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Measurement of Bone Quality in Growing Male Rats Using Dual Energy X-ray Absorptiometry and Bone Ash Content

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Abstract

Growing male rats have been considered and used as a model for bone growth and prevention of osteoporosis because of their high bone turnover and demand for calcium. Dual Energy X-ray Absorptiometry (DEXA) is a useful tool for identifying minimal changes in bone mineral density and has recently been adapted for use in small animal models. The objective of this trial was to identify the changes in Bone Mineral Density (BMD) in relation to age and to identify how BMD varies from site to site.

Sixty male Sprague-Dawley rats were split into six groups to allow measurements at one, two, three, four, five and six months of age (n=10 per group). At each time point a group of rats was scanned using a QDR4000 DEXA machine from Hologic. Duplicate BMD measurements were obtained for the whole body, spine and both femurs *in vivo*. The rats were then euthanased and the spine and both femurs were excised for *ex vivo* DEXA scanning and ashed calcium analysis.

BMD increased almost linearly to four months and then formed a plateau. This indicates that from weaning to four months is an especially sensitive time for manipulating bone growth in male rats. There was a significant difference in BMD between groups ($P<0.001$), which is to be expected in growing rats. There was also a significant difference in BMD within groups ($p<0.001$), believed to be due to variation at two and five months of age. There was a very strong positive correlation between weight and BMD and age and BMD at all sites, indicating that BMD is a strongly related to both weight and age. All sites were strongly correlated to each other and to the ashed calcium values. The excised femur had a lower BMD value than the *in vivo* femur, although the two values were strongly correlated. This is believed to be due to differences in positioning and indicates that the two methods cannot be used interchangeably.

These results indicate that bone mineral density is the gold standard for following changes in bone growth over time in the growing rat. Alternatively, ashed bone calcium content can be used, but only as a once off endpoint.

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List of Abbreviations

ACP = Acepromazine

Ashed Ca = Ashed calcium content

BMC = Bone Mineral Content

BMD = Bone Mineral Density

DEXA = Dual energy x-ray absorptiometry

LF = Left Femur BMD

Lfex = Ex vivo Left Femur BMD

Mth = Age in months

PBM = Peak Bone Mass **RF** = Right Femur BMD

RFex = Ex vivo Right Femur BMD

Spine = Spine BMD

Spex = Ex vivo spine BMD

Wt = Rat weight (g)

General Introduction

Osteoporosis

Osteoporosis is a wide spread disease in postmenopausal women and the elderly. It is estimated that in the U.S.A alone 15-20 million women over the age of 45 have osteoporosis (Baran *et al*, 1989; Petley *et al*, 1996). Osteoporosis describes a condition of low bone mass that results from excessive loss of bone after maturation or from inadequate development of the skeleton during maturation. Osteoporosis and its consequences have become one of the highest costs to our society (Aufdemorte *et al*, 1993).

With new advances in science and medicine, the average life expectancy has increased. Diseases that were previously fatal are now treated effectively by a range of drugs and practices. This has led to an increase in the number of elderly people in the population, resulting in an increase in the number of cases of osteoporosis. Loss of bone mass is an almost universal occurrence in the elderly and leads to an increased risk of fracture (Baran *et al*, 1989).

The World Health Organisation (WHO) definition of osteoporosis is "A systemic skeletal disease characterised by low bone mass and micro-architectural deterioration of bone tissue, with a consequent increase in bone fragility and susceptibility to fracture" (Kanis, 1994; Petley *et al*, 1996)). There is a rapid loss of trabeculae in spongy bone and a slower loss in cortical bone. This gives the bone a porous look. Although bone mass is decreased, mineralisation of the remaining bone is normal.

Bone is a living tissue that is constantly being remodelled. This happens in two phases. The first is bone resorption. Once activated, osteoclast precursor cells clump together and become an active multi-celled unit, which chews through bone. Tunnels are formed in cortical bone and lacunae are formed in trabecular bone. This process takes about one to three weeks. The osteoclasts then disappear and are replaced by osteoblasts. The osteoblast's role is to repair the tunnels and lacunae by filling them in with new bone. This process takes several months (Kanis, 1994). In healthy young adults the net balance is zero.

There are two types of osteoporosis, senile and postmenopausal. In senile osteoporosis, it appears that the cavities that are formed in bone are only being partially filled. This indicates a decrease in bone formation. It is likely that senile osteoporosis is a natural part of the aging process. Senile osteoporosis could act through an increase in bone resorption, a decrease in bone formation or a combination of both factors. Women are affected at a ratio of 2:1 compared to men. There are two main reasons for this. Firstly women are smaller in stature than men, so they have less bone mass to start with (refer to Figure 1). Secondly there is a definite hormonal link. The sex hormones have a protective role against the catabolic action of Parathyroid Hormone (PTH). Androgens are responsible for skeletal integrity in males (Rosen *et al*, 1995). Testosterone has a higher anabolic effect on bone than oestrogen although the levels of both decline with age. The circulating levels of estrogen decrease dramatically in women after menopause.

Growth of the skeleton

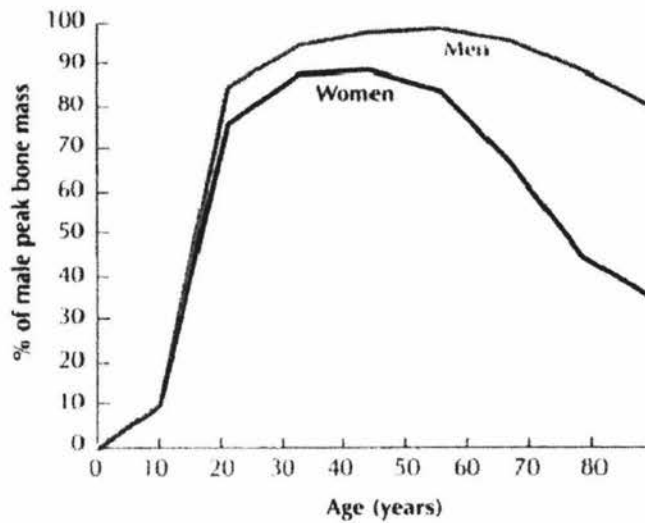


Figure 1 Growth of the skeleton

In postmenopausal osteoporosis, an increase in bone turnover occurs due to a lack of oestrogen (Ke *et al*, 1995). The circulating levels of oestrogen decrease after menopause. Oestrogen has an inhibitory effect on PTH, which is known to stimulate osteoclasts (Bagi, 1997). In postmenopausal osteoporosis the number of osteoclasts present increases. This results in very large cavities forming in the bone. Unfortunately there is no subsequent increase in the activity of the osteoblasts. This leads to a thinning of the bone's structure, especially in trabecular bone (Kanis, 1994).

Secondary osteoporosis may develop as a result of other factors (Nordin, 1984). Some genetic disorders such as Pagets disease and hypogonadism can lead to osteoporosis. Alcoholism and several drug therapies, such as the use of glucocorticosteroids, also lead to osteoporosis and increase the risk of fracture (Loré, 1989).

While treatment can increase bone mineral density (BMD), the damage to the architectural structure of the bone may never mend completely. The weakening in the bone's structure means that fractures may still occur despite positive responses in BMD.

Osteoporosis is a serious medical condition. While brittle bones in themselves do not cause a problem, there is a subsequent increase in fracture risk. Low bone density increases the risk of a debilitating fracture. A loss of height is the most recognisable sign of osteoporosis. In normal cortical bone, 95% of the area is taken up by bone material; in osteoporosis as little as 30% of the area may consist of bone (Sissons, 1962). This gives the bone a porous appearance and can lead to weakening and collapse of the bone structure. The lumbar spine is usually the first area to be affected, as it has a small area in comparison to its weight bearing capacity. This often results in a collapse of the vertebrae and causes a reduction in the patient's height.

The hip is probably the most serious site of fracture and is associated with a higher degree of morbidity and mortality (Sissons, 1962). It is usually the result of a fall and thin women with little padding around their hips are particularly susceptible. It is associated with a high cost to our society, as patients are in need of immediate hospitalisation. There is also a loss of mobility and increased pain, which can lead to a loss of independence. Patients may need to go into a nursing home or be cared for by family members.

The recommended course of action is a hip replacement. The patient is mobilised almost immediately. This stops muscle atrophy, which is associated with a decrease in blood supply to the bone. This course of action is usually highly successful (Kanis, 1994). If left in traction the fracture can heal of it's own accord but long-term

immobilisation is linked with a greater loss of bone mass, an undesirable side effect in osteoporosis. Hip fracture is unlikely to be a main cause of death. The higher degree of mortality is probably associated with patients who are too unhealthy to undergo hip replacement surgery or who acquire secondary complications unrelated to the actual fracture (Sissons, 1962).

The risk of osteoporosis can be significantly reduced by:

1. Maximising the development of high peak bone mass (PBM, see chapter 1.1.1).
2. Maintaining bone mass after PBM is achieved
3. Reducing bone loss as much as possible.

Chapter 1

Literature Review

1.1 Factors which Influence Bone density

1.1.1 Peak Bone Mass

PBM is the amount of bone tissue present at the end of skeletal maturation. After this, bone mass may be maintained or lost but is rarely gained without significant drug intervention (see figure 1). Thus bone density is a function of the amount of bone gained versus the amount of bone lost (Kanis, 1994; Klein, 1998).

PBM has a strong genetic component (Klein, 1998). Some people will have a higher bone density when corrected for height and weight than others. As people age, genetic differences tend to decrease and environmental factors play a bigger part in the retention of bone mass. By manipulating lifestyle patterns, such as diet and exercise, PBM may be increased and osteoporosis prevented (Kanis, 1994).

In adolescence there is a high rate of bone turnover, so despite the lengthening of bone, there is not a large increase in BMD. As calcium is being liberated from the bone, additional calcium at this stage may not be utilised fully. There is a period between the attainment of full longitudinal skeletal growth and PBM, known as consolidation (Kanis, 1994). This may be the ideal time to try and impact on bone PBM. The common belief is that PBM is achieved during the early twenties in humans.

There is a familial tendency towards osteoporosis and several lifestyle factors are thought to increase the likelihood of this disease. An overlap between post menopause and the natural process of aging is quite common and in many cases, it may be impossible to single out which of these two factors, is the major contributing factor to the disease.

1.1.2 Familial Tendency

The genetic link is the strongest predictor of low bone density. If a close family member has osteoporosis then the chance of other relatives being afflicted is much higher. Up to 50% of bone variation in youth may be attributed to genetics (Kanis, 1994, Klein, 1998). This has been linked to differences in the Vitamin D receptor. As age increases there is a decrease in these differences, which suggests that the environment may then play a bigger part in BMD. Racial differences in bone density also occur. African women tend to have higher bone density than Caucasian women.

Mice have been used as a model to identify and characterise genes that are linked to osteoporosis. PBM is precisely measurable and 80% heritable. Genetic researchers are trying to identify groups of genes (markers) that will identify people who are predisposed to osteoporosis (Klein, 1998).

1.1.3 Sex Hormones

In humans the level of androgens and estrogens fall with increased age. The sex hormones have a protective role against bone resorption by blocking the catabolic action of PTH (Bagi, 1997). Although the action of prolonged doses of PTH is

catabolic, intermittent doses of PTH combined with oestrogen, will stimulate bone formation (Shen, 1993). The administration of oestrogen completely blocks the activation of increased bone turnover leading to bone loss. Hormone replacement therapy (HRT) is effective in reducing the impact of menopause on bone. Unfortunately HRT has undesirable side effects such as an increase in breast and uterine cancer (Ke *et al*, 1995; Nuttall *et al*, 1998). Progestogens are often used in combination with oestrogen to counter these side effects (Kanis, 1994).

1.1.4 Calcium

Bone contains 98-99% of the body's calcium, which can be called upon in times of need. A negative balance in circulating calcium ions can lead to bone resorption (Loré, 1989). Calcium is required for many physiological functions, such as the control of muscle contraction and as the intracellular signal in the action of many hormones. If dietary levels of calcium are inadequate, calcium will then have to be recruited from bone.

High levels of dietary calcium (above 1400mg/day) are positively related to bone density (Michaëlsson *et al*, 1997; Baran *et al*, 1989). However, there is only a minute fraction of the population who would be obtaining this high level of calcium.

In postmenopausal osteoporosis, subjects often display a negative calcium balance. Most osteoporotic women have low calcium intakes and/or poor calcium absorption, which correspond with an increase in faecal calcium losses (Loré, 1989).

If dietary calcium is low, the body adapts by increasing calcium absorption in the intestine. As humans age, there is a decrease in calcium absorption and in women

with osteoporosis this adaptation mechanism may not operate at all. This defect in absorption cannot be overcome by increasing the amount of calcium in the diet alone.

1.1.5 Vitamin D

The addition of vitamin D has been shown to improve the efficiency of calcium treatment, by enhancing the active transcellular transport of calcium in the duodenum. 1,25-dihydroxyvitamin D₃ is likely to be the main regulator for intestinal absorption of calcium (Hugi *et al*, 1979; Wood *et al*, 1998). Hugi *et al* (1979) found that supplementing vitamin D is only effective in people who are already vitamin D deficient.

Vitamin D is also important in the calcification stage. Without vitamin D the matrix is too stable, thus blocking the change in the matrix that precedes calcification (Loré, 1989).

The elderly population is often vitamin D deficient (Wood *et al*, 1998). The absorption of this vitamin does not seem to be affected by age (Loré, 1989). This deficiency could be due to a lack in the diet or to a lack of sunlight exposure. Humans are capable of synthesising vitamin D in the skin. The reaction is dependent on sunlight exposure. The elderly spend more time indoors instead of outdoors in the sun. This could be through frailty or susceptibility to heat or cold. By giving vitamin D supplementation when vitamin D status is inadequate, an increase in the amount of calcium absorbed can be demonstrated.

1.1.6 Malnutrition

There is a greater incidence of osteoporosis in people suffering from eating disorders such as anorexia (Loré, 1989). Their starvation diet produces excess acid, which could contribute to bone loss. By starving themselves, anorexics rob the body of nutrients necessary for essential functions. Therefore the building blocks for healthy bone, such as protein and calcium, will not be provided in large enough quantities to achieve or maintain maximal bone formation.

When the body drops below a certain fat percentage, it fails to provide enough oestrogen and a condition similar to postmenopause occurs, known as amenorrhea (Kanis, 1994). Fat cells synthesise oestrogen by peripheral conversion of androstendione. This is one of the reasons why obesity plays a protective role against osteoporosis.

1.1.7 Obesity

Obesity is a protective factor against osteoporosis (Loré, 1989). It is not a recommended lifestyle choice to treat osteoporosis due to other health concerns. It has three modes of action. The first is a cushioning effect when women fall, as there is extra padding around the hips. The extra weight carried by obese people also provides weight training to strengthen bone. Finally fat cells manufacture oestrogen, which has a protective role against bone resorption (Loré, 1989).

Leptin is a polypeptide hormone produced and secreted primarily by adipocytes. Leptin deficient (ob/ob) and Leptin receptor deficient (db/db) mice have high bone density despite hypogonadism and hypercortisolism (Fleet, 2000). Low levels of sex

hormones and high levels of cortisol are linked to low bone density. Obese adults could also have a Leptin resistance resulting in high serum levels of Leptin. High bone mass in obese adults may be related to serum Leptin levels. The action of Leptin is unknown as yet but it is suggested that there is a neural pathway in the brain through which it acts. The regulation of body weight and bone mass by Leptin is apparently mediated through different signalling pathways.

1.1.8 Exercise

A moderate amount of exercise is believed to strengthen bone but prolonged and excessive exercise can also lead to amenorrhea. Over-exercising can cause micro fractures and stress fractures, which often have difficulty in healing (Loré, 1989).

Humans who are bed ridden or astronauts who are not subject to gravity have a significant decrease in their bone density (Wronski, 1998; Turner *et al*; 1998). Weight bearing is believed to impact positively on bone density by strengthening bone. Weight bearing exercise places stress on specific areas of bone. The body adapts by remodelling bone at this site to become stronger, reducing the risk of subsequent fracture.

The amount of bone present depends on the nutrients supplied by the blood flow to the bone. Blood flow to the bone is regulated equally by two different actions. The first is the heart pumping blood into the bone and the second is the muscles surrounding the bone pumping blood out of the bone. If blood supply is negatively affected, there will be a lack of building blocks for bone formation (Loré, 1989).

Exercise has an effect on several aspects of bone metabolism.

1. Exercise increases the partial pressure of oxygen in the blood supply by increasing the efficiency of the lungs. Oxygen is important for energy deriving pathways in bone.
2. Exercise also increases the size and efficiency of the heart and muscles. This increases the blood flow to the bone and subsequently increases the amount of bone tissue present.
3. There is a marked reduction in the level of corticosteroids present in those who exercise regularly. Corticosteroids have an anti-anabolic affect on bone.
4. Too little exercise causes atrophy of the muscles, which will affect blood supply to the bone.

Weight bearing exercise has a positive effect on bone mass in children and young adults. However, this adaptation declines with age and postmenopausal women do not display this response to exercise. Therefore, increased activity in these women will not increase bone mass (Loré, 1989).

Elderly people who are active do have a lower incidence of fracture than those who are inactive, despite comparable bone density. This could be due to an increase in muscle strength and coordination in the active population. Better coordination means that they are less likely to fall and cause a fracture. Cardiovascular exercise has not shown any bone strengthening properties (Loré, 1989).

1.1.9 Secondary Osteoporosis

Osteoporosis can also develop as a secondary outcome of some other disease. Diseases affecting the duodenum and jejunum will automatically reduce calcium absorption from the diet. If calcium cannot be obtained from the diet, bone will be

resorbed to supply the body with the calcium it needs for essential functions. Calcium is needed for muscular contractions of the heart and limbs and for the regulation of several key hormones (Loré, 1989).

