, given intra-peritoneal injections of calcein and marked on the back of the head with non-toxic marker spray. The same birds were given a second calcein injection at 13 days old, and the following day, all the marked birds, and a further 3 random non-injected birds per pen were blood sampled. At 15 days of age, all 6 birds per pen were culled by cervical dislocation, weighed, and both tibiotarsi were removed. At 28 days, and again at 40 days old, a further 3 birds per pen were injected with calcein and marked. Six birds per pen (including the marked birds) were blood sampled at 41 days, and culled and weighed at 42 days of age, and both tibiotarsi were again removed.

Plate 8.1:

Restricted fed selected birds in a typical pen. Birds marked with green dye have been injected with calcein

8.3.3: Sample Processing

Ionised Ca content was measured in the blood samples, which were then centrifuged and the plasma removed for use in assays to measure total Ca and inorganic P concentration. Technical problems with the Ca^{++}/pH analyser at 14 days meant that the ionised Ca results from this age were unreliable, and they were not used. The left tibiotarsi were stored at 4°C until use. They were x-rayed, and measurements of total and marrow width in the mid-diaphyseal segment, and radiodensity in the proximal shaft segment, were made using the NIH image analysis software. Cortical width in the mid-diaphyseal segment was subsequently calculated, as was the area moment of inertia (MI, an estimate of resistance to bending; Chapter 3, Biewener1992). The bones were then subjected to a three-point bending test to measure maximum load before rupture, and stiffness. From the right tibiotarsi, proximal growth plates were placed in cassettes and stored in buffered neutral formalin until they were processed through wax for assessment of the incidence of pathologies such as TD and hypocalcaemic rickets. The proximal shaft segments were stored at -20°C until use in ash analysis for bone ash, Ca, P, and Mg content. The mid-diaphyseal segments from birds injected with calcein were stored in cassettes in 70% alcohol in the dark until they were processed for measurement of the rate of periosteal bone formation. The stored segments from 42 days were found to be too short to mount for cutting, and sections were therefore taken from the surface of the adjoining distal segment, which had been stored unfixed at -20°C in the dark. Mid-diaphyseal segments from birds which were not injected with calcein were stored in buffered neutral formalin until they were processed through wax for the measurement of endosteal and periosteal cortical bone solidity using the NIH image analysis software. The distal shaft segments from the non-injected birds were frozen and stored at -70°C until cryostat sections were cut for use in ALP and TRAP reactions, and subsequent qualitative assessment of osteoblastic and osteoclastic activity respectively.

8.3.4: Statistical Analysis

Strains and feeding regimes were combined to give 4 bird groups: *ad lib* control, commercial, and selected, and restricted-fed selected. Data were analysed by split-plot analysis of variance, fitting block and pen as random factors, for differences between bird groups (between pens), and ages and calcein-injected / non-injected birds (within pens). The differences between bird groups were split into differences between the three groups of ad-lib fed birds (selected, commercial and control), and differences due to restricted feeding (selected *ad lib* versus restricted). Tibiotarsus dimensions and mechanical properties are highly related to body weight, and analysis of these measures was repeated

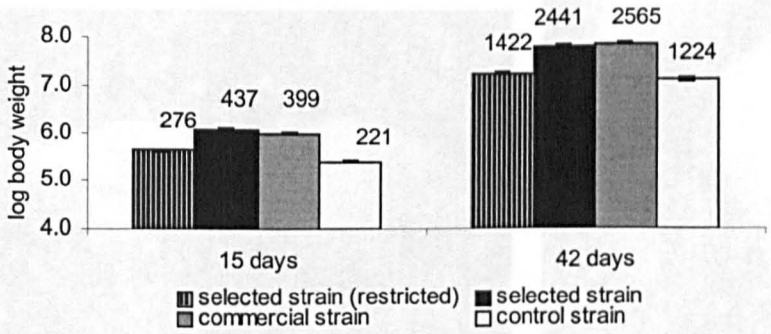
with the addition of weight into the model as a covariate. Possible differences in the slopes of the allometric relationships of these measures to body weight between strains, feeding regimes, or calcein injected and non injected birds, were also examined by linear regression of logarithmically transformed data. The usefulness of radiodensity and MI as predictors of strength and stiffness, were examined by simple linear regression.

8.4: RESULTS

8.4.1: Body Weight

Within the *ad lib* birds, at 15 days the selected strain was heavier than both other strains, and the two modern strains were considerably heavier than the control strain (Figure 8.1). By 42 days, the two modern strains had reached similar final weights, again much heavier than the control strain (strain.age interaction F 4.83, df 2,118, P=0.01). At both ages, restricted feeding considerably reduced the body weight attained by the selected strain, closer to that of the control strain (F 217.64, df 1,6, P<0.001).

Figure 8.1: Comparison of mean body weight between strains, feeding regimes and ages, along with standard errors



(Figures are back-transformed means in g)

8.4.2: Circulating Ca and P

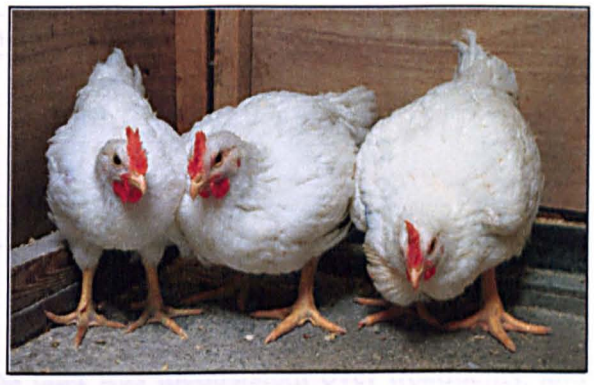
Mean total plasma calcium was 1.32 mg/dl at 15 days of age, rising to 1.45 mg/dl by 42 days (sem 0.034, F 6.99, df 1,118, P<0.01). Variation in plasma inorganic P was not equally distributed over the treatments, and the data were logged for analysis. Inorganic P dropped from 7.29 mg/dl (log 2.0) at 15 days to 5.19 mg/dl (log 1.6) at 42 days (log sem 0.026, F 85.34, df 1,118, P<0.001). There was no effect of feeding regime on these measures, and there were no differences in them between strains. There were no significant effects on circulating ionised Ca (grand mean 1.48 mMol/l, sem 0.016).

Plate 8.2:

Comparison of body size between strains and feeding regimes at 40 days

a.

b.



c.

d.

a. Overhead view: top selected strain, bottom right commercial strain, bottom left control strain.

b. Front view left control strain, middle commercial strain, right selected strain

c. Side view of selected strain, left restricted fed, right ad lib. fed.

d. Overhead view of selected strain, left restricted fed, right ad lib. fed.

8.4.3: Pathology

Overall pathology incidence was too low to be statistically analysed. No pathology was seen in the restricted fed selected strain (Table 8.2). In the *ad lib* fed birds, incidence of TD was generally low, with only 1-2 incidences seen at each age in the control and selected strains; however, incidence was higher in the commercial strain. Hypocalcaemic rickets (HC) was not seen at 15 days. At 42 days, there was no incidence in the control and restricted fed selected birds, and a low incidence in the ad-lib fed modern birds.

Table 8.2: Pathology incidence: number of birds out of an 18 bird sample

	15 days			42 days		
	TD	HC	normal	TD	HC	normal
selected strain (R)	0	0	18	0	0	18
selected strain	2	0	16	1	2	15
commercial strain	5	0	13	4	2	12
control strain	1	0	17	2	0	16

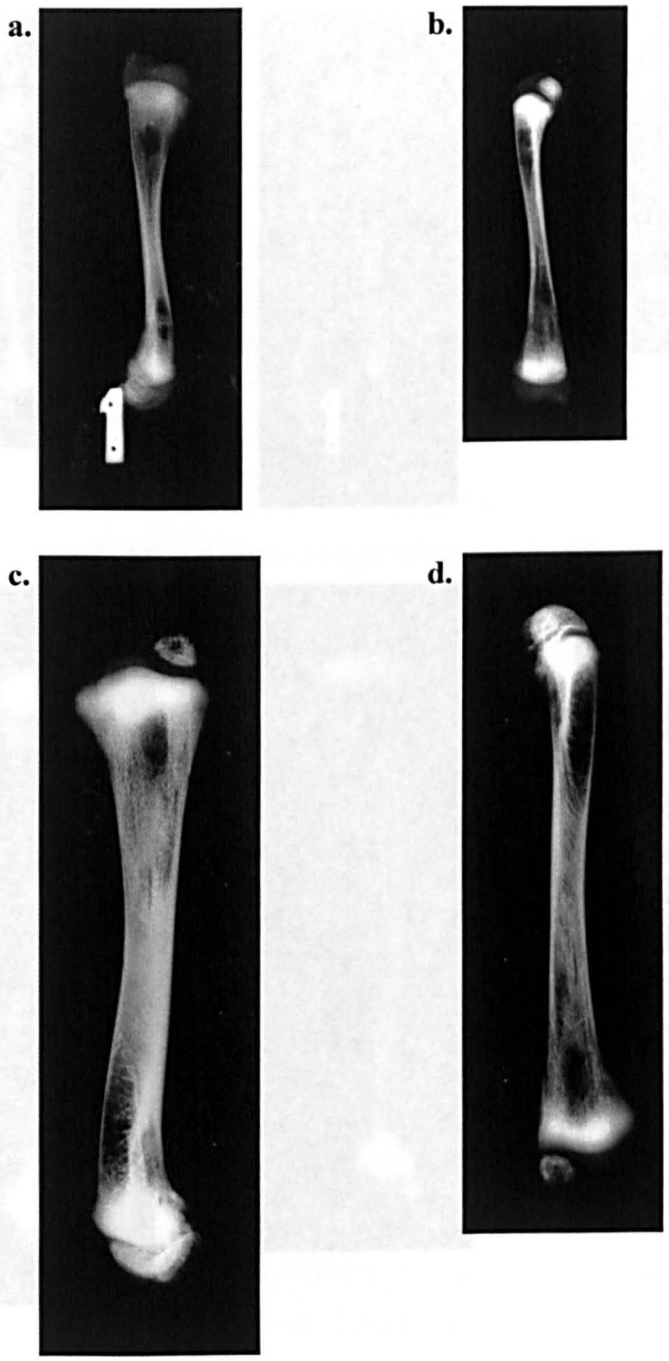
8.4.4: Bone Growth

The variation in bone width and thickness data was inconsistent over treatments, and log transformations were used in their analysis. Cortical thicknesses were similar in the modern strains and greater than in the control strain at both ages (F 37.52, df 2,6, $P<0.001$; Figure 8.2a). Restricted feeding considerably reduced cortical thickness at 15 days, however, this effect was lessened by 42 days due to a slight reduction in the cortical width of the *ad lib* birds (feeding.age interaction F 4.31, df 1,118, $P<0.05$). When covariate analysis was performed to account for body weight, there was a trend for the control strain to have a thicker cortex than the modern strain, but this was not significant. However, by 42 days, cortical thickness at a given weight in the selected strain was found to be considerably increased by restricted feeding (feeding.age interaction F 27.21, df 1,117, $P<0.01$; Figure 8.2b), reaching values very similar to the control strain.

Total tibiotarsus width gave a similar pattern of results to cortical thickness, in that the heavier modern strains had wider bones than the control strains. However, width increased faster with age in the modern strains than the control strain, and the difference was greater at 42 days (strain.age interaction F 3.74, df 2,118, $P<0.05$; Figure 8.3). Restricted feeding reduced bone width in the selected strain (F 164.52, df 1,6, $P<0.001$). When covariate analysis was performed to account for weight, all age, strain, and feeding effects disappeared.

Plate 8.3:

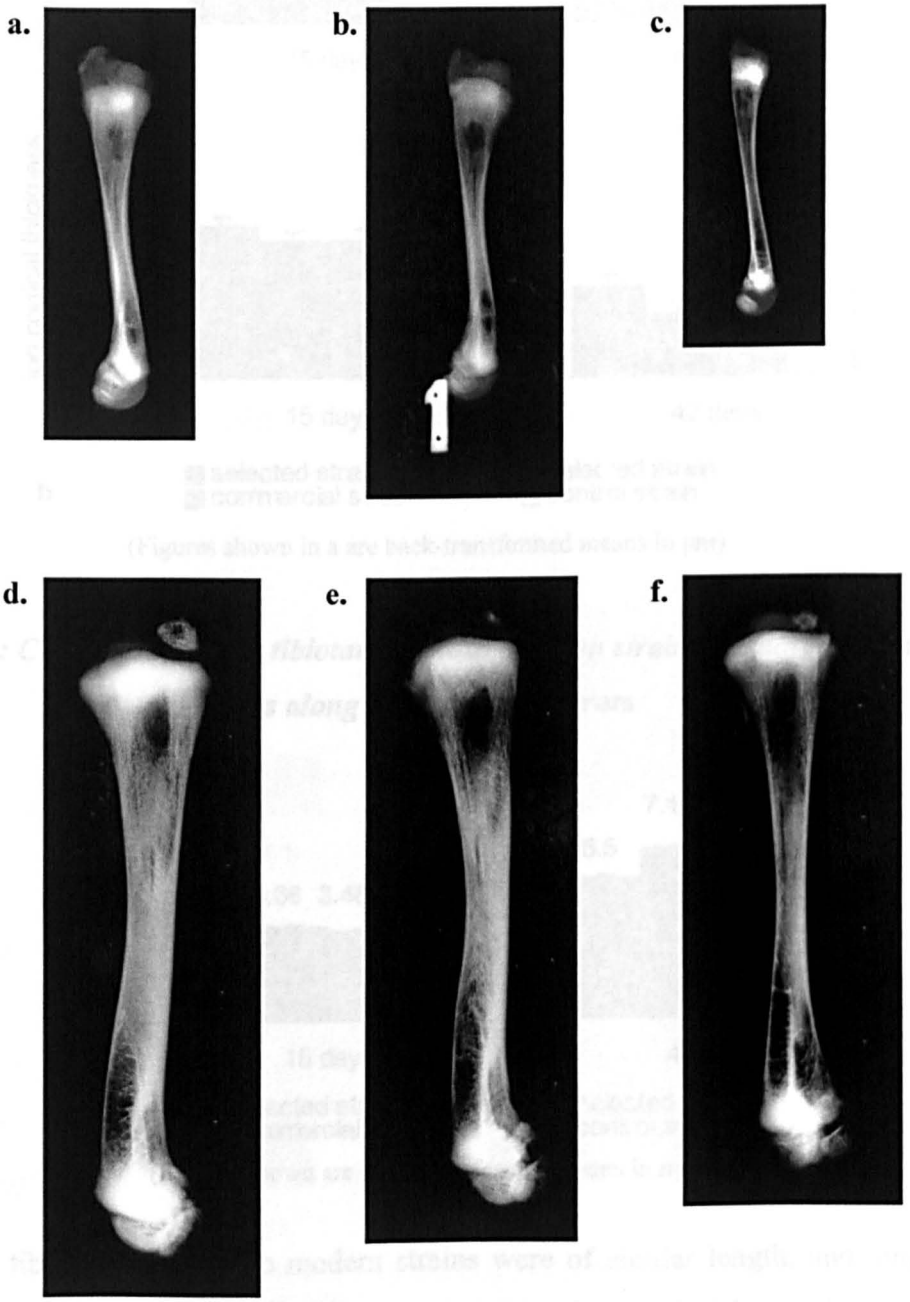
Comparison of bone x-rays at 2 and 6 weeks between ad lib and restricted fed birds of the selected strain



a. ad lib fed at 2 weeks
b. Restricted fed at 2 weeks
c. ad lib fed at 6 weeks
d. Restricted fed at 6 weeks

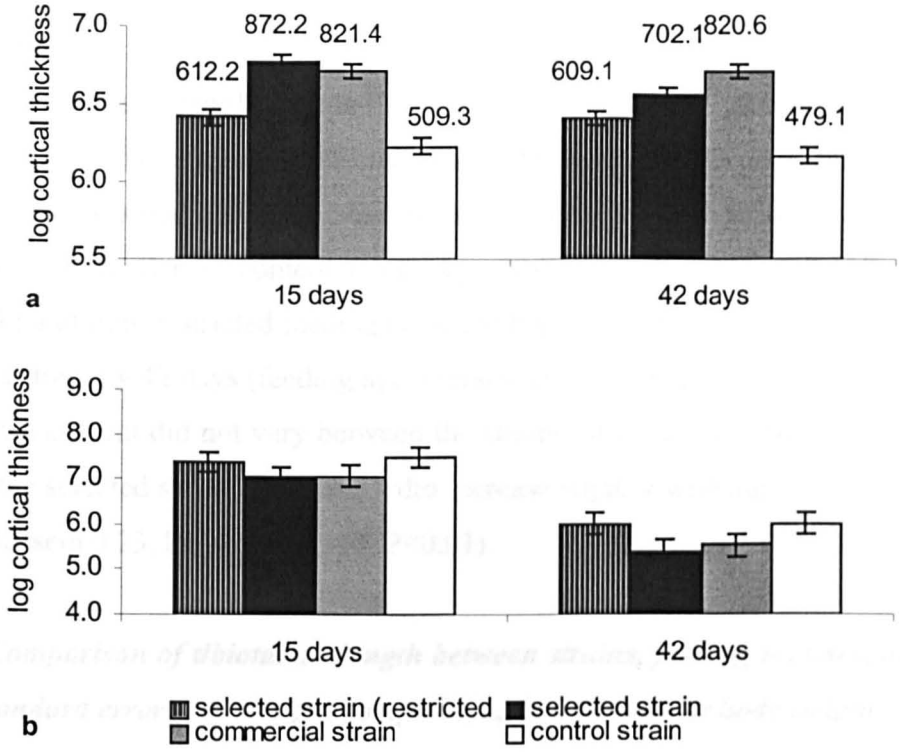
Plate 8.4:

Comparison of bone x-rays between ad lib fed strains at 2 and 6 weeks



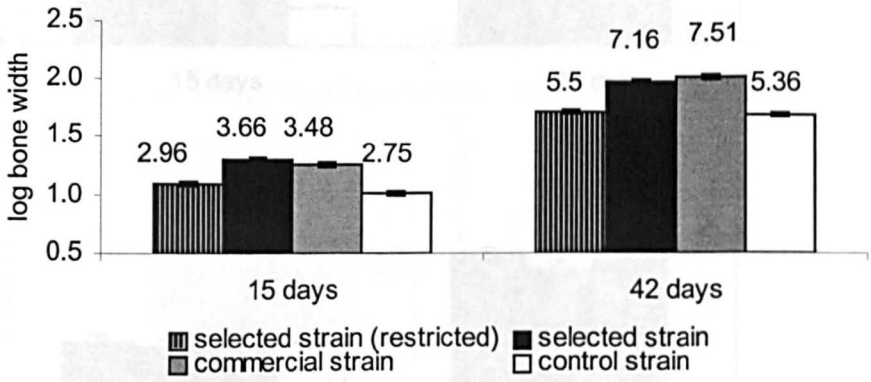
a. Commercial strain at 2 weeks
b. Selected strain at 2 weeks
c. Control strain at 2 weeks
d. Commercial strain at 6 weeks
e. Selected strain at 6 weeks
f. Control strain at 6 weeks

Figure 8.2: Comparisons of log cortical width between strains, feeding regimes, and ages along with standard errors, a. straight comparison, b. adjusted for body weight.



(Figures shown in a are back-transformed means in μm)

Figure 8.3: Comparison of log tibiotarsus width between strains, feeding regimes, and ages along with standard errors



(Figures shown are back-transformed means in mm)

At 15 days, tibiotarsi of the two modern strains were of similar length, and longer than those of the control strain. By 42 days, the commercial strain had longer bones than the selected strain, but again, both modern strains had longer bones than the control strain (strain.age interaction F 12.04, df 2,118, $P<0.001$; Figure 8.4a). Restricted feeding reduced bone length in the selected strain at 15 days, and this effect was larger by 42 days (feeding.age interaction F 29.09, df 1,118, $P<0.001$). When covariate analysis was used to account for the effect of body weight, restricted feeding showed no effect; however, by 42

days, the control strain was found to have longer bones at a given weight than the modern strains (strain.age interaction F 11.40, df 2,117, P<0.001; Figure 8.4 b).

Bone Mineralisation

At 15 days, total bone mineral content (% ash) was similar in all strains, and was not affected by restricted feeding in the selected strain. However, by 42 days, tibiotarsi from the control strain contained a considerably higher proportion of ash than the two modern strain, which were similar in content (strain.age interaction F 3.23, df 2,118, P<0.05; Figure 8.5). In addition, restricted feeding considerably increased ash content in the bones of the selected strain by 42 days (feeding.age interaction F 17.09, df 1,118, P<0.001).

Bone Ca content did not vary between the strains, and was not affected by feeding restriction in the selected strain, however, it did increase slightly with age (15 days, 15.1%, 42 days 16.1%, sem 0.23, F 9.67, df 1,118, P<0.01).

Figure 8.4: Comparison of tibiotarsus length between strains, feeding regimes, and ages along with standard errors, a. straight comparison, b. adjusted for body weight.

