In Utero Sources of Skeletal Variation: the Role of Maternal Prenatal Stress

by

Sarah Kigamwa Amugongo

A dissertation submitted in partial satisfaction of the

requirements for the degree of

Doctor of Philosophy

in

Integrative Biology

in the

Graduate Division

of the

University of California, Berkeley

Committee in charge:

Professor Leslea J. Hlusko, Chair
Professor Tim D. White
Professor Thomas J. Carlson
Professor Katharine Milton

Fall 2010

In Utero Sources of Skeletal Variation: the Role of Maternal Prenatal Stress

by

Sarah Kigamwa Amugongo

Abstract

In Utero Sources of Skeletal Variation: the Role of Maternal Prenatal Stress

by

Sarah Kigamwa Amugongo

Doctor of Philosophy in Integrative Biology

University of California, Berkeley

Professor Leslea J. Hlusko, Chair

Osteoporosis is one of the most important health conditions affecting aging humans (≥ 60 years in age), particularly women in North America and Europe (Jordan and Cooper, 2002). One of the risk factors for osteoporosis is not attaining the maximum peak bone mass density for which an individual has the genetic potential. This risk factor is ascribed to poor environmental conditions and is irreversible (Cooper et al., 2009). Individuals who do not attain peak bone mass are at a significant disadvantage when aging sets in, with its accompanying loss of bone mass density. This places them at a higher risk for osteoporosis.

Psychological stress has been demonstrated to predispose pre-menopausal women to osteoporosis (Eskandari et al., 2007). Considering that environmental factors acting in pre-natal life have been known to influence adult health (Barker, 1995a, b, c, d; Gluckman et al., 2005), I hypothesized that psychological stress during pregnancy could result in high levels of cortisol that would affect bone formation in the fetus. This would compromise early bone development of the fetus and diminish the potential for attaining peak bone mass density in young adults, and therefore be associated with a higher risk for osteoporosis later in life.

To test this hypothesis I induced immobilization stress to pregnant Wistar rats at different gestational stages: Group 1 mothers were stressed during gestation week 1 (GW1), Group 2 during gestation week 2 (GW2), Group 3 during gestation week 3 (GW3); the Control Group was not stressed in any week. During gestation I monitored dams' cortisol hormone levels through fecal sampling, food intake, and maternal weight gain. After birth the pups were raised in a stress-free environment with adequate access to food and water and minimal human handling. Different sets of pups were euthanized at 4, 8, 12 and 16 weeks old. At necropsy the tibia was removed and fixed in 10% phosphate buffered formalin at 4°Celsius for 24 hours. The proximal part of the tibia (1 cm from the proximal end) was dehydrated in graduated series of ethanol and embedded in methyl methacrylate. Longitudinal sections, 4-μm thick were obtained using Leica 2165 Microtome (Leica, Heidelberg, Germany) with a tungsten carbide knife and placed on 2% gelatinized slides. The sections were stained using Von Kossa method with McNeal's tetrachrome counterstain. Bone histomorphometry was performed using semi-automated image analysis (Bioquant Image Analysis Corporation, Nashville, TN, USA) linked to a microscope to assess the size of the growth plate, trabecular total tissue area, trabecular bone

area, trabecular bone perimeter, osteoblast surface, osteoid surface, erosion surface and number of osteoclasts.

The means and standard deviations were calculated for all outcome variables. Statistical differences between the stressed groups and control group were analyzed using t-test, F-test and Tukey-Kramer Honestly Significant Difference test (JMP. 2008. Version 8. SAS Institute Inc., Cary, NC). Linear regression analysis was performed to establish the relationship between stress *in utero* and indicators of bone development in offspring born to stressed mothers (StataCoRP. 2009. Stata; Release 11. Statistical Software. College Station, TX: StataCorp LP). *P* values equal to or less than 0.05 were considered significant.

The mean cortisol hormone levels in controls were consistently lower than those of all stressed groups. However, cortisol levels in the control group were found to increase over the duration of the pregnancy. The animals stressed in gestation week 1 had the highest cortisol hormone levels and were significantly different from controls during gestation week 1 (GW1 = 3.72μg/g, controls = 1.66μg/g, difference = 2.06μg/g), followed by those stressed in gestation week 2 (GW2 = 3.34μg/g, controls during GW2 = 1.73μg/g, difference = 1.62μg/g) and those stressed in gestation week 3 (GW3 = 3.36μg/g, GW3 controls = 2.38μg/g, difference = 0.98μg/g).

During the pregnancy period, stressed animals consumed 3 grams (12.5%) less food per day compared to the controls. It was noted that on the day before delivery, all the animals (stressed and controls) increased their food intake, almost doubling their norm.

The pregnant dams that were stressed during weeks 1 and 2 of their pregnancies gained significantly less weight over the duration of the pregnancy (GWI = 263.03 grams, GW2 = 277.64 grams) than did those stressed in week 3 or in the control group (GW3 = 315.40 grams, controls = 311.46 grams). The average number of pups born to females stressed in weeks 1 and 2 was greater (13 and 14 respectively) than for the controls or those stressed in week 3 (11 and 12 respectively). Both male and female offspring born to mothers stressed in GW3 were heavier compared to all the other groups, but the weight difference was not statistically significant.

Histological analysis was done on the offspring born to the dams stressed in gestation week 3 and the control group only. The decision to initially focus on GW3 offspring was based on the fact that this is the week during which rats' bones mineralize. Data collection for the histology phase of the project was very time-intensive. As such, the other experimental groups will be studied at a later date and the results reported elsewhere.

Histological analysis showed that males have larger bones compared to females starting at the age of 8 weeks for both offspring groups. Controlling for sex, there was no significant difference in trabecular total tissue area or the trabecular bone perimeter between the GW3 offspring and control offspring. The GW3 offspring had a higher bone formation rate as indicated by their higher trabecular bone area at the age of 8 weeks (GW3 = 2.16mm^2, controls = 1.27mm^2), higher number of osteoblasts, which are the bone forming cells, at the age of 12 weeks (GW3 = 21.66mm, controls = 12.14mm) and a bigger area of the osteoid surface, which is the collagen matrix laid down by osteoblasts that eventually calcifies to form the bone, at the age of 8 weeks (GW3 = 4.98mm, controls = 1.92mm) and 12 weeks (GW3 = 5.50mm, controls = 0.99mm). There was no significant difference in bone resorption rate between the two groups.

The control offspring had a thicker upper zone of the growth plate, consisting of resting and proliferative chondrocytes, at the age of 4 weeks (GW3 = 302.83mcm, controls = 210.29mcm) and 8 weeks (GW3 = 195.73mcm, controls = 123.56mcm).

This project confirms that stress during pregnancy has negative consequences on both the mother and the offspring. The caloric intake in the mother is reduced, potentially due to the excess cortisol that alters the hypothalamic control of food intake. As a result, the mother does not accrue as many nutrients to support the growing fetuses. Having been nutrient-restricted and exposed to high cortisol levels *in utero,* the offspring appear to be born with an altered metabolism that results in faster growth and higher weight gain compared to controls. The positive effect of this fast growth is that the offspring born to stressed mothers ended up with a higher bone volume compared to the control offspring.

This study also shows that exposure to high cortisol levels *in utero* negatively affects the growth plate in offspring. Growth plate analysis showed that at the age of 4 weeks, control offspring had a significantly thicker area of resting and proliferative chondrocytes in the growth plate compared to GW3 offspring. Therefore, the negative effect of prenatal stress was evident in the upper zone of the growth plate even at the age of 4 weeks when the seemingly catch-up growth is expected to have occurred in all measured aspects of bone development. This seems to be the most sensitive part of bone development in relation to prenatal cortisol exposure.

In conclusion, given the negative effects of prenatal stress on the mother and offspring as noted above, this research shows that osteoporosis may have some fetal-origin roots influenced by maternal stress (and elevated cortisol levels). Healthy bones in adulthood require a healthy start. The growth plate is the center for bone growth and any adverse effects during early development would eventually affect the entire skeletal development. A likely result of not attaining the maximum peak bone mass density for which an individual has the genetic potential is a higher risk for osteoporosis.

TABLE OF CONTENTS

LIST OF SYMBOLS AND ABBREVIATIONS

α	Alpha
β	Beta
Δ	Delta
ε	Epsilon
μ	Micro
μg/g	Micrograms per gram
μm	micrometer
%	Percent
°C	Degrees Celsius
>	Greater than
≥	Greater than or equal to
≤	Less than or equal to
<	Less than
$1,25(OH)_3$	1,25-dihydroxyvitamin D_3
ACTH	Adrenocorticotropin hormone
ACUC	Animal Care and Use Committee
AVP	Arginine vasopressin
ANOVA	Analysis of Variance
BMC	Bone mineral content
BMD	Bone mass density
BMPs	Bone morphogenetic proteins
Cat. #	Catalogue number
Cbfa1	Core-binding factor α-1
$CD4^+$	Cytotoxic T-lymphocyte with CD4 surface protein
$CD8^+$	Cytotoxic T-lymphocyte with CD4 surface protein
Cm	Centimeter
CO_2	Carbon dioxide
ControlWk1	Control dams during the fist week of gestation
ControlWk2	Control dams during the second week of gestation
ControlWk3	Control dams during the third week of gestation
CRH	Corticotropin releasing hormone
CT	Computed tomography
DF	Degrees of Freedom
DNA	Deoxyribonucleic acid
DXA	Dual energy X-ray absorptiometry
EIA	Enzyme immunoassay
EtOH	Ethanol
FDA	Food and Drug Administration
FGF	Fibroblast growth factors
GCs	Glucocorticoids
GH	Growth Hormone
Gm	Grams
GW	Gestational Week

GW 1	Dams stressed in gestation week 1
GW 2	Dams stressed in gestation week 2
GW 3	Dams stressed in gestation week 3
Hg	Pressure unit
HPA	Hypothalamic-pituitary-Adrenal
HRT	Hormone replacement therapy
HSD	Honestly Significant Difference
IGF	Insulin like growth factors
IgG	Immunoglobulin G Cytotoxic T-lymphocyte
Ihh	Indian hedgehog
Lb	Pounds
mcm	A thousand circular mils
MI	Michigan
mm	Millimeter
mm^2	Millimeters square
MMA	Methyl methacrylate
ml	Milliliter
NK	Natural killer cell
TGF	Transforming growth factor
TN	Tennessee
TNF	Tumor necrosis factor
OC/mm	Osteoclasts per millimeter
PDGF	Platelete derived growth factors
PTH	Parathyroid hormone
PTHrP	Parathyroid hormone related peptide
QCT	Quantitative computed tomography
RANK	Receptor activated NF-kappaB
RANKL	Receptor activated NF-kappaB ligand
SXA	Single energy X-ray absorptiometry
USA	United States of America
WHO	World Health Organization
X	Multiplication

LIST OF FIGURES

LIST OF TABLES

DEDICATION

This dissertation is dedicated to my parents, Henry Mushiling'ani Amugongo and Margaret Kedeng'ani Amugongo for their love and support. As a young girl my father cultivated a seed of hope in me ensuring me that I could be anything I wanted to be as long as I worked hard in school. He encouraged me to take risks with the promise that he would always stand by my side. A promise he has kept so far. Having traveled all the way from Kenya to attend my commencement ceremony, as we walked into the campus on the quite clear morning, he said these words that I will forever treasure "May 17, 2010, I prayed day and night and hoped for this day to come, now that I have seen it, if I die any moment from now, I will die in peace."

