MATERNAL EXERCISE DURING PREGNANCY AFFECTS THE RAT MUSCULOSKELETAL SYSTEM AND INDICES OF ENERGY METABOLISM

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ABSTRACT

The developmental origins of health and disease hypothesis postulates that environmental cues perceived by the developing organism during early life program long-term health outcomes. A series of studies were undertaken to examine the developmental programming effects of maternal exercise during pregnancy on offspring musculoskeletal health and energy metabolism using a rat model. Firstly, an exercise that did not cause a potentially confounding stress response in the exercising animal was identified. Secondly, pregnant dams then performed this exercise and its effects on fetal growth and the maternal stress response were quantified. Finally, the offspring of dams that exercised throughout pregnancy were allowed to grow to maturity, and the effects of maternal exercise on their musculoskeletal health and energy metabolism were assessed. Throughout these experiments, body composition was assessed by dual-energy X-ray absorptiometry, and tibial parameters were measured using peripheral quantitative computed tomography. Maternal stress was quantified by measurement of faecal corticoid metabolites. Serum concentrations of the fully and undercarboxylated forms of the bone-derived hormone osteocalcin, and expression of genes related to osteocalcin carboxylation, were measured to explore their role in the response of offspring bone and energy metabolism to maternal exercise.

Two exercise types, rising to an erect bipedal stance and tower climbing, were initially tested in non-pregnant rats. Both rapidly caused changes in the tibias of exercised animals without inducing stress. In pregnant rats, both exercises increased fetal growth relative to controls, and neither caused a physiological stress response in the dams. Since rising to an erect bipedal stance had the greater effect on fetal growth, it was selected for use in the final study in which the offspring were grown to maturity. Maternal exercise throughout pregnancy was associated with sex-dependent changes in

the bone and body composition of the mature offspring. Male offspring of exercised dams had increased adiposity and serum undercarboxylated osteocalcin concentrations, while offspring of both genders had lower volumetric bone mineral density at the tibial diaphysis, relative to controls. These results suggest that maternal exercise has long-term effects on the musculoskeletal system and energy metabolism, and that undercarboxylated osteocalcin may play a role in these effects.

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LIST OF PUBLICATIONS

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Chapter 1

INTRODUCTION

Background

The developmental origins of health and disease (DOHaD) hypothesis is based on the concept of developmental plasticity, or the ability of a single genotype to result in multiple possible phenotypes in response to environmental cues perceived by the developing organism *in utero* or during early life. The DOHaD model speculates that the fetus makes predictive adaptations in response to intrauterine cues, resulting in permanent adjustments in homeostatic systems to aid immediate survival and improve success in an adverse postnatal environment. However, inappropriate interpretations of prenatal cues or changes to that immediate environment may result in a mismatch between prenatal predictions and postnatal reality. As a result, these adaptations, known as predictive adaptive responses (PARS) [1,2], may ultimately be disadvantageous in postnatal life, leading to an increased risk of chronic non-communicable disease in adulthood and/or the inheritance of risk factors and a cycle of disease transmission across generations. The most often cited example of this effect is the association between poor early life nutrition, low birth weight, and subsequent development of coronary heart disease, hypertension, and insulin resistance in offspring [3].

To date, much of the research investigating the DOHaD hypothesis has focused on maternal nutrition during pregnancy, its effects on fetal and post-natal growth and development, and subsequent effects on adult energy metabolism. A strong link has been found between nutrient deprivation during the periconceptional and gestation periods and postnatal metabolism; with maternal undernutrition resulting in hyperleptinaemia, hyperinsulinaemia, and obesity in the mature offspring [4,5,6,7,8]. The persistence of these effects in adult life can be amplified, or in some cases

mitigated, by the postnatal environment acting as a secondary "trigger". For example, feeding the pups of undernourished rat dams a hypercaloric diet after weaning enhanced the hyperphagia, hyperinsulinaemia, and high systolic blood pressure observed following maternal undernutrition [6]. Conversely, voluntary wheel running daily from 60 days of age prevented the development of obesity in the offspring of maternally undernourished rats [9].

It has recently been proposed that musculoskeletal development and physical performance may also fit the DOHaD concept, and there is evidence that this is true across species. The bone strength of an individual in later life is directly related to the peak bone mass obtained by that individual during skeletal growth. In humans, there is a relationship between birth weight and adult bone mass [10], which may reflect the associations of birth weight and infant growth with muscle size and strength in later life [11,12]. Height and weight gains before and during puberty have been shown to affect adult physical performance and grip strength in humans, with sex-specific differences [13,14]. A maternal "junk food" diet (high in sugar, fat, and salt) during pregnancy results in rat offspring with impaired skeletal muscle development [15] and strength [16]. In sheep, the bone: muscle ratio in fetal lambs is affected by the nutrition level of the dam [17]. These findings suggest that developmental influences might affect not only the strength of the muscles that act on the bones, but also the response of the skeleton to the forces to which it is subjected.

Such developmental effects on the musculoskeletal system may also influence adult health and disease risk. Osteoporosis is a skeletal disorder characterised by decreased bone strength and increased risk of fracture [18], and evidence suggests that the risk of developing osteoporosis may be at least partially determined during fetal and early postnatal life [10]. The bone strength of an individual in old age is directly related

to the peak bone mass obtained by that individual during skeletal growth [18]. In humans, there is a relationship between birth weight, weight in infancy, and adult bone mass [10,19]. The relative contributions of weight at birth, 1 year, and adulthood to the bone area of the proximal femur, based on data from 468 women and 498 men who underwent bone densitometry while in their sixties, is 2.8, 6.8 and 8.2% in men and 6.7, 4.2, and 3.9% in women [20]. Poor growth during infancy in humans is associated with altered proximal femoral geometry in adult life [21], which has been shown to predict hip fracture risk in women suffering from osteoporosis [22]. Early life influences also affect long-term bone health in animals. Pregnant rats fed a protein-restricted diet produced offspring with altered osteoblast activity [23], changes in bone structure and mineral density [24], and delayed skeletal maturity [25].

In addition to the possible implications for musculoskeletal diseases, changes to musculoskeletal development may also have effects on the metabolism of other tissues. Recent research has demonstrated that osteocalcin (OC), the most abundant non-collagenous protein produced by osteoblasts [26], acts as a bone-derived endocrine hormone when in its undercarboxylated state. Undercarboxylated osteocalcin (uOC) has been shown in mice to have effects on insulin production and sensitivity through effects on multiple tissues, thus affecting whole body glucose metabolism [27]. Whether OC plays a similar role in regulating human glucose handling has yet to be fully elucidated, but the literature currently available suggests interplay between the skeleton and energy metabolism in at least some species. Environmental influences during development that affect the osteoblast phenotype, such as the effects of protein restriction on osteoblast activity reported by Lanham et al. [23], may result in long-term changes to both bone and energy metabolism in the affected organism.

The investigation of the effects of maternal nutrition and fetal growth on subsequent development and disease risk is of unquestionable importance. However other factors, such as physical activity during gestation, might also exert developmental effects on the fetus *in utero*--effects which may be evident in altered fetal growth or may become evident later in life though changes to phenotype and/or the emergence of adult-onset disease. At this time, little is known about the effects of maternal activity levels during pregnancy on the musculoskeletal system and metabolism of the offspring. Before proceeding with human studies, which are difficult to control and implement, proof of concept is needed using animal models in carefully controlled experiments. Such studies may involve aspects of developmental biology, musculoskeletal and metabolic physiology, and stress research. The following literature review presents relevant available information in these areas, in order to identify gaps in the literature, and to facilitate design of the experiments described in the subsequent chapters.

Literature Review

The Developmental Origins of Health and Disease (DOHaD)

In 1989 Dr. David Barker published his seminal paper, "Weight in infancy and death from ischaemic heart disease," in *The Lancet*. Dr. Barker's observation that men with lower birth weights and weights at one year of age had a higher risk of dying from ischaemic heart disease in old age [28] fundamentally changed the way we look at disease risk. The non-contagious diseases of senescence could now be seen not just as the result of unfortunate genetics and less-than ideal lifestyle, but as the closing chapter of a story begun during gestation and childhood through interactions between the developing organism and its environment.

The fundamental concept of the DOHaD hypothesis is the ability of the developing organism to respond to perceived environmental cues by permanently altering its phenotype. This 'developmental programming' occurs during periods of plasticity, times during which the expression of the genotype may be altered to result in any of multiple possible mature phenotypes [29]. Periods of plasticity, which vary between body systems, may be limited to periods of organogenesis during *in utero* development or may extend into early postnatal life. As mentioned above, alterations in gene expression and morphology of the organism that occur during either period are termed 'predictive adaptive responses', aimed at better suiting the developing organism to its anticipated environment [1]. However, these changes can also lead to increased risk of disease in later life if the actual and anticipated environments differ. Barker's initial observation that poor fetal growth (as evidenced by low birth weight) resulted in increased risk of ischaemic heart disease has been repeated in multiple studies in multiple countries [30,31,32]. It is now recognized that poor fetal growth can increase the risk of hypertension and renal failure [33,34,35], type 2 diabetes [4,33,36],

hypercholesterolaemia [37], and the endothelial dysfunction that may be the cause of the observed cardiovascular effects [38,39,40]. Maternal diet (both undernutrition and overnutrition) can have profound effects on the metabolism of the offspring, mediated in part by effects on leptin and insulin sensitivity [4,5,6,41,42,43].

Recognition of these metabolic effects led to the "thrifty phenotype" hypothesis, which proposed that undernutrition in early life inhibits pancreatic ß-cell development, ultimately resulting in an increased likelihood of developing type 2 diabetes. Poor early insulin production in response to rising glucose levels is proposed to be a "mechanism of nutritional thrift" that is beneficial in times of undernutrition, but increases disease risk in times of adequate nutrition [44]. Developing this further, the mismatch hypothesis proposed by Gluckman and Hanson [45] theorizes that the degree of 'match' between the biology of an organism and its environment, established by a combination of genetic legacy and epigenetic marks established *in utero*, influences that organism's ability to thrive and to reproduce in its postnatal and adult environment. Drastic environmental change, or a discrepancy between the environment the developing organism 'expected' based on *in utero* cues and the one into which it is born, leads to greater risk of failure to thrive, disease, failure to reproduce, and even death [45]. However, a mismatch is not the only circumstance that can lead to adverse effects as a result of developmental programming; in some cases it is a 'match' between the *in utero* experience and later life that results in the most detrimental outcomes. For example, rats whose dams were fed a diet high in energy, fat, sugar, and salt develop a preference for "junk food" and, if given access to a similarly obesogenic diet, become fatter than rats fed the same diets whose dams ate normal rat chow during pregnancy [46].

Permanent phenotypic changes that result from environmental 'programming' influences have been demonstrated in human epidemiological studies and well

characterised in animal models, but the mechanisms underlying these effects have not yet been fully elucidated. Most research has focused on the maternal nutritional milieu as an environmental factor capable of programming adult phenotype in the developing fetus. Maternal undernutrition and overnutrition have both been shown to result in a metabolic syndrome in the offspring, characterized by the development of hypertension, adiposity, dyslipidaemia, and reduced glucose tolerance. That two very different nutritional environments (overabundance and scarcity) during *in utero* development result in the same adult phenotype may indicate that the range of possible responses to programming effects is limited [47,48]; in other words, perturbations to the environment experienced by the developing organism lead to the same adult outcomes, regardless of the nature of those perturbations.

Potential mechanisms of DOHaD

Changes to adult phenotype induced by developmental programming effects during development are likely due to either permanent (or transient) changes in gene expression and/or cell differentiation, or to changes in organ development that result in an altered functional capacity of the affected organ systems. Discussion of the specific mechanisms underlying developmental programming is still largely speculative, and associations drawn between changes in gene expression and later-life programming effects may be secondary to the initial, acute response of the developing organism to its environment [48]. Mechanisms that have been proposed to underlie the influence of the environmental milieu on adult phenotype and disease risk include tissue remodelling (e.g. cardiac and renal remodelling), epigenetic changes (e.g. DNA hypomethylation), and alterations in placental glucocorticoid metabolism (e.g. increased fetal glucocorticoid exposure).

Tissue Remodelling

A simple explanation for the mechanism underlying the programming process is tissue remodelling, which occurs when the normal cycles of cell proliferation and differentiation during organ formation are disrupted resulting in an organ of altered size and/or functional capacity. Since the timing of organogenesis is tightly controlled during development, external environmental influences may affect different body systems depending on when they occur. Studies have demonstrated that tissue remodelling occurs during kidney development in rats [49,50] and may also occur in humans [51]. These studies suggest that certain environmental factors experienced during pregnancy, such as a low protein (LP) diet in rats and adverse factors evident in low birth weight in humans, result in reduced nephron numbers in the offspring. This then leads to hypertension, and may result in subsequent renal and cardiovascular disease during later life. Tissue remodelling is also evident in other organs, such as the pancreas and muscles; the offspring of rat dams fed a LP diet during pregnancy have fewer pancreatic ß-cells than controls [52] and rats whose dams were fed a LP diet during the stages of gestation critical for muscle fibre development (mid-gestation) show changes in muscle fibre number and type [53]. However, although tissue remodelling offers a compelling explanation for some DOHaD-type effects, it is likely that other instances of developmental programming are due to changes in the expression of specific genes that do not initially affect whole organ morphology and, in some cases, adult changes at the organ level may be secondary to alterations in gene expression. For this reason, research into the DOHaD hypothesis has turned to epigenetic modifications as a potential mechanism underlying the effects of developmental programming during gestation.

Epigenetics

The term epigenetics refers to changes in gene expression caused by chromatin modifications that are mitotically stable but do not affect the DNA sequence [54]. Most epigenetic changes are reset with each generation, although some might be stably inherited. Although there has been some controversy surrounding the types of chromatin modifications that can be considered 'epigenetic' [55], for the purposes of this review we will consider the major types of epigenetic mechanisms to be DNA methylation, histone modification, and non-coding RNAs. These potentially heritable changes in gene expression may underlie the programming effects of environmental influences during development. For example, undernutrition may reduce the availability of methyl groups for methylation, or may reduce the activity of enzymes necessary for epigenetic modifications to occur [48]. However, epigenetic changes likely also occur as the result of disease or altered phenotype rather than as its cause [56,57], as has been demonstrated *in vitro* by increased histone acetylation in response to a hyperglycaemic environment [58]. Thus, the importance of epigenetic changes associated with early environmental influences as potential causative mechanisms for later-life phenotypic alterations must be evaluated with caution.

Methylation

DNA methylation regulates gene transcription [59,60]. Methylation patterns are tissue-specific [61], and result primarily from the addition of methyl groups to cytosine residues in CpG dinucleotides. Regions of the genome rich in CpG dinucleotides are known as CpG islands, and progressive methylation of these islands within gene promoter regions is associated with repression of transcription [59,62]. Since cytosine and guanine form nucleotide pairs with each other, CpG dinucleotides occur symmetrically on both strands of DNA and thus methylation patterns are maintained in

both daughter cells resulting from subsequent mitotic division of the methylated precursor cell [63]. However, in mammalian development, methylation patterns are erased during the peri-implantation period of embryonic development and then remethylation occurs throughout fetal development [64]. This allows reprogramming of epigenetic marks, but intergenerational inheritance of methylation patterns might occur if some genes escape this early embryonic demethylation.

There is evidence that methylation is one of the mechanisms underlying developmental programming [60,65]. In humans, differences in placental methylation have been associated with birth weight [66], and DNA methylation patterns in cord blood have been associated with height and with lean and fat mass in childhood [67]. Although as yet there is little evidence linking methylation status to later-life disease in humans, these associations of birth and childhood outcomes with methylation status suggest that methylation might play a role in the epigenetic programming of the human genome during development. In animals, changes in tissue-specific methylation patterns in response to maternal nutritional status during pregnancy have been shown in several species, including rats, mice, pigs, and sheep [65]. For example, at 34 days of age the offspring of rat dams fed a LP diet during pregnancy have reduced methylation of the hepatic glucocorticoid receptor gene promoter and correspondingly increased expression of glucocorticoid receptor mRNA [68]. Similarly, pigs had altered global methylation of the liver in response to the protein content of the maternal diet [69]. Of note, in the rat model, supplementation of LP dams with the methyl donor folate reversed the epigenetic abnormalities observed in offspring [70].

Chromatin modification

DNA is packaged around histone proteins within the eukaryotic nucleus to form a DNA-protein complex called chromatin. The basic unit of chromatin is the

nucleosome, which consists of 145–147 DNA base pairs wound around an octamer of histone proteins (two each of the histone proteins H2A, H2B, H3 and H4) [71]. Allowing further chromatin organization, individual nucleosomes are linked by the histone protein H1 [71]. Various post-translational modifications to histone proteins can either promote or reduce transcription by changing the interaction between the histone and the DNA to either facilitate or hinder the access of transcription enzymes to the genetic code [72]. Histone proteins are subject to many different modifications, including the addition of chemical structures such as acetyl, methyl, ADP-ribose, and phosphate groups, as well as glycans and small ubiquitin-like modifier (SUMO) proteins [73]. Acetylation of histone proteins tends to promote transcription, whereas histone methylation can increase or decrease transcription depending on the methylation site. In addition, replacement of any of the histone proteins with a histone variant (variants exist for all histones except H4) can also influence transcription [72]. Although the mechanisms by which histone modifications may be maintained throughout successive cell divisions have not yet been fully elucidated, there is evidence from animal models that specific histone modifications are associated with the maternal environment during development. For example, the female offspring of rat dams fed a LP diet during pregnancy have increased acetylation of H3 and H4 in the CCAAT/enhancer-binding protein ß (C/EBPß) promoter region of skeletal muscle chromatin with corresponding increased expression of C/EBPß mRNA and protein [74]. Similarly, histone modifications resulted in reduced skeletal muscle glucose transporter (GLUT) 4 transcription in adult female rats that were subject to intrauterine growth restriction (IUGR) [75].

Non-coding RNA

A third mechanism by which environmental influences can affect gene expression are small non-coding RNAs known as microRNAs (miRNA). These do not affect transcription but do inhibit translation of their target mRNA transcripts, either by binding directly to the mRNA to form double stranded RNA that is subsequently degraded, or through the formation of the RNA-induced silencing complex [54]. miRNA can be endogenous, existing within the introns of protein-coding genes or as miRNA-specific genes [76], or can be exogenous. A recent study demonstrated the presence of rice miRNA in the sera of both humans and mice, and showed that the rice miRNA could bind to the mouse low-density lipoprotein receptor adaptor protein and thus reduce the clearance of low-density lipoprotein from mouse plasma [77]. This surprising finding of gene regulation across phylogenetic kingdoms suggests a means by which nutritional influences can directly alter gene expression. Although direct alteration of miRNA coding has not been shown to be an epigenetic mechanism, there is significant crosstalk between miRNA expression and DNA methylation, with methylation influencing the transcription of endogenous miRNA and some miRNA altering the expression of methyltransferases [78].

Epigenetics summary

A series of progressive epigenetic changes involving initial histone deacetylation followed by changes in the methylation of the pancreatic and duodenal homeobox 1 transcription factor (*Pdx1*) promoter in IUGR rats result in markedly reduced transcription of the *Pdx1* gene, which regulates pancreatic ß-cell differentiation and function, and leads to diabetes [79]. That epigenetic alterations might underpin the long-term health effects of developmental influences suggests a possible opportunity for early identification of individuals likely to suffer from chronic, adult-onset disease, and

for pharmacological management of DOHaD-type diseases through targeting of specific epigenetic processes [80]. In addition, a recent study by Godfrey et al. [81] offers compelling evidence of an epigenetic mechanism underlying later adiposity: children from two different cohorts (ages 6 and 9 years) underwent dual-energy x-ray absorptiometry (DXA) scanning to determine adiposity, and results were correlated with the methylation of specific genes in umbilical cord samples. Greater methylation of the retinoid X receptor-α gene at birth was strongly associated with later adiposity during childhood. Despite this evidence, it is important to bear in mind that specific epigenetic changes may not be the only mechanism underlying the effects of developmental programming, and alterations in epigenetic marks may be secondary to other factors mediating the influence of the environmental milieu on development. The gatekeeper hypothesis suggests there may be specific genes that are altered by, for example, any nutritional stress (overnutrition, undernutrition, or lack of a macro- or micronutrient); changes to these genes or pathways then lead to downstream epigenetic modifications or changes in gene expression that result in the programmed phenotype of (in the case of nutritional stress) metabolic syndrome [48]. One organ that fits the description of a gatekeeper is the placenta, which controls nutrient transfer from mother to fetus, and also regulates the transfer of many hormones between the mother and fetus.

The glucocorticoid programming hypothesis

The placenta regulates the environment experienced by the fetus by controlling the passage of nutrients and hormones from the mother to the developing baby. Placental 11ß-hydroxysteroid dehydrogenase (11ß-HSD) inactivates maternal glucocorticoids, thus protecting the fetus from the effects of maternal stress hormones [82]. Pharmacological inhibition of 11ß-HSD in pregnant rats resulted in offspring with

low birth weight and reduced glucose tolerance [83], and feeding a LP diet to rat dams produced a glucocorticoid-dependent hypertension in male offspring [84]. Treatment of pregnant sheep with synthetic glucocorticoids such as dexamethasone affects the renin-angiotensin system of the offspring; synthetic glucocorticoids are not inactivated by placental 11ß-HSD [85]. These results suggest that exposure to maternal glucocorticoids can have developmental programming effects, and thus it is important to consider maternal stress when examining the effects of environmental influences during pregnancy on offspring outcomes. External influences that decrease placental 11ß-HSD activity may lead to tissue remodelling and/or epigenetic changes, resulting in subsequent phenotypic changes consistent with the DOHaD hypothesis [86]. However, lasting changes can also be induced by environmental exposures that occur before the placenta has formed, and evidence suggests that the pre-implantation embryo is particularly susceptible to environmental influences [87]. This susceptibility might be due to the lack of the placental "gatekeeper", dependence of the embryo on nutrients within the intrauterine environment, the extensive epigenetic reprogramming that occurs shortly after fertilization, or changes in placental development that are induced by pre-implantation factors. Certainly, given the large number of children conceived *in vitro*, the effects of the pre-implantation environment on later health is a topic of great interest.

Conclusions regarding mechanisms of DOHaD

Whether any or all of the above-described mechanisms are responsible for the long-term consequences of environmental exposures during development remains to be proven. The role of epigenetic mechanisms in DOHaD-type effects has been championed by some, but seriously questioned by other authors. Ptashne, in a scathing

opinion piece [88] rebutting a recent Core Concepts article on epigenetics published in the Proceedings of the National Academy of Sciences [89], questioned whether epigenetic signals were integral to cellular differentiation and cell-specific gene expression and instead credited persistent stimulation or repression of gene expression by transcription factors regulated by positive-feedback loops with maintaining cells in their differentiated state [88]. Ptashne dismisses DNA methylation because "…its possible role in development remains unclear, and it does not exist in, for example, flies and worms…"; however, this statement is not quite accurate as presented. Although it is true that free-living worms such as *Caenorhabditis elegans* lack genomic methylation, there is now evidence of DNA methyltransferase genes in the *C. elegans* genome suggesting that methylation played a role in the evolution of this commonly used model organism [90]. In addition, the nematode *Trichinella spiralis* does utilize DNA methylation during certain life cycle stages [90]. More important is Ptashne's assertion in an earlier article, using examples from yeast, that epigenetic events that regulate transcription differ for individual processes and will need to be understood on an individual basis [91]. This certainly may be true of the regulation of DOHaD-type responses, whether they are due to tissue remodelling, epigenetic alterations, placental modulation of stress hormone exposures, or other regulatory mechanisms.

The epigenetic mechanisms described above may explain the remarkable and truly Lamarckianesque transgenerational inheritance of DOHaD-type effects, such as the risk of type 2 diabetes and obesity that has been documented in multiple species. A study in rats demonstrated that *in utero* exposure to undernutrition resulted in a slightly reduced insulin response in the F_1 offspring, but in marked insulin resistance in the F_2 generation [92], suggesting that the effects of undernutrition persist over several subsequent generations even when subsequent nutrition is normal. Similarly, maternal

exercise stress (2 hours of forced swimming until day 19 of pregnancy and from day 3–21 of lactation) in rats resulted in smaller pups for subsequent generations, and the F_2 pups were small even if the F_1 dams were not exercised [93]. Feeding a LP diet to pregnant rats resulted in similar levels of hypomethylation of the glucocorticoid receptor and peroxisome proliferator-activated receptor-α promoters in male F_1 and F_2 offspring [94]. These results, particularly the similar level of hypomethylation seen in both generations, suggest that at least some epigenetic changes induced by developmental influences can be inherited by subsequent generations, although the mechanism of the preservation of epigenetic marks throughout germ cell development and early embryonic demethylation has not yet been determined.

However, the transmission of DOHaD-type effects to subsequent generations might not be the result of inheritance of epigenetic changes, but might result from the altered intrauterine environment experienced by the offspring of the F_1 generation causing similar *de novo* developmental changes resulting in a disease phenotype similar to that of their parents and grandparents. Although the F_2 offspring did not experience the initial environmental exposure (such as undernutrition) that triggered the phenotypic change in their mother, it may be that their exposure to her altered physiology while *in utero* leads to a similar adult outcome. In the example of type 2 diabetes, the offspring of diabetic mothers are more at risk of themselves developing diabetes if their mother is diabetic during pregnancy than if their mother's diagnosis occurs after their birth [95]. This suggests that it is an intrauterine influence that leads to subsequent disease, and not an inherited genetic (or epigenetic) factor. Many Native American populations are currently troubled by inordinately high rates of type 2 diabetes (>30% of the population affected); these affected populations experienced nutritional deprivation during the late 1800s and early-mid 1900s [96]. In contrast, those Native American populations, such

as the Aleut and Yupik Inuit, that did not experience severe malnutrition during this time have a type 2 diabetes prevalence of <3% [96]. Such dramatic differences in disease prevalence suggest that the developmental programming effects of nutrient scarcity have persisted for over a century in these human populations.

Exercise, DOHaD, and the musculoskeletal system

Although maternal nutrition, and to a lesser extent maternal stress, have dominated research into the developmental origins of disease hypothesis, there is evidence from many mammalian and non-mammalian species of environmental programming of developing organisms by non-nutritional cues [97]. One example of this is coat thickness in vole pups, which is influenced by the photoperiod length experienced by dams prior to gestation [98]. Another organism that responds to non-nutritional environmental cues during development is the crustaceous water flea, *Daphnia cucullata*, which grows a larger helmet when its mother is exposed to predator pheromones while it is developing in her brood pouch [99]. Many external factors besides maternal nutrition during gestation might result in DOHaD-type responses, and one such factor is physical activity. Since in humans the amount and type of physical activity performed is usually under the control of the individual, understanding the implications of exercise during pregnancy on offspring development might lead to useful and inexpensive interventions to reduce the risk of later-life disease. Since the focus of this thesis is on the effects of maternal exercise on the offspring bone and energy metabolism, the sections of the literature review that follow focus on bone physiology, energy metabolism, and the effects of exercise on these systems.

Physiology of bone

The functions of the skeleton include providing structural support for the body, enabling locomotion, and maintaining calcium and phosphorous homeostasis. In spite of these multiple roles bone is relatively less cellular than many other tissues. Bone cells, which include osteoblasts, osteocytes, and osteoclasts, exist embedded within and on the surfaces of the mineralised matrix that forms the hard physical structure of the skeleton. Osteoblasts are the bone-forming cells that secrete the bone matrix (osteoid) that after mineralisation forms the physical scaffolding of the bone. This bone matrix is composed of approximately 90% collagen and 10% non-structural proteins, such as osteocalcin and proteoglycans. After secretion, the bone matrix is subsequently mineralised by deposition of calcium and phosphate in the form of hydroxyapatite crystals [$Ca_{10}(PO_4)6(OH)_2$] [100]. The composition and organization of the mineralised matrix determines the ability of the bone to withstand loads; the mineral content of bone provides strength and stiffness, while the non-mineral content provides elasticity or "toughness" [101,102]. When osteoblasts become surrounded by bone they terminally differentiate to become osteocytes, which convert mechanical stimuli to cellular signals by sensing fluid shifts in their canalicular networks and mediate the skeletal response to external stresses through the production of signalling molecules that regulate osteoblast and osteoclast activity [103]. Osteoclasts resorb bone, release matrix proteins and mineral into the circulation, and (working in concert with the actions of osteoblasts) remodel bone in response to load [103].

The delicate balance between bone formation by osteoblasts and bone resorption by osteoclasts is maintained by selective differentiation and maturation of these cell types. Osteoblastogenesis and osteoclastogenesis involve different molecular signalling pathways; however, considerable molecular communication between these cells is

necessary to maintain appropriate bone mass and their differentiation pathways are linked [104]. Osteoclast precursors are haematopoietic cells of the monocyte lineage, whereas osteoblasts are derived from mesenchymal stem cells [100]. Dysregulation of the differentiation, maturation, or apoptosis of either cell type can result in alterations in bone mass and structure. This is the case in osteoporosis, in which decreased osteoblastogenesis and/or osteoblast lifespan, coupled with increased osteoclastogenesis and/or osteoclast lifespan, leads to loss of bone mineral [105].

Mesenchymal stem cell differentiation into osteoblast precursors is regulated by the Runt-related transcription factor Runx2 (also known as CBFA1 and AML3) [106]. Haploid mutations in Runx2 result in cleidocranial dysplasia, and Runx2 knockout mice have a complete lack of skeletal ossification at birth and die due to inability to breathe [107]. Runx2 is involved in both the differentiation of osteoblasts and development of their mature phenotype; Runx regulatory elements are found in the promoters of all major osteoblast genes, including osteocalcin, osteopontin, bone sialoprotein, and type 1 collagen [106]. Osteoclastogenesis is controlled primarily by the receptor activator of nuclear factor κ B (RANK), its ligand (RANKL), and osteoprotegerin (OPG) [104]. RANK is expressed on the membrane of osteoclast progenitor cells. Binding of RANK by RANKL (in either its transmembrane or soluble form) causes osteoclast differentiation and stimulates bone resorption. RANKL is selectively expressed by cells committed to the osteoblast lineage, thus linking osteoblast formation with osteoclast formation. Soluble OPG is produced by osteoblasts and acts to block the interaction between RANK and RANKL and inhibit osteoclast formation [104]. However, the molecular regulation of osteoblastogenesis and osteoclastogenesis is not limited to the pathways described above. Many other molecules are involved in both stimulation and inhibition of bone formation and resorption. Additionally, the more traditional

regulators of calcium homeostasis—parathyroid hormone [108], calcitonin, and vitamin D3 [109]--each play their role in maintaining bone mineral and serum calcium concentrations.

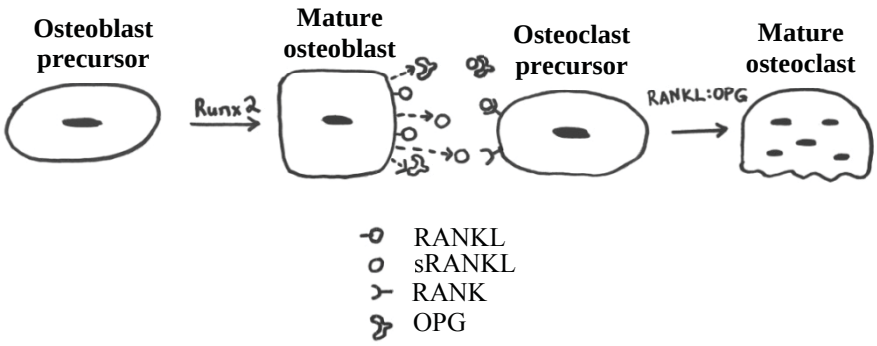

Figure 1. Aspects of osteoblast and osteoclast differentiation. Differentiation of osteoblast precursors into mature osteoblasts depends upon expression of Runt-related transcription factor 2 (Runx2). Osteoclast precursors express receptor activator of nuclear factor κ B (RANK) on their cell membranes and their differentiation to mature osteoclasts is dependent on osteoblast expression of RANK ligand in both its transmembrane (RANKL) and soluble (sRANKL) forms. Interactions of RANK with RANKL triggers osteoclast differentiation. Osteoprotegerin (OPG) is released by osteoblasts and competitively binds RANKL, blocking its interaction with RANK on the surface of osteoclast precursors and thus inhibiting differentiation. The ratio of RANKL: OPG determines osteoclast differentiation. (Adapted from [145] with permission from the *Clinical Journal of the American Society of Nephrology*; permission conveyed through Copyright Clearance Center, Inc.).

The adipokine leptin, which is a key regulator of metabolism [110], appears to mediate the effects of maternal undernutrition in rats on offspring metabolic outcomes [6,111]. In rats, maternal undernutrition during pregnancy results in a postnatal male offspring phenotype characterized by hyperphagia, hyperleptinaemia, and obesity [6]. Treatment of neonatal male rat offspring of undernourished dams with exogenous leptin resulted in normalisation of body composition in the offspring that were subsequently fed a normal diet, but not in offspring fed a high fat diet [112]. Leptin also exerts both central and peripheral effects on the skeleton. Binding of leptin receptors in the ventromedial hypothalamic nuclei affects whole body bone mass [113] and destruction of these receptors, or deletion of the leptin gene, results in a high bone mass phenotype in mice [114]. This is controlled at least in part through the sympathetic nervous system, which acts on β2-adrenergic receptors expressed by osteoblasts. These results highlight

the potential importance of leptin in both skeletal and metabolic regulation, and as a potential mediator of long-term effects of environmental influences on developing organisms. The regulation of both energy metabolism and bone mass by the adipokine leptin suggests a relationship between these two physiological processes. The alterations in leptin associated with the developmental programming of maternal undernutrition indicate that the relationship between bone and energy metabolism might be established *in utero*.

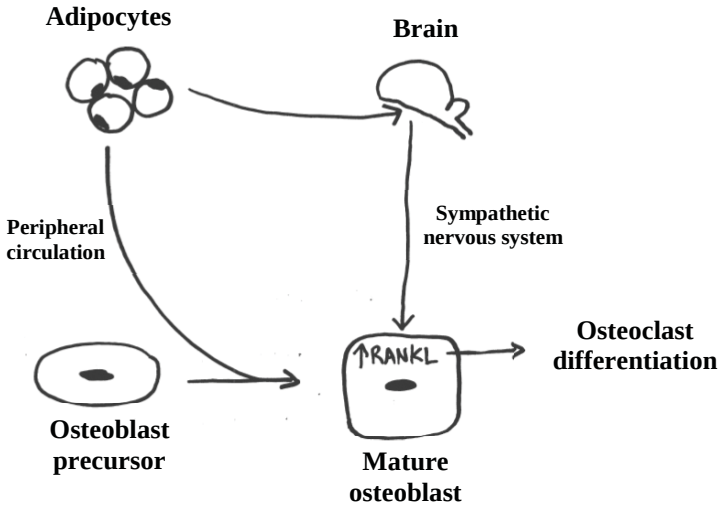

Figure 2. A simplified, schematic diagram of the role of leptin in bone formation and resorption. Leptin is produced by adipocytes. It acts peripherally to stimulate osteoblast differentiation and subsequent bone formation. Centrally, leptin binding of receptors in the ventromedial hypothalamus activates the sympathetic nervous system, which increases production of RANKL by osteoblasts and promotes osteoclast differentiation and bone resorption.

Osteocalcin: a potential link between bone and energy metabolism

Recently, another potential link between bone and energy metabolism has been discovered. The results of studies using knockout mice suggest that the bone-specific protein osteocalcin (OC) acts as an endocrine hormone regulating energy metabolism. Osteocalcin is the most abundant non-collagenous bone matrix protein produced by osteoblasts, and it is believed to have evolved around the same time as the first true bones appeared as it is present in birds, bony fish, and mammals but not in the calcified cartilages of elasmobranchii [115]. Fully carboxylated osteocalcin (cOC) is bound

within the bone matrix and the formation of the crystal structure of hydroxyapatite is inhibited by OC, suggesting that it plays a key role in bone structure [115]. Surprisingly, OC knockout mice do not show early skeletal abnormalities, but do develop increased cortical thickness and density and have less remodelled mineral in their cortices than do controls [116]. Until recently OC was thought of primarily as a marker of bone formation or turnover [26]; however, in 2007 Lee *et al*. reported that $OC^{-/-}$ mice were fatter and had higher blood glucose, and lower insulin secretion and sensitivity, than wild type mice [27]. This suggested that OC acts to regulate glucose handling. Following translation, OC is subject to the vitamin K-dependent addition of three carboxyl groups, which increase the affinity of OC for calcium and hydroxyapatite [26]. Most cOC is bound within the bone matrix, with the remainder being released into circulation [117]. Undercarboxylated osteocalcin (uOC) lacks one, two, or all three carboxyglutamyl residues and thus is not bound to the bone matrix. Further work revealed that it is the undercarboxylated form of OC that regulates glucose handling in mice, and that osteotesticular protein tyrosine phosphatase encoded by the Esp gene decreases the bioactivity of osteocalcin by promoting OC carboxylation and through interactions with the insulin receptor that increase OPG expression in osteoblasts and ultimately inhibit osteoclastic bone resorption and the associated acidic decarboxylation of OC in bone [118]. The Forkhead family transcription factor O1 (FoxO1) decreases the endocrine activity of bone by decreasing OC, and increasing Esp, expression [27,119]. Raising the possibility that OC might have therapeutic benefits for diabetics, Ferron *et al.* recently demonstrated that intermittent injections of recombinant uncarboxylated OC improves glucose tolerance and insulin sensitivity in wild type and high fat-fed mice, and increases skeletal muscle mitochondrial number and metabolic energy expenditure (not physical activity) thus reducing obesity in the high fat-fed

animals [120]. Although the data from mouse models provide convincing evidence of the capability of uOC to affect energy metabolism in that species, whether physiologic levels of uOC play an important role in regulating energy metabolism in normal mice remains to be shown.