Pagets disease of bone can also affect bone density measurements. It is characterised by excessive resorption and deposition of bone. The precise cause is unknown but it seems to be viral in nature. Instead of the normal lamellar arrangement of collagen fibres, which provides a strong framework for bone there is a mosaic pattern (Nordin, 1984). This affects the nature of the bone, causing it to become soft and chalky.

There may be no outward symptoms of this disease but many people who suffer from it will be prone to fractures (Nordin, 1984). In severe cases deformity will occur due to excessive formation of bone or due to bowing of long bones.

There are several lifestyle factors that increase bone loss. Drinking excessive amounts of caffeine leads to higher bone loss, although moderate amounts of caffeine do not have an adverse effect (Loré, 1989).

Smoking is also related to lower bone mineral density. The mechanism of action is unsure but there are three possible theories (Loré, 1989).

1. Due to lower weight in smokers. There is less of a weight bearing effect on bone and less oestrogen produced by fat cells.
2. Secondary to chronic pulmonary disease with acidosis. This would have a negative effect on blood flow to the bone. Therefore the bone is robbed of nutrients.
3. Smokers tend to go through menopause earlier than non-smokers. Thus they are exposed to the protective role of oestrogen for a shorter period of time.

Alcoholism is also linked to low bone density. Alcohol has a negative effect on calcium absorption. Steatorrhoea occurs in the alcoholics with subsequent malabsorption of calcium and vitamin D (Loré, 1989). Alcohol also has a direct effect on the cells lining the stomach. This results in the abnormal appearance of microvilli and mitochondria under the microscope (Loré, 1989).

1.1.10 Corticosteroids

Stress is associated with an increase in plasma levels of cortisol. Cortisol is a corticosteroid and has a negative effect on bone formation. Research suggests that glucocorticoids have a direct effect on osteoblast function. By administering short doses of glucocorticoids, there is a marked suppression in alkaline phosphatase and osteocalcin, which are osteoblast markers (Kanis, 1994).

Prolonged stress can lead to a constriction of arteries and an increase in granulation tissue present in the arteries (Loré, 1989). The size of the arteries is reduced and consequently the volume of blood that can flow through them is also reduced. This leads to a reduction in the amount of blood being pumped into the bone.

Corticosteroids also have an anti-anabolic action on muscle fibres. Both cortisol and cortisone reduce protein synthesis in the muscle by interfering with the incorporation of amino acids into protein (Loré, 1989). This weakens the muscle fibres and interferes with their ability to pump blood out of the bone. By reducing blood supply to the bone, nutrients necessary for the maintenance of bone diminish. This would interfere with bone formation and may lead to the death of portions of bone tissue.

Cortisone is a common drug used for treatment of certain diseases. Although cortisone is not harmful to cells in itself, about a third of it is converted to cortisol. The use of cortisone is associated with bone metabolic disorders (Loré, 1989).

1.2 Tools for Diagnosing Osteoporosis

1.2.1. Singh Index

The Singh Index was used for a long time to determine osteoporosis (Kanis, 1994). The proximal femur has a distinctive pattern of trabecular architecture, which is disturbed during the course of osteoporosis. The Singh Index is based on five categories, ranging from normal to severely osteoporotic. There are six grades. The risk of fracture increases with each decrease in grade. This method is still of use in epidemiological studies.

1.2.2 Quantitative Computed Tomography

Quantitative Computed Tomography (QCT) is a useful tool in bone measurements and gives the highest diagnostic sensitivity (Grier *et al*, 1996; Cardenas *et al*, 1997). It is the only instrument that measures pure trabecular bone. Trabecular bone is particularly sensitive to many factors that influence osteoporosis. It is said to be the most sensitive predictor of age related bone loss. The disadvantage of this method is that it is expensive and has 30 times the radiation of Dual-Energy X-ray Absorptiometry DEXA (definition on page 17).

1.2.3 Single Photon Absorptiometry and Dual Photon Absorptiometry

Single Photon Absorptiometry (SPA) was developed in order to combat the problems involved in QCT (Grier *et al*, 1996). Although it was less expensive and had lower radiation levels, it still had some problems of its own. SPA was limited to sites that could be immersed in water or surrounded by a constant soft tissue thickness. This made it difficult to measure important sites in bone resorption such as the lumbar regions. The lumbar region is the most common site for osteoporosis related pain. As humans are bipedal, the centre of gravity is shifted so that the lumbar region supports a large amount of weight for its size. The large surface area to volume ratio also means that at the same rate of resorption, the spine will decrease its volume at a greater rate than cortical bone. This makes it a prime site for diagnosing osteoporosis.

This led to the development of Dual Photon Absorptiometry (DPA). This allowed bone that was not surrounded by a constant tissue thickness to be evaluated, but meant that scan times became longer (20-30 min).

There were additional problems to using SPA and DPA. Resolution was poor and precision errors were high at around 2-4% (Grier *et al*, 1996). The radioisotopes also had a short duration and their use was strictly regulated, which limited their use and availability.

1.2.4 Ultrasound

Ultrasound measurements are also gaining in popularity. While SPA, DPA and DEXA are able to account for 60-80% of the variation in bone strength they do not provide much indication of bone structure and material properties (Antich, 1993). The advantage of ultrasound is that bone elasticity can be measured (Njeh, 1997). A combination of bone elasticity, structure and density information could be used to provide a more sensitive indicator of fracture risk (Gluer, 1997). There has been good correlation between Ultrasound and DEXA measurements (Amo *et al*, 1996). These measurements have been able to significantly differentiate between groups of osteoporotic and normal people. Future improvements in technology may increase the popularity of ultrasound use in osteoporosis detection.

1.2.5 Dual Energy X-ray Absorptiometry (DEXA)

The most popular technique used to measure bone density is Dual-Energy X-ray Absorptiometry (DEXA). DEXA uses a more stable radiation source than SPA or DPA. It uses two different x-ray energy levels to distinguish bone from soft tissue. The amount of energy absorbed or refracted by bone allows for the bone density to be calculated. The greater difference between the two energy sources allows for better resolution. With better resolution a clearer print out can be obtained. Algorithms are used to calculate and quantify the type of tissue found in each pixel scanned (Grier *et al*, 1996).

DEXA is also relatively inexpensive and gives reliable bone density measurements (Grier *et al*, 1996). Scanning times are reduced to 10-20 minutes for whole body measurements. There is a compare function that allows for serial measurements.

The baseline scan can be brought up on the screen next to the most recent scan. An identical region of interest can then be taken from the baseline scan and drawn around the most recent scan. The DEXA is sensitive enough to pick up relatively small changes (Gala Paniagua *et al*, 1998). This allows quick detection of osteoporosis and comprehensive monitoring of treatment regimes. Patient acceptance of DEXA is also high, as there is minimal radiation and there is a permanent record of their bone density (Grier *et al*, 1996).

The main problem with DEXA is that it measures areal density rather than true volumetric density (Jiang *et al*, 2000). This means that a large osteoporotic bone can have a higher bone density than a normal small bone. This has to be taken into account, especially in research when comparing different bones. It is also unable to give any indication of the bone mechanical properties, so it is unable explain why some patients with low bone density may never suffer from fractures, while others will (Cunningham *et al*, 1996). In addition DEXA is unable to discriminate between osteopenia related to osteoporosis and osteopenia caused by other disorders. Osteopenia is a condition relating to low bone mass. It is not as serious as osteoporosis. It is the middle ground between normal BMD and osteoporosis. It may lead into osteoporosis.

Positioning is extremely important as DEXA takes a three-dimensional object into a two-dimensional image (Griffin *et al*, 1993). Different aspects of the bone during scanning will give different bone density measurements, as will bones that overlap. This will affect the precision of the measurement and means that comparisons cannot be made with previous or subsequent scans.

DEXA tends to over estimate bone mineral content but it has a strong correlation with ashed values (Grier *et al*, 1996). This means that although it is not the most accurate

method of obtaining BMD, it still has the ability to accurately predict differences within a sample population.

As mentioned above algorithms are used to calculate and quantify the type of tissue found in each pixel scanned (Grier *et al*, 1996). These algorithms are based on human subjects. Different software allows for rats to be scanned on the same machine as humans. The recommended weight range for optimal results in rats is 200-750 grams (QDR 4000 User Guide, 1999). In rats weighing less than 200g, the difference between low-density bones and soft tissue is small and DEXA may have trouble defining them. It is recommended that rats below 200g be scanned three times (Lu *et al*, 1994)

1.3 The Value of Reference Data

A database is an important research tool (Jiang *et al*, 2000). It provides information on a normal population against which trial subjects can be compared. This allows for the researcher to determine how effective the treatment is and how much of the result can be attributed to normal variation.

The use of different databases in the U.K. has led to different classifications of similar bone densities. A customised database uses raw unprocessed data, while the manufacturer applies a regression fitting technique to at least some of their normal ranges. Depending on the population, people with low bone densities may be classified as normal by one database and osteoporotic by another (Simmons *et al*, 1995). This can lead to discrepancies over treatment and means that comparisons cannot be made between studies in different areas. It is important for a database to

be a good representation of the normal population (Petley *et al*, 1996). Standardisation of the population normal used in clinical trials is therefore important.

In research the emphasis moves from diagnosing osteoporosis to finding differences in BMD between treatment groups. As these differences can be very small it is useful to have a custom-made database for the population that is being studied.

Rats of different strains and ages have different bone metabolism. As rats from an animal production unit tend to have a degree of inbreeding, their phenotype and genotype may be very close. As BMD has a genetic link, a similar genotype would narrow the range of BMD values within the population. A database from another rat colony, with a wider normal range, could lead us to mistake an animal with a low BMD as normal. For this reason we have chosen to create our own database rather than buy the commercial database from Hologic.

Hologic provides a package for human software, which allows the researcher to draw customised normal curves. These curves can be used as a default setting against which trial subjects can be analysed. Due to a lack of demand Hologic no longer supplies a software package to allow a normal curve to be created for a rat population (Binkes, 2000). As researchers tend to have their own particular criteria for analysing trials, most clients tend to build up a database on the statistical program of their choice.

To be a credible database the results must be repeatable in the same population. If the trial was repeated in a year's time it should give a similar result. The database must be a true representation of the population without traces of bias. If the integrity of the database is in question, then it will be useless to assess subsequent trials in relation to it.

The use of the database built in this trial would be irrelevant in any other research facility.

1.4 The Rat as a Model for Osteoporosis

The use of animal models allows researchers to simulate osteoporosis (Amman *et al*, 1992; Amman *et al*, 1999; Aufdemorte *et al*, 1993; Turner *et al*, 1998). Diet and hormone function can be artificially modified in order to cause bone loss. Treatment can then be administered to try and replace lost bone. With improved knowledge on how these manipulations affect the bone, treatments can be refined and improved.

There are several advantages to using rats as a model for studies on bone. It is very easy to control external stimulus on rats. They can all be housed in one room and subjected to the same temperature and light patterns. Large numbers can be used, as rats are cheap to house and feed. Written consent from the participant is not required (Mosekilde, 1995). There is no trouble with compliance, as rats are not going to pull out, move to another city or simply forget to turn up. Although rats may object to the treatment, they have little or no choice to reject it.

Rats also have a relatively short life span, so a treatment effect can be seen in weeks rather than months or years. Their anatomy is similar to ours, including lamellar bone structure and comparable remodelling sites in cancellous bone (Mosekilde, 1995; Aufdemorte *et al*, 1993).

The ovariectomised rat is a good model for postmenopausal osteoporosis (Omi *et al*, 1995; Turner *et al*, 1998; Yamauchi *et al*, 1995). In order to gain approval from the

United States Food and Drug Administration, they require that new bone therapies be tested on an ovariectomised rat model and a large animal model (Thompson *et al*, 1995). Female rats have a good oestrus cycle with oestrogen (E2) levels spiking every four days. The epiphysis is closed at 6-8 months in females. After one-year of age bone loss occurs as the spikes in E2 end. This follows a similar pattern to postmenopausal humans. (Amman *et al*, 1999; Aufdemorte *et al*, 1993; Omi *et al*, 1995).

Unlike humans, rats with low bone density do not suffer from osteopenia related fractures. Fracture likelihood is a strong factor in predicting osteoporosis (Amman *et al*, 1999). Tools used to measure bone density and biomechanical strength have been adapted for use in rats.

DEXA measurements can be performed *in vivo* on the spine and femurs to obtain BMD measurements without the need to sacrifice the animals (Vanderschueren, 1993).

Machines are also available to test bone-breaking strength in the femur. Force is applied to the femur until it breaks. The machine then measures the force needed to break the bone. Osteoporosis is defined as a disease, which leads to fractures due to minimal trauma. The ease at which the bone is broken can indicate damage to the micro-architectural structure that is common in osteoporosis. The femur breaking strength becomes a surrogate measurement for human hip fracture likelihood (Amman *et al*, 1999).

There are also disadvantages to using rats as a model. Rat bones are more active than human bones, with differences in bone turnover at many skeletal sites. Unlike humans, the epiphysis remains open well past the attainment of maturity and there is

pronounced bone remodelling throughout the life of the rat. Rats also have a different loading pattern to humans, which could affect nutritional studies in particular (Mosekilde, 1995).

It is unlikely that a perfect animal model will be developed for human studies that is not an actual human. Therefore, as long as the disadvantages of the rat model are taken into account when analysing results, they seem to be an excellent model (Mosekilde, 1995).

The growing rat is a sensitive model for bone studies. Bone remodelling is occurring at a particularly high rate, so bone density is vulnerable to changes. The diet can be manipulated to artificially lower bone density. For example the demand for calcium is particularly high and by limiting the amount of calcium in the diet, bone resorption can be increased to meet these demands.

1.5 Factors Affecting Bone Density in Rats

Rats from a small breeding colony tend to be inbred. The variation in bone density should be quite small within this population due to reduced genetic diversity (Klein *et al*, 1998). There may be a negative effect on bone density by selecting rats for their ease of handling in the laboratory.

Between the ages of one and six months, the level of testosterone in rats should be within normal levels for their stage of maturity. A normal pattern through puberty and good growth trends would indicate that androgen levels are normal. Only male rats are used for growing rat studies. The interaction of hormones with bone during puberty, mean that growing females are not suitable for this type of study.

If rats were unable to eat adequate amounts of rat chow, malnutrition may occur. This could be due to several factors. Diseases of the duodenum or jejunum could affect absorption of the diet. This would mean that the rat was unable to absorb nutrients in large enough quantities to satisfy growth or maintenance. Occasionally rats will suffer from poorly presented teeth. If a tooth is poking out sideways or backwards it may make it painful or impossible to eat. Growth rates need to be monitored, in order to assess general animal health.

Rats in shoebox cages, without the opportunity to run on a treadmill or wheel, may suffer from lack of exercise, leading to some form of muscle atrophy that could affect bone. These rats have a sedentary lifestyle, which may be uncommon to their ancestors. Wild rats tend to have a small territory, which they are reluctant to stray from. Within this territory they need to scavenge for food. This means at night they go through periods of quite high activity. Studies have been undertaken to assess the affect of exercise on BMD in rats (Iwamoto, 1998). Exercise has been shown to have a positive effect on BMD in rats (Raab *et al*, 1990).

Stress can affect bone density so it was important that our rats were kept in a stress free environment. Very low or very high temperatures could put our animals under stress. The rats were kept at 22°C in temperature controlled rooms, which were monitored daily. As there were no large changes in environmental temperature we can assume this was not a problem.

Rats can also become stressed in response to loud noises. Handlers are trained to be quiet when working in animal rooms. The radio was left on in the room to accustom rats to constant background noise and talking.

Lab rats have been selectively bred for common traits and ease of handling. It would be interesting to see whether this selection has had a positive or negative effect on bone density.

Any problems should be picked up in the daily health checks, as unhealthy animals would lose a considerable amount of condition. The animal would be removed from the trial and euthanased.

1.6 Objectives

The main objective of this trial was to establish a methodology for using Dual Energy X-ray Absorptiometry (DEXA) in small animal trials.

Specific questions related to this objective were:

1. What is the best anaesthetic to use and what are the side effects
2. How best to position the animal on the scan table for a high level of precision, which is easy to reproduce.
3. Which sites give the best precision?
4. How many animals can be scanned during a specified time?

The secondary objective of this study was to assess how accurate DEXA is for measuring bone quality in small animals.

Specific questions relating to this objective were:

1. How does DEXA vary with the age of the animal?
2. How does each specific site change with age?
3. How reproducible are the scans within and between animals?
4. What are the differences between *in vivo* and *ex vivo* DEXA measurements and do they correlate?
5. How well does DEXA correlate with bone ash mineral content.

Chapter 2

Materials and Methods

2.1 Animals and Housing

Sixty male Sprague-Dawley rats were obtained from the Small Animal Production Unit at Massey University. They were bred under the same conditions and maintained *ad lib* on standard rat chow and water. They were randomised into six age groups (n=10). The groups corresponded to the ages at which they were to be DEXA scanned and consisted of one, two, three, four, five and six months. Each rat was scanned at its appropriate age and then sacrificed. Renovations of the room containing the DEXA machine delayed the start of the trial by three months. This necessitated the use of two separate batches of rats. The first batch was from 1-3 months and the second was from 4-6 months.

Rats were weaned at three weeks of age and were randomised so that litters were distributed evenly amongst the 6 groups. At three weeks of age the rats were too small to randomise by weight. There is a strong genetic component to bone density (Kanis, 1994), so it was important to separate siblings to avoid bias within the groups.

At the time of weaning the rats were housed in cages of three. By two months they were housed in pairs. At three months they were housed separately. The rats had *ad lib* access to food and water and were maintained on standard rat chow. The room they were housed in was maintained at 22°C +/- 2°C and had a 12-hour light/dark cycle.

Correct handling of the animals was maintained to reduce fear and distress within the rats (Marshall *et al*, 1994). Animals were handled each week when cages were changed. Animals were touched on their back so as not to surprise them and then restrained. They were picked up in a similar manner to a cat, with a hand supporting the animal under its stomach. They were then placed onto the caretakers arm with a restraining hand on their back. Being picked up by the tail can be a stressful experience for rats, as picking large rats up by their tail can cause them to twist and injure or even dislocate their tail. A pain response can increase levels of cortisol.