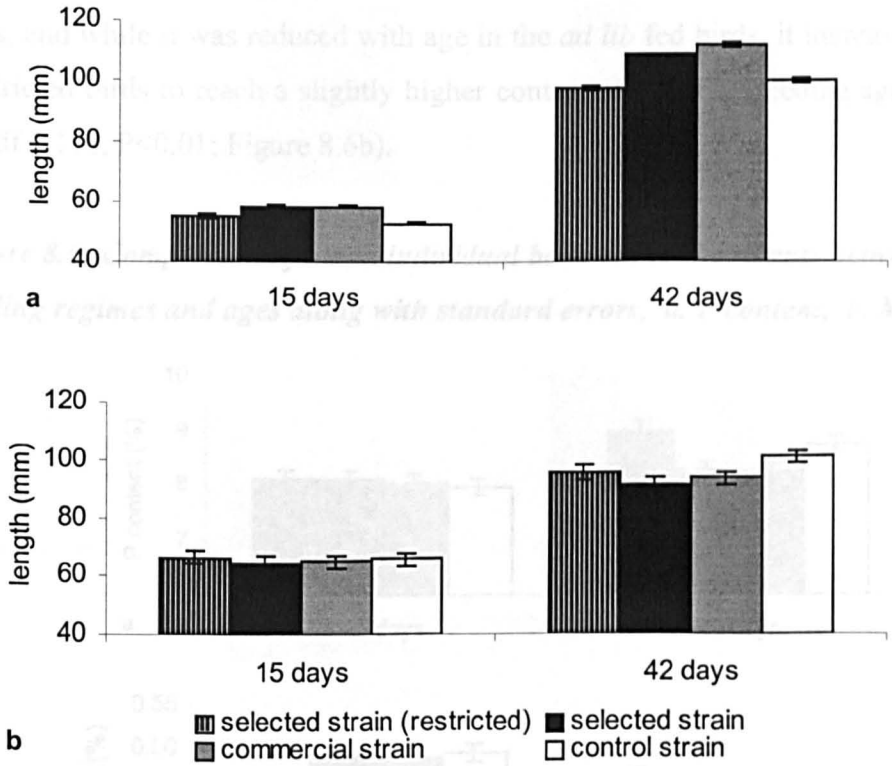
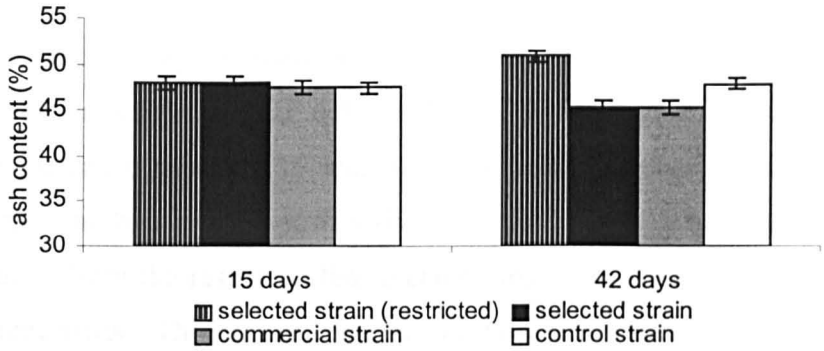
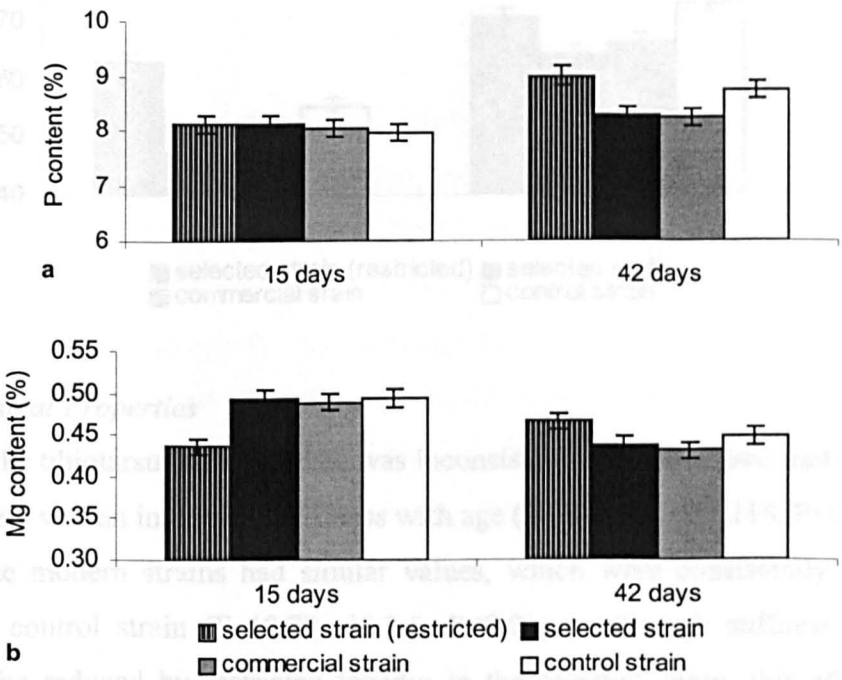


Figure 8.5: Comparison of tibiotarsus total mineral content between strains, feeding regimes and ages along with standard errors



Bone P content was also similar in all treatments at 15 days, and also increased slightly with age; there was a trend for the control strain to have higher a P content, but this was not significant. Restricted feeding resulted in higher bone P contents in the selected strain by 42 days (feeding.age interaction F 5.27, df 1,118, P<0.05; Figure 8.6a). There were no effects on the molar bone Ca:P ratio, however, and the values observed were lower than expected (grand mean 1.45, sem 0.05) . There were no strain differences in tibiotarsus Mg content. However, content was considerably lower in the restricted birds at 15 days, and while it was reduced with age in the *ad lib* fed birds, it increased with age in the restricted birds to reach a slightly higher content at 42 days (feeding.age interaction F 20.13, df 1,118, P<0.01; Figure 8.6b).

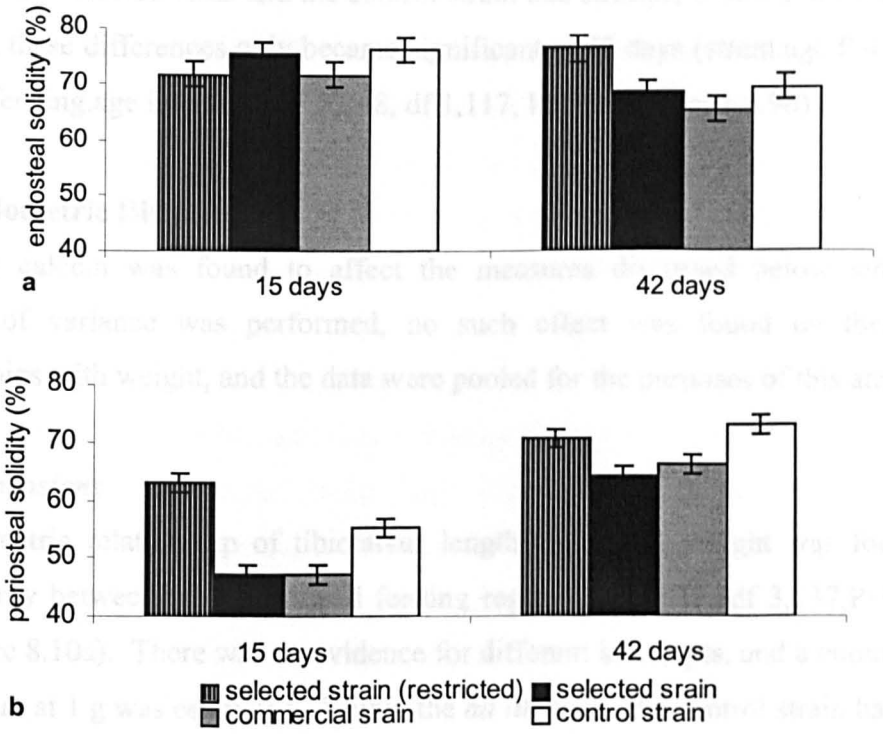
Figure 8.6: Comparison of mean individual bone mineral contents between strains, feeding regimes and ages along with standard errors, a. P content, b. Mg content.



Bone Solidity

There was no difference in bone solidity in the endosteal area between strains. Restricted feeding had no effect at 15 days, however, while solidity was reduced in all the *ad lib* fed birds between 15 and 42 days, solidity increased in this area in the restricted fed selected strain birds (feeding.age interaction F 6.34, df 1,54, P<0.05; Figure 8.7a). Solidity in the periosteal area increased between 15 and 42 days, and was consistently higher in the control strain than the two modern strains (F 12.10, df 2,6, P<0.01; Figure 8.7b). At 15 days, cortical bone from the restricted fed selected birds had a much greater solidity than their *ad lib* counterparts. This was still true at 42 days, however, the effect was lessened (feeding.age interaction F 33.90, df 1,54, P<0.01) due to the smaller increase in solidity over the period 15-42 days seen in the restricted fed birds (improved by 12% of original solidity) when compared to the *ad lib* fed birds (improved by 35% of original solidity).

Figure 8.7: Comparison of bone solidity between strains, feeding regimes and ages along with standard errors, a. endosteal area, b. periosteal area



Bone Mechanical Properties

Variation in the tibiotarsus stiffness data was inconsistent, and the square root was used for analysis. There was an increase in stiffness with age (F 2645.68 df 1,118, P<0.001; Figure 8.8a), and the modern strains had similar values, which were consistently greater than those of the control strain (F 10.79, df 2,6, P<0.01). Although stiffness did show a tendency to be reduced by restricted feeding in the selected strain, this effect was not

significant. When covariate analysis was used to adjust for body weight, the two modern strains again displayed similar values, however, bones of these strains were considerably less stiff than those of the control strain at an average body weight; this effect was greater at 42 days due to a reduction in the stiffness of the bones from the modern strains (strain.age interaction F 12.38, df 2,117, P<0.001; Figure 8.8b). Restricted feeding of the selected strain resulted in greater stiffness at the average body weight, and again, this effect was greater at 42 days (feeding.age interaction F 17.98, df 1,117, P<0.001).

Data on the maximum load endured by the tibiotarsi before rupture were logged before analysis due to inconsistent variation. Maximum load showed a very similar pattern to stiffness. Strength increased with age, and bones from the control strain were consistently weaker than those from the modern strains (F 42.70, df 2,6, P<0.001; Figure 8.9a). The tendency shown by stiffness to be reduced in the selected strain by restricted feeding was echoed by a significant drop in bone strength; this effect was more apparent at 15 days (feeding. age interaction F 7.65, df 1,118, P<0.01). When covariate analysis was used to adjust for body weight, the maximum load pattern again resembled stiffness, the restricted fed selected birds and the control strain had stronger bones at an average weight; however, these differences only became significant at 42 days (strain.age F 4.41, df 2,117, P<0.05; feeding.age interaction F 23.48, df 1,117, P<0.001; Figure 8.9b).

8.4.5: Allometric Differences

Although calcein was found to affect the measures discussed below when split-plot analysis of variance was performed, no such effect was found on their allometric relationships with weight, and the data were pooled for the purposes of this analysis.

Bone Dimensions

The allometric relationship of tibiotarsus length with body weight was found to differ significantly between the strains and feeding regimes (F 126.7, df 3,137,P<0.001; Table 8.3, Figure 8.10a). There was no evidence for different intercepts, and a common intercept of 7.24 mm at 1 g was estimated. Within the *ad lib* birds, the control strain had the longest bones at a given weight (slope 0.369), followed by the commercial strain (slope 0.349), and the selected strain had the shortest bones (slope 0.346). Restricted feeding considerably increased the slope of the relationship, giving the birds longer tibiotarsi than selected birds fed *ad lib* (slope 0.359), however they were still significantly shorter than tibiotarsi from the control strain.

Table 8.3: Comparison of weight effect on tibiotarsus length between strains and feeding regimes

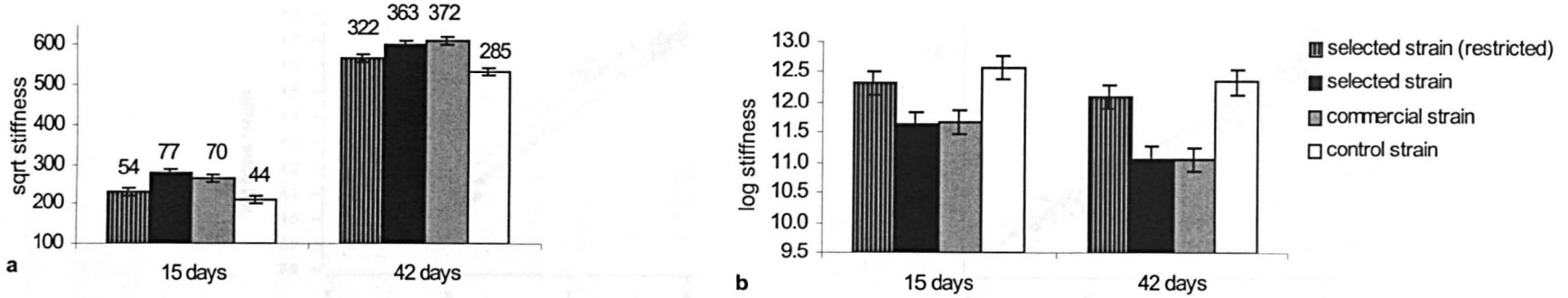
	t_{137}	P<
selected adlib – restricted	10.73	0.001
control – selected restricted	-7.70	0.001
selected – control	17.96	0.001
selected – commercial	2.78	0.01
commercial – control	15.45	0.001

The slope of the allometric relationship of tibiotarsus width with weight also varied (F 3.23, df 3,137, P<0.001; Figure 8.10b). Again, no evidence of different intercepts was observed, and a common intercept of 0.32 mm at 1 g was estimated. No difference was found between the strains (selected slope 0.398; commercial slope 0.400; control slope 0.396), however restricted feeding of the selected strain reduced the slope (slope 0.392, t – 2.33, df 137, P<0.05), giving these birds narrower tibiotarsi indistinguishable from those of the control birds.

Bone Mechanical Properties

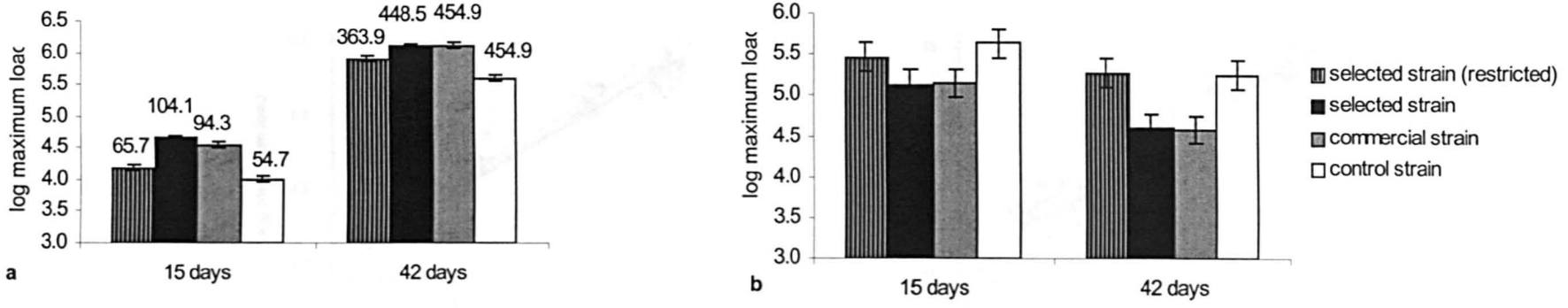
The allometric relationships of maximum load and stiffness with body weight varied between groups (F 7.43 and F 27.72 respectively, df 3,137, P<0.001; Table 8.4, Figure 8.11). The results presented are based on the assumption of common intercepts, however, there was some evidence of differing intercepts, hence they should be treated with caution. The estimated intercept for maximum load was 0.37 N at 1 g. The control birds (slope 0.926) had considerably stronger bones at a given weight than the commercial strain (slope 0.913). The strength of bones from the selected strain was intermediate to both of these at a given weight (slope 0.916), and was indistinguishable from that of either the control or commercial strains. Restricted feeding of the selected strain resulted in much stronger bones (slope 0.938), supporting similar maximum loads to the tibiotarsi of the control strain. The estimated intercept for stiffness was 176.6 N/m. Stiffness was similar in the two modern strains (selected slope 0.985, commercial slope 0.982; Figure 8.11b), and was considerably less than that of the control strain (slope 1.032). Restricted feeding also resulted in much stiffer bones in the selected strain (slope 1.026), again giving values similar to the control strain.

Figure 8.8: Comparison of mean tibiotarsus stiffness between strains, feeding regimes and ages along with standard errors, a. straight comparison, b. adjusted for body weight.



(Figures shown in a are back transformed means in 1000 N/m)

Figure 8.9: Comparison of mean tibiotarsus strength between strains, feeding regimes and ages along with standard errors, a. straight comparison, b. adjusted for body weight.



(Figures shown in a are back transformed means in N)

Figure 8.10: Comparison of allometric relationships of tibiotarsi dimensions with body weight between strain and feeding regime, a. length, b. width

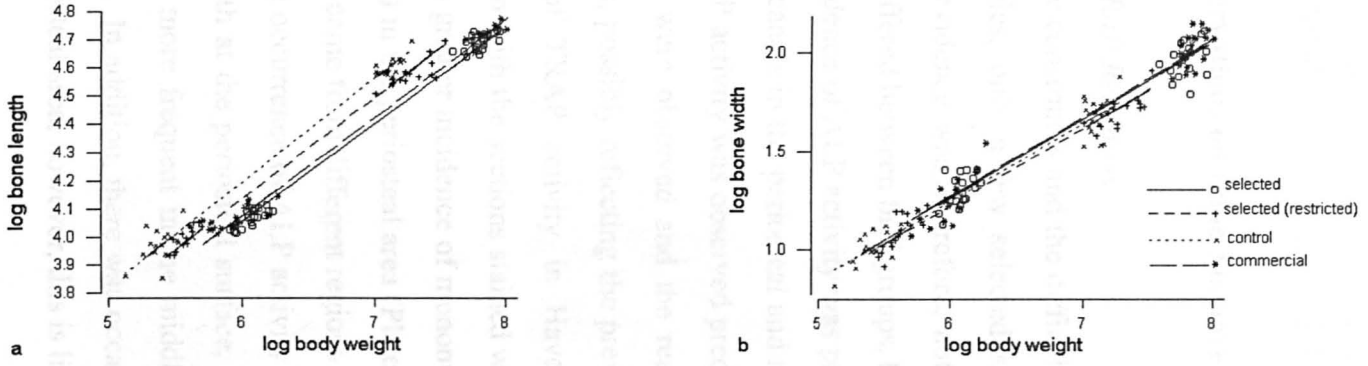


Figure 8.11: Comparison of allometric relationships of bone mechanical properties with body weight between strains and feeding regimes, a. strength, b. stiffness

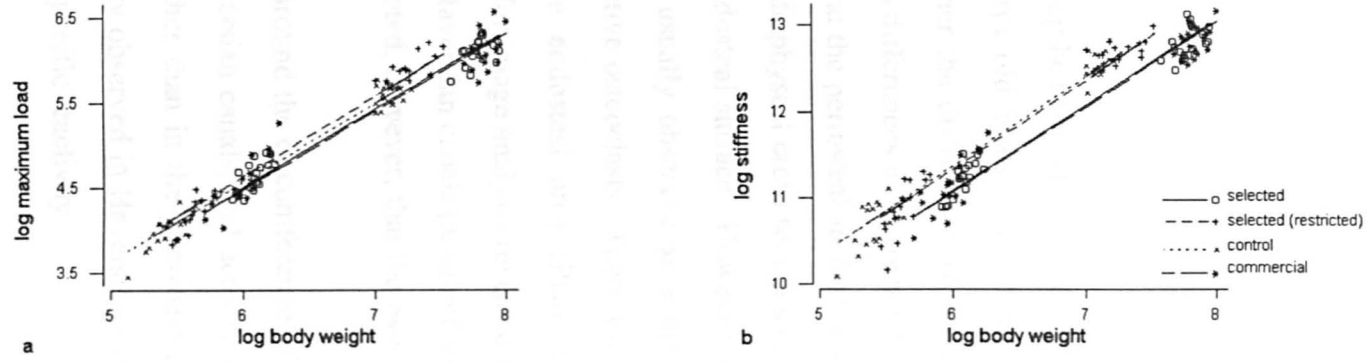


Table 8.4: Comparison of the weight effects on bone strength and stiffness between strains and feeding regimes

	maximum load		stiffness	
	t_{137}	P<	t_{137}	P<
selected *ad lib* – restricted	3.80	0.001	6.11	0.001
control – selected restricted	1.92	NS	-0.92	NS
selected - control	1.71	NS	6.76	0.001
selected – commercial	-0.60	NS	-0.34	NS
commercial - control	2.24	0.05	7.06	0.001

8.4.6: Observations on Bone Turnover

ALP and TRAP Reactions

Due to time constraints and the difficulties inherent in cutting heavily mineralised cortical bone samples, only a few selected samples from 15 days old birds were examined. Complete evidence was, therefore, not available on whether the distribution of reaction products differed between bird groups, however, no obvious differences were apparent.

Evidence of ALP activity was predominantly found at the periosteal surface, and in Haversian canals in the periosteal and middle areas of the diaphyseal cross sections (Plate 8.3). TRAP activity was observed predominantly at the endosteal surface. However, few osteoclasts were observed and the reaction product was usually observed as a diffuse colouration, possibly reflecting the previous presence of active osteoclasts. There was no evidence of TRAP activity in Haversian canals in the endosteal area (Plate 8.3). Comparison with the sections stained with Toluidine Blue for image analysis revealed that there was a greater incidence of mononuclear cells within Haversian canals (assumed to be osteoblasts) in the periosteal area (Plate 8.4). It must, be noted, however, that the two sets of sections came from different regions of the diaphysis.