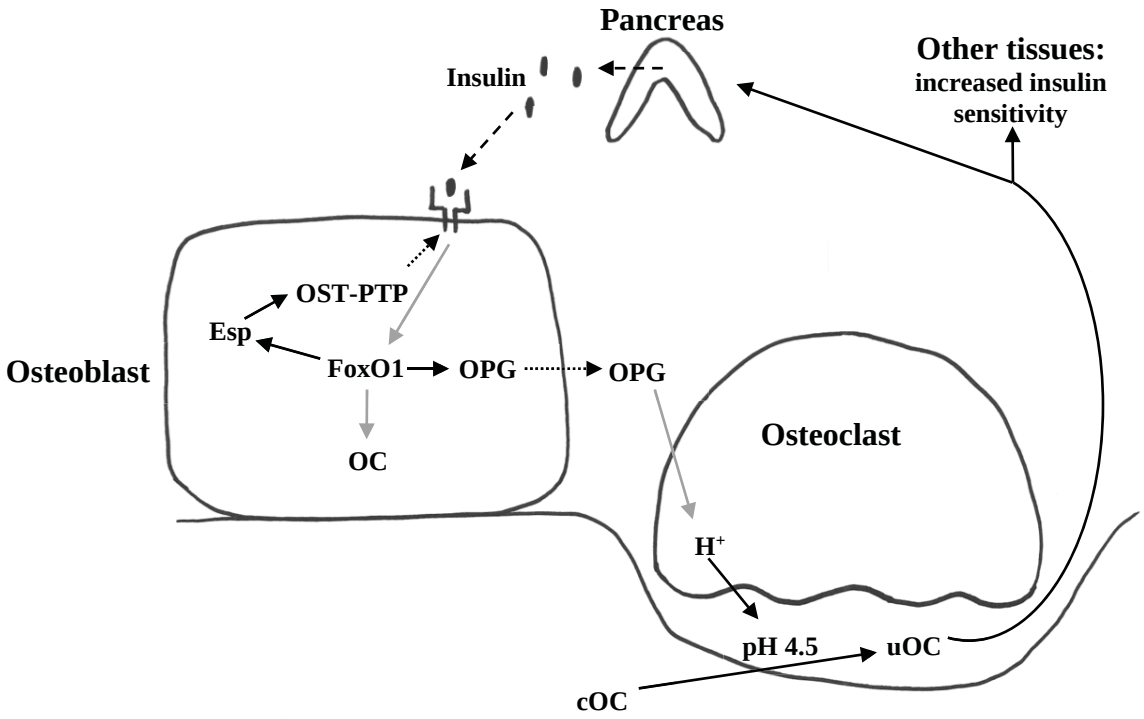

Figure 3. Control of osteocalcin production and carboxylation. Osteoblasts produce osteocalcin. Expression of the Forkhead family transcription factor O1 (FoxO1) inhibits osteocalcin gene expression, promotes production of osteotesticular protein tyrosine phosphatase (OST-PTP) by its gene (Esp), and increases osteoprotegerin (OPG) expression. Binding of insulin to its receptor inhibits FoxO1 expression by osteoblasts, but OST-PTP dephosphorylates the insulin receptor blocking this inhibition. OPG release inhibits osteoclast differentiation. During bone resorption by osteoclasts, fully carboxylated osteocalcin (cOC) is released from the bone matrix and decarboxylated by the acidic pH of the resorption lacuna, becoming undercarboxylated osteocalcin (uOC), which acts on the pancreas to promote insulin production, and on other tissues to increase insulin sensitivity. (Adapted from [118] with permission).

Since the initial report by Lee *et al*. in 2007 [27], there have been numerous human studies that have attempted to discover a metabolic role for OC. The results of these studies have been inconsistent and have failed to yield a clear picture of the relationship, if any, between OC and energy metabolism in humans. In general, total OC has been inversely correlated with measures of glycaemia in multiple human studies,

but a consistent relationship between uOC and energy metabolism has been distinctly lacking [121]. One recent study showed an inverse correlation between cOC concentrations and measures of insulin resistance in older men and women, suggesting a role for the fully carboxylated form of OC in human glucose handling [122].

Extrapolating the results of osteocalcin studies in knockout mice to humans is complicated by several factors. First, knockout mice lack expression of the osteocalcin gene and thus do not express the OC protein, a much greater derangement than would ever occur physiologically even in diseased animals. For obvious ethical reasons knockout human studies are not possible, and although knockout human cell lines can be grown *in vitro* these do not allow assessment of the integrated effects of gene deletion at the organism level. Second, OC carboxylation requires vitamin K as a cofactor. Laboratory rats fed commercial diets have more than adequate levels of vitamin K, and the majority of their OC is fully carboxylated [123]. Humans, however, have variable levels of vitamin K and dietary restriction of phylloquinone (vitamin K_1) intake rapidly increases the proportion of blood OC that is undercarboxylated [124]. Conversely, dietary supplementation with phylloquinone decreases the concentration of uOC found in blood [124,125], but this reduction in uOC does not consistently translate into changes in glucose, insulin, measures of insulin resistance, or adiposity. A 2008 study showed that vitamin K supplementation and decreased uOC were associated with decreased measures of insulin resistance in men only [126] and a 2011 study demonstrated that relatively short-term (4 weeks) vitamin K supplementation resulted in decreased uOC and increased cOC with improvements in indices of insulin sensitivity [127]. However, other studies have failed to show changes in body weight [128] or insulin resistance [129] associated with changes in uOC concentration in response to vitamin K supplementation, although a cross-sectional study showed an inverse

correlation between serum cOC concentrations and body weight [128]. Dietary fluctuations in vitamin K levels can result in large changes to uOC concentrations, eating the equivalent of 2 servings of leafy green vegetables can reduce uOC by 15% [121]. Also, in addition to its effects on the carboxylation of OC, vitamin K itself may improve insulin sensitivity through suppression of inflammation [130]. It is therefore clearly necessary to account for vitamin K status when examining the role of uOC in humans, but few studies have done this [121]. Third, the measurement of osteocalcin is itself fraught with complexity as the available assays measure different forms of the molecule and osteocalcin exists in circulation as both the intact molecule and multiple fragments, as well as in its fully carboxylated and undercarboxylated forms [26]. Careful consideration of assay efficacy is necessary to interpret the results of measurements of OC, and inconsistency in assay type and interpretation between studies complicates comparison of the available literature.

However, although the role of OC in human energy metabolism is unclear, the observation that uOC can act as a bone-derived hormone that regulates glucose handling through effects on insulin secretion and sensitivity, energy expenditure, and fat mass in mice has changed our perspective on the role of the skeleton in metabolic health and disease. Exercise has beneficial effects on both the skeleton and energy metabolism, and OC offers an intriguing potential link between these effects. Although the extent to which the various benefits of exercise are achieved depends on the type, amount, frequency, and intensity of the exercise, in general physical activity enhances muscle metabolism [131], induces fatty acid oxidation [132], decreases fat mass, increases lean mass, and reduces the risk of type 2 diabetes [133]. In addition, exercise directly improves cellular glucose uptake through translocation of GLUT4 to the cell membrane, and by increasing GLUT4 expression through hyperacetylation of the GLUT4 promoter

[134]. Regular exercise may also, to some extent, protect against the increased disease risk associated with an adverse intrauterine environment. In a recent study [9], prenatally undernourished rats that would have become obese under standard laboratory conditions performed 56 metres of voluntary treadmill exercise daily from 60 days of age. This moderate amount of exercise prevented the programmed obese phenotype. In addition, although the prenatally undernourished rats remained small and hyperleptinaemic, their body fat was normalised to a percentage indistinguishable from that of controls [9]. Whether maternal exercise during pregnancy can alter offspring musculoskeletal health or energy metabolism remains to be shown.

The response of bone to exercise

Exercise has many beneficial effects on both the musculoskeletal system and metabolism, and the benefits of exercise extend from childhood through to old age. The World Health Organization currently recommends at least 60 minutes of moderate to vigorous physical activity per day for children 5–17 years old, and at least 150 minutes/week of moderate activity or 75 minutes/week of vigorous activity for healthy adults ages 18+ [135]. Exercise, depending upon the type, quantity, and intensity performed, increases the size and strength [136], mitochondrial content and respiratory capacity [131], and neural activation of the exercised muscles [137]. Similarly, the skeleton also becomes stronger in response to some forms of exercise as the bones adapt to the novel loads imposed upon them [138] by the skeletal muscles, which are the major contributors of those loads [139]. For this reason athletes participating in sports requiring maximal muscle force, such as weight lifting and gymnastics, have more bone mineral than long-distance runners, whose sport requires muscle endurance but not maximal strength [140,141]. In humans who underwent three months of bed rest,

recovery of lost bone mineral density was preceded by recovery of muscle mass [142], demonstrating the important role of muscle in maintaining bone mineralisation. These observations are explained by the concept of bone and muscle as a functional unit [143] and by the Utah paradigm, which postulates that the primary function of bone is to provide only enough structural strength to keep voluntary physical loads from resulting in fracture [138,139]. This relationship between bone and muscle may be subject to developmental programming effects. For example, that the bone mineral content: lean mass ratio of fetal lambs was influenced by the maternal plane of nutrition during pregnancy indicates that the relationship between bone and muscle is at least partially established *in utero* [17].

Bone modelling and remodelling

The basic shape of an individual bone is genetically determined, as evidenced by the ability of the femur to take on a normal shape when the lower limb bud of a chick is removed from its normal anatomic position and grafted onto the chorioallantoic membrane of another chick embryo [144]; however, bones can alter their shape to allow them to better cope with the forces that they experience. This process is called modelling, and involves the independent actions of osteoblasts and osteoclasts [145]. Modelling of long bones often includes widening of the bone, which allows an increase in bone strength without an equivalent increase in bone material or weight. This occurs because the shafts of long bones are primarily subject to bending forces and the ability of a tubular structure to resist bending is inversely proportional to $\pi/4(r_p^4-r_e^4)$, where r_p is the periosteal radius and r_e is the endosteal radius [101]. Since the periosteal radius is always greater than the endosteal radius, increasing the periosteal radius will inevitably enhance the resistance of the bone to bending, even if the endosteal radius also increases

[101,146]. In contrast to modelling, bone remodelling involves tightly-coupled osteoblast and osteoclast activity, which allows bone renewal to maintain strength and mineral homeostasis through the coordinated removal and deposition of bone [101]. Different bones experience different forces during physical activity, depending upon their alignment relative to ground reaction forces and the location and force characteristics of the muscles that act upon them. This will lead to varying responses of individual bones, and specific regions within those bones, depending upon the type, amount and intensity of the physical activity performed.

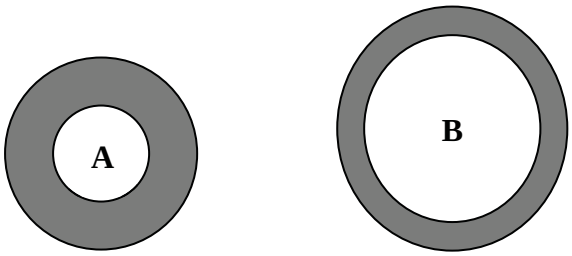

Figure 4. Expansion of the periosteal radius increases resistance to bending. The circles represent cross-sections through the diaphysis of a long bone. The diaphyseal bone represented by circle B is more resistant to bending than the diaphyseal bone represented by circle A, even though it has a thinner cortex, because of its greater periosteal radius.

Cortical bone is dense and forms the outer surface of bone. Trabecular bone consists of a honeycomb-like bony structure surrounded by bone marrow [145]. The ratio of cortical: trabecular bone differs between species, individuals, and individual bones; for example, human vertebrae have a ratio of 25:75, while the radial diaphysis has a ratio of 95:5 [145]. Long, or appendicular, bones usually consist of a diaphyseal region comprised of cortical bone, two metaphyses that contain trabecular bone surrounded by a cortical shell, and two epiphyses that consist of trabecular bone with a more compact 'shell' of subchondral bone underlying the articular surfaces at either end of the bone. The different morphology of the various anatomic regions of long bones reflects their mechanical adaptations to the forces that act upon them. Analyses of serial

pQCT scans of human tibias suggest that the distal end, which contains trabecular bone, is more adapted to compression forces, while the mid/diaphyseal region of the bone is more resistant to bending and torsion [147,148]. Diaphyseal cortical bone responds to strain in various ways. It can increase in mineral content, thickness, or diameter through bone formation on the periosteal surface, which is often coupled with resorption on the endosteal surface [145], allowing cortical drift and alteration of the shape of the bone. This latter change in bone architecture redistributes the mineralised tissue to enhance resistance to bending without greatly increasing weight, and may result in a lower overall bone mineral density (BMD) of the region due to the presence of newly formed lower density bone, even though the bone has gained in strength [146,149]. Trabecular bone has a higher surface area and is said to be more metabolically active than cortical bone [150]; it can respond to strains by increasing the number of, size of, mineral density of, and connections between the trabeculae. The trabecular response to loading has been graphically demonstrated in Thoroughbred race horses by Boyde *et al*. [151]; horses that had undergone race training had significant new bone formation infilling the marrow spaces between the trabeculae in the distal third metacarpus. Although this new bone was of lower mineral density than the more mature trabecular bone, its addition likely resulted in increased bone strength [151]. The response of an individual bone to strain is site-specific and dependent upon the type of stress applied to the bone [150].

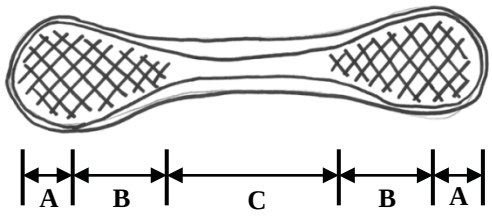

Figure 5. Anatomy of a long bone. A. epiphysis; B. metaphysis; C. diaphysis.

The mechanostat: a reflection of the bone-muscle relationship

Along with the understanding that bone is shaped by the forces it experiences comes the concept of the mechanostat, which postulates that a strain threshold exists within the bone that, once exceeded, stimulates the bone to increase its stiffness and thus reduce the strain to below threshold levels [138]. Thus young, growing animals, whose increasing body size and weight constantly result in progressively greater bone strains, are in a state of bone formation. However, once maturity is reached and body size stabilises, stimulation of bone formation requires novel strains from loads greater than those imposed on the bone through daily living. Hence the necessity of new or maximal exercises to increase bone mineral in adults [139]. However, this description of the mechanostat is perhaps too simplistic [152], and it is important to recognize that different bones and different regions within a bone may have differing strain thresholds [153]. How these are established has not yet been discovered.

The bone-muscle relationship is reflected within the mechanostat concept, and this can be altered by systemic factors such as hormones. Oestrogen is a critical regulator of bone mass and its actions, coupled with the effects of mechanical strain, are the major factors required for maintenance of bone mass [154]. Oestrogen decreases osteoclast formation, activity and lifespan, and may increase the actions of osteoblasts [154]. It also lowers the strain threshold above which bone is formed, resulting in rapid accrual of bone mineral in girls around the time of puberty [155]. This is demonstrated by the difference in the relationship between whole body BMC (an index of bone strength) and LM (an index of muscle strength) that is evident between boys and girls around the time that girls enter puberty. At that time, girls rapidly gain BMC, and gain more BMC than boys with the same LM [156]. Since muscles are the primary contributors of bone strain, this indicates that the bone formation threshold has been

lowered in pubescent girls. This rapid gain in bone mineral plateaus after a few years, which suggests that the stronger bones now reduce the strains to below the threshold required for bone formation. Bone loss occurs when oestrogen concentrations decline (as occurs at menopause in women or following ovariectomy in rats), possibly as a consequence of an increase in the remodelling threshold [155]. Psychological stress and its associated hormonal responses can also change the bone: muscle relationship. Chronically elevated levels of glucocorticoids result in loss of bone mass [157] through inhibition of osteoblast formation and stimulation of osteoblast and osteocyte apoptosis [158]. This can result from exogenous or endogenous glucocorticoid excess. Depression, with its associated hypercortisolism, has also been suggested to be a risk factor for osteoporosis [159]. Dysfunction of the bone-muscle relationship, even without obvious hormonal influences, may also lead to disease. Children suffering from idiopathic juvenile osteoporosis have a markedly lower BMC than expected for their lean mass (LM). As they recover, their BMC: LM ratio becomes more normal [160].

Maternal exercise during pregnancy

Human studies on exercise during pregnancy

The effects of exercise during pregnancy on the developing offspring are difficult to define in humans. Since the effects of exercise differ with the type, amount, frequency, and intensity of the activity performed, differing exercise regimens will likely lead to different effects on offspring development. The types of physical activities studied in pregnant women are almost as varied as the studies themselves, ranging from low-intensity walking [161] to long-distance running [162,163], and thus it is difficult to compare their outcomes. Many studies also use self-reported exercise rather than a standardized, supervised exercise intervention/programme [164,165]; such

questionnaire assessments of physical activity have limited reliability and can lead to both under- and over-reporting of the amount of exercise performed. The small size and methodological inconsistencies (such as problems with randomization) of the studies available led the authors of a 2010 Cochrane Review to conclude that more work is needed before evidence-based recommendations could be made regarding aerobic exercise during pregnancy [166]. In addition, due to the long life-span of our species there are very real practical difficulties in examining the effects of maternal exercise during pregnancy on, for example, the risk of osteoporosis during senescence of the offspring.

In spite of these difficulties, most reports indicate that exercise during normal low-risk pregnancy does not harm pregnancy outcome [167], and that it may reduce the risks of being born either small or large for gestational age, both of which are associated with adverse later-life health outcomes [168]. Recent work has shown that there are no acute detrimental effects on the human fetus after moderate treadmill exercise (30 minutes at 40–59% of aerobic capacity reserve) by habitually inactive women, or after more vigorous treadmill exercise (30 minutes at 60–84% of aerobic capacity reserve) by habitually active women [169]. It is increasingly suggested that maternal exercise in gestation can be beneficial to the health of both mother and baby [168,170]. Although there is some evidence of a slight increase in miscarriage rate in women who engage in non-swimming exercise in early gestation [171], beginning moderate exercise in early pregnancy enhances placental growth and increases birth weight [172]. This evidence suggests that, given the modern problem of obesity, exercise during pregnancy may in many cases carry more benefits than risks [173].

Links between birth weight (as a proxy for fetal growth) and future disease risk have historically been a fundamental part of the DOHaD theory, and as such the effects

of exercise during pregnancy on birth weight are of great interest. Birth weight correlates with total placental volume and placental villous volume, and the effects of exercise on these placental parameters (and subsequently birth weight) varies with the intensity and timing of exercise during pregnancy [174]. Several studies have shown no negative effect of exercise on birth weight [175,176,177]. However, others have found small decreases in birth weight associated with heavy exercise, especially in late gestation [174,178,179,180]. This difference is largely due to decreased fetal fat in the offspring of heavily exercising mothers [174,181]. A recent study found that pregnant women who performed 15 weeks of regular, non-weight bearing exercise at approximately 65% of aerobic capacity had babies that were slightly, but significantly, lighter than those of non-exercised controls although their birth weights remained within the normal range [182]. This result raises the question of whether a small reduction in birth weight is detrimental or beneficial to the offspring. Although the definitive answer to this question will have to wait many years for follow-up data to be collected from the adult offspring, the reduction in birth weight may carry increased risk of metabolic dysfunction [183] or may be associated with decreased future adiposity, since higher birth weight has been associated with higher BMI in childhood and adulthood [184]. Maternal carbohydrate intake also has a strong influence on birth weight [185], and mothers who eat a highly glycaemic diet have large babies regardless of physical activity [181]. Low birth weight (<2500 g) can be the result of IUGR or pre-term delivery [186], but exercise (including structured light resistance training and running programmes, as well as all types of leisure time physical activity) does not increase rates of pre-term delivery in healthy pregnant women [177,180,187,188,189]. Recent Cochrane Reviews have concluded that there is currently insufficient evidence to determine whether exercise during pregnancy reduces the risk of gestational diabetes

[190] or pre-eclampsia [191], but the results of some studies suggest that exercise may decrease the risk of these conditions in pregnant women [189,192,193]. Labour and delivery are also shorter and less likely to require intervention in women who have exercised throughout their pregnancy [180].

There are very few human studies of the effects of maternal exercise during gestation on offspring outcomes in later life. In a series of two studies examining morphometric and neurodevelopmental outcomes in children born to gestationally exercising women at 1 and 5 years of age, the 1 year old children did not differ from the children of non-exercising control mothers [194]; however, the 5 year old children of exercising mothers were thinner, but not shorter, than the children of non-exercising mothers, and performed better on tests of oral language skills [195].

Rat studies on exercise during pregnancy

Animal studies of the developmental effects of exercise during pregnancy have primarily utilised sheep and rat models; we have confined this review to discussion of studies that utilised pregnant rats because most of the data available on offspring outcomes after maternal exercise and other DOHaD effects come from rat studies, and these are the most applicable to our work. Rats are advantageous because of their small size, short gestation period, and the similarity of the rat placenta to the human placenta. However, rats are a polyovulatory species and their simultaneous gestation of numerous offspring may have developmental effects that complicate extrapolation of data from rat studies to humans. In addition, rats are born at an earlier stage of development than human babies. In spite of this, much of our knowledge about the developmental origins of health and disease has come from studies using rats, and findings in rats have provided proof of the concept that prenatal influences may lead to alterations of adult

phenotype [196]. For example, research in rats has provided partial explanation of the mechanism behind Barker's initial finding that poor prenatal growth leads to an increased risk of cardiovascular disease. Undernourished pregnant rats produce offspring with endothelial dysfunction and reduced ability to cope with reactive oxygen species [38,39], and when such vascular changes occur in humans they predispose to atherosclerosis and coronary heart disease [197]. Similarly, studies in rats have begun to expose the mechanisms for the increased risk of type 2 diabetes associated with maternal malnourishment [4]. Thus, in spite of differences between rat and human pregnancy, rats remain an acceptable model organism for controlled studies investigating the effects of environmental influences during pregnancy on offspring health.

Studies of exercise in the pregnant rat have utilized swimming [198,199,200], treadmill training [201,202,203], and voluntary wheel running [198]. Potential problems with the use of these exercises to study the developmental effects of exercise during pregnancy include the likelihood that they induce a maternal physiological stress response and the possibility of causing IUGR through their effects on maternal nutrient distribution and/or uterine blood flow. Swimming and treadmill running have both been shown to cause increases in stress hormones in exercise-habituated adult rats [204] and prenatal stress in rats induces IUGR and leads to postnatal metabolic dysfunction [36]. Strenuous exercise in pregnant rats has been demonstrated to reduce both pup size and number [205,206], and two hours of maternal swimming daily through pre-pregnancy, pregnancy and lactation has been shown to cause growth retardation in the pups of two subsequent generations [93]. However, a study using more than 100 rats found that low-intensity exercise did not change birth weight or litter size [202]. Since IUGR is known to produce developmental effects [8,36,112,207], it is important that any exercise

performed by pregnant rats be sufficiently moderate so as to not restrict growth of the fetal pups.

Investigations of the long-term effects of dam exercise during pregnancy on offspring outcomes beyond the perinatal period are few in the literature and most of those that exist have, likely because of evidence of improvements in neurodevelopment in the children of exercising women [195], focused on brain effects. These report improved memory and hippocampal neurodevelopment in the pups of exercised dams [199,201], which seems to be mediated by serotonergic and noradrenergic systems [198]. The only report (other than the published papers included as chapters in this thesis) describing the effects of maternal exercise during pregnancy on the musculoskeletal system of the offspring in later life is that of Monteiro *et al.* [208]. They reported no differences in the growth or bone mineral content of the femur in 90 day old pups from exercised or sedentary dams.

Summary of the literature in areas pertinent to the doctoral research

This review of the literature suggested the potential of *in utero* influences to cause long-term alterations to the musculoskeletal system and energy metabolism of the developing offspring. Effects on energy metabolism (in terms of increased obesity and diabetes risk) have already been well demonstrated in the offspring of undernourished human and animal mothers. Adult differences in musculoskeletal health resulting from programming factors during development have been less well established, but evidence suggests that these too may be programmed by the intrauterine environment. Although nutrition has been the most widely studied factor resulting in developmental programming effects, maternal physical activity may also influence long-term offspring health. However, there are very few studies examining the long-term consequences for

the offspring of maternal exercise during pregnancy, particularly in terms of musculoskeletal outcomes.

Since physical activity performed *ex utero* affects both the musculoskeletal system and energy metabolism of the exercising individual, maternal exercise during pregnancy might also have long-term effects on both of these physiological systems in the offspring. Undercarboxylated OC, a bone-produced protein that affects glucose handling in mice, is an intriguing possible link between the effects of exercise on the musculoskeletal system and energy metabolism, but few studies have examined this. In addition, the influence of the adipokine leptin on both energy metabolism and bone mass suggests that there is considerable interplay between these systems. Leptin is involved in the *in utero* programming of metabolic outcomes in a rat maternal undernutrition model, but the role of uOC in DOHaD-type effects has not been investigated.

These gaps in the literature led to the series of experiments that follow in **Chapters 2** through **5**, which were designed to validate a suitable model of exercise in the pregnant rat and then use this model to investigate the effects of maternal exercise during pregnancy on bone and energy metabolism in the mature offspring.

Aims and Hypotheses

This thesis aims to examine the effects of maternal exercise during pregnancy on the long-term musculoskeletal health and body composition of offspring using the rat as an animal model. Based on the information available in the literature, we hypothesised that voluntary maternal exercise during pregnancy would result in long-term improvements to the offspring musculoskeletal system and energy metabolism. We recognize that the effects of maternal exercise during pregnancy will vary with the type, intensity, frequency, and timing of the exercise; the scope of this thesis is limited to investigation of one type of moderate, voluntary exercise and the results may not be applicable to all types of exercise that could be performed by a gravid female. Because of the particular interest in bone, we selected exercises that were thought likely to have a significant effect on maternal bone, but unlikely to cause a systemic maternal stress response. We then validated our exercise model before applying it to pregnant rats.

The specific aims and hypotheses addressed in the chapters of this thesis are as follows:

Chapter 2 aims to validate potential voluntary exercise models in non-pregnant rats. The rats performed one of two exercise types for a 3 week period (equivalent to the length of gestation in the rat). Faecal corticoids and bone parameters were assessed to determine both the physiological stress and bone response to exercise.

> ***Hypothesis***: Short-term voluntary exercise will cause a measureable increase in BMC, bone area, or BMD of the tibia without causing a concomitant rise in faecal corticoid excretion.

Based on the results of the previous chapter, **Chapter 3** aims to validate these same exercise models in pregnant rats, to assess the physiological stress and bone response of pregnant dams to exercise, and to determine effects on fetal growth *in utero*.

> ***Hypothesis***: Voluntary maternal exercise during pregnancy will not adversely affect fetal outcomes at day 19 of pregnancy or increase maternal stress hormone concentrations, but will alter maternal bone relative to that of control dams.

In **Chapter 4** we select one exercise and allow pregnant rat dams to perform it for the duration of their pregnancy. The aims of this chapter were to determine the effects of maternal exercise during pregnancy on early life outcomes of the pups, and on maternal bone over the combined pregnancy and lactation period. In addition, we also further explored maternal faecal corticoid excretion in order to confirm that the exercise we selected did not result in a maternal physiological stress response during pregnancy.

> ***Hypothesis***: Voluntary maternal exercise during pregnancy will not affect litter size, will result in heavier offspring relative to those of non-exercised controls, and will attenuate the loss of maternal bone mineral throughout pregnancy and lactation.

Chapter 5 aims to evaluate the long term effects of maternal exercise during pregnancy on the bone and body composition of the adult offspring living under control laboratory conditions, as well as to explore the potential role of uOC as a mechanism of these effects.

> ***Hypothesis***: Adult offspring of dams that exercised during pregnancy will show improvements in musculoskeletal health and body composition relative to the adult offspring of control dams, and the bone-derived hormone uOC will play a role in these effects.

Chapter 6 aims to place our results within the context of the currently available literature, to highlight the original contributions to knowledge presented in this thesis, and to suggest directions for further work.

Appendix A provides detailed information about the various laboratory techniques used in these experiments. In addition, validation work was done to confirm that serum concentrations of osteocalcin do not vary significantly with estrous cycle stage in female rats; this work is presented in **Appendix B**. Furthermore, as the rat offspring aged we encountered some cases of chronic progressive nephropathy, a condition that affects laboratory rats. This is further discussed in **Appendix C**.

Since the experiments described in each chapter tested specific hypotheses related to this investigation, each chapter contains its own introductory material and discussion of results. Thus, further background can be found within the chapters of this thesis. Specifically, **Chapter 2** contains additional discussion about exercise models in rats and the effects of physiological stress on the musculoskeletal system; **Chapter 3** provides additional material on exercise, calcium handling, and physiologic stress responses during pregnancy; **Chapter 4** further discusses the response of bone to exercise and to the calcium demands of pregnancy and lactation, as well as the effects of exercise during pregnancy on offspring birth weight; and **Chapter 5** provides more background on exercise during pregnancy and the potential role of uOC in bone and energy metabolism.

This thesis includes five published papers, as well as an introductory chapter, a discussion and conclusions chapter, and several appendices. The contents of the published manuscripts have not been altered, but the reference citation style has been formatted to provide consistency throughout the thesis. Presenting a thesis by publication may result in some repetition of the material in the literature review and the introduction and discussion sections of the chapters. For this reason, the references are provided at the end of each chapter rather than as a separate reference section for the entire thesis. As with most scientific endeavours, the work in the published manuscripts

represents a collaborative effort between my coauthors and myself; however, in all cases my input was the greatest. Contribution forms detailing the amount of my contribution to each manuscript are provided in **Appendix D**.

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Chapter 2

SHORT-TERM VOLUNTARY EXERCISE IN THE RAT CAUSES BONE MODELING WITHOUT INITIATING A PHYSIOLOGICAL STRESS RESPONSE

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Abstract

Recent research has revealed a neuroendocrine connection between the skeleton and metabolism. Exercise alters both bone modeling and energy balance, and may be useful in further developing our understanding of this complex interplay. However, research in this field requires an animal model of exercise that does not cause a physiological stress response in the exercised subjects. In this study we develop a model of short-term voluntary exercise in the female rat that causes bone modeling without causing stress. Rats were randomly assigned to one of three age-matched groups: control, tower climbing, and squat exercise (rising to an erect bipedal stance). Twenty one days of exercise resulted in bone modeling as assessed by peripheral quantitative computed tomography. Fecal corticosterone output was used to assess physiological stress at three time points during the study (pre-exercise, early exercise, and late in the exercise period). There were no differences in fecal corticosterone levels between groups or time points. This model of voluntary exercise in the rat will be useful for future studies of the influence of exercise on the relationship between skeletal and metabolic health, and may be appropriate for investigation of the developmental origins of those effects.

Introduction

Recent advances in our understanding of the dynamic relationship between body structure and metabolism have revealed a neuroendocrine connection between bone remodeling and energy balance, with both bone and fat acting as endocrine organs [1]. The osteoblast-specific protein osteocalcin regulates glucose handling through effects on insulin secretion and sensitivity [2]. This action is in turn mediated by the adipokine leptin, which inhibits insulin secretion through direct effects on pancreatic ß-cells [3] and through inhibition of osteocalcin activity [4]. The interplay between bone and metabolism has recently been further elucidated by the discovery that the Forkhead family transcription factor FoxO1, a key regulator of insulin handling in multiple tissues, also acts within the osteoblast to control osteocalcin expression and bioactivity [5].

Exercise stimulates modeling of the musculoskeletal system [6,7,8], alters metabolism [9] and enhances brain function [10], and thus exercise provides an excellent means of exploring the neuroendocrine connection between bone, fat and energy balance. The rat model of exercise has been used extensively to study the physiological effects of physical activity using such exercise modalities as swimming [11,12], running [13,14,15,16,17], jumping [18], tower climbing [19], rising to an erect bipedal stance [20,21], and weight lifting [22]. The effects of exercise on bone have been widely characterized in the rat. Ovariectomized rats undergo changes in bone structure that are nearly identical to those seen in human osteoporosis [23,24]; and these animals have proven useful for the study of exercise interventions in osteoporosis treatment [25].

In examining exercise effects on the interrelationship between bone and metabolism it is important to consider other potential effects on both systems. One such

potential confounder is the stress response induced by the exercise model. Increased hypothalamic-pituitary-adrenal (HPA) axis activity and stress hormone levels are known to affect metabolism, causing alterations in visceral adiposity and insulin sensitivity. Chronic stress also affects bone turnover, leading to "low-turnover" osteoporosis [26,27,28,29]. Involuntary forms of exercise such as swimming and forced treadmill running increase serum biomarkers of stress in rats [30], and even voluntary wheel running has been shown to increase corticosterone secretion [31,32]. Conversely, exercise that causes increased corticosteroid levels may also reduce adrenal activity in response to external stressors [17,33]. As the connection between the HPA axis, the adipoinsular axis, and the skeletal system is further elucidated, separating the biomechanical effects of exercise on bone from the systemic neuroendocrine effects of exercise on the skeleton becomes increasingly challenging. A model of exercise in the rat that does not cause an associated stress response is needed.

The purpose of this study was to establish a model of short-term exercise in the rat that would be useful for examination of exercise effects on the musculoskeletal system and metabolism. An exercise model that does not cause a stress response in exercised rats would also be potentially useful for examining the developmental effects of exercise during gestation on the offspring's musculoskeletal system and metabolism. To reduce the likelihood of the confounding effects of stress, voluntary exercises of moderate intensity were selected. Voluntary exercises shown to induce bone modeling in the rat include tower climbing [19] and inducing rats to rise to an erect bipedal stance in order to reach food [20,34]. This study was conducted using sexually mature, nonpregnant female rats to test the hypothesis that short-term (21 days) tower climbing or rising to an erect bipedal stance (in this paper termed "squat") exercise in the rat

would cause a measurable increase in bone mineral content (BMC), bone area, or bone mineral density (BMD) without a concomitant rise in fecal corticosterone.

Materials and Methods

Animals. Twenty nine female Wistar rats were habituated to their surroundings and to a low phytoestrogen diet (AIN-93G, Research Diets Inc) for 19–22 days prior to commencing the exercise protocols. All rats were housed in the same room in a climate-controlled dedicated animal research facility with a 12: 12-h light-dark cycle. Rats were bedded on kiln-dried wood shavings, and all were housed in pairs during the study period. Feed and water were provided *ad libitum* and intake was monitored by weighing initial and residual feed and measuring initial and residual water volume every 1–2 days throughout the exercise trial. Body weight was measured two times weekly. The study protocol and all animal procedures were approved by the Massey University Animal Ethics Committee.

Exercise. After the habituation period, when they were 100 +/-8 days of age, the rats were randomly assigned to one of three age-matched exercise groups: control, squat exercise, and tower climbing.

Control rats were housed conventionally for the duration of the trial in a 419 mm x 279 mm x 152-mm high cage (Fig. 1A), with their feed and water at a height of ~90 mm from the cage floor.

Rats in the squat exercise group were housed in a modified cage, the sides of which were gradually raised in height over a 5-day period. At full height the food and water available to the rats was ~220 mm above the cage floor (Fig. 1B), requiring the rats to stand on fully extended hind limbs to obtain food and water. This model has been previously shown to induce bone modelling in sexually intact rats and to reduce bone loss in gonadectomized rats [34].

For the tower climbing group, the cage lid was replaced with a 2-m-high tower made of wire mesh (12 x 12 mm welded stainless steel 1.6 mm) as shown in Fig. 1C.

Over a 5 day period the water bottle in the cage was incrementally raised from an initial height of 90 mm to a final height of 2 m. Tower climbing has been previously shown to induce bone modeling in rats [19].

During their 5-day gradual introduction to exercise, the rats were observed daily to ensure they were able to reach the food and water and that they were maintaining body weight and feed and water intake. Any rats with reduced water intake were examined by a veterinarian to ensure there were no clinical signs of dehydration. Following this orientation period, the rats remained in their exercise cages for 21 days.