Rats were weighed monthly to obtain growth rates. The Massey University Animal Ethics Committee approved all procedures.

2.2 Bone Densitometry

Bone mineral measurements were taken using a Hologic QDR4000 bone densitometer. It was a pencil beam unit from Hologic (Bedford USA). A daily Quality Control (QC) was performed using a hydroxyapatite spine phantom, to ensure the precision of the machine. The QC was rejected if the measurement strayed outside the accepted coefficient of variation (see Appendix 1)



Figure 2 *In vivo* whole body scan:
Positioning of the rat.

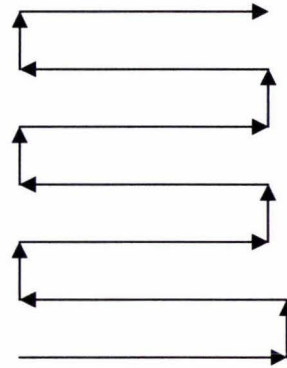


Figure 3 Scan Pattern of the
DEXA machine

The machine scanned from the tail region up towards the head, moving across the body in progressive sections (see Figures 2 and 3). The whole body scan used a 0.3 inch diameter collimator with a point resolution of 0.076 inches and line spacing of 0.1511 inches and the regional hi-resolution scans have a 0.06 inch diameter collimator and line spacing and point resolution default of 0.0127 inches and 0.0254 inches respectively (QDR 4000 User Guide, 1999).

A literature search, along with veterinary consultation was undertaken in order to select the best anaesthetic to use for DEXA procedures. There were several requirements for the anaesthetic. Rats needed to be immobilised for at least 80 minutes, in order to complete the scans. The rats also needed to be unconscious to minimise stress levels.

The rats were weighed and then anaesthetised with an intra-peritoneal injection. The dose administered was 0.1ml/100g of body weight. The anaesthetic was a mixture of 0.2ml Acepromazine (ACP) +0.5ml ketamine + 0.1ml Xylazine + 0.2ml H₂O per ml (Parton, 2000). Rats attained a suitable level of anaesthesia between five and ten minutes of injection. They were under anaesthesia for approximately two hours before they were euthanased by overdose.

Rats have a large surface area compared to their size, which means that they lose body heat quickly. Once they are anaesthetised their metabolism slows down and they are no longer able to generate enough heat to keep warm. Heat lamps were used to stop the rats from becoming hypothermic. A lubricating eye ointment was used to stop the eyes from drying out.

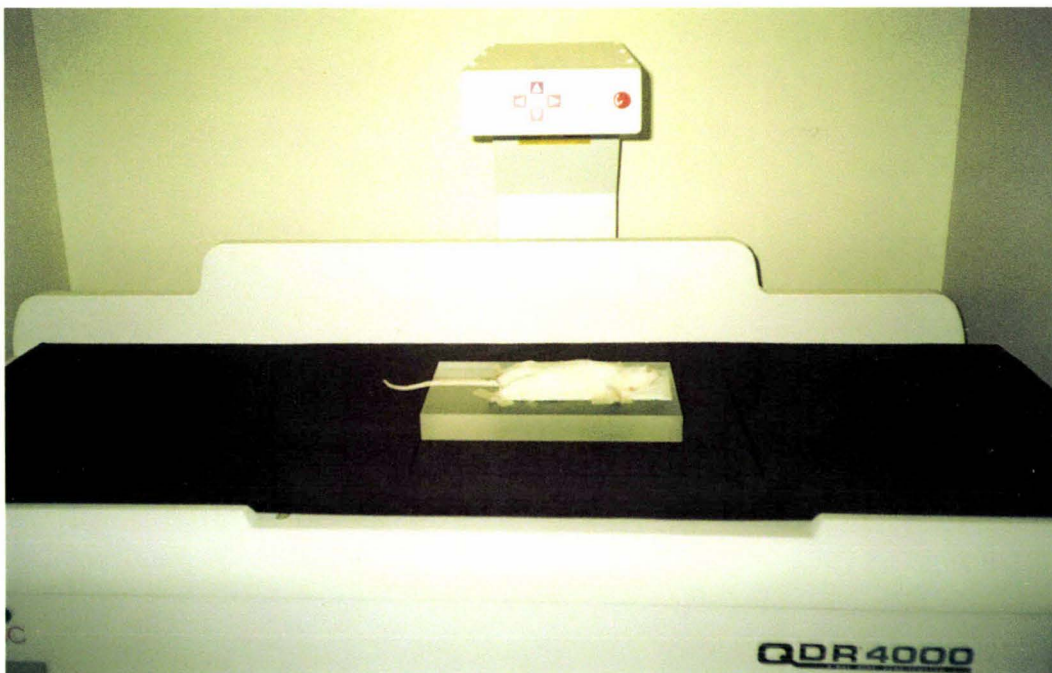


Figure 4. Rat positioned for a Spine BMD measurement.

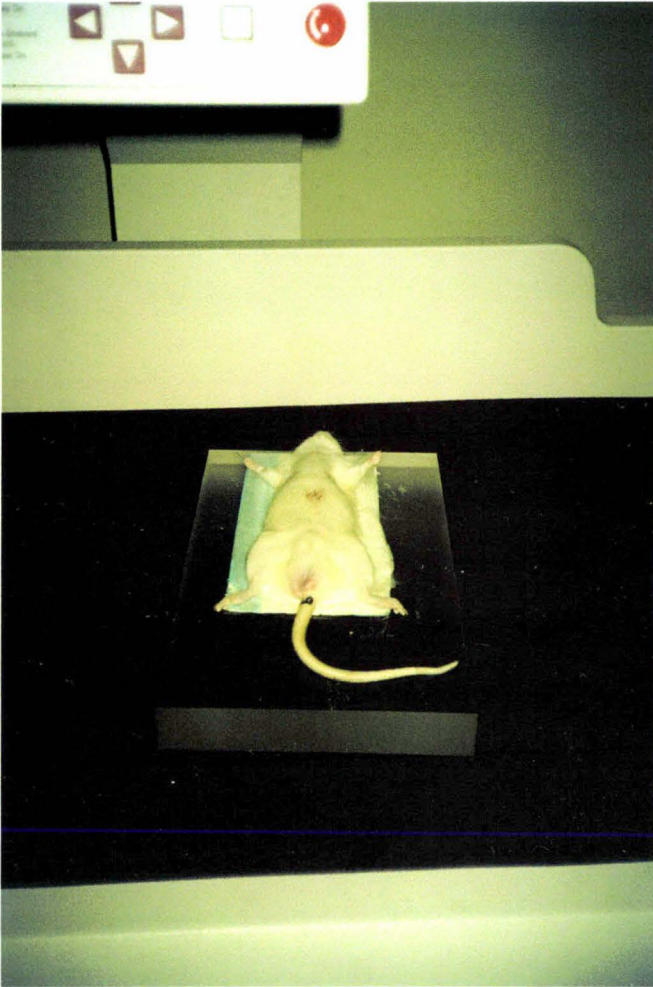


Figure 5. Rat positioned for a right femur BMD measurement.

Rats were placed on an acrylic platform of uniform thickness of 1.5 inches. Each rat underwent a whole body scan and three regional high-resolution scans of the spine and both femurs. Any rats under 200 grams were scanned three times successively, while those over 200g were scanned twice (Bertin *et al*, 1998). Rats were repositioned between each scan, to ensure the precision of the machine.

For the whole-body scan the rats were placed prone on the scan table (Rose *et al*, 1997). For the regional high-resolution scans the rats were placed in a supine position (see Figure 4). The rats were positioned on the table with right angles between the spine and the femur and femur and the tibia (see Figure 5). Great care

was taken to position the rats accurately each time so that the same region of interest could be investigated in each scan.

The rats were then euthanased by an overdose of the anaesthetic. The two femurs and the spine were excised and frozen in PBS to scan at a later date. Bones were defrosted and scanned using the hi-resolution software. The bones were scanned with a uniform covering of 3cms of PBS (Amman *et al*, 1992 Yamauchi *et al*, 1995). PBS was used rather than water to prevent minerals from being leached from the bone. *Ex vivo* femurs were scanned with the ball of the femur at the top left (LF) or top right (RF) of the scan window so that the bones were scanned from the same aspect.

2.3 Ashing of the Right Femur

After scanning, the bones were scraped and dried for 12hrs at 105°C in the oven. Length was measured using callipers and bones were weighed prior to preparation for calcium analysis. They were ground using an Industriestr 8 6580 Odar-Oberstein hammer mill made by Fritsch (Germany), weighed and split into two duplicates. The following day they were ashed in a muffle furnace at 600°C for 12hrs. The sample was weighed before and after ashing. Duplicates were then dissolved in dilute nitric acid and analysed using a Vista model Inductively Coupled Plasma Optical Emission Spectroscopy (ICPOES) machine made by Varian for calcium analysis.

Calcium values were in mg/kg. Ash percentage was calculated using the equation

$$\text{Ash \%} = \frac{\text{wt ash} \times 100}{\text{wt sample}}$$

The amount of calcium in the bone was calculated using the equation

$$\text{Bone Ca} = \frac{(\text{bone wt} \times \text{ash } \%) \times \text{Ca(mg/kg)}}{100}$$

2.4 Data analysis

Descriptive statistics were performed using Minitab version 13.

A coefficient of variation was performed on the data from each site in order to assess whether the level of variation within the group was too high. The coefficient of variation assesses variability when repeat measures are used over time. The coefficient of variation is calculated using the equation $\text{CV\%} = \frac{\text{SD} \times 100}{\text{mean}}$.

mean

SAS version 6.12 was used to run a split plot analysis of variance, with month as a fixed effect in the main plot and repetition as a fixed effect in the split plot, was used to assess whether there was any difference between months and between repetitions within months.

Correlations were performed to assess the relationships between BMD at the three different sites and the relationship between rat weight and BMD. Correlations were performed between the ashed right femur and the bone density and bone mineral content measurements of the femur.

Chapter 3

Results

3.1 Choice of Anaesthesia for *in vivo* DEXA scanning

A literature search, along with veterinary consultation was undertaken in order to select the best anaesthetic to use for DEXA procedures. There were several key requirements for the anaesthetic. Rats needed to be immobilised for at least 80 minutes in order to complete the scans, and also needed to be unconscious to minimise stress levels. Although not imperative for this trial, a reasonably short recovery time from the anaesthesia is desirable for trials involving serial measurements on live animals.

When combined with other compounds, ketamine produces light to medium planes of anaesthesia (Waynforth & Flecknell, 1992). The most widely used ketamine combination is ketamine and xylazine at a level of 90mg/kg + 10mg/kg respectively. Xylazine is an α_2 -adrenergic agonist, which has sedative and analgesic properties. A Ketamine/Acepromazine (ACP, a phenothiazine tranquilliser) mixture provides light surgical anaesthesia (75mg/kg + 2.5mg/kg).

After consultation with a veterinarian, it was decided that a combination 2 parts ACP + 5 parts ketamine + 1 part Xylazine (10%) + 2 parts sterile MQ water per ml, would be used, administered at a dose rate of 0.1ml/100g body weight. This mixture has a shelf life of one month if stored at room temperature and two months if refrigerated.

The rats used in this study were euthanased after scanning in order to harvest the spine and both femurs, thus recovery times and long-term side effects from the

anaesthesia were not examined. It took between 5 and 10 minutes for a suitable level of anaesthesia to be achieved. This was considered to be a failure of the righting reflex and a lack of response to the pedal withdrawal test (Brammer *et al*, 1993; Waynforth *et al*, 1992; Wixon *et al*, 1987; Whelan *et al*, 1993).

The mixture provided a good plane of anaesthesia for the 80 minutes that the rats were on the scan table. Marked respiratory depression was observed during scanning, and periods of apnoea were noted in about 80% of all rats. This combination of drugs is known to cause respiratory depression, among other adverse side effects, such as marked hypotension, hyperglycaemia and a diuretic effect (Waynforth *et al*, 1992; Wixon *et al*, 1987). In order to reduce periods of apnoea the dose rate was reduced to 0.07ml/100g body weight for smaller rats.

3.2 Accurate Positioning of Animals for *in vivo* DEXA scanning

Animals were positioned prone for whole body scans (Rose *et al*, 1997), with a right angle between the spine and the femur, and the femur and the tibia. This positioning could be quickly and easily reproduced between scans. Animals can be removed from the scan platform and subsequently repositioned within two minutes. It also allowed the scan window to remain the same size for each of the duplicate or triplicate scans. This method provides a standard methodology that can be used by anyone running small animal trials using the DEXA machine.

The coefficient of variation (CV), expressed as a percentage, measures the variability of repeated measures on the same samples. It is an easy method of assessing the precision of the operator. A low coefficient of variation suggests that the operator is

accurate in repositioning and that the method of positioning gives good reproducibility.

The variability between different animals within the same age group was measured. Triplicate scans were performed for each rat weighing <200g, with a CV for whole body scans of 4.1%. Duplicate scans were performed on each rat weighing >200g. The CV for whole body scans ranged from 2.1 to 5.4%.

Rats were positioned supine for hi-resolution scans, again with a right angle between the spine and the femur, and the femur and the tibia. This was a quick and easy method of positioning that allowed bones to be scanned from the same aspect each time.

For rats weighing <200g, which corresponds to the one month old group, the CV between animals of that age was 13.2 % for the spine, and 43.6% and 36.1% for the left and right femur respectively. This is extremely high, and indicates a large degree of variation within rats at this age. The CV for the spine ranged from 3.4 to 7.7% in rats weighing >200g and the CV for the femurs ranged from 2.1 to 5.1% (see Table 1 below).

A coefficient of variation between 1 to 2% can be achieved within groups using Hologic machines (Ammann *et al*, 1992; Casez *et al*, 1994). However, these results were obtained using rats that had been allocated to groups on a weight basis, therefore there was less variation in animal weight within rats of the same group. For this study, the main priority was to separate siblings, so that a genetic bias did not occur. Unfortunately a large variation in weight occurred. This resulted in a higher coefficient of variation.

Table 1. The coefficient of variation (percentage) of repeat measurements between animals of the same age at each *in vivo* site.

age of rat variable	1mth CV%	2mth CV%	3mth CV%	4mth CV%	5mth CV%	6mth CV%
WB	4.1	4.8	2.8	3.1	5.4	2.1
Spine	13.2	7.7	3.4	5.7	6.0	4.8
LF	43.6	4.6	2.1	2.5	3.7	4.3
RF	36.1	5.1	2.9	2.2	4.4	2.2

3.3 Accurate positioning of animals for *ex vivo* DEXA scanning

The spine and both femurs were excised and frozen in PBS, so that they could be scanned *ex vivo* at a later date. A layer of tissue about 0.5cm in depth was left covering the bone. Without the presence of this tissue the DEXA machine had trouble detecting the individual lumbar regions of the spine. The spine was positioned supine in a thin walled dish, and covered with PBS to a depth of 3cms. It was necessary that the spine thawed in a straight line, to ensure that the region of interest boxes could be drawn around the lumbar region correctly. This positioning was easily replicated. The CV for the spine ranged from 3.4 to 7.7% and the CV for the femurs ranges from 2 to 6% in the 2 to 6-month group. The CV for the one-month old group is still unacceptably high (refer Table 2).

Table 2. The coefficient of variation (percentage) of repeated measurements between animals of the same age at each *ex vivo* site.

age of rat variable	1mth CV%	2mth CV%	3mth CV%	4mth CV%	5mth CV%	6mth CV%
Spineex	10.7	6.0	2.5	4.5	4.9	4.4
LFex	9.2	3.1	2.7	2.4	5.6	3.6
RFex	7.5	4.1	2.0	3.8	4.0	3.4

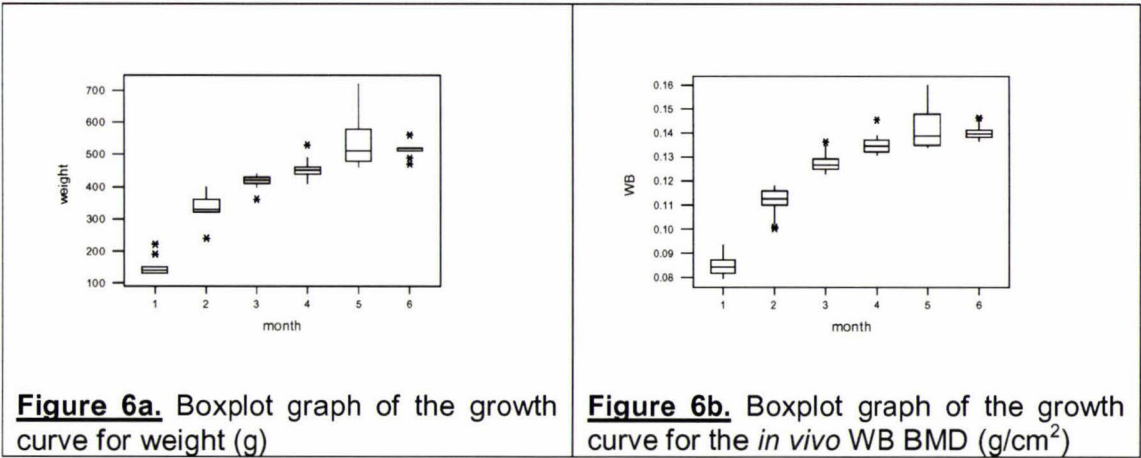
In general CV values were lower for the femurs, indicating that they have the highest precision. The *ex vivo* femurs were positioned flat, with the ball of the femur in the top left of the scan window for the left femur, and the top right of the scan window for the right femur. Again tissue was trimmed to a uniform thickness of 0.5cm and the bones were covered in PBS to a depth of 3cms. This guaranteed that the bone was scanned from the same aspect each time and allowed for quick positioning.