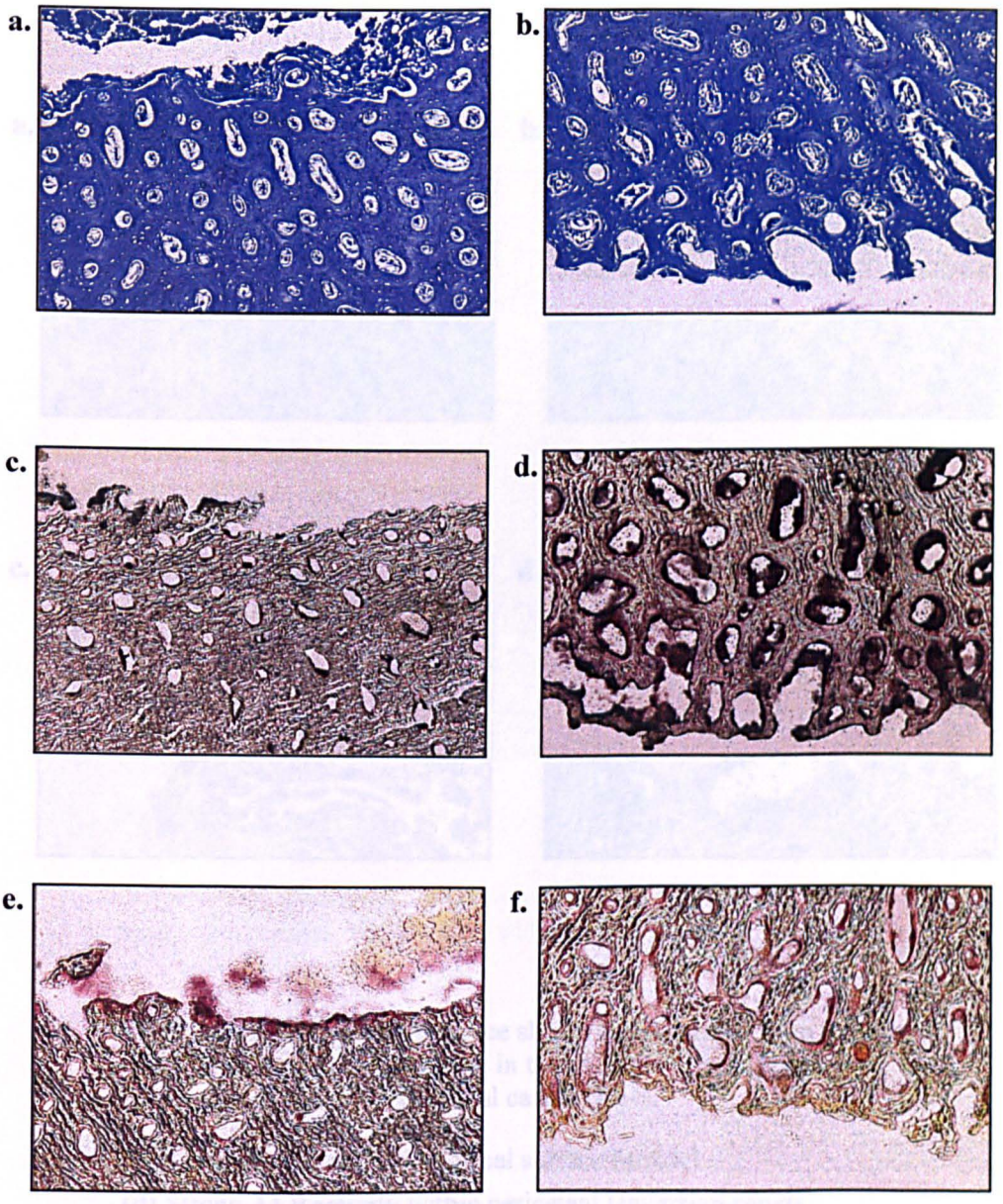
The occurrence of ALP activity was found to vary around the circumference of the section, both at the periosteal surface, and within the Haversian canals, and activity was sometimes more frequent in the middle of the cortex, rather than in the periosteal area (Plate 8.5). In addition, there was occasional TRAP activity observed in Haversian canals in the periosteal area, however, this is likely to reflect non-specific reactivity.

Calcein

The data on bone formation rate from the double calcein injections was not as complete as was hoped for, and no statistical analyses were performed. Some samples had rings which were too faint to measure (classed as missing), some had only one ring; either the inner ring, or occasionally the outer ring, was missing.

Plate 8.5:

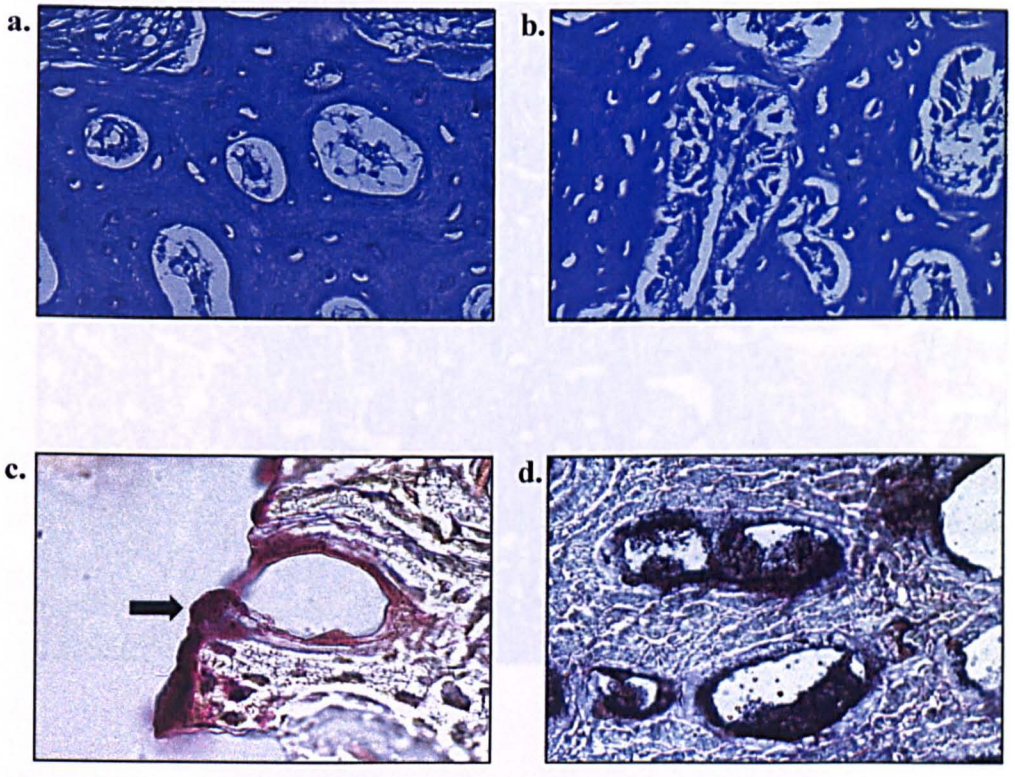
Comparing bone formation and resorption in periosteal and endosteal areas at 2 weeks of age.



a. Endosteal surface (Toluidine Blue)
b. Periosteal surface (Toluidine Blue)
c. Endosteal surface (ALP)
d. Periosteal surface (ALP)
e. Endosteal surface (TRAP)
f. Periosteal surface (TRAP)

Plate 8.6:

Cells present on the surface of bone in the periosteal and endosteal areas.



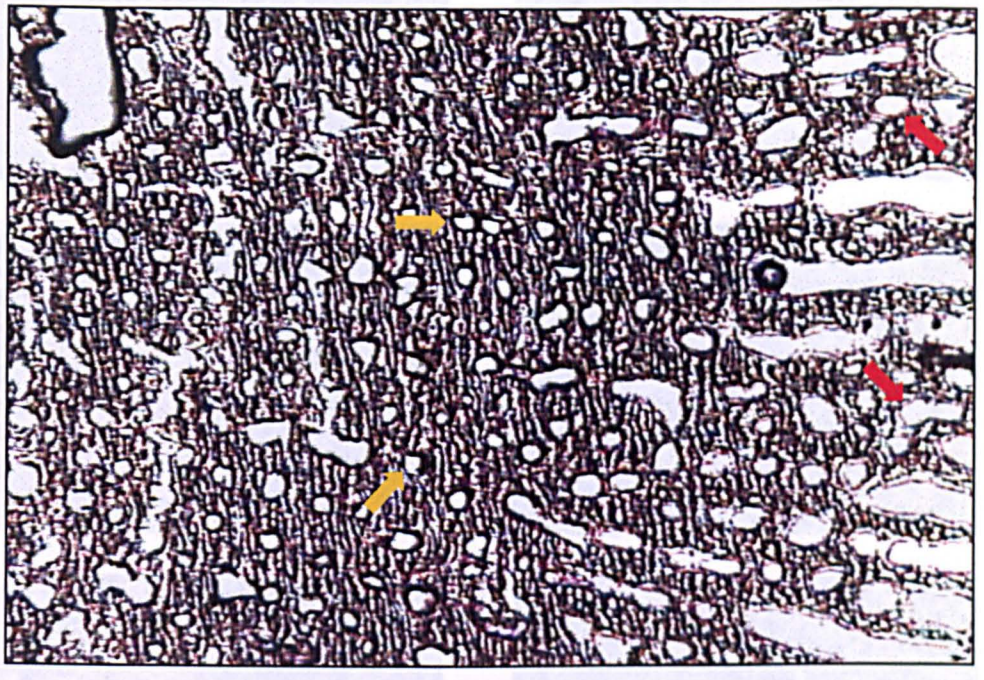
The major histological difference shown by Toluidine Blue staining is the reduction of cell numbers in the endosteal Haversian canals **(a)** compared with the periosteal canals **(b)**.

(c) An osteoclast on the endosteal surface (arrow)

(d) Strong ALP activity within periosteal Haversian canals

Plate 8.7:

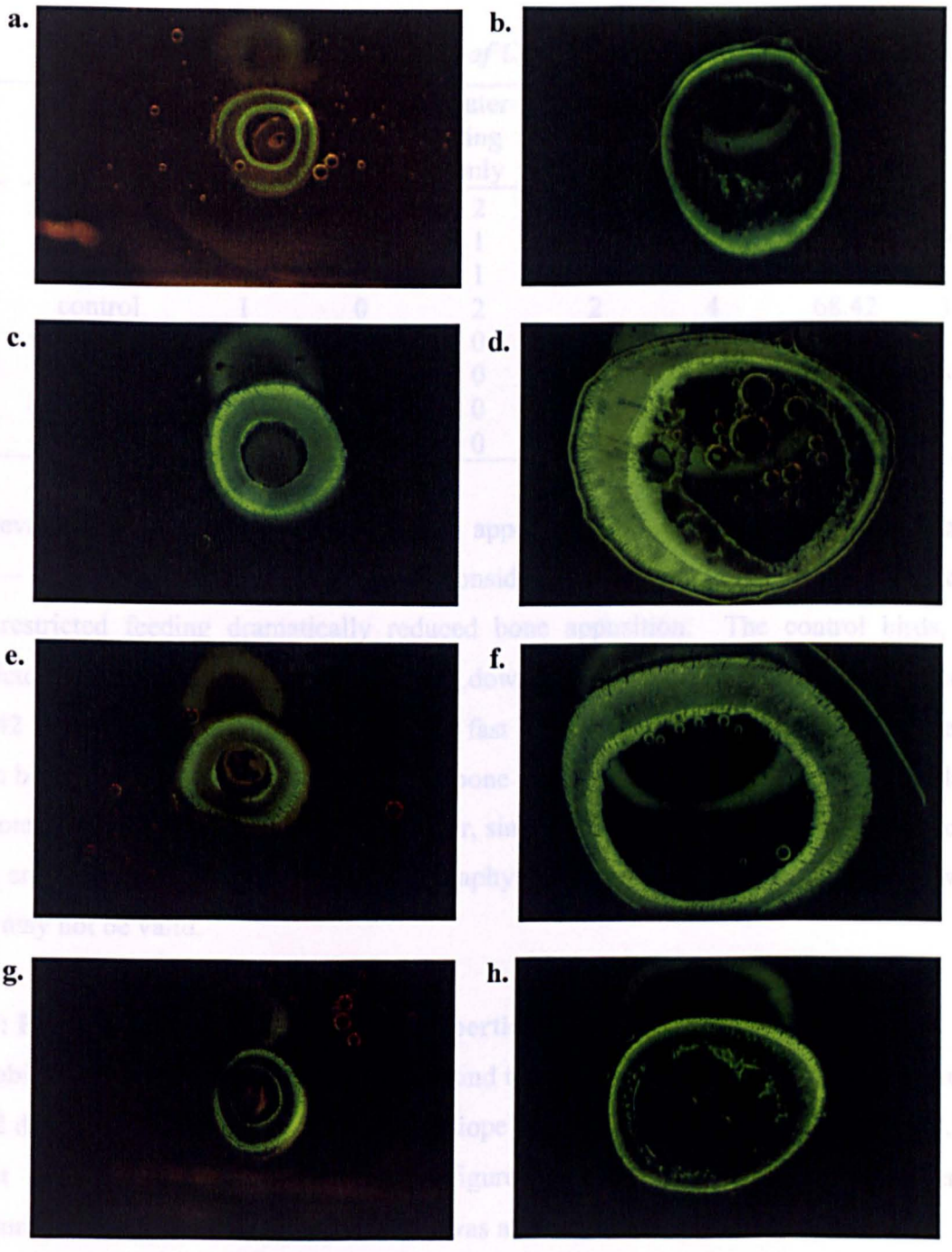
Unusual ALP distribution



ALP staining predominant in the middle of the cortex (yellow arrows) rather than towards the periosteal surface (orange arrows).

Plate. 8.8:

A comparison of bone growth between strains and feeding regimes from 15-42 days, using calcein as a fluorescent marker of the calcification front



a. Restricted-fed selected strain at 15 days.
b. Restricted-fed selected strain at 42 days.
c. Selected strain at 15 days.
d. Selected strain at 42 days.
e. Commercial strain at 15 days.
f. Commercial strain at 42 days.
g. Control strain at 15 days.
h. Control strain at 42 days

The latter samples are assumed to have come from birds where endosteal resorption was occurring so fast that the original marker had been totally resorbed when sampling took place. This is supported by the observation of some samples where resorption of some portions of the inner marker had taken place by the time of sampling. The number of samples falling into each category (out of a total of 9 per group at each age), and the mean thickness of bone laid down per day in those samples with two rings is shown in Table 8.5.

Table 8.5: Results of Calcein injections

age	group	missing	inner ring only	outer ring only	some inner missing	two full rings	mean (μm/day)	SE
15 days	selected (R)	3	0	2	0	4	60.29	2.2
	selected	0	2	1	2	4	98.03	9.69
	commercial	2	1	1	2	3	91.34	13.2
	control	1	0	2	2	4	68.42	3.62
42 days	selected (R)	2	1	0	2	4	61.73	12.2
	selected	1	2	0	1	5	56.14	4.34
	commercial	1	0	0	1	7	101.74	13.7
	control	1	1	0	6	1	55.63	-

However, even this limited amount of data appears to follow the expected pattern at 15 days – the two modern strains laid down considerably more bone than the control strain, and restricted feeding dramatically reduced bone apposition. The control birds, and restricted fed selected birds appeared to lay down bone at a slow but steady rate over the full 42 days, and the commercial bird at a fast but steady rate. The *ad lib* fed selected strain birds appeared to reduce their rate of bone apposition by 42 days, to levels similar to the control and restricted fed birds. However, since data from 15 and 42 day samples came from cross-sections of the mid and distal diaphysis respectively, this comparison between ages may not be valid.

8.4.7: Predictors of Bone Mechanical Properties

The ability of MI to predict stiffness was found to be high, especially at 15 days, however, by 42 days the data became more variable (slope 0.621, SD 0.022 t 28.77, df 140, $P<0.001$; t test assuming a common variation; Figure 8.12a). The ability of radiodensity measurements to predict breaking strength was also high, and apparently consistent at both 15 and 42 days (slope 2.273, SD 0.050, t 45.58, df 140, $P<0.001$; Figure 8.12b)

8.4.8: Effect of Injection With Calcein

The injection of calcein as a marker for bone formation was found to result in tibiotarsi which were narrower at 15 days (calcein.age interaction F 7.67, df 1,118, $P<0.01$; t test

assuming a common variation; Figure 8.13a). These bones were also weaker and less stiff at 15 days (calcein.age interaction, maximum load F 5.49, stiffness F 4.57, df 1,118, $P<0.05$; Figure 8.13b and c). Bone Ca and Mg content were found to be increased by calcein injection, again only at 15 days (calcein.age interaction, Ca F 4.74, Mg F 5.41, df 1,118, $P<0.05$; Figure 8.14). None of these measures were affected by injection with calcein for the 42 day sample.

Figure 8.12: Predictors of bone mechanical properties, a. MI for stiffness, b. radiodensity for breaking strength

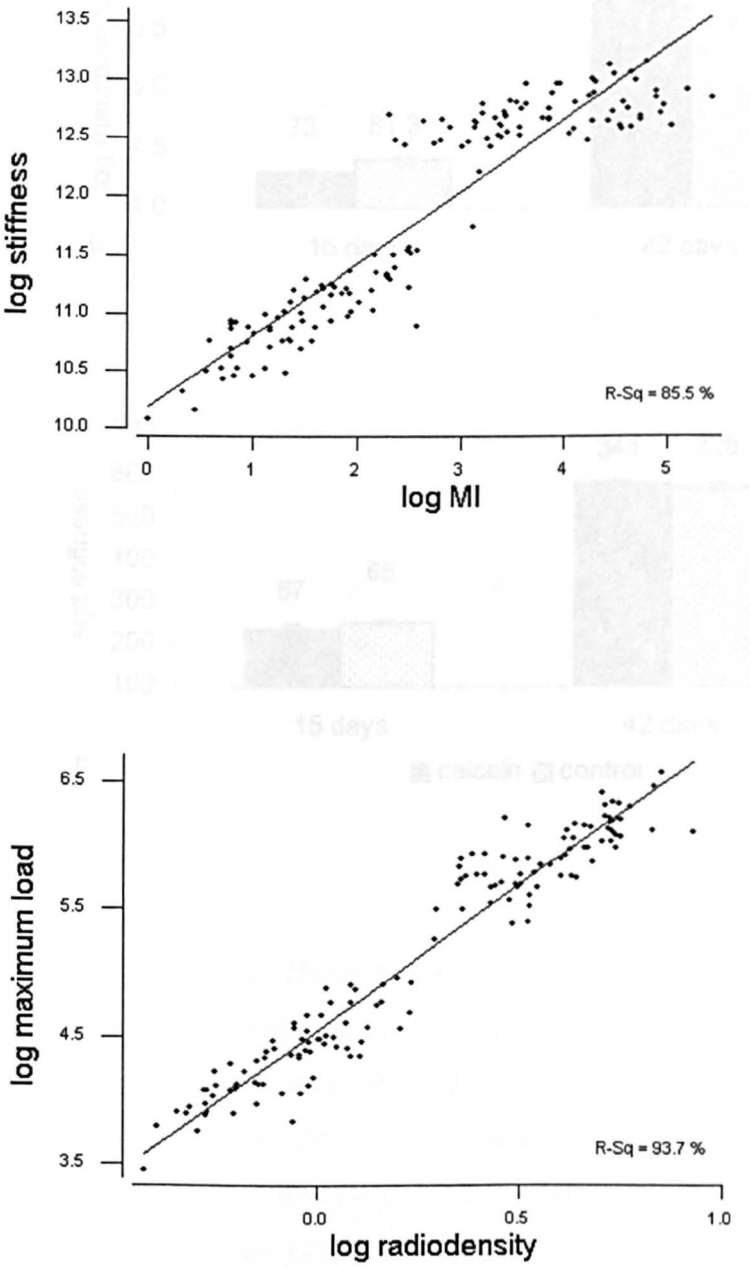


Figure 8.13: The effects of calcein injections on tibiotarsi dimensions and mechanical properties, a. width, b. breaking strength, c. stiffness.

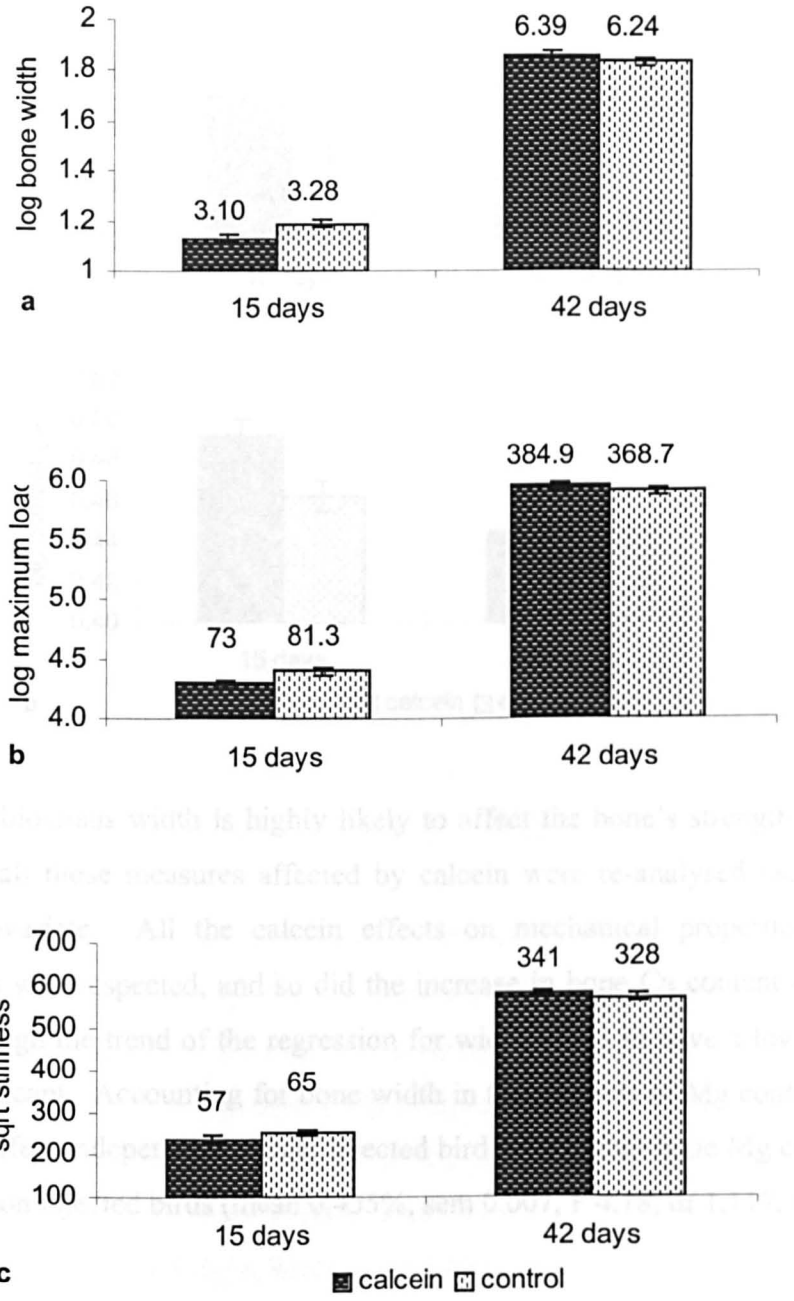
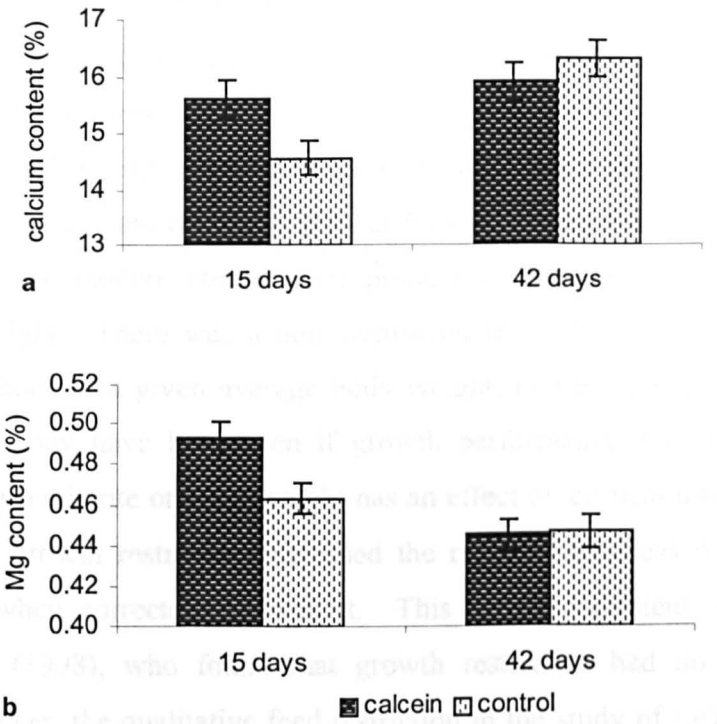


Figure 8.14: The effects of calcein injections on tibiotarsus mineral content along with standard errors, a. calcium, b. magnesium.