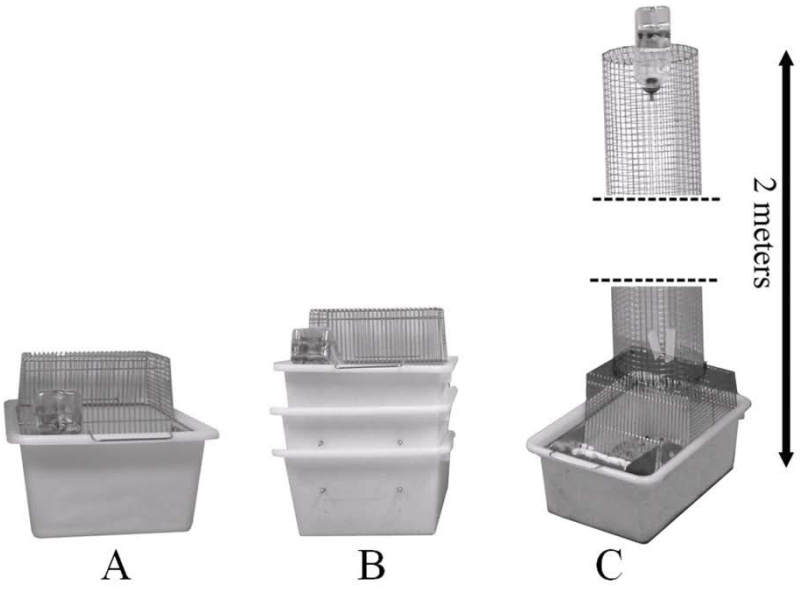

Figure 1. A. Control cage. B. Squat exercise cage. C. Tower exercise cage.

Imaging. Peripheral quantitative computed tomography (pQCT) and dual energy X-ray absorptiometry (DXA) were performed two times during the trial, at 6 days prior to beginning the orientation period (baseline) and on day 26 (5 days of orientation plus 21 days of exercise) after which the rats were killed. Prior to imaging the rats were anesthetized with a mixture consisting of 0.5 ml ketamine (100 mg/ml) + 0.2 ml acepromazine (2 mg/ml) + 0.1 ml xylazine (100 mg/ml) + 0.2 ml sterile water injected

intraperitoneally at a dose rate of 0.6 ml/100 g via a 25-gauge needle. A level of anesthesia suitable for non-invasive imaging procedures was obtained within 5–10 minutes and was maintained for ~1 h.

Peripheral quantitative computed tomography of the right tibia of each rat was performed (XCT2000 pQCT scanner; Stratec, Pforzheim, Germany). Tibial length was measured from the palpable lateral aspect of the tibial plateau to the distal end of the lateral malleolus, using callipers. The rat was then placed in right lateral recumbency and affixed to a cardboard platform with adhesive tape. Scans were made 5mm distal to the tibial plateau (proximal metaphysis) and at 50% of the tibial length (mid-diaphysis) [35] with a voxel size of 0.1 mm and scan speed of 10 mm/sec. Scans were analyzed using the manufacturer's software. Metaphyseal bone was analyzed by contour mode 3, peel mode 2, with an outer threshold of 214 g/cm^3 and an inner threshold of 606 g/cm^3 as described elsewhere [36], and diaphyseal bone was analyzed using cortmode 1 with a threshold of 710 g/cm^3. The coefficient of variation (CV) for total density ranged from 0.48 to 1.47% at the mid-tibial diaphysis and from 1.32 to 2.23% at the proximal tibial metaphysis, and the CV for total area ranged from 1.47 to 1.72% at the mid-tibial diaphysis and from 3.73 to 7.11% at the proximal tibial metaphysis without and with repositioning between scans.

BMC, areal bone mineral density (BMD_a), and whole body composition data were determined with a fan beam densitometer (Hologic Discovery A, Bedford, MA, USA) using the small animal application. Anesthetized rats were placed in dorsal recumbency on an acrylic platform with their hind limbs in a frog-legged position with the femurs fully abducted and a femorotibial joint angle of 90 degrees, and held in position with adhesive tape. Regional high-resolution scans of both hind limbs (femur and tibia) and the lumbar spine were performed, as well as whole body scans. Quality

control scans were performed daily to ensure that the precision met the required DXA manufacturer's CV, which was 0.98–1.01%. High-resolution scans of the femurs had a CV of 0.60% and 1.20% without and with repositioning between scans.

Fecal sample collection and corticosterone measurement. To measure the stress response to voluntary exercise, fecal samples were collected from all groups during three periods: preexercise, during early exercise (day 6–9), and towards the end of the exercise period (day 18–21). Feces were collected by a protocol similar to that described by Boggiano et al. [37]. Briefly, the cage bedding was changed one hour before the start of the dark period. All feces were collected from the bedding 4 hours after the start of the dark period. Urine-soaked feces were discarded (this occurred only once as the high quality bedding instantly absorbed any voided urine). The first four hours of the dark period is approximately the time of the fecal corticosterone nadir [37,38], at which time between-group differences in corticosterone would be most apparent [39]. Female rats produce varying levels of fecal corticoids during the different phases of their estrous cycle [38]. To minimize the effects of the estrous cycle on corticosterone levels, samples were collected for four consecutive days during each sampling period.

Fecal samples were stored at -20 degrees Celsius until the completion of the study period. Samples were then freeze-dried, ground, and ethanol extraction was performed by the nonboiling method described by Fraisse and Cockrem [40] with the following slight modifications. The dried ethanol extracts were reconstituted in 1 ml of 0.1M phosphate-buffered saline with 0.1% gelatin, pH 7.0 (PBSG), and the final supernatant diluted in PBSG by a factor of two prior to freezing. The recovery of corticosterone following extraction was measured as previously described [40]. The mean recovery of corticosterone from spiked control samples was 70.7 $\pm$ 0.8% (n=15). The coefficient of variation for the mean percentage recovery was low (5.2%), and the

mean percentage recovery was used to calculate results for all the samples. All samples were assayed in duplicate by a commercially available radioimmunoassay kit (Double Antibody Corticosterone ^{125} I RIA kit for rats and mice; MP Biomedicals) and radiation counts were determined in a LKB Wallac 1261 Multigamma gamma counter for 3 min each. The sample and reagent volumes that were used in the assay were all 1/10 those of the kit protocol. Following addition of the precipitant and vortexing, 20 µl starch (25 g/l starch (Sigma) plus 0.05 g/l neutral red (BDH) in PBSG) were added to increase adhesion of the pellet to the tube. The intra-assay CV for corticosterone were 8.4, 6.0, and 7.2% and interassay CV for corticosterone were 7.8, 8.4, and 11.5% for low-, medium- and high-concentration solutions, respectively. Fecal samples were weighed after drying and before extraction. Corticosterone is expressed as total nanograms excreted over the collection period, calculated as the fecal corticosterone concentration (ng/g) multiplied by the weight of the fecal sample.

Statistical analysis. All statistical analysis was performed with SAS 9.1 and an alpha level of 0.05. Significance of final imaging bone parameters was determined by covariate analysis using Proc GLM. Fixed effects initially tested were exercise group and age, and covariates were baseline parameter value and initial body weight at the start of the exercise training period. The final model included exercise group, baseline bone parameter value and their interaction. Baseline differences between groups were assessed by simple ANOVA. Fecal corticosterone was assessed by repeat measures ANOVA. The final model included exercise group, cage, collection period and the interaction of exercise group and collection period. A covariance analysis was conducted to analyze the relationship between cumulative feed intake and body weight gain during the experiment. A linear model (Proc GLM; SAS), with exercise as a fixed effect, cage within exercise as a random effect, the covariates body weight gain and

body weight gain squared, and the interactions between the covariates and the fixed effect (to test for homogeneity of the linear and quadratic regression coefficients), was fitted to the cumulative feed intake data. Where appropriate, 95% confidence intervals for the linear and quadratic regression coefficients were calculated to show differences between exercise groups. All data are expressed as least square means ± standard error unless otherwise indicated.

Results

Animals. All rats successfully performed the exercise required by their group without injury or compromised health. Rats in all groups gained weight over the study period as shown in Fig. 2, with control rats gaining the most weight and tower-climbing rats gaining the least. Feed intake was very similar between groups (Fig. 3) although the relationship between feed intake and weight gain differed between exercise groups. This relationship was most variable in the tower-climbing group, with an initial decrease in weight gain per gram of feed intake followed by a marked increase in feed efficiency (the ratio of weight gain to feed intake [41]). Figure 4 shows the relationship between weight gain and cumulative feed intake. The regression coefficients of the line demonstrating the relationship between intake and weight gain in the tower-climbing rats were significantly different from that of the controls. Feed efficiency over time is shown in Fig. 5. While the feed efficiency of rats in the control group remained unchanged over the exercise period, rats in the squat exercise and tower-climbing groups demonstrated an improvement in feed efficiency over the course of the trial.

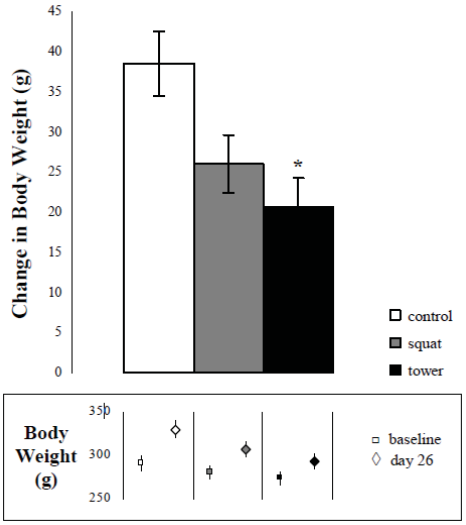

Figure 2. Bar graph of mean change in body weight over the experiment for each group. * Significant difference from control value ($p<0.05$). Below each bar the baseline (weight at preexercise scan) and postexercise (day 26) mean body weight is shown for each exercise group. There was no significant difference in baseline body weight between groups.

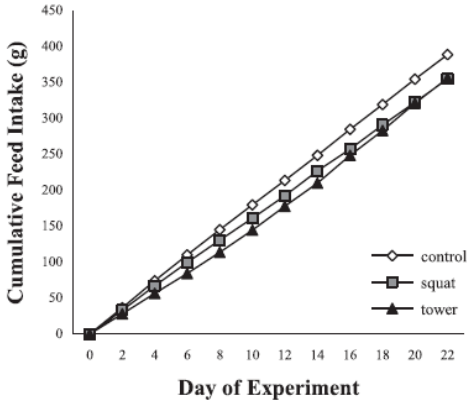

Figure 3. Mean cumulative feed intake over the exercise period for each group.

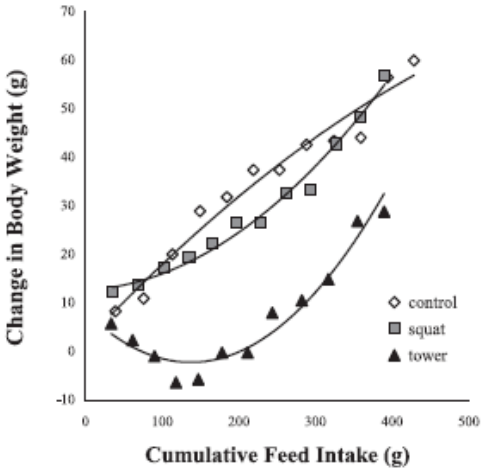

Figure 4. Mean change in body weight per gram of cumulative feed intake on a per cage basis (2 rats per cage) throughout the exercise period.

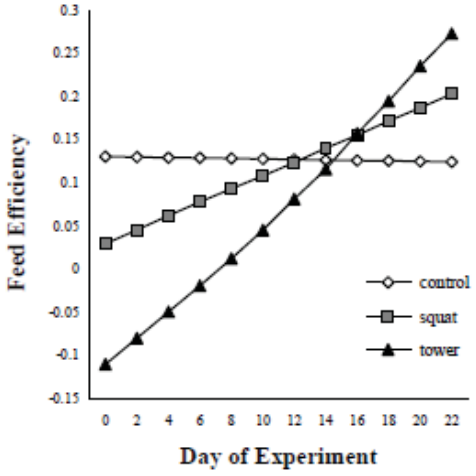

Figure 5. Feed efficiency by day of experiment. The feed efficiency is calculated as the first derivative of the functions represented by the lines in Figure 4.

Imaging

The results of pQCT imaging of the proximal tibial metaphysis are presented in Table 1. Postexercise (day 26), the tower-climbing group had a significantly larger total BMC, total bone area, and trabecular and cortical/subcortical (c/sc) BMC than did the control group. Squat exercise rats gained total BMC and c/sc BMC without gaining significant total bone area, leading to a significantly greater volumetric bone mineral density than that of day 26 controls.

Table 1. *In vivo* pQCT values at the proximal tibial metaphysis

	Baseline			*Post-Exercise (Day 26)*		
	Control	**S**quat	**T**ower	**C**ontrol	**S**quat	**T**ower
Total BMC (mg)	11.1 ± 0.3	11.0 ± 0.3	10.6 ± 0.3	11.5 ± 0.2	12.5 ± 0.2*	12.6 ± 0.2*
Total area (mm^2)	16.2 ± 0.5	16.4 ± 0.5	16.0 ± 0.5	15.6 ± 0.3	16.5 ± 0.3	17.0 ± 0.3*
Total $_v$BMD (mg/cm^3)	688.2 ± 12.5	668.3 ± 11.2	662.4 ± 11.2	727.8 ± 5.5	745.9 ± 4.4*	730.3 ± 4.6
Trabecular BMC (mg)	2.21 ± 0.15	2.29 ± 0.14	2.31 ± 0.14	1.74 ± 0.07	1.96 ± 0.06	2.01 ± 0.06*
Trabecular area (mm^2)	6.1 ± 0.4	6.4 ± 0.3	6.6 ± 0.3	5.3 ± 0.1	5.4 ± 0.1	5.6 ± 0.1
Trabecular BMD (mg/cm^3)	361.9 ± 12.4	360.3 ± 11.1	346.0 ± 11.1	336.3 ± 9.6	365.1 ± 8.5	361.2 ± 8.7
Cort/subcort BMC (mg)	8.9 ± 0.2	8.7 ± 0.2	8.3 ± 0.2	9.7 ± 0.2	10.5 ± 0.2*	10.6 ± 0.2*
Cort/subcort area (mm^2)	10.1 ± 0.3	10.0 ± 0.2	9.4 ± 0.2	10.5 ± 0.3	11.2 ± 0.2	11.4 ± 0.3
Cort/subcort $_v$BMD (mg/cm^3)	883.7 ± 11.9	865.5 ± 10.7	880.5 ± 10.7	938.3 ± 8.7	942.6 ± 7.9	914.0 ± 7.4
Periosteal Circumference (mm)	14.2 ± 0.2	14.4 ± 0.2	14.2 ± 0.2	14.0 ± 0.1	14.4 ± 0.1	14.6 ± 0.1*

Values are least-square means ± SE. All data are from the proximal tibial metaphysis 5mm distal to the proximal tibial plateau. There were no significant between group differences in baseline values.
*Significant difference from control values at day 26 ($p<0.05$).

The results of pQCT imaging of the midtibial diaphysis are presented in Table 2. Following the exercise period both exercise groups had significantly greater cortical BMC and cortical bone area than did the control group. The periosteal circumference also increased over the exercise period in both exercised groups, whereas it decreased in

control animals. Volumetric BMD was significantly greater at the end than at the beginning of the experiment in all three groups.

Table 2. *In vivo* pQCT values at the midtibial diaphysis

	Baseline			*Post-Exercise (Day26)*		
	Control	**S**quat	**T**ower	**C**ontrol	**S**quat	**T**ower
Cortical BMC (mg)	5.7 ± 0.1	5.6 ± 0.1	5.6 ± 0.1	6.2 ± 0.04	6.5 ± 0.04*	6.4 ± 0.04*
Cortical area (mm^2)	4.5 ± 0.1	4.4 ± 0.1	4.3 ± 0.1	4.6 ± 0.04	4.9 ± 0.03*	4.8 ± 0.04*
Cortical BMD (mg/cm^3)	1280.1 ± 7.4	1287.5 ± 6.6	1289.8 ± 6.6	1343.3 ± 7.2	1335.4 ± 6.2	1342.5 ± 6.2
Endosteal circumference (mm)	5.0 ± 0.1	5.1 ± 0.1	5.1 ± 0.1	4.7 ± 0.1	5.0 ± 0.1	4.9 ± 0.1
Periosteal circumference (mm)	9.0 ± 0.1	9.0 ± 0.1	9.0 ± 0.1	8.9 ± 0.1	9.3 ± 0.1*	9.2 ± 0.1*

Values are least square means ± standard error. All data are from the mid tibial diaphysis. There were no significant between group differences in baseline values. * Significant difference from post-exercise control value ($p<0.05$).

DXA imaging revealed no significant differences in BMD_a values between groups. Due to a malfunction of the densitometer the baseline soft tissue values were unusable, so only day 26 values were available for analysis. The control group mean body fat percentage on day 26 was 30.8 ± 1.8%, which was numerically (but not significantly) higher than that of either the squat exercise group (24.8 ± 1.6%, P=0.05) or the tower exercise group (25.7± 1.6%).

Fecal corticosterone. Mean daily fecal corticosterone excretion during the first 4 h of the dark cycle over each 4-day collection period is shown in Fig. 6. There were no significant differences between groups during any collection period, and there were no significant differences across collection periods in any group.

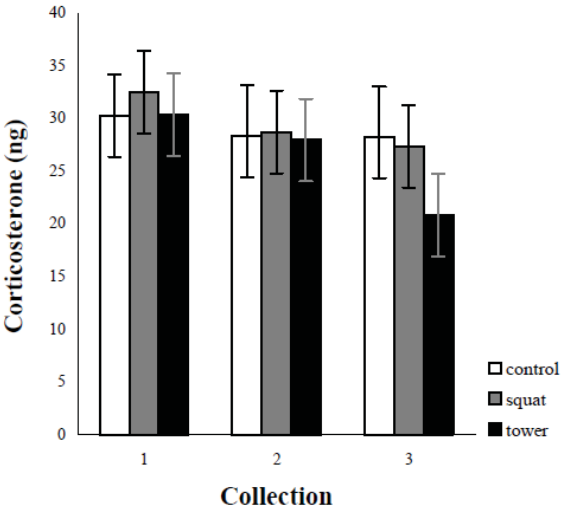

Figure 6. Mean total fecal corticosterone excreted during the first 4 hours of the dark period by each group during each 4 day collection period. Collection 1 = pre-exercise, collection 2 = day 6–9 of exercise, collection 3 = day 18–21 of exercise. No significant differences were found between the groups during any collection period.

Discussion

Weight gain and feed efficiency. Over the exercise period tower-climbing rats gained less weight than either control or squat exercise rats, which is expected given the increased opportunity for exercise available to the tower-climbing rats. The rats were observed to climb the towers frequently, often without drinking, and at times spent extended periods (up to 45 minutes observed) at the top of the tower clinging to the wire mesh. It is thus likely that the metabolic effects of exercise were greatest in the tower-climbing rats. This is also suggested by the rapid improvement in feed efficiency seen in tower-climbing rats over the exercise period. At the start of the exercise period the tower-climbing rats' very low feed efficiency is reflective of a sudden increase in activity level when they were first put into the tower cages. As they adjusted to exercise, their feed efficiency improved rapidly, until by day 15 it was equal to that of the control rats. After day 15, the weight gain per gram of feed intake of the tower-climbing rats exceeded that of the control animals. The less rapidly, but still greatly, improving feed efficiency of the squat exercise rats suggests a more moderate but still present metabolic effect of exercise in this group. Control rats experienced no change in feed efficiency over the study period.

Studies of exercise and feed efficiency in the rat have yielded varying results ranging from decreases in feed efficiency with exercise [42] to significant improvements in feed efficiency [43]. Interpretation of these studies is complicated by the use of varying types and amounts of exercise, strains and ages of rats, diets, and experimental conditions. It is possible that more strenuous exercise may decrease efficiency, while moderate exercise such as performed in our trial may increase it. A similar response has been seen in broiler chickens that were encouraged to exercise moderately through provision of ramps and toys; the improvement in feed efficiency

was sufficient for the authors to suggest that such interventions might be commercially worthwhile [44]. Certainly very moderate amounts of exercise have been shown to have profound effects on metabolism in the rat; intrauterine growth restriction (IUGR) in the rat results in obesity as the animals mature; however, when IUGR rats were allowed only 56 meters of treadmill running/day they did not become obese [9]. Such findings indicate that the metabolic effects of exercise are not limited to an increase in the number of calories expended.

Imaging parameters. The change in pQCT parameters at the proximal tibial metaphysis from baseline in exercised groups compared with controls reveals a significant effect of the exercise on bone modeling in both squat and tower rats. Both exercised groups had significantly greater day 26 total BMC than controls, but the distribution of the increased bone mineral varied with the exercise type. The tower group responded to exercise with a greater gain in bone area than the control group, resulting in bones of comparable density but greater cross-sectional area than those of control rats. Squat rats did not increase their proximal tibial metaphyseal bone cross-sectional area in response to exercise, but did gain total BMC and thus increased their total bone density. These differences in bone response to exercise may reflect the differing forces acting on the tibia caused by the two exercise forms, one of which involves only the hind limbs (squat exercise) while the other requires the rat to use all four limbs (tower climbing). All groups had a lower mean trabecular area (marrow area) at the day 26 scan (reflecting endocortical bone formation and thus a decrease in the marrow area).

At the level of the mid-tibial diaphysis the two exercised groups responded to exercise similarly with an increase in bone area, periosteal circumference, and BMC. There was no difference between the day 26 values for cortical bone mineral density

between exercised rats and controls; however, exercise resulted in rats with larger cortices of the same density as control rats and thus effectively stronger bones.

Several studies have demonstrated that exercise causes bone formation in the rat [19,45,46,47], and prevents bone loss in gonadectomized animals [18,25,34]. However, in most of these studies the animals exercised for longer periods of time than in our current study. Through the use of pQCT we have been able to demonstrate a significant bone response to a relatively short exercise exposure. This brief exercise period was selected because it approximates the length of pregnancy in the rat. A nonstressful exercise model that rapidly induces metabolic and bone responses will be essential for studying intergenerational effects of exercise during gestation.

Stress. One possible criticism of any animal exercise model is that exercise may cause stress to the animal, which may affect the outcome measures. Thus we chose voluntary exercises for our rats, and measured fecal corticosterone levels at three time points to determine if either exercise protocol induced stress in the rats. Fecal corticosterone levels have been shown to reflect response to a stressor in rats, with fecal levels peaking 7–9 hours after a plasma corticosterone peak [48]. By collecting feces over the first 4 h of the dark period we obtained samples at the approximate fecal corticosterone nadir, which is the time most likely to reveal a stress response [37,39,49]. Although plasma corticosterone levels may be more indicative of acute stress, measurement of fecal corticoids is well-suited to analysis of longer-term stressors (hours to days) as it quantifies a summated index of systemic corticoids proven to reflect stress responses [50,51]. Reporting total fecal corticoids over time, instead of fecal corticoid concentrations, provides a more accurate representation of systemic corticoid production by taking into account the amount of feces produced during the sampling time period, which varies over the light: dark cycle [38]. Corticosterone and

its metabolites enter the small intestine via the biliary system. The total amount of corticoids excreted into the feces reflects the plasma corticoid concentration and is independent of fecal mass; thus the measured concentration of corticoids in feces will vary with fecal output [38,52]. As even some forms of voluntary exercise may induce a stress response in rodents [31] it was important to determine whether the moderate-intensity voluntary exercises we selected caused a stress response in the exercised rats. The lack of significant differences suggests that the exercised animals experienced no greater stress than the unexercised controls.

Research into the interplay between the HPA axis and metabolism suggests that the relationship between these systems is established during the prenatal and early postnatal period [53,54,55,56], and maternal stress experienced by the fetus during gestation has proven developmental and metabolic effects [57,58,59,60]. Persistent stress also influences bone function, with chronic elevations of cortisol leading to low bone mineral in humans [29]. Although little work has been done to elucidate the early life factors affecting long term skeletal health, it has recently been proposed that osteoporosis risk may also be linked to interactions between the genotype and the environment during development [61]. The bone mass of an individual in later life is directly related to the peak bone mass attained by that individual during skeletal growth [62]; in humans birth weight influences future adult bone mass [61]. Pregnant rats fed a protein-restricted diet produce offspring with altered osteoblast activity [63], changes in bone structure and mineral density [64], and delayed skeletal maturity [65]. As the connections between the skeleton and metabolism become further clarified, it seems likely that the foundations of this chemical and mechanical interplay may also be established during early life.

Perspectives and Significance

A model of moderate exercise that does not cause stress is critical for investigations into exercise effects on the interrelationship between bone and metabolism. Recent research suggests that the basis of this relationship may be established in early life, even prior to birth. The results of this study demonstrate that both tower climbing and rising to an erect bipedal stance are suitable exercise models for examining the connection between skeletal and metabolic health, since both forms of exercise resulted in bone modeling without causing a physiological stress response. The differences in metabolic effect of the two exercises, as demonstrated by their varying effects on feed efficiency, may prove useful for future examination of the bone-metabolism relationship. Further study in pregnant animals is needed to confirm that these exercises do not cause stress under the different physiological conditions of gestation. If this proves to be the case, these exercise models will be suitable for examining the effects of exercise during gestation on the relationship between bone and energy balance in both mother and offspring and the effects of gestational exercise on the future metabolic and skeletal health of the next generation.

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Chapter 3

VOLUNTARY EXERCISE IN PREGNANT RATS POSITIVELY INFLUENCES FETAL GROWTH WITHOUT INITIATING A MATERNAL PHYSIOLOGICAL STRESS RESPONSE

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Abstract

The effects of increased physical activity during pregnancy on the health of the offspring in later life are unknown. Research in this field requires an animal model of exercise during pregnancy that is sufficiently strenuous to cause an effect but does not elicit a stress response. Previously, we demonstrated that two models of voluntary exercise in the nonpregnant rat, tower climbing and rising to an erect bipedal stance (squat), cause bone modeling without elevating the stress hormone corticosterone. In this study, these same models were applied to pregnant rats. Gravid Wistar rats were randomly divided into three groups: control, tower climbing, and squat exercise. The rats exercised throughout pregnancy and were killed at day 19. Maternal stress was assessed by fecal corticosterone measurement. Maternal bone and soft tissue responses to exercise were assessed by peripheral quantitative computed tomography and dual-energy X-ray absorptiometry. Maternal weight gain during the first 19 days of pregnancy was less in exercised than in nonexercised pregnant control rats. Fecal corticosterone levels did not differ between the three maternal groups. The fetuses responded to maternal exercise in a uterine position-dependent manner. Mid-uterine horn fetuses from the squat exercise group were heavier ($P<0.0001$) and longer ($P<0.0001$) and had a greater placental weight ($P=0.001$) than those from control rats. Fetuses from tower-climbing dams were longer ($P<0.0001$) and had heavier placentas ($P=0.01$) than those from control rats, but fetal weight did not differ from those of controls. These models of voluntary exercise in the rat may be useful for future studies of the effects of exercise during pregnancy on the developmental origins of health and disease.

Introduction

Events that occur during fetal development can have long-lasting effects on the health and later-life outcomes of the developing organism [1]. Low birth weight in humans is associated with an increased risk of later-life diseases, such as coronary heart disease, hypertension, and insulin resistance [2]. Exercise during pregnancy may significantly impact both birth weight and later-life health, but the effects of maternal exercise during gestation on fetal growth are unclear, and studies in humans and animals have yielded varying results. Studies in humans have found that exercise during pregnancy was associated with birth weight that was reduced [3,4,5], unaffected [6], or increased [7,8] in the offspring of exercising women. Comparison between studies is complicated by differing exercise regimens, and many used self-reported exercise, rather than a standardized, supervised exercise program. Timing during pregnancy, intensity, and type of the exercise may influence its effects on the fetus. For example, moderate weight-bearing (treadmill, stair stepper, or step aerobics) exercise begun in early pregnancy has been shown to increase fetoplacental growth and birth weight [8], whereas a high volume of the same exercises in late gestation reduced fetoplacental growth [9]. No data are available on the long-term effects of exercise during pregnancy on adult human offspring health outcomes.

Research in animals has been similarly difficult to interpret, with different types and amounts of exercise complicating comparison of results. For example, birth weight of pups from treadmill-exercised dams was lower than from nonexercised dams in some studies [10,11,12] and treadmill exercise had no effect on birth weight in others [13]. Birth weight of pups from pregnant rats that swam for 60 min/day was lower than [14], whereas birth weight of pups from pregnant rats that swam for only 10 min/day was not different from, that of pups from control animals [15]. In addition, exercise during

pregnancy may cause stress in experimental animals, providing a major confounder in the interpretation of results. Swimming [15,16] and treadmill running [16,17,18] increase plasma corticosterone levels in pregnant rats; and the effects of swimming-induced stress during pregnancy on birth weight persist through two subsequent generations [19].

Investigation of the effects of maternal exercise during pregnancy in the rat on later-life health of the pups requires a model of exercise that neither elicits a stress response nor negatively affects fetal growth. Tower climbing and rising to an erect bipedal stance (squat) are voluntary resistance-type exercises that have been shown to cause bone modeling in rats [20,21,22,23]. Both of these exercises require muscle contraction against gravity, although the dynamic and repeated muscular contractions of all four limbs during tower climbing may give it a greater aerobic component than the more isometric bipedal "squat" exercise, which requires the rats to attain a "standing" position on extended hind limbs and then maintain that position while they eat and drink. The "squat" exercise we describe is essentially the reverse of the squat exercise performed by body builders: for humans maintaining limb flexion while weight-bearing requires more muscular work than maintaining limb extension, for rats standing on extended hind limbs demands more effort than maintaining their normal, quadripedal, flexed-hindlimb posture. Additionally, neither exercise requires a noxious stimulus to the rats. This is in contrast to the treadmill running and swimming extensively described in pregnant rats [14,24,25,26]; these often involve noxious stimuli to the animals and are likely to require greater energy use than squat exercise or tower climbing. Previously, we demonstrated that voluntary tower climbing and squat exercise to obtain food are sufficiently strenuous to cause modeling of the tibia in nonpregnant rats without a concomitant rise in fecal corticosterone levels [23], a measure of stress in the

rat. In this study we used these same exercises in pregnant rats to test the hypothesis that neither tower climbing nor squat exercise would increase maternal fecal corticosterone levels or adversely affect fetal outcomes at day 19 of pregnancy. We concurrently tested the hypothesis that pregnant rats that performed either tower climbing or squat exercise would respond with greater bone changes than pregnant control animals.

Methods

Animals. Twenty-four female Wistar rats were habituated to their surroundings and to a low phytoestrogen control diet (AIN-93G, Research Diets) for ≥2 wk prior to commencing the exercise protocols. All rats were housed in the same room in a climate-controlled dedicated animal research facility with a 12:12-h light-dark cycle. Rats were bedded on kiln-dried wood shavings, and all were housed in pairs during the study period. Feed and water were provided *ad libitum* and initial and residual feed and initial and residual water volume were measured three times weekly throughout the exercise trial to monitor intake. Body weight was also measured three times weekly. Body length (from the occipital crest to the base of the tail: crown-rump length) was measured twice: once under anesthesia for imaging and again immediately following study termination. Maternal feed efficiency was calculated as the ratio of weight gain to feed intake on a per-cage (2 rats per cage) basis, as described previously [23]. The study protocol and all animal procedures were approved by the Massey University Animal Ethics Committee.

Exercise. After the habituation period, at 88–95 days of age, the rats were randomly assigned to one of three age- and weight-matched groups: control, squat exercise, and tower climbing. We have previously shown that these exercise models induce bone modeling in nonpregnant rats without initiating a physiological stress response [23].

Briefly, control rats were housed conventionally for the duration of the trial in a 419 mm x 279 mm x 152 mm high cage, with their food and water at a height of ~90 mm from the cage floor. Rats in the squat-exercise group were housed in a modified cage, the sides of which were gradually raised over a 5-day period; at full height, the rats had to obtain an erect bipedal stance in order to reach their food and water, which was ~220 mm above the cage floor. For the tower-climbing group, the cage lid was

replaced with a 2-m-high tower made of wire mesh (12 x 12 mm welded stainless steel, 1.6 mm) and the water bottle was gradually raised over a 5-day period to a final height of 2 m.

During their 5-day gradual introduction to exercise, the rats were observed daily to ensure they were able to reach the feed and water and that they were maintaining intake and body weight. After this orientation period, all rats were mated; their age at successful mating was 97 ± 4 days. On confirmation of mating by visualization of a plug, the rats were returned to their exercise cages until day 19 of pregnancy. The day the plug was visualized was designated day 0 of pregnancy.

Imaging. Peripheral quantitative computed tomography (pQCT) and dual-energy X-ray absorptiometry (DXA) were performed twice during the trial: at 4 days prior to beginning the orientation period (baseline) and following terminal sampling on day 19 of pregnancy. Prior to the initial imaging, the rats were anesthetized with a mixture consisting of 0.5 ml ketamine (100 mg/ml) + 0.2 ml acepromazine (2 mg/ml) + 0.1 ml xylazine (100 mg/ml) + 0.2 ml sterile water injected intraperitoneally at a dose rate of 0.06 ml/100 g via a 25-gauge needle. A plane of anesthesia suitable for noninvasive imaging procedures was obtained within 5–10 minutes and was maintained for ~1 h.

pQCT (XCT2000 pQCT scanner, Stratec, Pforzheim, Germany) of the right tibia of each rat was performed as described previously [23]. Scans were made 5 mm distal to the tibial plateau (proximal metaphysis) and at 50% of tibial length (middiaphysis) [27], with a voxel size of 0.1 mm and scan speed of 5 mm/sec. Scans were analyzed using the manufacturer's software. Metaphyseal bone was analyzed by contour mode 3, peel mode 2, with an outer threshold of 214 g/cm^3 and an inner threshold of 606 g/cm^3, and diaphyseal bone was analyzed using cortmode 1 with a threshold of 710 g/cm^3. The coefficient of variation (CV) for total density ranged from 0.48 to 1.47% at the midtibial

diaphysis and from 1.32 to 2.23% at the proximal tibial metaphysis. The CV for total area ranged from 1.47 to 1.72% at the midtibial diaphysis and from 3.73 to 7.11% at the proximal tibial metaphysis without and with repositioning between scans.

Bone mineral content (BMC), areal bone mineral density (BMD_a), and whole body composition data were determined with a fan beam densitometer (Hologic Discovery A, Bedford, MA, USA) using the small animal application, as described previously [23]. Prior to imaging at day 19 of pregnancy, all rats were killed and their uteri, left fore and hind limbs and brain were removed. Whole body scans were analyzed, with the skull and associated tissues excluded from the region of interest. Quality control scans were performed daily to ensure that the precision met the required DXA manufacturer's CV, which was 0.98–1.01%. High resolution scans of the femurs had a CV of 0.60% and 1.20% without and with repositioning between scans.

Fecal sample collection and corticosterone measurement. To measure the stress response to voluntary exercise, fecal samples were collected from all groups during three periods: before exercise, during the exercise introduction period (immediately before mating), and during the exercise period (days 10–16 of pregnancy). Feces were collected as described previously [23]. Samples were freeze-dried and ground, and ethanol extraction was performed by the nonboiling method [28] with the following slight modifications. The dried ethanol extracts were reconstituted in 2 ml of 0.1M phosphate-buffered saline with 0.1% gelatin, pH 7.0 (PBSG), and the final supernatant was used without further dilution. The recovery of corticosterone following extraction was measured as previously described [28]. The mean recovery of corticosterone from spiked control samples was 76.6 $\pm$ 0.7% (n= 20). The CV for the mean percent recovery was low (4.3%), and the mean percent recovery was used to calculate results for all the samples. All samples were assayed in duplicate by a

commercially available RIA kit (double-antibody corticosterone ^{125}I-RIA kit for rats and mice, MP Biomedicals), and radiation counts were determined in a gamma counter (1261 Multigamma, LKB Wallac) for 5 min each. All the sample and reagent volumes used in the assay were 1/10th those of the kit protocol. Following addition of the precipitant and vortexing, 20 µl of starch (25 g/l starch (Sigma) + 0.05 g/l neutral red (BDH) in PBSG) were added to increase adhesion of the pellet to the tube. The intra-assay CV for corticosterone were 8.4, 6.0, and 7.2% and interassay CV for corticosterone were 7.8, 8.4, and 11.5% for low-, medium-, and high-concentration solutions, respectively. Fecal samples were weighed after drying and before extraction. Corticosterone is expressed as total nanograms excreted over the collection period, calculated as the fecal corticosterone concentration (ng/g) multiplied by the weight of the fecal sample.