ACKNOWLEDGEMENTS

I thank my advisor Prof. Leslea J. Hlusko for her wisdom and support throughout my time at Berkeley and for giving me the opportunity to accomplish this thesis. It has been a pleasure working under the guidance of Leslea as she gave me the freedom to pursue a research topic of my interest even though initially I didn't know much about it. Through this I came to discover the potential in me that I wouldn't have realised were it not for an advisor that had great confidence in me. She challenged me to work harder and think more critically, and she has led me by example. I thank my committee members Prof. Tim D. White, Prof. Thomas J. Carlson and Prof. Katharine Milton for their advice and support throughout my time as graduate student. They have inspired me and helped me grow as a scientist. I am truly honored to have been mentored by them.

I thank Prof. Tyrone Hayes for being on my qualifying examination committee and for his support. He has been a great inspiration to me.

I thank Dr. Peter Gakunga for his support, critical technical advice, constant feedback on my work, inspiration, friendship and many stimulating discussions. When things became tough he encouraged me to hang in there and be persistent.

I thank Prof. Daniela Kaufer and Darlene Francis for the training and advice they offered me on live animal research. They opened the doors of their laboratories to me and allowed me benefit from their expertise. They also introduced me to their postdocs and graduate students; Dr. Christian Mirescu, Dr. Annalliese Berry, Matt Reid and Samuel Sakhai who trained me on the various techniques that I needed for my research. I thank Dr. Christian Mirescu for technical advice, friendship and support.

I thank Dr. Lorena Havill for her technical advice and facilitating my research. She introduced me to Dr. David Burr's laboratory where I got my histology training. I thank Dr. David Burr for allowing me to get technical training in histology in his lab. I thank Dr. Keith Condon for the thorough histology training he offered me and his support. I thank Dr. Marvelee H. Wake for her technical advice on histology and support.

I thank Dr. Nancy Lane and Dr. Wei Yao for allowing me to use the equipment in their laboratory for histology analysis and for their technical assistance and support. I thank Dr. Mohammad Shahnazari for his technical assistance, advice, stimulating conversations and friendship.

I thank Liz Bates, Kara Timmins and Alicia Murua-Gonzalez for their tremendous help with my data collection as Undergraduate Research Assistants. The long hours we spend in the animal facility would have been unbearable were it not for these three former students.

I thank Dr. Jacqueline Moustakas, Oliver Rizk and Theresa Grieco of the Hlusko laboratory for their tremendous help with my data collection, advice, friendship and discussion throughout my time at Berkeley.

I thank Dr. Maureen Lahiff, Tendro Ramaharitra Tondrasoa and Kehinde Ajayi for their tremendous help with my data analysis.

I thank Dr. Henry Gilbert, Dr. Rebecca Jabbour and Dr. Gary Richards of the Human Evolution Research Center for their advice, friendship, and support.

I thank Dr. Kelley Jensen for her technical advice on animal care and cortisol hormone level analysis. I thank Montey Hill for his tremendous help with taking care of the rats used in this project.

I thank Julie Woodruff for training me in how to carry out enzyme-linked immunosorbent assays. I thank Dr. Palme Rupert for his help with fecal cortisol hormone analysis.

I thank Paul Falso and Sherrie Gallipeau of the Hayes laboratory for their technical support, friendship and advice.

I thank Dr. Travis Brown, Paul Falso, Kyle Brudvik, Michael Wasserman and Andrew Ritchie for their help with my qualifying exam preparation, stimulating discussions, friendship and support.

I thank Katie Brakora, Abby Moore and Lorraine Casazza for the friendship and support during the writing phase of my dissertation. It has been great to have colleagues who are also friends encouraging each other and working together towards a common goal.

I thank the Wenner-Gren Foundation, the Leakey Foundation, the National Science Foundation (grant number BCS 0925788 DDIG), the University of California Berkeley Museum of Vertebrate Paleontology, the University of California Berkeley Museum of Vertebrate Zoology, the University of California Berkeley Department of Integrative Biology, the University of California Berkeley International House and the Southwest Foundation for Biomedical Research, San Antinio Texas, for financial support throughout my time at Berkeley.

CHAPTER 1: INTRODUCTION

The appendicular skeleton provides the human body with structural support, aids in locomotion, and provides protection to vital organs (White, 2000). Other functions include mineral storage and homeostasis, formation of blood cells, and facilitation of the auditory process because the bones of the middle ear (malleus, incus and staples) transmit and amplify sound vibrations to the eardrum and inner ear (Marieb et al., 2005). Bone health is essential for all these functions and any significant change in bone mass density (BMD), bone mineral content (BMC) and bone quality leads to compromised function.

> "*Bone quality describes aspects of bone composition and structure that contribute to bone strength independently of BMD…These include bone turnover, microarchitecture, mineralisation, microdamage and the composition of bone matrix and mineral*" (Compston 2006; p. 579).

Over the course of an individual's lifetime, BMD, BMC and bone strength decline with age (Kanis, 2002; Martin et al., 1985; Martin, 1993; Burstein et al., 1976; Mosekilde and Mosekilde, 1986; Martin and Atkinson, 1977). This is a natural phenomenon in all mammals including humans. However, most mammals do not lose their bone mass to the extent that their bones become fragile and fracture following relatively low impact falls, as is the case in humans (Turner et al., 2001). On the other hand, it is unclear as to whether female mammals in the wild live long enough past their reproductive age to end up with fragile bones as do humans. "The condition that results from this systemic decrease in bone mass and microarchitectural deterioration, with consequent increase in bone fragility and susceptibility to fracture, is called osteoporosis" (Watts, 2002; p. 1148). According to the World Health Organization (WHO), osteoporosis is diagnosed when a value for BMD or BMC is 2.5 standard deviations or more below the mean of the young adult reference range (Jee and Yao, 2001; Kanis, 1994; Kanis et al., 2005; Lelovas et al., 2008). This condition is most prevalent in Caucasian women in Europe and the United States (Cooper et al., 1992; Walker-Bone et al., 2002; Dennison et al., 2005; Kanis et al., 1994).

Osteoporosis is a complex disease with multiple non-genetic risk factors as well as multiple genetic effects (Styrkarsdorttir et al., 2003; Kammerer et al., 2004; Walker-Bone et al., 2002; Denninson et al., 2005). Considerable effort is being focused on the investigation of the genetic factors contributing to bone loss (e.g., Ralston, 2005; Styrkarsdorttir et al., 2003; Kammerer et al., 2004; Karasik et al., 2003; Havill, 2007; Havill et al., 2007). The non-genetic risk factors studied to date include age (Schuit et al., 2005), sex (Gold, 1996; Ensrud et al., 1997), low level of physical activity (Geiser and Trueta, 1958), insufficient dietary vitamin D_3 and calcium (Gallagher et al., 1979; Okumura et al., 1987; Koshihara et al., 2004), the effects of diet more generally (Jørkov et al., 2009, Walker et al., 2008), alcohol abuse (Saville, 1965; Bikle et al., 1985), cigarette smoking (Wong et al., 2007; Fusby et al., 2010), and mechanical loading (Collard and Wood, 2007).

A review of the literature reveals that the majority of studies about these risk factors are focused on elderly populations, specifically people who are 60 years of age and above (Wengreen et al., 2004; Grados et al., 2004). Considerable research has been done on the elderly because the most distressing clinical manifestation of osteoporosis is bone fracture mostly in this

age group (Finsen et al., 2004; Boufous et al., 2004). Osteoporosis is complicated because peak BMD is attained at about the age of 30 in women, and around age 40 in men. Therefore, if an individual is diagnosed as osteoporotic at age 60, he or she has been slowly losing their BMD for about 30 years by the time they present with the disease clinically. Furthermore, several studies have demonstrated that various methods that have been suggested to prevent bone loss in adults, such as regular bone challenging exercise, are not as effective in adults once they pass the age of acquiring peak BMD (Welten et al., 1994; Kemper et al., 2000; Carter and Beaupre, 2001).

The two main factors that determine susceptibility to osteoporosis are:

1) The amount of bone accrued during intrauterine life, childhood and puberty, which translates into peak bone mass density at age 30-40 years (Walker-Bone et al., 2002)
2) The rate of bone loss once the growth period is over (Walker-Bone et al., 2002).

Current research is primarily concentrated on bone loss after the growth period. Hence, factors that influence early skeletal development, especially prenatal effects, are much less understood. It is important to consider the differences in skeletal development in human populations during the intrauterine and childhood growth periods given that the developmental process greatly affects the peak bone mass density attained (Lloyd et al., 1992; Ondrak et al., 2007; Doğan et al., 2009). If an individual attains the maximum peak mass for which they have the genetic potential during the growth period, then when they start to lose bone mass, they have relatively more bone to lose before becoming osteoporotic.

My dissertation research investigates maternal stress during pregnancy as a non-genetic factor that may affect fetal bone formation and development, and as such predispose an individual to osteoporosis as an adult. Ultimately, this work seeks to understand the possible role of prenatal stress as a possible risk factor for human osteoporosis.

> "*Stress refers to the consequence of failure of an organism to attain the internal dynamic equilibrium, homeostasis, to psychological or physical threats*" (De Kloet et al., 2005; p. 463).

In this dissertation project physical stress, which is an external challenge to homeostasis (Sapolsky, 2005), was induced in pregnant rats with the aim of understanding its effect on fetal bone development. Studies in humans have demonstrated that stress activates the excessive release of cortisol hormone, which is known to inhibit bone growth and has been shown to be a risk factor for osteoporosis in pre-menopausal women (Eskandari et al., 2007; Cizza et al., 2001). Given the significant long-term impact that the intrauterine environment has on an individual's life (e.g Barker, 1995c; Morton, 1955; Hales and Barker, 1992; Langley and Jackson, 1994; Lumey, 1992), it is entirely feasible that maternal health and well being during gestation may play a key role in the acquisition of peak bone mass density by the offspring. A review of the literature reveals a significant lack of information on this subject. This dissertation project helps to fill this gap in our knowledge.

I. RESEARCH OBJECTIVE AND HYPOTHESIS

The objective of my study is to determine how the timing of the negative affects of prenatal stress, and presumably high cortisol levels *in utero*, influences bone development and impacts peak bone mass density in the offspring. The hypothesis tested was that

environmentally induced stress on a pregnant female has a significant and negative impact on the skeletal development of her offspring, and ultimately on the offspring's peak bone mass density, even if the offspring's postnatal environment is stress-free. This hypothesis was tested using data collected during a twelve-month experimental protocol on rats.

Prior to discussing the details of my study design *(Chapter 2: Materials and Methods),* I will review the broad literature that provides the background and justification for this research.