Since tibiotarsus width is highly likely to affect the bone's strength and stiffness, the data from all those measures affected by calcein were re-analysed using tibiotarsus width as a covariate. All the calcein effects on mechanical properties at 15 days disappeared, as was suspected, and so did the increase in bone Ca content due to calcein injection, although the trend of the regression for wider bones to have a lower Ca content was non- significant. Accounting for bone width in the analysis of Mg content revealed a direct calcein effect independent of age: injected birds had higher bone Mg contents (mean 0.469%) than non injected birds (mean 0.455%, sem 0.007, F 4.18, df 1,117, $P<0.05$).

8.5: DISCUSSION

8.5.1: Body Weight and Bone Dimensions

As expected, the modern strains achieved much greater body weights than the control strain, and restricted feeding reduced the body weight of the selected strain towards that of the control strain (Figure 8.1). However, the mean weight achieved by the selected strain at 42 days in the present experiment (2441 g) was again lower than that achieved on the Ross farms in the initial study (2902 g by 39 days; Chapter 3). As discussed previously (Chapter 5), this is likely to be due to the superior environmental control on modern commercial broiler farms compared with most research facilities (Nicholson 1998), which

reduces or eliminates the effect of environmental variation on feed conversion and growth rate (Ross Breeders 1996). The modern strains had longer (Figure 8.4a) and wider tibiotarsi (Figure 8.3), with a thicker cortex (Figure 8.2a) than the control strain in line with their greater weight, and restricted feeding of the selected strain resulted in a reduction in all these bone measurements.

However, when analysis of cortical thickness data was repeated with log body weight as a covariate, strain differences in thickness disappeared (Figure 8.2b), hence, in this experiment, the modern strains were producing bone of a sufficient thickness to support their weight. There was a non significant trend for the control strain to have thicker cortical bone at a given average body weight, however, and it is possible that a strain difference may have been seen if growth performance had been optimal. The suggestion that growth rate or body weight has an effect on cortical thickness is supported by the fact that growth restriction increased the relative thickness of the cortex in the selected strain when corrected for weight. This is not consistent with the results of LeTerrier *et al* (1998), who found that growth restriction had no effect on cortical thickness. However, the qualitative feed restriction in the study of LeTerrier *et al* (1998) was relatively mild, using a 28% reduction of dietary energy and protein contents, and it is possibly that the growth restriction was not large enough to produce a detectable effect on cortical thickness.

In the present study, covariate analysis of total bone width data also revealed no difference in width at an average weight between strains, or between feeding regimes. However, regression analysis showed that restricted feeding reduced the slope of the allometric relationship of width with body weight in the selected strain; reducing the growth rate, therefore, reduced the width of the tibiotarsus at any given weight. In a previous study (Chapter 5) control birds were found to have wider tibiotarsi than the selected strain at any given weight, which would suggest the opposite relationship between growth rate and tibiotarsi width, if such a relationship existed. Although the estimated intercepts were very similar in both studies, such a difference was not observed in this study, or in Chapter 3. The reasons for these discrepancies are unknown, as is the mechanism by which the thicker cortex was achieved in the slower growing birds when there was no difference in bone width.

When tibiotarsus length data were subjected to covariate analysis, length was found to be similar at an average weight in all groups of birds at 15 days, however, the control strain had significantly longer bones by 42 days. Regression analysis also revealed that the slope of the allometric relationship of bone length with body weight was larger in the control strain than in the modern strains, however, this relationship varied considerably

between all the groups. The longest bones at any given weight were found in the control strain, followed by the growth restricted birds, then those of the commercial strain, with the selected strain having the shortest bones at any given weight. These results confirm the findings of Chapters 3 and 5 that modern strains of broilers have shorter tibiotarsi than the older, slower growing control strain, and give a similar estimated intercept to Chapter 5. A shorter bone will have a smaller bending moment exerted on it for any given force (Biewener 1992), and intermittent loading regimes have been found to reduce growth in the length of ulnas in rats (Mosely *et al* 1997). It was, therefore, previously suggested (Chapter 5) that the difference in length might be mediated by loading effects on the bone resulting in modified activity in the growth plate. This would reduce the bending moment, and perhaps compensate stiffness for the poorer bone quality seen in fast growing modern strains. Alternatively, the *ad lib* fed modern birds may be reaching a limit to the rate at which bone production can occur at the growth plate. The results from this experiment point to a combined effect of growth rate and/or body weight and genetics in the control of bone length; the two modern strain had similar growth performances, yet different bone lengths, and while growth restriction did give relatively longer bones, they were still significantly shorter than those of the control strain. A similar effect of growth restriction on bone length was observed by Corr (1999).

8.5.2: Bone Pathology

There was considerable variation in pathology incidence (Table 8.2), however it should be pointed out that the small sample sizes preclude any firm conclusions from these data, and may give a misleadingly high impression of the overall incidence of pathology. Hypocalcaemic rickets was not seen at all at 15 days, and was only present in the faster growing birds (*ad lib* selected and commercial) at 42 days. Although this is predominantly a nutritional disorder (Nonindez 1928, Thorp 1994), it is known that there is a genetic component to rickets (Austic *et al* 1977), and it may be that chromosome regions (trait loci) associated with fast growth or body weight contain the genes responsible for susceptibility to rickets. No evidence, however, of such a link has been documented to date to the authors knowledge. Since restricted feeding eliminated the incidence of this pathology in the present experiment, it appears that growth rate, or body weight, may affect its incidence directly, perhaps through a marginal deficiency in dietary calcium which only becomes critical when rapidly producing large bones. Previous work on starter mineral requirements showed that the selected strain required more Ca than currently recommended to reduce growth plate pathology during the starter period (Chapter 4, Williams *et al* in press). No such evidence was found for the finisher diets (Chapter 6),

however, the diets with low Ca in that experiment also contained low P, high levels of which are known to exacerbate the rickets-inducing effect of low Ca (Thorp 1994).

The incidence of TD in this experiment followed the expected pattern. The disorder tends to occur early in development when growth is proceeding most rapidly (Lilburn 1994, Marks 1979), and in the present experiment, incidence was higher at 15 than 42 days. TD is also known to be associated with larger breast weights, longer tibiotarsi (Yalcin *et al* 1996), and faster-growing long bones (Riddell 1975b), all hallmarks of the modern broiler. Both the control birds and the restricted fed selected birds were smaller, and grew more slowly than the commercial and *ad lib* selected birds, and they had much lower incidences of TD; in fact the restricted fed birds displayed no TD at all. The results therefore confirm those of other workers (e.g. Edwards and Sorenson 1987, Lilburn *et al* 1989, Poulos *et al* 1978) that restricted feeding reduces the incidence of TD. The lack of any TD in the growth restricted selected birds, and the fact that their *ad lib* counterparts had less than half the incidence of TD than the current commercial strain, suggests that the Ross selection process for breeding is successfully reducing the portion of the population with genetic susceptibility for TD. However, a larger study would be needed to confirm this.

8.5.3: Circulating Mineral Status

There were no differences in circulating mineral content between broiler strains, hence it appears that all the strains achieve similar homeostatic end-points despite their likely differences in mineral demands, and that restricted feeding does not disrupt this in the selected strain. The reason for the rise in total Ca and drop in inorganic P between 15 and 42 days is unknown, and especially surprising in view of the pathology results (Table 8.2). The *ad lib* selected and commercial birds only displayed hypocalcaemic rickets at 42 days, when the bloods results suggest they were *less* likely to be hypocalcaemic at this age. However, blood measurments are instantaneous, while the incidence of pathology is due to the culmulative effect of mineral deficiencies over a period of time. The results do tell us that the fast growing birds are not hypocalcaemic, or hypophosphataemic when compared with the control strain and restricted fed groups.

8.5.4: Bone Turnover

Unfortunately there was not time to produce enough good quality sections for a detailed comparison of ALP and TRAP activity between groups. In addition, a large number of bone apposition rate measurements were lost due to the sheer speed of bone growth in all the bird groups, resulting in the partial or total resorption of many of the inner calcein

rings; in hindsight, shorter intervals between calcein injections may have been more informative.

Despite the limitations, it was possible to obtain a view of what was occurring during diaphyseal bone growth. It is known that periosteal apposition and endosteal resorption occur simultaneously to increase the size of both the bone and the marrow cavity, while keeping the shape of the bone constant (Recker 1992). If we assume that bone apposition rate is similar in the mid and distal regions of the diaphysis, apposition rate appeared to be fairly constant over both ages in all bird groups except the *ad lib* selected group (Table 8.5). Since cortical width was also very similar at both ages in the same three groups, endosteal resorption must also have been occurring at a steady rate. At a younger age, however, a higher proportion of the cortical bone existing at any one point in time would be replaced by this expanding process, leading to the observed higher incidence of inner ring resorption at 15 days. A comparison between the two ages using sections from the same region of the diaphysis would be needed to confirm this. Apposition proceeded at a slower rate in the control and restricted fed selected birds, as expected by the narrower bones achieved, yet the smaller size of the bones meant that the smaller apposition rate had the same relative effect on inner ring presence.

The partial resorption of the inner calcein rings observed even at 42 days suggests that the expansion in bone and marrow cavity width occurred at such a high rate in all these strains of chicken that there should be no need for bone remodelling within osteons – the majority of the cortex was replaced within 12 days before the 42 day sample, simply by the expansion process. Again assuming such processes are similar in all regions of the diaphysis, this is supported, at least at 15 days, by the fact that TRAP activity in non-calcein injected birds was observed only at the endosteal surface; there was no resorption occurring within any of the osteons (Plate 8.3). There was occasionally some reaction product observed in periosteal Haversian systems, however, it seems highly unlikely that this represents osteoclastic activity in an area of intensive osteoblastic bone formation. The reactions used to observe osteoblast and osteoclast activity both utilise the substrate napthol-phosphate, with the biggest difference in the reactions being the pH at which they are performed (ALP pH 9.4, TRAP pH 6.2; Chapter 2). It is possible that areas of particularly intense ALP production were occasionally reacting during the TRAP procedure, despite the non-optimum pH, giving a false positive result. Some diaphyseal sections, stained with Toluidine Blue for solidity measurements, were examined under a polarised light. The images were not of great quality, however, there again appeared to be no evidence of secondary osteon formation – no obvious reversal lines as described by Banks (1986) were observed around any of the osteons, and there appeared to be none of

the surrounding interstitial bone lamellae consisting of the remains of remodelled osteons described by Pritchard (1972). The combination of all this evidence appears to rule out a role for bone resorption in the increased porosity seen in some modern broilers.

Bone formation appeared to be occurring over a much wider area of the distal cortex, with ALP activity being observed predominantly at the periosteal surface (consistent with the positioning of the most recent calcein ring in calcein injected birds), and within Haversian canals in the periosteal- and mid-regions of the cortex (Plate 8.3). When compared with Toluidine Blue stained mid diaphyseal sections, the ALP-active Haversian canals were in regions which also contained large numbers of mononuclear cells, whereas the canals in the endosteal region contained few observable cells of any type (Plate 8.4). This again suggests that within the cortex, bone formation is the major activity. Moving from the periosteal surface towards the medullary cavity, the Haversian canals become smaller as the osteoblasts within them actively lay down new bone which is then mineralised, fitting in with the process of primary osteon formation described by Banks (1986, Chapter 1 p21). The incidence of ALP activity differs around the circumference of the bone, presumably in response to loading patterns. This is consistent with the results of other workers who showed that different areas of the diaphysis display varied rates of bone apposition (e.g. Rodenhoff and Bronch 1971). It can be seen from the calcein sections (Plate 8.6) that bone cross-sections change from circular to oval shapes with age, with the frontal region becoming gradually thicker than other areas, an observation also made by other workers (e.g. LeBlanc *et al* 1986). Occasionally, areas were seen where there was intense ALP activity in the middle of the cortex, with relatively little in the periosteal region (Plate 8.5). It is possible that this represents a change in the loading pattern, and a consequential change in the regions important for bone production. Osteons already produced would be filled in as usual, thus ALP is observed in central Haversian systems. However, new osteons may then have been produced at a lower rate, by fewer osteoblasts, thus less reaction occurred outside this central region. Without serial ALP reacted and Toluidine Blue stained sections, however, it is impossible to confirm this.

At 15 days, the *ad lib* selected and commercial birds were forming new mineralised bone by apposition at a rate (98 and 91 μm/day respectively) approximately 50% greater than that of the control and restricted birds (68 and 60 μm/day respectively). This was still true for the commercial birds at 42 days, however, the ad-lib selected birds appeared to dramatically slow their apposition rate (Table 8.5). It is uncertain whether this is a true representation, since the calcein data was limited and from different diaphyseal regions, however, there was also a drop in cortical width in the ad-lib selected birds at this age

(Figure 8.2a). This may represent an altered bone production profile in the selected strain, however, further work with a larger data set is needed to come to any conclusion.

The mineral apposition rates observed here for the control and restricted fed birds are comparable to the 50 μm/day observed during the first week of chick growth by Sandhu and Jande (1981). However, they are considerably greater than rates observed by these workers at older ages (23-27 μm/day), and the rates of 3-5 μm/day observed by Hudson *et al* (1993) and LeTerrier *et al* (1998). The use of different chicken strains and ages might be expected to lead to some variation in the rates observed between studies, however, such large discrepancies appear strange, and the reason for them is unknown.

8.5.5: Calcein Effects

Calcein is a relatively low affinity Ca ion chelator, which forms fluorescent complexes with Ca, Mg and several other metal ions (Molecular Probes Inc. 1996). The injection of up to 20 mg calcein per 1 kg body weight was not found to affect body weight, and was therefore assumed not to be a toxic dose (Hudson *et al* 1993). However, injected birds were found to have narrower tibiotarsi, which, since bone width is a major factor in these properties, also had lower stiffness and breaking strengths. It is unknown exactly how calcein affects bone growth by apposition, however, it is likely that the binding of ionised Ca disrupts a number of biological processes, hence the toxicity of calcein at high doses. A similar effect on bone width has been seen using injections of tetracycline (Compston pers. comm.), which also binds to the Ca ions at the surface of newly formed bone mineral crystals (Urist and Ibsen 1963). Bone content of both Ca, and Mg which can substitute for Ca (Posner 1987), was higher in injected birds. The effect of calcein on Ca content was found to be due to the reduction in bone width; a trend within treatments was found for narrower tibiotarsi to have higher Ca contents, perhaps due to the slower apposition rate allowing better mineralisation to occur. However, calcein was found to have a direct effect on bone Mg content. The reasons for these results are currently unknown, however, they emphasise the importance of including non-injected birds as controls in any experiment involving calcein as a marker for bone formation.

8.5.6: Bone Solidity

As in the previous study (Chapter 3, Williams *et al* 2000), periosteal solidity increased with age, alongside a reduction in growth rate, and was lower in the fast growing modern strains than in the slower growing control strain. In addition, growth restriction increased solidity considerably, and it was confirmed that the slow growing (control strain and growth restricted selected) birds, with considerably more solid cortical bone, produced

bone by apposition at a much slower rate (Figure 8.7b, Table 8.5). This difference in solidity was also especially obvious at 15 days, during the early period of rapid growth. It is therefore suggested that the differences in growth rate and bone apposition rate are likely to be largely responsible for the differences observed in periosteal bone porosity between the groups.

It is possible that there is a limit to the rate of osteoblast differentiation, or osteogenic cell production, perhaps through competition for limited resources with other body systems, also undergoing rapid growth and development. The growth restriction in the selected strain, and the naturally slower growth rate in the control strain, would then allow more of these limited cell numbers to be directed to filling in the new osteons. However, since bone width is of great importance in bone strength (Schwartz and Biewener 1992), in order to increase the load capacity as rapidly as possible in line with the rapidly increasing body weight, the *ad lib* fed selected birds, and the commercial strain, might be forced to concentrate on expanding the width of the bone by more periosteal apposition, over an ever larger periosteal surface, reducing the osteoblast numbers available for apposition within the new osteons formed. This hypothesis could perhaps be verified by quantitative measurements of the number and activity of osteoblasts within different regions of the cortex, however, such information is not available at present. Perhaps a more simple hypothesis for the higher periosteal solidity in slower growing birds is that when bone is formed more slowly at the periosteal surface, new osteons take longer to become progressively closer to the center of the cortex. This would result in an osteon spending more time in the periosteal area of the cortex being filled in, thus increasing periosteal solidity.

As in the previous study (Chapter 3, Williams *et al* 2000), endosteal solidity decreased with age; unlike the previous study there was no difference between strains. This appears to be due to the selected strain (*ad lib*) achieving greater solidity values than it did in the previous study, which, if endosteal solidity is also dependant on growth rate, may be due to the lower growth performance in the selected strain in the present study. By 42 days, restricted fed selected birds had considerably more solid endosteal bone than any of the *ad lib* fed birds, hence growth rate does appear to significantly affect endosteal porosity. It was previously suggested that the decrease in endosteal solidity observed with age might be due to increasing resorption occurring in these endosteal osteons to augment the rapid resorption at the endosteal surface (Chapter 3, Williams *et al* 2000). It was also suggested that the greater endosteal porosity observed in the selected strain might be due to the need for more rapid medullary expansion, and greater Haversian augmentation. The TRAP staining distribution in the current experiment does not support the augmentation

hypothesis, since no activity was seen in any Haversian canals; however, only a few frozen sections from 15 days samples were prepared. Recent work has shown that the porosity of cortical bone in the endosteal area of ulna cross-sections also increases with age between 1-5.5 years in the rooster; although low levels of bone remodelling were occuring at these ages, again, no evidence of increased resorption in this area was found (Srinivasan *et al* 2000). The mechanism for this increase in porosity with age, which is also seen in humans (Martin *et al* 1980), is uncertain. Impaired osteoblastogenesis or inefficient osteoblasts (Jilka *et al* 1996, Manolagas 1998), osteocyte apoptosis (Tomkinson *et al* 1997) and hypoxia (Dodd *et al* 1999), proximity to osteoclast progenitor pools (Perkins *et al* 1994), and increased osteoclast activity (Okamoto *et al* 1995) have all been suggested as factors.

8.5.7: Bone Mineral Content

There was no difference in bone Ca content between strains or feeding regimes, however, there was an increase in content between 15 and 42 days, consistent with the results of the previous study (Chapter 3, Williams *et al* 2000), and the theory that bone mineral matures by the addition of Ca (McLean and Urist 1968, Glimcher 1976). P content also increased with age, again consistent with the previous study. The increase was larger in the slower growing birds, giving them higher bone P contents by 42 days, although this difference was not quite significant for the control strain (Figure 8.6a). Neither the change in Ca content with age, nor that in the P content with feeding regime, was large enough to affect bone molar Ca:P ratio. The reason for the very low ratios observed, even at 42 days when bone mineral was expected to be mature with a ratio approaching 1.67 to 1 (Pellegrino and Biltz 1968, Legros *et al* 1987), is unknown.

In the previous study (Chapter 3, Williams *et al* 2000), the control strain had consistently better mineralised bones than the selected strain from 4 days onwards. In the present experiment, there was no difference in ash content between strains or feeding regimes at 15 days; again, a role for growth performance in this discrepancy cannot be ruled out. Despite the increase in Ca content with age in all treatments, total bone mineral content in the *ad lib* fed modern strains was reduced between 15 and 42 days; only ash content in the restricted fed selected birds increased. The reason for this is uncertain, however, bone mineral is a complex substance containing much more than Ca and phosphate. Complete elemental analysis of bone mineral would be required for the clarification and explanation of such changes; unfortunately this was not possible with the equipment available.

By 42 days, bones from the control strain and restricted fed selected birds had a much higher total mineral (ash content) than those from the *ad lib* fed modern strains

(Figure 8.5), and again, growth rate appears to be a major factor, this time in the ability of birds to mineralise the bone produced. The blood results suggested that fast growing birds have no difficulty taking up enough minerals for normal homeostatic levels (based on the control strain) to be achieved. Bigger, faster growing birds are likely to have a larger circulatory system, and higher metabolic demands for Ca and P. The fact that their circulatory systems contained similar concentrations of Ca and P to those of smaller, slower growing birds must mean that mineral uptake from the diet has been increased significantly, whether by a simple quantitative mechanism (they ingest more), or by a selective, active process. However, it is not possible to determine from the available data whether the fast growing birds were able to take up enough extra mineral to allow for adequate bone mineralisation in addition to maintaining homeostatic levels, which biologically, have precedence over bone quality. A significant role for dietary mineral content as a limiting factor in total mineral content was not found in a previous experiment (Chapter 4, Williams *et al* in press). To determine whether the low mineralisation is due to a limited mineral uptake would require comparisons between strains and feeding regimes of mineral transport from the gut into the circulation. There is some evidence to suggest such a possibility, for example, differences in gut size and length (Cherry *et al* 1987, Nestor *et al* 1996), enzyme activity (Nir *et al* 1978), and nutritional absorption efficiency (Nir *et al* 1974, Weiss and Diamond 1998) have been found between heavy and light breeds of broiler. In addition, genetic based differences in the efficiency of Ca absorption from the gut have been found in mice (Chen and Kalu 1999). However, this is a complex subject area which could not adequately be entered into within the time frame of this project. If a role for a limited rate of mineral uptake could be ruled out, this would leave the possibility of a limiting rate of bone mineralisation. Assuming substrate supply is not limiting, all chemical processes occur at a specific rate under certain temperature and pH conditions. Since temperature and pH conditions within birds are tightly regulated, it may be that new bone is being produced at a faster rate than the chemical reactions involved in forming bone mineral can occur.