Sample collection. On day 19 of pregnancy, the rats were killed by cardiac puncture and terminal blood draw while under anesthesia. The uterus and its contents were removed immediately using a standardized procedure as follows: a ventral midline incision was made, and the proper ligaments of both ovaries were transected; the mesometrium was dissected so that all fat remained within the abdomen; the cervix was then transected, and the gravid uterus removed from the abdomen and weighed. The fetuses were then removed from the uterus, weighed, and measured, their sex was determined, and their position within the uterus was recorded. The discoid placentas were freed of amniotic membranes and umbilical cord and then weighed. The brain and liver were removed from three male and three female mid-uterine horn fetuses per litter, and the individual weights of these organs were obtained; if three fetuses of each gender were not available from the mid-uterine horn region, then two were used. Fetal sex was initially determined by assessment of ano-genital distance [29] and, when questionable,

confirmed by visualization of the gonad under a dissecting microscope after sample collection. The single fetus closest to the cervix was classified as having the position adjacent to the cervix, the single fetus nearest the ovary was classified as located at the ovarian end of the horn, all other fetuses were considered to be in a mid-uterine horn position. Prior to imaging, the left fore- and hindlimbs of the dams were removed at the shoulder and hip, respectively, and the bones were harvested and stored.

Statistical Analysis. All statistical analysis was performed with SAS 9.1 using proc GLM and Tukey-Kramer post hoc analysis. Significance of differences in fetal outcome parameters was determined using a general linear model with exercise group, fetal sex, dam nested within exercise group, and position of the fetus within the uterus as fixed effects. The interaction between exercise group and position of the fetus within the uterus was also included in the model. Fecal corticosterone was assessed using repeated-measures ANOVA, with the effects exercise group, cage nested within exercise group, collection period, and the interaction of exercise group and collection period. To achieve normal distribution the fecal corticosterone data were logarithmically transformed prior to analysis. Baseline differences in imaging parameters were assessed by simple ANOVA. Day 19 values for imaging parameters were analyzed using covariate analysis, with exercise group, baseline parameter value, and total weight of the fetuses carried by the dam as covariates. All data are expressed as least-square means ± SE unless otherwise indicated. Differences are considered significant at the $P \leq 0.05$.

Results

Animals. All rats successfully performed the exercise required by their group without injury or compromised health. Two animals (1 control and 1 squat exercise) failed to become pregnant and were excluded from the analysis. The 22 gravid dams (control n=7, squat n=7, tower climbing n=8) collectively contained 294 live fetuses. Mean litter size was 13.3 fetuses per litter, and there were no significant between-group differences in the number of fetuses per litter, the male: female ratio, or the number of resorptions at day 19 of pregnancy.

There were no significant differences in feed intake between groups, and rats in all groups gained weight throughout pregnancy. However, rats in the exercised groups gained significantly less weight (excluding the weight of the uterus and its contents) than rats in the control group (Fig. 1). Maternal crown-rump length at day 19 of pregnancy did not significantly differ between groups (17.40 ± 0.39, 16.57 ± 0.33, and 16.86 ± 0.31 cm for the control, squat-exercise, and tower-climbing groups, respectively). Mean overall feed efficiencies [23] for the control, squat-exercise, and tower-climbing groups over the pregnancy period did not significantly differ at 0.27 ± 0.02, 0.24 ± 0.02, and 0.25 ± 0.01 g body wt gained per gram of feed intake, respectively.

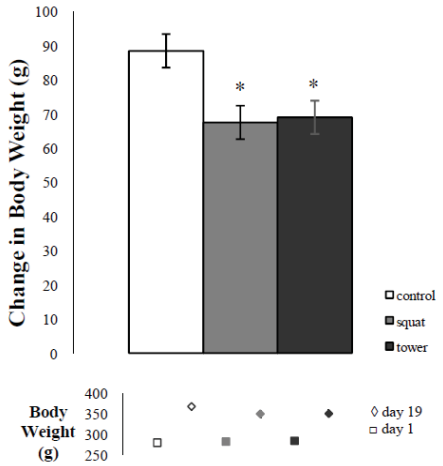

Figure 1. Change in dam body weight over 19 days of the pregnancy (excluding the weight of the uterus and its contents). *Significantly different from control (P≤0.05).

Imaging. pQCT of the right proximal tibial metaphysis and midtibial diaphysis of the dams revealed no significant differences in any parameters between groups before, or at completion of, the exercise period (Tables 1 and 2).

Table 1. *In vivo* pQCT values at the right proximal tibial metaphysis

	Baseline			*Post-Exercise (Day 19 of pregnancy)*		
	Control	**S**quat	**T**ower	**C**ontrol	**S**quat	**T**ower
Total BMC (mg)	9.48 ± 0.30	9.31 ± 0.30	9.41 ± 0.28	10.80 ± 0.18	11.38 ± 0.21	10.93 ± 0.19
Total area (mm^2)	13.91 ± 0.51	13.86 ± 0.51	14.04 ± 0.48	14.66 ± 0.28	14.95 ± 0.33	14.68 ± 0.31
Total BMD_v (mg/cm^3)	682.80 ± 18.38	673.51 ± 18.38	673.25 ± 17.19	738.99 ± 8.53	760.09 ± 9.29	747.56 ± 8.78
Trabecular BMC (mg)	1.67 ± 0.13	1.71 ± 0.13	1.79 ± 0.12	1.66 ± 0.07	1.72 ± 0.08	1.52 ± 0.08
Trabecular area (mm^2)	5.52 ± 0.36	5.65 ± 0.36	5.66 ± 0.34	5.38 ± 0.14	5.22 ± 0.16	5.09 ± 0.15
Trabecular BMD_v (mg/cm^3)	300.16 ± 18.61	302.47± 18.61	321.25 ± 17.41	315.26 ± 8.89	340.93 ± 9.76	293.13 ± 9.30
Cort/subcort BMC (mg)	6.67 ± 0.19	6.49 ± 0.19	6.43 ± 0.17	8.02 ± 0.14	8.39 ± 0.15	8.36 ± 0.14
Cort/subcort area (mm^2)	8.39 ± 0.29	8.21 ± 0.29	8.38 ± 0.28	9.30 ± 0.20	9.79 ± 0.22	9.52 ± 0.20
Cort/subcort BMD_v (mg/cm^3)	933.99 ± 16.76	929.04 ± 16.76	910.08 ± 15.68	987.14 ± 11.34	983.94 ± 12.41	991.28 ± 11.95
Periosteal Circumference (mm)	13.20 ± 0.24	13.19 ± 0.24	13.27 ± 0.23	13.57 ± 0.13	13.70 ± 0.15	13.57 ± 0.14

Values are least-square means ± SE (n = 7 control, 7 squat, and 8 tower). All data are from the right proximal tibial metaphysis 5 mm distal to the proximal tibial plateau. pQCT, peripheral quantitative computed tomography; BMC, bone mineral content; BMD_v, volumetric bone mineral density; cort/subcort, cortical/subcortical. There were no significant between-group differences in baseline or day 19 values.

Table 2. *In vivo* pQCT values at the right midtibial diaphysis

	Baseline			*Post-Exercise (Day19 of pregnancy)*		
	Control	**S**quat	**T**ower	**C**ontrol	**S**quat	**T**ower
Cortical BMC (mg)	5.62 ± 0.10	5.42 ± 0.10	5.41 ± 0.09	6.33 ± 0.07	6.41 ± 0.07	6.31 ± 0.06
Cortical area (mm^2)	4.46 ± 0.07	4.29 ± 0.07	4.29 ± 0.07	4.80 ± 0.05	4.89 ± 0.05	4.80 ± 0.05
Cortical BMD_v (mg/cm^3)	1262.34 ± 10.26	1262.76 ± 10.26	1261.44 ± 9.59	1323.74 ± 8.12	1311.50 ± 8.87	1310.98 ± 8.31
Endosteal circumference (mm)	4.99 ± 0.14	5.00 ± 0.14	5.02 ± 0.13	4.78 ± 0.09	4.93 ± 0.10	4.83 ± 0.09
Periosteal circumference (mm)	9.0 ± 0.12	8.89 ± 0.12	8.90 ± 0.11	9.12 ± 0.06	9.26 ± 0.06	9.14 ± 0.06

Values are least-square means ± SE (n = 7 control, 7 squat, and 8 tower). All data are from the right midtibial diaphysis. There were no significant between-group differences in baseline or day 19 values.

The results of whole body DXA imaging, excluding the skull and associated tissues, are shown in Table 3. There were no prepregnancy differences in body composition. All animals gained fat, increasing their body fat percentage and decreasing

their percent lean mass, over the trial period. Total lean mass was significantly lower in squat-exercise than control rats on day 19 of pregnancy; however, percent lean mass of these two groups did not differ. Whole body BMC was significantly lower in both exercised groups than in controls (P=0.02–0.03), and bone area was significantly lower in the tower-climbing group than in the control group (P=0.02), with a similar trend (P=0.06) in the squat-exercise group. However, when expressed per gram of body weight, these differences were no longer significant.

Table 3. Dam whole body (excluding the skull and associated tissues) DXA imaging results.

	Baseline (Non-Pregnant)			*Post-Exercise (Day 19 of Pregnancy)*		
	Control	**S**quat	**T**ower	**C**ontrol	**S**quat	**T**ower
Lean mass (g)	189.05 ± 3.15	182.72 ± 3.15	187.50 ± 3.15	192.70 ± 2.76	178.12 ± 2.95*	190.75 ± 3.18
Fat mass (g)	62.76 ± 5.96	72.51 ± 5.96	68.81 ± 5.96	105.13 ± 5.20	100.50 ± 5.51	90.87 ± 5.54
Percent lean (%)	73.12 ± 1.66	69.83 ± 1.66	71.34 ± 1.66	63.41 ± 1.33	62.36 ± 1.37	65.08 ± 1.53
Percent fat (%)	24.07 ± 1.70	27.39 ± 1.70	25.89 ± 1.70	34.09 ± 1.44	34.90 ± 1.52	30.93 ± 1.52
BMC (g)	7.20 ± 0.20	7.26 ± 0.20	7.24 ± 0.19	7.69 ± 0.11	7.24 ± 0.11*	7.14 ± 0.11*
BMC/BW	0.03 ± 0.0008	0.03 ± 0.0008	0.03 ± 0.0008	0.019 ± 0.0004	0.018 ± 0.0004	0.018 ± 0.0003
Bone Area (cm^2)	50.54 ± 1.15	51.39 ± 1.15	51.59 ± 1.07	51.46 ± 0.72	48.88 ± 0.73	48.41 ± 0.72*
Bone Area /BW	0.18 ± 0.005	0.18 ± 0.005	0.18 ± 0.005	0.13 ± 0.002	0.13 ± 0.002	0.12 ± 0.002
log BMD_a (g/cm^2)	-0.85 ± 0.005	-0.85 ± 0.005	-0.85 ± 0.005	-0.83 ± 0.005	-0.83 ± 0.005	-0.83 ± 0.005

Data are least-square means ± SE. Prior to scan at day 19 dams were euthanized and their uteri and left fore- and hindlimbs were removed. DXA, dual-energy absorptiometry; BW, live weight on the day of scanning. *Significant difference from control day 19 value (P≤0.05).

The results of DXA imaging of the first four lumbar vertebrae (L_1-L_4) are shown in Table 4. There were no preexercise/prepregnancy differences in BMC, bone area, or BMD_a. The bone area of L_1-L_4 was significantly lower in squat-exercise rats at day 19 of pregnancy than in control or tower-climbing animals. There was a trend toward a lower BMC of L_1-L_4 at day 19 in squat-exercise than control rats (P=0.06). As with whole body BMC and bone area, when expressed per gram of body weight, these

differences were no longer significant. There were no between-group differences in BMD_a following the pregnancy/exercise period.

Table 4. DXA imaging results of the first four lumbar vertebrae of the dams.

	Baseline (Non-Pregnant)			*Post-Exercise (Day 19 of Pregnancy)*		
	Control	**S**quat	**T**ower	**C**ontrol	**S**quat	**T**ower
BMC (g)	0.43 ± 0.01	0.45 ± 0.01	0.42 ± 0.01	0.52 ± 0.01	0.47 ± 0.01	0.51 ± 0.01
BMC/BW	0.0014 ± 0.00006	0.0016 ± 0.00006	0.0015 ± 0.00005	0.0013 ± 0.00004	0.0012 ± 0.00004	0.0013 ± 0.00004
Area (cm^2)	1.89 ± 0.04	1.95 ± 0.04	1.84 ± 0.04	2.11 ± 0.04	1.98 ± 0.04*	2.10 ± 0.04
Bone Area/BW	0.007 ± 0.0002	0.007 ± 0.0002	0.007 ± 0.0002	0.005 ± 0.0001	0.005 ± 0.0001	0.005 ± 0.0001
BMD_a (g/cm^2)	0.23 ± 0.004	0.23 ± 0.004	0.23 ± 0.004	0.25 ± 0.003	0.24 ± 0.003	0.24 ± 0.003

Values are least-square means ± SE (n = 7 control, 7 squat, 8 tower). * Significant difference from control day 19 value ($P \leq 0.05$).

Regional scans of the right hind limb did not reveal any significant differences between groups in bone or soft tissue parameters.

Fecal corticosterone. Mean daily fecal corticosterone excretion on a per-cage basis (2 rats/cage) during the first 4 h of the dark cycle over each 4-day collection period is shown in Fig. 2. There were no significant differences between groups during any collection period, and there were no significant differences across collection periods in any group. When data from all groups were pooled, fecal corticosterone was significantly lower during midpregnancy than during the preexercise or the exercise adjustment period.

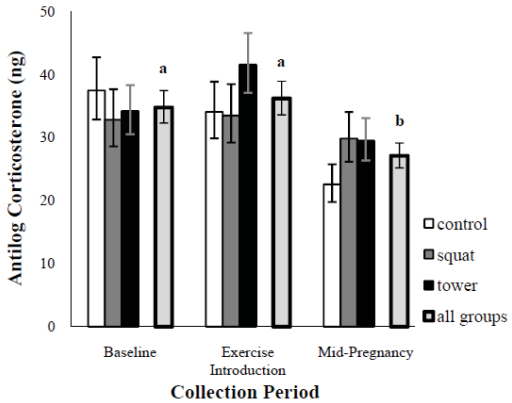

Figure 2. Mean total fecal corticosterone excretion over the first 4 h of the dark period for each exercise group on a per-cage basis (2 rats/cage) and for all groups combined during each 4-day collection period. Data are presented as antilogarithm of least-square means to facilitate comparison of corticosterone output between studies. The baseline and exercise introduction collection periods are prior to mating. There were no significant differences between groups at any time point or between time points for any exercise group; however, when data for all groups were combined, fecal corticosterone was significantly less at midpregnancy less than at baseline or during exercise introduction. Different letters (a, b) indicate a significant difference in fecal corticosterone ($P \leq 0.05$).

Fetal outcomes. Fetal weight and length and placental weight are shown in Table 5. Fetuses of squat-exercise rats were heavier than those of the other groups (effect of exercise group on fetal weight, $P = 0.05$); however, the interaction between exercise group and position of the fetus within the uterus significantly affected fetal weight (effect of position on fetal weight, $P < 0.0001$; effect of the interaction between exercise group and position, $P = 0.03$), with fetuses at the ovarian ends of the uterine horns failing to show a difference with exercise. The effect of exercise on fetal crown-rump length was also related to the intra-uterine position of the fetus, with mid-uterine horn fetuses from both exercise groups being longer than those from control rats ($P = <0.0001$). Similarly, placentas of mid-uterine horn fetuses from both exercise groups were heavier than those from control rats ($P = 0.001$ and $P = 0.01$ for squat-exercise and tower-climbing groups, respectively). Fetuses located at the cervical end of the uterine horns in the squat-exercise dams were also heavier than control fetuses ($P = 0.04$). When data from all groups were combined, fetuses at the cervical end of the horns were significantly heavier than those in the mid-uterine horn region, which were in turn, significantly heavier than those at the ovarian end of the uterine horns (1.50 ± 0.03, 1.45 ± 0.02, and 1.39 ± 0.03 g body wt for cervical end, mid-horn, and ovarian end fetuses, respectively; $P < 0.0001$–0.01). Fetal brain and liver weights and their ratio did not differ between groups. The single dead fetus (in a control dam) was not included in the weight, length and placental weight calculations. Two dams had a combined total of three fetal resorption sites in the control and squat-exercise groups, while three tower-climbing dams had a total of four resorption sites.

Table 5. Fetal weight and length and placental weight by exercise group and position within the uterus.

		Position of fetus within uterus		
		Adjacent to cervix	*Mid-uterine horn*	*Ovarian end of horn*
Fetal Weight (g)	**Control**	1.48 ± 0.03	1.40 ± 0.03	1.36 ± 0.03
	Squat	1.60 ± 0.03*	1.51 ± 0.02*	1.42 ± 0.03
	Tower	1.42 ± 0.03	1.43 ± 0.02	1.40 ± 0.03
Fetal Length (mm)	**Control**	26.38 ± 0.41	25.27 ± 0.20	25.53 ± 0.40
	Squat	27.90 ± 0.42	27.61 ± 0.20*	26.75 ± 0.40
	Tower	26.47 ± 0.46	26.85 ± 0.23*	26.78 ± 0.44
Placental Weight (g)	**Control**	0.29 ± 0.014	0.30 ± 0.010	0.28 ± 0.014
	Squat	0.34 ± 0.014	0.32 ± 0.010*	0.32 ± 0.014
	Tower	0.31 ± 0.014	0.32 ± 0.009*	0.31 ± 0.014

Values are least-square means ± SE (n = 7 control, 7 squat, and 8 tower dams). * Significant difference from control at same uterine position ($P \leq 0.05$).

Discussion

The purpose of this study was to test the hypothesis that, in rats, voluntary exercise during pregnancy (tower climbing and squat exercise) would not adversely affect fetal outcomes or cause a systemic maternal stress response and that these exercises would alter bone parameters in the exercised dams relative to nonexercised pregnant controls. Our results indicate that there were no adverse effects of either exercise type on fetal outcomes and that neither exercise resulted in elevated maternal fecal corticosterone levels. However, neither exercised group demonstrated the bone changes relative to controls that we anticipated.

Both exercises enhanced fetal growth, but in slightly different ways. Squat exercise increased weight and length of the fetuses and placental weight; tower climbing increased fetal length and placental weight, but not fetal weight. These differences may be due to the different nature of the exercises. Squat exercise is a resistance exercise that requires extended muscle contractions against gravity as the rats stand on their hind limbs to eat and drink. Tower climbing involves more dynamic movement and, likely, a greater aerobic component. Enhancement of offspring growth with exercise during pregnancy has also been reported in humans; offspring of women who began a program of moderate weight-bearing exercise in early gestation were heavier and longer than offspring of sedentary controls [8]. This improved fetal growth was attributed to enhanced placental function, resulting from adaptation to the transient decreases in placental oxygen and nutrient delivery that occur during exercise [30]. Although placental function was not specifically assessed in this study, the larger placental mass of the fetuses of exercised dams suggests that a similar mechanism might be present in rodents.

The position of the fetus within the uterus modified the exercise effects on fetal outcomes. Response to maternal exercise was observed predominantly in fetuses located in the mid-uterine horn region. This may be due to regional differences in fetoplacental blood flow and concomitant regional differences in blood flow alteration with exercise. In rats, fetuses at the ovarian ends of the uterine horns are lighter than those in the mid-horn region [31]. However, the smaller mass of fetuses located at the ovarian ends of the uterine horns does not correlate with decreased blood flow; fetuses in the mid-uterine horn region receive less blood flow than fetuses at either the ovarian or cervical ends of the horns. This difference in blood flow is most pronounced at day 15 of pregnancy, and blood flow becomes more similar between regions of the uterus by day 21 (during the period of rapid fetal growth) [32]. Placental blood flow in the rat is constrained by the necessity of maintaining intraplacenta pressure at a level low enough to avoid compression of the labyrinthine vessels of the placenta [33]. Fetuses receiving less blood flow under control conditions (the fetuses in the mid-uterine horn) may experience a greater adaptive increase in blood flow with maternal exercise. Acute strenuous exercise causes a reduction in placental blood flow in rats [34]; but regular exercise may lead to enhanced placental blood flow at rest and, thus, an increase in overall delivery of oxygen and nutrients to the fetus [30]. This would suggest that the different responses to exercise of the fetuses from the squat-exercise and tower-climbing dams may reflect different effects on blood flow of the two exercises.

The lower maternal weight gain over pregnancy in both exercised groups is unsurprising: in humans and rats, exercise has been shown to reduce weight gained by the exercising mother [35,36]. However, the lesser weight gain of rats in the exercised groups did not reflect a lower fat gain as might be expected. Instead, whole body DXA imaging revealed lower lean mass in the squat-exercise group than controls, a lower

BMC in both exercised groups, and less bone area in the tower-climbing group than control animals. When expressed per gram of body weight, these differences in BMC and bone area became nonsignificant, suggesting that the exercised rats had appropriate BMC and bone area for their body weight, which was less than that of the control rats. Rats at 100 days of age are sexually mature but still growing in size; these results suggest that the exercised young adult rats grew less than control rats over the course of their pregnancies. These findings are similar to those of Mottola *et al*. (1983), who found that pregnant rats that performed exercise weighed less, even when all skin and subcutaneous fat had been removed [35]. Thus perhaps placental adaptations to exercise result in improved nutrient delivery to the fetuses at the expense of maternal growth.

We anticipated that squat exercise and tower climbing would cause modeling of the tibia of pregnant rats, as we previously reported in nonpregnant animals [23], but this did not occur. Analysis of pQCT results showed no differences in BMC, bone area, or volumetric bone mineral density between control and exercised groups at the proximal tibial metaphysis or the midtibial diaphysis on day 19 of pregnancy. The lack of a tibial bone response to exercise in these pregnant animals suggests that the physiological state of pregnancy has superceded the bone's response to the external forces imposed by the exercises we utilized. Rats store calcium during early pregnancy in preparation for the calcium demands of late gestation and lactation [37]. In late pregnancy, as the maternal bone meets the increased fetal demand for calcium, maternal bone formation and resorption vary in a site-specific manner. At the end of pregnancy, bone formation decreases in the maternal lumbar vertebral bodies, but periosteal bone formation increases in the maternal femur [38]. It may be that the enhancement of periosteal bone formation in the appendicular skeleton that occurs with pregnancy overwhelmed any increase that would have resulted from the physical strains of our

moderate exercises. Women normally lose bone mineral during pregnancy [39]; however, there are few published data on the effects of exercise during pregnancy on bone parameters in humans. One study examined BMD_a (DXA) and bone stiffness index as measured by ultrasound at 12 wk gestation and several weeks postpartum in women who played tennis during pregnancy compared with sedentary pregnant controls. The researchers found that tennis attenuated the loss in bone stiffness index that occurred over the pregnancy and early lactation period, but not the loss in BMD_a [40].

Fecal corticosterone was assessed to determine if tower climbing or squat exercise would initiate a stress response in pregnant rats. Although tower climbing and squat exercise have been shown to be nonstressful in nonpregnant animals [23], we decided to investigate the stress response to these exercises in rats in the very different physiological state of pregnancy. Fecal corticosterone levels have been shown to reflect a stress response in rats, with fecal levels peaking 7–9 hours after a plasma corticosterone peak [41]. Measurement of fecal corticoids is well suited to the analysis of chronic stressors, as it provides a summated index of systemic corticoid production [42,43]. We chose to assess fecal corticosterone over the first four hours of the dark period, at the approximate nadir of its circadian cycle, as this is the time most likely to reflect a stress response [44,45]. Reporting total fecal corticoids over time, rather than fecal corticoid concentration, provides a more accurate representation of systemic corticoid production by taking into account the amount of feces produced during the sampling time period [46].

There were no differences in fecal corticosterone between exercise groups at any time point, indicating that neither exercise caused a stress response sufficient to chronically elevate corticosterone to levels greater than those of pregnant control

animals. This is unsurprising, given the voluntary nature of the exercise and its incorporation into the daily routine of the rats (the exercises were performed while the rats were accessing food and water). Humans also can perform certain moderate exercises during pregnancy without an increase in stress hormone levels. In pregnant women, plasma cortisol was elevated immediately after 40 minutes of treadmill walking but not after 40 minutes of aerobic dance [47]. A study in men found that subjects needed to exceed ~60% of their maximal O_2 consumption to induce an acute rise in cortisol levels [48]; although care must be taken when extrapolating data from human to rodent studies, it is unlikely that either tower climbing or squat exercise would have utilized a significant portion of the rats' aerobic capacity.

When data from all groups were pooled, fecal corticosterone output during pregnancy (days 10–16) was significantly lower than prior to pregnancy. Similar reductions of plasma corticosterone concentration have been shown in early pregnancy and midpregnancy in rats, with corticosterone decreasing sharply in early pregnancy and then slowly increasing from day 10 until they are significantly greater than nonpregnant levels by day 22 of pregnancy [49]. We chose to assess corticosterone in our pregnant rats in midgestation, when corticosterone levels are least in flux; however, it is important to note that our experimental design may have caused us to miss between-group differences in corticosterone output in early or late pregnancy. The reduction in corticosterone that occurs in midpregnancy in rats differs from the situation in humans, in which pregnancy is a period of "normal" hypercortisolemia, with blood cortisol concentrations rising gradually throughout pregnancy to a peak in the third trimester [50]. However, regardless of differences in normal levels of stress hormones during pregnancy, chronic maternal stress has been shown to have long-term deleterious effects on human and rat offspring [51].

Events that occur during fetal development can have long-lasting effects on the health and later-life outcomes of the developing organism [1]. Few studies have examined the effects of maternal exercise during pregnancy on the offspring beyond the neonatal period. To the authors' knowledge, no study has examined the long-term effects of nonstressful exercise in pregnant rats on offspring health. In one study, offspring of women who exercised throughout pregnancy were lighter at birth than those of controls and were leaner, but not shorter, than those of controls at 5 years of age [52]. This suggests that physiological changes induced by exercise during pregnancy can persist into childhood, but further study is needed to examine the effects of maternal exercise during gestation on the later-life health of the offspring.

Perspectives and Significance

Exercise during pregnancy may have long-lasting effects on offspring health. Investigation of this issue is complicated by the effects of exercise-induced stress on fetal development. The results of this study demonstrate that tower climbing and rising to an erect bipedal stance may be suitable for examining the effects of exercise during pregnancy on the future health of the offspring in the rat. Both exercises enhance fetoplacental growth without causing a concomitant rise in corticosterone output. Our observation that the maternal exercise effects on fetal growth at day 19 of gestation are dependent upon position of the fetus within the uterus suggests that the selection of the appropriate offspring for study of long-term effects may be important. We found that fetuses in the mid-uterine horn region were most likely to respond to maternal exercise with enhanced growth. Fetuses in the mid-uterine horn region were closer to the mean fetal weight of the litter than fetuses at either the cervical or ovarian end of the uterine horns, which suggests that offspring of average weight should be selected for later-life evaluation. The different bone response to exercise of the pregnant animals in this study

compared with the increased bone modeling in response to exercise of nonpregnant animals reported previously [23] also warrants further investigation.

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Chapter 4

VOLUNTARY EXERCISE IN PREGNANT RATS IMPROVES POST-LACTATION MATERNAL BONE PARAMETERS BUT DOES NOT AFFECT OFFSPRING OUTCOMES IN EARLY LIFE

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Abstract

Objectives: The objectives of this study were to examine the effects of voluntary exercise during pregnancy on maternal post-lactation bone parameters and offspring growth. **Methods:** Pregnant Wistar rats were housed in conventional cages (control), or were housed in raised cages requiring them to rise to an erect, bipedal stance to obtain food/water, throughout pregnancy. Dual energy X-ray absorptiometry and peripheral quantitative computed tomography scans were performed pre-mating and post-weaning. Maternal stress was assessed by fecal corticosterone measurement. Offspring weights were assessed at postnatal days 1 and 25 (weaning). **Results:** Changes in bone mineral over the pregnancy/lactation period were site-specific. Exercise did not affect loss of bone mineral from the lumbar spine, but did attenuate the loss of trabecular bone mineral from the tibial metaphysis and enhance the strength strain index and cross-sectional moment of inertia at the tibial diaphysis ($P \leq 0.05$) in dams in the exercised group. Fecal corticosterone did not differ between dam groups. There were no significant differences in offspring weight between the exercised and control group at either time point. **Conclusions:** Voluntary exercise in the pregnant rat can improve some post-lactation bone parameters and does not adversely affect early postnatal outcomes of the offspring.

Introduction

Exercise during pregnancy may affect both the mother and offspring [1,2]. In mothers, exercise reduces the risk of pregnancy-associated disorders such as gestational diabetes and pre-eclampsia [1], and may have effects on the maternal skeleton. Pregnancy and lactation are times of increased bone turnover in both humans and rodents [3]. The fetal demand for calcium in late pregnancy and the postnatal demand for calcium during lactation are met primarily from the trabecular rather than the cortical component of the skeleton [4,5,6]. This loss of maternal bone mineral is usually recovered after lactation has ended, but low bone mass in the puerperal period may be predictive of later osteoporosis risk in women [7]. It is unclear how maternal loss of bone mineral during pregnancy and lactation may affect attainment of peak bone mass and subsequent risk of osteoporosis in later life.

Mechanical loading increases bone mineral in a strain-dependent and site-specific manner [8,9,10]. Thus exercise during pregnancy may have the potential to attenuate loss of maternal bone mineral over the pregnancy and lactation period, but little work has been done to examine this. A study of women who played tennis during pregnancy showed that exercise eliminated the loss in an ultrasound-assessed bone stiffness index that occurred at the calcaneus during the pregnancy and early lactation period, but not the decrease in bone mineral density measured by dual energy X-ray absorptiometry (DXA) [11]. When examining the effects of exercise on bone it is very important to control for effects of stress (mediated by the hypothalamic-pituitary-adrenal axis) as this can influence osteoblast activity and bone formation [12]. We previously demonstrated that voluntary exercise causes bone modeling in non-pregnant female rats without elevating the stress hormone corticosterone [13]. When we applied those same exercises to pregnant rats they still did not induce a stress response.

However, there were no differences between exercised and control rats in any of the assessed parameters of bone mineral or area at day 19 of gestation when expressed per gram of body weight [14]. Pregnant rats store calcium in early pregnancy in preparation for the high calcium demands of the pups during late gestation and lactation [3]. The bone mineral increases associated with the physiological state of pregnancy through day 19 appeared to supersede the effects of our mild, voluntary exercise regime. In this study, we examined whether effects of exercise during pregnancy would be evident at the end of the lactation period, or whether the increased calcium demands in late pregnancy and lactation [15] would also mask the effects of exercise on bone.

Exercise during pregnancy can affect the offspring, but comparison of studies is complicated by differing types, intensities, and timing of exercise interventions. The focus of recent work has been on birth weight. Studies in animals and humans have yielded varied results including increases [16] and decreases [17,18,19,20], as well as no change [21,22], in birth weight following maternal exercise during pregnancy. A recent review of the work in humans suggests that the long-term impact of pregnancy exercise on the offspring is likely to vary with maternal fitness, health and diet, as well as type and timing of the exercise during pregnancy [23]. Our previous work showed that voluntary exercise during pregnancy in rats increased fetal growth to day 19 of pregnancy, with the fetuses of exercised dams being longer and heavier than those of controls [14]. As birth weight is considered a reflection of the intrauterine environment, these results may indicate that voluntary exercise during pregnancy enhanced the intrauterine environment for the offspring; this may have long-term health effects in later life.

In this study we utilized the bipedal stance ("squat") exercise first described by Yao *et al.* [24], which positively influences fetal growth without inducing a maternal

stress response [14], to test the hypothesis that voluntary exercise during pregnancy in rats will not affect litter size, will attenuate the loss of maternal bone mineral throughout pregnancy and lactation, and will result in heavier offspring relative to those of non-exercised controls.

Materials and Methods

Animals

Twenty virgin female Wistar rats were housed in individual cages in a climate-controlled room in a dedicated animal research facility with a 12:12 hour light:dark cycle. All rats were allowed a minimum of two weeks to habituate to their surroundings before beginning exercise. Feed and water were provided *ad libitum* and initial and residual feed was weighed daily from gestational day (GD) 0 until parturition. Body weight was also measured daily. Body length (nose-tail, nose-ischiatic tuberosity, and nose-atlanto-occipital junction) was measured once under anesthesia (for imaging) and once immediately following euthanasia at study termination. Maternal feed efficiency over the pregnancy period was calculated as total body weight gain from GD0 to GD21 divided by total feed intake over the same period. The Massey University Animal Ethics Committee approved the study protocol and all animal procedures.

Exercise

After the period of habituation to the environment, when they were 92 to 96 days old, the rats were randomly assigned to one of two age- and weight-matched groups. Rats in the exercise group were housed in raised cages from GD0 to lactation day (L) 1 so that they had to achieve an erect bipedal stance to obtain food and water as described previously [13]. Rats in the control group were housed in cages of conventional height for the duration of the trial. Rats in the exercise group were gradually introduced to exercise in the five days immediately prior to mating as described previously [14].

Reproduction

Rats were mated between 98 and 104 days of age. Each sire mated with one exercised and one control dam. Mating was confirmed by visualization of a semen plug

and the rats were then returned to their exercise cages for the duration of pregnancy. The day the plug was visualized was designated GD0. All rats that became pregnant gave birth on GD22. The day after parturition was designated L1.

On L1 all offspring were weighed and litter sizes were reduced to 8 per litter. When possible, four male and four female pups close to the mean pup weight were allowed to remain with the mother, but in two litters 6 males and 2 females were selected as there was an insufficient number of female pups. Exercise was stopped on L1, and all mothers and pups were housed in control housing throughout the lactation period. Food intake of the mothers and pups combined, and individual body weights of all animals, were recorded daily. Pups were weaned at L25 and dam body composition and bone parameters were assessed on the day following weaning. We chose to stop exercise on the first day of lactation in order to limit our study to the effects of exercise during pregnancy, as opposed to the combined pregnancy/lactation period. Dam body composition and bone parameters were assessed on the day following weaning to examine whether exercise during pregnancy resulted in physical changes to the dams that persisted throughout the lactation period.

Imaging

Peripheral quantitative computed tomography (pQCT) and dual-energy X-ray absorptiometry (DXA) were performed twice during the trial, once 6 days prior to the beginning of the gradual introduction to exercise (age 86-90 days) and once immediately prior to euthanasia on the day following weaning (age 146–152 days). Anesthesia, DXA and the baseline pQCT scans were performed as described previously, except that DXA whole body scans included the skull in the ROI in both the baseline and post-lactation scan analyses [13]. Post-weaning pQCT scans were performed *ex vivo*. The right tibias were fixed in 4% paraformaldehyde for one week and then stored

in 70% ethanol until scanning. The CV for *ex vivo* pQCT bone parameters ranged from 0.5%–2.6%.

Fecal corticosterone

Fecal samples were collected from all rats over three collection periods of four consecutive days each: baseline (pre-exercise/pre-scanning/pre-mating), GD3–GD6, and GD17–GD20. The method for fecal collection and processing was as previously described [13]. The mean recovery of corticosterone from spiked control samples was 76.6 ± 1.9% (n=20) with a CV of 11.3%. The mean percentage recovery was used to calculate results for all the samples.

Statistical analysis

Statistical analysis was performed with SAS 9.2 using PROC GLM. Significance of difference of offspring weights was tested using a nested model, with dam exercise group and offspring sex as fixed effects and dam nested within exercise group as a random effect. Offspring weights reported at L1 are mean weights for all live pups, offspring weights reported at L25 are for 8 pups per litter. Between-time point and between-group differences in dam imaging parameters, feed intake, body weight, and fecal corticosterone levels were determined using repeat measures analysis with dam exercise group, time point and their interaction as fixed effects, dam nested within exercise group as a random effect, and Tukey-Kramer post-hoc adjustments. Fecal corticosterone data were logarithmically transformed prior to analysis to achieve normal distribution. All data are expressed as lsmeans ± SE unless otherwise indicated. Differences are considered significant at $P \leq 0.05$.

Results

Animals

All animals successfully performed the exercise required by their group. Fifteen of the twenty animals were included in the final analysis. Of the 5 excluded animals, 3 (1 control and 2 exercised) failed to become pregnant, and two (both controls) were excluded for failing to have eight or more live offspring; final group sizes were control n=7, exercised n=8. There were no significant between-group differences in litter size or the male: female ratio of the live pups. Litter size, defined as the number of live offspring on L1, ranged from 9–17 with a mean of 13.5 ± 2.2 pups/litter. Mean litter size was 14.3 ± 0.8 for the control group and 12.9 ± 0.8 for the exercised group (P=0.22). As the animals were not under constant observation and the mothers may (often) eat any dead pups immediately, the number of pups born dead could not be accurately assessed; however, there were no differences between groups in the number of uneaten dead pups found in the cages.