3.4 *In vivo* and *ex vivo* scans of animals aged from one to six months

3.4.1 Comparisons of age, weight and bone density using *in vivo* and *ex vivo* DEXA scans

The descriptive statistics of the *in vivo* & *ex vivo* DEXA scans are shown in Appendix 3. The animals used in this study ranged from one-month old rats that had only recently been weaned to six-month-old mature rats. Weight was plotted against age to obtain a growth curve (Figure 6a). The mean weight of each group increased from 148g at month one, to 512g at month six. On dissecting the spine and both femurs, it was noted that the rats were obese.

Figures 6a-h Increase in body weight & BMD, relative to age, in one to six month old male rats.



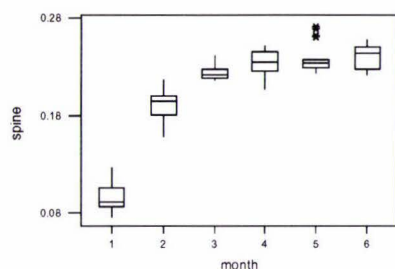


Figure 6c. Boxplot graph of the growth curve for the *in vivo* spine BMD (g/cm^2)

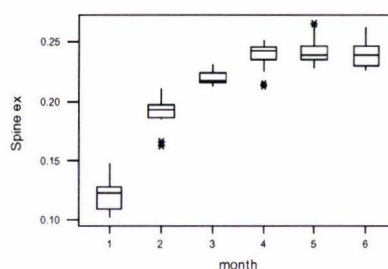


Figure 6d. Boxplot graph for the *ex vivo* spine BMD (g/cm^2)

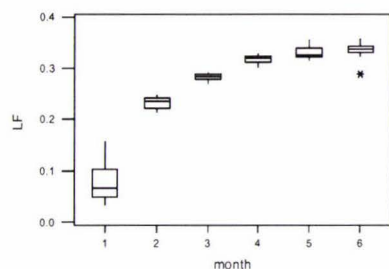


Figure 6e. Boxplot graph of the growth curve for the *in vivo* LF BMD (g/cm^2)

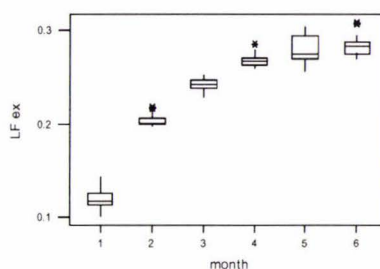


Figure 6f. Boxplot graph for the *ex vivo* LF BMD (g/cm^2)

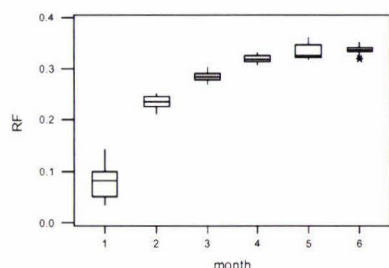


Figure 6g. Boxplot graph of the growth curve for the *in vivo* RF BMD (g/cm^2)

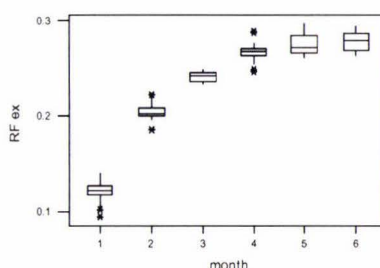


Figure 6h. Box plot graph for the *ex vivo* RF BMD (g/cm^2)

Whole body, spine, and left and right femur BMD increase from one month to six months of age. These graphs show that the five-month old group has a higher mean BMD for their whole body scans than any other group, however this is not statistically significant (Figure 6b). The large variation in whole body BMD for the five-month-old group (Figure 6b) mirrors the large variation in weight at this age (Figure 6a).

There is an almost linear relationship between weight and age to about four months. From four to six months, a plateau begins to form. The growth curve for the spine

(Figure 6c) begins to plateau at three months of age, which is a month earlier than other sites. Figure 6d shows the growth curve for the *ex vivo* spine. The BMD values are almost identical to Figure 6c, which shows the growth curve for the *in vivo* spine. The scans of the *in vivo* femurs (Figures 6e & 6g) have higher BMD values than the scans of the *ex vivo* femurs (Figures 6f & 6h).

The descriptive statistics (Appendix 3), show that the *in vivo* and *ex vivo* scans of the spine have very similar values, with the BMD measurements increasing from one to six-months of age. The values for the *in vivo* femurs are higher than those of the *ex vivo* femurs, most likely due to differences in positioning, although they do follow the same trend as the animals age. The standard deviation of the *ex vivo* femurs is also lower than that of the *in vivo* scans (Appendix 3). This is confirmed by the growth curves, which are almost identical for the *in vivo* and *ex vivo* scans (Figures 6a-h).

The BMD for the hi-resolution scans of the spine and both femurs do not show such a large variation in the five-month old group. The impact of weight on BMD would appear not to be as pronounced in the hi-resolution scans as it is in the whole body scans. The six-month-old group has the highest mean BMD for each of the high resolution scans. There is a distinct narrowing in the range of BMD values as the rat's age.

3.4.2 Frequency Distribution of the data

Frequency distribution graphs were plotted to assess whether the data was normally distributed. Figures 7a-h show the frequency distribution graphs for weight and BMD. All frequency distribution graphs followed the same trend, showing a bimodal distribution. The one-month old group forms a separate population, while the two to six-month old group displays a typical bell shaped curve.

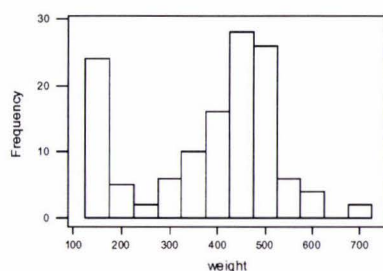


Figure 7a. The frequency distribution of the weight (g) from 1 to 6 months of age

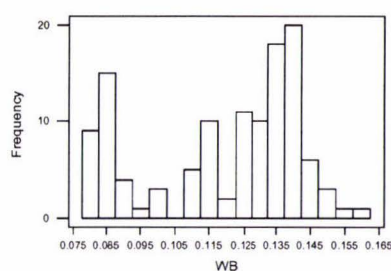


Figure 7b. The frequency distribution of the WB BMD (g/cm^2) from 1 to 6 months of age

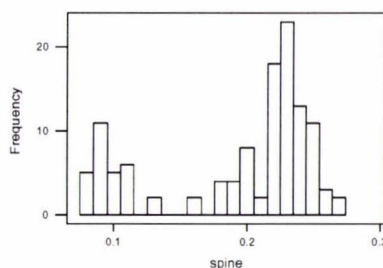


Figure 7c. The frequency distribution of the *in vivo* spine BMD (g/cm^2) from 1 to 6 months of age

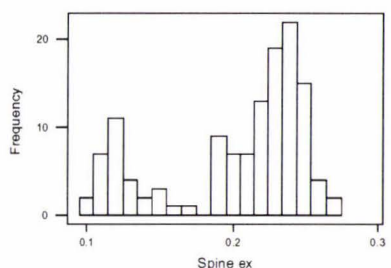


Figure 7d. The frequency distribution of the *ex vivo* spine BMD (g/cm^2) from 1 to 6 months of age

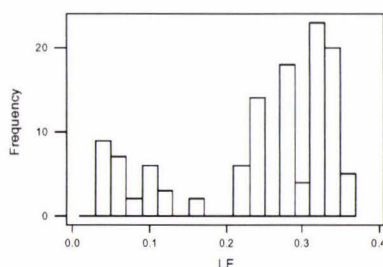


Figure 7e. The frequency distribution of the *in vivo* LF BMD (g/cm^2) from 1 to 6 months of age

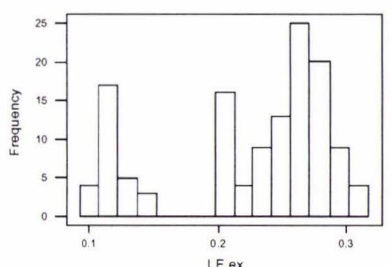


Figure 7f. The frequency distribution of the *ex vivo* LF BMD (g/cm^2) from 1 to 6 months of age

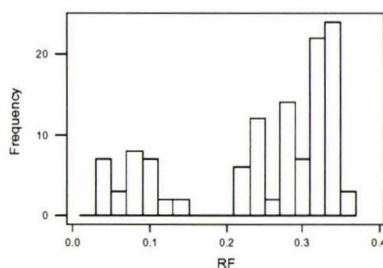


Figure 7g. The frequency distribution of the *in vivo* RF BMD (g/cm^2) from 1 to 6 months of age

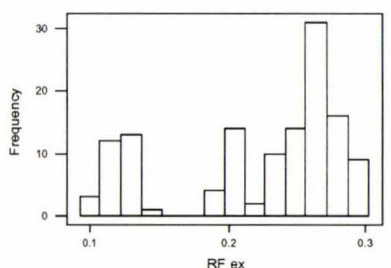
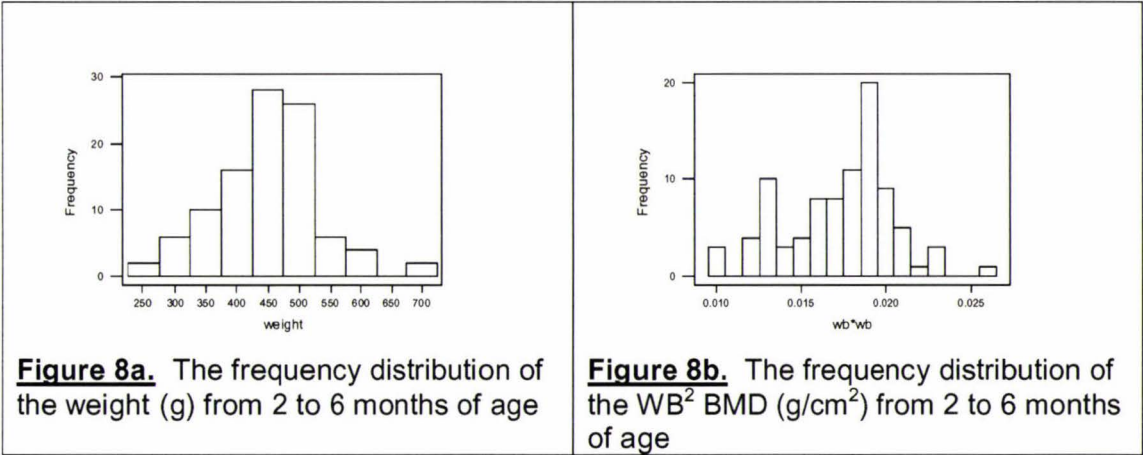


Figure 7h. The frequency distribution of the *ex vivo* RF BMD (g/cm^2) from 1 to 6 months of age

Rats weighing <200g (corresponding to the one-month-old group) were removed from the frequency distribution graphs. At one month of age calcification of bones does not seem to be complete. This seems to be an effect of age rather than weight as previous trials have successfully scanned rats below 200g (Lu *et al*, 1994). For this reason and because of the very high coefficient of variation, the one-month-old group is not considered suitable for baseline scans and has been removed from further analysis. Subsequent data focuses on the 2 to 6 month old group (figures 8a-8h).

Transformations were performed in order to improve the shape of the bell curve. By squaring the values for whole body and *in vivo* spine BMD the normal distribution was improved (Figures 8b & 8c). Transformations of the left and right femur BMD did not improve the distribution. However, the assumption was made that the distribution is close enough to normal to make ANOVA results valid.

The frequency distribution of the excised femurs shows a more normal distribution than the *in vivo* femurs (Figures 8e-h). The positioning of the femurs *ex vivo* seems to be more accurate.



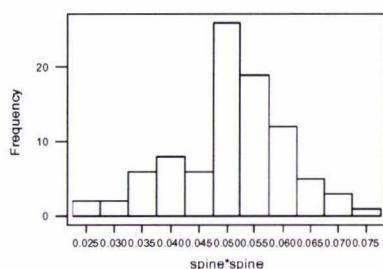


Figure 8c. The frequency distribution of the *in vivo* spine² BMD (g/cm²) from 2 to 6 months of age

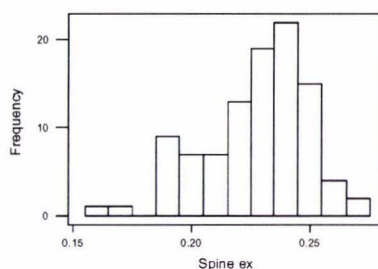


Figure 8d. The frequency distribution of the *ex vivo* spine BMD (g/cm²) from 2 to 6 months of age

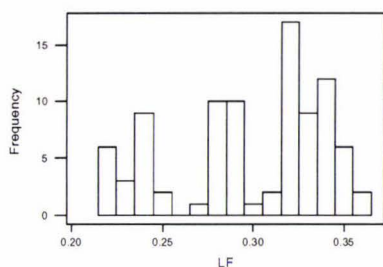


Figure 8e. The frequency distribution of the *in vivo* LF BMD (g/cm²) from 2 to 6 months of age

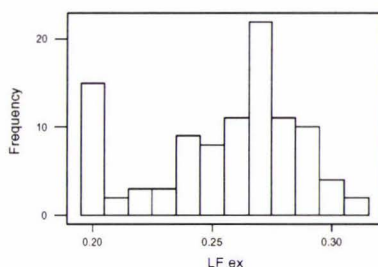


Figure 8f. The frequency distribution of the *ex vivo* LF BMD (g/cm²) from 2 to 6 months of age

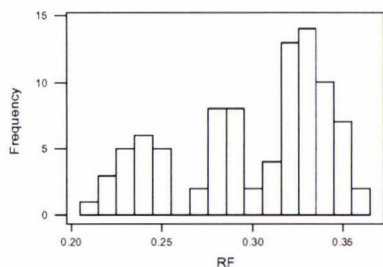


Figure 8g. The frequency distribution of the *in vivo* RF BMD (g/cm²) from 2 to 6 months of age

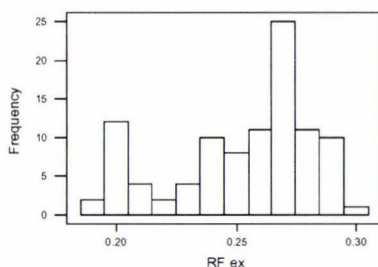


Figure 8h. The frequency distribution of the *ex vivo* RF BMD (g/cm²) from 2 to 6 months of age

3.5 ANOVA

3.5.1 Analysis of Variance of the *in vivo* DEXA scans

An analysis of variance was performed to determine whether there were significant differences between the BMD of rats at different ages and to assess whether there

was a significant difference between the duplicate scans performed on each animal *in vivo*.

Table 3. The split plot analysis of variance of *in vivo* DEXA for the 2 to 6 month-old age groups of rats.

variables	Effects Fitted			
	month	Ratno(month)	repetition	Month*repetition
WB ²	<0.0001*	<0.0001*	0.9447	0.9681
Spine ²	<0.0001*	<0.0001*	0.9162	0.6086
LF	<0.0001*	<0.0001*	0.5269	0.1546
RF	<0.0001*	<0.0001*	0.5450	0.1724

Table 3 shows the results of the ANOVA of the 2-6 month old rats. The p-values for the effects fitted of the *in vivo* scans are given. P is considered significant at $p<0.05$ and is designated by an asterisk *.

The first column (month) deals with the variation in BMD at different ages. There is a highly significant difference in the BMD at each site between months ($p=0.0001$). As this study assessed growing rats, a significant difference between a two-month old rat and a six-month old rat is to be expected.

The second column (Ratno(month)) deals with the variation in BMD between different animals within each month. There is a significant difference between the individuals within each age group. This is an unexpected result. There is a large variation between animals at two and five months of age, which resulted in a significant difference within these groups. At two months of age the animals are growing at a fast rate. Five months of age coincides with puberty and this could explain individual differences due to different developmental stages.

The third column (repetition) deals with the variation between the duplicate scans of each animal. There is no significant difference between the repetitions. This

confirms that animals can be accurately repositioned and scanned by the same operator.

The fourth column (Rep(month)) deals with the repetition between duplicate scans of the same rat within each age group. Again there is no significant difference between the repeated scans for each animal, confirming the precision of the operator.

Age has a significant effect on BMD and it is necessary to ascertain which ages differ from each other, in order to calculate start and endpoints for trials. Looking at Figure 6c the spine begins to plateau at four months of age, a month earlier than all of the other sites. Results of post-hoc testing (Tukey t-test, Table 4), confirms this trend, with no significant difference in spine BMD between the 4 and 5 month-old groups.

Table 4. Least Squares means of weight and BMD at the different sites, for the two to six-month old groups.

	2 months	3 months	4 months	5 months	6 months
Weight (g)	335 ^a	416 ^b	458 ^c	537 ^d	512 ^d
WB (g/cm²)	0.112 ^a	0.127 ^b	0.135 ^c	0.141 ^d	0.141 ^d
Spine (g/cm²)	0.191 ^a	0.224 ^b	0.234 ^c	0.238 ^{cd}	0.243 ^d
LF (g/cm²)	0.233 ^a	0.284 ^b	0.319 ^c	0.331 ^d	0.336 ^d
RF (g/cm²)	0.235 ^a	0.285 ^b	0.319 ^c	0.334 ^d	0.336 ^d

Values with a different superscript denote a significant difference between groups (P<0.05).

There is a significant difference between the two, three and four month old groups at all sites (Appendix 4). There is no significant difference between the four and five month old groups at the spine, although there is a significant difference between these two ages at all other sites. This appears to indicate that the spine matures at a faster rate than the rest of the body. There is no significant difference between the data for the five and six month old groups at any site. This indicates that growth is slowing at five and six months of age.

3.5.2 Analysis of Variance of the *ex vivo* DEXA scans

An analysis of variance was performed to determine whether there were significant differences between the BMD of rats at different ages and to assess whether there was a significant difference between the duplicate scans of each animal *ex vivo*.

Table 5. Analysis of variance for the *ex vivo* scans.