Bone magnesium content was reduced between 15 and 42 days in all the *ad lib* fed birds, including the control strain, but was increased from an initially much lower level in the restricted fed selected birds (Figure 8.6b). Mg has been suggested to have a role in the formation of early bone crystals (Casciani *et al* 1979, Kakei *et al* 1997), and to positively affect bone strength, possibly through effects on osteoblasts, bone formation, and mineralisation (Kenney *et al* 1994). However, the reasons for the results observed in the present experiment are still a matter of conjecture.

8.5.8: Bone Mechanical Properties

The modern strains fed *ad lib* had considerably stiffer (Figure 8.8a) and stronger (Figure 8.9a) tibiotarsi than the control strain in line with their greater weight, and growth restriction of the selected strain reduced both these measures accordingly. However, covariate analysis revealed that at an average weight, the control strain and the restricted fed selected birds had bones with considerably higher breaking strength (Figure 8.9b) and stiffness (Figure 8.8b). This is highly likely to be due to the much lower cortical solidity and mineral content achieved by the modern *ad lib* fed birds, for example, Schaffler and Burr (1988) found that increasing bone porosity reduced the elastic modulus of bone, although in a non-linear manner. Despite evidence of problems with the assumption of a common intercept, the allometric relationships of stiffness with body weight directly mirrored the covariate analysis results; the control and restricted fed selected birds had similar slopes, and the two *ad lib* fed modern strains had similar but much smaller slopes. However, although the commercial strain was found to have a smaller slope of breaking strength with weight than the control strain, the slope of the selected strain was found to be intermediate, and regression analysis revealed no difference in its allometric relationship from the control strain. The model did confirm the covariate analysis finding that growth restriction significantly improved breaking strength.

8.5.9: X-Ray Predictors of Mechanical Properties

Radiodensity is currently being promoted within clinical science as a useful way of predicting bone mineral density (Cosman *et al* 1991) and probability of fracture (Yates *et al* 1995). However, it is uncertain exactly what radiodensity is measuring, since predominantly bone size, but also shape, porosity, mineral content, and marrow cavity content will all affect it in a complex manner (Chapter 3, Appendix 1). Despite this, there was a consistently close relationship of radiodensity with maximum load before rupture (Figure 8.12b), consistent with the results of Fleming *et al* (1994) in laying hens. This confirms it as a useful non-invasive predictor of bone breaking strength. MI (estimated resistance to bending), also had a good relationship with bone stiffness at 15 days (Figure 8.12a), despite the fact that its calculation assumes a regular, circular, hollow tube of solid material. However, the data were more variable in the 42 day samples, and the predictive power of MI was therefore lessened at this age. This might be due to greater variation at 42 days in the porosity and mineralisation of cortical bone, or in the contents of the medullary cavity; however probably the major factor affecting MI's ability to predict stiffness at older ages is the change in bone shape. At younger ages, a cross-section of mid-diaphyseal bone is approximately circular, with cortical width being similar around

the whole section. However, as seen from the calcein sections (Plate 8.6), with age, the shape changes to a more oval one, with bone thickness varying dramatically around the section. The calculation of MI could be modified at older ages to account for the oval shape, which would probably increase the estimates predictive powers significantly.

At present, selection of breeding birds for improved bone mechanical properties is confined to retrospective selection of hens for breeding by testing bone mechanical properties in their progeny (Bishop *et al* 2000). Non-invasive methods of predicting such properties might make the selection process quicker, and simpler. If the techniques could be adapted for use in live birds, the analysis of x-rays, or the digitised fluoroscopy images used by Fleming *et al* (1998), for MI and radiodensity might be useful in aiding the selection process for improved leg health.

8.5.10: Conclusions

This experiment confirmed many of the differences in aspects of bone quality previously observed between the selected and control strains, and the data from the restricted fed selected birds strongly suggests that many of these differences are due to either growth rate or body weight. The high growth rate and/or body weights seen in the *ad lib* fed modern strains were found to negatively affect bone length, cortical thickness, solidity, and mineralisation, bone breaking strength and stiffness, and to increase the incidence of growth plate pathologies when compared with the control and weight restricted birds. In the case of length, the reduction is suggested to be due to a combination of genetic factors, and either a limit in the rate at which the growth plate can produce an increase in length, or a response at the growth plate to improve stiffness and load capacity at higher weights. Further work is needed to distinguish whether growth rate or weight is responsible for the shortening effect in these modern strains of broiler; if growth rate is the important factor, this may herald problems with skeletal size in the future as broilers continue to be selected for faster growth.

All birds achieved similar circulating Ca and P concentrations, hence the fast growing modern strains do not appear to be hypocalcaemic or hypophosphataemic. Although the extent of bone mineralisation is affected by growth rate, it is still uncertain whether this is due to limitations in mineral uptake, or in mineral utilisation. No evidence was found of a role for bone resorption in the high degree of porosity observed in the modern strains, and bone remodelling appears to occur as part of the radial expansion process. The increased porosity at higher growth rates is suggested to occur as a result of a limit in the rate of bone matrix production by osteoblasts, perhaps due to limits in cell

numbers, and the need to concentrate on the rapid expansion in bone width to increase load capacity in the bone as quickly as possible.

Growth restriction in the selected strain considerably improved many aspects of bone quality, however, this was also accompanied by a dramatic reduction in the final weight gained. Further work using a shorter period of restriction to allow compensatory growth to occur is needed to assess whether bone quality at slaughter could be improved in the commercial situation by this method.

CHAPTER 9
GENERAL DISCUSSION

9.1: COMMON ASPECTS OF BROILER BONE DEVELOPMENT

It is known that long bones are initially formed as solid cartilaginous models. During embryonic development, the cartilage undergoes cellular changes characterised by alterations in cell size and matrix deposition; the matrix becomes calcified and is ultimately replaced by bone. In the chick embryo, active osteoclasts have only been observed after 12 days of incubation, hence the replacement of the cartilaginous model and the formation of a marrow cavity must occur after this time. Evidence presented in this thesis suggests that rapid cartilage resorption in the tibiotarsus started before day 16 of incubation (Chapter 7). At this age a marrow cavity was already visible, and cross-sections of diaphyseal bone displayed a maze-like arrangement of thin struts, presumably formed by resorption at a number of foci within the cartilage designed to become cortical bone.

Although tibiotarsus width increased from this stage of incubation to hatch and beyond (Chapters 3 and 7), cortical thickness was actually reduced between incubation and hatch (Chapter 7). In chick embryos, pulmonary respiration begins a few hours before hatching, and an adequate supply of red blood cells, formed in bone marrow, will be essential at this time. It was suggested that the reduction in cortical width between day 16 of incubation and 1 day old may be a mechanism to attain an adequate volume of bone marrow for red cell production. As previously discussed, this reduction in cortical thickness has also been observed in human embryonic tibia, possibly for a similar reason.

During the same developmental period, the maze-like structure of the cortex was filled in by new bone, and diaphyseal cross-sections displayed large Haversian canals by 1 day of age, within osteons which were rapidly mineralised, as evidenced by the rapid increase in bone mineral content. However, the composition of bone mineral in the day-old chick did not have the low molar Ca:P expected for immature bone mineral, rather the ratio was higher than that of the 1.67 to 1 expected for mature hydroxyapatite-like mineral. Large Ca:P ratios have been observed in the medullary bone of hens, which is subject to rapid turnover. These high ratios may reflect a mineralisation strategy applied during periods when the quantity, rather than the composition of mineral is more important in terms of increasing bone strength rapidly. Further work in this area would have been interesting, however, the time, equipment and expertise required was not available, and the topic of embryonic bone mineralisation is peripheral to the main aims of the study.

By 6 days of age, bone mineral content had increased further, and the bone molar Ca:P ratio had regained a low value consistent with the young age of the chick (Chapter 7). In addition, further bone apposition within Haversian canals had resulted in diaphyseal cross-sections displaying the standard pattern displayed in older birds, when two distinct regions are recognisable (Chapter 3). In the periosteal area, Haversian canals were large, and sometimes oval with the long axes radially arranged; this was the area of active bone formation, as supported by the distribution of cells and ALP activity (Chapter 8). New osteons, formed at the periosteal surface by differential osteoblast activity, were gradually filled in by further bone apposition as they approached the centre of the cortical bone. In contrast, in the endosteal area, Haversian canals were much smaller, contained far fewer cells, showed no evidence of either ALP or TRAP activity, and were assumed to be resting, with neither bone formation nor resorption occurring.

Throughout this process, mineralisation of the new bone continued to occur at a rapid rate, until it reached a plateau by approximately 11 days (Chapter 3). After this age, there was only a slight increase in total mineral content with age; cortical bone contents from 14 days onwards were mainly in the range of 55-65%. Concurrent with this mineralisation and the continuous increase in tibiotarsus length and width, there was a rapid increase in cortical thickness from day old until approximately 18 days of age (Chapters 3 and 7). This implies that once the bone marrow volume was sufficient, the balance between endosteal resorption and periosteal apposition shifted in favour of a period of intensive bone formation to provide adequate skeletal support for the growing bird. From day 18, cortical thickness remained fairly constant; further increases in bone strength as the birds gained more weight appeared to be achieved through an increase in tibiotarsus width, and the resulting increase in cortical cross-sectional area.

There was a more gradual change over time in the solidity of bone. An increase in periosteal solidity was seen between 4-42 days of age (Chapters 3 and 8). It is possible that as the growth rate of the birds reduces with age, and the rate of bone formation at the periosteal surface slows, more resources can be directed towards filling in the new osteons, resulting in a natural increase in bone solidity in this area as the birds reach heavier weights. Perhaps a more simple hypothesis is that as bone is formed more slowly at the periosteal surface as the growth rate reduces, new osteons take longer to become progressively closer to the center of the cortex. This would result in an osteon spending more time in the periosteal area of the cortex being filled in, thus increasing periosteal solidity. Neither hypothesis was supported by the data on bone formation rate from calcein injections in Chapter 8, except, perhaps, in the case of the selected strain, however, the data were limited. Conversely, there was a gradual decrease in endosteal solidity with age

(Chapters 3 and 8). It was proposed in Chapter 3 that there is a natural increase in bone resorption in endosteal Haversian canals to augment the resorption occurring over an ever increasing endosteal surface. No evidence of such a process was seen at 15 days (Chapter 8), however, this does not rule out the existence of such a process since only a few samples at one age were assessed for TRAP activity. Recent work by other authors has shown that endosteal solidity in ulna cross-sections also increases with age between 1-5.5 years in the rooster; although low levels of bone remodelling were occuring at these ages, again, no evidence of increased resorption in this area was found. This increase in porosity with age is also seen in humans, however, the mechanism is uncertain; further investigation is needed to fully understand these changes in bone solidity with age.

9.2: FACTORS AFFECTING BONE QUALITY

9.2.1: Bone Length and Width

In general, all the modern broiler strains investigated appeared to produce tibiotarsi of an adequate width to support a given weight when compared with a slower growing control strain (Chapters 3 and 8). However there was some evidence that the fast growing selected strain had narrower bones at a given weight than the control strain (Chapter 5). The reason for this discrepancy is not clear, and it may simply be due to chance. Under normal circumstances, a narrower bone is likely to be less strong, and further investigation might be advisable to rule out a problem in bone width in the modern strains.

Although longer in absolute terms, tibiotarsi in modern strains were found to be considerably shorter than those from the control strain at a given weight (Chapters 3, 5, and 8); this did not appear to be due to current dietary mineral contents being inadequate for the modern strains (Chapter 4). It was suggested that this shortening might be due to either a limit in the rate at which new bone production at the growth plate can occur, or to an adaptive response occurring at the growth plate to reduce the bending moment, and increase bone strength. Growth restriction resulted in a significant lengthening of tibiotarsus in the selected strain at a given weight (Chapter 8), which supports both these hypotheses. Further investigation using a combination of growth restriction and artificial loading is needed to distinguish whether is was the reduced growth rate (indicating a problem) or the reduced weight (indicating an adaptation) that resulted in this lengthening.

However, there was also some evidence for the involvement of genetic factors in tibiotarsus length. Although growth restriction did lengthen tibiotarsi in the selected strain, they were still shorter than the control strain. Furthermore, although both current

commercial strains investigated grew at a similar rate, and achieved similar body weights to the selected strain, the Ross commercial strain had longer tibiotarsi than the selected strain (Chapters 5 and 8). This implies a progressive genetic change towards shorter tibiotarsi, possibly as a side effect of selection against susceptibility to TD, since this disorder is associated with longer, faster growing tibiotarsi, or possibly as an independent genetic adaptation to the larger loads the modern broiler skeleton is expected to carry.

9.2.2: Cortical Bone Mineral Content and Composition

Some of the bone mineral data were inconsistent. For example, in Chapter 3, total bone mineral content was found to be consistently lower in the selected strain than in the control strain from 4 days of age, yet in Chapters 5 and 8, the modern strains achieved similar bone mineral content to the control strain at 14 and 15 days. The reason for this is uncertain; it may be due to variations in bird sampling between the studies, but variation in growth performance between the studies can not be ruled out as a potential factor. However, the bulk of evidence presented in this thesis does point to the bone of modern broilers being under-mineralised with respect to the control strain, possibly from 4 days onwards, and definitely by 45 days.

The possibility that this might be due to inadequate mineral contents in current diets, in terms of the requirements of the modern strains, was investigated, but again, the results were inconsistent. In Chapter 2, no significant dietary mineral effect was observed, although there was a trend for cortical bone ash to be slightly higher in birds fed high Ca diets. However, in Chapter 6, higher dietary mineral content gave higher cortical bone mineral content, despite a smaller range of dietary contents being used; the reasons for this discrepancy are again uncertain, but they may be the same as those for the variation in ash results. The results do tend to suggest that it might be possible to improve bone mineralisation in the modern strains by increasing dietary mineral content, however the lack of a consistent effect may point to the involvement of other, perhaps more important factors. For example, growth rate appears to have an effect in the older bird, as continuous growth restriction of the selected strain from an early age resulted in cortical bone ash contents even higher than those of the control strain by 42 days (Chapter 8).

The mechanism for this improvement in bone mineralisation at a slower growth rate is unknown. As discussed above, the role of a dietary mineral supply limiting to the fast growing birds cannot be ruled out; it is possible that the studies discussed in this thesis were simply not large enough to consistently reveal a dietary effect on cortical bone mineral. Other potential factors include a limitation in the rate of mineral uptake, or a limitation in the rate of uptake into bone, neither of which have been adequately explored

during this project. Further work on a larger scale is needed to confirm and expand the results presented herein, perhaps involving a more comprehensive combination of dietary mineral manipulation and growth restriction to ascertain if the two factors act together or independently. Unfortunately, short term growth restriction during the starter period had no detectable effect on bone mineral content at slaughter age, and a long term approach could not be used commercially due to the dramatic reduction in the body weight.

Data on bone mineral composition were the least consistent. Between days 11-18, abnormally high molar Ca:P ratios were observed in cortical bone of the selected strain in Chapters 3, 4, and 6. However, such high ratios were conspicuously absent in Chapter 5, where it was intended to investigate the reason behind them, and in Chapter 8, the ratios were much lower than expected. A dietary affect on bone mineral content was found in Chapter 4: higher dietary P contents reduced the ratio, however, they also gave high incidences of hypocalcaemic rickets and TD. Although dietary mineral content had a definite effect on bone mineral composition, it seems highly unlikely that the high ratio was caused by a P deficiency in the current diets. High ratios have been observed in medullary bone in hens, which is rapidly turned over, and were also seen in the rapidly mineralising tibiotarsi of day-old chicks of a variety of strains (Chapter 7). It was, therefore, suggested that the high ratios could be due to a mechanism to rapidly increase bone mineral content in the short term; differences in growth performance between studies might then explain some of the discrepancies observed. However, what this mechanism might be, and why, other than in day old chicks, it was only observed towards the end of the period of rapid cortical bone formation, after the rate of mineralisation has slowed, are unknown. Both the development and composition of bone mineral have been the subject of intense debate for a number of years, and that debate appears no closer to a conclusion. It is, therefore, not entirely surprising that with the limited time available to this project, and the limitations in local equipment and expertise in this field, that no clear conclusions can be drawn on this subject. In addition, although considered unlikely, the possiblity of analytical problems in the laboratory cannot be ruled out. For a more useful exploration of broiler bone mineral composition, a collaborative project with a lab which has experience in crystalline chemistry, and access to all the necessary equipment would be required.

9.2.3: Cortical Thickness and Bone Solidity

Cortical thickness at a given weight (Chapter 8) and cortical solidity (Chapters 3 and 8) were significantly lower in the modern strains. Dietary influence on bone solidity was not investigated during the course of this project, however, no evidence was found for an effect on cortical width (Chapter 4). Growth restriction of the selected strain increased both these

measures to values comparable with or better than the control strain. The mechanism of the effect on cortical thickness is uncertain, since no similar effect on bone width was detected. The increase in bone solidity is likely to be directly related to the lower periosteal bone apposition rates observed in the restricted birds. It is possible that there is a limit to the rate of matrix formation by the osteoblast, the rate of osteoblast differentiation, and/or the rate of osteogenic cell proliferation, perhaps due to competition with other rapidly developing body systems. Bone strength is highly related to bone width, and in the fast growing *ad lib* fed modern strains, the best way to increase bone strength quickly is likely to be a rapid expansion in bone width. It is proposed than in the face of limited resources, this periosteal apposition is performed at the expense of bone apposition within the newly formed osteons. The slower growth rate imposed on the restricted selected strain birds, and that naturally occurring in the control strain, resulted in a slower periosteal apposition rate, and perhaps a greater availability of resources, or a more suitable amount of time for bone apposition within the osteons.

9.2.4: Bone Strength and Stiffness

At a given body weight, modern strains of broiler had tibiotarsi which were less stiff, and ruptured under smaller loads than those of the control strain (Chapters 5, and 8). In Chapter 6, both breaking strength and stiffness were found to be affected by dietary mineral content, presumably through the higher bone ash content achieved with higher dietary mineral content in this experiment. In the case of bone strength this was a relatively weak effect in comparison with that of body weight, and therefore bone size, and no dietary effect was detected in Chapter 4. However the effect observed on bone stiffness in Chapter 6 was stronger, and it is suggested that bone composition has a stronger effect on stiffness than on strength. This may mean that a higher dietary mineral content could help reduce the occurrence of some bone deformities, since stiffer bones are less likely to bend constantly under the weight of the heavy modern birds, however, further work is needed to confirm this. Growth restriction was found to considerably increase both breaking strength and stiffness in the selected strain, these measures reaching values comparable to those observed in the control strain at a given weight (Chapter 8). It is highly likely that this was related to the effect of growth rate on cortical solidity, since solidity was found to strongly affect both strength and stiffness, and was reduced in the fast growing *ad lib* modern strains. This may explain why the improvement in strength and solidity achieved by growth restriction in the starter period in Chapter 6 was lost after an *ad lib* finishing period. Due to the process of radial expansion, the relatively solid bone present in the cortex at 15 days was probably replaced by 45 days, and since the birds were

undergoing a period of rapid compensatory growth, the rate of periosteal apposition would be increased, and the new bone would be less solid, and therefore less strong and stiff. It is currently unknown whether bone solidity could be influenced by dietary manipulation, or what aspects of nutrition are important in the production of bone matrix, and further investigations are needed. It is also unknown whether there is a genetic basis to bone solidity in broilers; a retrospective selection programme could be set up in order to examine this possibility, as has been done in laying hens.

9.3: GENERAL CONCLUSIONS

There were some discrepancies in the results presented; these may have been related to the relative lack of environmental control available at Roslin and the resulting inconsistencies in growth performance between studies. Alternatively, they may have been due to sample size and/or experimental design affecting the sensitivity of statistical tests. However, the work contained in this thesis describes the developments of tibiotarsus dimensions, inner structure, and mineral content in the broiler chicken. The processes described are consistent with, and expand on, previously documented information on bone development in birds. It has been confirmed that birds of modern broiler strains adhere to the main developmental process, but it has been shown that there are significant impairments in aspects of cortical bone quality. These included a thinner and more porous cortex, with a lower mineral content, and resulted in the bones of modern birds being significantly weaker and more elastic at a given body weight than those of the control strain. It is likely that these impairments contribute to the high incidence of fracture in birds of slaughter age. The differences between strains were not detected in the egg, and probably develop during rearing. Some evidence for the involvement of a dietary mineral limitation was found in the incidence of growth plate pathologies, and to a lesser extent, cortical bone mineralisation and mechanical properties, and it is recommended that dietary levels of Ca should be increased to 1.1-1.3% for the selected strain broilers. However, no evidence of a dietary effect on other aspects of bone quality was found. Short term periods of growth restriction were found to have no effect on bone quality at market age, despite growth rate significantly affecting most aspects of bone quality. It is possible that the only way to truly improve bone quality in the modern broiler is to slow the growth rate by reversing genetic selection. However, larger dietary trials would be needed to rule out the possibility of a nutritional solution, and the further possibility of selecting for improved bone solidity should be investigated.

APPENDIX 1

RADIODENSITY: FACTORS AFFECTING IT, AND THE QUESTION OF STANDARDISATION

A 1.1: INTRODUCTION

Over the years, a number of non-invasive techniques have been developed to measure bone mass. This was generally done with the purpose of assessing the risk of development of osteoporosis in humans, to help in its diagnosis, and to establish the usefulness of treatment and prevention of the disorder. Currently, the most commonly used methods include single-photon absorptiometry, dual-photon absorptiometry, quantitative computed tomography, and more recently, dual energy x-ray absorptiometry (Cosman *et al* 1991).

Hand radiography preceded all of the above as a way of estimating bone density and bone loss (Albanese *et al* 1969, Mack *et al* 1949). X-ray utilisation in medical science has a long history - scientists were experimenting with quantitative evaluation of skeletal density as early as 1897 (Vose 1966). The technique used in the present study, in its basic principle, is over 50 years old (Mack *et al* 1939). Early versions of the technique, known as photodensitometry, used a reference standard wedge (usually of Aluminium) to compensate for differences in apparent bone density due to variations in voltage settings, exposure times, film quality, and processing (Mack and Vogt 1971). A point on the wedge image comparable to that of the bone image was identified, and the corresponding wedge thickness (mmAl) was used to give an estimate of bone mineral density (Huddleston 1988).