Rats in the exercised group gained significantly (P=0.01) less weight over pregnancy than did control rats. Weight gain in both groups followed a similar trajectory, but body weights, which include the weight of the uterus and its contents until the end of pregnancy, differed significantly from day 15 of pregnancy onwards as shown in Figure 1. Daily food intake during pregnancy did not significantly differ between groups (Figure 2). Total offspring weight also was not different between groups (P=0.35). The difference in dam weight between GD21 and L1 was taken as an approximation of the weight of the uterine contents, which includes the fetuses, placentas, and associated fluid. Mean body weight change during this time was -100.7 ± 5.4 g and -88.6 ± 5.0 g for the control and exercised rats, respectively (P=0.12). On L1, dams in the control group weighed 370 ± 2.8 g and dams in the exercised group

weighed 349.3 ± 2.6 g (P<0.0001). During lactation, exercised dams recovered some, but not all, of their body weight deficit relative to controls so that at weaning the groups did not significantly differ but the exercised group was still numerically lighter than control rats. Feed efficiency of the dams over the pregnancy period was 0.35 ± 0.01 and 0.32 ± 0.01 for the control and exercised groups respectively; this difference was not statistically significant (P=0.08). Measures of dam length did not differ between groups prior to, or following, the pregnancy/lactation period.

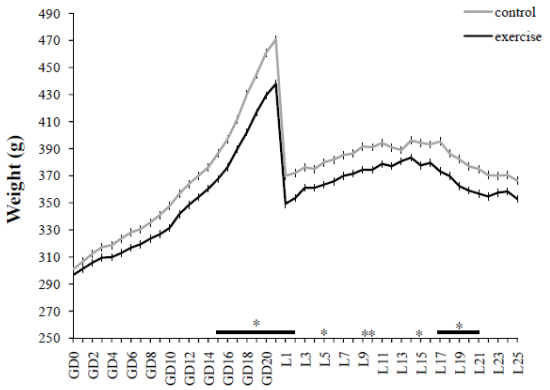

Figure 1. Dam body weight throughout pregnancy and lactation. A * above the horizontal axis indicates that the mean body weight of exercised dams was significantly different from that of control dams on that day, P≤0.05. Body weight of the exercised dams differed significantly from that of controls on days GD15-L2, and L5, 9, 10, 15, and 17-21.

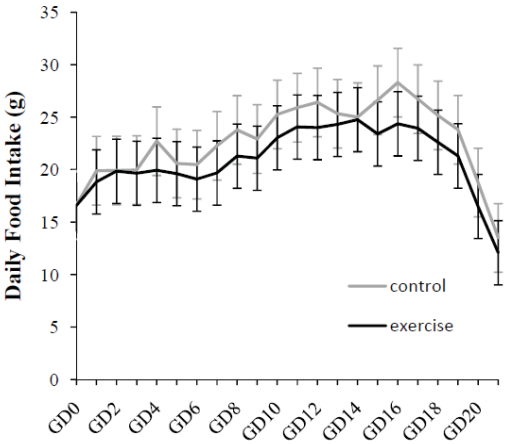

Figure 2. Daily food intake of exercised and control dams during pregnancy. There were no significant differences in daily food intake between groups on any day of pregnancy.

Male offspring were heavier than female offspring at lactation day 1 and at weaning regardless of exercise group (P<0.001; shown in Table 1). However, there were no significant differences in offspring weight between the exercised and control groups at either time point (Table 1), and the interaction between sex and exercise group was also not significant (P=0.47 and P=0.77 at L1 and L25, respectively).

Table 1. Offspring weights at 1 and 25 days after birth

	Weight at L1 (g)	*P*	Weight at L25 (g)	*P*
Male	6.5 ± 0.04	0.0007	82.0 ± 0.6	<0.0001
Female	6.2 ± 0.05		78.0 ± 0.6	
Control	6.2 ± 0.1	n.s.	78.4 ± 1.6	n.s.
Exercised	6.4 ± 0.1		81.6 ± 1.4	

Values are least square means ± standard error. n.s. = not significant

DXA-assessed changes in dam body composition and bone parameters

Both groups gained whole body BMC and bone area over the pregnancy/lactation period, with a proportionally greater increase in area than BMC resulting in a decrease in whole body areal bone mineral density (BMD_a). Although total fat, lean and body mass increased over pregnancy and lactation, the percentages of fat and lean mass did not change from baseline in either group (Table 2). Thus, when their offspring were weaned, the dams were larger than at mating but did not differ in body composition. When corrected for body weight, both whole body BMC and bone area were significantly lower at the time of weaning than at mating in both exercised and control rats. The decrease in bone area per gram of body weight was significantly less in the exercised rats relative to the controls (P=0.02).

Table 2. Results of *in vivo* DXA whole body scans

	Baseline		*Post-lactation*		*P*		
	Control	**E**xercise	**C**ontrol	**E**xercise	**Ex**	**T**ime	**Ex*T**
Fat Mass (g)	46.38 ± 3.28	52.82 ± 3.07	60.92 ± 3.28	66.20 ± 3.07	n.s.	0.0007	n.s.
Lean Mass (g)	215.91 ± 3.98	208.39 ± 3.72	291.59 ± 3.98	277.84 ± 3.72	n.s.	<0.0001	n.s.
Total Mass (g)	271.85 ± 3.60	270.32 ± 3.37	363.12 ± 3.60	354.27 ± 3.37	n.s.	<0.0001	n.s.
% Fat	17.06 ± 0.92	19.63 ± 0.86	16.78 ± 0.92	18.63 ± 0.86	n.s.	n.s.	n.s.
% Lean	79.42 ± 0.92	77.00 ± 0.86	80.30 ± 0.92	78.48 ± 0.86	n.s.	n.s.	n.s.
Bone Area (cm^2)	60.41 ± 0.45	57.85 ± 0.42	70.71 ± 0.45	69.63 ± 0.42	n.s.	<0.0001	n.s.
BMC (g)[a]	9.56 ± 0.11	9.11 ± 0.11	10.6 ± 0.11	10.23 ± 0.11	n.s.	<0.0001	n.s.
BMD_a (g/cm^2)[a]	0.16 ± 0.001	0.16 ± 0.001	0.15 ± 0.001	0.15 ± 0.001	n.s.	<0.0001	n.s.
Bone Area/BW	0.22 ± 0.002	0.21 ± 0.002	0.19 ± 0.002	0.20 ± 0.002	n.s.	<0.0001	0.02
BMC/BW	0.035 ± 0.0004	0.034 ± 0.0004	0.029 ± 0.0004	0.029 ± 0.0003	n.s.	<0.0001	n.s.

Values are least square means ± standard error. Baseline is 6 days prior to starting exercise training. Post-lactation is 26 days after parturition. DXA, dual-energy X-ray absorptiometry; Ex, exercise group; Time, time of measurement (baseline or post-lactation); Ex*T, interaction between exercise group and time of measurement; BMC, bone mineral content; BMD_a, areal bone mineral density; BW, body weight; n.s., not significant. [a]This unit replaces unit used in original publication.

Table 3 shows the results of high resolution DXA scans of the first four vertebrae of the lumbar spine. Bone area in both groups increased over the study period, but BMC did not change, resulting in a lower BMD_a at weaning than at baseline. When corrected for body weight, bone area and BMC were significantly lower post-lactation than at baseline in both groups, and this reduction was not affected by exercise.

Table 3. Results of high resolution DXA scans of the lumbar spine

	Baseline		*Post-lactation*		*P*		
	Control	**E**xercise	**C**ontrol	**E**xercise	**E**x	**T**ime	**E**x*T
Bone Area (cm^2)	1.90 ± 0.02	1.86 ± 0.02	2.06 ± 0.02	2.00 ± 0.02	n.s.	<0.0001	n.s.
BMC (g)[a]	0.41 ± 0.01	0.39 ± 0.01	0.39 ± 0.01	0.38 ± 0.01	n.s.	n.s.	n.s.
BMD_a (g/cm^2)[a]	0.21 ± 0.004	0.21 ± 0.003	0.19 ± 0.004	0.19 ± 0.003	n.s.	<0.0001	n.s.
Area/BW	0.0070 ± 0.00006	0.0069 ± 0.00005	0.0057 ± 0.00006	0.0057 ± 0.00005	n.s.	<0.0001	n.s.
BMC/BW	0.0015 ± 0.00003	0.0014 ± 0.00003	0.0011 ± 0.00003	0.0011 ± 0.00003	n.s.	<0.0001	n.s.

Values are least square means ± standard error. Baseline is 6 days prior to starting exercise training. Post-lactation is 26 days after parturition. DXA, dual-energy X-ray absorptiometry; Ex, exercise group; Time, time of measurement (baseline or post-lactation); Ex*T, interaction between exercise group and time of measurement; BMC, bone mineral content; BMD_a, areal bone mineral density; BW, body weight; n.s., not significant. [a]This unit replaces unit used in original publication.

In the right femur both groups gained bone area and BMC, resulting in no net change in BMD_a. As seen at the other sites, when bone area and BMC are corrected for body weight both groups were lower post-lactation than at baseline (Table 4).

Table 4. Results of high resolution DXA scans of the right femur

	Baseline		*Post-lactation*		*P*		
	Control	**E**xercise	**C**ontrol	**E**xercise	**E**x	**T**ime	**E**x*T
Area (cm^2)	1.24 ± 0.02	1.20 ± 0.02	1.41 ± 0.02	1.38 ± 0.02	n.s.	<0.0001	n.s.
BMC (g)[a]	0.32 ± 0.01	0.29 ± 0.01	0.34 ± 0.01	0.34 ± 0.01	n.s.	0.0039	n.s.
BMD_a (g/cm^2)[a]	0.26 ± 0.01	0.24 ± 0.01	0.24 ± 0.01	0.24 ± 0.01	n.s.	n.s.	n.s.
Area/BW	0.0045 ± 0.0006	0.0045 ± 0.0006	0.0039 ± 0.0006	0.0039 ± 0.0006	n.s.	<0.0001	n.s.
BMC/BW	0.0012 ± 0.00003	0.0011 ± 0.00003	0.0009 ± 0.00003	0.0009 ± 0.00003	n.s.	<0.0001	n.s.

Values are least square means ± standard error. Baseline is 6 days prior to starting exercise training. Post-lactation is 26 days after parturition. DXA, dual-energy X-ray absorptiometry; Ex, exercise group; Time, time of measurement (baseline or post-lactation); Ex*T, interaction between exercise group and time of measurement; BMC, bone mineral content; BMD_a, areal bone mineral density; BW, body weight; n.s., not significant. [a]This unit replaces unit used in original publication.

pQCT-assessed changes in dam bone parameters

When changes in the right tibia over the study period were examined using pQCT, both pregnancy/lactation and exercise affected bone parameters in a site-specific fashion. The results of pQCT scans at the right proximal tibial metaphysis are shown in Table 5, and at the right mid-tibial diaphysis are shown in Table 6. At the proximal tibial metaphysis exercise attenuated the loss of bone mineral. The change in trabecular BMC from baseline to post-lactation was significantly different between groups. The decrease in total BMC at this site was also greater in the controls than in the exercised animals, and this difference approached significance (P=0.07). Both groups increased in total and trabecular bone area; with the exercised animals gaining more total bone area than the controls. The relative magnitude of these changes in bone area and BMC led to lower volumetric bone mineral density (BMD_v) at all sites post-lactation. At the mid-tibial diaphysis, BMC and bone area increased in both groups. Relatively larger changes in both of these parameters resulted in a greater increase in strength strain index (SSI) in exercised animals relative to controls over the pregnancy/lactation period. Similarly, the change in the cross-sectional moment of inertia in the frontal plane (CSMI-x) from baseline to post-lactation also differed significantly between groups.

Table 5. pQCT results at the right proximal tibial metaphysis (baseline results are *in vivo*, post-lactation results are *ex vivo*)

	Baseline		*Post-Lactation*		*P*		
	Control	**E**xercise	**C**ontrol	**E**xercise	**E**x	**T**ime	**E**x*T
Total BMC (mg)	9.26 ± 0.21	8.64 ± 0.20	8.34 ± 0.21	8.51 ± 0.20	n.s.	0.02	n.s.
Total area (mm^2)	13.78 ± 0.27	13.01 ± 0.26	15.79 ± 0.27	16.15 ± 0.26	n.s.	<0.0001	0.05
Total BMD_v (mg/cm^3)	672.66 ± 13.61	667.61 ± 12.73	529.61 ± 13.61	529.29 ± 12.73	n.s.	<0.0001	n.s.
Trabecular BMC (mg)	1.68 ± 0.06	1.36 ± 0.06	1.47 ± 0.06	1.48 ± 0.06	n.s.	n.s.	0.02
Trabecular area (mm^2)	5.74 ± 0.28	5.51 ± 0.26	7.87 ± 0.28	8.03 ± 0.26	n.s.	<0.0001	n.s.
Trabecular BMD_v (mg/cm^3)	294.20 ± 9.81	252.50 ± 9.18	186.56 ± 9.81	184.85 ± 9.18	n.s.	<0.0001	n.s.
Cort/subcort BMC (mg)	7.58 ± 0.22	7.27 ± 0.20	6.86 ± 0.22	7.04 ± 0.20	n.s.	0.04	n.s.
Cort/subcort area (mm^2)	8.05 ± 0.23	7.51± 0.21	7.93 ± 0.23	8.12 ± 0.21	n.s.	n.s.	n.s.
Cort/subcort BMD_v (mg/cm^3)	943.31 ± 8.28	971.30 ± 7.74	865.19 ± 8.28	866.93 ± 7.74	n.s.	<0.0001	n.s.

Values are least square means ± standard error. All data are from the right proximal tibial metaphysis 5 mm distal to the proximal tibial plateau. Baseline is 6 days prior to starting exercise training. Post-lactation is *ex vivo*, bones were collected 26 days after parturition. pQCT, peripheral quantitative computed tomography; Ex, exercise group; Time, time of measurement (baseline or post-lactation); Ex*T, interaction between exercise group and time of measurement; BMC, bone mineral content; BMD_v, volumetric bone mineral density; cort/subcort, cortical/subcortical; n.s., not significant.

Table 6. pQCT values at the right mid-tibial diaphysis (baseline results are *in vivo*, post-lactation results are *ex vivo*)

	Baseline		*Post-Lactation*		*P*		
	Control	**E**xercise	**C**ontrol	**E**xercise	**E**x	**T**ime	**E**x*T
Cortical BMC (mg)	5.57 ± 0.07	5.45 ± 0.07	6.04 ± 0.07	6.17 ± 0.07	n.s.	<0.0001	n.s.
Cortical area (mm^2)	4.45 ± 0.07	4.40 ± 0.06	4.63 ± 0.07	4.75 ± 0.06	n.s.	0.001	n.s.
Cortical BMD_v (mg/cm^3)	1253.43 ± 9.70	1237.43 ± 9.07	1304.57 ± 9.70	1298.86 ± 9.07	n.s.	<0.0001	n.s.
Endosteal circumference (mm)	4.94 ± 0.10	5.16 ± 0.09	5.03 ± 0.10	5.16 ± 0.09	n.s.	n.s.	n.s.
Periosteal circumference (mm)	8.96 ± 0.07	9.05 ± 0.07	9.14 ± 0.07	9.29 ± 0.07	n.s.	0.01	n.s.
SSI	3.26 ± 0.07	3.20 ± 0.06	3.69 ± 0.07	4.03 ± 0.06	n.s.	<0.0001	0.01
CSMI-x ($mm^{4)}$	2.71 ± 0.08	2.71 ± 0.07	2.46 ± 0.08	2.80 ± 0.07	n.s.	n.s.	0.04
CSMI-y (mm^4)	2.59 ± 0.15	2.78 ± 0.14	3.79 ± 0.15	4.25 ± 0.14	n.s.	<0.0001	n.s.
CSMI-p (mm^4)	5.31 ± 0.17	5.48 ± 0.16	6.25 ± 0.17	7.05 ± 0.16	n.s.	<0.0001	n.s.

Values are least square means ± standard error. All data are from the right mid-tibial diaphysis. Baseline is 6 days prior to starting exercise training. Post-lactation is *ex vivo*, bones were collected 26 days after parturition. pQCT, peripheral quantitative computed tomography; Ex, exercise group; Time, time of measurement (baseline or post-lactation); Ex*T, interaction between exercise group and time of measurement; BMC, bone mineral content; BMD_v, volumetric bone mineral density; SSI, strength strain index; CSMI-x, cross-sectional moment of inertia in the frontal plane; CSMI-y, cross-sectional moment of inertia in the sagittal plane; CSMI-p, torsional cross-sectional moment of inertia; n.s., not significant.

Fecal corticosterone

Mean daily fecal corticosterone excretion during the first four hours of the dark cycle over each four day collection period is shown in Figure 3. There were no significant between-group differences at any time point or between-time point differences in any group. Although the largest within-group numerical change in fecal corticosterone levels was in the exercised group from the GD3–6 to the GD17–20 collection periods this change was not significant (P=0.25). However, when data from both groups were combined mean fecal corticosterone excretion was significantly lower in early than in late gestation.

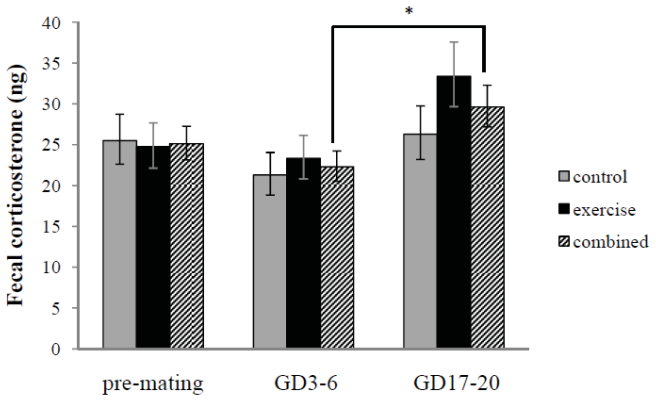

Figure 3. Mean total fecal corticosterone excretion over the first four hours of the dark period for each exercise group and for all groups combined during each 4-day collection period. Data are presented as antilog of lsmeans to facilitate comparison of corticosterone output between studies. The baseline collection period is prior to baseline imaging (pre-mating). There were no significant differences between groups at any time point or between time points for any exercise group. However, when data for all groups were combined, excreted fecal corticosterone at the GD17-GD20 collection period was significantly greater than at GD3-GD6.*Significant difference, P≤0.05.

Discussion

We anticipated that the offspring of exercised dams would be significantly heavier than those of controls during the early postnatal period, but they were not. Exercised dams had heavier fetuses relative to controls at GD19 in our previous study [14]. That this heavier fetal weight did not persist into early postnatal life may reflect a number of factors. Day 19 of gestation is after the primary period of organogenesis (from implantation to closure of the hard palate) but before the period of greatest total gain of mass in the fetal rat [25,26]. The effects of exercise on fetal growth may differ during the different periods of pregnancy, resulting in different effects on offspring weight. In humans this is known to be the case; exercise in early pregnancy enhances placental function and offspring growth in humans, but continued moderate to high volumes of exercise in later pregnancy is associated with lower birth weight through reduction in fetal fat [2,20]. It is also important to note that in our previous study we found the greatest effects of exercise on fetal weight at GD19 in fetuses located in the mid-uterine horn. In our current study the L1 weights reported are from all live offspring; since the animals had already been born there was no way to be certain of their intra-uterine position. Additionally, as we are interested in the long-term effects of exercise on offspring health, we chose not to disturb the dams and pups on the day of birth so as to allow proper bonding and maternal care, so the early postnatal weights reported are from the day following parturition. At birth rat pups weigh approximately 4.5 g [25], whereas by L1 our pups had grown considerably and weighed approximately 6.5 g. This rapid increase in weight in the immediate postnatal period may also have obscured subtle effects of exercise on pup weight. However, birth or early postnatal weight is only a crude reflection of the intra-uterine environment, and intra-uterine effects on fetal organogenesis during pregnancy can alter organ development without

changes in postnatal body weight. For example, it has been reported that pups whose dams underwent exercise during pregnancy had birth weights equivalent to those of control pups, but had increased cell numbers in the hippocampus and better short term memory [27]. Animal models of cardiovascular and renal disease have also shown that later-life disease can result from prenatal influences that do not affect birth weight [28]. In addition, differences in maternal weight during pregnancy, and growth rate and quality in the postnatal period, have also been linked to long-term health effects in both humans and animals [29]. Thus, differences in growth during early fetal life that do not result in differences in postnatal body weight may still have later-life health consequences.

Rats that exercised during pregnancy had a greater post-lactation SSI of the right tibia than non-exercised controls. This was unexpected, as we had previously demonstrated that bipedal stance exercise throughout pregnancy did not significantly alter bone parameters at GD19. The fact that between-group differences were evident after lactation in this study, although exercise was stopped on day 1 of lactation, may indicate that rising to an erect bipedal stance coupled with the rapid weight gain of late gestation increased the strain on the tibia sufficiently to trigger modeling of the bone greater than that induced by pregnancy alone. During pregnancy in the rat, maternal bone meets the demand of the fetuses for calcium by altering bone formation and resorption in a site-specific manner; for example, at the end of pregnancy trabecular bone formation in the lumbar vertebrae has decreased, while cortical bone formation has increased at the periosteal surface of the femur [15]. Since we did not measure bone parameters during and immediately after parturition we do not know if between-group differences were present at that time. However, the increase in the tibial SSI of the dams

that exercised during pregnancy clearly shows that there are skeletal benefits of exercise during pregnancy that can persist through lactation.

That the diaphyseal CSMI-x also increased in the exercised dams indicates that the changes reflected by the larger SSI are primarily due to modeling drift, as opposed to changes in bone material. CSMI is a measure of bone geometry, and indicates the bone's ability to resist frontal, sagittal, or torsional bending [30,31]. The increase in CSMI-x indicates that the bone has altered its geometry to resist the bending forces imposed by the caudal muscles of the hindlimb acting to extend the tarsus when the rat rises to a bipedal stance. The torsional CSMI (CSMI-p) also had a greater numerical increase in exercised dams than in controls, although this difference did not reach significance (interaction of exercise group and time, P=0.08). In humans, action of the soleus muscle places both bending and torsion forces on the tibia [30]; given the similar anatomic arrangement of this muscle in the rat we may suppose that both bending and torsion stresses are also applied by soleus activity in this species. The CSMI results seen in our study are similar to the findings of other studies in both rats and humans. Yao *et al.* saw increases in CSMI-x in male rats that performed bipedal stance exercise [32], and Miyagawa *et al*. found that rats allowed to perform walking exercise had a greater CSMI-x than those that were prevented from walking [33]. In a study examining the relationship between bone structure and distance running in human athletes, Feldman *et al*. found greater CSMIs in the diaphyseal region of the tibia in runners than in non-runners [30]. Modeling effects, as opposed to material changes, have also been seen in bones at other anatomic sites after exercise. For example, the increased bone strength in the dominant arm of tennis players is primarily due to increases in bone size rather than BMD_v [34].

The differences in bone properties between exercised and control dams are more evident at the tibial diaphysis than at the metaphysis. This site-specificity reflects the forces placed upon the bone by bipedal stance exercise. The forces applied to the bone by this non-impact exercise are primarily those of muscle contraction, which would tend to cause bending forces in the mid-tibia region and not compressive forces at the proximal and distal bone ends. That bones respond to exercise in a site-specific manner is both inherent in Wolff's Law, which holds that bone adapts to the specific loads placed upon it, and has been shown and well-discussed in relation to human runners [30]. The site-specific response of bone to exercise may have implications for the utility of exercise as a therapy for osteoporotic patients.

Dams in our study did not lose whole body bone mineral over pregnancy and lactation. Both groups gained whole body bone mineral content (BMC) and bone area over the study period, in absolute terms, as would be expected in these sexually mature but still growing animals. However, due to a greater increase in bone area than BMC, BMD_a decreased. BMD_a is the ratio of bone mineral to bone area; and can decrease when the BMC decreases or when bone area increases, or both. Increased bone area can increase bone strength (through geometry changes, and also increased total bone mineral content) while leading to a BMD_a decrease. This highlights the fact that decreases in areal bone mineral density do not equal loss of whole body or whole organ bone mineral (reviewed in [35]). It is for this reason that high resolution DXA scans of specific regions, such as the primarily trabecular lumbar vertebrae or the more predominantly cortical femur, are used to make inferences about the state of trabecular or cortical bone. Used in this way DXA can provide useful measures of whole body or regional composition. However, pQCT allows separate analysis of the cortical and

trabecular compartments of bone, and can provide a more biologically accurate picture of the bone's response to the physiological states of pregnancy and lactation.

Exercise during pregnancy attenuated the loss of bone mineral over the pregnancy/lactation period from the trabecular bone of the proximal tibial metaphysis. That both groups maintained total, and lost BW-corrected, BMC (measured by DXA) at the primarily trabecular lumbar spine, may be due to the nature of the exercise, which might cause more strain in the bones of the hind limbs than of the spine. Alternatively, it may be due to a physiological tendency to release calcium from the vertebrae rather than the bones of the appendicular skeleton, possibly because of the greater fraction of trabecular bone, which contributes more to meeting the increased calcium demands of gestation and lactation than cortical bone [36]. In humans, exercise during lactation may reduce associated changes in BMD_a of the lumbar spine [37], but little data are available as to whether exercise during gestation, but not lactation, has a similar effect. Although in both humans and rats bone parameters tend to return to "normal" levels after weaning of the offspring and resumption of cycling, this does not occur in all cases [38,39] and low BMD_a in puerperal women has been associated with later osteopenia [7]. The relationship of pregnancy/lactation-associated bone loss with later-life osteoporosis is unclear. Studies have yielded varying results, but little work has been done to examine the long-term effects of pregnancy and lactation on the skeleton of two potentially higher risk groups of women: adolescent mothers and older mothers near the end of their reproductive years [38]. The attenuating effects of exercise during gestation on bone loss that we have seen in this study suggest that even mild exercise during pregnancy could positively influence bone health, perhaps especially in these higher risk populations. Since numerous studies have shown that parental physical activity tends to decrease after having children (reviewed in [40]), and that mothers tend to replace

leisure-time exercise with household activities, first pregnancies may present a unique opportunity for targeted exercise interventions at a time when there are fewer perceived barriers to physical activity.

In both the DXA and pQCT scans there were several unexpected between-group differences in imaging parameters at baseline. Whole body bone area (DXA) and trabecular BMC (pQCT) were both significantly less in the exercised group than the controls prior to any experimental intervention. This was surprising, as there were no between-group differences in weight or length. Rats were randomly allocated to the exercise and control groups prior to baseline scanning, and we therefore conclude that these initial differences are due to random chance. Additionally, our repeated measures analysis with dam nested within exercise group accounts for individual dam variability. Therefore, we believe that significant between-group differences in the change in bone parameters from baseline to post-lactation (as indicated by significance of the interaction between exercise and time as shown in tables 2–6) reflect exercise-induced differences in the bone response to lactation, rather than accidental pre-treatment between-group differences.

Building on our previous work, we have now shown that bipedal stance exercise in the pregnant rat does not cause a physiological stress response sufficient to chronically elevate fecal corticosterone levels in early, mid or late pregnancy [14]. We used fecal corticosterone to assess the maternal stress response to exercise as it is well-suited to analysis of chronic stressors. We report total corticosterone excreted over the collection period rather than concentration as this provides a more accurate representation of adrenal corticoid production, and our fecal collections were timed to maximize our chances of detecting a stress response, as we have described previously [13]. Maternal stress during gestation can have long-term implications for both humans

and rats [41]. Some types of exercise, such as swimming [27] and treadmill running [42], cause a stress response in pregnant rats. However, by choosing a voluntary, moderate exercise we avoided the confounding effects of maternal stress. Although in our model the rats must rise to an erect bipedal stance in order to reach their food, they are able to perform the exercise at their own discretion and without human handling. Additionally, all the rats were monitored to ensure that they were able to reach their food without difficulty. Thus we consider our exercise model to be voluntary in comparison to models such as forced treadmill running or swimming, in which the rat must exercise at a time and intensity not of their own choosing, and which often involve aversive stimuli. An exercise model that does not cause stress in pregnant animals is necessary to examine the effects of exercise during gestation on offspring health without the confounding effects of a stressed uterine environment. The rat model has played a key role in establishing that there are long-term effects of other environmental influences, such as nutrition and stress, during development. The bipedal stance model of exercise that we have utilized does not cause a physiologic stress response in pregnant rats, and thus differences between the offspring of exercised and non-exercised dams can be attributed to the effects of the exercise alone. Thus, this model may be potentially useful for examination of the effects of exercise during pregnancy on the later-life health outcomes of offspring.

Conclusions

Exercise during pregnancy may affect the later-life health of both the mother and her offspring. In this study we demonstrate that very moderate, voluntary exercise in the pregnant rat can improve indices of bone strength measured after lactation and attenuate the loss of trabecular bone mineral, without adversely affecting early postnatal outcomes of the offspring. We have further confirmed that this exercise model does not

cause a physiological stress response in pregnant animals, thus allowing its use to examine the effects of exercise during pregnancy on offspring health without the confounding developmental effects of a stressed intrauterine environment. Although we did not see differences in mean early postnatal weights between the offspring of exercised and control dams, previous studies demonstrating differences in fetal growth to GD19 indicate that further evaluation of the long-term effects of this exercise model on offspring health are warranted.

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Chapter 5

MODERATE EXERCISE DURING PREGNANCY IN WISTAR RATS ALTERS BONE AND BODY COMPOSITION OF THE ADULT OFFSPRING IN A SEX-DEPENDENT MANNER

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Abstract

Exercise during pregnancy may have long-lasting effects on offspring health. Musculoskeletal growth and development, metabolism, and later-life disease risk can all be impacted by the maternal environment during pregnancy. The skeleton influences glucose handling through the actions of the bone-derived hormone osteocalcin. The purpose of this study was to test the effects of moderate maternal exercise during pregnancy on the bone and body composition of the offspring in adult life, and to investigate the role of osteocalcin in these effects. Groups of pregnant Wistar rats either performed bipedal standing exercise to obtain food/water throughout gestation but not lactation, or were fed conventionally. Litters were reduced to 8/dam and pups were raised to maturity under control conditions. Whole body dual-energy x-ray absorptiometry, and *ex vivo* peripheral quantitative computed tomography scans of the right tibia were performed. At study termination blood and tissue samples were collected. Serum concentrations of fully and undercarboxylated osteocalcin were measured, and the relative expression levels of osteocalcin, insulin receptor, Forkhead box transcription factor O1, and osteotesticular protein tyrosine phosphatase mRNA were quantified. Body mass did not differ between the offspring of exercised and control dams, but the male offspring of exercised dams had a greater % fat and lower % lean than controls ($p=0.001$ and $p=0.0008$, respectively). At the mid-tibial diaphysis, offspring of exercised dams had a lower volumetric bone mineral density than controls ($p=0.01$) and in the male offspring of exercised dams the bone: muscle relationship was fundamentally altered. Serum concentrations of undercarboxylated osteocalcin were significantly greater in the male offspring of exercised dams than in controls ($p=0.02$); however, the relative expression of the measured genes did not differ between groups. These results suggest that moderate exercise during pregnancy can result in lasting

changes to the musculoskeletal system and adiposity in offspring, in a sex-specific manner.

Introduction

The Developmental Origins of Health and Disease (DOHaD) paradigm suggests that the environment to which an organism is exposed during prenatal development and early life can have lasting health consequences [1]. Maternal undernutrition during pregnancy has been widely studied in relation to later-life health, and animal models used in controlled studies have confirmed the long-lasting effects of undernutrition during development. The pups of undernourished pregnant rats are hypertensive, hyperphagic, and obese in mature life [2], and both under- and over-nutrition during gestation result in earlier reproductive maturation in female rat pups [3]. However, while the effects of nutritional stress during fetal development on long-term health have been well-proven, the effects on offspring development of other environmental influences during pregnancy have not been clearly defined.

Exercise during pregnancy may also affect later offspring health. In humans, exercise during pregnancy affects fetal growth through effects on placental size and blood flow in a type-, frequency-, and intensity-dependent manner [4]. Thus mothers who begin a moderate exercise program in early pregnancy will have larger babies [5], while those who continue rigorous exercise throughout pregnancy have thinner, lighter offspring [6]. Whether these effects on birth size result in later-life health effects is not yet known, as no data are currently available on the effects of exercise during pregnancy on human offspring during adulthood. The results of studies that investigated the effects of exercise during pregnancy on childhood outcomes suggest that the children of exercising mothers are cognitively advanced relative to controls and at 5 years old are slightly lighter than, but otherwise similar to, controls in terms of physical development [7,8,9].

Research using animal models has shown a dose-response effect of exercise on uteroplacental blood flow similar to that seen in humans [10]. The effects of maternal exercise during pregnancy on birth weight in rats have also varied with the type and intensity of the exercise performed, with results ranging from no change [11,12,13] to significant decreases [14,15,16] in birth weight, the latter being associated with higher intensity maternal exercise. Studies examining the effects of exercise during pregnancy on factors other than birth weight have shown increased brain-derived neurotrophic factor and improved learning and memory in the pups of exercised rat dams relative to those of controls [11,17]. Studies examining the effects of maternal exercise during pregnancy on adult health in rats are few, but the available data suggest that maternal exercise can indeed have a long-term effect on the health of the offspring: a recent study showed that maternal treadmill running during pregnancy enhances insulin sensitivity in the adult offspring [18].

Although there has been great interest in the long-term metabolic effects of early life influences, the musculoskeletal system may also be affected by environmental factors during development. Recent research in mice has revealed that bone is a key regulator of metabolism, and thus changes to the skeleton may also affect metabolic health. Osteoblasts produce osteocalcin (OC), the undercarboxylated form of which can act as an endocrine hormone and regulate glucose handling by stimulating insulin production and sensitivity [19,20]. Developmental influences that alter osteoblast activity may result in changes to whole body glucose handling. It has also been suggested that the skeletal disorder osteoporosis may fit the DOHaD concept [21] and in humans there is a relationship between birth weight and adult bone mass [23]; this may reflect the associations of birth weight and infant growth with muscle size and strength in later life [24,25]. However, there has been very little research examining the effects

of exercise during pregnancy on the musculoskeletal system of the offspring in adulthood. To our knowledge, the only data available on this topic are those of Monteiro *et al*. [22], who investigated the effects of both exercise during pregnancy and protein malnutrition during pregnancy and lactation, and found that only protein malnutrition, and not maternal exercise, affected femur length in adult offspring.

Since maternal exercise during pregnancy may have beneficial effects on the metabolism and musculoskeletal system of the offspring in later life, we hypothesized that the adult offspring of dams that exercised during pregnancy would have improved musculoskeletal and metabolic health relative to the adult offspring of control rats. Since bone and metabolism are linked by the endocrine actions of the undercarboxylated form of OC, we further surmised that serum concentrations of this bone-derived hormone would differ in the adult offspring of exercised and control dams. In this study, we utilized rising to an erect bipedal stance [23], which we have previously shown to be a non-stressful exercise for pregnant rats [13,24], to test the long-term effects of maternal exercise during pregnancy on the musculoskeletal and metabolic health of the offspring, and to investigate the role of OC in these effects.

Materials and Methods

Animals

Twenty virgin female Wistar rats were randomly assigned to one of two age- and weight-matched groups (exercise and control). Rats in the exercise group (DAMEX) were habituated to their bipedal stance exercise over a 5 day period, during which the height of their cages was raised incrementally each day until they had to stand on extended hindlimbs to reach the feeder and water bottle in the cage lid, and then mated. Mating took place in standard control height cages with a wire mesh floor to facilitate identification of the semen plug. Once the plug was observed the DAMEX rats were housed in raised cages throughout gestation so that they had to achieve an erect bipedal stance to obtain food and water as described previously [25]. Rats in the control group (DAMCON) were housed in cages of conventional height for the duration of the trial. Fifteen (7 control and 8 exercised) of the 20 females gave birth to litters of 8 or more live pups. Of the remaining 5 mated females, three (1 DAMCON and 2 DAMEX) did not become pregnant, and two (both DAMCON) had less than eight live pups each. A detailed description of the dam exercise regime and early-life pup outcomes from birth until weaning is provided in Rosa *et al*. (2012) [13].

On the day after parturition litter sizes were reduced to 8 per litter and bipedal stance exercise was stopped. All mothers and pups were then housed in control housing throughout the lactation period. Pups were weaned at lactation day 25. Male pups were pair housed from weaning until day 98 ± 2, at which time they were separated into individual cages to allow more space for their larger body size and to prevent fighting. Female pups were pair housed from weaning until study termination. All rats were housed in a climate-controlled dedicated animal facility with a 12:12 hour light:dark cycle. Feed (Research Diets AIN-93G) and water were provided *ad libitum*. Rats were

bedded on kiln-dried wood shavings, and after weaning all offspring were provided with PVC tubes to allow sheltering behaviour and with marbles for enrichment.

Ethics Statement

This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The Massey University Animal Ethics Committee approved the study protocol and all animal procedures (permit number: 10/72). All rats were anesthetized prior to imaging procedures with a mixture consisting of 0.5 mL ketamine (100 mg/mL), 0.2 mL acepromazine (2 mg/mL), 0.1 mL xylazine (100 mg/mL), and 0.2 mL sterile water injected intraperitoneally at a dose rate of 0.6 mL/100 g live weight via a 25 g needle. Every effort was made to minimize animal suffering.