II. REVIEW OF BONE DEVELOPMENT

Bone is a dynamic tissue during both pre- and post-natal development of an individual. "It has three main types of cells: the osteoblast whose role is to form bone tissue, the osteoclast whose role is to resorb bone tissue, and the osteocyte whose role is to maintain bone tissue by sensing bone damage and signaling for the osteoblasts and osteoclasts to repair the damage" (White, 2000; p. 27). The bone cells therefore play crucial roles in changing the shape and morphology of bone, and subsequently results in the ontogenetic variations observed in the skeleton as one grows older (Pearson and Lieberman, 2004; Parfitt, 1984).

II.a. Gross Anatomy of Bone

The skeleton can be divided into two major parts: the appendicular skeleton (consisting of all the bones of the lower body from the neck region to lower extremities) and the cranial skeleton (consisting of the bones that make up the head). At a macroscopic level, bones are made of two main parts. The first is the cortical bone (compact bone)—the dense external tissue. The second part is the spongy trabecular bone found within the cortical bone (White, 2000; p. 23). These two bone tissue types are molecularly and cellularly identical despite the differences in macroscopic appearance (White, 2000; p. 23).

II.b. Cellular Components of Bone

Bone is made of two distinct components: organic and inorganic (Frost and Stratmeyer, 1977; White, 2000; Marieb et al., 2005). The organic component is composed of protein and is largely collagen, giving bone flexibility and elasticity. The inorganic component of bone is made of calcium and phosphorus minerals, which embed into the collagen matrix making bone hard. There are three main cell types in bone whose roles are to form, resorb and maintain it.

II.b.i. Osteoblasts

Osteoblasts are the cells that form bone (Ducy et al., 2000). These originate from mesenchymal precursor cells (Ducy et al., 2000) and line the bone surface beneath the periosteum (White, 2000; p. 27). The gene family known as the Bone Morphogenetic Proteins (BMPs) are critical for the differentiation of mesenchymal cells into mature osteoblasts (Yamaguchi et al., 2000; p. 397). "Osteoblasts are mononucleated cells with a basic cell structure like that found in other cells types except for the presence of extensive rough surfaced membranes of the endoplasmic reticulum and numerous free ribosomes which are crucial for synthesizing protein" (Cameron, 1961; p. 590). They synthesize and deposit osteoid (pre-bone

tissue), an organic matrix rich in collagen (Parfitt, 1984; Parfitt, 1994; Pearson and Lieberman, 2004). The matrix is calcified by deposition of hydroxyapatite.

II.b.ii. Osteocytes

These are essentially osteoblasts that have completed their role of secreting osteoid (Nakahama, 2010). They are surrounded by a bony matrix as a result of the calcification of the collagen matrix. Osteocytes are stellate-shaped cells that "reside in the lacunae and are connected to the neighboring cells by long slender cytoplasmic processes, which radiate from the cell bodies in all directions" (Klein-Nulend et al., 2003; p. 5). The cytoplasmic processes pass through the bone matrix through the canaliculae.

Osteocytes serve as mechanosensors of bone (Adachi et al., 2010). They sense mechanical loading through the canalicular flow of interstitial fluid that result from the loading event on the bone (Klein-Nulend et al., 2003). "The osteocytes convert the mechanical stimuli into an intracellular signal mechanotransduction. The extracellular matrix receptors on the osteocytes are the mechanotransducers that signal the recruitment of osteoclasts and osteoblasts by producing signal molecule like nitric oxide" (Klein-Nulend et al., 2003; p. 7). The mechanically induced flow of the interstitial fluid is important because it transports metabolites to and from the osteocytes, which ensures their viability.

II.b.iii. Osteoclasts

These are large (~100μm) multinucleated cells whose main role is to resorb bone tissue (White, 2000; Pearson and Lieberman, 2004). The osteoclasts are part of the macrophage cell lineage and each one is derived from 10-20 mononuclear phagocytes in hematopoietic marrow (Boyle et al., 2003). "When activated, osteoclasts form a ruffled surface that adheres to a bone surface, creating a seal necessary for resorption to take place" (Pearson and Lieberman, 2004; p. 72). This is followed by secretion of a highly concentrated hydrochloric acid, which dissolves the mineral part of the bone and releases calcium ions and phosphorus ions into the blood (Parfitt, 1984; Parfitt, 1994; Pearson and Lieberman, 2004). They also secrete proteolytic enzymes that digest the bone's collagen matrix (Boyle et al., 2003). "Finally, the osteoclasts take up the degradation products, including soluble calcium and phosphate as well as collagen fragments, by phagocytosis, and release them into circulation" (Boyle et al., 2003; p. 337).

Modeling is the initial process of bone formation during growth whereas remodeling is the process of continuous removal and replacement of bone during life and serves a maintenance purpose after the growth period is complete (Frost, 1990; Erben, 1996). Since there is continuous formation and resorption during both the modeling and remodeling processes, bone formation is a process that continues throughout life. The ultimate result is change or maintenance of bone shape and size over an individual's lifetime.

The antagonism action of osteoclasts and osteoblasts replaces old bone with new and repairs micro damage, which is important in maintaining the strength and integrity of bone (Jee and Frost, 1992).

II.c. Bone Development in Humans

The process by which bone tissue is formed is called osteogenesis or ossification (White, 2000; Marieb et al., 2005). "It starts off as modeling at 8 weeks post-conception in the embryo and continues in childhood and through adolescence" (Marieb et al., 2005; p. 132-133). During the first two months after conception, the embryonic skeleton is mainly made of hyaline cartilage and mesenchyme (Marieb et al., 2005). This skeletal precursor is then replaced by mineralized tissue through intramembranous and endochondral ossification (Marieb et al., 2005; p. 133). As such, the modeling process slows down towards the end of the second decade in life and the remodeling process takes over to maintain the skeleton during most of adulthood, occurring at a much slower rate than did modeling.

II.c.i. Intramembranous Ossification

The bones formed by intramembranous ossification develop from mesenchymal tissue and include the clavicles and all bones of the skull except the mandible, temporal, occipital, sphenoid and ethmoid bones (White, 2000; Ducy, 2000; Yamaguchi et al., 2000).

Intramembranous ossification begins during week 8 of embryonic development (Marieb et al., 2005). "Mesechymal cells aggregate in the connective tissue membrane and transform into osteoblasts, the bone forming cells" (Marieb et al., 2005; p.133). This transformation is triggered by bone morphogenetic proteins (BMPs). BMPs also up-regulate the transcription factor core-binding factor α-1 (Cbfa1; also known as Runx2) that is important in determining osteoblast cell lineage and maturation (Yamaguchi et al., 2000; Ahrens et al., 1993; Ducy, 2000; Ducy et al., 2000). The osteoblasts secrete osteoid, the pre bone organic matrix tissue that is rich in collagen (Pearson and Lieberman, 2004; Ducy, 2000). Calcium and phosphorus minerals are then deposited into the collagen matrix. "Once collagen and mineral matrix surround the osteoblasts they are known as osteocytes… Since the new bone formation takes place between embryonic blood vessels, which are randomly woven, the bone that results is woven with the trabeculae arranged in networks" (Marieb et al., 2005; p.133). The lamellae that have a regular parallel alignment as a result of slower bone formation in mature spongy bones are not normally present in embryonic tissue.

II.c.ii. Endochondral Ossification

"Endochondral ossification begins towards the end of the second month post-conception and continues to early adulthood, a period in which bones elongate and widen" (Marieb et al., 2005, p.133). This is the process during which hyaline cartilage that is first laid down as a template is replaced by bone tissue (Marieb et al., 2005). The resulting bones are referred to as endochondral bones or cartilage bones. The bones that are formed by this process include all bones in the appendicular skeleton except clavicles (Marieb et al., 2005). In the craniofacial skeleton some bones (namely the mandible, temporal, occipital, sphenoid and ethmoid) are also of endochondral ossification origin (White, 2000; Ducy, 2000; Yamaguchi et al., 2000).

II.c.ii.1. Stage 1: Formation of a bone collar around the diaphysis

This stage begins in week 8 of embryonic development when the cartilate template is laid down and is surrounded by perichondrium (Marieb et al., 2005). This is followed by vascular invasion into the perichondrium surrounding the diaphysis. The perichondrium transforms into bone, forming periosteum at the end of week 8. Osteoblasts in this new periosteum lay down a collar of bone tissue around the diaphysis. This is preceded by expression of nuclear transcription factor Sox9 which is required for expression of cartilage specific matrix proteins (Goldring et al., 2006). The osteoblast is regulated indirectly by members of all major families of growth factors known to affect Cbfa1 (Ducy, 2000; Ducy et al., 2000; Yamaguchi et al., 2000). These include BMPs, fibroblast growth factors (FGF-1 and 2), insulin like growth factors (IGF-1, IGF-2 and GFBP 3/5), transforming growth factors-β (TGF-β-1 and -2) and the platelet derived growth factors (PDGF –AA, -AB, -BB) (Ducy, 2000; Ducy et al., 2000; Yamaguchi et al., 2000; Goldring et al., 2006). Their main role is in differentiation of mecenchymal progenitor cells into mature osteoblasts as well as osteoblast differentiation (Yamaguchi et al., 2000).

Osteoblasts are also regulated by the endocrine system through:

a) parathyroid hormone (PTH) which stimulates production of RANKL (receptor activator of nuclear factor-kB ligand), a cytokine that is expressed in pre osteoblastic cells (Rubin et al., 2002);

b) $1,25(OH)_3$ which stimulates intestinal absorption which indirectly promotes calcification of the collagen secreted by osteoblasts;

c) calcitonin that is secreted by the thyroid and up-regulates osteoblasts, and

d) estrogen that stimulates osteoblast protein synthesis.

II.c.ii.2. Stage 2: Cartilage calcifies in the center of the diaphysis

As the bone collar forms the chondrocytes the center of the diaphysis begin to hypertrophy, increasing cellular fluid volume by almost 20 times (Marieb et al., 2005). The prehypertrophic chondrocytes express Indian hedgehog (Ihh), a gene required for endochondral bone formation (Goldring et al., 2006) and signal the surrounding matrix to calcify. The calcified matrix blocks the diffusion of interstitial fluid to the core of the bone (Marieb et al., 2005). Since the viability of the chondrocytes residing in the middle of the bone is dependent on the nutrients from the interstitial fluid, the calcified matrix leads to their death and disintegration, resulting in cavities in the cartilage (Marieb et al., 2005). As a result, the cartilage matrix deteriorates. This process is unique to the center of the diaphysis and results in the formation of a hollow medullary cavity in the center of the bone (Marieb et al., 2005). The rest of the cartilage continuous to grow normally.

II.c.ii.3. Stage 3: The periosteal bud invades the diaphysis and the first bone trabeculae form

"During the third month of development, the cavities formed in the center of the diaphysis are invaded by a collection of elements called periosteal bud" (Marieb et al., 2005; p. 134). The bud contains nutrients, a vein, cells that will form the marrow, pre-osteoblasts and pre-osteoclasts. The matrix of the calcified cartilage is partially eroded by the invading osteoclasts while the pre-osteoblasts synthesize and secrete osteoid (Ducy, 2000; Ducy et al.,

2000). "This results into bone-covered trabeculae, which marks the initial formation of spongy bone within the diaphysis" (Marieb et al., 2005; p. 134).