Photodensitometry as it stood then was of limited use due to poor accuracy and reliability (Cosman *et al* 1991, Huddleston 1988). However, although once considered obsolete, recent developments in the basic technique are said to be providing a cheap, practical, and rapid way of determining bone mineral density (Cosman *et al* 1991). Radiographic images are now 'captured' by video camera, and the grey levels of the digitised image are subjected to sophisticated mathematics to combine wedge and bone image data to determine bone mineral density (Adami *et al* 1996, Kleerekoper *et al* 1994).

Referred to as radiographic absorptiometery, the refined technique appears comparable to other current medical techniques in terms of precision, accuracy, and the ability to predict fracture (*e.g.* Kleerekoper *et al* 1994, Yates *et al* 1995). It was therefore decided to utilise this method during the course of the project as an alternative estimate of bone mineral

content. However, initial results gained from Experiment 1 showed little correspondence of radiodensity measurements with bone ash results.

A 1.2: AIMS

Radiodensity values reflect a combination of many factors; the solidity of the bone, its size in terms of the depth of material through which x-rays must pass, and the proportion of mineral and organic contents within that material. There follows an attempt to disentangle the various factors to determine exactly what radiodensity is best at measuring in the broiler tibiotarsus. In particular, the technique of ashing to determine mineral quantity is considerably simpler and less time-consuming than the image analysis currently performed to measure bone solidity; therefore some investigation was made into the possibility of using radiodensity as an estimate of bone solidity.

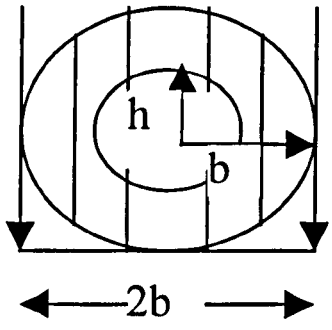
A 1.3: MATERIALS AND METHODS

Information on x-ray image analysis for radiodensity, histological image analysis for bone solidity, and ash analysis can be found in Chapter 2. Data used here were taken from the mid-shaft segment of bones from Chapter 3. Simple linear regression analysis was performed to assess relationships between mid-shaft bone radiodensity and bone width, cortical thickness, mid-shaft mineral content, and bone solidity. The same approach was used to investigate the relationship of radiodensity with the average depth of cortical bone between the x-ray source and the film. If a sample had a rectangular cross-section, its depth could be calculated by dividing its cross-sectional area by its width. In the same manner, the average depth of cortical bone was calculated as:

$$\frac{\pi\left(b^2 - h^2\right)}{2b}$$

where h is the radius of the marrow cavity, b is the radius of the whole bone, and the bone is assumed to be a cylindrical tube (Figure 10.1). Thus, the cross-sectional area of cortical bone is divided by the bone's total width.

Figure 10.1: Calculating the average depth of cortical bone



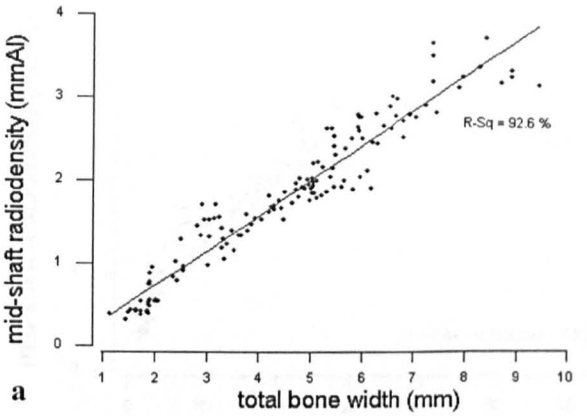
A quadratic regression model was used to assess the relationship of total bone cross-sectional area (πb^2) and cortical bone cross-sectional area ($\pi b^2 - \pi h^2$) with radiodensity. Linear regression analysis was then used to assess how successful some methods of standardising radiodensity were in the prediction of bone mineral content and porosity values. Standardisation was done in a simple manner, by dividing radiodensity by the factor in question.

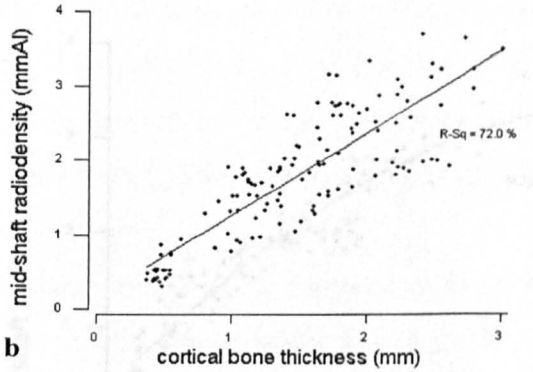
A 1.4: RESULTS

A 1.4.1: Relationship of Bone Dimensions to Radiodensity

All width-, depth- or thickness-based measures of bone dimensions gave positive linear relationships with radiodensity. Total width had the closest relationship to raw radiodensity (slope 0.42, sd 0.01, t_{136} 41.2, $P<0.001$; Figure 10.2a), followed by cortical thickness (slope 1.10, sd 0.06, t_{135} 18.6, $P<0.001$; Figure 10.2b) and the average depth of cortical bone between the x-ray source and film (slope 0.73, sd 0.03, t_{135} 23.72, $P<0.001$; Figure 10.1c). Cross sectional area has a quadratic relationship with radiodensity. Both total ($F_{2,135}$ 832, $P<0.001$; Figure 10.3a) and cortical cross-sectional area ($F_{2,134}$ 695, $P<0.001$; Figure 10.3b) had fits similar in closeness to the linear relationship with total width.

Figure 10.2: The relationship of bone radiodensity with a. total bone width, b. cortical bone thickness, c. average cortical bone depth





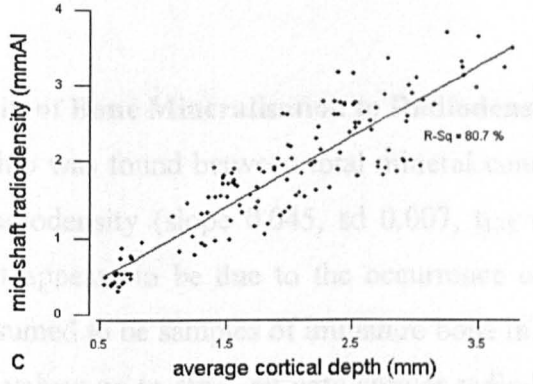
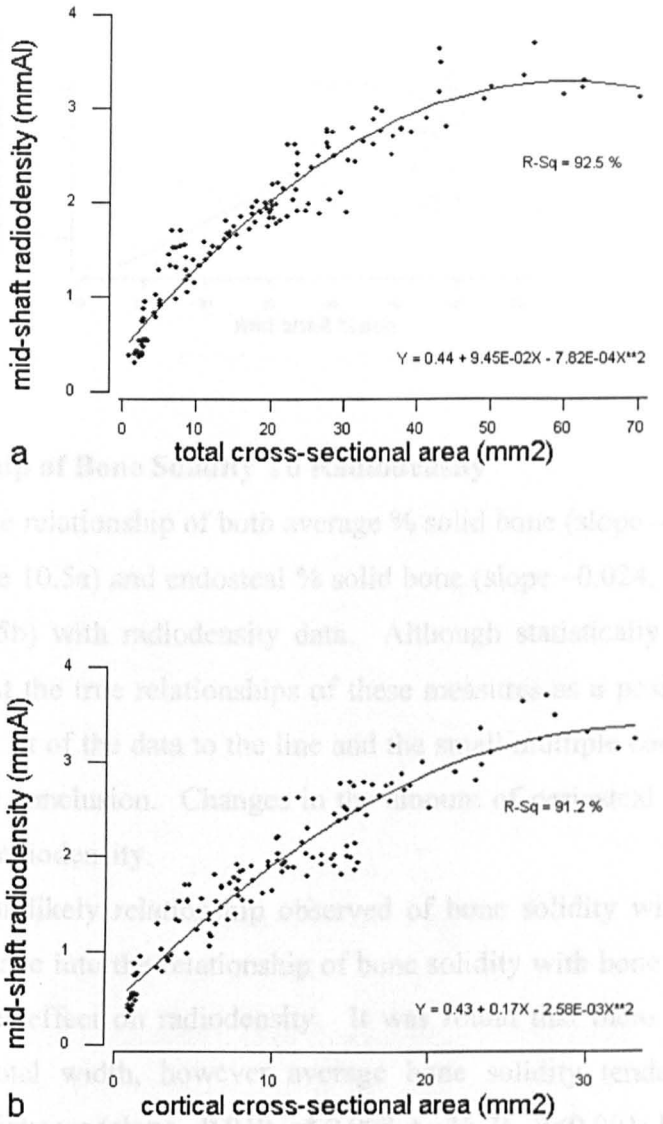


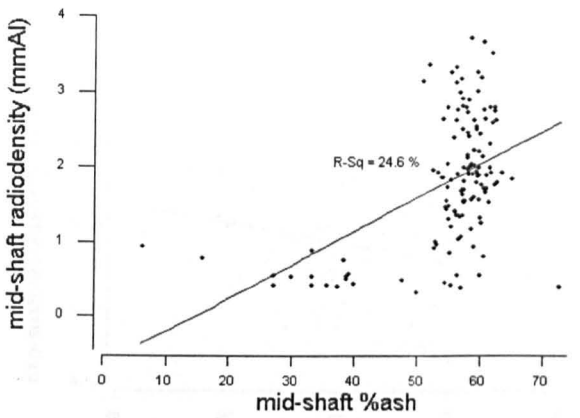
Figure 10.3: The relationship of bone radiodensity with a. total cross-sectional area, b. cortical cross-sectional area



A 1.4.2: Relationship of Bone Mineralisation to Radiodensity

A positive relationship was found between total mineral content (% ash) of the mid-shaft bone section and radiodensity (slope 0.045, sd 0.007, t_{135} 6.64, $P<0.001$; Figure 10.4). However, this result appears to be due to the occurrence of two sub-groupings of data points. What are assumed to be samples of immature bone in the process of mineralisation have a range of ash values up to 40%, all with similar radiodensity. Conversely, samples from mature bone all have similar ash contents (50-60%), but a much larger range of radiodensity values. The fit did not improve if a quadratic model was used, and linear analysis using only data with an ash content value of over 45% gave no significant effect.

Figure 10.4: The relationship of bone radiodensity with ash content



A 1.4.3: Relationship of Bone Solidity To Radiodensity

There was a negative relationship of both average % solid bone (slope –0.03, sd 0.01, t_{116} –2.32, P<0.05; Figure 10.5a) and endosteal % solid bone (slope –0.024, sd 0.008, t_{95} –3.05, P<0.01; Figure 10.5b) with radiodensity data. Although statistically significant, this is unlikely to represent the true relationships of these measures as a positive relationship is expected. The poor fit of the data to the line and the small multiple correlation coefficient (R-sq) supports this conclusion. Changes in the amount of periosteal % solid bone were not found to affect radiodensity.

Due to the unlikely relationship observed of bone solidity with radiodensity, an investigation was made into the relationship of bone solidity with bone dimensions, which had a much stronger effect on radiodensity. It was found that there was no significant relationship with total width, however average bone solidity tended to reduce with increased cortical thickness (slope –0.030, sd 0.008, t –36.76, P<0.001; Figure 10.6).

A 1.4.4: Predicting Bone Solidity and Mineral Content Using Standardised Radiodensity

Standardising by Total Bone Width

Significant negative linear relationships were found between radiodensity standardised in this manner and both average bone solidity (slope –0.003, sd 0.0009, T –3.16, P<0.01; Figure 10.7a) and periosteal bone solidity (slope –0.001, sd 0.0005, T –2.43, P<0.05; Figure 10.7b). Again, any such relationship was expected to be positive, and the fit of the data to the predicted line was poor. There was no relationship observed with endosteal bone solidity.

Figure 10.5: The relationship of bone radiodensity with a. average bone solidity, b. endosteal bone solidity

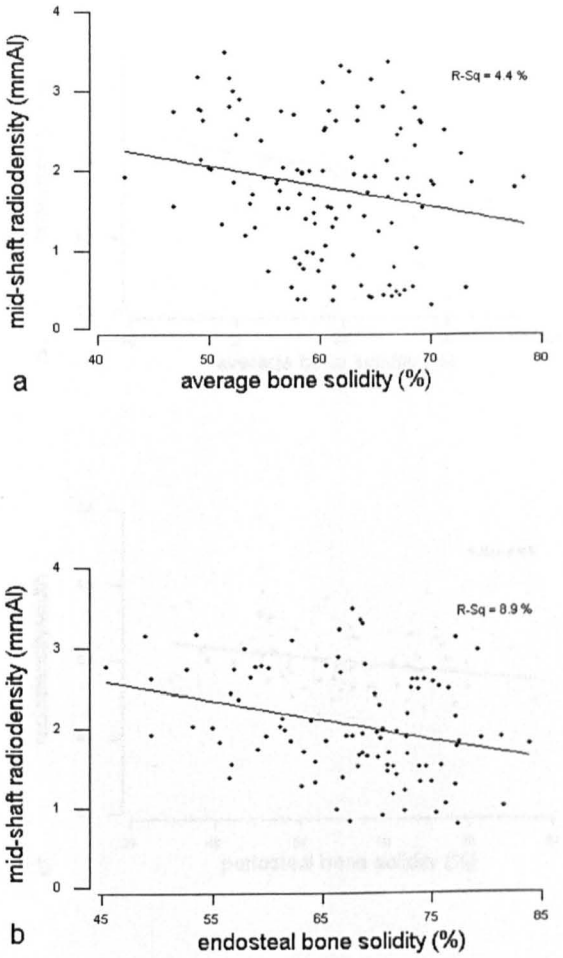


Figure 10.6: The relationship of bone solidity with cortical thickness

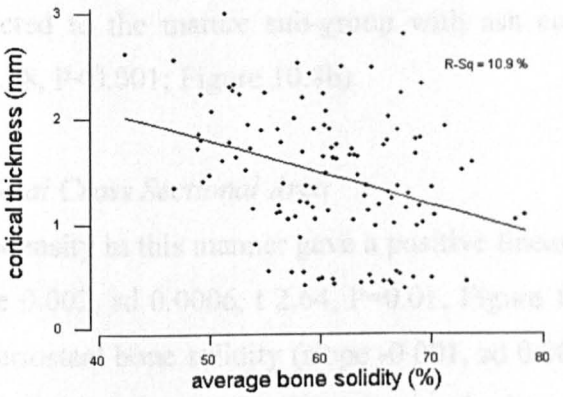
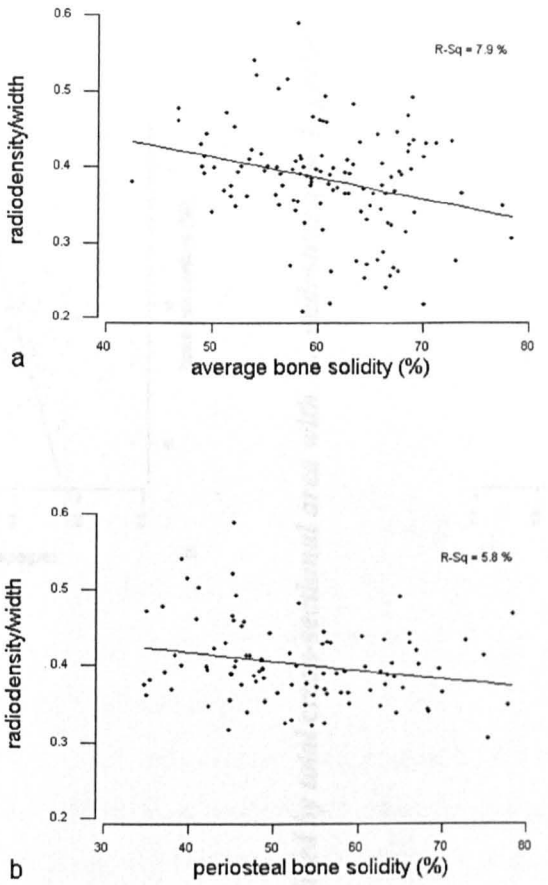


Figure 10.7: The relationship of mid shaft bone radiodensity standardised by tibiotarsus width with a. average bone solidity, b. periosteal bone solidity



A positive linear relationship with bone ash content was observed using this method of standardisation (slope 0.003, sd 0.0006, t 4.55, P<0.001; Figure 10.8a). Again, this relationship appeared to be due to mature bone having a wider range of radiodensity values than immature bone. However, in this case, the linear relationship was maintained if analysis was restricted to the mature sub-group with ash contents of over 45% (slope 0.006, sd 0.002, t 3.68, P<0.001; Figure 10.8b).

Standardising By Total Cross Sectional Area

Standardising radiodensity in this manner gave a positive linear relationship with endosteal bone solidity (slope 0.002, sd 0.0006, t 2.64, P=0.01; Figure 10.9a), and a negative linear relationship with periosteal bone solidity (slope -0.001, sd 0.0004, t –2.88, P<0.01; Figure 10.9b). However, in both cases the fit of the data to the line was poor, and the proposed relationships are likely to be due to the increased spread of radiodensity values corresponding to the high radiodensity end of the predicted line. There was no relationship observed with average bone solidity.

Figure 10.8: The relationship of mid shaft bone radiodensity standardised by tibiotarsus width, with a. bone ash content, b. bone ash content over 45%

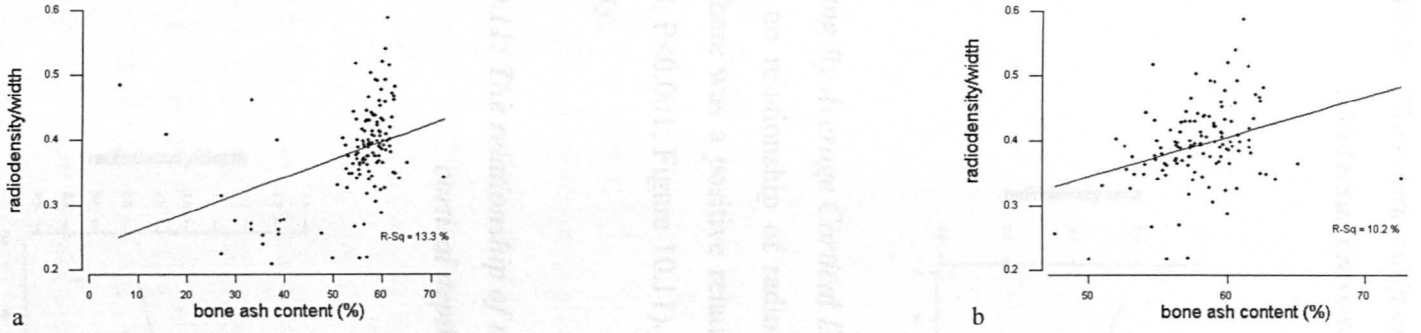
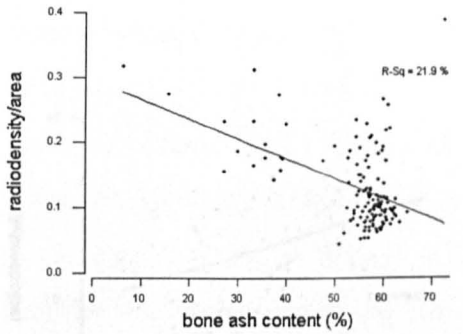


Figure 10.9: The relationship of mid shaft bone radiodensity standardised by total cross-sectional area with a. endosteal and b. periosteal bone solidity

This method of standardisation also gave a negative linear relationship with bone ash content (slope –0.003, sd 0.0005, t –6.16, P<0.001; Figure 10.10). This appeared to be due to an approximate vertical inversion of the raw data points, showing the same large spread of radiodensity/width data at the mature ash values. No relationship was present when ash data over 45% was examined.

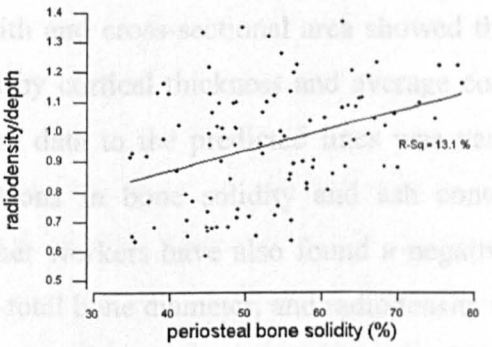
Figure 10.10: The relationship of mid shaft bone radiodensity standardised by total tibiotarsus cross-sectional area with bone ash content



Standardising By Average Cortical Depth

There was no relationship of radiodensity standardised in this way with bone mineral content. There was a positive relationship with periosteal bone solidity (slope 0.007, sd 0.002, t 3.8, P<0.001; Figure 10.11). There was no relationship with average or endosteal bone solidity.

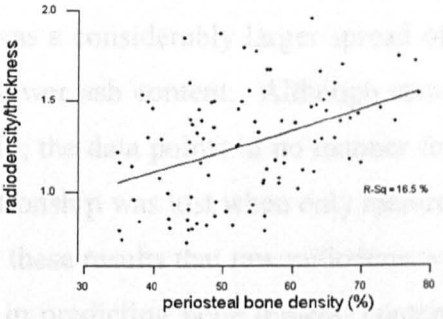
Figure 10.11: The relationship of mid shaft bone radiodensity standardised by average cortical depth with periosteal bone solidity



Standardising By Cortical Thickness

Since cortical thickness was found to have a negative linear relationship with bone solidity, it was decided to investigate the effect of standardising by this measure. There was no relationship between radiodensity standardised by cortical thickness and average or endosteal bone solidity, or with bone ash content. However, there was a positive linear relationship with periosteal bone density (slope 0.012, sd 0.002, t 4.36, P<0.001; Figure 10.12).

Figure 10.12: The relationship of periosteal bone solidity with bone radiodensity standardised by cortical thickness.