Feed Intake and Puberty Assessment

The female offspring were visually inspected once daily from day 27 until vaginal opening (indicating the onset of puberty) was observed. The rats were weighed weekly except when the daily feed intake and live weight gain of all rats was recorded over a one week period beginning when the rats were 98-101 days old; measurement of food intake was started two days after the males were moved to single housing to allow them to adjust to the cage change. Since the females were pair-housed, individual feed efficiencies were obtained for only male rats.

Grip Strength

Forelimb grip strength was measured using a grip strength meter for rats (Columbus Instruments, Columbus, Ohio, USA) when the rats were 167 – 172 days old. Rats were allowed to grasp a metal bar connected to the force meter and were then held at the base of the tail and pulled slowly backwards until they released their grasp. Grip strength testing was repeated 5 times with a rest period of 15-30 seconds between each

test. All tests were performed by the same handler, and grip strength was defined as the mean value of all successful measurements.

Imaging

Dual-energy X-ray absorptiometry (DXA) was used to measure bone and soft tissue parameters as described previously [25]. Male rats were scanned twice during the trial, once at 114–118 days old and a second time 2 weeks prior to euthanasia (age 187–193 days). Female rats underwent only one DXA scan at age 227–232 days; also 2 weeks prior to euthanasia. Peripheral quantitative computed tomography (pQCT) scans of the right tibia were performed *ex vivo*. Scans were made at the proximal tibial metaphysis 5 mm distal to the tibial plateau, and at the midpoint of the tibia as described previously [13]. The CV for *ex vivo* pQCT bone parameters ranged from 0.5%–2.6%.

Sample collection

Male rats were euthanized at 201–207 days of age, and female rats were euthanized at 242–249 days of age. The rats were fasted for 12 hours prior to euthanasia and were killed by anesthetic overdose followed by terminal blood collection as described previously [13]. All sample collection was performed between 0800 and 1200 hours to minimize the effects of circadian variation on measured parameters. Blood glucose was immediately assessed using an Accu-check Advantage blood glucose meter (Roche Diagnostics GmbH, Mannheim, Germany). Blood samples were collected into plain and Ca-ethylenediaminetetraacetic acid vacutainer tubes and immediately placed on ice; both serum and plasma were separated and stored at -80^{0}C within one hour of collection. Immediately following euthanasia, nose-tail, head and spine length (from the occipital condyles to the palpable ischiatic tuberosity of the pelvis) were measured, and samples of bone and soft tissues were collected for future gene expression analysis; these were snap frozen in liquid nitrogen and stored at -80^{0}C. The right tibia and both

kidneys were fixed in 4% paraformaldehyde for one week and then stored in 70% ethanol. All tissue samples were collected within 20 minutes of death. Because several rats had evidence of renal pathology on post mortem examination, the kidneys of all rats were examined by a veterinary pathologist and scored from 1–4 for degree of nephropathy where 1 = 0–10% glomeruli affected, 2 = 10–40% glomeruli affected, 3 = 40–80% glomeruli affected, 4 = end-stage renal disease (80–100% glomeruli affected). Rats with nephropathy scores ≥ 3 were excluded from all post-weaning analyses.

Osteocalcin assay

Serum levels of carboxylated (cOC) and undercarboxylated (uOC) osteocalcin were measured using commercially available, highly sensitive, rat-specific EIA kits (MK 126 and 146, Takara Bio Inc., Otsu, Japan). All samples were assayed in duplicate and the average result for each sample was used for statistical analysis. The intra-assay CVs were 5.3% and 3.5% for the cOC and uOC assays, respectively.

Gene Expression

At euthanasia the left femur was removed, cleaned of soft tissue, cut into equal length thirds with a small hacksaw, flushed clean of bone marrow using saline, and snap frozen in liquid nitrogen. Prior to RNA extraction the cleaned femoral diaphyses were pre-crushed using a MicroCryoCrusher (BioSpec, Oklahoma, USA) with liquid nitrogen cooling, and then 50 mg samples were homogenized by agitation in a Mini-Beadbeater-16 (BioSpec, Oklahoma, USA) in 1 ml of Tri-Reagent (T9424, Sigma-Aldrich, Auckland, New Zealand). RNA was extracted using chloroform with isopropanol precipitation and the RNA pellet resuspended in 50 µL of diethylpyrocarbonate treated water. The extracted RNA was DNase-treated with TURBO-DNA free (Ambion, Life Technologies, Texas, USA) according to the manufacturer's instructions. RNA concentration and purity was determined using a Nanodrop ND-1000

Spectrophotometer (Thermoscientific, Wilmington, USA) and Qubit 2.0 (Life Technologies, Carlsbad, USA) followed by storage at -80^{0}C prior to further analysis.

The RNA was converted to cDNA using the Roche Transcriptor cDNA synthesis kit (Roche Applied Science, Mannheim, Germany) as per the manufacturer's instructions. A mix of 2.5 µM oligo(dT), 60 µM random hexamers, 1 mM each dNTP, 20 U RNase Inhibitor, 10 U reverse transcriptase, 5X reaction buffer, 250 ng RNA, and water, up to a final volume of 20 µL, was added to each tube. Samples were incubated at 25^{0}C for 10 min, 55^{0}C for 30 min and 85^{0}C for 5 min. Real time quantitative polymerase chain reaction (qPCR) analyses for hydroxymethylbilane synthase (Hmbs), ß-actin (ActB), osteocalcin (OC), insulin receptor (InsR), Forkhead box transcription factor O1 (FoxO1), and osteotesticular protein tyrosine phosphatase (Esp) were performed using the StepOne Real-Time PCR system (Applied Biosystems, California, USA) and Taqman® primer/ probe sets (Applied Biosystems, Life Technologies, Texas, USA). Each qPCR reaction mix contained 1X Taqman® gene expression assay (Applied Biosystems, Life Technologies, Texas, USA), 1X Taqman® gene expression master mix (Applied Biosystems, Life Technologies, Texas, USA), 3 µL cDNA and H_2O, up to a final volume of 10 µL. Thermal cycling conditions included an initial hold at 50^{0}C for 2 minutes, 95^{0}C for 10 minutes, and then 40 cycles of 95^{0}C for 15 seconds and 60^{0}C for 1 minute. All samples were assayed in duplicate. Standard curves were performed to determine the efficiency and R^2 of the primer/probe combinations, which were as follows: OC (assay ID Rn00566386_g1) 95.6% efficiency, R^2=1.00; InsR (assay ID Rn00690703_m1) 94.0% efficiency, R^2=0.98, FoxO1 (assay ID Rn01494868_m1) 95.1% efficiency, R^2=0.98; Esp (assay ID Rn00583620_m1) 101.6% efficiency, R^2=0.99; Hmbs (assay ID Rn00565886_m1) 95.1% efficiency, R^2=0.99; and ActB (assay ID Rn00667869_m1) 95.4% efficiency, R^2=1.00. Target genes were

normalized to the reference genes Hmbs and ActB. The real-time data were analysed using StepOne plus software (Applied Biosystems, Life Technologies Corp., Carlsbad CA, USA) to produce relative expression ratios.

Statistical analysis

Offspring were excluded from analysis if they had a nephropathy score ≥3 or any health problems that might have adversely influenced growth. Several rats also died during or after anesthesia. For the statistical analysis of body weight, grip strength, and imaging data, 1–4 male offspring per dam and 2–4 female offspring per dam were used; the number of animals used in the analyses is given below each table. For serum OC testing, 1 male and 2 females per litter were randomly selected from the eligible animals, females were in any stage of their estrous cycle as this has been shown to have little effect on serum concentrations of uOC and cOC [26]. The same male and one of the females used for OC testing were selected for gene expression testing. Between-group differences in pQCT, DXA, serum OC and gene expression results were assessed using a nested model, with sex, dam exercise group, and their interaction as fixed effects and dam nested within exercise group as a random effect. Log transformation was performed prior to analysis when required to achieve a normal distribution. Day of puberty attainment and the cOC:uOC ratio were not normally distributed, even after transformation, and for these variables the differences between dam exercise groups for each sex were assessed using the non-parametric Wilcoxon test. Pearson's correlation coefficients were determined using the residuals of the variables after fitting the linear model. All data are expressed as lsmeans ± SE unless otherwise indicated. Differences are considered significant if $p \leq 0.05$.

Results

Body composition and size

We previously reported that there were no between dam exercise-group differences in the body weights of these offspring at birth or weaning [13]. This initial lack of difference in body weight persisted throughout the study. At study termination, the weights of the DAMCON offspring versus the DAMEX offspring were 434.37 ± 47.08 g versus 421.68 ± 73.52 g (p=0.14) and 709.93 ± 55.76 g versus 699.45 ± 62.08 g (p=0.97) for female and male animals, respectively. However, body size did differ significantly between the female offspring of exercised and control dams: the mean spine length of the DAMCON female offspring was 16.54 ± 1.61 cm and of the DAMEX female offspring was 15.88 ± 0.53 cm (p=0.005); whereas the mean spine lengths of the males were 18.19 ± 0.52 and 17.99 ± 0.49 cm (p=0.25) for the DAMCON and DAMEX offspring, respectively. Analysis of the DXA scans performed 2 weeks prior to euthanasia revealed significant differences in the body composition of the male DAMEX and DAMCON offspring, but not the females; male DAMEX offspring had a greater percent body fat (p=0.001) and lesser percent lean tissue (p=0.0008) than the male DAMCON offspring (Table 1). Dam exercise group did not significantly influence whole body bone mineral content (BMC), bone mineral density, or bone area as assessed by DXA scanning. The first DXA scan (performed at 114–118 days of age in males only) showed no significant differences between the DAMEX and DAMCON offspring in bone or body composition. Spine length also did not differ between the male DAMCON and DAMEX offspring at that time.

Table 1. Body composition of the offspring of control and exercised dams

	Male		**Female**		***P-values***		
	DAMCON	DAMEX	DAMCON	DAMEX	*Ex*	*Sex*	*Ex*Sex*
% Fat	32.48 ± 1.06	37.55 ± 1.08	35.47 ± 0.98	36.27 ± 0.93	0.25	0.41	0.04
% Lean	64.78 ± 1.03	59.74 ± 1.04	61.47 ± 0.94	60.51 ± 0.90	0.23	0.20	0.04

Data are lsmeans ± SE.
N = 95 offspring from 15 dams.
DAMCON = offspring of control dams, DAMEX = offspring of exercised dams, Ex = dam exercise group.
Significance of difference between % Fat in male and female offspring, p = 0.001 and 0.56, respectively.
Significance of difference between % Lean in male and female offspring, p = 0.0008 and 0.46, respectively.

Puberty attainment and feed efficiency

The age at which the female offspring attained puberty (vaginal opening) was almost significantly different between groups (DAMCON 31.67 ± 2.04 days vs. DAMEX 30.59 ± 1.59 days, p=0.06). Individual feed intake and feed efficiency of the male rats did not differ between the DAMEX and DAMCON offspring at week 16 of life (p=0.41 and 0.42 for intake and efficiency, respectively). Since the female rats were pair-housed individual feed intake and efficiency were not measured.

Peripheral quantitative computed tomography

The results of analysis of *ex vivo* pQCT images of the right proximal tibial metaphysis and mid-diaphysis are shown in Tables 2 and 3. At the proximal tibial metaphysis there were no significant effects of exercise on any parameters, but total BMC trended lower in both male and female DAMEX offspring than in DAMCON offspring (p=0.10). At the mid-tibial diaphysis, the DAMEX offspring had lower cortical volumetric bone mineral density (BMD_v) than the DAMCON offspring (p=0.01) of both genders. Correcting for body weight or spine length in the model did not change the significance of the values obtained.

Table 2. pQCT results at the right proximal tibial metaphysis

	Male		**Female**		***P-values***		
	DAMCON	DAMEX	DAMCON	DAMEX	*Ex*	*Sex*	*Ex*Sex*
Total BMC (mg)	14.59 ± 0.22	13.61 ± 0.22	11.22 ± 0.20	10.79 ± 0.23	0.10	<0.0001	0.19
Total area (mm^2)	24.65 ± 0.52	22.81 ± 0.53	16.23 ± 0.48	15.85 ± 0.54	0.16	<0.0001	0.15
Total BMD_v (mg/cm^3)	595.87 ± 8.20	596.79 ± 8.36	693.97 ± 7.50	686.21 ± 8.40	0.77	<0.0001	0.59
Log Trabecular BMC (mg)	0.62 ± 0.07	0.46 ± 0.07	0.44 ± 0.07	0.36 ± 0.07	0.21	0.04	0.52
Log Trabecular area (mm^2)	2.39 ± 0.04	2.31 ± 0.04	1.82 ± 0.04	1.81 ± 0.04	0.42	<0.0001	0.33
Trabecular BMD_v (mg/cm^3)	174.55 ± 9.93	158.97 ± 9.98	257.57 ± 9.14	241.93 ± 10.23	0.37	<0.0001	0.998
Cort/subcort BMC (mg)	12.52 ± 0.14	12.01 ± 0.14	9.60 ± 0.12	9.26 ± 0.14	0.20	<0.0001	0.53
Cort/subcort area (mm^2)	8.05 ± 0.23	7.51± 0.21	7.93 ± 0.23	8.12 ± 0.21	0.11	<0.0001	0.38
Cort/subcort BMD_v (mg/cm^3)	934.30 ± 12.22	944.82 ± 12.29	964.48 ± 11.26	972.41 ± 12.60	0.55	0.02	0.91

Data are lsmeans ± SE.
N = 94 offspring from 15 dams.
DAMCON = offspring of control dams, DAMEX = offspring of exercised dams, Ex = dam exercise group, BMC = bone mineral content, BMD_v = volumetric bone mineral density.

Table 3. pQCT results at the right mid-tibial diaphysis

	Male		**Female**		***P-values***		
	DAMCON	DAMEX	DAMCON	DAMEX	*Ex*	*Sex*	*Ex*Sex*
Cortical BMC (mg)	10.24 ± 0.14	9.72 ± 0.14	7.08 ± 0.13	6.87 ± 0.14	0.15	<0.0001	0.23
Cortical area (mm^2)	7.50 ± 0.10	7.20 ± 0.10	5.23 ± 0.09	5.15 ± 0.10	0.26	<0.0001	0.25
Cortical BMD_v (mg/cm^3)	1363.96 ± 3.45	1350.17 ± 3.52	1352.29 ± 3.18	1337.33 ± 3.56	0.01	0.0004	0.86
Endosteal circumference (mm)	6.28 ± 0.08	6.17 ± 0.08	5.00 ± 0.07	5.07 ± 0.08	0.89	<0.0001	0.21
Periosteal circumference (mm)	11.56 ± 0.08	11.34 ± 0.09	9.52 ± 0.08	9.51 ± 0.09	0.47	<0.0001	0.21
Log SSI	2.08 ± 0.02	2.01 ± 0.02	1.52 ± 0.02	1.51 ± 0.03	0.17	<0.0001	0.33

Data are lsmeans ± SE.
N = 94 offspring from 15 dams.
DAMCON = offspring of control dams, DAMEX = offspring of exercised dams, Ex = dam exercise group, BMC = bone mineral content, BMD_v = volumetric bone mineral density.

Because the CT scans were done after the tibias had been cleaned of soft tissue we were unable to compare the BMC at the tibial diaphysis directly with the muscle mass in that region. Therefore, we used the DXA values for total body BMC and lean mass to examine the relationship between bone mineral content and muscle. When the head was excluded from the region of interest, the BMC: lean mass ratio of the male DAMEX offspring was significantly greater than that of the male DAMCON offspring

(p=0.0003), but the relationship between BMC and lean mass did not differ between female DAMEX and DAMCON offspring (p=0.23).

Grip strength

Mean forelimb grip strength differed significantly between genders (p<0.0001), but did not differ between dam exercise groups (p=0.24). The average mean grip strengths were 618.87 ± 15.02 g for females and 757.43 ±16.05 g for males. Forelimb grip strength was correlated with total BMC of the tibial diaphysis (R=0.22, p=0.03), total BMD_v of the tibial metaphysis (R=0.22, p=0.03), and total body lean mass (R=0.20, p=0.05). Total body lean mass was also correlated with BMC of the tibial diaphysis (R=0.53, p<0.0001).

Serum osteocalcin

There was large variation between individuals in serum OC concentrations (Table 4), with coefficients of variation of 21.0 and 38.6% for uOC, and 20.7 and 27.8% for cOC, in female and male animals, respectively. However, dam exercise significantly affected offspring serum uOC (p=0.02), with concentrations markedly greater in the male DAMEX offspring relative to male DAMCON offspring.

Table 4. Serum carboxylated and undercarboxylated osteocalcin concentrations in the offspring of exercised and control dams

	Male		**Female**		***P-values***		
	DAMCON	DAMEX	DAMCON	DAMEX	*Ex*	*Sex*	*Ex*Sex*
cOC (ng/mL)	137.62 ± 13.22	152.71 ± 12.36	127.93 ± 9.85	130.30 ± 8.54	0.30	0.19	0.63
uOC (ng/mL)	17.86 ± 2.12	25.42 ± 1.98	17.47± 1.58	18.32 ± 1.37	0.02	0.05	0.08
cOC + uOC (ng/mL)	155.48 ± 14.91	178.14 ± 13.95	145.40 ± 11.12	148.61 ± 9.63	0.19	0.15	0.49
cOC:uOC*	8.77 ± 4.86	6.24 ± 1.27	7.31 ± 0.056	7.23 ± 0.86	0.15 (male)		0.30 (female)

Data are lsmeans ± SE unless otherwise indicated.
N = 44 offspring from 15 dams (1 male, 2 female/dam except for 1 excluded female).
DAMCON = offspring of control dams, DAMEX = offspring of exercised dams, Ex = dam exercise group, cOC = fully carboxylated osteocalcin, uOC = undercarboxylated osteocalcin.
*Not normally distributed therefore values shown are unadjusted means ± SD and non-parametric analysis was performed. P-values were determined by two-sided Wilcoxon test.

Gene expression

The log transformed relative expression levels of OC, FoxO1, InsR, and Esp mRNA are shown in Table 5 and the antilog of the mean expression levels are shown in Figure 1. Dam exercise group did not significantly affect the relative expression of these genes in either gender. Sex was a significant factor in the relative expression of Esp, with the expression levels in the males being approximately twice that in the females. In both males and females, the relative expression levels of OC and FoxO1 were highly correlated ($R=0.79$, $p<0.0001$; Figure 2), as were the relative expression levels of FoxO1 and Esp ($R=0.59$, $p=0.0006$; Figure 3). The relative expression level of InsR trended towards a correlation with the expression levels of OC ($R=0.34$, $p=0.07$), FoxO1 ($R=0.34$, $p=0.07$), and Esp ($R=0.35$, $p=0.06$), but these relationships did not reach significance. There were no significant correlations between the mean expression levels of OC, FoxO1, InsR, and Esp mRNA and serum concentrations of cOC and uOC.

Blood glucose

Blood glucose at euthanasia was lower in females than males (12.41 ± 0.25 versus 14.91 ± 0.27 g/dL, $p<0.0001$), but did not differ between dam exercise group in either gender ($p=0.91$ and 0.26 for males and females, respectively). Blood glucose concentrations were significantly correlated with percent body fat ($R=0.28$, $p=0.007$), but were not correlated with the relative expression of any of the measured genes, or any measure of serum osteocalcin.

Table 5. Log transformed relative expression levels of target genes in the offspring of exercised and control dams

	Male		**Female**		***P-values***		
	DAMCON	DAMEX	DAMCON	DAMEX	*Ex*	*Sex*	*Ex*Sex*
OC	1.26 ± 0.33	0.25 ± 0.31	0.13 ± 0.33	0.18 ± 0.31	0.19	0.09	0.12
InsR	-0.16 ± 0.22	-0.81 ± 0.21	-0.53 ± 0.22	-0.47 ± 0.21	0.18	0.93	0.13
FoxO1	0.094 ± 0.20	-0.41 ± 0.19	0.053 ± 0.20	0.024 ± 0.19	0.38	0.32	0.24
Esp	2.29 ± 0.31	1.67 ± 0.29	0.98 ± 0.31	1.06 ± 0.29	0.33	0.0067	0.26

Data are lsmeans of log transformed relative expression levels ± SE.
N = 30 offspring from 15 dams (1 male and 1 female/dam).
DAMCON = offspring of control dams, DAMEX = offspring of exercised dams, Ex = dam exercise group, OC = osteocalcin, FoxO1 = Forkhead box transcription factor O1, InsR = insulin receptor, Esp = osteotesticular protein tyrosine phosphatase.

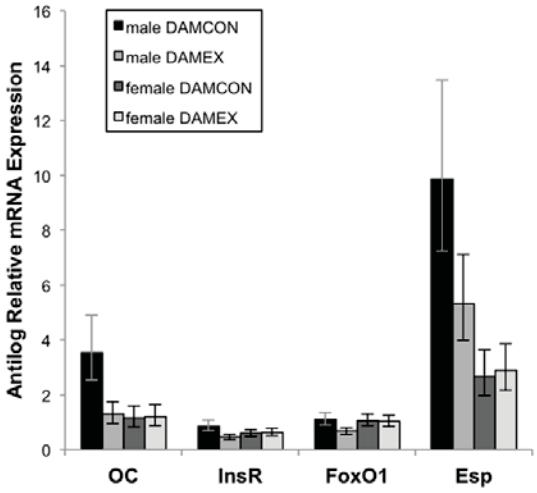

Figure 1. Antilog of relative expression of osteocalcin (OC), insulin receptor (InsR), Forkhead box transcription factor O1 (FoxO1), and osteotesticular protein tyrosine phosphatase (Esp) mRNA. There were no significant differences in the relative expression of these genes in the offspring of exercised and control dams. Males expressed approximately twice as much Esp mRNA as females (p=0.007). Error bars are antilog of mean ± SE on a log scale (as shown in Table 5).

Discussion

To our knowledge these are the first results that demonstrate long-term effects on both body composition and bone in the offspring of dams that performed exercise during pregnancy. The long-term effects of dam exercise were evident in both the male and female offspring, but were greater in the males. Sex differences in programming effects have been demonstrated previously in both humans and animal models [27], and may be mediated by the expression of placental genes that change in response to environmental influences in both a gender- and timing-dependent manner [28]. The differences in the percentages of lean and fatty tissue in the male DAMEX and DAMCON offspring were not significant at the initial DXA scan performed at 114–118 days of age, but were highly significant by 187–193 days of age. That the lower percentage lean mass and higher percentage fat mass seen in the male DAMEX offspring are present without any differences in body weight or length, suggests that our intervention during pregnancy altered a fundamental aspect of the musculoskeletal system of the male offspring so that, when raised under control (non-exercising) conditions, the male DAMEX offspring developed less muscle than controls. This may be due to differences in their basal level of physical activity, or to an increased propensity for their stem cells to differentiate to fat instead of muscle, and may indicate a mismatch between the environment that they perceived during development and the postnatal environment that they experienced. Further research is needed to clarify the mechanisms underlying this alteration in body composition.

Similarly, the differences in the cortical BMD_v of the DAMEX and DAMCON offspring suggest that the exercise performed by the dams during pregnancy resulted in a persistent change to the skeletons of their pups. At the mid-tibial diaphysis, the bones of the DAMEX offspring were both smaller in cross-sectional area and lower in BMC

than the bones of the DAMCON offspring. A proportionally greater difference in between-group BMC than in bone area resulted in the significant between-group difference in BMD_v. Both bone area and BMC change in response to the forces that act on the skeleton, and muscles are the primary source of these forces [29]. The relationship between muscle and bone can be assessed by examining the ratio of bone mineral and lean mass. In this study, the whole body BMC: lean mass ratio was greater in the male DAMEX offspring once the head, which has a much greater BMD than long bones and thus could obscure subtle whole-body changes, was excluded from the region of interest. These results indicate that the relationship between bone and muscle was fundamentally altered in the male DAMEX offspring, and that (although they had lower BMD_v and BMC) they had more bone mineral per gram of lean tissue than the DAMCON offspring. That such an alteration resulted from a relatively mild exposure to exercise during gestation lends support to previous reports that the bone: muscle relationship is affected by early-life influences [30].

Further evidence for long-lasting effects of early-life circumstances on the skeleton is the significant between-group difference in serum uOC concentrations, which also suggests a fundamental alteration in the bone biology of the DAMEX offspring. Osteocalcin, the most abundant non-collagenous protein produced by osteoblasts, is subject to the post-translational addition of three carboxyl groups to its amino acid chain; these increase the affinity of OC for calcium and hydroxyapatite [31]. Undercarboxylated OC lacks one, two, or all three of these carboxyl groups, and has recently been shown to act as a bone-derived hormone that regulates glucose handling (reviewed in [32]). In mice, the amount of uOC released from bone depends upon the relative expression of the genes FoxO1 [33] and Esp [20,33] within the osteoblast, as well as insulin receptor activity [34], and the vitamin K status of the animal [35]. The

release of uOC from bone is at least partially linked to bone resorption [36], as the acidic pH of the resorption lacuna during osteoclastic bone resorption stimulates the decarboxylation of the cOC freed from the dissolved bone matrix [34,37]. In our study, male DAMEX offspring had significantly higher uOC concentrations than male DAMCON offspring; however, their blood glucose levels did not differ. Since the expression levels of OC, FoxO1 and Esp also did not differ between groups, this suggests a difference in the balance between bone formation and resorption, with the balance shifted slightly more towards resorption in the DAMEX than the DAMCON offspring. This might be due to a mismatch between their control environment and the exercise environment that the DAMEX offspring expected.

There is now considerable evidence that uOC can act as a bone-derived endocrine hormone regulating metabolism in mice. This was first demonstrated in OC knockout mice, which develop a metabolic syndrome that includes high blood glucose, low serum insulin concentrations, and poor glucose tolerance [20]. A recent study found that injections of uOC reduced blood glucose and improved insulin sensitivity in normal mice and mice fed a high fat diet [38], providing further evidence of the important role of uOC in regulating glucose handling in the mouse. However, data from human studies have been less conclusive [39,40], and there have been no previous studies that report on the role of uOC in the metabolism of the rat. In the current study we found no correlations between blood glucose concentrations and the serum concentrations of uOC or cOC in Wistar rats. However, the studies that defined the relationship between uOC and glucose handling used knockout mice, which would have had much more severe perturbations in OC levels than our normal rats. In our animals the effects on blood glucose concentrations of changes in serum uOC or cOC levels might be too small to detect, especially considering the large individual variation in serum OC concentrations.

We chose to investigate the expression of the OC, FoxO1, Esp, and InsR genes because FoxO1, Esp, and InsR are components of a recently described positive feedback loop that regulates the endocrine function of OC through effects on OC gene expression and carboxylation [34,41]. However, the relative expression levels of OC, FoxO1, Esp, and InsR mRNA in the mid-femur were not associated with serum cOC or uOC concentrations. There are several possible explanations for this lack of correlation. First, protein expression is determined by mRNA synthesis and degradation, and protein synthesis and degradation. A large scale study, which investigated the correlation between mRNA and protein levels *in vitro* in several thousand mouse fibroblast genes, found that mRNA levels accounted for only slightly more than 40% of the variation in protein levels [42]. Although this correlation is higher than correlations previously reported in mammalian studies [42,43,44], it is still low enough to suggest that regulation of protein expression is primarily post-transcriptional. Second, the lack of correlation between the expression of genes involved in OC production and regulation with serum OC concentrations in this study may also be due to the limited number and size of samples that we used for gene expression analysis. Reverse transcription qPCR was performed only on samples from the mid-femur, thus the results may not be indicative of the relative gene expression levels of the entire skeleton. Perhaps if whole skeleton expression of OC, InsR, FoxO1, and Esp were assessed, or if we had used a larger number of animals, there would have been significant correlations between expression levels and serum concentrations of cOC and uOC. However, in spite of the lack of association between gene expression and serum OC levels, the significant correlations between FoxO1 and both OC and Esp mRNA expression provides further verification of their involvement in a common pathway; as do the almost significant correlations between InsR and OC, FoxO1, and Esp mRNA expression. This, coupled

with the increase in serum uOC concentrations seen in the male DAMEX offspring, suggests that the role of uOC in the metabolism of rats may be similar to its role in mice, and that it may also be similarly regulated. However, further studies examining both mRNA and protein expression are needed to clarify the role of, and regulation of, osteocalcin expression and carboxylation in rats.

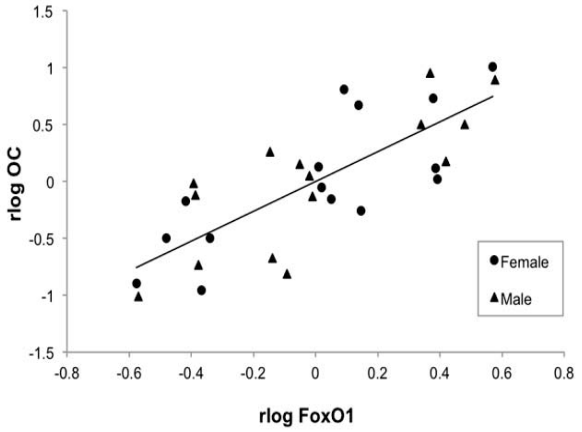

Figure 2. Correlation between the relative expression of Forkhead box transcription factor (FoxO1) and osteocalcin (OC) mRNA**.** The expression of FoxO1 and OC were significantly correlated (R=0.79, p<0.0001). rlog FoxO1 = residuals of log of FoxO1 relative expression. rlog OC = residuals of log of OC relative expression.

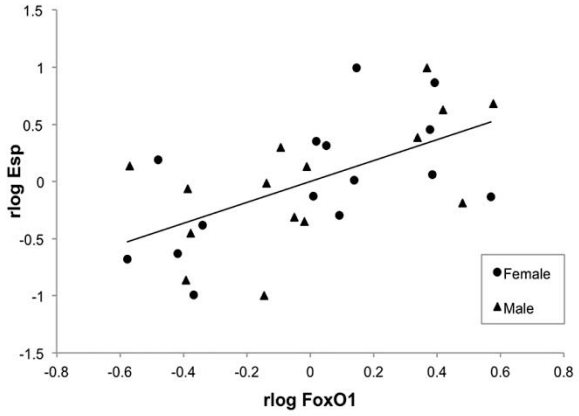

Figure 3. Correlation between the relative expression of Forkhead box transcription factor (FoxO1) and osteotesticular protein tyrosine phosphatase (Esp) mRNA**.** The expression of FoxO1 and Esp were significantly correlated (R=0.59, p=0.0006). rlog FoxO1 = residuals of log of FoxO1 relative expression. rlog Esp = residuals of log of Esp relative expression.

Other limitations of our study include the relatively small number of dams used. Although the number of offspring used in the analyses is fairly large, the exercise intervention was performed on the dams and thus a nested analysis was used to account for the fact that the dams were the actual experimental unit. In many of the statistical analyses the effect of dam within exercise group was highly significant, indicating that it is very important to include dam as a factor when analyzing data from studies such as

this one. By including dam within exercise group in our statistical models when testing whether exercise significantly affected the outcome variables, we controlled for the influence of individual dams on offspring outcomes and accounted for the hierarchical nature of our data [45]. In addition, it is important to recognize that the female offspring were approximately 40 days older than the males at the time of scanning and at sample collection. This was done for logistical reasons, but the age difference must be acknowledged when considering the effects of sex on our results. However, since all rats were over 200 days old (fully mature but not geriatric) at the time of sample collection we consider it unlikely that the difference in the ages of the male and female offspring at scanning and sample collection had a significant effect on our findings. Also of note are the number of rat offspring that were affected by nephropathy in later life. Although all of the rats appeared to be healthy at birth, by the end of the study period a total of 8 rats had significant nephropathy (histologic score ≥ 3). Of these, 6 were male, 2 were female, and all were DAMEX offspring from one of three dams. Chronic progressive nephropathy in laboratory rats is a known entity in many strains of laboratory rats, and is most commonly seen in aging males [46]. Whether the occurrence of this condition in only the DAMEX offspring in our study is related to the maternal exercise or to genetic predisposition of the dams who happened to be in the exercise group is difficult to determine with the number of animals per group that we used. Certainly nephron development occurs during gestation and the early postnatal period, and the number of nephrons that the offspring have is affected by environmental conditions during pregnancy, effects which may not be evident in altered birth weight [47]. However, most of the DAMEX offspring in our study did not have significant nephropathy, suggesting that there are other factors underlying the development of this condition. Because of the relationships between kidney disease, growth, parathyroid

hormone and OC concentrations, and bone mineralization, we decided to screen all animals for histologic evidence of nephropathy and to exclude from the analysis any with a nephropathy score of ≥ 3. Thus, the animals that we have included in our analyses were physiologically "normal" to the best of our knowledge, and our results reflect the effects of maternal exercise during pregnancy on these normal offspring. Whether the maternal exercise itself predisposes the rats to develop nephropathy is a topic for future research.

The differences that we observed between the DAMEX and DAMCON offspring were triggered by a minor intervention during development, but the actual factor or factors associated with the exercise performed by the dams in this study that resulted in these changes is difficult to determine. The increase in fatness seen in the DAMEX offspring is similar to the changes seen in the offspring of rat dams that were undernourished during pregnancy. Our exercising dams did have to stand to reach their food and water, and did have numerically (but not significantly) lower food intakes during pregnancy than did their control counterparts [13]. Perhaps this small reduction in food intake may have resulted in a subtle nutritional stress on the developing offspring that, over time, resulted in relatively mild but detectable changes in body composition. Nutritional stress during pregnancy and around the time of conception can also result in a reduced activity level in the offspring, as has been demonstrated in both rats [48] and sheep [49].

It is also possible that the between-group differences in our study resulted from predictive adaptive responses of the DAMEX offspring to an anticipated living situation that included exercise, and that the subsequent lack of exercise in their postnatal environment (control conditions for laboratory rats) resulted in a mismatch. A mismatch situation is one in which an organism makes predictive adaptive changes during its

development in response to environmental cues, responses that would aid its survival in the environment that it expects to encounter, but then is born into an environment in which its developmental adaptations are no longer advantageous and may even be deleterious [50]. In the current study, possibly the DAMEX offspring developed anticipating a greater amount of exercise than they were allowed during postnatal life, and thus became fatter and had lower bone density than the DAMCON offspring in identical living conditions; in the latter group, the environmental conditions perceived during gestation and experienced postnatally were the same. Interpreting the differences in uOC in the male offspring, and the altered bone: muscle relationship (also more pronounced in males), is difficult at this time. Future studies that examine the effects of dam exercise during pregnancy on long-term outcomes in offspring that are also exercised postnatally will provide more insight into the specific factors underlying the effects of maternal exercise during pregnancy on adult offspring health.

In conclusion, the results of our study provide the first evidence that very moderate voluntary exercise during pregnancy can result in lasting changes to the musculoskeletal system and adiposity in the offspring without differences in birth weight. Our results provide further support to the concept of the skeleton as an organ that can be permanently altered by fetal programming, and suggest a link between uOC and metabolism in the rat.

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Chapter 6

GENERAL DISCUSSION AND CONCLUDING REMARKS

Preface

Chapters 2 through **5** of this thesis present the results of a series of experiments that examine the relationship between maternal exercise during pregnancy and adult offspring musculoskeletal health and body composition in a rat model. As each of these chapters has been previously published, the results are discussed within the chapter in which they are presented. Within this general discussion in **Chapter 6** the *Principal Findings* of the experimental work are summarised, *Discussion* of important aspects of the results is further developed, the overall *Limitations and Strengths* of this work are presented, general *Conclusions* are drawn, and *Future Directions* are suggested. Since this thesis includes publications that were written to stand independently there will inevitably be some repetition of points from the discussion within individual chapters, but every effort has been made to avoid this when possible.

Principal findings

The experiments conducted for this PhD research were iterative in nature, and tested the hypotheses presented in **Chapter 1**. The results of the experiment described in **Chapter 2** confirmed our hypothesis that both bipedal stance and tower climbing exercise were sufficiently strenuous to cause a measurable change in the tibia of non-pregnant rats when performed over a short period of time (equivalent to the length of rat gestation) without causing a physiological stress response in the exercising animal [1]. Since these results indicated that both exercise types were suitable for extrapolation to work in pregnant rats, the exercise paradigms were then tested in pregnant animals (**Chapter 3**). As hypothesised, both forms of exercise enhanced fetal growth through day 19 of pregnancy, and neither was associated with increases in excretion of faecal corticosterone metabolites in gravid female rats. However, although we had hypothesised that exercise would cause significant effects on dam bone parameters, pQCT did not reveal significant differences between the tibias of exercised and control dams [2]. The exercise that resulted in greater fetal effects, rising to an erect bipedal stance, was chosen for use in the third study. Pregnant rats were allowed to exercise throughout pregnancy, and offspring were raised to mature adulthood. The results reported in **Chapter 4** confirmed our hypotheses that exercise during gestation would not impact upon litter size but would improve the dams' bone parameters at the end of lactation. However, contrary to our expectations, maternal exercise did not affect offspring birth or weaning weight [3]. Offspring outcomes during mature adulthood are reported in **Chapter 5**. Maternal exercise was associated with alterations in bone and body composition, as well as between-group differences in undercarboxylated osteocalcin (uOC) concentrations, but the nature and gender-specificity of these differences was novel and unexpected. In the adult male offspring, maternal exercise

was associated with increased adiposity and serum uOC concentrations, while maternal exercise was associated with lower volumetric bone mineral density (BMD_v) of the mid-tibial diaphysis in mature offspring of both genders [4]. Thus, although maternal exercise did lead to differences in bone, body composition, and serum uOC concentrations as hypothesised, these differences were not as clearly beneficial as we had predicted.