> "*Therefore the appearance of bone tissue around and in the center of diaphysis takes place in the third month of development. The cartilage of the epiphysis continuous to grow rapidly for the remaining 6 months of fetal development. Mineralization continually takes place in the part closest to the diaphysis. The cartilage is replaced by bone trabeculae of the growing primary ossification center. On the other hand, the ends of the trabeculae are resorbed by osteoclasts. This results in the formation of a hollow cavity in the center of the bone called the medullary cavity. The cavity increases in size as the bone grows*" (Marieb et al., 2005; p. 134).

II.c.ii.4. Stage 4: Secondary ossification centers form in the epiphyses

The formation of secondary ossification centers occurs shortly before birth.

> "*Just like the formation of the primary ossification center, the cartilage in the center of each epiphysis calcifies and degenerates due to lack of maintenance by chondrocytes. A bud containing the epiphyseal vessels invades the epiphysis and bone trabeculae appear as described earlier in primary ossification which forms the primary ossification centers. Once the formation of ossification centers is complete and the epiphysis has ossified, hyaline cartilage mainly remains in two places… [1.] On the epiphyseal surface where it forms the articular cartilage… [and 2.] Between the diaphysis and epiphysis where it forms the epiphyseal plate also called the epiphysis disc or growth plate*" (Marieb et al., 2005; p. 134).

The continuous growth and calcification of the cartilage in the epiphyseal disc is responsible for longitudinal bone growth during the first two decades of postnatal life.

II.c.ii.5. Anatomy of epiphyseal growth areas

In order for adult bones to reach the required length and proportions, the growth of the skeleton must be tightly regulated. This regulation of growth is achieved at the growth plates and occurs through the interaction of circulating systemic hormones, locally produced growth factors and gene expression (Goldring et al., 2006). These molecules coordinate changes in chondrocyte size, extracellular matrix components, secreted enzymes and growth factors, and receptor expression (Goldring et al., 2006). The end result is the calcification of the matrix and chondrocyte apoptosis (Ballock and O'Keefe, 2003).

The growth plate is organized in a way that allows for quick and efficient growth (Howell and Dean, 1992). The growth plate can be divided into a series of microanatomical zones that delineate unique stages during the process of chondrocyte differentiation (Howell and Dean, 1992; Miralles-Flores and Delgado-Baeza, 1992; Snow and Keiver, 2007). In the resting zone, the ratio of extracellular matrix to cell volume is high and the cells are in a relatively quiescent state (Howell and Dean, 1992). In the proliferating zone, chondrocytes become flattened, begin to divide, and become organized into columns (Howell and Dean, 1992). In the zone of

maturation, the synthesis of extracellular matrix allows the cells to separate from each other (Howell and Dean, 1992).

II.c.iii. Postnatal Growth of Endochondral Bone

Growth of the epiphyseal plates is central in the elongation of endochondral bones during childhood and adolescence (Marieb et al., 2005).

> "*As the bone elongates, the epiphyseal plate maintains a constant thickness since the rate at which cartilage is replaced with bone tissue on the diaphysis is equal to the rate at which it grows. Towards the end of the growth period, the rate of chondrogenesis slows down and the growth plate begins to close. The growth plate eventually exhausts its supply of mitotically active cartilage cells, the cartilage stops growing and is replaced by bone tissue*" (Marieb et al., 2005; p. 135).

The diaphysis and epiphysis fuse, signifying the closure of the growth plate (Juul, 2001; Cutler, 1997). In humans, this closure occurs at different times for different bones. For example, the proximal end of the tibia fuses at approximately age 15 years in males and 14 years in females (Ogden, 1984). The distal end of the tibia on the other hand fuses at approximately age 15-18 years in males and 12-14 years in females (Ogden and McCarthy, 1983; White et al., 2008). The closure of the growth plate in long bones marks the end of appendicular growth (Juul, 2001; Cutler, 1997).

Other than elongation, endochondral bones also increase in width during growth. This is achieved by a process called appositional growth (Marieb et al., 2005). In appositional growth, osteoblasts in the periosteum lay down bone on the external surface whilst osteoclasts in the inner surface of the periosteum resorb it (Howell and Dean, 1992; Miralles-Flores and Delgado-Baeza, 1992).

II.d. Hormonal Regulation of Bone Growth

Bone growth is regulated by a number of hormones. These include growth hormone (GH) that is produced by the pituitary gland (Marieb et al., 2005). GH regulates bone growth directly by stimulating the growth of epiphyseal plate (Lanes et al., 2010; Shalet and Rosenfeld, 1998). GH also stimulates the multiplication of cycling cells in the germinal layer of the epiphyseal plate.

Growth is also influenced by insulin like growth factors I and II (IGF-I and IGF-II) produced from the liver and other tissues under stimulation of GH. IGF-I and IGF-II trigger the first step of chondrocyte maturation and promote proliferation of chondrocytes (De Los Rios and Hill, 2000).

Thyroid hormone stimulates growth and maturation of cartilage by initiating chondrocyte hypertrophy (Scanes et al., 1987; Burch and Lebovitz, 1982). This is a normal process in growth plates where mature cells prepare the tissue for ossification (Böhme et al., 1992).

Estrogen at low concentration promotes bone growth during the growth spurts at adolescence. Its effect is indirect because it first binds to intracellular estrogen receptors stimulating GH secretion (Lanfranco et al., 2008). Estrogen may subsequently stimulate chondrocyte growth in the proliferation zone. By contrast, high doses of estrogen inhibit clonal

expansion in the hypertrophic zone and later induce the epiphyseal plates to close, ending growth (MacGillivray et al., 1998; Börjesson et al., 2010).

II.e. Bone Remodeling

Bone is a very dynamic and active tissue. "In an individual's lifetime, the bone matrix and osteocytes are continually removed and replaced within the skeleton. This results in a continual change in the small-scale architecture of bones" (Marieb et al., 2005, p. 136). In adults, bone deposition and removal occur at the periosteal and endosteal surfaces. These two processes constitute bone remodeling (Parfitt, 1984; Parfitt, 1995).

Bone remodeling has two primary functions, first to repair micro damage within the skeleton to maintain skeletal strengths, and second, to supply calcium ions and phosphorus ions from the skeleton to maintain constant concentration of the two ions in serum.

Several circulating hormones regulate bone remodeling. These include estrogens, androgens, vitamin D_3 and parathyroid hormone (Frenkel et al., 2010; Anderson et al., 2008; Parfitt, 1976). Also locally produced growth factors influence bone remodeling, such as IGF-I and II, transforming growth factor (TGF)β, parathyroid hormone related peptide PTHrP, ILS, Prostaglandins, and Tumor necrosis factor (TNF) (Ducy et al., 2000; Yamaguchi et al., 2000; Hoekman et al., 1991; Fazzalari, 2008).

An essential part of remodeling is the communication between osteoblast and osteoclast. Three factors are involved in this process: NF-kappaB ligand (RANKL), NF-kappaB (RANK) and Osteoprotegerin. Increased activation results in bones that are more porous, which does not appear to affect bone strength unless the overall diameter of the bone is significantly changed. See Braunwald et al. (2001) for a more detailed description.

III. REVIEW OF OSTEOPOROSIS

Once the skeleton is full grown bone strength is maintained through remodeling. Bone mineral density (BMD) is the most commonly used proxy for bone health in humans. BMD level has long been used for diagnosis of osteoporosis and osteopenia in humans and can be measured in various ways.

III.a. Measurement of Bone Mass Density (BMD)

Several non-invasive techniques have been developed for estimating skeletal mass or density. These include dual energy X-ray absorptiometry (DXA), single energy X-ray absorptiometry (SXA), quantitative computed tomography (QCT), and ultrasound (Genant and Jiang, 2006; Albanese et al., 2006).

DXA is a highly accurate X-ray technique used to measure BMD and has become the standard in most centers. The units of measurement are grams per square centimeters (g/cm^2) (Lewiecki, 2010). In this technique, ionizing radiation with photon beams of two different energy levels is used (Lewiecki, 2010; Kalkwarf et al., 2010). "The difference in attenuation of the beams passing through body tissues of variable composition allows the instrument to provide a quantitative measurement of BMD" (Lewiecki 2010; p. 124). The mineral content is divided by the area, which partially corrects for body size. "However this correction is only partial

because DXA is a 2 dimensional scanning technique and cannot estimate the depths of postero-anterior length of the bone. Thus, small people tend to have a lower than average BMD" (Jameson, 2006).

Quantitative Computed Tomography (QCT) is used primarily to measure BMD at the spine, neck of the femur, forearm and tibia (Engelke et al., 2008). It utilizes X-rays to provide an image, based on the linear X-ray absorption coefficients of the tissues through which it passes. The QCT image is generated by acquisition of scan data followed by tomographic reconstruction by a mathematical process of calculating the image from the acquired data (Adams, 2009) giving a more detailed characterization of bone than does DXA (Kalkwarf et al., 2010; Khoo et al., 2009). Some of the parameters that are measured by QCT that cannot be determined by DXA include volumetric BMD, bone geometry, and distribution of minerals within the bone cross-section. However, QCT is expensive, involves greater radiation exposure, and is less reproducible (Genant and Jiang, 2006; Breen et al., 1998; Njeh et al., 1997).

"Ultrasound is used to measure bone mass by calculating the attenuation of the signals as it passes through bone or the speed with which it traverses the bone" (Jameson, 2006; p. 471). Compared to DXA, ultrasound devices are less expensive, more portable and radiation free, which makes the technique more valuable for use in screening procedures (Krieg et al., 2008; Hans and Krieg, 2009).

The neck of the femur is the preferred site of measurement for most people since it predicts the risk of hip fracture, the most important consequence of osteoporosis (Finsen et al., 2004; Yan et al., 1999; Memon et al., 1998). However, "[i]n younger individuals, such as perimenopausal or early postmenopausal women, spine measurements may be the most sensitive indicator of bone loss" (Jameson, 2006; p. 472").

The National Osteoporosis Foundation recommends bone mass measurements for all women beginning at age 65. This is based on the assumption that by approximately a decade after menopause, age is a risk factor for osteoporosis in addition to sex and estrogen deficiency.

The World Health Organization (WHO) suggests that patients be considered for treatment when bone mass density (BMD) is >2.5 below mean value for young adults (T-score ≤ 2.5) of the same sex and race (Jameson, 2006). In postmenopausal women the risk factor is a hip (neck of the femur) BMD of < 2.0 (Jee and Yao, 2001; Kanis, 1994; Kanis et al., 2005; Lelovas et al., 2008).