A 1.5: DISCUSSION

A 1.5.1: Raw Radiodensity

The use of radiodensity measurements as an estimate of bone mass is essentially based on a simple concept: assuming an absorber consists of only one element, the mass of material present is proportional to the absorbance of an x-ray beam of known quality and intensity (Vose 1966). Although bone is not made up of a single element, this basic theorem appeared to be upheld well in the present study. Bone mass is likely to be strongly related to bone size; total width and cross-sectional area showed the strongest relationships with radiodensity, followed by cortical thickness and average cortical depth (Figures 10.2 and 10.3). The fit of these data to the predicted lines was very close, despite complicating factors such as variations in bone solidity and ash content, and the contents of the medullary cavity. Other workers have also found a negative linear relationship between the ratio of marrow-to-total bone diameter, and radiodensity (Fleming *et al* 1994), *i.e.* for a given total width, an increase in marrow cavity diameter (which reduces cortical thickness) gives a lower radiodensity value.

The observed negative relationships of radiodensity with bone solidity measurements (Figure 10.5) were unconvincing, not least because they implied that

radiodensity was reduced by an increase in bone solidity. However, there was a negative relationship between bone solidity and cortical thickness. This may be due to some sort of compensation effect; thin solid bone may have similar strength to thicker, more porous bone; however, further investigation would be needed to confirm this. As mentioned previously, a positive relationship between cortical thickness and radiodensity was identified. The effect of cortical thickness on radiodensity is likely to be stronger than the smaller difference in bone mass due to variation in porosity, hence it is possible that the negative relationship of bone solidity with radiodensity is due to the concurrent relationship of porosity with cortical thickness.

The relationship observed between radiodensity and bone ash content, although positive, was similarly unconvincing (Figure 10.3). The majority of bone samples contained 55-65% ash, and within this range, which was assumed to correspond to mature mineralised bone, there was a considerably larger spread of radiodensity values than there was for samples with a lower ash content. Although statistically there was a significant positive linear relationship, the data points in no manner followed the pattern of a straight line. In addition, the relationship was lost when only mature samples were investigated.

It is obvious from these results that raw radiodensity measurements from the broiler tibiotarsus are of no use in predicting bone mineral content, or bone solidity; any effects these factors have on radiodensity are swamped by the much stronger dimensional effects. It would be possible to use radiodensity to predict bone dimensions; however, if the original bone sample was available there seems little point, as direct measurement would be simpler and more accurate.

A 1.5.2: Standardising Radiodensity

It is obvious from the results above that in order to estimate changes in bone mineral content or solidity, standardisation by some dimensional factor is necessary. The usual method of standardisation is to divide radiodensity by total bone width (*e.g.* Hagiwara 1998, Hayashi *et al* 1990, Ross 1997), however, cross-sectional area has also been used (*e.g.* Adami *et al* 1996).

Bone is essentially comprised of two major fractions in terms of x-ray absorption: mineral, and water and organic compounds. These have two widely different x-ray absorption co-efficents, and the mean absorption co-efficient of bone therefore depends on the wavelength of the x-ray beam (which was constant in the present study), and the relative proportions of organic and inorganic material (Vose 1966). Thus if radiodensity measurements are standardised for bone dimensions, in a solid bone sample it should be possible to estimate the proportion of mineral present in the sample on this basis; after

standardisation, any change in x-ray absorption is assumed to be accounted for by a difference in mineral content. This has been shown to work well on the metacarpal and phalangeal bones of hands from human cadavers, where correlation co-efficients of over 0.9 have been obtained between bone mineral content estimated from radiodensity data, and bone ash content (*e.g.* Hagiwara *et al* 1998, Yang *et al* 1994).

In the present study, standardising radiodensity by most dimensional factors produced no convincing relationships with bone ash content. Standardisation by total bone width did give a positive linear relationship with bone ash, which also held when investigating only mature bone samples. However, the data fitted the line relatively poorly, and this method could not be used to accurately predict bone mineral content. The shaft of the avian tibiotarsus is more complex in structure than that of the bones of the human hand. The latter are solid cylinders of spongy bone with a thin crust of cortical bone. The tibiotarsus consists of a tube of cortical bone of varying thickness and solidity, within which is a variable mixture of trabecular bone and marrow (Davies and Coupland 1969). Although there is negligible trabecular bone in the mid diaphysis of the tibiotarsus, variability in the shape, solidity, and organic composition of the cortical bone, and variability in the marrow cavity contents, do serve to complicate matters. It is therefore obvious that even after standardising by total width, there are still many other factors in addition to bone mineral content that could account for any variation in radiodensity. Rather than using a cylindrical segment, analysis of a section of the tibiotarsus cortical wall, or of a cross section of the diaphysis, might improve the technique's predictive powers; however, there would still be some interference by varying degrees of cortical porosity. For accurate prediction of bone mineral content in broilers using radiodensity measurement, it may, therefore, be more useful to use solid bones from the feet.

Concerning the prediction of cortical solidity, standardising radiodensity by total width did not overcome the effect of the cortical width–solidity relationship outlined previously, and negative relationships were still observed. A positive relationship was seen with endosteal bone solidity after standardisation of radiodensity with total cross-sectional area. However, this was not a convincing relationship, since it appeared to be due mainly to an increased spread of radiodensity values at higher bone solidity. The most convincing positive linear relationships observed were those with periosteal bone solidity after radiodensity had been standardised by cortical thickness or average cortical depth. However, again, the relationship was not close enough to be able to accurately predict cortical bone solidity. This technique's predictive capability might again be improved by the analysis of a section of the cortical wall or a cross-section of the diaphysis, and the standardisation of radiodensity measurements by both cortical thickness and ash content,

possibly using a multiple regression approach. However, further investigation would be needed to confirm whether such modifications of the method would result in a technique useful in the estimation of bone solidity.

A 1.5.3: Conclusions

Raw radiodensity data showed a close relationship with bone dimensions, particularly total width and total cross-sectional area. However, it showed a very poor relationship with both bone ash content and bone solidity, as any effect that variations of these measures might have on radiodensity were masked by much stronger dimensional effects.

Standardisation of radiodensity data by some form of dimensional data is therefore necessary before any attempt at predicting bone solidity or mineral content can be made. For the purpose of predicting mineral content, standardisation of radiodensity data by total bone width gave the best relationship with bone ash content. However, this relationship was not strong enough to allow accurate prediction of bone mineral content, probably due to variations in cortical solidity and thickness. It is possible that a better prediction of bone mineral content might be achieved by radiodensity measurements of the solid toe bones, however, it is recommended that traditional ashing techniques be employed for this purpose in the broiler tibiotarsus.

It would have been most useful to develop a technique of standardising radiodensity for the estimation of bone solidity. Although standardising by cortical width or average cortical depth gave reasonable relationships with periosteal bone solidity, again these were not close enough to allow accurate prediction. It is evident from this work that the assumption of a symetrical tubular bone shaft is not sufficiently accurate when standardising tibiotarsus radiodensity; more precise dimensional measurements are needed for standardisation in order to better reveal the relationship of radiodensity with both bone ash content and solidiy. It is possible that further modifications to tissue preparation and standardisation techniques might result in a useful alternative method for estimating bone density, however, at present, histological image analysis remains the best option in the tibiotarsus.

APPENDIX 2

ASSESSING METHODS OF PHOSPHORUS EXTRACTION FOR NON-PHYTATE PHOSPHORUS ANALYSIS OF POULTRY DIETS

A 2.1: INTRODUCTION

The amount and availability of minerals in feedstuffs can vary for a number of reasons. For example, soils may vary in their trace mineral content, and plants in their uptake, hence feedstuffs from a particular geographic area may be marginal or deficient in a specific element (NRC 1994). Due to interactions occurring between minerals, an excess of one mineral in the diet may also result in a deficiency in the amount available of another (Mertz 1986). For example, an excess of dietary calcium interferes with the availability of phosphorus, magnesium, manganese, and zinc (NRC 1994). Undigested highly saturated fatty acids may also complex with minerals to form unabsorbed soaps, which are then excreted (Atteh and Leeson 1984). A major consideration in the availability of P in a diet is its form. That P present as ATP or phospholipids in plants, and as P from animal products and supplements is considered to be well utilised (NRC 1994). However, approximately 66% of the P in plant tissue is in the form of phytate P ($_{p}P$; myo-inositol hexabiphosphate), which has a low availability for monogastric species (Williams 1997), and can also affect the availability of other minerals which form a complex with it. Only about 10% of the $_{p}P$ in corn and wheat is digested by poultry (Nelson 1976). Its exact availability is determined by a number of factors. The cations fixed on the phytate ion determine its solubility, while the presence of intestinal phytases, either endogenous or supplemental microbial enzymes, affects its digestion. The presence of minerals such as calcium affects the activity of such phytases, as does the processing the feedstuff has undergone (Nelson 1980).

It is clear then that the factors affecting mineral availability are complex, and its accurate assessment is likely to involve measurements of P intake and excretion from a large number of individually fed and housed animals. This is generally not practical, and therefore for most purposes the majority of minerals in the diet, including non-phytate P ($_{np}P$), are assumed to be fully available, whereas $_{p}P$ is assumed to be totally unavailable. There are now 'book values' available for the proportion of $_{np}P$ in most feed ingredients (e.g. NRC 1994), and these are used in the formulation of many diets. However, since

mineral content can vary between batches of the same ingredient, for those to whom an accurate knowledge of mineral content is important, direct measurement is the preferred option. There is now a wealth of methods that have been developed for the measurement of phytate. These range from the long established phytate precipitation technique using $FeCl_3$ (e.g. Foyand and Thompson 1943, Thompson and Erdman 1982), to improved HPLC methods which can distinguish between the related isomers of myo-inositol produced by phytate hydrolysis, and containing varying proportions of P (e.g. Skoglund *et al* 1997). The majority of these methods include an extraction phase, whereby the total P content ($_{tot}P$) in the feed is extracted using an acid solution (see Table 11.2 for a selection of published methods), and a second phase where $_{p}P$ or $_{np}P$ content is measured.

In a previous large scale experiment on the effect of dietary Ca and available P content on bone development in chicks during the starter period (Chapter 4), an attempt was made to analyse the diets for $_{np}P$ in addition to total Ca and P. After considerable research into the options available, a method using the $FeCl_3$-precipitation technique was adapted from Thomson and Erdman (1982). The results obtained were not what were expected. Samples of the diets had previously been ashed and analysed for total Ca and P by atomic spectrophotometry and colorimetric means respectively (Chapter 2). Samples from the extract phase of $_{np}P$ analysis were analysed for $_{tot}P$, and were found to contain considerably less P than the ashed samples; in fact the extract samples contained $_{tot}P$ at concentrations which closely matched the expected $_{np}P$ content, rather than the $_{tot}P$ expected from ash results. To the best of our knowledge, the precipitation phase for the removal of $_{p}P$ and the subsequent measurement of the $_{np}P$ left in the supernatant appeared to work. However, the estimation of $_{np}P$ was biased due to the lack of a reliable estimation of $_{tot}P$. For the purposes of Chapter 4, and subsequent experiments, it was decided to use the expected values (as per feed formulation) for the $_{np}P$ contents of the diets.

As mentioned previously, most methods of measuring the $_{p}P$ or $_{np}P$ content of a diet include an extraction phase; however, acid type, strength and volume used vary, as does the length of time of the extraction procedure (see Table 11.2). The present four-part experiment was therefore designed to both assess some of the previously published extraction methods, and also to look at how changing various aspects of the methodology and diet composition affects $_{tot}P$ extraction efficiency, and the measurement of $_{np}P$.

A 2.2: AIMS

The main aim of this experiment was to put forward a hypothesis to explain why attempts to analyse diets used in Chapter 4 for $_{np}$P were unsuccessful, and to identify a solution. The first part aimed to assess which, if any, of the selected published methods gave efficient extraction of $_{tot}$P from a standard broiler starter diet. The second part was intended to identify which of the variety of acid types and concentrations suggested in the literature might best extract $_{tot}$P from the same standard diet. A preliminary investigation into the effects of the length of extraction time, and the method used to remove feed particles from the extract solution was also made. The third part used the optimum acid solutions identified in part 2 to investigate further the effect of a variety of factors (extraction time, method of particle removal, acid type, and dietary composition and Ca:P ratio) on the efficacy of $_{tot}$P extraction and $_{np}$P measurement. Some of the results from this investigation suggested a potential problem with the use of filter paper in the analysis of P content. Since this could have major implications for the whole project, ashing analysis of the diets was repeated in the last part of this experiment, and an alternative method of ash removal was investigated.

A 2.3: MATERIALS AND METHODS

A 2.3.1: Feed Samples

Three broiler starter diets were used during this experiment: S, a standard starter diet; H, containing a high calcium content; and L, containing a low calcium content. All three diets were formulated to contain 0.5% $_{np}$P, and samples were ashed and analysed for $_{tot}$P by colorimetric means previous to this experiment. Based on these figures, an arbitrary acceptable range for $_{tot}$P content in this experiment was set as the value obtained by ashing ± 0.05 (Table 11.1).

Table 11.1: Dietary $_{tot}$P contents obtained by ash analysis and accepted ranges

diet	mean $_{tot}$P (%)	accepted range of $_{tot}$P (%)
H	0.81	0.76 – 0.86
S	0.74	0.69 – 0.79
L	0.79	0.74 – 0.84

A 2.3.2: Selected Published Methods

A representative selection of extraction methods available in the literature (Table 11.2) was tested using duplicate samples of diet S. Extraction was performed in 50 ml Falcon tubes on motorised rollers at room temperature. After extraction, samples were either vacuum filtered using Whatman's No. 1 filter paper, or centrifuged, before the solutions were analysed for $_{tot}P$ content by colorimetric means in the same manner as for the ashed samples.

Table 11.2: Selected extraction methods

Source	sample size	amount & type of extracting agent	time extracted	feed removal
AOAC 1990	2 g	20 ml 2.4% (2.2 M) HCl	3 hrs	VF
Thompson & Erdman 1982	2 g	100 ml 1.2% (1.1 M) HCl	2 hrs	VF
Skoglund *et al* 1997	0.5 g	20 ml 0.5 M HCl	3 hrs	C[b]
March *et al* 1995[a]	(1) g	(50) ml 3% (0.7 M) H_2SO_4	1 hr	C[c]

(C centrifugation; VF vacuum filtration; a. amount of feed and volume of acid not specified, values used shown in brackets; b. speed and length not specified, used 2000 g for 5 minutes; c. 6000 rpm for 15 minutes specified – our centrifuge maximum speed of 4500 rpm was used)

Any extracted samples which contained $_{tot}P$ at levels close to the accepted range for diet S were taken on to the second phase of analysis (after Thompson and Erdman 1982). Ten millilitres of the extracted solution were pipetted into a 50 ml falcon tube. Ten millilitres of distilled water were added, followed by 12 ml of $FeCl_3$ (0.2% $FeCl_3.6H_2O$ in 0.53 M HCl). The tubes were placed in a boiling water bath for 75 minutes, allowed to cool for 1 hour, and centrifuged for 15 minutes at 1000 g at room temperature. The supernatant was filtered through No. 1 Whatman's filter paper and analysed for $_{np}P$ content as above.

A 2.3.3: Varying Type and Strength of Acid

Three different acid types at a concentration of 0.5 M were used as extractants: HCl, H_2SO_4, and Trichloroacetic acid (TCA: $C_2HO_2Cl_3$). Two other concentrations of HCl were also used: 2 M and 1 M. The pH of each acid solution was measured using a pH meter (HI 8520, Hanna Instruments). Samples of the diets were mixed with each of the 5 acid solutions for a range of extraction times: 1, 1.5, or 3 hours. A preliminary comparison of the effect of vacuum filtration versus centrifugation as a method of removing the feed from the extract solution was made at 3 hours for all the acid solutions. Duplicate 1 g samples of diet S were extracted in 50 ml of the relevant acid solution. Extraction was again performed in 50 ml Falcon tubes on motorised rollers at room temperature. Samples were then either vacuum filtered as before, or centrifuged at 2000 g for 3 minutes at room temperature to remove the feed, and analysed for $_{tot}P$. Again, any extracted solutions that

fell within the accepted range of values were used in the precipitation phase as above, and the supernatant was analysed for $_{np}$P content.

A 2.3.4: Filtration Versus Centrifuging, Dietary Effect

Duplicate 1 g samples of diets S, H, and L were used. Two acid types, HCl and H_2SO_4, at a concentration of 0.5 M were used as extractants. Fifty millilitres of an extractant was added to each feed sample in a 50 ml Falcon tube, and the tubes were placed on a roller at room temperature for either 1, 2 or 3 hours. For each time-acid combination, samples were either vacuum filtered as above, or centrifuged at 2000 g for 5 minutes at room temperature to remove the feed. The extracted solutions were analysed for $_{tot}$P, and then used in the precipitation phase as above. Supernatants were then analysed for $_{np}$P content.

A 2.3.5: Re-Ashing of Feed Samples

Four 2 g samples of the three diets were weighed out into separate beakers and ashed at 550°C for 16 hours. They were then digested and reconstituted in 6 M HCl as normal, before 2 of each of the four samples per diet were filtered using No. 1 Whatman's filter paper, and the other two were centrifuged at 2000 g for 5 minutes to remove the ash. All solutions were then diluted to 100 ml and analysed for $_{tot}$P as usual.

A 2.3.6: Statistical Analysis

Data from both the extract and supernatant phases of analysis were analysed for main effects and any interactions between factors by ANOVA for unbalanced data in Genstat.

A 2.4: RESULTS

A 2.4.1: Selected Published Methods

The Thompson and Erdman (1982) extraction method gave a mean $_{tot}$P of 0.67% for diet S, just below the accepted range. The subsequent precipitation phase gave a mean $_{np}$P of 0.41%, lower than the 0.5% expected from the formulation. The extraction method of March *et al* (1995) gave a mean $_{tot}$P content of 0.66%, also just below the accepted range. The precipitation phase gave a mean $_{np}$P of 0.43%, again lower than the 0.5% expected. The extraction method of Skoglund *et al* (1997) gave a mean $_{tot}$P content of 0.84%, above the accepted range. The subsequent precipitation phase gave a mean $_{np}$P content of 0.50%, which was exactly the content expected. The official extraction method of the AOAC

(1990) gave a value of only 0.49% for $_{tot}$P content, considerably below the accepted range; the precipitation phase was not performed on these samples.

A 2.4.2: Varying Type and Strength of Acid

The pH of the acid solutions were found to be: 0.5 M TCA, pH 1.18; 0.5 M H_2SO_4, pH 0.97; 0.5 M HCl, pH 0.88; 1 M HCl, pH 0.71; 2 M HCl, pH 0.50. There was no significant effect of the length of extraction time on $_{tot}$P content of extract solutions. The $_{tot}$P content of centrifuged extract solutions was consistently higher than that of those that were vacuum filtered (F 29.39, df 1, P<0.001; Table 11.1). There was also a considerable effect exerted on $_{tot}$P content of the extract by the acid type used ($F_{2,19}$ 100.29, P<0.001), as TCA was highly ineffective at extracting P from the feed samples. Finally, the 0.5 M solution of HCl extracted significantly more P from the samples than either of the other two stronger concentrations ($F_{2,19}$ 8.29, P<0.01). Of the range of acids and times used in this part of the experiment, only 2 centrifuged treatments gave observed results within the accepted range; 0.5 M HCl (0.69% $_{tot}$P), and 0.5 M H_2SO_4 (0.72% $_{tot}$P). These extract samples were precipitated with $FeCl_3$, and the supernatants contained mean observed $_{np}$P contents of 0.48% and 0.46% respectively, both within 0.05 of the expected value of 0.5% $_{np}$P. All other treatments gave observed $_{tot}$P contents below the accepted range.

A 2.4.3: Filtration Versus Centrifugation, Dietary Effect

In this part of the experiment an effect of extraction time was found on both $_{tot}$P content in the extract solution, and on $_{np}$P content in the supernatant after precipitation ($F_{2,36}$ 6.77, P<0.01, and $F_{2,32}$ 3.62, P<0.05 respectively). In both cases, the 3 hour extraction period gave the highest predicted contents, and the 2 hour period gave the lowest predicted P contents (Tables 11.4 and 11.5). No time effect was found on the ratio of total-to-non-phytate P .

The method of feed removal was again found to have a significant effect on $_{tot}$P content of the extracted solution ($F_{1,36}$ 242, P<0.001; Table 11.4), with centrifuged samples of all three diets having higher contents than equivalent vacuum filtered samples. This was also the case for $_{np}$P content in the supernatant for all three diets ($F_{1,32}$ 94.51, P< 0.001; Table 11.5). Furthermore, the ratio of total-to-non-phytate P measured in the feed samples was also significantly higher in centrifuged samples of all three diets than in those that were vacuum filtered ($F_{1,32}$ 18.18, P<0.001; Table 11.6).