Variables	Effects Fitted			
	month	Ratno(month)	repetition	Month*repetition
Spineex	<0.0001*	<0.0001*	0.9507	0.8813
LFex	<0.0001*	<0.0001*	0.9470	0.7804
RFex	<0.0001*	<0.0001*	0.9932	0.3076

The p-values for the ANOVA of the *ex vivo* scans are given. P is significant at $p<0.05$ and is designated by an asterisk *.

Table 5 corresponds with Table 3 for the *in vivo* scans. The first column (month) deals with the variation between each age group. The BMD values for the *ex vivo* scans of each month are significantly different.

The second column (Ratno(month)) deals with the variation of the rats within each month. There is a significant difference between the *ex vivo* scans within each month. This follows the same trend as the *in vivo* scans. This is an unexpected result. The large variation between animals at two and five months of age is believed to be responsible for this.

There is no significant difference between the duplicate scans of the *ex vivo* spine and both femurs between or within months (repetition & Rep(month)).

Table 6. Least Squares means for the *ex vivo* BMD at the different sites, for the two to six-month-old groups.

	2 months	3 months	4 months	5 months	6 months
Spineex (g/cm²)	0.191^a	0.220^b	0.239^c	0.243^c	0.240^c
Lfex (g/cm²)	0.204^a	0.242^b	0.268^c	0.279^d	0.284^d
RFex (g/cm²)	0.203^a	0.241^b	0.266^c	0.275^d	0.277^d

Values with a different superscript denote a significant difference ($P < 0.05$).

Table 6 shows which age groups have a significant difference in their *ex vivo* BMD values. The BMD values follow the same pattern as seen in the *in vivo* scans. There is no significant difference between the four-month old group and the five and six month old groups at the spine, although there is a significant difference between the femurs at this age. This indicates that growth of the spine has slowed down at four months of age, a month earlier than all other sites. There is no significant difference between the five and six month group. This agrees with the results of the *in vivo* scans.

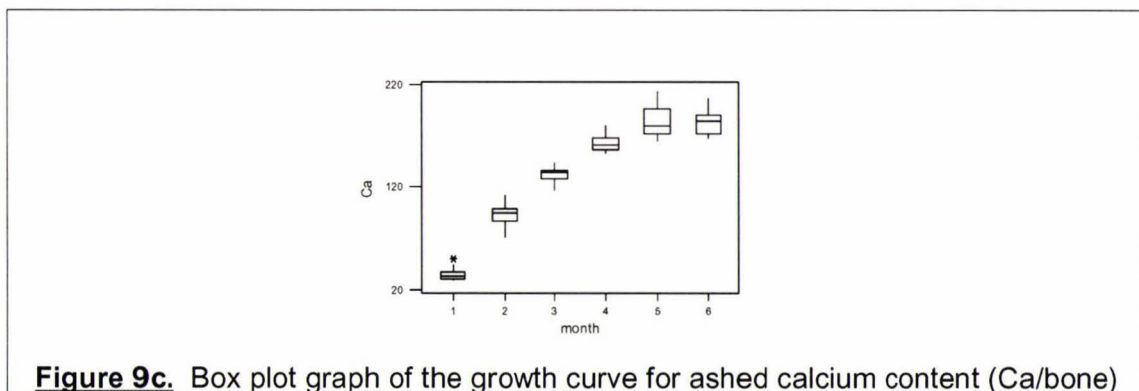
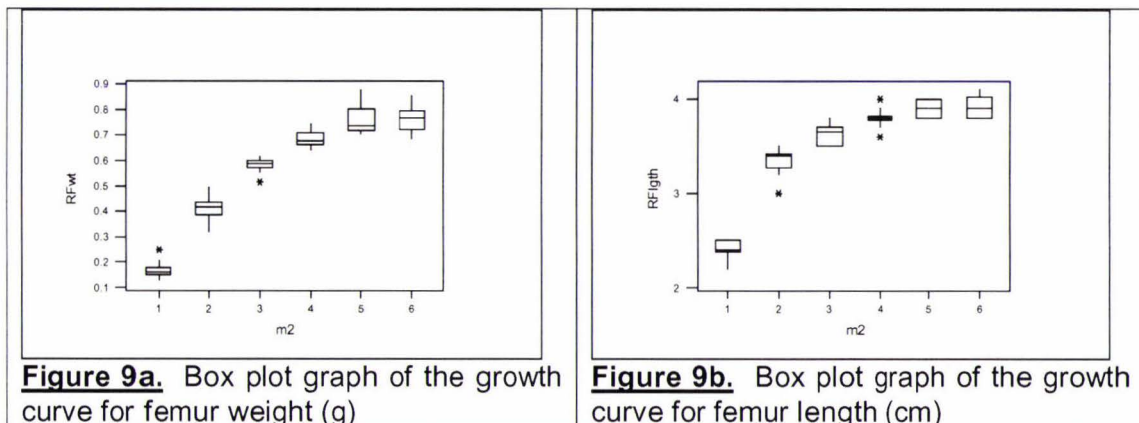
Table 7. The analysis of variance of the *in vivo* versus the *ex vivo* scans

	P-value
Spine vs spineex	0.561
LF vs LFex	0.000*
RF vs RFex	0.000*

There is no significant difference in BMD for the *in vivo* and *ex vivo* spine ($p = 0.561$). Positioning of the spine *in vivo* and *ex vivo* was identical, with the rat scanned in a supine position. However there is a significant difference between the *in vivo* femurs and the *ex vivo* femurs $p = 0.000$ (Table 7). The BMD for the *ex vivo* femurs is significantly lower than the BMD for the *in vivo* femurs. The P-value is considered to be significant at $P < 0.05$. This is denoted by an asterisk *.

3.6 Right femur weight, length and ashed calcium content

The right femur was ashed in order to ascertain whether the same relativities could be obtained using either ashed calcium content or DEXA BMD/BMC. The average femur weight increased from 0.1698g at month one to 0.7657g at month six (Appendix 3). Average femur lengths increased from 2.41 cm at month one to 3.92 cm at month six. As is to be expected, the increase in femur length and weight follows the same trend with increasing age, as seen in Figures 6a-h with weight and BMD. The growth curves begin to plateau at around four months of age (refer Figure 9a and Figure 9b).



The growth curve for femur weight and ashed calcium content follow the same trend. There is a linear relationship until about four months, and then a plateau forms at five

and six months. Femur length seems to plateau at around four months of age. This indicates that the longitudinal growth of the femur slows at four months, although bone mass continues to increase.

3.6.1 Analysis of variance of excised right femur

An analysis of variance was performed on the variables for the right femur to identify the pattern of growth, and whether it should be assessed using DEXA or ashed calcium content. Bone weight and ashed calcium content follow the same trend, but the growth of femur length looks to slow down a month earlier than the other two variables (Table 8).

Table 8 Least Squares means for the excised right femur

	2 months	3 months	4 months	5 months	6 months
RFwt (g)	0.41 ^a	0.58 ^b	0.69 ^c	0.76 ^d	0.77 ^d
RFIgh (cm)	3.34 ^a	3.63 ^b	3.80 ^c	3.89 ^{cd}	3.92 ^d
Ash Ca (mg/bone)	92 ^a	131 ^b	162 ^c	182 ^d	180 ^d

Values with a different superscript are significantly different ($P < 0.05$)

There is a significant difference in the bone weight and ashed calcium values between the two, three, four and five-month old rats, but there is no significant difference in femur length between the four and five month old groups. This indicates that as far as length is concerned, bone growth slows down at four months of age, and this could coincide with a period known as consolidation in humans. There is no significant difference between the femurs of the five and six month old group.

3.7 Pearson Correlations

3.7.1 Pearson Correlation between age and BMD, weight and BMD, and *in vivo* and *ex vivo* BMD

Correlations were performed in order to assess whether the same results could be obtained using different sites, such as spine, LF and RF. The use of *in vivo* DEXA against *ex vivo* DEXA was also evaluated, to ascertain whether the two methods were comparable. A strong positive correlation between two sites indicates that they have the same ranking. If they have the same ranking, then the assumption can be made that the same relativities can be achieved by using either site.

Referring back to the growth curves, each site follows the same trend. It is obvious that weight will increase with age up until maturity, when a plateau will form. The BMD follows this trend at all sites. In nearly all cases, excluding the spine, the plateau forms at around four months of age. From this age onwards it appears that there is an increase in bone density but no increase in size. This may be the point at which peak skeletal area is achieved. There is a strong positive correlation between age and weight (0.89), and age and BMD (ranging from 0.84 to 0.91 refer to Table 9). A strong positive correlation indicates that an increase in age, weight, or BMD, at a particular site, will subsequently lead to an increase in BMD at all other sites.

Age was constant in each group, thus age cannot be responsible for the large variation in BMD present within some groups. There was a large variation in weight amongst some groups, which corresponded with the large variation in BMD. Weight is strongly linked to BMD and has a stronger correlation than age (Table 9), indicating that weight is a better predictor than age.

The *in vivo* and *ex vivo* spine were positioned in exactly the same manner, so the correlations are expected to be high. The positioning of the femurs *ex vivo* was different to that *in vivo*. The data at the *ex vivo* sites had lower values than the data for the *in vivo* sites (refer to appendix 3). Despite the differing values there is a strong positive correlation between *in vivo* and *ex vivo* BMD measures, indicating that the ranking is the same. The fact that the growth curves for the *in vivo* and *ex vivo* femurs follow exactly the same trend indicates that the same relativities remain with both methods.

Table 9 Pearson Correlations between age, weight and BMD are shown. For these correlations values of all ages were pooled (n = 60).

	Mth	wt	WB	spine	LF	RF	LFex	Spex
Wt	0.894 0.000							
WB	0.905 0.000	0.974 0.000						
spine	0.837 0.000	0.955 0.000	0.974 0.000					
LF	0.877 0.000	0.958 0.000	0.971 0.000	0.975 0.000				
RF	0.880 0.000	0.962 0.000	0.974 0.000	0.977 0.000	0.981 0.000			
LFex	0.910 0.000	0.958 0.000	0.983 0.000	0.968 0.000	0.981 0.000	0.981 0.000		
Spex	0.864 0.000	0.960 0.000	0.982 0.000	0.981 0.000	0.970 0.000	0.974 0.000	0.977 0.000	
RFex	0.903 0.000	0.966 0.000	0.981 0.000	0.972 0.000	0.981 0.000	0.979 0.000	0.985 0.000	0.976 0.000

3.7.2 Pearson Correlations for Ashed Calcium against *in vivo* and *ex vivo* Right Femur BMD & BMC

Pearson correlations were performed between ashed calcium results and the descriptive statistics for the right femur, in order to assess whether the DEXA machine accurately predicted the relationship between bone ash content, bone mineral content and bone mineral density (Table 10). Although the DEXA machine overestimates bone mineral content, there is a strong correlation between the ashed

calcium results and the DEXA BMD and BMC measurements of the right femur, indicating that they have the same relativity to the bone.

Table 10, Pearson correlations between ashed femur calcium and right femur BMD & BMC *in vivo* and *ex vivo* in the 2-6 month old group.

	Body weight	Femur length	Ashed calcium	Bone wt	RF BMD	RFex BMD	RF BMC
Femur length	0.874 0.000						
Ashed calcium	0.925 0.000	0.926 0.000					
Bone wt	0.929 0.000	0.930 0.000	0.933 0.000				
RF BMD	0.892 0.000	0.908 0.000	0.975 0.000	0.972 0.000			
RFex BMD	0.874 0.000	0.915 0.000	0.952 0.000	0.948 0.000	0.954 0.000		
RF BMC	0.871 0.000	0.881 0.000	0.944 0.000	0.933 0.000	0.943 0.000	0.907 0.000	
RFex BMC	0.934 0.000	0.931 0.000	0.994 0.000	0.996 0.000	0.977 0.000	0.958 0.000	0.935 0.000

Correlations between liveweight and femur weight and length are also shown here. The correlation between Bone Mineral Content assessed by DEXA, and BMC measured by ashed calcium results are also very strong. These correlations indicate that the similar results could be obtained using ashed calcium content or DEXA. Therefore, either method can be used to assess bone quality.

Chapter 4

Discussion

Introduction

Growing rats have been used as a model for bone resorption studies. They have a high demand for calcium, which means that they are particularly sensitive to dietary manipulations. New technology has made it possible to measure bone mineral density without the need to sacrifice animals. DEXA allows for serial BMD measurements to be made on live animals. The objective of this trial was to create a methodology for scanning small animals using a QDR4000 DEXA unit from Hologic. This trial endeavours to answer several key questions, pertaining to time frames, positioning and accuracy, so that a quick and easy method for BMD measurements can be recommended. Reference data on the growth pattern of male rats was also collected in order to allow researchers to estimate rat size and feed intake requirements for future trials.

4.1 Choice of Anaesthesia for DEXA scanning

A combination of 0.2ml ACP + 0.5ml ketamine + 0.1ml Xylazine + 0.2ml H₂O per ml was used to anaesthetise the rats, administered at the recommended dose rate of 0.1ml/100g of body weight. This dose rate gave a good plane of anaesthesia for the 100 minutes that the rats were on the scanning table but caused periods of apnoea in around 80% of rats, which was a concern for future trials where animals would be expected to recover. Waynforth *et al* (1992) noted apnoea as a side effect of this drug combination.

Recovery from the anaesthetic was not assessed in this trial but follow up trials have noted death in approximately 7% of rats. This is quite distressing for the researcher and poses a problem with animal ethics. If animals are dying from a seemingly non-invasive procedure, the question should be asked as to why this is happening. The most obvious answer is that there is a problem with the anaesthetic, as it is the only manipulation performed on the animal, that could possibly be life threatening.

This trial looked at the precision of the operator and of the method of positioning; hence scans were repeated at each site. This meant that the scanning time was about 100 minutes. In general, for most studies not all scan sites would be investigated. If the researcher chose to use hi-resolution scans only, scan times would be reduced to between thirty and forty minutes, depending on the operator. If the rat only needs to be anaesthetised for half the time, then it is possible that a reduced dose of the anaesthetic could be administered.

Further trials carried out at this research centre, have shown that a dose rate of 0.05ml/100g body weight provides good anaesthesia for about 40 minutes. The induction time for the anaesthetic is increased to 10-15 minutes, but sleep time is reduced to 2hrs, and animals recover well enough to eat normally within the 24hr period following anaesthesia. Veterinarians recommend a dose rate of 0.1ml/100g body weight to provide a level of anaesthesia suitable for surgical procedures. However such a deep level of anaesthesia is not required for DEXA scanning, as the animals are not undergoing invasive procedures. Therefore, the researcher can confidently reduce the level of anaesthetic without raising ethical or welfare concerns.

4.2 Positioning of Animals

4.2.1 Accurate Positioning of Animals for In Vivo DEXA Scanning

A standard procedure for positioning rats on the scan table was required, so that comparisons between animals could be made. Positioning the rats with a right angle between the spine and the femur and the femur and the tibia (Ammann *et al*, 1993) was a quick and easily reproducible method but it also needed to be accurate. The coefficient of variation between animals within each group for the two to six month old rats was generally low (Table 1), which suggests that the operator was accurate in repositioning and that the method of positioning gave good reproducibility.

The coefficient of variation for the one-month old group was very high (Table 1 & 2). This seems to be due to a large variation between animals in this group. At one month of age the animals were still growing rapidly. Looking at the growth curves (Figures 6a-h), at one month the slope is still steep and has not yet begun to plateau. Individual differences in metabolism will affect growth rates, and will be more noticeable in periods of fast growth. This may explain the large range between values at this age when compared to older animals. A high coefficient of variation could be expected within this group.

Gala Paniagua *et al* (1990) believed that the best DEXA precision in rats was from the femur and femoral subregions. In this study the femurs generally have a better coefficient of variation than any other site (Table 1). This indicates that there is less variation in the femur within these age groups. The femurs seem to have a slower growth rate than the spine, which means the animals are grouped more tightly around the mean.

The femur may not be the most sensitive site for detecting changes in BMD, as the shaft of the femur is made up largely of cortical bone, rather than trabecular bone. Trabecular bone is particularly sensitive to the effects of bone metabolism (Grier *et al*, 1996). Specific sites of the femur such as the distal metaphysis are rich in trabecular bone (Pastoureau *et al*, 1995). Therefore when using the femur for evaluating BMD, it may be beneficial to target subregions rich in trabecular bone rather than looking at the femur as a whole.

4.2.2 Accurate Positioning of Animals for *ex vivo* DEXA scanning

Apart from at one month of age the coefficient of variation for the *ex vivo* scans ranges from 2% to 6% (Table 2). The coefficient of variation is slightly better for the femurs than for the spine, which follows the trend seen *in vivo*. The level of variation within the sample is acceptable. If the coefficient of variation is too high then differences due to sampling error could be attributed to a treatment effect.

Coefficients of variation between 1-2% can be achieved within groups of rats of the same age using Hologic DEXA machines (Ammann *et al*, 1992; Casez *et al*, 1994). However the results obtained by Casez *et al*, were of rats that had been allocated to groups on a weight basis, therefore there was less variation in animal weight than what was seen in this trial. Unfortunately animals in this trial were allocated into groups at three weeks of age and the most important concern was that the animals were designated into groups on a genetic basis, in order to separate siblings. There is a strong genetic component to BMD and growth rate. If rats with the same genetic predisposition are grouped together, it may create a bias and results could be unnaturally higher or lower than they should be.

In order to reduce the chance of operator variation being mistaken for a treatment effect, the same operator was used for all scans. Further trials to investigate the precision of different operators scanning the same bone are needed. It would be convenient if different operators could be used on the same trial. This would minimise the burden on the operator, who otherwise may need to spend long days scanning animals, and would decrease delays in scanning due to unforeseen circumstances, such as the operator becoming ill or relocating.