III.b. Risk Factors for Osteoporotic Fracture

Osteoporosis is a condition with multiple risk factors (Pinheiro et al., 2010; Pinheiro et al., 2009; Blazkova et al., 2010). The risk factors can be divided into genetic (non modifiable) and nongenetic (largely due to lifestyle and are therefore potentially modifiable) (2006 position statement of The North American Menopause Society; 2010 position statement of The North American Menopause Society; Pinheiro et al., 2010; Pinheiro et al., 2009; Blazkova et al., 2010). I list these below:

Non-modifiable	**Potentially Modifiable**
Personal history of fracture	Current cigarette smoking
History of fracture in first-degree relative	Low body weight (< 58 lb-127lb)
Female sex	Estrogen deficiency

Advanced age	Early menopause (<45 years or bilateral)
Caucasian race	Ovariectomy
Dementia	Prolonged premenstrual amenorrhea (> 1 year)
	Low calcium intake
	Alcoholism
	Impaired eyesight despite adequate correction
	Recurrent falls
	Inadequate physical activity
	Poor health/fragility
	Stress

This list was compiled from Braunwald et al, (2001; p. 2228), 2006 and 2010 position statements of The North American Menopause Society, Pinheiro et al. (2009 and 2010), and Blazkova et al. (2010).

Some of the risk factors for osteoporosis have been studied comprehensively. These include for example, diet, level of physical activity, menopause, cigarette smoking, alcoholism, genetics, and low body weight. I will only discuss in more detail stress, since it is the focus of my research.

Stress is one of the risk factors that has not been well investigated. However, there are significant reasons to suspect that stress plays an important role in bone health (Sapolsky, 2005; De Kloet et al., 2005). The body responds to stress by physiological and behavioral mechanisms that reinstate homeostasis (Sapolsky, 2005; De Kloet et al., 2005). Behavioral response, depending on the type of stressor, may range from fighting to fleeing.

Physiological response involves a chain of events. When an event is interpreted as stressful, the hypothalamus part of the brain produces corticotropin-releasing hormone (CRH) and arginine vasopressin (AVP) (Hadley and Levine, 2007). These are delivered to the anterior pituitary gland through the hypophysial portal blood vessels and activate the synthesis of adrenocorticotropin hormone (ACTH). ACTH stimulates the adrenal cortex to secrete cortisol in humans, and corticosterone in rats and mice. "Corticosteroids reach every organ by way of circulation, which allows the coordination of brain and body functions geared towards coping with stress, recovery and adaptation" (De Kloet et al., 2005; p 464).

Stress activates the excessive release of cortisol hormone, which is known to inhibit bone growth in humans and has been demonstrated to be a risk factor later in life for osteoporosis (Eskandari et al., 2007, Cizza et al., 2001, Yirmiya et al., 2006, Ilias et al., 2006).

Stress
↓
Hypothalamus (Corticotropin Releasing Hormone)+ Arginine Vasopressin (AVP)
↓
Anterior Pituitary Gland (Adenocorticotropin Hormone, ACTH)
↓
Adrenal cortex (Cortisol in humans, corticosterone in rats) → Released to circulation.

Figure 1.1 Stress hormone axis (following Hadley and Levine, 2007; p. 344).

Cholesterol

↓Desmolase enzyme cleaves the terminal 6 carbons of the side chain of cholesterol

Pregnenolone

↓ 17α-Hydroxylase

17α-Hydroxypregnenolone

↓ Δ5, Δ4 Isomerase

17α-Hydroxyprogesterone

↓ 21 Hydroxylase

II Deoxycortisol

↓ IIβ Hydroxylase

Cortisol
↓↑
Cortisone

Figure 1.2 Pathway of cortisol hormone biosynthesis (following Hadley and Levine, 2007; p. 341).

In humans, "[g]lococorticoid-induced osteoporosis occurs in two phases: a rapid early phase in which bone mineral density [BMD] is reduced possibly as a result of excessive bone resorption, and a slower, progressive phase in which [BMD] declines because of impaired bone formation" (Mazzioti et al., 2006; p. 144). Below is a figure showing the influence of glucocorticoids (GCs) on bone cells.

		+Osteoclasts	↑Cell recruitment	→ Increased bone resorption
			↑Cell differentiation	→ Increased bone resorption
Glucorcorticoids	→	-Osteoblast	↓Proliferation	
			↓Differentiation	
			↑Apoptosis	→ Decreased bone formation
			↓Osteocalsin	
			↓Osteoprotegerin	
		-Osteocytes	↑Apoptosis	→ Decreased repair of bone micro-damage

Figure 1.3 Effects of glucocorticoids on bone cells. GCs have general inhibitory (-) effects on osteoblasts and osteocytes which results in decreased bone formation and repair of microdamage. GCs also have a stimulatory (+) effects on osteoclasts, which enhance bone resporption (Mazziotti et al., 2006; p.145)**.**

GCs have direct effects on bone through an increase in the expression of receptor activation of nuclear factor kappaB ligand (RANK-L), a cytokine responsible for communication between osteoclasts and osteoblasts (Canalis et al., 2004; Mazziotti et al., 2006; Li et al., 2000) in stromal and osteoblastic cells. On the other hand, GCs decrease the expression of osteoproteregin, a cytokine that serves as a decoy receptor for osteoprotegerin ligand. Osteoprotegerin ligand is a factor that stimulates osteoclastogenesis. This series of events leads to a reduction in osteoblast activity (Canalis et al., 2004; Mazziotti et al., 2006). Additionally, GCs enhance the expression of macrophage colony stimulating factor (M-CSF), a cytokine which influences the hemopoietic stem cells to differentiate into macrophages and other related cell lineages (Maziotti et al., 2006). The osteoclasts are part of the macrophage cell lineage (Boyle et al., 2003). Therefore M-CSF in presence of RANK-L induces osteoclastogenesis which enhances bone resorption (Canalis et al., 2004; Mazziotti et al., 2006). GCs also recruit more osteoclasts that resorb bone by upregulating subunits for osteoclastogenic cytokines of glycoprotein 130 family (Richards et al., 2000; Mazziotti et al., 2006). This recruits more osteoclasts that resorb bone. Excess GC also results in a reduction in osteoblast number, which leads to a decrease in bone formation (Mazziotti et al., 2006).

The indirect effects of GCs on bone include the inhibition of calcium absorption from the gastrointestinal tract to serum (Mazziotti et al., 2006; Canalis and Delany, 2002). Calcium binding proteins are required to actively transport calcium across from the gut to serum (Emtage et al., 1974). The formation of calcium binding proteins is regulated by vitamin D_3 hormone. GCs oppose vitamin D action and by so doing affect calcium deposition in bone (Mazziotti et al., 2006; Canalis and Delany, 2002). GCs also inhibit renal tubular calcium reabsorption which results in loss of calcium through urine (Mazziotti et al., 2006; Canalis and Delany, 2002), and influence the production and action of Parathyroid, Calcitonin and Vitamin D hormones, which

regulate bone, calcium and phosphorus metabolism (Mazziotti et al., 2006). GCs can lead to a reduction in secretion of Gonadotropin hormone which in return leads to a negative downstream effect, i.e., reduced production of sex steroids (Manelli and Giustina, 2000; Mazziotti et al., 2006). Estrogen in particular is essential for bone maintenance (Manolagas et al., 2002) and its deficiency enhances bone resorption, exposing the individual to a greater risk of bone loss (Manelli and Giustina, 2000; Mazziotti et al., 2006). And last, GC also regulates growth hormone (GH) and the insulin-like growth factor-1 (IGF-1) axis (Giustina, and Wehrenberg, 1992; Mazziotti et al., 2006). IGF-I is produced from the liver and other tissues under stimulation of GH. IGF-I triggers the first step of chondrocyte maturation and promotes proliferation of chondrocytes (De Los Rios and Hill, 2000). Since GCs weaken the secretion of GH, this leads to a reduction in IGF-1 production (Giustina and Wehrenberg, 1992; Malerba et al., 2005) and a decrease in IGF-1 transcription in osteoblasts (Delany et al., 2001; Mazziotti et al., 2006).

GCs negatively affect the quality of bone through their inhibitory effect on osteoblasts and osteocytes, as well as through their stimulatory effect on osteoclasts (Mazziotti et al., 2006). This inhibition and stimulation occurs through various molecular mechanisms. GCs inhibit the replication and differentiation of osteoblasts by opposing Wnt β catenin signaling (Ohnaka et al., 2005). Wnt signaling is a key pathway for promoting osteoblastogenesis (Ohnaka et al., 2005; Smith and Frenkel, 2005; Canalis, 2005; Mazziotti et al., 2006). Another mechanism through which GCs inhibit osteoblastogenesis is by repressing bone morphogenetic protein-2, (Luppen et al., 2003). GCs also exert their inhibitory effect on bone formation and repair of microdamage by enhancing the apoptosis of osteoblasts and osteocytes (Canalis, 2005; O'Brien et al., 2004; Mazziotti et al., 2006). The apoptosis process is upregulated by activation of caspase 3 (Liu et al., 2004). Members of this protein family are sequentially activated in order to play a key role in cell apoptosis (Thornberry and Lazebnik, 1998). Also, GCs indirectly reduce the number of osteoblasts by redirecting differentiation of bone marrow stromal cells from cells of osteoblastic lineage to those of adipocyte lineage (Mazziotti et al., 2006).

III.c. Current Treatment for Osteoporosis

In the United States alone it is estimated that 10 million people are affected by osteoporosis (Becker et al., 2010). It is therefore necessary to come up with management measures for the affected population. These can be changes in lifestyle for the risk factors that are modifiable (such as increasing levels of physical activity, eating a balanced diet which adequate calcium and vitamin D, cessation of cigarette smoking, and reduced consumption of alcohol). The change in lifestyle strategy works better for postmenopausal women who are at a lower risk for osteoporotic fracture. However, in patients with advanced osteoporosis who have had fractures a number of drugs are currently available on the market to help in treatment.

Three treatments slow down bone resporption: estrogen, calcitonin, and bisphosphonates. Estrogen hormone replacement therapy decreases the rate of bone resorption but does not increase rate of bone formation, and as such plays a major role in prevention of osteoporosis in postmenopausal women rather than a role in treatment (Gallagher et al., 1993; Spelsberg et al., 1999). Calcitonin inhibits bone resorption by acting directly on osteoclasts, the cells that are responsible for bone resorption. Calcitonin alters osteoclast morphology and inhibits production of protease, which is the enzyme that breaks down bone. And last, bisphosphonates are a class of drugs that reduce bone resorption by inhibiting the activity of osteoclasts and shortening their

lifespan. By so doing they reduce the rate of bone resorption, causing an increase in bone density and decrease incidents of fracture (McClung, 2003; McClung, 2006).

There are two treatments that do improve osteoblastic bone formation: PTH and sodium fluoride ions. Parathyroid hormone (PTH) stimulates osteoblastic bone formation and therefore has an initial anabolic effect (Hosking et al., 2009; Hildreth et al., 2010). This results in an increase in trabecular bone density and connectivity, especially when taken intermittently. PTH stimulates IGF-I and collagen production, and appears to increase osteoblast number by recruitment, replication, and inhibition of apoptosis (Canalis, 2010). Sodium flouride ions can also be deposited into the skeleton, where they are incorporated into the crystal lattice of hydroxyapatite, substituting for hydroxyl ions (Vestergaard et al., 2008). This process results in a mineral phase of greater crystallinity. Sodium fluoride and intermittent low doses of PTH are the only agents that are currently known to stimulate osteoblastic proliferation, a function that increases bone formation.