In addition to the ordered testing of the hypotheses presented in **Chapter 1**, further hypotheses were tested in an effort to determine possible mechanisms underlying the programming effects of maternal exercise. The work on gene expression, puberty attainment, and feed efficiency described in **Chapter 5** was aimed at elucidating the factors underlying the observed phenotypic changes in the mature adult offspring. In addition, our work regarding nephropathy in our rats (**Appendix C**) was not hypothesis driven but was done as a result of finding some affected animals in our study population.

Discussion

As described in **Chapter 2**, in non-pregnant female rats both bipedal stance ('squat') and tower climbing exercise resulted in measurable gains in bone mineral content (BMC) relative to that of non-exercised control rats. Faecal corticoid content did not differ between groups, indicating that the neither exercise initiated a physiological stress response in the exercising animals. These results suggest that both bipedal stance and tower climbing exercise may be suitable for use in studies examining the early life foundations of bone health and energy metabolism. The work described in **Chapter 2** was particularly concerned with determining the corticoid response, or lack thereof, associated with the specific exercise modalities tested because stress needed to be eliminated as a potential confounder. Evidence suggests that a relationship exists between the hypothalamo-pituitary-adrenal axis and energy metabolism [5], and that the nature of this interplay is established during early life [6]. Disruption of placental 11ß-hydroxysteroid dehydrogenase-2 inactivation of maternal corticosterone (cortisol in humans), results in increased exposure of the fetus to active maternal glucocorticoids. This leads to aberrant developmental programming effects as per the glucocorticoid programming hypothesis described in the **Literature Review** [7]. The lack of difference in faecal corticoid output between the exercising and non-exercising animals confirmed that both exercises were suitable for further validation in pregnant animals.

The tibial response to the moderate voluntary exercises tested in **Chapter 2** indicated that the loads generated by the performance of both exercises were sufficient to stimulate bone formation, most likely because the exercise caused novel bone strains. This is unsurprising given the lack of previous opportunity for exercise that the rats would have experienced while housed in standard laboratory rat cages. Since bones adapt to cope with the forces that they experience [8], prior to beginning the exercises in

this study the rats would have had bones adapted to a relatively sedentary lifestyle. Of note is the rapidity of the tibial response to exercise. The rats were exercised for only 26 days (including a 5 day acclimation period), yet they gained substantial bone mineral during that time. Although many studies have demonstrated positive effects of exercise on bone formation in the rat [9,10,11,12], few have examined the effects of exercise of such limited duration on bone. Holy *et al.* did demonstrate increased mineral in the ashed tibias of 9-week old rats that had performed 4 weeks of voluntary wheel running exercise immediately prior to sample collection [13]. However, the results of their study are not directly comparable to the results described in **Chapter 2** because the rats they exercised were younger, and thus still growing at a faster linear rate, than our rats; they would have been undergoing greater bone formation even in an unexercised state. In addition, their rats ran mean weekly distances ranging from 20 ± 3 to 50 ± 8 km/week. Although we did not directly measure the amount of exercise performed by our rats, it is unlikely to have been even remotely equivalent to running distances of this magnitude. Acute changes in serum markers of bone formation may be evident after a single session of exercise in humans [14,15], but measurable enhancements of BMC have not been demonstrated at such an early stage.

More relevant to the rapid bone response to both bipedal stance and tower climbing exercise described in **Chapter 2** are the increases in bone mass and modelling changes identified by Mosley *et al*. using an *in vivo* ulna loading model in rats [16]. Ten days of dynamic loading of the ulna of anaesthetised rats at strain magnitudes within the normal physiological range resulted in changes in bone length and site-specific periosteal bone formation, as well as modelling drifts resulting in reduced curvature of the ulna. However, although the authors considered the strains they used to be within the normal physiological range, they based this range on the strains experienced by the

rat ulna during landing from a 30 cm drop. While similar strain magnitudes likely do occur in wild rats, they would be unlikely to occur 1,200 times in rapid succession as applied in the ulna loading model, and thus the response of the ulna described by Mosley *et al.* is more indicative of a response to a supraphysiological loading pattern than to normal physiological loading. Another, more recent, study demonstrated a reduction in femoral strength and cortical area after 5 days of free fall loading (10 or 30 free fall landings per day from a height of 40 cm) [17]. This may be a transient response of the bone to loading, which would be followed by subsequent increase in bone strength and associated parameters, or may be indicative of physiological stress induced by the loading protocol with subsequent negative glucocorticoid-related effects on bone. The results presented in **Chapter 2** provide evidence of more rapid bone formation in response to moderate exercise than other work available in the literature, without the potential confounding factor of an exercised-induced corticoid response.

Based on the findings presented in **Chapter 2**, we hypothesised that bipedal stance and tower climbing exercise in pregnant rats would result in tibial modelling similar to the response observed in non-pregnant rats, but this did not occur. The data obtained from pQCT of the proximal tibial metaphysis and mid-tibial diaphysis (presented in **Chapter 3** Tables 1 and 2, respectively) showed no differences in either absolute values or delta change in the mean BMC of the tower climbing or bipedal stance exercise groups versus controls. This lack of a tibial response in pregnant rats to exercises which had induced a significant increase in BMC in non-pregnant rats may have been due to the tendency of rats to accrue bone during pregnancy in order to meet the calcium demands of the fetuses during late gestation and lactation. Bone formation during pregnancy may be due to alterations of the bone formation/resorption thresholds in response to hormones or other physiological factors associated with pregnancy. In

addition, absorption of calcium by the small intestine increases by mid-pregnancy in the rat, well before the periods of intense calcium demand that occur during late gestation and lactation [18]. This allows the pregnant dams to store calcium within their skeleton [19,20], and isotope studies have demonstrated that approximately 92% of the calcium found in the fetal skeleton was absorbed from the maternal diet during pregnancy [21]. Calcium accretion within maternal bone during early/mid pregnancy results in net maintenance of the calcium content of the maternal skeleton over the pregnancy period [20]. If dietary calcium restriction prevents early accrual of bone mineral during pregnancy, a net loss of skeletal bone mineral will occur over gestation [22]. Since the accretion of bone mineral during pregnancy would result in reduced exercise-related bone strain, and bone adapts its material composition and architecture in response to strain, the ability of the exercises performed by the pregnant rats in our study to stimulate bone formation was likely reduced by the pregnancy-associated increase in bone mineral.

However, while the dam tibias did not significantly respond to the exercises tested in **Chapter 3**, both fetal size and weight were significantly affected by maternal exercise. Fetuses from dams that performed bipedal stance exercise were heavier, longer, and had heavier placentas than control fetuses. Tower climbing dams had longer fetuses with heavier placentas than controls, but fetal weight did not differ from that of control fetuses. Importantly, these effects of exercise were most evident in mid-uterine horn fetuses, suggesting that regional differences in blood flow or other factors amplified or reduced the effects of dam exercise on the fetuses. This has important implications for studies investigating the developmental origins of health and disease (DOHaD) theory using the rat model. In these types of studies, litter sizes are typically reduced to a standard number of offspring (often 8 or 10) after birth to minimize

differences in pre-weaning nutrition and maternal care between litters. Since intrauterine position during gestation may affect the degree to which the neonate was affected by potential programming influences during pregnancy, culling of offspring that developed in specific intrauterine regions may alter the results, particularly in studies that use only a small number of dams. Unfortunately, it is difficult to know where in the uterus a specific pup developed once it has been born; however, significant intrauterine position-related differences in fetal weight at day 19 of pregnancy were evident, with the heaviest fetuses being located near the cervix and the lightest at the ovarian ends of the uterine horns. This difference may not persist until birth, at which time the pups will weigh approximately 3 times their weight at gestational day 19 [23], but the results reported in **Chapter 3** suggest that rat pups of mid-range birth weights will be more sensitive to the environment experienced by their dams during pregnancy than their high- or low-end birth weight litter mates.

Although the biological imperative to add bone during pregnancy superseded the effects of exercise on the tibia in the experiment described in **Chapter 3**, the absence of a measurable effect on tibial bone parameters assessed by pQCT at day 19 of pregnancy did not preclude the possibility of a significant effect of pregnancy exercise on maternal bone later in the reproductive cycle. In **Chapter 4** the effects of maternal exercise throughout pregnancy on maternal bone post-lactation and on early offspring outcomes was examined. Bipedal stance exercise was selected based on the greater fetal response to this exercise than to tower climbing, coupled with the lack of a significant maternal corticoid response to either exercise. Indeed, the findings reported in **Chapter 4** demonstrate that exercise during pregnancy did benefit the maternal skeleton, with exercised dams having a greater tibial strength strain index and cross-sectional moment of inertia in the frontal plane at the end of lactation than non-exercised control dams. To

our knowledge, no other studies have examined the effects of exercise during only pregnancy on maternal post-lactation bone parameters.

In **Chapter 4** we also report further evidence that bipedal stance exercise did not increase the maternal physiological stress response. Faecal corticoid output measured during early (days 3–7) and late (days 17–20) pregnancy did not significantly differ between exercised and control animals. However, as shown in Figure 3 in **Chapter 4**, there was a numerical but non-significant difference in faecal corticoid excretion between groups during late pregnancy, with exercised dams having numerically greater faecal corticoid excretion than controls. Although this difference was not significant, it merits further discussion because of the importance of determining a potential stress response to maternal exercise. The non-significant increase in faecal corticoid excretion of exercised dams when compared with that of controls was small enough in relation to other results presented in the literature that we believe it is not indicative of a level of maternal stress that would be of consequence to the developing offspring. To put these results into context, Contarteze *et al.* reported nearly 4-fold higher plasma corticosterone concentrations relative to controls in rats sacrificed shortly after the last of several sessions of treadmill exercise, and nearly 8-fold higher levels shortly after the last of several sessions of forced swimming [24].

Female rats exhibit a more rapid stress response to exercise than males, and it has been reported that non-pregnant female rats had plasma corticosterone concentrations nearly triple that of sedentary controls after only 5 minutes of forced wheel running, while male rat plasma corticosterone concentrations after performing the same exercise were only 37% higher than controls [25]. The response of pregnant rats to exercise is of a similar magnitude, and post-exercise plasma corticosterone in treadmill-trained late pregnant rats was approximately 3–4 times greater than in sedentary late

pregnant control animals; fetal plasma corticosterone concentrations were also significantly elevated [26]. Voluntary exercise also increases corticosterone release in rats. For example, when 7-week-old male rats were allowed 4 weeks of voluntary wheel running their plasma corticosterone concentrations, which were measured at three different times on the last day of each week, were 2–3 times greater in the morning (the time with the greatest difference) than those of sedentary controls—these differences were significant for the first two weeks of exercise [27]. In contrast, in the evening the differences in plasma corticosterone were significant at the end of week 1, when the exercised group had concentrations almost twice that of the control group, but by week 4 the difference between the plasma corticosterone concentrations of the two groups had declined to approximately 11% [27]. These results indicate that even voluntary exercise can induce an increase in plasma corticosterone in rats and that this effect is greatest in the morning; faecal corticoid excretion would thus reflect this increase in the evening since there is a mean time lag of 16.7 hours between the release of corticosterone into plasma and excretion of its metabolites in the feces [28]. The numerical, but non-significant, corticoid increase that we observed in the exercised rats described in **Chapter 4** was of a much lesser magnitude than the corticosterone responses to exercise described in previous studies. Although it is possible that there was a small physiological stress response to the bipedal stance exercise, our results suggest that this response, if it even existed, was much less than the response seen with other forms of exercise and thus bipedal stance exercise was quite suitable to be used to determine the effects of maternal physical activity during gestation on offspring outcomes without the confounding effects of stress-mediated programming of the fetuses.

The offspring outcomes reported in **Chapters 4 and 5** did not differ between the offspring of exercised and control dams in neonatal or early adult life, but there were

significant differences in BMD_v and adiposity in later life. The greater weight of the fetuses of exercised dams relative to that of the fetuses of control dams at day 19 of pregnancy (reported in **Chapter 3)** was not evident in neonatal body weight at day 1 of lactation or at weaning (**Chapter 4**, Table 1). Thus the between-group differences seen in the mature adult offspring at the time of study termination may reflect differences in the intrauterine environments experienced by the offspring that were neither sufficiently adverse nor enhanced as to cause birth weight differences. Since day 19 of pregnancy is after the period of fetal organogenesis, but before the period of greatest absolute fetal weight gain, the differences observed at day 19 suggest that maternal exercise may have had effects on organ development or metabolism even in the absence of differences in weight shortly after birth. Similarly, the lack of differences in the body composition and length of the male offspring at 114–118 days of age (in contrast to the increased adiposity seen at 187-193 days of age) also suggests that early growth of the male offspring was not affected by maternal exercise during pregnancy.

However, the situation in female rats was slightly different. The first indication of a difference between the female offspring of exercised and control dams was the trend ($p=0.06$) towards earlier vaginal opening (indicating puberty) in the offspring of exercised dams; these animals attained vaginal opening almost 1 day earlier than control offspring. The onset of puberty is related to body weight and leptin in rats [29], and the mean body weights were 110.7 ± 4.7 g and 115.5 ± 5.0 g ($p=0.50$) for the female offspring of exercised and control dams at the time of vaginal opening; the lack of a statistically significant difference suggests that maternal exercise did not significantly affect the weight at which the female offspring attained puberty. The almost 1 day reduction in age at puberty that we observed in our rats is somewhat less than the 1.3 day reduction in age at puberty seen by Sloboda *et al.* in the female offspring of dams

that were undernourished during pregnancy [30]. However, the latter undernourished dams were fed only 50% of a control diet, and the authors speculated that the earlier onset of puberty in prenatally undernourished animals might be a predictive adaptive response to an anticipated shorter life span [30]. Such food restriction is an intervention of much greater magnitude and likely programming effect than the very moderate exercise that we imposed during pregnancy or than the non-significant difference in maternal food intake that we observed. The offspring of undernourished dams in two studies achieved vaginal opening at a lower body weight than control offspring.[30,31] contrasting with the non-significant difference in the body weights of our rats at that time, despite puberty being reached almost 1 day earlier in the offspring of exercised dams than controls. This suggests that the female offspring of exercised dams in our study attained a weight and metabolic status sufficient to allow puberty slightly earlier than the offspring of control dams, possibly indicating a slightly faster rate of maturation. The spine length of the female offspring of exercised dams was also slightly shorter than that of controls, which is consistent with more rapid maturation. Since we have demonstrated that the maternal exercise did not initiate a significant corticoid response in the dam, the underlying reason for a change in maturation rate is unknown. The adipokine leptin signals metabolic information to the reproductive system [32] and also exerts regulatory effects on bone [33]. It is thus tempting to speculate that differences in leptin expression or sensitivity may underlie both the timing of puberty and spine length differences that we observed between the offspring of exercised and control dams. In our study we did not assess the timing of puberty onset in male rats, but sex differences in the pubertal response to undernutrition during gestation have been reported [31].

In the adult offspring there were sexually dimorphic responses to maternal exercise during pregnancy in adiposity, BMC: lean mass (LM) ratio, and serum concentrations of uOC. Male offspring of exercised dams had a higher percentage body fat and BMC: LM ratio than the male offspring of control dams, whereas the females did not (Table 1, **Chapter 5**). Similarly, male offspring of exercised dams had significantly higher concentrations of serum uOC than male offspring of control dams, but females did not (Table 4, **Chapter 5**). Sexually dimorphic responses have been reported in response to many different developmental influences. A recent review described gender differences in the offspring response to maternal overnutrition, maternal undernutrition, placental insufficiency, maternal renal insufficiency, angiotensin II receptor insufficiency, and excess glucocorticoids [34]. Differences between the sexes appear very early in development: pre-implantation embryos have sex-dependent transcriptome differences and, in some mammalian species, embryos produced *in vitro* exhibit gender differences in their rate of development [34]. Such differences between genders might result in different programming responses to *in utero* stimuli. Alternatively, or as well, the actions of sex hormones and other postnatal gender differences might result in altered progression of the phenotypic response to initially similar developmental alterations, resulting in different long-term outcomes. A recent study found that the methylation status of certain genes in humans exposed to famine during the periconceptual period not only differed from that of unexposed siblings, but were also significantly different between men and women in the famine-affected group [35,36]. Since epigenetic changes may be established by *in utero* programming, or may be secondary to the progression of a programmed phenotype [37,38], this does not clarify at what stage of development the sexual dimorphism arose. However, fetal mice whose dams were fed a high-fat diet during pregnancy exhibited

sexually dimorphic changes in the placental expression of multiple genes at 15.5 days gestation [39], which suggests that innate gender differences during *in utero* development might play a role in the between-sex differences in adult outcomes resulting from fetal programming influences.

A sexually dimorphic response to maternal physical activity during pregnancy was reported in a cohort of 439 offspring of women who had answered a series of questions regarding their physical activity during the second trimester [40]. More time spent walking or bike riding was associated with lower concentrations of high density lipoprotein, and higher body mass index, blood pressure, and leptin concentrations in the twenty-year-old male offspring. In the female offspring, only the association with high density lipoprotein levels was evident. Although surveys of physical activity have limited reliability and validity [41], it is interesting to note that the results of this survey-based study are similar to those reported in **Chapter 5**. Following our controlled pregnancy exercise intervention we observed increased adiposity of the male offspring of exercised dams, suggesting adverse metabolic consequences of *in utero* exposure to exercise, while females showed only a slight reduction in length but had equivalent body composition to that of controls [4].

The authors of the survey-based study, Danielsen *et al*., suggest that their results may be due to adaptation of the fetus to an intrauterine environment characterised by intermittent periods of reduced oxygen and glucose availability as a result of maternal physical activity, and that this may cause effects similar to those of maternal undernutrition [40]. The diversion of blood flow away from the viscera and towards the muscles and skin during exercise results in decreased availability of nutrients to the fetoplacental unit, but blood flow and nutrient availability quickly return to normal once exercise stops [42]. Although in the study described in **Chapters 4 and 5** the exercised

dams performed only very moderate exercise and did not significantly differ in maternal food intake and feed efficiency, intermittent periods of reduced nutrient availability as a result of maternal physical activity, although likely mild in severity and brief in duration, might have resulted in developmental programming for greater fatness. The disparity between the environment anticipated, based on prenatal experiences *in utero* (nutrient scarcity), and the nutrient abundance of the postnatal environment may represent a mismatch situation, in which a predictive adaptive response results in a phenotypic disadvantage in an unexpected postnatal environment [43]. Alternatively, the observed difference in fatness between the male offspring of exercised and control dams that we observed might be due to differences in the physical activity level of the offspring, which we did not quantify due to resource constraint. Maternal periconceptual undernutrition in sheep, and undernourishment throughout pregnancy in rats, both reduce the voluntary physical activity of the adult offspring [44,45].

The phenotypic differences between the offspring of exercised and control dams may be indicative of an exercise mismatch situation in the offspring of exercised dams. Perhaps the offspring of exercised dams "expected" more exercise in postnatal life than they were allowed. All offspring in our study were kept in a control laboratory rat environment, which provides very limited opportunities to exercise. Had these rats been permitted more physical activity, such as through group housing in open rooms or through access to running wheels or other means of exercise, the results might have demonstrated more positive effects of maternal exercise on later offspring body composition. It is unfortunate that no data are available on the physical activity level of the offspring in the Danielsen study, or from our own work.

In contrast to the observation that maternal exercise during pregnancy may negatively affect aspects of male energy metabolism [4,40], Carter *et al*. [46] reported in

2013 that the female offspring of rat dams allowed voluntary wheel running before and throughout pregnancy and early lactation demonstrated lower fasting plasma insulin concentrations and improved glucose disposal (lower area under the curve after oral glucose challenge) relative to controls at 15 months of age. There were also tissue-specific differences in glucose uptake between the female offspring of exercised and control dams, with the offspring of exercised dams showing greater glucose uptake in the extensor digitorum longus and gastrocnemius muscles and lesser uptake in cardiac muscle when in a hyperinsulinemic euglycemic steady state. No such data were available from male offspring in the Carter study, as the authors chose to use only females to conserve resources; this precludes comparison of the response to maternal exercise between the sexes. Also, notably, no attempt was made in the Carter study to account for dam effect in the analysis of the results; however, their data from the female offspring suggest a beneficial influence of exercise on offspring glucose disposal. Whether male offspring of the dams in their study would have shown similarly beneficial effects of maternal exercise is unknown. Certainly the mechanisms underlying the sex differences in the effects of maternal exercise remain to be elucidated, but males and females have been shown to be differently susceptible to the programming influences of maternal nutrition [34], and exercise may also act to permanently alter organism development in a similar and sex-specific manner.

Several mechanisms have the potential to explain the significantly lower bone mineral density of the tibial diaphysis in both the male and female offspring of exercised dams, as well as the higher serum concentration of uOC in males only. One is a lower physical activity level of the male offspring of exercised dams relative to controls, with an associated increase in fatness and reduction in muscle mass. The correlations between muscle area and BMC, total, and cortical area of the humerus as

assessed by magnetic resonance imaging in tennis players [47] indicate a relationship between muscle size and the force applied by the muscle to the skeleton. However, the differences in muscle area explained only 12–16% of the differences in the aforementioned bone parameters, suggesting that factors other than increased muscle size also play a role in the bone response to exercise [47]. The bones of less active animals may be subject to stresses of lower frequency and magnitude because of the lower activity level, smaller muscle size, and other possible factors such as differences in neuromuscular activation. Since bones maintain their strength through material (gain or loss of bone mineral) and architectural adaptations to the loads imposed upon them [48], lower forces result in lower bone strength. An extreme example of this is the bone resorption and accompanying reduction in bone strength that occurs after extended limb immobilization [49]. Bone is a labile organ and the balance between bone formation and resorption is necessary for both its structural and homeostatic functions; even slightly reduced physical activity might shift that balance towards resorption. Since uOC is released from resorbed bone, increased resorption would lead to increased serum concentrations of uOC similar to those observed in the male rat offspring of exercised dams (**Chapter 5**). That raised uOC concentrations were not also evident in the female siblings of these male rats, in spite of their similarly lower BMD_v at the mid-tibial diaphysis, suggests possible gender differences in the effects of maternal exercise on offspring bone.

Leptin is another potential mediator of the observed effects of maternal exercise on the male offspring. Leptin is produced primarily by adipocytes [50], transported across the blood-brain barrier [51], and regulates energy metabolism through appetite suppression and enhancement of energy expenditure [52,53]. Leptin also has both osteogenic and antiosteogenic effects [54]: in the peripheral circulation it stimulates

differentiation of mesenchymal stem cells to osteoblasts, while centrally leptin acts in the hypothalamus to stimulate the sympathetic nervous system, leading to increased osteoblast expression of the receptor activator of nuclear factor kappa B ligand (RANKL) and thus stimulating osteoclast differentiation and promoting bone resorption [54]. However, although enhanced resorption seems to be the main central effect of leptin on bone, leptin may also stimulate bone formation centrally through interactions with the cocaine amphetamine regulated transcript (CART), which decreases RANKL expression within osteoblasts and thus inhibits osteoclast differentiation [55]. Leptin also inhibits serotonin production by the brain stem; brain-derived serotonin acts to increase bone mass and appetite [56]. In addition, leptin has been suggested to regulate sex-specific differences in bone development, since male leptin knockout mice have femoral bone parameters (including BMC, and periosteal and endosteal circumference) that are significantly lower than those of male wild type mice and similar to those of female mice [57]. These differences are evident in only post-pubertal male mice, indicating that the effects of leptin may be androgen-dependent [57]. A classic experiment demonstrated that leptin knockout mice have a high bone mass phenotype, in spite of hypogonadism and hypercortisolism that would be expected to result in low bone mass, and that intracerebroventricular infusion of leptin reverses this high bone mass phenotype [58]. This suggests that the primary function of leptin in skeletal regulation is to enhance bone resorption through central pathways. If the maternal exercise described in **Chapter 4** resulted in alterations in offspring leptin production, sensitivity, or interactions with central pathways regulating bone, appetite, and activity, then the offspring phenotype could have been altered to be higher in fat and more prone to bone resorption. Future work measuring both the physical activity level and the leptin

production and sensitivity of the offspring of dams exercised during pregnancy will clarify the extent of involvement of these factors in determining offspring phenotype.

Limitations and Strengths

The series of studies reported in this thesis first investigated potential exercises for use during pregnancy in rats, and then used the exercise that had been found most suitable to test the effects of maternal exercise during gestation on offspring musculoskeletal health and body composition. The exercise performed by the pregnant animals was carefully chosen based on its proven effects on bone parameters and fetal development. However, these studies had several limitations.

The first is that, due to logistical considerations, only one type of exercise was ultimately used to evaluate the effects on offspring outcome of dam exercise during pregnancy, and that exercise was performed throughout the entire gestation. The maternal and fetal response to exercise is likely to vary with the type, intensity, and frequency of the exercise, and its timing during pregnancy [59]. Effects of the timing of maternal exposure to environmental influences on the offspring's musculoskeletal system were seen in a study evaluating the programming effects of a maternal low-protein diet on muscle fibre type and number in rats. Moderate protein restriction throughout the entire pregnancy had very little effect, but protein restriction during only portions of the pregnancy changed both the type and number of the muscle fibres [60]. In **Chapter 3** we report that maternal exercise enhanced fetal growth through day 19 of pregnancy [2], but in **Chapter 4** we report no difference in offspring weight at birth. Perhaps if maternal exercise had been stopped at day 19 of pregnancy, before the period of most rapid absolute fetal weight gain, there would have been between-group differences in neonatal weight and growth trajectories. In humans, mothers that performed a reduced amount of exercise after 20 weeks of pregnancy had larger babies than those who either continued a moderate amount of exercise throughout their entire gestation or increased their exercise volume in late gestation [61]. It is possible that the

rapid increase in size of the fetuses during late pregnancy coupled with the exertions associated with the bipedal stance exercise created sufficient demands on nutrient resources to undo the previously beneficial effects of exercise through day 19 of pregnancy.

A technical limitation of this study was the use of pQCT to assess between-group differences in bone parameters. Although pQCT (discussed further in **Appendix A**) allows precise, high-resolution imaging of the peripheral skeleton, the ability of this technique to detect small variations in bone mineral content, area and density is limited by the size of the voxels used to integrate the summated attenuation data into a transverse image of the scanned region. Projecting a continuous image onto a defined grid, such as occurs when attenuation data from pQCT scans are integrated into discrete voxels, results in a partial volume effect that occurs at the edges of the image [62]. In pQCT analysis of bone, this partial volume effect occurs when a bone edge is included within (but does not fill) a voxel, thus causing the density of the voxel to be lower than the density of the bone included within a portion of that voxel [63]. Since the pQCT program uses density thresholds to determine whether a voxel is categorised as cortical or trabecular bone or soft-tissue, voxels that include only a small area of bone may be excluded from the bone analysis or misclassified as trabecular when in fact they contain cortical bone. In the studies reported in **Chapters 2** through **5**, a small voxel size (for a clinical machine) of 0.1 mm was used to provide highest possible resolution images and minimise the partial volume effect in the small bones that we scanned. However, the partial volume effect may result in some loss of resolution, and thus some effects of maternal exercise during pregnancy on maternal and offspring bone parameters may not have been detected using this technique.

The choice of the rat as a mammalian model for these studies is another potential weakness. Although rats have been used extensively for bone studies and for pregnancy and DOHaD-related research, they differ importantly from humans in a number of ways. Rats are polyovulatory animals and often gestate more than 10 fetuses. In **Chapter 3** we report differences in fetal growth related to intrauterine position, thus not all of the offspring experience identical conditions during gestation. Our finding that intrauterine position influences the effects of the maternal environment on offspring development could have important implications for studies examining developmental programming influences, particularly if only a few offspring from each litter are used to evaluate offspring outcomes. Also, rats are born at an earlier stage of development than humans, and thus may be more susceptible to programming effects *ex utero*. As an animal model for studies of bone physiology rats also differ importantly from humans in that the growth plates of many rat long bones remain open throughout much of adulthood, although longitudinal bone growth slows greatly as the rat ages [64]. Rats have also been suggested to be poor models for osteoporosis research because of a lack of Haversian remodelling of cortical bone, but this is actually not the case [65]; as in other species, levels of remodelling increase with age and fatigue loading in the rat [65,66].

There are also many reasons why rats are good animal models. Ease of handling and a relatively short generation interval and life span facilitate their use in physiology research, particularly studies of DOHaD-type effects in which a rapid generation time and quick maturation to adulthood is very beneficial. In addition, the multiple offspring produced by rat dams allows the use of statistical methods that account for the effect of dam in the offspring results, something that cannot be as easily done using monotocous species. Much of our understanding of the DOHaD hypothesis has come from research

using rats [67], which are often used in pregnancy research because the rat placenta is similar to that of humans [68]. Similarly, there has been a wealth of research on the rat skeleton, and its response to exercise, pregnancy, lactation, and almost any other physiological state has been well-characterised. Rats are easily trained to exercise [68], and are a useful model of human osteoporosis [64]. No animal will be a perfect model of the human condition, and the results of all research using animal models must be interpreted in light of the specific similarities and differences between the model species and humans. However, by using a highly standardised outbred animal model, high-quality open-source feeds, and robust statistical analysis we have ensured to the best of our ability that our results are a reflection of the biological processes associated with our maternal exercise intervention.

An unanticipated potential limitation of this study was the number of offspring affected by chronic progressive nephropathy (CPN). This condition is known to occur in older laboratory rats [69], but we did not anticipate clinical CPN in our study animals at less than one year of age. The existence of this disease in our offspring population was brought to our attention when several rats became ill throughout the course of the study and were diagnosed with CPN on post-mortem examination. Because of this, we decided to perform gross necropsy and histological examination of both kidneys on all offspring prior to inclusion in the data analysis. This was to ensure that all were "normal" and did not have any lesions that could have affected growth or skeletal parameters. Because of the potential influence of kidney-related elevations in serum parathyroid hormone on bone, all offspring were scored for degree of nephropathy and only those with scores low enough to indicate normal kidney function were included in the final data analysis. Such detailed examination, particularly of the kidneys, of study animals is not commonly performed in the literature. However, particularly in DOHaD

studies in which study animals live through a large proportion of their life span, it is important to ensure that the animals included in the statistical analysis are as "normal" as possible and that differences detected are not due to physiological conditions unrelated to the experiment. Thus the careful post-mortem examination of each rat became a strength of this work, as the animals included in the analysis were confirmed to be grossly normal and to have no morphological evidence that renal function would be abnormal. In addition to nephropathy, animals were also excluded because of kyphoscoliosis, necrosis of the femoral head, and chronic malocclusion.

Besides careful post-mortem examination of each rat included in the analysis, there are several additional strengths that distinguish this series of studies from much of the available literature. The extensive work done to determine that the exercise chosen for use in pregnant rats did not induce a physiological stress response allows the offspring outcomes to be considered without the potential confounding effects of exercised-induced maternal glucocorticoid elevations on developmental programming. This was critical because of the potential for stress-related effects on bone, and also for programming effects due to maternal stress. In addition, the statistical analyses of offspring outcomes included dam within treatment group as a random effect, thus accounting for the hierarchical nature of the data. As discussed in **Chapter 5**, this type of analysis is critical to ensure that between-group differences are not due to individual dam effects [70], but is not always done in the DOHaD literature. The screening of rats for lesions prior to inclusion in the analysis, careful selection of the type of exercise performed by the pregnant animals, and stringent statistical analyses provide confidence that the later-life offspring outcomes are the result of maternal exercise during pregnancy, and not some other confounding factor.

Conclusions

There were significant effects of maternal exercise during pregnancy on offspring outcomes but these effects were not clearly beneficial, particularly for the male offspring. While further research is needed to clarify the effects of exercise during pregnancy on the developing organism, the potential for negative long-term influences of maternal exercise on offspring health should be considered when designing prospective human trials.

The results of the research presented in this thesis provide the first evidence that:

- Maternal exercise during pregnancy has permanent and sex-specific effects on the offspring skeleton and adiposity;
- Undercarboxylated osteocalcin may play a role in energy metabolism in rats through similar pathways to those recently reported in mice;
- Maternal exercise during pregnancy has positive effects on fetal growth through day 19 of pregnancy and on post-lactation bone parameters;
- Position within the uterus modulates the fetal response to maternal exercise during rat pregnancy;
- Neither rising to an erect bipedal stance to obtain food/water nor tower climbing cause a physiological stress response in pregnant or non-pregnant female Wistar rats, but both cause significant tibial modelling in non-pregnant females.

Future Directions

The results presented in this thesis raise a number of further questions, which include:

1. What is the role of leptin in mediating the effects of maternal exercise during pregnancy on offspring outcomes?
2. Are there differences in postnatal physical activity related to maternal exercise during pregnancy, and can these explain the phenotypic differences between offspring of exercised and non-exercised dams?
3. What role does the increase in serum uOC concentration in the male offspring of exercised dams play in modulating the observed phenotypic outcomes?
4. Do the offspring of exercised and control dams differ in their own response to exercise imposed during adult life?
5. Does the effect of maternal exercise on long-term offspring outcomes vary with the timing of the maternal exercise during pregnancy?

The answers to these questions will further clarify the role of maternal physical activity in developmental programming, and the potential mechanisms underlying its effects.

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Appendix A

REVIEW OF LABORATORY TECHNIQUES

Introduction

As discussed in the **Background** and **Literature Review**, many studies demonstrate that environmental stimuli during gestation can affect postnatal body composition, and evidence suggests that the maternal environment experienced *in utero* may affect offspring musculoskeletal outcomes in later life. The work described in this thesis utilised a number of laboratory techniques to test the hypothesis that voluntary maternal exercise during pregnancy would result in long-term improvements to the musculoskeletal system and energy metabolism of the offspring. In order to investigate the effects of maternal exercise during pregnancy on offspring bone and body composition we used dual energy X-ray absorptiometry (DXA) and peripheral quantitative computed tomography (pQCT). We also used serum enzyme immunoassays (EIA) to measure serum concentrations of fully carboxylated osteocalcin (cOC) and undercarboxylated osteocalcin (uOC), and reverse transcriptase-quantitative polymerase chain reaction (RT-qPCR) to assess the expression of genes related to osteocalcin expression and carboxylation. In order to determine whether the exercise performed by the pregnant rats caused a physiological stress response we utilized radioimmunoassay (RIA) to measure faecal corticosterone metabolites. All of these techniques are described, and relevant coefficients of variation are given, in the thesis chapters in which the techniques were utilised. Further background information about these laboratory techniques is provided here.

Dual energy X-ray absorptiometry (DXA)

Dual energy X-ray absorptiometry uses two x-ray beams of different energies to allow determination of the densities of multiple components of a non-homogenous absorber (in our studies, the multiple tissue types in a rat). The subject being scanned is placed between the x-ray source and detector, and when the x-ray photons pass through the subject they either emerge unaffected, scatter (Compton scattering), or are absorbed (photoelectric effect). Those that are scattered or absorbed are not detected by the DXA radiation detector, and the reduction in the x-ray intensity caused by passing through the absorber is called attenuation. The ability of a material to absorb or scatter radiation is called the mass attenuation coefficient; this varies at different photon energies, but is constant and known for individual elements. By calculating the ratio of the mass attenuation coefficients (R) for the low and high-energy x-ray beams as they pass through a non-homogenous absorber, and using the known mass attenuation coefficients for the components of that absorber, it is possible to determine the fractional masses of the components of a two-component mixture. This is done using the equation: $R = f_1 \times R_1 + f_2 \times R_2$, in which R is the measured mass attenuation ratio, R_1 is the known mass attenuation ratio of component 1, R_2 is the known mass attenuation ratio of component 2, f_1 is the fractional mass of component 1, and f_2 is the fractional mass of component 2. Although an animal body contains more than 2 components, the DXA computer considers the body to be composed of either "bone mineral + soft tissue" or "fat + bone mineral-free lean" [1]. In order to calculate body composition, the DXA computer first separates each pixel in the image into two categories, those containing only soft-tissue and those containing bone and soft tissue, using an R threshold value and image processing methods that delineate skeletal boundaries. Experimentally determined R values for fat and lean mass are then used to determine the fractional mass

of fat and lean tissue in the non bone-containing pixels. In bone-containing pixels, the soft tissue content of the pixel is estimated and then the fractional mass of the bone is determined. Thus DXA is able to calculate bone mass, fat mass, and lean mass simultaneously [1]. Areal bone mineral density (BMD_a) is determined based on the calculated bone mass and skeletal area, yielding a value based on a two-dimensional image of the skeleton. Although this technique for measuring bone mineral density has limitations, DXA is currently the most commonly used method for measuring body composition and assessing osteoporotic changes in BMD in humans, and has been clinically validated in many studies [2].