4.3 Comparisons of age, weight and bone density using *in vivo* and *ex vivo* DEXA scans

4.3.1 Increases in Weight and BMD as male rats age.

The growth curves (Figures 6a-h) are almost linear till about four months of age when a plateau begins to form. This plateau indicates that growth has slowed down after four months of age. The most sensitive time for manipulating bone growth would be from weaning until four months of age, when bone is growing at a fast rate and there is a high demand for nutrients such as calcium. Once the growth rate has slowed down, the researcher would need to employ a more aggressive approach, such as ovariectomy, in order to manipulate changes in bone.

The growth curve for the spine (Figures 6c&6d) plateaus a month earlier than the growth curve for the WB and Femurs (refer Figures 6b&6e-h). This could indicate that the femurs are slower to mature than the spine. The extra outliers that are present in the *ex vivo* box plots (Figures 6d,f&h) may indicate that the *ex vivo* scans give a more accurate BMD measurement, as there is less interference between the bone and soft tissue.

4.3.2 In vivo versus ex vivo BMD measurements

Appendix 3 displays the descriptive statistics for the *in vivo* and *ex vivo* hi-resolution scans. The *in vivo* and *ex vivo* spines have very similar BMD values. This is reflected in the growth curves (Figures 6c&d). Figure 6d shows the growth curve for the *ex vivo* spine, which is almost identical to Figure 6c, which shows the growth curve for the *in vivo* spine. The curve for the spine is almost linear until 3 months of age and then forms a plateau from four months of age. This is a month earlier than any other site. The spine was positioned supine for both *in vivo* and *ex vivo* scanning. The tissue around the *ex vivo* spine was trimmed to a uniform thickness of 0.5cms and for scanning it was submersed in PBS to a depth of 3cms. The fact that the *in vivo* and *ex vivo* spine measurements are so close indicates that removal of surrounding tissues has not affected the BMD measurement.

The BMD values for the *in vivo* and *ex vivo* femurs are significantly different (Table 7). The values for the *ex vivo* femurs are lower than the *in vivo* femurs, although they do follow the same trend in the growth curves (Figures 6e-h). The femurs grow almost linearly to about four months and then plateau from five to six months. The difference between the *in vivo* and *ex vivo* femurs was a concern, as the *ex vivo* scans are of bones that were excised directly after *in vivo* scanning, and were expected to be similar.

The most obvious reason for the difference between the *in vivo* and *ex vivo* femurs was that they were positioned differently. The DEXA machine would have analysed the two different types of scans from two very different perspectives. *In vivo* the femurs were scanned from a lateral view, while *ex vivo* bones were placed supine, so that they were scanned flat (refer figures 11 & 12). This will change the area of bone that is being scanned. BMD is a function of BMC over area, so a change in area will

result in a change in BMD. To ensure precision and to enable serial scans to be made, it is imperative that the bone is positioned in the same manner each time it is scanned, and that the region of interest is defined correctly. The region of interest is defined by outlining the specific area for analysis using computer software.

Direct comparisons between *in vivo* and *ex vivo* scans cannot be made. *Ex vivo* scans can be used as a once off endpoint or to confirm *in vivo* relativities. However they cannot be used as the endpoint of a series of *in vivo* scans. In serial scans the object is to plot the changes in BMD against time. The *ex vivo* BMD values are significantly lower than the *in vivo* values. If the *ex vivo* scans were plotted against the *in vivo* scans there would be a significant drop in BMD at the end of the trial, which would not be a true reflection of what is actually happening.

In addition, tissue thickness is believed to have some influence on BMD measurements (Ammann *et al*, 1992; Casez *et al*, 1994). When scanning the femur *in vivo*, the dual energy rays would have to pass through layers of skeletal muscle and adipose tissue that were several times thicker than the actual bone. In the *ex vivo* scans, the femurs were dissected out and cleaned to a uniform tissue covering of about half a centimetre. This could contribute to the significant difference between scans of the *in vivo* and *ex vivo* femurs (Table 7).

The excised spine and both femurs were scanned in a uniform covering of 3cms of Phosphate Buffered Saline (PBS) (Amman *et al*, 1992). It is important that the PBS solution is at a constant thickness, to prevent differences in BMD. Tissue thickness is believed to have a small impact on total body *calcium* (Amman *et al*, 1992; Casez *et al*, 1994). PBS would more closely resemble lipid tissue and organs than skeletal muscle.

4.3.3 The impact of Obesity on BMD

On dissecting the spine and both femurs, it was noted that the rats were obese. Obesity is linked with higher bone density (Loré, 1989; Fleet, 2000). Adipose tissue is known to produce oestrogen through peripheral conversion of androstenedione (Loré, 1989) and the extra weight bearing strengthens bone. This may have led to an increase in BMD in these rats.

There are two possible reasons relating to feed intake as to why the rats on this trial were obese. Firstly, the protocol for this trial called for *ad lib* feeding. The rats may have been bored and therefore ate more than their daily requirements for maintenance. In future trials it may be useful to limit feed intake to prevent rats from becoming obese. Secondly, the rats were fed on standard rat chow. Feed companies tend to use the cheapest ingredients to formulate their diets. There is no precise control on the levels of fat, protein and carbohydrate. High levels of fat, protein or carbohydrate could have led to the obesity seen in this trial. Appropriate formulation of synthetic diets with lower calorie content could avoid this problem.

Obesity is also linked to hypercortisolism (Loré, 1989). Cortisol has a negative effect on bone metabolism. Research suggests that glucocorticoids have a direct effect on osteoblast function. By administering short doses of glucocorticoids, there is a marked suppression in alkaline phosphatase and osteocalcin, which are osteoblast markers (Kanis, 1994). It is possible that the rats in this trial had elevated levels of cortisol. The rats all appeared healthy and had no trouble with the anaesthetic. Indicators of stress, include the excretion of poriferan from the eyes and nose, harshness of the coat and a drop in appetite. None of the rats on this trial displayed these traits upon daily health checks, so hypercortisolism could be ruled out.

4.3.4 The impact of age on weight and BMD

The five-month old group had a large variation in both weight and BMD values with a slightly higher weight and whole body BMD than the six-month group, although this was not statistically significant. Weight may have a stronger influence on WB scans than hi-resolution scans. Five months of age is around the time puberty occurs in male rats. Not all rats will go through puberty at the same time. Thus at five months of age the rats were probably at different developmental stages. Around puberty there will be an increase in the circulating levels of testosterone. Sex hormones have a positive effect on BMD (Kanis, 1994), which may have resulted in the higher BMD seen in some rats within this group.

Excluding the spine, there are no outliers in any of the graphs for the five-month old group, which suggests that the data is normal. Littermates were split so that they were scanned at different ages and were then randomised into groups. Each group contained animals with varying body weights. In order to avoid this large variation, rats could have been randomised into age groups on a weight basis. Alternatively BMD could be normalised for weight (Jiang, 2000).

If the five-month old group is considered separately, the growth curves show a distinct narrowing in the range of weights and BMD (Figures 6a-h) as the animal's age. The animals in this trial were from a commercial breeding facility. They are bred to have a similar phenotype so that they can be used for experimental research. Although the majority of rats will grow to a similar size, they will tend to grow at

different rates. This highlights the differences in metabolism between individuals, especially due to rapid onset of puberty.

4.4 Frequency Distribution of the data

Figures 7a-h show the frequency distribution graphs for weight, WB and the *in vivo* and *ex vivo* hi-resolution scans. The frequency distribution graphs follow the same trend *ex vivo* (Figures 7d, 7f & 7h) as they do *in vivo* (Figures 7c, 7e & 7g), displaying a bimodal distribution.

The one-month old group forms a separate population. At this stage their bones are still immature. At one month of age, the rats had only been weaned for one week. The rats would have consumed some of the mother's rat chow already, but would have primarily been consuming milk. Rat chow is high in fat, making it a high-energy source. Once the rats started consuming the rat chow, they would have grown at a much faster rate. By two months of age there was a considerable difference between the one and two-month old groups both in terms of weight and bone density. For this reason the one-month old group was removed and analysed separately. By assessing the 2 to 6 month groups inclusive, the graphs for weight whole body and spine BMD (Figures 8a-d), displayed the typical bell-shaped curve.

There are three distinct humps in the frequency distribution graphs of the *in vivo* femurs. It is interesting to note that the *ex vivo* femurs (Figures 8f&h) have a more normal distribution than the *in vivo* femurs (Figures 8e&g). This method of positioning may be more accurate. The *ex vivo* femurs had a uniform covering of

tissue of about half a centimetre and their standard deviation is smaller than the *in vivo* scans (Appendix 3). If there is less variation within the group the bars of the histogram are wider and the curve is smoothed. The *ex vivo* femurs show the least variation of any site. For trials using young animals (up to 2 months), where serial measurements are not appropriate, scanning the *ex vivo* femurs as an endpoint may be the most accurate method of measuring of BMD.

The growth pattern for the femur may also be slightly different from the spine and the whole body. The femur may not grow linearly but may go through a phase of growth, a phase of remodelling, which includes bone resorption, and then carry on through another phase of growth.

4.5 Rats at One month of age

The objective of this study was to set up a methodology for running DEXA trials using small animals. One of the major considerations was to find a suitable age at which rats could be used for a baseline scan. Rats at one month of age were tested for their suitability as a baseline group. At one month of age there is a large variation between animals. This can be seen in the high coefficient of variation at nearly all sites (Table 2).

At one month of age the rats were growing at a fast rate, represented by the steepest part of the growth curves (Figures 6a-h). The rats had been weaned and had had one week on standard rat chow. Differences in weight may have been due to the quality of the mother's milk and the size of the litter the rat came from. A large litter would have led to competition between the pups for the limited amount of milk their mother produced. The weaker pups would have to wait for their stronger siblings to

feed and may have missed out on adequate quantities of milk. This may have led to the initial differences in weight.

How well the rats had acclimatised to rat chow may have also led to differences in weight. Some rats would have had to adjust from a milk diet to a solid food diet. It is likely that most of the rats would have sampled chow from their mother's provision before they were weaned. The rats were also caged in threes so the bossier or heavier rats may have dominated the feeder preventing the smaller rats from eating.

The frequency distribution for the BMD at each site was plotted (Figure 7a-h). Instead of the bell-shaped curve associated with a normal distribution, the graph had a bimodal distribution. The one-month old group formed a separate population. In order to give a more normal distribution the one-month group was analysed separately.

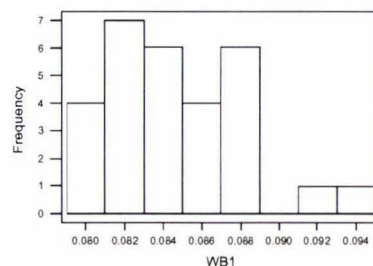


Figure 10 Frequency distribution graph of WB (g/cm^2) for the one-month group

At one month of age the frequency distribution graph for whole body BMD is not normally distributed. Figure 10 is representative of all other sites. Transformations were unable to correct for this.

There was no significant difference between the triplicate scans performed on each animal at one month of age. This indicates that the operator is repositioning the

animals accurately and that the method of positioning is good. The variation looks to be due to variation within the group rather than variation between the scans.

On dissection and scraping of the right femur it was noted that the bones of the one-month old group were extremely soft, and were easily damaged with the scalpel blade. This would indicate that calcification of the bone matrix is not complete at this age. The DEXA machine had trouble distinguishing poorly calcified bone from soft tissue.

There is a poor correlation between the *in vivo* and *ex vivo* scan of the right femur at one-month of age. There is also a poor correlation between the ashed calcium result and the DEXA BMD measurements of the right femur (refer Appendix 4). The correlations for the one-month old group were approximately 0.3-0.4. The DEXA machine was only measuring 30-40% of the BMD at this age.

The correlation between the BMC estimated by DEXA and the ashed calcium content of the one-month old group also shows a positive correlation (0.772, refer Appendix 4). This correlation is statistically significant (RF, $p=0.009$ & Rfex, $p=0.017$). It would appear that the machine was able to estimate the bone mineral content of the one-month old group but was unable to estimate bone area. Based on these findings rats of this age are unsuitable for DEXA trials. It is recommended that animals should not be scanned at one month of age.

The DEXA machine used is built for human scans but has software that enables rat's to be scanned (Lu *et al*, 1994). The BMD measurement is calculated using a series of algorithms that are based on humans. If very small rats are used, these algorithms may not be accurate and the machine will not be able to distinguish bone

from soft tissue (Grier *et al*, 1996). In the final stage before calculation of the BMD, the machine creates a bone map. The area that the machine reads as bone is shown on the screen, and is filled out in yellow. If the bone is very low density the machine will not fill the bone area out completely. The operator must then manually fill in the bone area. In the one-month old group none of the bone maps were completely filled out. This suggests that the bones were of very low density and the machine was having trouble distinguishing bone from soft tissue.

A study by Lu *et al* (1994) showed that rats between 150 and 200g could be scanned accurately. This was in agreement with a study performed by Bertin *et al* (1998). Both of these studies had several differences to the one that created this reference data. The major difference was that female rats were used instead of males (Lu *et al*, 1994) or along with males (Bertin *et al*, 1998). Female rats grow more slowly than males. Bertin *et al*'s study also used Wistar rats, which are smaller than the Sprague-Dawleys used in this trial. The rats in Lu *et al*'s study were aged between 6 and 12 weeks, while Bertin *et al*'s were aged between 2 and 24 months. The rats weighing less than 200g in this trial were just over four weeks (one month) of age.

The study by Lu *et al* centred on the rat whole body scans, with regional areas of interest. No regional high-resolution scans were performed. In the study reported here the coefficient of variation for the whole body was lower than for other sites (Table 1).

The purpose of this reference data is to provide the researcher with a tool to make a reasonably accurate prediction of the "normal" BMD of a rat at a certain age. The great variation between animals at one month of age makes this difficult. Therefore it is recommended that the one-month old group is not included in the reference data, and that rats are suitable for baseline scans from about two months of age onwards.

4.6 Analysis of Variance

4.6.1 Analysis of Variance of the in vivo DEXA scans

Table 3 shows the ANOVA of results from the 2-6 month old rats. There is a significant difference in BMD values between months ($p=0.0001$). The trial looked at growing rats, so it is to be expected that there is a difference between each age group. The two-month old rats were still juveniles and were growing at a fast rate, while the six-month old group had attained maturity.

There is a significant difference between the individuals within the age groups (Table 3). The variation within the groups may be due to differences in weight, as weight is believed to be the strongest predictor for BMD (Loré, 1989). The two and five-month old groups have the largest range between the biggest and smallest rats. In the case of the two-month old rats, genetics, nutrition and metabolism will affect the initial size and growth rate of the rats. Some rats will grow at a faster rate, this is similar to what is seen in primary school children.

Five months of age coincides with puberty. As in humans, not all rats will attain puberty at the same time. Attainment of puberty depends on similar factors to those affecting growth rate, such as nutrition and genetics. As the rats were separated into groups on a genetic basis, it is logical that there will be differences in the way the rats mature within the groups. The large variation in the 5-month old group may be due to rats being at different developmental stages.

There is no significant difference between the repetitions of each scan site either between or within groups (Table 3). This is to be expected, as the precision of the machine is good and a significant difference would indicate operator error or a poor

method of positioning. In this study the method of positioning was therefore accurate and repeatable, and the operator was accurately repositioning the rat each time.

Age has a significant effect on BMD. Growing rats are used as a model for studying bone, because they are growing at a fast rate and have a high demand for calcium. It is important for the researcher to know which ages differ significantly from each other and when growth slows down, so that they can predict when the bone will be particularly sensitive to manipulation. Post hoc testing (Tukey) shows that there is a significant difference between age groups up until five-months of age (Table 4), when growth seems to slow down.

Looking at Figure 6c the spine begins to plateau at four months of age, a month earlier than all of the other sites. The post hoc testing (Tukey, Table 4) confirms this trend, with no significant difference in spine BMD between the 4 and 5 month-old groups ($P=0.434$, Appendix 4). It seems that the spine matures faster than the rest of the body. This could be due to a physiological mechanism to protect the spinal cord from damage. The spine is believed to be the most sensitive site for factors that affect bone metabolism in humans. It would be interesting to examine whether this holds true for rats. In humans the spine must support a great deal of weight for its surface area, since humans are bipedal. In quadrupeds, such as the rat, the weight would shift from the spine to the front legs.

It would appear that the most sensitive period for manipulating bone growth in a rat would be from weaning to four or five months of age, depending on which site you are interested in.

4.6.2 Analysis of Variance of the ex vivo scans

It is possible that tissue thickness can affect BMD. The algorithms that are used to define soft tissue from bone are based on humans. In small animals these algorithms may no longer be as accurate. The split-plot ANOVA of the data for the *ex vivo* scans follows exactly the same trend as the *in vivo* scans (Table 5). The BMD values for the *ex vivo* scans of each month are significantly different. There is also a significant difference between the *ex vivo* scans within each month (Table 5). This is an unexpected result believed to be due to the variation within the two and five month old groups.

There is no significant difference between the duplicate scans of the *ex vivo* spine and both femurs (Table 5) or between the four and five month old group at the spine (Table 6), which also follows the trend seen *in vivo*.

There is no significant difference between the BMD for the *in vivo* and *ex vivo* spine ($p=0.561$, Table 7). Positioning of the spine *in vivo* and *ex vivo* was identical, with the spine scanned in a supine position both times. There is a significant difference between the *in vivo* femurs and the excised femurs $p=0.000$ (Table 7). It is important to get the same positioning each time, as scanning bones on different angles will give different BMD results. *In vivo* the femurs were scanned from a lateral view, while *ex vivo* bones were placed supine, so that they were scanned flat.