And last but not least, adequate calcium supplementation and vitamin D_3 intake before age 30 to 35 enhances peak bone mass acquisition. Vitamin D preparations have been used in osteoporosis because calcium absorption is impaired if serum levels of vitamin D_3 are marginally low.

IV. FETAL-ORIGINS HYPOTHESIS

Fetal development is important for ensuring the future health of an individual. A number of studies have linked diseases of aging, including osteoporosis, to harmful alterations that happen in critical periods during fetal development (Barker, 1993; Barker, 1995a, b; Dennison et al., 1997; Cooper et al., 2006). Given the significant long-term impact that intrauterine environment has on an individual's life (Barker, 1995c, d; Morton, 1955; Hales and Barker, 1992; Langley and Jackson, 1994; Lumey, 1992), I hypothesize that **maternal prenatal stress will have a negative effect on the offspring's bone development and peak bone mass density, and subsequently predispose the individual to osteoporosis.** This may be a result of exposure of the fetus to high levels of cortisol.

IV.a. Effects of Prenatal Stress in Public Health Studies

During pregnancy some women experience daily stress. These stresses include depression, anxiety, anger, day-to-day challenges, sudden change of environment, social isolation, and pathological conditions (Lazinski et al., 2008; Field and Diego, 2008; Talge et al., 2007). The typical somatic response to these and other similar stressors is elevation of cortisol hormone. Cortisol is known to cross the placenta and consequently influence various aspects of development in the human fetus (Field and Diego, 2008). The effects of elevated cortisol levels on the fetus may vary from defective development (Obel et al., 2005) to spontaneous abortion (Nepomnaschy et al., 2006).

Timing of the stress also plays a crucial role. The outcomes are dependent on whether the mother was exposed to stress during the first, second or third trimester (Talge et al., 2007). The first trimester is characterized by rapid organ development and therefore stress exposure in this period leads to widespread, global effects like cognitive dysfunction, heart malformation, cataracts, deafness and genital and intestinal abnormalities. In the second and third trimesters the

organs mainly enlarge and undergo refinement. Stress exposure in this period leads to low birth weight, skeletal abnormalities and hearing loss (Talge et al., 2007).

Psychological stress in mothers has been found to decrease the gestation period, which results in low birth weight (Wadhwa, 2005). For example, in 1994 when an earthquake of magnitude 6.8 struck the city of Northridge, California USA, women who were pregnant at this time delivered one week earlier on average (Glynn et al., 2001). On the other hand, after the September 11, 2001, terrorist attack in the United States, pregnant women who were present at or in close proximity to the World Trade Center gave birth to babies with low birth weight, suggesting their growth *in utero* had been affected (Berkowitz et al., 2003). In a different study, maternal perception of negative life events between 24 and 29 weeks gestation was demonstrated to increase the risk of preterm birth (Dole et al., 2003).

Prenatal stress has also been demonstrated to increase the risk of abnormal neurodevelopment and behavior and has been shown to be one of the determinants of infant temperament (Davis et al., 2007; Buitelaar et al., 2003). Several studies have found stressful life events in the prenatal period to be associated with poor mental development, delayed motor skills, and poor language abilities (Sarkar et al., 2008; Lazinski et al., 2008; Huizink et al., 2003). According to Talge and colleagues (2007), these infants spend more time in deep sleep, less time in quiet and active alert status, and show a less optimal performance on the Neonatal Behavioral Assessment.

"During the third trimester, maternal cortisol level was shown to predict increased negative reactivity, e.g. startle reactions or distress, in response to novel surprising stimuli as measured by the fear subscale of an infant behavior questionnaire" (Davis et al., 2007; p. 742). One recently published study conducted a 10-month follow-up of behavioral reactivity to prenatal stress exposure and found it to persist throughout this period (Leung et al., 2010). However these results were not consistent with those from an earlier study in which the most significant differences between infants born of stressed mother and those born of non-stressed mothers disappeared by the age of 4 to 5 months (De Weerth et al., 2003).

De Bruijin and colleagues (2009) went further and tested cortisol levels of preschool children born of stressed and non-stressed mothers. Children who were prenatally exposed to high cortisol levels had higher cortisol levels when compared to non-exposed children. Levels were higher in girls compared to boys. Different behavioral outcomes have been reported for boys and girls as well, depending on the timing of the stress. Maternal prenatal stress in the first trimester has been found to be associated with behavioral problems in boys, whereas stress in the third trimester is associated with behavioral problems in girls (De Bruijn et al., 2009).

IV.b. Effects of Prenatal Stress in Anthropology

Anthropologists have had a longstanding interest in how events in early life affect the long-term development of an individual. This is not easy to investigate because it most usually involves the design of longitudinal studies with their attendant challenges. However, retrospective studies done in a cross-sectional design have provided significant insights. For example, Owsley and Jantz (1985) conducted a study on an archeological sample of Arikara Indian skeletal populations from South Dakota to determine how the arrival and settlement of Europeans affected fetal growth. The Europeans' arrival in America was a great source of stress to the native populations because they introduced new diseases like smallpox and measles that

wiped out most of the indigenous populations. There was also increased warfare. In their study, Owsley and Jantz (1985) compared the length of long bones of two infant samples of early and late postcontact period. Retarded growth was reported among the populations that lived in poor socioeconomic conditions with chronic diseases and inadequate food.

The effects of war, famine and disease on birth outcomes have also been investigated. The Dutch famine, for example, was a five-month period of severe food shortage during the last year of World War II. A study on Dutch women born between 1943 and 1947 reported that women exposed to prenatal famine reported a significantly increased history of breast cancer (Painter et al., 2006a).

Barker and colleagues (Barker, 1995a; Barker et al., 1989a; Barker et al., 1991) have conducted studies on people born between 1911 and 1930 in six districts of Hertfordshire, England, to determine whether there was any pattern linking early life conditions to various diseases at old age. Some of their findings include an association between below average birth weight and below average weight at one year of age with poor adult lung function (Barker et al., 1991), ischaemic heart disease in men (Barker et al., 1989b), impaired glucose tolerance, non-insulin dependant diabetes, beta cell dysfunction, high blood pressure (Hales et al., 1991), and death from cardiovascular disease (Osmond et al., 1993).

In Sweden, a parish population comprising individuals born in 1805-1845 and still alive at age 40 was found to be at a higher risk of sudden death from cerebral and cardiovascular disease if their mothers were struck by a poor harvest during either early or late stages of pregnancy (Bygren et al., 2000). From this study, it is evident that a stable nutrient supply is essential to the mother and fetus during gestation.

In a living population, Godoy and colleagues (2008) looked at how environmental perturbations like rainfall variability affect native Amazonians in Bolivia. They found rainfall variation during years 2-5 of childhood to be associated with lower adult height. Their results also suggested that adverse climatic conditions harm women more than men. "Possible explanations for this finding could be household allocation of resources in favor of boys, or the effect of environmental perturbations on path variables that only affect females, for example, the onset of menarche" (Godoy et al., 2008; p. 32).

From these studies, it is clear that the conditions that prevail during pregnancy can and do affect the offspring. These are mostly situations that induce stress to the mother during pregnancy. Since most of the studies cited above are retrospective, the researchers mainly gathered the information from available records and interviews. These studies have, however, laid a strong foundation on which to conduct further research on this important topic.

IV.c. Effects of Prenatal Stress in Animal Studies

In animal studies, "the effects of maternal stress… have been shown to vary based on the type and intensity of stress applied, gender of the offspring, age, strain of the experimental subject used, the timing of stress relative to the critical developmental period of the organ under investigation and nature of the dependent variables evaluated" (Bellinger et al., 2008; p. 420). Since most of these effects last for a long time, the results suggest that stress *in utero* may lead to a permanent change in gene expression, which results in permanent physiological effects to the individual. A number of animal studies have demonstrated that most defects reported in offspring due to stress *in utero* occur when stress is induced during the last trimester of gestation.

This is the period when the gonads, reproductive tract, lymphoid organs, and the brain differentiate (Bellinger et al., 2008). The results are contrary to the human studies reviewed in the previous section, in which more global defects are manifested in offspring when exposed to stress *in utero* in the first trimester, and lower birth weight associated in maternal stress in the third trimester.

Kapoor and colleagues (2009) set up an experiment in guinea pigs to help understand the effects of the timing of prenatal stress on learning and memory. In their study, they looked at the effect of early, late, and mid stress on hippocampal-dependent spatial memory and non-spatial discrimination learning. They demonstrated that maternal stress in late gestation resulted in adult male offspring that were faster to develop a search strategy. On the other hand, males whose mothers were exposed to stress in mid gestation, a period of rapid brain development in guinea pigs, exhibited impaired spatial learning.

In non-human primates, prenatal stress in early stages of gestation has been shown to be associated with low birth weight and decreased activity in rhesus monkeys (Schneider et al., 1999), whereas inducing stress in mid to late stages of gestation had no impact on birth weight. At 6 months of age, prenatally stressed rhesus monkey infants were reported to have low level of exploratory behavior, more disturbance behavior, and abnormal response to novelty in the form of falling asleep (Schneider et al., 1999). Other effects of maternal stress observed in rhesus monkeys include compromised physical growth, short attention span, and retarded motor development (Buitelaar et al., 2003). In squirrel monkeys, social stress in pregnant mothers resulted in infants with slow motor maturity and activity, short attention span, and less well developed balance control (Clarke and Schneider, 1993).

Prenatal stress has been associated with lower weight of pups and poor early development in rats (Weinstock et al., 1988). Prenatally stressed rodents also show decreased exploration and more defecation, which are typical signs of stress in rodents (DeSantis and Schmaltz, 1984; Grimm, 1987; Wakshlak and Weinstock, 1990). Other studies have demonstrated the opposite results, with the prenatally stressed rodents being more active and explorative when exposed to novel stimulation (Deminiere et al., 1992). Genes may be essential contributing factors to these differences since differences in activity levels in offspring have been observed in different strains of mice following stress exposure *in utero* (Stöhr et al., 1998). "For example, prenatally stressed male offspring from a low activity strain of mice were more active than control males, whereas prenatally stressed male offspring from a high activity strain were less active" (Buitelaar et al., 2003; p. 54). Female offspring from both strains were less active (Stöhr et al., 1998).

Proper early development of the immune system is crucial for all animals. Animal studies have demonstrated that maternal stress in general compromises the development of innate, acquired, and passive immunity. For example, exposing pregnant mice to psychological stress during the last third stage of gestation inhibits macrophage and neutrophil functions (spreading and phagocytosis) (Palermo-Neto et al., 2001; Fonseca et al., 2002). In rats, prenatal stress in gestation week three weakens the natural killer cell (NK) cytotoxicity and increases growth of experimentally induced tumors *in vivo* (Klein and Rager, 1995; Kay et al., 1998; Palermo-Neto et al., 2001). Reduction in activity of NK cells has also been observed in rhesus monkey infants exposed to stress *in utero.*

The thymus is a critical organ in the immune system. Its function is production of T-lymphocytes, which are important cells of the acquired immune system. In prenatally stressed

pigs, the size, morphology, and function of the thymus were found to be affected at birth (Tuchscherer et al., 2002). Other studies demonstrated that the total number of peripheral blood lymphocytes, $CD4^+$, and $CD8^+$ lymphocytes were reduced in adult rats stressed *in utero* (Llorente et al., 2002; Gotz and Stefanski, 2007).