Figure 1. An anaesthetized rat undergoing DXA scanning.

Peripheral quantitative computed tomography (pQCT)

Bone strength is determined by the combination of the mechanical properties and structural arrangement of the bone material [3]. While DXA is limited by the two-dimensional nature of its images and does not provide much information about bone structure, particularly the distribution of the bone material into trabecular and cortical compartments, computed tomography (CT) is well-suited to provide this information. In addition, the volumetric bone mineral density (BMD_v) measured by CT is a three-dimensional density expressed in mg/cm^3, however DXA can calculate only an areal bone mineral density (BMD_a) expressed in g/cm^2. Peripheral quantitative CT allows high resolution imaging of the peripheral skeleton. During pQCT, an X-ray source and detector rotate 180˚ around the object being scanned to create a series of thin transverse projections of the scanned object from different angles. The detector measures the attenuation of the radiation as it passes through the slice of tissue and the attenuations from the multiple projections are used to generate the CT image [4,5]. The magnitude of the x-ray attenuation depends upon the type of material it passes through; in peripheral limbs, bone mineral generates the highest attenuation while soft tissues generate different, and lesser, attenuations. A computer integrates the attenuation data into an image of the transverse slice of tissue scanned by dividing the scan area into voxels, which are essentially volumetric or 3-dimensional pixels, and calculating the attenuation of each voxel. These attenuations are then compared to the attenuation values generated by scanning a manufacturer-provided hydroxyapatite calibration phantom, with the attenuation of fat set to zero [4]. The Stratec XCT 2000 generates 90 projections/image with a slice thickness of 2.0 mm [6]; for our purposes (scanning small rat bones) the voxel size was set to 0.1 mm. It is important to recognize that, since the voxels are 3-dimensional, the attenuation for each voxel is representative of the total attenuation

caused by all of the tissues within that voxel; in bone that may include mineral, matrix, fat, blood, etc. However, in spite of this, BMD_v is considered a good indication of the material quality of cortical bone [4], and pQCT-generated indices of bone strength have been shown to correlate well with the results of mechanical testing in rats [3,7].

Figure 2. An anaesthetized rat undergoing pQCT of the right tibia.

Enzyme immunoassay (EIA)

Commercially available, highly sensitive, rat-specific EIA kits (MK 126 and 146, Takara Bio Inc., Otsu, Japan) were used to determine serum cOC and uOC concentrations. These assays utilise a capture antibody to bind the osteocalcin (OC) protein to the assay plate, and then detect OC in its carboxylated or undercarboxylated form using a second, enzyme-labelled detection antibody. The MK 126 kit detects cOC by sandwich ELISA using a capture antibody specific to the C-terminus of rat OC and an enzyme-labelled detection antibody specific to the carboxylated glutamate residue at position 17 (Takara Inc., personal communication). Since the carboxylation of osteocalcin is an ordered process with carboxylation of positions 21 and 24 occurring before carboxylation at position 17 [8], this assay is specific for cOC. The MK 146 kit uses the same capture antibody as the MK 126, but the detection antibody recognizes uncarboxylated glutamate residues at positions 21 and 24 of the OC molecule. Since these positions are the first to be carboxylated following OC synthesis within the osteoblast endoplasmic reticulum, and since the carboxyglutamyl residue at position 13 in mice (analogous to position 17 in rats) is very prone to decarboxylation during osteoclastic bone resorption [9], this assay detects primarily fully uncarboxylated OC. However, because uOC may result from both incomplete carboxylation in the endoplasmic reticulum and decarboxylation (incomplete or complete) in the osteoclastic resorption lacuna, and the detection antibody for the MK 146 assay will detect fully uncarboxylated OC or OC molecules that are carboxylated at position 17, we have chosen to use the term undercarboxylated OC rather than uncarboxylated OC when discussing the results of this assay. The C-terminal fragment is more easily cleaved from the intact OC molecule than the N-terminal fragment and the carboxylation sites are located on the N-terminal fragment (17) and the mid-region (21 and 24) of the

amino acid chain. Thus, use of a C-terminal specific capture antibody ensures that both assays primarily detect the intact form of OC. This avoids the potential overestimation of OC concentrations that can occur with the use of assays that detect OC fragments [10].

Reverse transcriptase quantitative polymerase chain reaction (RT-qPCR)

Polymerase chain reaction (PCR) uses thermostable DNA polymerases (usually *Taq* polymerase) to exponentially amplify a select region of DNA from a DNA template. Double-stranded DNA is first denatured (split into single strands) by incubation at a high temperature (95 ^{0}C) and then oligonucleotide primers are allowed to bind to the DNA strands by lowering the temperature to an optimal annealing temperature (60 ^{0}C in our study). The DNA polymerase then adds nucleotides starting from the primer to produce a complementary DNA strand. The advantage of PCR is that it allows the detection of specific DNA sequences in a very small amount of starting material; in fact, PCR is so sensitive that there is significant risk of erroneous detection due to contamination, and strict procedures to minimize DNA contamination of the sample must be adhered to.

Reverse transcriptase (RT)-PCR allows detection of specific mRNA sequences by first converting the mRNA into complementary DNA (cDNA), and then following the standard PCR procedure. Quantitative (q)-PCR, also called real-time PCR, allows quantification of the amount of amplicon produced by the PCR reaction. There are several methods used to achieve this, one is to add SYBR^{TM} Green to the PCR mixture. SYBR^{TM} Green fluoresces when it binds to double-stranded DNA allowing detection of the amount of double-stranded DNA created. This is a non-specific method, as the SYBR^{TM} Green will bind to any double-stranded DNA, and it is important to confirm the amplification of the correct target through sequencing of the DNA amplicon and melting curve analysis after the PCR reaction. A more specific technique uses a specific oligonucleotide probe to detect a sequence within the amplicon. The TaqMan method is the most commonly used probe-based qPCR technique. TaqMan probes contain a fluorophore at their 5' end and a quencher at their 3' end. The quencher suppresses the

fluorescence of the fluorophore as long as both are within the same molecule; however, as the PCR reaction progresses the probe is degraded by the exonuclease activity of the polymerase, which leads to activation of the fluorophore. Since the sequences used in TaqMan probes are different from the sequences of the primers, this method avoids interference from primer binding and primer dimers. Probes which span an exon-exon junction also minimize the risk of detection of amplified genomic DNA [11].

In our study, we performed RT-qPCR using TaqMan probes. All probes spanned exon-exon junctions; however, to further minimize the risk of genomic DNA detection all samples were DNase-treated with TURBO-DNA free (Ambion, Life Technologies, Texas, USA) prior to cDNA creation.

Radioimmunoassay (RIA)

Measurement of the immunoreactive metabolites of corticosterone excreted in the faeces is a non-invasive means of assessing stress in rodents [12]. In **Chapters 2–4**, faecal corticosterone metabolites (referred to simply as faecal corticosterone in these chapters) were measured using a commercially available double antibody RIA kit specific for rat and mouse corticosterone (Double Antibody Corticosterone 125I RIA kit for rats and mice, MP Biomedicals, Orangeburg, NY, USA). In this competitive RIA, ^{125}I-labelled corticosterone competes with unlabelled corticosterone and/or corticosterone metabolites in the biological sample for binding sites on rabbit anti-corticosterone antibodies. If no corticoids are present in the biological sample, then the antibodies will bind 50–60% of the ^{125}I-labelled corticosterone, which is present in the assay in a known quantity. Any corticoids in the biological sample will compete with the labelled corticosterone for binding sites on the antibody and as the amount of corticoids in the biological sample increases the amount of radiolabelled corticosterone bound by the antibody will decrease. A mixture of polyethylene glycol and goat anti-rabbit gamma globulins is then used to precipitate all of the antibody-bound corticoids, which are separated from the supernatant by centrifugation. Radiation counts are determined using a gamma counter and the mean radiation counts of blank tubes to which no sample, calibration solution, or antibodies have been added are subtracted from the mean values for tubes containing biological samples or calibration solution to obtain corrected values. These are then divided by the corrected mean radiation count of the tubes containing the zero calibrator solution (which contains no unlabelled corticosterone) to determine the percent of bound ^{125}I-labelled corticosterone. A standard curve is generated using the percent of bound ^{125}I-labelled corticosterone in six calibration solutions of known corticosterone concentration, and values obtained from

the biological samples are plotted against this standard curve to determine their corticoid concentrations. (Details from product data sheet, MP Biomedicals).

Although this kit is intended for measuring corticosterone in plasma or serum, faecal corticoid immunoreactive metabolites can be measured [12] after extraction from dried faecal samples using a previously validated technique [13]; the extracts are then reconstituted in phosphate buffered saline with 0.1% gelatin prior to use in the assay. The concentrations of corticoids in the reconstituted samples were above the minimum detection limit of the assay, which was 7.7 ng/mL.

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Appendix B

SERUM CONCENTRATIONS OF FULLY AND UNDERCARBOXYLATED OSTEOCALCIN DO NOT VARY BETWEEN ESTROUS CYCLE STAGES IN SPRAGUE-DAWLEY RATS

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Introduction

Osteocalcin (OC) is the most abundant non-collagenous protein produced by osteoblasts. Following translation, it is subject to vitamin K-dependent addition of three carboxyl groups, which increase the affinity of OC for calcium and hydroxyapatite [1]. Undercarboxylated osteocalcin (uOC) lacks one or more of these carboxyl groups. The results of recent studies in mice suggest that uOC acts as a bone-derived hormone that regulates glucose handling through effects on insulin secretion and sensitivity, energy expenditure, and fat mass (reviewed in [2]).

The role of uOC in human metabolism is not yet clear [2]. The rat OC protein is 73% homologous to the human OC protein, and expression of the OC gene is up-regulated by 1,25-dihydroxyvitamin D_3 in both rats and humans [3]. Thus rats may be a useful model for further studies of the mechanisms of OC action and its potential therapeutic uses. However, in order to develop proper sampling protocols to assess serum OC concentrations in rats, it is necessary to know if these concentrations are influenced by estrous cycle stage. Haruyama *et al*. (2002) showed that the concentrations of total serum OC in young adult female Wistar rats varied with stage of the estrous cycle, with peak concentrations occurring during diestrus and the lowest concentrations during estrus [4]. However, Sengupta *et al*. reported no effect of stage of estrous cycle on total serum OC concentrations in comparable Sprague–Dawley rats [5]. We could find no studies of the effects of the estrous cycle on serum concentrations of fully carboxylated osteocalcin (cOC) and uOC in female rats. In this pilot study, we utilized commercially available enzyme immunoassays to determine whether serum concentrations of cOC and uOC vary with stage of estrous cycle in young adult, virgin, female Sprague–Dawley rats.

Methods

Eighty virgin female Sprague–Dawley rats were euthanized by CO2 inhalation at 200-g body weight for purposes unrelated to this study. All rats had been housed under standard breeding colony conditions and were fed a standard rat chow produced by Massey University containing 3 mg vitamin K per kg of diet. None had undergone any experimental interventions. Euthanasia was performed 3.5–4.5 h after the start of the light cycle. Rats were not fasted prior to euthanasia. Since the decision to euthanize the animals was unrelated to this study and all samples were collected after euthanasia, approval by the Massey University Animal Ethics Committee was not required.

Immediately following euthanasia, cardiac blood and vaginal cytology samples were obtained. Blood was collected into plain Vacutainer tubes, placed on ice, and centrifuged for 10 min at 2,000 x *g* within 2 h of collection; serum was stored at -80 C until assay. Stage of estrous cycle was determined by vaginal cytology as described in Goldman *et al.* [6].

Samples from 30 animals that could be unequivocally assigned to proestrus, estrus or diestrus (n=10/estrous cycle stage) were used for the OC assay. Since only a small number of animals were considered definitively in metestrus, this phase was not included. We chose to use 10 animals/group because this is a typical group size used in many bone-related studies. Serum cOC and uOC concentrations were determined using commercially available, highly sensitive, rat-specific EIA kits (MK 126 and 146, Takara Bio Inc., Otsu, Japan). Samples were assayed in duplicate according to the manufacturer's instructions and the mean values of cOC and uOC for each sample were used. Intra-assay CVs were 5.3 and 3.5% for the cOC and uOC assays, respectively, and we obtained concentrations similar to those reported in the product manual.

Data were analyzed using SAS 9.2. All data were tested for normal distribution. *P* values for stage of estrus for cOC, uOC, and uOC+cOC were determined by simple ANOVA using PROC GLM. The values for cOC and uOC+cOC were log transformed prior to analysis. The values for the cOC:uOC ratio were not normally distributed after transformation; therefore, the *p* value was determined by non-parametric analysis using the Kruskal–Wallis test.

Results and Discussion

Overall mean serum concentrations of uOC, cOC, their sum, and ratio, as well as mean concentrations at each stage of the estrous cycle, are shown in Table 1. Serum concentrations of cOC were approximately 7 times greater than concentrations of uOC. There were no significant differences in any parameter at different estrous cycle stages; however, all parameters were non-significantly greater during diestrus than during other cycle stages. This is consistent with the results for total OC reported by Nielsen *et al*. in humans [7] and by Jackson *et al*. in horses [8]. The Nielsen study used only 8 women whereas Jackson *et al*. used 47 horses, but in both of these studies individuals were sampled multiple times. Perhaps if we had sampled our rats multiple times, or had used a larger number of animals, we would have also detected significant differences. However, the number of animals that we used is consistent with the numbers used in many studies of bone and metabolism.

Table 1. Serum concentrations of carboxylated (cOC) and undercarboxylated (uOC) osteocalcin during the estrous cycle in rats

	Proestrus N=10	**Estrus N=10**	**Diestrus N=10**	**P (Estrous)**	**Overall Mean**	**Overall Range**
uOC (ng/ml)	30.78 (5.60) [22.00–37.64]	34.29 (6.58) [20.64–43.92]	35.76 (7.38) [24.70–44.84]	0.24	33.61 (6.68)	20.64-44.84
cOC (ng/ml)	226.10 (40.24) [168.64–296.81]	245.76 (54.25) [165.05–363.58]	267.93 (83.37) [166.24–453.88]	0.40	246.60 (62.25)	165.05-453.88
uOC+cOC (ng/ml)	256.88 (44.58) [190.64–333.31]	280.05 (59.32) [194.79–405.66]	303.70 (88.42) [191.66–491.20]	0.37	280.21 (67.18)	190.64-491.20
cOC:uOC	7.40 (0.89) [6.17–9.40]	7.24 (1.24) [5.55–9.79]	7.48 (1.68) [6.53–12.16]	0.64	7.37 (1.27)	5.55-12.16

Values are unadjusted means (SD) [Range]

The between-individual CVs for cOC and uOC at different cycle stages ranged from 17.8–31.1% and 18.2–20.6%, respectively. This variability in normal OC concentrations suggests that a larger number of individuals per group would be required to detect a between-group difference in the concentration of cOC or uOC than the 10/group that we used. Although there were no significant differences in mean values

between different estrous cycle stages, the differences in individual serum concentrations of cOC and uOC may still have been related to individual levels of endocrine hormones associated with the reproductive cycle. This is an area for further study. In humans, uOC concentrations vary with both age and diet [9]; thus using rats of different ages, or feeding a different diet, could also have affected our results. However, in this study, to have detected as significant the different concentrations of uOC, cOC, their sum, and ratio (as shown in Table 1) would have required sample sizes of 34, 44, 19, and 499 animals per group, respectively (based on overall SD, and calculated using G*Power3) [10] .

This report is the first to present data examining the influence of stage of estrous on serum uOC and cOC concentrations in rats. The physiological changes associated with estrous cycle stage were not sufficient to cause a detectable difference in cOC or uOC concentrations in this study. It is our hope that the data we present will assist researchers in performing power calculations and designing sampling protocols for experiments examining cOC and uOC concentrations in female rats.

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Appendix C

FURTHER DISCUSSION OF CHRONIC PROGRESSIVE NEPHROPATHY IN LABORATORY RATS

Background

Chronic progressive nephropathy (CPN) is a degenerative disease of the kidney that occurs spontaneously in all commonly-used strains of laboratory rats [1]. Also called chronic nephrosis, chronic nephritis, spontaneous glomerular sclerosis, and progressive glomerulonephrosis [2], its many names reflect the lack of understanding of its pathogenesis that persists in the literature today. Although a condition similar to CPN was recognised in albino rats as early as the 1920s, the underlying causes of this degenerative disease are still not fully understood [3]. Chronic progressive nephropathy is not directly comparable to any of the major human renal diseases [4], and it is a potential confounder in trials using laboratory rats [1].

Pathology

The kidneys of rats with mild CPN are grossly normal in appearance, although proteinuria will be evident [3]. Increasing proteinuria occurs commonly with aging, in male Sprague-Dawley rats the percentage of animals with detectable proteinuria increased from 0% at 2 weeks of age to 94% at 2 years of age [5]. The degree of the proteinuria correlates roughly with the severity of the histological changes associated with CPN [5]. As the disease progresses, the kidneys become pale and enlarged with pitted surfaces [6]. Casts are present in the renal tubules [3]; at least some of these are comprised of albumin, although Tamm Horsfall protein may also contribute to cast formation [1]. In some cases, parathyroid hypertrophy and fibrous osteodystrophy are evident in the late stages of the disease [1].

The basic histological lesion of CPN is thought to be degeneration of the basement membrane of the nephron [3]. It has been generally accepted that the glomerulus is the primary target of CPN [1]. Progressive glomerulosclerosis is thought to develop as a result of functional overload of the glomeruli [7] by a high protein diet or overfeeding, with resultant changes in renal perfusion and glomerular filtration rate leading to hyperfiltration of protein [1]. However, the work underlying this theory was based on renal ablation studies, and it was subsequently demonstrated that the age-related renal dysfunction that occurs spontaneously in laboratory rats is not related to haemodynamic changes within the glomerular capillaries [8].

More recently, it was demonstrated that the flux of albumin through the glomerular capillary wall was greater than had previously been thought [9]. Thus, the proteinuria associated with CPN might be due to failure of protein reuptake mechanisms in the proximal tubular epithelium rather than protein leakage through the glomerulus [1,10]. The presence of basophilic tubules in the renal cortex is a very early histological change

associated with CPN [1], which suggests that changes at the level of the proximal tubule may in fact be the primary factor underlying its development.

Risk factors

The cause of CPN is unknown, but several predisposing factors have been identified. These are strain of rat, age, sex, and diet. Sprague-Dawley rats are among the most susceptible strains, Long-Evans rats are relatively insusceptible, and Wistars have an intermediate susceptibility [3]. However, within these strains there are likely subpopulations that are more and less prone to the disease. As rats age the risk of their developing CPN increases, and CPN has been referred to as an age-related nephropathy [2]. Rats of greater than 1 year of age are at greatest risk [3], although the age of onset may be related to strain susceptibility. Intact males are more susceptible than castrated males or females, and it appears to be the presence of androgens, rather than the absence of oestrogens, that results in increased risk since ovariectomised females are also protected from the disease [8].

The one modifiable risk factor for CPN in laboratory rats is diet. High protein and high calorie diets have been implicated in its progression, and restricting the intake of calories, with or without protein restriction, appears to be protective [11]. Feeding a high protein diet may increase the risk of CPN, and rats fed a 14.4% protein diet for two years had lower nephropathy scores than rats fed a diet containing 23.5% protein [12]. This contrasts with earlier work in which reducing the protein content of the ration from 26% to 14.4% did not change the incidence of disease in 9–18-month-old Sprague-Dawley rats [3]. The results of several studies suggest that casein as a protein source is more damaging to the kidneys than vegetable-based protein sources, such as soy [3,13]. However, casein is a very useful source of protein in the diets of rats used for bone studies, because of the potential skeletal effects of the phytoestrogens found in soy.

Implications for research using rats

The potential implications of CPN for toxicology studies have been discussed in the literature [2]. However, although CPN is a recognized limitation of the use of the rat as a toxicology model, its potential implications for DOHaD-type studies, especially those examining offspring outcomes in later adult life, have not been acknowledged. Literature searches failed to reveal any references that mentioned CPN as a potential confounder of long-term DOHaD-type studies. A recent review of animal models for the study of DOHaD [14] also does not even mention the existence of this disease in rats. However, it seems likely that the presence of a spontaneous age-related disease, such as CPN, in laboratory animal populations could potentially confound studies using these animal models to investigate the long-term health effects of early life influences.

Our experiences with CPN

The first indication that any rat in our study population was clinically affected by CPN was the death of a breeding male at approximately 200 days of age. Necropsy of this rat revealed bilaterally enlarged kidneys with moderately roughened surfaces. Histologically, interstitial and periglomerular fibrosis was evident and the renal architecture was diffusely disrupted. Proteinaceous material was evident within ectatic tubules, and tubular epithelial cells were variably attenuated or swollen. Based on these histological findings, a diagnosis of CPN was made.

Although this particular male had failed to impregnate either of the two females that he was mated with, several offspring from the same cohort (**Chapter 5**) also died or were euthanised due to severe CPN prior to the end of the study. One female rat was euthanised at only 140 days of age for severe illness (inappetance, weight loss, diarrhoea) and subsequently diagnosed with CPN, and three males and one additional female began showing clinical signs of severe renal failure at approximately 200 days of age. This early onset of end-stage CPN was surprising, as CPN is considered to be an age-related disease and incidence is reported to be greatest in male rats over 1 year of age [3]. In addition, the rats were fed a diet that was only 17.8% protein (AIN-93G, Research Diets) [15]. The source of the dietary protein was casein because the diet was selected in part for its low phytoestrogen content.

Because of the potential for chronic disease to affect growth, and for the secondary hyperparathyroidism and 1,25-dihydroxyvitamin D_3 deficiency associated with renal disease to affect bone mineralisation [16,17] and serum osteocalcin levels [18], once it was evident that there were clinical cases of CPN in our study population it was decided that all rats would undergo histological screening of their kidneys prior to inclusion in data analysis (Table 1). Kidneys were scored from 1–4 for degree of

nephropathy, where 1 = 0–10% glomeruli affected, 2 = 10–40% glomeruli affected, 3 = 40–80% glomeruli affected, 4 = end-stage renal disease (80–100% glomeruli affected). There were 8 rats with kidney scores ≥ 3, and these were excluded from statistical analyses. We also conducted gross necropsy on all rats and excluded any animals with lesions that might have affected growth or skeletal development. The lesions discovered during post-mortem examination, and all other reasons for exclusion of animals from the statistical analyses described in **Chapter 5**, are shown in Table 2.

Table 1. Number of male and female rats in each kidney score category

Kidney Score	# Males	# Females
1	38	46
2	12	6
3	3	0
4	3	2

Animals are the offspring of exercised and control dams described in Chapter 5

Table 2. Animals excluded from post-weaning analyses in Chapter 5

Reason for Exclusion	Male		Female	
	DAMCON	DAMEX	DAMCON	DAMEX
Anaesthetic Death	**5**	**3***	**0**	**0**
Nephropathy	**0**	**6**	**0**	**2**
Chronic Malocclusion	**1**	**1**	**0**	**1**
Necrosis of Femoral Head	**1**	**0**	**0**	**0**
Kyphoscoliosis	**1**	**0**	**1**	**0**
Kidneys not collected	**0**	**1**	**0**	**0**
Osteocalcin concentrations 10-fold > normal	**0**	**0**	**1**	**0**
Total number of offspring before any excluded	**30**	**34**	**26**	**30**

Animals were excluded from post-weaning analyses if they did not have a kidney score, if they had a kidney score of ≥3, or if they had another abnormality that might have influenced their growth.
*Note that these animals died under anaesthesia during the second DXA scan and kidney scores were obtained for them. Therefore, their data have been used for the DXA analysis but they are not included in the final blood glucose, body weight, and pQCT analysis.
DAMCON = offspring of control dams, DAMEX = offspring of exercised dams

Of the 8 rats with kidney scores ≥ 3, all were the offspring of 1 of 3 dams in the group that exercised during pregnancy. Whether this indicates an effect of maternal exercise on the incidence or severity of CPN, or a genetic predisposition of the parents

of the affected offspring, is not clear (discussed in **Chapter 5**). However, what is clear from our results is that thorough post-mortem examinations of all rats allowed exclusion of animals with lesions that could have affected growth; without careful necropsy following euthanasia these animals might have gone undetected. This type of screening of animals is not routinely performed in most studies, but perhaps should be considered, particularly in studies using strains of rats that are prone to CPN.

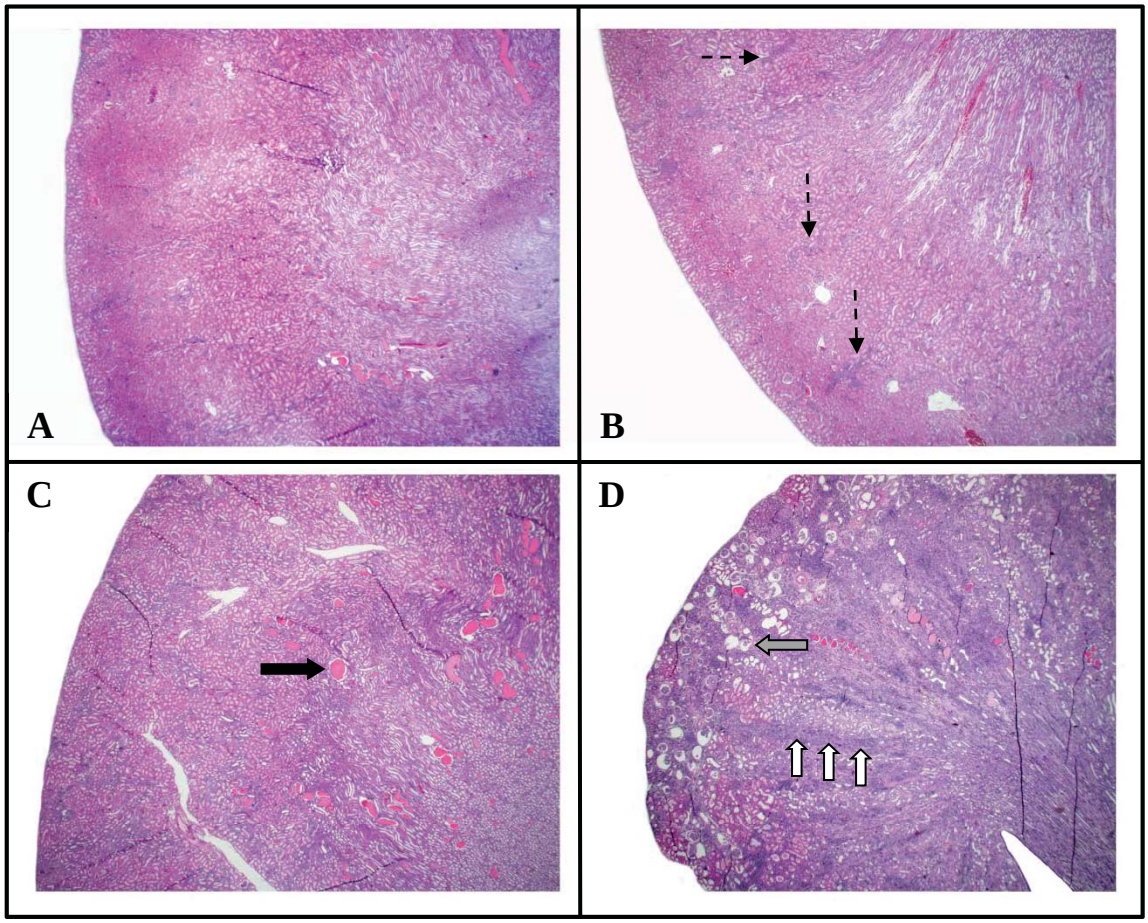

Figure 1. Example histological images of kidneys with nephropathy scores 1–4. A. Nephropathy score = 1, less than 10% of kidney affected; B. Nephropathy score = 2; 10–40% of kidney affected; C. Nephropathy score = 3, 40–80% of kidney affected; D. Nephropathy score = 4, end-stage kidney. Dashed arrows indicate basophilic tubules, solid black arrow indicates a dilated tubule filled with proteinaceous material, solid grey arrow indicates a dilated urinary space secondary to fibrosis of the kidney, white arrows indicate areas of lymphoplasmacytic infiltration and fibrosis.

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Appendix D

STATEMENTS OF CONTRIBUTION TO DOCTORAL THESIS CONTAINING PUBLICATIONS

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STATEMENT OF CONTRIBUTION
TO DOCTORAL THESIS CONTAINING PUBLICATIONS

(To appear at the end of each thesis chapter/section/appendix submitted as an article/paper or collected as an appendix at the end of the thesis)

We, the candidate and the candidate's Principal Supervisor, certify that all co-authors have consented to their work being included in the thesis and they have accepted the candidate's contribution as indicated below in the *Statement of Originality*.

Name of Candidate: Brielle Rosa

Name/Title of Principal Supervisor: Elwyn Firth

Name of Published Research Output and full reference:

SHORT-TERM VOLUNTARY EXERCISE IN THE RAT CAUSES BONE MODELING WITHOUT INITIATING A PHYSIOLOGICAL STRESS RESPONSE
Brielle V. Rosa, Elwyn C. Firth, Hugh T. Blair, Mark H. Vickers, Patrick C. H. Morel, and John F. Cockrem.
American Journal of Physiology: Regulatory Integrative and Comparative Physiology (2010) 299: R1037-1043.

In which Chapter is the Published Work: Chapter 2

Please indicate either:

- The percentage of the Published Work that was contributed by the candidate: 80%

  and / or

- Describe the contribution that the candidate has made to the Published Work:

  The candidate designed the study, collected the data, performed the laboratory techniques, analysed the data, and wrote the manuscript. All of this work was done in collaboration with the coauthors, but the candidate's contribution was the greatest.

Brielle Rosa — Digitally signed by Brielle Rosa DN: cn=Brielle Rosa, o=IVABS, Massey University, ou=NRCGD, email=B.V.Rosa@massey.ac.nz, c=NZ Date: 2013.11.28 22:53:09 -0700'

Candidate's Signature

28 November 2013

Date

Elwyn Firth — Digitally signed by Elwyn Firth DN: cn=Elwyn Firth, o=University of Auckland, ou=Exercise Science, email=e.firth@auckland.ac.nz, c=NZ Date: 2013.12.09 15:02:19 +13'00'

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Name of Candidate: Brielle Rosa

Name/Title of Principal Supervisor: Elwyn Firth

Name of Published Research Output and full reference:

VOLUNTARY EXERCISE IN PREGNANT RATS POSITIVELY INFLUENCES FETAL GROWTH WITHOUT INITIATING A MATERNAL PHYSIOLOGICAL STRESS RESPONSE
Brielle V. Rosa, Elwyn C. Firth, Hugh T. Blair, Mark H. Vickers, and Patrick C. H. Morel. American Journal of Physiology: Regulatory Integrative and Comparative Physiology (2011) 300: R1134-1141.

In which Chapter is the Published Work: Chapter 3

Please indicate either:

- The percentage of the Published Work that was contributed by the candidate: 80%

  and / or

- Describe the contribution that the candidate has made to the Published Work:

  The candidate designed the study, collected the data, performed the laboratory techniques, analysed the data, and wrote the manuscript. All of this work was done in collaboration with the coauthors, but the candidate's contribution was the greatest.

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Elwyn Firth — Digitally signed by Elwyn Firth DN: cn=Elwyn Firth, o=University of Auckland, ou=Exercise Science, email=e.firth@auckland.ac.nz, c=NZ Date: 2013.12.09 15:33:31 +13'00'

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STATEMENT OF CONTRIBUTION
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Name of Candidate: Brielle Rosa

Name/Title of Principal Supervisor: Elwyn Firth

Name of Published Research Output and full reference:

VOLUNTARY EXERCISE IN PREGNANT RATS IMPROVES POST-LACTATION MATERNAL BONE PARAMETERS BUT DOES NOT AFFECT OFFSPRING OUTCOMES IN EARLY LIFE
Brielle V. Rosa, Hugh T. Blair, Mark H. Vickers, Patrick C. H. Morel, John F. Cockrem, and Elwyn C. Firth.
Journal of Musculoskeletal and Neuronal Interactions (2012) 12: 199-208.

In which Chapter is the Published Work: Chapter 4

Please indicate either:

- The percentage of the Published Work that was contributed by the candidate: 80%

  and / or

- Describe the contribution that the candidate has made to the Published Work:

  The candidate designed the study, collected the data, performed the laboratory techniques, analysed the data, and wrote the manuscript. All of this work was done in collaboration with the coauthors, but the candidate's contribution was the greatest.

Brielle Rosa — Digitally signed by Brielle Rosa DN: cn=Brielle Rosa, o=IMBS, Massey University, ou=NRCGD, email=B.V.Rosa@massey.ac.nz, c=NZ Date: 2013.11.28 23:02:28 -07'00'

Candidate's Signature — 28 November 2013 — Date

Elwyn Firth — Digitally signed by Elwyn Firth DN: cn=Elwyn Firth, o=University of Auckland, ou=Exercise Science, email=e.firth@auckland.ac.nz, c=NZ Date: 2013.12.09 15:34:46 +13'00'

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(To appear at the end of each thesis chapter/section/appendix submitted as an article/paper or collected as an appendix at the end of the thesis)

We, the candidate and the candidate's Principal Supervisor, certify that all co-authors have consented to their work being included in the thesis and they have accepted the candidate's contribution as indicated below in the *Statement of Originality*.

Name of Candidate: Brielle Rosa

Name/Title of Principal Supervisor: Elwyn Firth

Name of Published Research Output and full reference:

MODERATE EXERCISE DURING PREGNANCY IN WISTAR RATS ALTERS BONE AND BODY COMPOSITION OF THE ADULT OFFSPRING IN A SEX-DEPENDENT MANNER
Brielle V. Rosa, Hugh T. Blair, Mark H. Vickers, Keren E. Dittmer, Patrick C. H. Morel, Cameron G. Knight, and Elwyn C. Firth. PLOS ONE (2013) Accepted.

In which Chapter is the Published Work: Chapter 5

Please indicate either:

- The percentage of the Published Work that was contributed by the candidate: 80%

  and / or

- Describe the contribution that the candidate has made to the Published Work:

  The candidate designed the study, collected the data, performed the laboratory techniques, analysed the data, and wrote the manuscript. All of this work was done in collaboration with the coauthors, but the candidate's contribution was the greatest.

Brielle Rosa — Digitally signed by Brielle Rosa DN: cn=Brielle Rosa, o=IVABS, Massey University, ou=NRCGD, email=B.V.Rosa@massey.ac.nz, c=NZ Date: 2013.11.28 23:05:40 -0700'

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Elwyn Firth — Digitally signed by Elwyn Firth DN: cn=Elwyn Firth, o=University of Auckland, ou=Exercise Science, email=e.firth@auckland.ac.nz, c=NZ Date: 2013.12.09 15:36:10 +13'00'

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STATEMENT OF CONTRIBUTION
TO DOCTORAL THESIS CONTAINING PUBLICATIONS

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We, the candidate and the candidate's Principal Supervisor, certify that all co-authors have consented to their work being included in the thesis and they have accepted the candidate's contribution as indicated below in the *Statement of Originality*.

Name of Candidate: Brielle Rosa

Name/Title of Principal Supervisor: Elwyn Firth

Name of Published Research Output and full reference:

SERUM CONCENTRATIONS OF FULLY AND UNDERCARBOXYLATED OSTEOCALCIN DO NOT VARY BETWEEN ESTROUS CYCLE STAGES IN SPRAGUE-DAWLEY RATS
Brielle V. Rosa, Hugh T. Blair, Mark H. Vickers, Cameron G. Knight, Patrick C. H. Morel, and Elwyn C. Firth. Endocrine (2013) 44: 809-811.

In which Chapter is the Published Work: Appendix B

Please indicate either:

- The percentage of the Published Work that was contributed by the candidate: 70%

  and / or

- Describe the contribution that the candidate has made to the Published Work:

  The candidate designed the study, collected the data, performed the laboratory techniques, analysed the data, and wrote the manuscript. All of this work was done in collaboration with the coauthors, but the candidate's contribution was the greatest.

Brielle Rosa — Digitally signed by Brielle Rosa DN: cn=Brielle Rosa, o=IVABS, Massey University, ou=NRCGD, email=B.V.Rosa@massey.ac.nz, c=NZ Date: 2013.11.28 23:13:05 -0700'

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Elwyn Firth — Digitally signed by Elwyn Firth DN: cn=Elwyn Firth, o=University of Auckland, ou=Exercise Science, email=e.firth@auckland.ac.nz, c=NZ Date: 2013.12.09 15:42:42 +13'00'

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