Figure 11 *in vivo* scan of femur

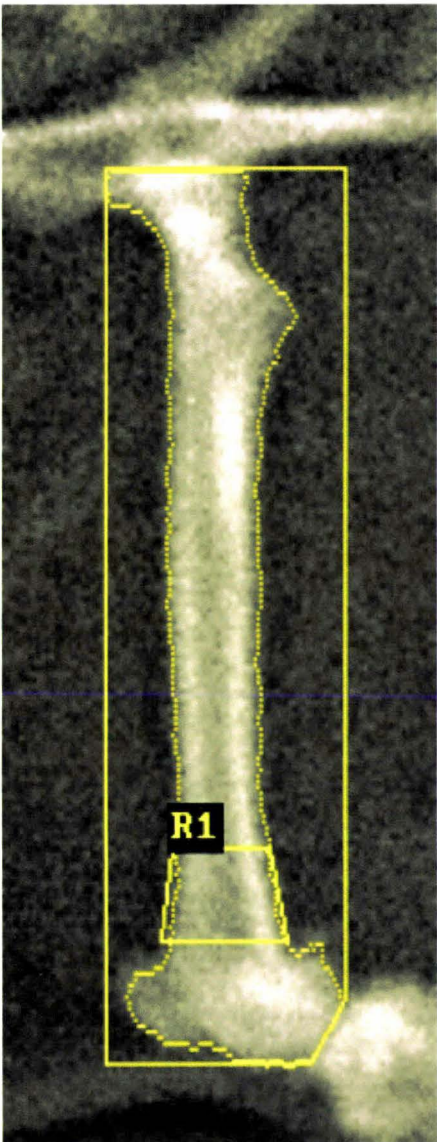
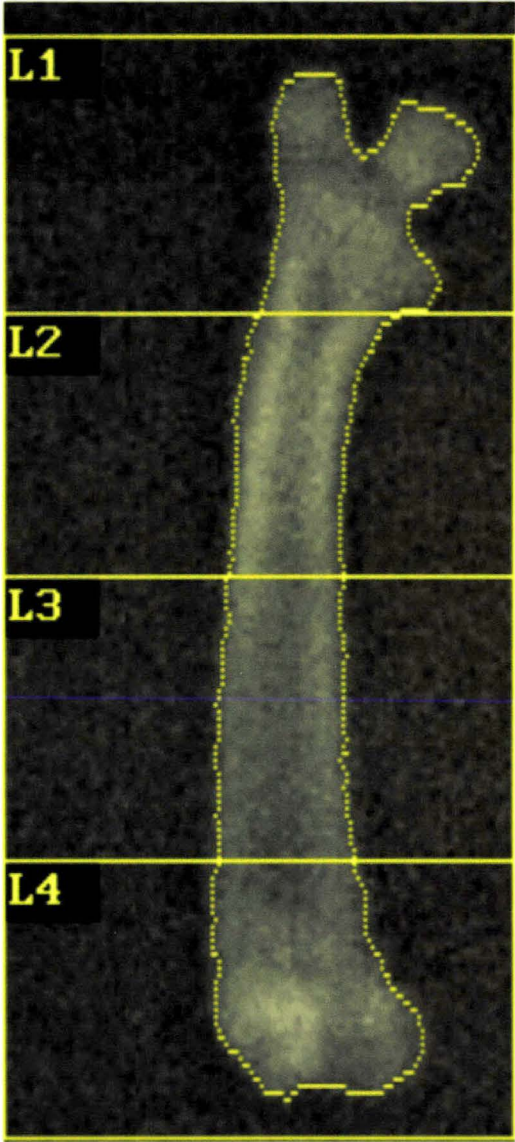


Figure 12 *ex vivo* scan of femur



Anatomically it is impossible to get the femurs flat *in vivo*. Positioning the femurs at right angles is the most easily replicable placement of the animal. In Table 3 and 4, the one-way ANOVA of repetition shows that there is no significant difference between the duplicate scans taken at each site. The weight distribution of the bone makes it almost impossible to get a clear lateral view of the bone *ex vivo*. By placing the bone in a prone position, the precision on subsequent scans is very high.

In the *in vivo* scan there is an overlap between the ball of the femur and the pelvic girdle. The top of the ball of the femur was removed from the scan to avoid the inclusion of the pelvis in the analysis. However, the kneecap was included in the scan. The inclusion of the kneecap and exclusion of the top of the ball will have influenced the BMD.

Despite the differences in BMD between the *in vivo* and *ex vivo* scans of the femur, the correlation between the two is high at 0.98 (Table 2). Any differences between animals would be detected using either of these two methods. This highlights the importance of positioning the animal correctly every time that it is scanned.

4.7 Femur Weight, Length and Ashed Calcium Content

The growth curves for femur length, weight and ashed calcium content (Figures 9a,b&c) follow the same trend with age, as the growth curves for weight and BMD (Figures 6a-h). The growth curves are almost linear until four months of age, after which a plateau forms. The growth curve for femur length (Figure 9b) has already reached a plateau at four months of age, a month earlier than the curves for femur weight and ashed calcium content (Figures 9a&c). This follows the same pattern as the spine (Figure 6c). It appears that by four months of age the growth in areal size of the bone is complete but that mineralization of the bone occurs for at least another month.

4.7.1 Analysis of variance of excised right femur

There is no significant difference in femur length between the 4, 5 and 6-month old groups (Table 8). There is a significant difference in the RF weight of the four-month

group compared with the five and six month groups. It looks like the femur stops growing in length at about four months of age. This follows the same trend as the spine BMD. It can be concluded that skeletal size is probably attained at four months of age. The increase in weight from four months is most likely due to an increase in mineral content rather than an increase in size. This time period in rats is similar to the consolidation phase in humans (Kanis, 1994).

4.8 Pearson Correlations

4.8.1 Pearson Correlations between age and BMD, weight and BMD, and *in vivo* and *ex vivo* BMD

The correlations between age and BMD, weight and BMD, and *in vivo* and *ex vivo* BMD are 0.97 to 0.98 (Table 9). This is a strong positive correlation. An increase in age, weight or BMD at a particular site will subsequently lead to an increase in BMD at all other sites. A correlation of 1 indicates that the ranking of the data would be the same using either of the two methods being compared. As there is such a high correlation between all sites, further trials should select the sites with the highest precision or sensitivity for use in research.

Despite differences between the raw data for the *in vivo* (Table 1) and *ex vivo* (Table 6) scans, correlations were excellent at all sites (Table 9). The two methods of positioning are complimentary. However as explained earlier they cannot be used in direct comparison with each other. The *in vivo* and *ex vivo* scans are for the same group of animals, so a high correlation is expected. A poor correlation would indicate operator error or that one of the two methods of positioning was inaccurate. Researchers can be confident that either method will detect differences in BMD. This strengthens the case for using serial DEXA scans as a valid method for monitoring treatments *in vivo*. The high correlation would indicate that tissue thickness is not

affecting the *in vivo* scans. If tissue thickness was a confounding factor, the correlation would be expected to be lower.

There was a very strong correlation between weight and BMD. This is to be expected as the machine looks at areal bone density rather than true density (Jiang *et al*, 2000). It is logical that larger rats will have bigger bones. Bigger bones have a larger area and hence a greater BMD.

This is a problem when using DEXA as a diagnostic tool for osteoporosis (Cunningham, 1996). A small healthy bone can have a lower BMD than a low-density large bone. There is a need for a quantitative system to take size into account when obtaining the BMD. It is recommended that the DEXA be used to assess differences in BMD between scans of the same rat. Groups can be analysed by assessing the differences within individuals rather than between individuals.

If start and endpoint scans of each rat are not possible then the importance of allocating rats to a trial on a weight basis cannot be emphasized enough. Researchers must take this factor into account when considering their results. An alternative could be to normalise BMD values on a weight basis. Jiang *et al* (2000) performed a trial in which BMD was normalised using measurement of bone size from a standard pelvic radiograph. If the BMD value is transformed mathematically using weight then direct comparisons between different sized animals might be possible.

Due to the length of time it takes to perform a scan, and the large number of animals used as trial subjects, it is not practical to look at each individual site during a trial. The strong correlation between sites means that all sites were following the same trend, and that not every site has to be scanned for good results.

The whole-body scans take twenty minutes per rat and each of the hi-resolution scans take 10 minutes. With repositioning it takes over an hour for each rat to be scanned. It is important that all rats are scanned as close together in time as possible to ensure time does not have an effect on BMD results. In general, with at least 40 rats per trial, it is not practical to scan all sites.

The whole body scan cannot target spine or specific sites of the femur accurately. There is no guarantee that the same subregion is being scanned each time, especially in young animals. The software used in conjunction with the DEXA machine allows for a "compare" function, which goes some way to alleviating this problem and can be used in serial measurements of older rats. The "compare" function recalls the most recent scan and places a region of interest the same size and shape, around the bone that is currently being scanned. Unfortunately this means that the scan window is exactly the same size each time. In growing rats the subregions that fit at two months will be too small at three months, as the rats are getting bigger.

The spine and whole body scans are highly correlated, so differences in BMD should be detected at either site. The results for the spine BMD appear to be a good representation of the changes occurring in young rats. Looking at Table 3 the femurs have the lowest coefficient of variation, which suggests that they have the highest precision when measuring BMD. These results suggest that the spine and at least one femur could be used to give accurate predictions of BMD either *in vivo* or *ex vivo*. *Ex vivo* scans of the spine and both femurs could also be performed at a later date and used to back up the *in vivo* hi-resolution results.

Regional hi resolution scans allow for sub regions of the femur to be analysed. The distal metaphysis is believed to be a sensitive region for bone metabolism (Pastoureau, 1995). This area is rich in trabecular bone, which is most sensitive to bone resorption.

The recommendation from this trial is to perform only the regional hi resolution scans. Without the whole-body scan, scanning times are reduced to 40 minutes. This would allow the dose rate of the anaesthetic to be reduced to 0.05ml/100g bodyweight, and the recovery time would then decrease from about four hours to only two hours. Thus animals will be measured within closer time lines, improving the accuracy of the measurements.

4.8.2 Pearson Correlations for Ashed calcium content against *in vivo* and *ex vivo* Right Femur BMD & BMC

The correlations between the DEXA BMD and BMC and the ashed calcium show a strong positive relationship for the 2-6 month group (Table 10). This agrees with the results found by Klein *et al* (1998) and Griffen *et al* (1993). Although DEXA bone density measurements tend to overestimate the amount of bone mineral present, they have a very good correlation with the ashed calcium content (Table 10). This confirms observations by Grier *et al*, in 1996. Either method could be used for identifying relativities in BMD/BMC. The disadvantage with ashed calcium content is that it can only be used as an endpoint measure, whereas DEXA has the advantage of being able to be used for serial BMD measurements.

The difference in BMD between the *in vivo* and *ex vivo* scans of the femurs in this trial was a concern. However, the strong correlations indicate that the two methods of positioning are both effective in measuring the BMD of the femur. Again, they

cannot be used together for direct comparisons, but may be used to reinforce the data from a given trial.

Chapter 5

Summary and Recommendations

5.1 Summary

The anaesthetic combination used in this trial met the two main requirements for DEXA scanning, i.e. immobility and insentience, but unfortunately caused periods of apnoea. In further trials it was found that as many as 7% of animals did not recover from the anaesthetic. This is unacceptable for a non-invasive procedure such as DEXA scanning. By reducing the dose rate to 0.05ml/100g bodyweight, the adverse side effects, as well as sleep time for the anaesthetic, were reduced. In conclusion, this anaesthetic combination is suitable for use in studies on bone using DEXA scanning provided that it is administered at the correct dose rate.

The method of positioning used in this study has proved to be accurate and easily reproduced by the operator. It is recommended as a standard operating procedure for the DEXA machine.

The femur has the highest precision of any site and is recommended for use in trials using young animals. With less variation than the spine, the results are likely to be clearer and significant differences are more likely to be identified.

If the researcher only performs the hi-resolution scans, it takes between 30 and 40 minutes for an animal to be scanned. For serial DEXA scans, where recovery times from the anaesthetic must be taken into account, 12 rats could be scanned in a normal 8hour day. It would require two full time people, one to scan and one to

monitor the rats in recovery. Where rats are to be euthanased, 14 to 16 rats could be scanned within 8 hours.

The growth curves for the BMD values increase linearly to about four months of age. At five and six months of age a plateau forms. This indicates that the most sensitive time to influence accretion of bone is between weaning and four months of age. Weight appears to be a strong predictor of BMD, and as the animal gets older, has more of an impact on BMD than age. Groups need to be segregated on a weight basis for DEXA measurements, or if this is not possible, BMD needs to be corrected for weight.

The spine matures at fourth months of age, a month earlier than any other site. In younger animals this tends to highlight the variation between animals. It is recommended that the femur be used for BMD measurements in young animals as it has the highest precision.

The split plot ANOVA indicates that the scans were highly reproducible within and between animals. This is reinforced by the low coefficient of variation for the 2 to 6 month old group. The method of positioning can be confidently employed to measure the same scan site within animals, for serial scans that measure changes in bone density and also to detect differences between animals, in order to monitor treatment effects.

The *in vivo* DEXA BMD values for the femurs are significantly different from the *ex vivo* BMD values. This is due to differences in positioning. The values correlate strongly, indicating that the same relativities can be achieved with either method. The *ex vivo* scans can be used to confirm the relativities seen *in vivo*, or as a stand-

alone endpoint but they cannot be used as the endpoint for serial *in vivo* DEXA measurements.

Although DEXA tends to overestimate bone mineral content, DEXA BMD and BMC correlate strongly with ashed calcium content. Either DEXA or bone ashing can be used as an endpoint to a trial. The advantage of DEXA is that it can be used without the necessity of sacrificing the animal, making serial measurements possible.

5.2 Recommendations

The key recommendations from this trial are listed below;

1. The anaesthetic mix of 0.2ml ACP + 0.5ml ketamine + 0.1ml Xylazine + 0.2ml H₂O per ml administered at 0.05ml/100g body weight provides good anaesthesia and has minimal side effects. It is recommended for use in DEXA scanning.
2. Rats should be positioned with a right angle between the spine and femur and femur and tibia. This method allows for quick and easy positioning of the scan arm and produces the highest precision *in vivo*.
3. Animals should be allocated to treatment groups on a weight basis, as weight is a strong predictor of BMD in this model. If this is not possible and/or variations in weight occur, individual BMD's should be corrected for weight before comparisons are made.
4. Hi-resolution scans provide more precise and accurate measures of BMD than the whole body scan. It is recommended that for further trials whole body scans

need not be performed. This will decrease the time taken to scan each animal and allow 12 animals to be scanned within 8 hours. The spine is believed to be the most sensitive site for bone resorption, while the femurs have the highest precision.

5. When planning future trials using DEXA measurements it is recommended that one-month old animals are not used. It appears that the bone has not been fully calcified at this stage and that BMD measurements are unlikely to be credible.
6. If DEXA is not available then bone ashed calcium content may be used as an endpoint to detect differences in BMC between groups.

References

Ammann P., Rizzoli R. & Bonjour J-P (1999). Modèles Animaux et Développement Préclinique des Médicaments Contre L'ostéoporose. Revue de Rhumatisme **66** (11), 607-608

Ammann P., Rizzoli R., Slosman D & Bonjour J-P. (1992) Sequential and Precise *In Vivo* Measurement of Bone Mineral Density in Rats Using Dual-Energy X-ray Absorptiometry. Journal of Bone and Mineral Research **7** (3), 311-316

Ammann P., Rizzoli R., Müller K., Slosman D., & Bonjour J-P. (1993). IGF-I and Pamidronate Increase Bone Mineral Density in Ovariectomised Adult Rats. American Journal of Physiology **265**, E770-E776

Amo C., Revilla M., Hernandez E.R., Gonzalez-Riola J., Villa L.F., Secoc., Rico H. (1996) Correlation of Ultrasound Bone Velocity with Dual-Energy X-ray Absorptiometry in Rat Bone Specimens. Investigative Radiology **31** (2), 114-117

Antich.P.P. (1993). Ultrasound study of bone *in vivo*. Calcified Tissue International **53** (s1), s157-s161

Aufdemorte T.B., Boyan B.D., Fox C.W & Miller D. (1993). Diagnostic Tools and Biological Markers: Animal Models in the Study of Osteoporosis and Oral Bone Loss. Journal of Bone and Mineral Research **8** (2), S529-S534

Bagi CM. (1997). Effect of estrogen deficiency on cancellous and cortical bone structure and strength of the femoral neck in rats. Calcified Tissue International **61**, 336-344

Baran D., Sorensen A., Grimes J., Low R., Karellas A., Johnson B. & Roche J. (1989). Dietary modification with Dairy Products for Preventing Vertebral Bone loss in Premenopausal Women: A three-year Study. Journal of Clinical Endocrinology and Metabolism **70** (1), 264-270

Brammer A., West C.D., & Allen S.L. (1993). "A Comparison of Propofol with Other Injectable Anaesthetics in a Rat Model for Measuring Cardiovascular Parameters. Laboratory Animals **27** 250-257

Cardenas J.L., Revilla M., Hernandez E.R., Aguado F., Villa L.F., Rico H. (1997). Comparison of Three Bone Density Methods in Osteoporotic Women. Calcified Tissue International **61**, 358-361

Casez J.P., Muehlbauer R.C., Lippuner K., Kelly T., Fleisch H., & Jaeger P.H. (1994). Dual-Energy X-ray Absorptiometry for Measuring Total Bone Mineral Content in the Rat: Study of Accuracy and Precision. Bone and Mineral **26**, 61-68

Cunningham J.L., Fordham J.N., Hewitt J.A. & Speed C.A. (1996). Ultrasound Velocity and Attenuation at Different Skeletal Sites Compared with Bone Mineral Density Measured using Dual –Energy X-ray Absorptiometry. British Journal of Radiology **69**, 25-33

Gala Paniagua J., Diaz-Curiel M., de la Piedra Gordo C., Castilla Peparez C. & Torralbo Garcia M. (1998) Bone Assessment in Rats by Dual Energy X-ray Absorptiometry. British Journal of Radiology **71**, 754-758.