Passive immunity, the transfer of ready-made antibodies from the mother to the fetus or infant through the placenta or milk, has also been shown to be affected by prenatal stress. In rats, transfer of maternal immunoglobulin G (IgG) was found to be reduced when the mother was exposed to inescapable electric shock in the last fourth of gestation (Sobrian et al., 1992). Sex specific differences have also been observed in squirrel monkeys, with the IgG levels being low in male offspring whereas the female offspring remain unaffected (Coe and Crispen, 2000). This is consistent with other human and animal studies on prenatal stress that demonstrate sex specific differences in behavioral and neuroendocrine outcomes in offspring that are prenatally stressed (Weinstock, 2007; de Bruijn et al., 2009).

While my research goals are focused on human health, the vast majority of research experiments on bone have been done on rats (Gasser et al., 2006; Bagi et al., 1993; Lane et al., 2003; Koshihara et al., 2004; Sampson et al., 1996; Kavuncu et al., 2003). My work is based on a rat model in order to be able to test specifically for the role that prenatal stress plays on bone development.

V. THE RAT AS AN ANIMAL MODEL FOR STUDYING OSTEOPOROSIS

From a biomedical perspective, basic research into the etiology of many important human conditions, including those involving the skeleton, is often initiated in animal models. Since 1994, the U.S. Food and Drug Administration (FDA) requires data from both the rat, and a well-validated larger animal model for preclinical evaluation of new experimental drug therapies (at a clinical dose and five times the dose (Turner et al., 2001). Mice and rats are often used because of the detailed knowledge of their genetics (Mouse Genome Sequencing Consortium et al., 2002; Gibbs et al., 2004). More specifically for this thesis, the rat has been extensively used as an animal model for osteoporosis research (Lelovas et al., 2008). This research spans studies ranging from risk factors to therapeutic options. These will be reviewed briefly in the following sections.

V.a. Menopause

In the 80's, Wronski and collegues established ovariectomized rats as a suitable model for studying postmenopausal osteoporosis (Wronski et al., 1988; Wronski et al., 1989a, b). In these studies, bilateral ovariectomy was performed on Sprague Dawley rats and bone quality assessed for the proximal tibial metaphysis and the vertebrae. "Data obtained indicated that ovariectomy induced an initial rapid phase, and later slow phase of bone loss, both associated with increased bone turnover" (Wronski et al., 1989b; p 365). Other studies corroborate the finding that the surgical removal of ovaries was associated with osteoporosis (e.g., Aitken et al., 1972; Lindgen and Lindholm, 1979; Lindgren and Deluca, 1982; Kalu, 1984). Ovariectmomy is still currently used in rats to mimic menopause in oteoporosis research (Palumbo et al., 2009; Ferretti et al., 2010).

Since surgical removal of ovaries is invasive and leads to inactivity in the animal during the recovery period, which is a confounding risk factor, alternative methods for studying postmenopausal osteoporosis have been developed. These consist of the administration of drugs that inhibit the protective role of estrogen on the rat skeleton. "For example, administation of ICI 182, 780, a pure estrogen antagonist in adult female rats leads to 30% reduction in bone volume" (Gallagher et al., 1993; p. 133). This reduction is associated with an increase in osteoclast surface, hence antagonizing estrogen's action on bone formation and resorption. Buserelin, an agent that lowers endogenous estrogen indirectly by reducing the secretion of gonadotropin releasing hormone, was also demonstarted to induce osteopenia as effectively as bilateral ovariectomy (Goulding and Gold, 1989). Inhibiting aromatase, an enzyme that is responsible for a key step in the biosynthesis of estrogen, has been demonstrated to reduce serum levels of estrogen in rats, leading to bone loss (Gasser et al., 2006).

V.b. Lack of Physical Activity

Skeletal remodelling responds to mechanical forces and as such, physical activity is crucial in maintaining bone health (Frost, 1992). To assess the lack of physical activity as a risk factor for osteoporosis, studies have been conducted on rats in which specific procedures are performed on the animal to limit its level of physical activity. For example, surgical removal of a small part of the sciatic nerve in rats greatly limits movement of the upper limbs. Abolishing movement in the lower limb has been demonstrated to lead to a reduction in both trabecular and cortical bone volume and inhibition of age related bone gain (Zeng et al., 1996). Severing knee tendons (Thompson and Rodan, 1988) and spinal cord injury (Okumura et al., 1987) are other surgical forms of inducing immobilization in rats that have been shown to increase bone resorption and and decrease bone apposition, inducing osteopenia in rats.

Physical restriction of limb movement such as bandaging the limb to the body with elastic tape (Bagi et al., 1993) or placing a cast on the rat limbs (Steinberg and Trueta, 1981) was also shown to lead to increased bone loss, decreased bone stiffness, and decreased bone growth in general. Physical restraint in small cages is the other means by which rats have been used to study the effects of immobility on bone (Zorbas et al., 1998).

V.c. Alcohol Consumption

Alcohol abuse is a significant contributing factor to osteoporosis and bone loss (e.g., Malik et al., 2009; Kim et al., 2003; Saville, 1965; Laitinen et al., 1991). Sampson and colleagues (1996) performed a study on Sprague-Dawley rats to understand the effects of alcohol consumption in young and actively growing bones. Their results show that chronic alcohol consumption during bone development results in reduced density and mass for both cancellous and cortical bone. Defective vitamin D metabolism in rats has been found to be one of the mechanisms through which chronic alcohol consumption affects bone health (Turner et al., 1988).

V.d. Dietary Calcium Intake

Calcium deficiency is one of the major risk factors for osteoporosis, and rats have been

used in studies exploring the effects of low levels of calcium in the diet. Low levels of dietary calcium have been found to result in a significant reduction of bone mineral density in the trabecular bone and increased rates of bone turnover (Koshihara et al., 2005), which results in rapid bone loss. The effect of the calcium-to -phosphorus ratio on bone mineralization has also been studied in rats (Koshihara et al., 2004; Koshihara et al., 2005).

V.e. Therapeutic Agents

Given the key role that estrogen plays in maintaining normal bone metabolism and skeletal integrity (Spelsberg et al.,1999), estrogen hormone replacement therapy (HRT) has been used in treating bone loss in postmenouposal women (Cummings, 1991, Judd et al., 1983, Nelson et al., 2002). However, given the negative effects that HRT has on the reproductive system, especially the increased risk of breast and uterine cancer (Jacobs, 2000), and migrane headaches (Silberstein, 1999), there has been a need to develop therapeutic agents for bone loss that do not have side effects on the reproductive system. Raloxifen has been tested on ovariectomized rats and found to be beneficial without negative effects on the reproductive system (Black et al., 1994). Calcitonin, an antiresorptive agent that attenuates the decline in bone mineral density in postmenopausal women without noticeable effects on reproductive tissue was also tested on rats (Kavuncu et al., 2003). A one-year trial on bisphosphonates, a class of drugs known to inhibit bone resportion in short term experiments of young growing animals, was found to improve functional bone quality in mature ovariectomized rats (Hornby et al., 2003). Increased bone strength has also been obtained in experiments involving use of parathyroid hormone in rats (Søgaard et al., 1994), and low doses of strotium were shown to stimulate bone formation as well improve bone strength and architecture in rats (Marie et al., 1985).

V.f. Evaluation of Bone Mass, Architechture and Metabolism

Methods of evaluating bone mass, architechture and metabolism have also been tested in rats. There are invasive and noninvasive methods of bone evaluation, and rats have need used in testing both. The noninvasive methods include:

- Measuring levels of biochemical markers of bone turnover, such as alkaline phosphatase, serum osteocalcin, pyridinoline, and telopedtide of type 1 collagen in urine or serum (Seibel, 2000; Bohatyrewicz, 1998; Shen et al., 1995; Vanderschueren et al., 1994).
- Quantitative assesment of bone macrostructure by use of dual X ray absorptiometry (DXA) and computed tomography (CT) (Tsujio et al., 2009; Petersen et al., 2000; Griffith et al., 2010).
- Quantitative assesment of bone microstructure by use of micro-computed tomography (micro-CT) (Boyd et al., 2006; Jiang et al., 2005).

Invasive methods of bone quality assesment used in rats include :

- Histomorphometry, which is quantitave histology based on stereology and evaluates micro-architecture in 2 dimensional sections (Yao et al., 2005; Parfit et al., 1987; Turner et al., 1987).
- Mechanical strength evaluation performed by compression testing (Sun et al., 2009; Yao et al., 2005).

Note that only the noninvasive methods of bone quality evaluation are used on living human beings. However, human cadavers have been used to assess bone strength and the results employed in improving the management and treatment of osteoporosis. For example Kayanja and colleagues (2004) investigated the distribution of anterior cortical strain by compression to determine the vertebral body at risk of secondary fracture using cadaveric thoracic spinal segments. Their results indicate that the "anterior cortical strain is concentrated at the apex of a thoracic kyphotic curve" (Kayanja et al., 2004; p. 76). "The vertebral body immediately above the index vertebral compression fracture has the next highest amount of strain and therefore the highest risk of secondary fracture" (Kayanja et al., 2004; p. 86). In a different study, Sutter and colleagues (2010) used human cadaveric femora to test whether femoroplasty (the injection of bone cement into the proximal femur in an attempt to prevent fragility fracture) improves the mechanical properties of bone. The results showed that "1) femoroplasty significantly increased fracture load and energy to fracture when osteoporotic femora were loaded in simulated fall conditions, and 2) cement filling in the femoral neck may have an important role in the extent to which femoroplasty affects mechanical strength of the proximal femur" (Sutter et al., 2010; p. 99).

V.g. Summary of Rationale for Animal Use

The experiments in my study are designed to determine the effect of pre-natal stress on bone development in growing rats. To accomplish this, it was imperative to maintain control over the life of the mother as well as the offspring. For the mother, the diet and external environment as well as the timing of mating and conception were standardized. During gestation, stress was induced with specificity in timing and duration. For the offspring, the postnatal environment was standardized to eliminate all other stress variables except the *in utero* stress. I also needed to work with short-lived animals in order to cover gestation as well as a significant portion of postnatal life. This experimental design precluded the study of human subjects. Basic research into the etiology of many important human conditions is often initiated in animal models. The study therefore used the rat model, specifically the Wistar strain, which is a well-characterized model for studying osteoporosis (Campbell et al., 2010; Tagil et al., 2010; Tami et al., 2009; Kavuncu et al., 2003; Koshihara et al., 2005). As such, the results are readily applicable and comparable to other studies in this discipline.

VI. SUMMARY

In this chapter I outlined bone development and the biology of bone remodeling, followed by a discussion of osteoporosis and its associated risk factors, which includes stress. Based on a survey of the literature relevant to the fetal-origins hypothesis for adult diseases (from public health, anthropological, and animal studies), there is significant reason to hypothesize that stress to a pregnant female can result in deleterious effects to the bone development of her offspring, even when that offspring does not suffer stress after birth. In order to test this hypothesis, I developed a controlled study using rats. These animals are commonly used in studies of bone development and osteoporosis. As such, they make a reasonable model for studying the various factors that may contribute to human osteoporosis. The methodology for this study is outlined in the next chapter.