Table 11.3: Extraction treatments used in part 2, predicted mean $_{tot}$P contents (%), along with standard errors and observed means

time (hrs)	feed removal	HCl			H_2SO_4	TCA
		2M	1M	0.5M	0.5M	0.5M
1	Vacuum filtration	0.54 ± 0.01 (0.55)	0.54 ± 0.01 (0.55)	0.60 ± 0.01 (0.56)	0.63 ± 0.01 (0.62)	0.42 ± 0.01 (0.45)
1.5	Vacuum filtration	0.53 ± 0.01 (0.54)	0.53 ± 0.01 (0.53)	0.58 ± 0.01 (0.58)	0.61 ± 0.01 (0.61)	0.41 ± 0.02 (0.43)
3	Vacuum filtration	0.55 ± 0.01 (0.56)	0.54 ± 0.01 (0.55)	0.60 ± 0.01 (0.61)	0.63 ± 0.01 (0.61)	0.43 ± 0.01 (0.41)
3	centrifugation	0.62 ± 0.01 (0.60)	0.62 ± 0.01 (0.61)	0.67 ± 0.01 **(0.69)**	**0.70 ± 0.01 (0.72)**	0.50 ± 0.01 (0.47)

(Centrifugation at 2000 rpm for 3 min, figures in bold are within the accepted range; mean observed values in brackets)

Table 11.4: Extraction treatments used in part 3, predicted mean $_{tot}$P contents (%), along with standard errors and observed means

time (hrs)	feed removal	0.5 M HCl			0.5 M H_2SO_4		
		Diet H	Diet S	Diet L	Diet H	Diet S	Diet L
1	C	0.88 ± 0.01 (0.89)	**0.73 ± 0.01 (0.73)**	0.87 ± 0.01 **(0.84)**	0.89 ± 0.01 (0.89)	**0.74 ± 0.01 (0.73)**	0.88 ± 0.01 (0.89)
	VF	**0.76 ± 0.01** (0.75)	0.61 ± 0.01 (0.60)	**0.75 ± 0.01** (0.73)	**0.76 ± 0.01 (0.78)**	0.61 ± 0.01 (0.64)	**0.76 ± 0.01 (0.78)**
2	C	0.87 ± 0.01 **(0.86)**	**0.72 ± 0.01 (0.74)**	0.86 ± 0.01 **(0.83)**	0.87 ± 0.01 (0.88)	**0.72 ± 0.01 (0.73)**	0.87 ± 0.01 (0.89)
	VF	0.74 ± 0.01 (0.74)	0.60 ± 0.01 (0.57)	**0.74 ± 0.01 (0.74)**	0.75 ± 0.01 (0.73)	0.60 ± 0.01 (0.60)	**0.75 ± 0.01 (0.74)**
3	C	0.90 ± 0.01 (0.89)	**0.75 ± 0.01 (0.76)**	0.90 ± 0.01 (0.90)	0.91 ± 0.01 (0.92)	**0.76 ± 0.01 (0.72)**	0.90 ± 0.01 (0.92)
	VF	**0.78 ± 0.01 (0.79)**	0.63 ± 0.01 (0.64)	**0.77 ± 0.01 (0.74)**	**0.79 ± 0.01 (0.75)**	0.64 ± 0.01 (0.64)	**0.78 ± 0.01 (0.76)**

(C centrifugation at 2000 rpm for 5 min, VF vacuum filtration; figures in bold are within the relevant accepted range; mean observed values in brackets)

Table 11.5: Extraction treatments used in part 3, predicted mean $_{np}P$ contents after precipitation (%), along with standard errors and observed means

time (hrs)	feed removal	0.5 M HCl			0.5 M H_2SO_4		
		Diet H	Diet S	Diet L	Diet H	Diet S	Diet L
1	C	**0.53 ± 0.01** (0.54)	**0.47 ± 0.01** (0.47)	**0.51 ± 0.01** (0.50)	**0.50 ± 0.01** (0.50)	0.44 ± 0.01 (0.44)	**0.49 ± 0.01** (0.48)
	VF	**0.48 ± 0.01** (0.49)	0.42 ± 0.01 (0.40)	**0.46 ± 0.01** (0.46)	**0.46 ± 0.01** (0.47)	0.40 ± 0.01 (0.39)	**0.44 ± 0.01** (0.45)
2	C	**0.52 ± 0.01** (0.51)	**0.46 ± 0.01** (0.46)	**0.51 ± 0.01** (0.50)	**0.50 ± 0.01** (0.52)	0.44 ± 0.01 (0.46)	**0.48 ± 0.01** (0.49)
	VF	**0.48 ± 0.01** (0.47)	0.42 ± 0.01 (0.44)	**0.46 ± 0.01** (0.44)	**0.45 ± 0.01** (0.44)	0.39 ± 0.01 (0.39)	0.44 ± 0.01 (0.44)
3	C	**0.54 ± 0.01** (0.52)	**0.48 ± 0.01** (0.48)	**0.52 ± 0.01** (0.53)	**0.51 ± 0.01** (0.51)	**0.46 ± 0.01** (0.44)	**0.50 ± 0.01** (0.50)
	VF	**0.49 ± 0.01** (0.50)	0.43 ± 0.01 (0.44)	**0.47 ± 0.01** (0.49)	**0.47 ± 0.01** (0.46)	0.41 ± 0.01 (0.40)	**0.45 ± 0.01** (0.45)

(C centrifugation at 2000 rpm for 5 min, VF vacuum filtration; figures in bold are within the relevant accepted range; mean observed values in brackets)

Table 11.6: Extraction treatments used in part 3, predicted ratios of total to non-phytate P along with standard errors and observed means

time (hrs)	feed removal	0.5 M HCl			0.5 M H_2SO_4		
		Diet H	Diet S	Diet L	Diet H	Diet S	Diet L
1	C	1.67 ± 0.03 (1.65)	1.57 ± 0.03 (1.55)	1.72 ± 0.03 (1.68)	1.77 ± 0.03 (1.78)	1.67 ± 0.03 (1.66)	1.82 ± 0.03 (1.85)
	VF	1.58 ± 0.03 (1.53)	1.46 ± 0.03 (1.50)	1.61 ± 0.03 (1.59)	1.67 ± 0.03 (1.66)	1.57 ± 0.03 (1.64)	1.71 ± 0.03 (1.73)
2	C	1.66 ± 0.03 (1.69)	1.55 ± 0.03 (1.61)	1.70 ± 0.03 (1.66)	1.76 ± 0.03 (1.69)	1.65 ± 0.03 (1.59)	1.80 ± 0.03 (1.82)
	VF	1.55 ± 0.03 (1.58)	1.45 ± 0.03 (1.30)	0.60 ± 0.03 (1.68)	1.65 ± 0.03 (1.66)	1.55 ± 0.03 (1.54)	1.70 ± 0.03 (1.68)
3	C	1.68 ± 0.03 (1.71)	1.57 ± 0.03 (1.58)	1.72 ± 0.03 (1.70)	1.78 ± 0.03 (1.80)	1.67 ± 0.03 (1.64)	1.82 ± 0.03 (1.84)
	VF	1.57 ± 0.03 (1.58)	1.47 ± 0.03 (1.60)	1.62 ± 0.03 (1.51)	1.67 ± 0.03 (1.63)	1.57 ± 0.03 (1.60)	1.72 ± 0.03 (1.69)

(C centrifugation at 2000 rpm for 5 min, VF vacuum filtration; mean observed values in brackets)

However, a significant difference was found between diets in the measured P contents achieved for equivalent treatments ($_{tot}$P: $F_{2,36}$ 158.47, P<0.001; $_{np}$P: $F_{2,32}$ 54.83, P<0.001). Vacuum-filtered extract solutions of diets H and L were generally on, or just below (4 out of 12 observed values) range for observed $_{tot}$P content. However, when samples of these diets were centrifuged, all but two of the extract solutions were found to be over-range for observed $_{tot}$P; the two samples which were on range were at the top end of it (Table 11.4). When the precipitation phase was performed on diets H and L, the supernatants of centrifuged samples were all on target for observed $_{np}$P content. The majority of those from samples that had been vacuum filtered were also on target; three out of 12 of these samples had observed P contents lower than expected (Table 11.5). The results from diet S were opposite to those for diets H and L. Centrifuged extract samples were consistently on range for observed $_{tot}$P, whilst those that were vacuum filtered were consistently below range (Table 11.4). As might be expected, the supernatant $_{np}$P contents observed were below the expected 0.5% in vacuum filtered samples, and higher in centrifuged samples. In addition, the ratio of total-non-phytate P in diet S was found to be consistently lower than the ratios predicted / observed in diet H and L ($F_{2,32}$ 12.92, P<0.001).

Samples extracted with HCl produced higher supernatant $_{np}$P contents than extraction with H_2SO_4 ($F_{1,32}$ 22.36, P<0.001). Within the centrifuged samples, those treated with HCl all had observed contents on target, while only 1 of 3 of those treated with H_2SO_4 were on target, the rest of the samples having below target observed contents (Table 11.5). There was no significant effect of acid type on $_{tot}$P content in the extracted solution, however, there was an effect on the ratio of total to non-phytate P ($F_{1,32}$ 17.67, P<0.001), with H_sSO_4 producing higher predicted ratios.

A2.4.4: Re-Ashing of Feed Samples

Table 11.7: Comparison of ash results

	diet H		diet S		diet L	
ash removal	previous	re-ash	previous	re-ash	previous	re-ash
filtration	0.81	0.79	0.74	0.57	0.79	0.81
centrifugation		0.79		0.75		0.70

Diets H and L showed good consistency between the previous ashing and the re-ashing using the normal filtration, however, the samples of diet S gave a much lower $_{tot}$P content the second time. Normally, this amount of variation from the expected would result in a re-test, however, there was no sample of this diet left. If we assume that the original analysis of diet S is correct (which seems most likely), centrifuging samples rather than

filtering them appeared to make no difference to the final results for diets H and S. However, the $_{tot}$P content of centrifuged samples of diet L appeared to be lower than that of samples that were filtered.

A 2.5: DISCUSSION

A 2.5.1: Selected Published Methods

All methods except that of Skoglund *et al* (1997) gave $_{tot}$P and $_{np}$P contents below the accepted range; the official AOAC (1990) method was particularly ineffective at extracting P from diet S. Conversely, while the Skoglund *et al* method gave exactly the $_{np}$P content expected from formulation, it also gave a $_{tot}$P above the accepted range. Since all these methods have been published, it is reasonable to assume that the authors had some evidence that they worked for the samples being measured at that time. It is equally reasonable to conclude that none of these methods gave the results expected here. These two statements are not mutually exclusive, however, since there is some evidence from both this work, and that of other authors (Thompson and Erdman 1982), that the exact composition of a feed sample can effect the efficacy of any particular extraction method.

A 2.5.2: Effect of Time of Extraction

Although there was a statistically significant time effect on both $_{tot}$P and $_{np}$P in the second part of the investigation, the non-linear form of this effect appears unlikely. Any real time effect would be expected to be linear, with more P being extracted from the sample gradually over time. There were no consistent patterns with time for the observed values (Tables 11.4 and 11.5), nor were there any time effects observed in the previous part of the investigation for $_{tot}$P content. Some doubt is therefore shed on the reality of this effect, and it is suggested that any extraction that is going to occur does so within 1 hour.

A 2.5.3: Effect of Acid Concentration

The first part of the experiment, the least concentrated HCl solution (0.5 M) gave $_{tot}$P results closer to the accepted range than either the 1 M or 2 M solutions for all four time periods. This was most evident when the extracted solutions were centrifuged, when the observed value was on range (Table 11.3). The occurrence of HCl in gastric juice is normal in birds (Farner 1960) as well as in mammals. In chickens, quotes for free H^+ and Cl^- are 123.1 mEq/l and 147.1 mEq/l respectively (Duke 1986). The equivalent weight of a

substance is that weight which is capable of evolving, replacing, or reacting with a 1 g atom, or an ion, of hydrogen during a reaction (Considine and Considine 1983). In the case of HCl, this corresponds to the molecular weight of the acid, 36.46 g, while the equivalent weight of H_2SO_4 is half of its molecular weight of 98.07 g, i.e. 49.04 g. In the gastric juice of a chicken, a free H^+ concentration of 0.123 Eq/l, if we assume all the ions are coming from HCl, roughly corresponds to a HCl concentration of 0.123 x 36.46 = 4.49g/l, or a 4.49/36.46 = 0.12 M HCl solution. Thus it appears that of the solutuions studied here, the 0.5 M acid solution is the closest to physiological HCl concentration in the stomach.

In addition, available data suggest that pure gastric juice in cormorants has a H^+ concentration of the order of pH 1-2 (van Dobben 1952); this is corroborated by Duke's (1986) observation that avian gastric juice generally has a pH of about 2. A pH of 2 has been found to be the optimum for maximum activity of enzymes in the stomach, such as pepsin (Duke 1986), and enzymes helpful to digestion and endogenous to the feed may also have coincident optimum pH for activity. Thus, while the pH of the 0.5 M HCl solution was only 0.88, of the 3 HCl solutions used, it also had the pH closest to the proposed optimum of 2.

A 2.5.4: Effect of Acid Type

Despite having a pH of 1.18, the closest of all the acid solutions to the proposed optimum of 2, it is clear that 0.5 M TCA was not effective as an extractant at any of the 4 time periods (Table 11.3). It may be that some other property of TCA affecting feed digestion differs from sulphuric and hydrochloric acids. For example, TCA is classed as a dangerous substance due to its precipitating effect on proteins (AOAC 1990). It is possible, therefore, that phospho-proteins in the diets were also being precipitated, trapping some of the $_{tot}P$ in the filter paper.

Sulphuric and hydrochloric acid (0.5 M) appeared to be equally efficient at extracting P from the diet samples when $_{tot}P$ was measured in the extracted solutions (Tables 11.3 and 11.4). Samples extracted in 0.5 M sulphuric acid gave a significantly lower $_{np}P$ content in the supernatant solutions than those given the equivalent time/removal treatment extracted in 0.5 M hydrochloric acid (Table 11.5). They also gave higher total to non-phytate P ratios (Table 6), which would correspond to a greater proportion of $_pP$ in samples extracted with sulphuric acid than in those extracted with HCl. Although for practical purposes the fact is ignored, it is known that some $_pP$ can be digested by birds (Nelson 1976), and as mentioned previously, HCl is the acid naturally present in gastric juice. Since $_{tot}P$ in the extracted solution was the same for the two acids, it is possible that

the hydrochloric acid was degrading some of the $_{p}$P, thus producing more $_{np}$P, while the sulphuric acid was unable to do the same. This could be due to the fact that the 0.5 M H_2SO_4 solution had a pH of 0.97, and was therefore less acidic, and possibly less corrosive, than the 0.5 M HCl solution. Alternatively it may be due to some other unknown property of HCl which made it nature's acid of 'choice' in gastric juice. This may mean that using hydrochloric acid as the extractant might mimic the biological system better than using sulphuric acid. However, the differences observed were small in this study, and confirmation from a larger study is needed.

A 2.5.5: Effect of Method of Feed Removal

Higher $_{tot}$P results were consistently obtained from extracted solutions that were centrifuged, rather than solutions given equivalent time and extraction treatments but vacuum filtered (Tables 11.3 and 11.4). The ratio of total to non-phytate P was also larger in centrifuged samples than vacuum filtered (Table 11.6), implying a greater proportion of $_{p}$P in centrifuged samples. This suggests that the filter paper may be retaining some of the $_{p}$P in some way. In addition, the $_{np}$P contents of the supernatants from vacuum filtered samples were also consistently lower than those from centrifuged samples (Table 11.5), suggesting that some $_{np}$P was also being retained by the filter paper. For centrifugation to give both higher $_{tot}$P and $_{np}$P, and still give a higher ratio, the filter paper must retain a larger proportion of the $_{p}$P than the $_{np}$P. However, the results still imply that some of both types of P were in a form large enough to be held back by filter paper, but light enough not to be removed by centrifugation – quite how this would be achieved is uncertain. Perhaps some of the $_{np}$P had formed a complex with, for example, Ca, and this complex was large enough to be retained on the filter paper. In addition, phytate (myo-inositol hexabiphosphate) can be hydrolysed to a range of different sized isomers containing varying proportions of P (inositol penta- to monophosphates), many of which are present in foodstuff (Skogland *et al* 1997). Perhaps the filter paper was retaining the larger isomers, and only allowing the smaller isomers to pass through. Finally, there may have been some adsorption to the hydroxyl and carboxyl groups in the cellulose-based filter paper, since cellulose is also the basis for ion exchange columns. Without further in depth study, it is impossible to come to any firm conclusions; however, it is obvious from these results that the use of filter paper appears to reduce the amount of $_{tot}$P and $_{np}$P measured in this type of feed analysis.

A 2.5.6: Variation in Results Between Diets

As might be expected from the above discussion, while $_{tot}$P results from extracted solutions of diet S were on range for most centrifuged samples (those from the Skogland method were over range), they were below range for vacuum filtered samples (0.5 M acid, Tables 11.3 and 11.4). However, $_{tot}$P results of solutions extracted from diets H and L were on range for vacuum filtered samples, and over range for centrifuged samples for all time periods, and for both hydrochloric and sulphuric acid (Table 11.4). How centrifuged samples of diets gave higher $_{tot}$P results than those obtained by ashing is unknown. It is possible that during ash analysis, some small portion of the $_{tot}$P was lost in residue stuck to the beaker, however, it is unlikely that this would involve amounts large enough to produce the differences seen here. In addition, if this type of error was the reason, one would expect a random distribution since the amount of any such residue occurring is unlikely to be consistent between beakers. The results observed here cannot be considered random. The discrepancy between the diets also seems unlikely to be due to their Ca:P ratio; diet H has a $Ca:P_{tot}$ ratio of 1.7, diet S 1.4, and diet L 1.0. If the $Ca:P_{tot}$ ratio of the diets was having an effect on the analysis, we would expect either a linear response with all 3 diets giving different patterns of results in order of ratios, or a curved response whereby two of the diets with adjacent ratios would give similar patterns. Neither of these scenarios was observed in the present study.

It was therefore postulated that perhaps the use of filter paper to remove the digested ash from the reconstituted solution before dilution might also have retained some form of P during the previously performed standard method of ash analysis. Why this might occur in only 2 of the 3 diets was again unclear, however, if such a problem was occurring in any of the diets, it could have major implications for both the feed and bone ash analysis for the whole project. However, when feed samples were re ashed to compare the effect of filtration and centrifugation, no convincing evidence of such a retention was seen; the method of ash removal had no effect in 2 out of the three diets after ash analysis. The lower mean observed in centrifuged samples of diet L could imply a potential problem with feeds with $Ca:P_{tot}$ close to 1, however, with such low sample numbers, it is probably as likely to have occurred by chance. During the process of ash analysis, samples are ashed at 550°C for 16 hours to remove all carbon, hydrogen, and nitrogen, thus destroying all organic molecules. Ash samples are then boiled in 6N HCl twice to dissolve the inorganic mineral remaining – it is considered unlikely that anything is left in a large enough form to be retained by filter paper. There is the possibility of losing some P by adsorption to the filter paper, however, no evidence was observed of this mechanism

having a large influence on P contents from ash analysis during the last part of this experiment.

The implication is, therefore, that the problem may be occurring with the extracted samples, probably at the P analysis stage, and it may not necessarily involve a true difference in P content between ashed and extracted samples. For example it is possible that the reaction of the P in the sample solution with the molybdovanadate reagent used is pH sensitive, although there is no mention of this in the AOAC method description (AOAC 1990). This possibility could potentially account for some of the differences between total P contents obtained by ashing (dissolved in 0.6 M HCl), and those obtained by extraction in a variety of other acid solutions. However the difference in pH between 0.5 M and 0.6 M solutions of HCl is unlikely to be large enough to produce such a drastic change in apparent P content, in any case the same acid treatments were used for all three diets.

Alternatively, there may be organic molecules present in the extracted sample solutions which interfere with the molybdovanadate reaction, or with the colorimetric reading of the reacted solution, giving higher than true P content readings. The latter suggestion might also explain the reason why in general only extract solutions from diets H and L demonstrated higher $_{tot}$P contents than ashed samples. These diets were produced previously for use in an experiment on dietary Ca and P content (Chapter 4), while diet S was produced for a different experiment (Chapter 5). The main ingredients in all the diets were wheat and soya, however, diet S also contained fishmeal. If and how the presence or absence of fishmeal would affect the digestion of organic material in a diet is not known, however, it is possible that its presence counteracted the suggested negative effects of the hypothetical organic molecule present in another feed ingredient. Another possibility is that while all the diets had been stored at 4°C, diets H and L had been stored for considerably longer than diet S; there may have been some degradation of the feed over this period, perhaps resulting in the formation of the hypothetical 'problem molecule'. For example, lipids are likely to be oxidised to their fatty acid components over time, resulting in both a change in the pH of the feed, and in the organic molecules contained within it (Skougal, pers. comm.). The proposed effect of storage length is supported to some extent by the fact that when the initial attempt was made to analyse all the diets from Experiment 2, including H and L, for $_{np}$P, these diets showed a similar response to diet S, i.e. lower than expected $_{tot}$P results when vacuum filtered. Again, further investigation with larger sample sizes would be needed to clarify which, if any, of these effects is in operation, and how they are manifested.

A 2.5.6: Conclusions

The present study was small and limited in design, however, it did appear to point to a number of potential pitfalls and some potential solutions in the analysis of feed for $_{np}P$. Vacuum filtration through filter paper gave consistently lower $_{tot}P$ results than centrifugation, and the filter paper appeared to retain both $_{p}P$ and $_{np}P$. If this is a true effect, it has significant implications for all methods of phytate analysis which currently use filter paper - they will be consistently underestimating the amount of $_{tot}P$, and therefore $_{p}P$ and $_{np}P$ in a sample. No convincing evidence of such retention was seen after employment of the standard ashing procedure, which means that the current standard ashing procedure has not been called into question. The least concentrated of the HCl solutions (0.5 M) was a more efficient extractant than stronger solutions. It was suggested that this might be due to its concentration and pH being closer to that found in chicken gastric juice than the pH of the stronger solutions. This might also be the optimum pH for endogenous enzyme activation in the feed, which could aid the extraction process. Hydrochloric acid and H_2SO_4 were equally efficient at extracting $_{tot}P$ at 0.5 M, however, HCl gave slightly higher $_{np}P$ results, and may be a better mimic of the biological system. Diets H and L consistently gave much higher results than diet S, and when centrifuged, gave higher total P results than ashing. There were differences in both the ingredients of the feed, and in the length of storage, and therefore the amount of potential feed degradation. It was proposed that this may have resulted in the presence of some organic compound in diets H and L which interfered with either the molybdovanadate reaction, or the reading of the reacted solution, thus giving higher than true P content readings. Again, due to the limitations of the present study, this evidence is not conclusive. However, it could have some disturbing implications on the consistency of phytate analysis performed on diets made from different ingredients, or stored for varying lengths of time.

During the course of the current project, discrepancies between the expected and analysed contents of total Ca and P in diets have been frequent. A reliable method of analysing available (non-phytate) P would, therefore, be a useful tool. However, during the course of this experiment, variations in response to particular methods have been found between diets. The results of this experiment can not, therefore, point towards a universally useful extraction method; even if methods work well on one set of feed samples, they will not necessarily work on another. However, the results do imply that some current extraction methods using more concentrated acid solutions, including the current AOAC method using 2.2 M HCl, are unlikely to give very accurate assessment of $_{tot}P$, and therefore $_{np}P$ content. It may, in fact, be beneficial to adjust extraction methods for particular target species by attempting to produce a HCl solution with a pH as close to

that of the species gastric juice as possible. While this would still not be as accurate as using a live animal, with is own mixture of endogenous enzymes and bacterial by-products, it might give results on P availability closer to those from animal studies. After consideration of all the above, it was decided that phytate analysis was far too complex to enter into further within the time frame allowed. It was concluded that, even given the observed variation in diet mineral contents from the expected, performing non-phytate P analysis on the diets was unlikely to give results any closer to the true contents than the expected contents as per formulation without a considerable amount of extra work.

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