Gluer C.C., for the international quantitative ultrasound consensus group. (1997) Quantitative Ultrasound Techniques for the Assessment of Osteoporosis: Expert Agreement on Current Status. British Journal of Bone Mineral Research **12**, 1280-1288

Grier S.J., Turner A.S. & Alvis, M.R. (1996) The Use of Dual-Energy X-ray Absorptiometry in Animals. Investigative Radiology **31** (1), 50-62

Griffin M.G. Kimble R., Hopfer W. & Pacifici R. (1993). Dual-Energy X-ray Absorptiometry of the Rat: Accuracy, Precision, and Measurement of Bone Loss. Journal of Bone and Mineral Research **8** (7), 795-800

Hugi K., Bonjour J-P. & Fleisch H. (1979). Renal Handling of Calcium: Influence of Parathyroid Hormone and 1,25-dihydroxy Vitamin D₃. American Journal of Physiology. **236** (4), F349-F356

Iwamoto J (1998). Effects of exercise on bone mineral density in mature osteopenic rats. Journal of Bone & Mineral Research **13** (8), 1308-1317

Jiang C., Giger M.L., Kwak S.M., Chinander M.R., Martell J.M. & Favus M.J. (2000) Normalized BMD as a Predictor of Bone Strength. Academic Radiology **7**, 33-39

Jiang Y (1997). Long-term changes in bone mineral and biomechanical properties of vertebrae and femur in aging, dietary calcium restricted, and/or estrogen-deprived/-replaced rats. Journal of Bone & Mineral Research **12** (97), 820-831

Kanis J.A. (1994). Osteoporosis, Blackwell Sciences Limited, Oxford, UK

Ke, H.Z., Simmons H.A., Pirie C.M., Crawford D.T., & Thompson D.D. (1995). Droloxifene, a New Estrogen Antagonist/Agonist, Prevents Bone Loss in Ovariectomised Rats. Endocrinology **136** (6), 2435-2441

Klein R.F., Mitchell S.R., Phillips T.J., Belknap J.K. & Orwoll E.S. (1998). Quantitative Trait Loci Affecting Peak Bone Mineral Density in Mice. Journal of Bone Mineral Research **13** (11), 1648-56

Loré F. (1989) Diet & lifestyle Factors in Osteoporosis. In Advances in Bone Regulatory Factors: Morphology, Biochemistry, Physiology & Pharmacology, (editors A Pecile & B de Bernard) NATOASI series, Plenum Press, New York, 1990

Lu P.W., Briody J.N., Howman-Giles R., Trube A. & Cowell C.T. (1994). DXA for Bone Density Measurement in Small Rats Weighing 150-250 Grams. Bone **15** (2), 199-202

Marshall S., Milligan A., & Yates R. (1994) Experimental Techniques and Anaesthesia in the Rat and Mouse. ANZCCART News **7** (1), 1-4

Michaëlsson K., Bergström R., Holmberg L., Mallmin H., Wolk A. & Ljunghall S. (1997). A High Dietary Calcium Intake is needed for appositive Effect on Bone

Mineral Density in Swedish Postmenopausal Women. Osteoporosis International **7**, 155-161

Mosekilde L. (1995). Assessing bone quality - animal models in preclinical osteoporosis research. Bone **17** (4), 343s-352s

Njeh.C.F. (1997) The role of ultrasound in the assessment of osteoporosis: A review. Osteoporosis International **7**, 7-22

Nordin B.E.C. (1984). Metabolic Bone and Stone Disease. (edited by B.E.C. Nordin) Churchill Livingstone, 1984, London, UK

Nuttall M.E., Bradbeer J.N., Stroup G.B., Nadeau D.P., Hoffman S.J., Zhao H., Rhem S. & Gowen M. (1998) Idoxifene: A novel Selective Estrogen Receptor Modulator Prevents Bone Loss and Lowers Cholesterol Levels in Ovariectomized Rats and Decreases Uterine Weight in Intact Rats. Endocrinology **139** (12), 5224-5234

Omi N. & Ezawa I. (1995). The effect of Ovariectomy on Bone Metabolism in Rats. Bone **17** (4), 163S-168S

Parton K. 2000. Institute of Veterinary and Biomedical Sciences (IVABS), Massey University, Palmerston North

Pastoureau P., Chomel A. & Bonnet J. (1995). Specific Evaluation of Localised Bone Mass and Bone Loss in the rat Using Dual-Energy X-ray Absorptiometry Subregional Analysis. Osteoporosis International **5**, 143-149

Pecile A. (1990). Advances in Bone Regulatory Factors: Morphology, Biochemistry, Physiology & Pharmacology, (editors A Pecile & B de Bernard) NATOASI series, Plenum Press, New York, 1990

Petley G.W., Cotton A.M., Murrills A.J., Taylor P.A, Cooper C., Cawley M.I.D. & Wilken T.J. (1996). Reference Ranges of Bone Mineral Density for Women in Southern England: the Impact of Local Data on the Diagnosis of Osteoporosis. The British Journal of Radiology **69**, 655-660

QDR 4000 User Guide (1999) Hologic Inc, Bedford, USA

Raab Diane M. (1990) Bone mechanical properties after exercise training in young and adult rats. Journal of Applied Physiology **68** (1), 130-134

Rose B.S., Flatt W.P., Martin R.J., & Lewis R.D. (1997) Whole body composition of Rats Determined by Dual Energy X-ray Absorptiometry Is Correlated with Chemical Analysis. Journal of Nutrition **128** 246-250

Rosen H.N., Tollin S., Balena R., Middlebrooks V.L., Moses A.C., Yamamoto M., Zeind A.J. & Greenspan S.L. (1995) Bone Density is Normal in Male Rats Treated with Finastrade. Endocrinology **136** (4) 1381-1387

Shen V., Dempster D.W., Birchman R., Xu R. & Lindsay R. (1993). Loss of Cancellous Bone Mass and Connectivity in Ovariectomised Rats Can be Restored By a Combined Treatment with Parathyroid Hormone and Estradiol. Journal of Clinical Investigation **91**, 2479-2487

Simmons A., O'Doherty M.J., Barrington S.F. & Coakley A.J. (1995) A Survey of Dual-Energy X-ray Absorptiometry (DEXA) Normal Reference Ranges Used Within the UK and Their Effect on Patient Classification. Nuclear Medicine Communications. **16**, 1041-1053

Sissons H.A. (1962) Bone Metabolism in Relation to Clinical Medicine: Proceedings of a Symposium Organised by the Association of Clinical Pathologists and the Association of Clinical Biochemists Held in London at the Royal College of Surgeons, September 28th –29th 1962, (edited by H.A. Sissons), Pitman Medical, 1963, London, UK

SCLAN. Subcommittee on Laboratory Animal Nutrition (1995), Nutritional Requirements of Lab Animals, Fourth Revised Edition. (Committee on Animal Nutrition, Board of Agriculture) National Academy Press, 1995, Washington DC, USA

Thompson D.D., Simmons H.A., Pirie C.M., & Ke H.Z. (1995) FDA Guidelines and Animal Models for Osteoporosis. Bone **17** (4), 125S-133S

Turner R.T., Evans G.L., Cavolina J.M. Halloran B., & Morey-Holton E. (1998). Programmed Administration of Parathyroid Hormone Increases Bone Formation and Reduces Bone Loss in Hindlimb-Unloaded Ovariectomised Rats. Endocrinology **139** (10), 4086-4091

Vanderschueren D. (1993). The Aged Male Rat as a Model for Human Osteoporosis: Evaluation by Nondestructive Measurements and Biomechanical Testing. Calcified Tissue International. **53**: 342-347

Waynforth H.B. & Flecknell P.A., (1992) Experimental and Surgical Technique in the Rat. Second Edition. Academic Press Limited London 1992

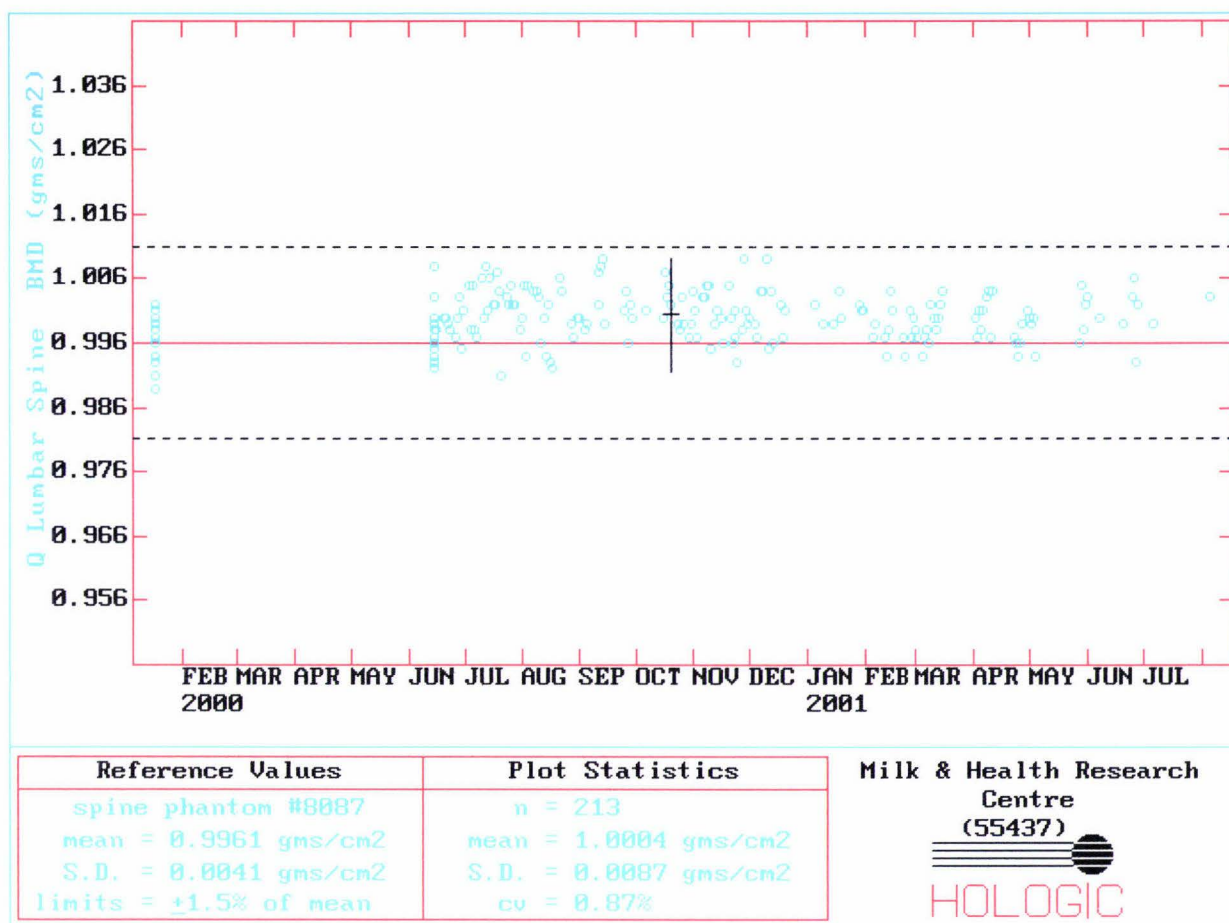
Whelan G., & Flecknell P.A. (1994). The Use of Etorphine/Methotrimeprazine and Midazolam as an Anaesthetic Technique in Laboratory Rats and Mice. Laboratory Animals **28**, 70-77

Wixon S.K., White W.J., Hughes H.C. Lang C.M., & Marshall W.K. (1987). A Comparison of Pentobarbital, Fentanyl-Droperidol, Ketamine-Xylazine and Ketamine-Diazepam Anaesthesia in Adult Male Rats. Laboratory Animal Science **37** (6), 726-735

Wood R.J., Fleet J.C., Cashman K., Bruns M.E., & Deluca H.F. (1998) Intestinal Calcium Absorption in the Aged Rat: Evidence of Intestinal Resistance to $1,25(\text{OH})^2$ Vitamin D. Endocrinology **139** (9) 3843-3848

Wronski T.J. (1998). Lack of effect of Spaceflight on Bone Mass and Bone Formation in Group Housed Rats. Journal of Applied Physiology **85** (1), 279-285

Yamauchi H., Kushida K., Yamazaki K., & Inoue T. (1995). Assessment of Bone Mineral Density in Ovariectomised Rats Using DEXA. Journal of Bone and Mineral Research **10** (7), 1033-1039



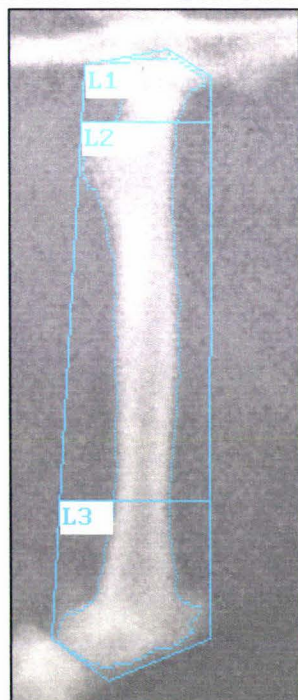
Appendix 1. Criteria for rejection of the daily QC scan.

The cut off limits are the black broken lines. If the QC (denoted by the blue dots) is outside the broken lines, the QC for that day is rejected and the scan must be repeated.

C.F.	1.0100	1.0580	1.0000
Region	Area (cm2)	BMC (grams)	BMD (gms/cm2)
GLOBAL	108.4625	16.1571	0.1490



Hologic

$$k = 1.330 \quad d\theta = 149.8(1.000H)$$


TOTAL BMD CV FOR L1 - L4 1.0%

C.F.	1.010	1.058	1.000
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Region	Area (cm ²)	BMC (grams)	BMD (gms/cm ²)
L1	0.2144	0.0968	0.4515
L2	1.1199	0.4376	0.3907
L3	0.6258	0.2058	0.3289
TOTAL	1.9602	0.7402	0.3776



Hologic

Table i. Descriptive statistics for weight and *in vivo* and *ex vivo* scans.

		<i>1 mth</i>	<i>2 mth</i>	<i>3 mth</i>	<i>4 mth</i>	<i>5 mth</i>	<i>6 mth</i>
Wt (g)	Mean	148.62	335	416	458	537	512
	SD	26.28	42.12	22.10	31.39	77.5	22.38
	SE	4.88	9.42	4.94	7.02	17.3	5.01
WB (g/cm²)	Mean	0.085	0.112	0.127	0.135	0.141	0.141
	SD	0.003	0.005	0.004	0.004	0.008	0.003
	SE	0.001	0.001	0.001	0.001	0.002	0.001
Spine (g/cm²)	Mean	0.096	0.191	0.224	0.234	0.238	0.244
	SD	0.013	0.015	0.008	0.013	0.014	0.011
	SE	0.002	0.003	0.002	0.004	0.003	0.003
LF (g/cm²)	Mean	0.077	0.233	0.284	0.319	0.331	0.336
	SD	0.033	0.011	0.006	0.008	0.012	0.015
	SE	0.006	0.002	0.001	0.002	0.003	0.003
RF (g/cm²)	Mean	0.079	0.235	0.285	0.319	0.334	0.336
	SD	0.029	0.012	0.008	0.007	0.015	0.007
	SE	0.005	0.003	0.002	0.002	0.003	0.002
Spineex (g/cm²)	Mean	0.122	0.191	0.220	0.239	0.243	0.240
	SD	0.013	0.012	0.006	0.011	0.012	0.011
	SE	0.002	0.003	0.001	0.002	0.003	0.002
Lfex (g/cm²)	Mean	0.120	0.204	0.242	0.268	0.279	0.284
	SD	0.011	0.006	0.007	0.006	0.016	0.010
	SE	0.002	0.001	0.001	0.001	0.003	0.002
RFex (g/cm²)	Mean	0.121	0.203	0.241	0.266	0.275	0.277
	SD	0.009	0.009	0.005	0.010	0.011	0.009
	SE	0.002	0.002	0.001	0.002	0.002	0.002

Values are given as mean, standard deviation and standard error. N = 10 per age group for weight and 20 (duplicate scans per rat) per age group for the BMD sites.

Table ii. Descriptive statistics for Right Femur Weight and Length.

		<i>1mth</i>	<i>2mth</i>	<i>3mth</i>	<i>4mth</i>	<i>5mth</i>	<i>6mth</i>
RFwt (g)	Mean	0.1698	0.4117	0.5807	0.0685	0.7560	0.7657
	SD	0.0340	0.0462	0.0298	0.0343	0.0567	0.0490
	SE	0.0107	0.0146	0.0094	0.0109	0.0179	0.0155
RFlgth (cm)	Mean	2.4100	3.3400	3.6300	3.8000	3.8900	3.9200
	SD	0.0994	0.1506	0.1059	0.1059	0.0876	0.1135
	SE	0.0314	0.0476	0.0335	0.0333	0.0277	0.0359
Ashed Ca (Ca/bone)	Mean	35.5	92.47	130.72	162.13	181.85	180.16
	SD	6.71	9.99	7.53	7.64	14.35	10.66
	SE	2.12	3.16	2.38	2.42	4.54	3.37

Table iii. Pearson Correlations between ashed femur calcium and right femur BMD *in vivo* and *ex vivo* in the one-month old group.

	RF1m	Rfex1m
Rfex1m	0.362 0.304	
Ca1	0.332 0.348	0.524 0.120

Table iv. Pearson Correlations between ashed femur calcium and right femur BMC *in vivo* and *ex vivo* in the one-month old group

	RF BMC 1-month	Rfex BMC 1-month
Rfex BMC 1-month	0.605 0.064	
Ashed Ca	0.772 0.009	0.726 0.017

Table v. Analysis of variance of BMD between each age

	mt h 2 vs mt h 3	mt h 3 vs mt h 4	mt h 4 vs mt h 5	mt h 5 vs mt h 6
Femur length	0.000	0.002	0.052	0.517
Femur wt	0.000	0.000	0.003	0.677
Ashed calcium content	0.000	0.000	0.001	0.768
WB	0.000	0.000	0.037	0.743
Spine	0.000	0.015	0.434	0.627
LF	0.000	0.000	0.007	0.287
RF	0.000	0.000	0.006	0.462
Spineex	0.000	0.000	0.261	0.393
Lfex	0.000	0.000	0.005	0.286
RFex	0.000	0.000	0.023	0.405