CHAPTER 2: MATERIALS AND METHODS

I. OVERVIEW

This project involved seven different parts. Protocols for animal breeding, stress induction to pregnant mothers (also referred to as pregnant dams in this dissertation, the terms are used interchangeably), fecal sampling for cortisol analysis, monitoring of food intake by the mothers, rearing of the offspring, euthanasia and bone histology. All procedures on live animals were performed according to protocol number R325-1009 approved by the University of California Berkeley, Animal Care and Use Committee (ACUC) on October 28, 2008.

The male and female animals used for breeding were purchased from Charles River, 1000 Park Center Drive, Hollister, California. Breeding was done at the University of California Berkeley animal care facility. After conception, the pregnant mothers were put in four different groups depending on the gestation period in which stress was to be induced. Stress induction was done by restraining the mothers in rat restrainers called decapicone bags (Braintree Scientific, Inc. PO Box 361 Braintree, MA 02185) product catalogue number DC-200 (Vyas et al., 2002).

To confirm whether restraining was stressful to the mothers, fecal samples were collected from the mothers and used to analyze their levels of the stress hormone, cortisol. This method was preferred because it is not invasive and as such would not cause additional and unquantifiable stress to the pregnant dams. To determine whether stress induction had an impact on the animals' caloric intake, the amount of food consumption by the mothers during pregnancy was measured every 24 hours. Upon delivery, the litters were culled to 8 pups for each mother. The pups were allowed to nurse for 3 weeks, following standard ACUC protocols. Weaning was done on day 21 postnatal. The offspring were raised under normal laboratory conditions, housed in same-sex pairs with *ad libitum* access to food and water. None of the offspring were stressed. At termination point, the animals were euthanized according to guidelines developed by the ACUC. The tibias were extracted at necropsy and processed for histology to examine morphological development. The remainders of the carcasses are in storage at -20 degrees Celsius for future study.

In summary, data was collected on maternal weight gain from gestation day 1 to day 21, cortisol hormone level in the mothers, amount of food intake by the mothers, offspring weight before sacrificing them and bone histomorphometry of offspring.

II. ANIMAL BREEDING

II.a. Animal Purchase

To investigate prenatal stress as a risk factor for reduced bone quality in the postnatal life of rats, this experiment was performed using a total of 283 Wistar rats (*Rattus norvegicus*). These rats are commonly used in osteoporosis research (Campbell et al., 2010; Tagil et al., 2010; Tami et al., 2009; Kavuncu et al., 2003; Koshihara et al., 2005). Since breeding was done at the University of California Berkeley animal care facility, only a total of 28 sexually mature females (9-10 weeks old weighing 200-250grams) and 7 males (established breeders weighing 400-500

grams) were purchased from Charles River, 1000 Park Center Drive, Hollister, California, USA. These 28 adults were bred to obtain 224 offspring.

Total rats purchased	28 females 7 males
Total rats bred	224
Total rats used	259

Table 2.1 Total number of animals used for the project.

II.b. Housing

As soon as the purchased animals arrived at the facility they were housed in 10 x 19 x 7 inch cages (136 inch^2 of floor space) according to the guidelines developed by ACUC summarized as follows:

- Adult males were housed no more than 2 per cage (older rats weighing greater than 600 grams were housed one animal per cage).
- Adult females were housed no more than 3 per cage.
- Breeding cages housed no more than one female and their litter.
- Pups were weaned at 21 days of age.
- Weanling rats were housed at 5 per cage, but housed as adults when they reached 6 weeks of age.

These guidelines were followed throughout the study. The number of animals per cage was determined based on estimated weight at various ages to ensure the rats had adequate cage-floor space per animal. Every cage had a card with identification information consisting of: animal group number, sex and date of birth. The cages were changed twice a week to ensure the animals were in a clean and comfortable environment.

The animal housing room was maintained under standard laboratory conditions:

- Light-dark cycle: 12:12 hours, lights on at 07:00h and off at 19:00h
- Temperature 22+/-1°C
- Relative humidity 50+/-10%

The rats seemed to enjoy the dark session very much as noted by the extra playing and excitement that took place as soon as the lights went off.

Most of the purchased adult animals were allowed a one-week conditioning period to their new environment. During this period, the rats were handled daily and weighed every other day. This was done to train the rats to get used to the new personnel and daily handling. The training helped ensure the animals were not scared every time they were weighed, fed and their cages changed for cleaning. The conditioning was crucial because given the aim of the study, to find out whether immobilization stress on the mother had an impact on bone development in offspring, experiencing handing stress would have been a confounding factor.

Seven of the females that came impregnated did not undergo the one-week conditioning period. This is because the experimental protocol, as described in detail below, was employed as soon as they arrived at the animal facility.

II.c. Feeding

Commercial rat diet (Harlan Telkad irradiated laboratory diets 2918-092809m, Madison, Wisconsin USA) and bottled tap water were available *ad libitum* except for the periods when the females were undergoing immobilization stress as later described. The basic composition of the diet was crude protein not less than 18%, crude oil (fat) not less than 6%, crude fiber not less than 3.8%, ash 5.9% and available carbohydrate not less that 50%.

II.d. Breeding of Animals for Timed Pregnancies

Before breeding, females were determined to be in estrus by behavioral observation. The female was moved to a male cage and observed for an average of 10 to 15 minutes. The behavioral signs indicating estrus are: darting around in the cage when chased around by the male, making a squeaking noise, wiggling of ears and arching of the back when mounted by the male. When not in estrus, the females resisted any approaches by the male by kicking him away any time he tried to mount.

Estrous females were transferred to the male cage for 24 hours. The beginning time was recorded. If the male was observed to not be very aggressive (i.e., not chasing the female around the cage and not trying to mount her many times), the female would be transferred to a different male cage.

After 24 hours, the female was singly housed and that particular date marked as breeding day 0. As is typical for rats, no vaginal plugs were observed on the females after the mating period and so confirmation of conception was mainly done by monitoring the weight gain. Since the animals were allowed to stay in the male cage for 24 hours and there was no continuous monitoring for the entire period, the assumption was that the females groomed themselves before the plug could be observed.

The normal weight gain for the females used in this study was 3 grams on average every 24 hours if not pregnant. However, if pregnant, the mothers' weight gain was as high as 6 grams per day from the beginning of the second week of pregnancy and went higher every day until the last day of pregnancy.

Because the weight gain in the first week of gestation was similar to that of non-pregnant animals (3 grams on average), it was difficult to determine whether the female was pregnant in the first week after breeding using the weight gain monitoring method. For this reason, the animals that were stressed in the first week of gestation were obtained from the vendors as timed pregnant females. The vendors, like Charles River, have various ways of determining whether the females conceived soon after breeding, such as constant monitoring to ensure copulation takes place and the female has a vaginal plug (http://www.criver.com/SiteCollectionDocuments/rm_rm_d_pregnant_rodent.pdf).

III. STRESS INDUCTION TO PREGNANT MOTHERS

III.a. Stress Induced at Various Times of Gestation

The pregnant females were divided into three stress groups and one control group (n=7 per group). Each maternal stress group experienced immobilization stress during a different

week of gestation. Stress was induced daily during Gestational Week (GW) 1 for the first maternal group, daily during GW 2 for the second maternal group, daily during GW 3 for the third maternal group, and not at all for the fourth maternal group (the control group). Only one control group of 7 females was used for the entire study. Since the animals were bred in a controlled environment, one control group was enough for the entire experiment. Stress and the time it was induced were the two main variables.

Stress period	Gestational week (GW) 1	GW 2	GW 3	No Stress (Control)	Total
Total number of dams	7	7	7	7	28

Table 2.2 Stress schedule for pregnant dams.

Note: Each pregnant dam in Table 2.2, apart from those in the control group, was stressed for 45 minutes, 3 times per day during the stress period. The control group was not stressed.

Fecal samples were collected twice a week from all 28 pregnant dams to determine whether or not the immobilization stress resulted in elevated cortisol levels (the details for how the feces were collected is described later). Two offspring per litter for each maternal group described above were sacrificed at either birth, 1 month, 2 months, 3 months, or 4 months postnatal.

III.b. Chronic Immobilization Stress

The procedure described below was applied to all pregnant animals, in the stress groups, during their designated stress week. The pregnant dams were stressed for just one of the three weeks of their pregnancies. None of the offspring were stressed.

Adult pregnant rats (2-3 months old) were placed in standard immobilization bags. This was done by holding the bag open with one hand resting on the bench and with the other hand holding the rat by the tail with its hind limbs suspended in the air and only the front limbs on the bench. This way, the animal could only move forward and so was directed to walk inside the immobilization bag. The immobilization bags were rodent-sized “Decapicone” bags (Braintree Scientific, Inc.) modified as follows: 1) the tip was snipped to allow the rat’s snout to protrude, 2) in order to minimize hyperthermia small holes were punched on the sides of the bag to increase ventilation 3) the open end of the bag was twisted around the tail of the animal, and the twisted area secured with standard 1/4" brightly colored laboratory tape. A second piece of tape was used across the top to secure the bag and animal to the floor or side of the empty cage, to prevent the animal from falling on its side while in the bag. Only their tails and snouts protruded from the bag. Thus, they did not have access to either food or water during this time. The animals were restrained in decapicone bags, which prevented them from making any movements other than shuffling their paws. They could not turn their heads to the side nor turn their bodies within the bags. However, they had sufficient room for their rib cage to expand with heavy breathing.

The rat in the bag was placed in an empty cage. A cage top was placed on the cage to prevent possible escape. The cages containing the animals were placed in a well-lit fume hood because stressed rats emit strong and unpleasant odors. By placing them in a fume hood, the odors were contained. In addition, the constant flow of air over the immobilized rats helped minimize hyperthermia during the procedure. The hood was well lit so that the animals could be easily observed and monitored.

The animals were restrained for three 45 minute periods per day (9AM, 1PM and 5PM). A 45-minute immobilization period was the minimum requirement for this study because it takes 30 minutes for the steroid hormone level to rise in the system. Since the study involved measuring of hormone metabolites in fecal matter normally observed after 14-17 hours (Lepschy et al., 2007; Bamberg et al., 2001), it was necessary that the animals had enough exposure time in order to detect the hormone rise in their feces.

The weight and behavior of the pregnant rats were monitored closely to ensure they were faring well during the study. During stress episodes, the animals were monitored every 15 minutes, or more frequently, from the time they were placed in the decapicone bags until they were returned to their home cages. Animals were examined for signs of strenuous breathing or distortion in the rat's body into a potential painful or harmful position. If the rat was not fully upright (all 4 paws on the ground) they were temporarily removed and repositioned in the bags. At the end of the 45 minutes, the rat was removed from the bag, and returned to its home cage, where monitoring of its behavior for possible lethargy and limb pain continued. Animals were observed for the following; chromodacryorrhea (red tears, nasal/eye porphyrin discharge) reduced feed consumption or total failure to eat/drink, difficulty in breathing, excessive weight loss, ruffled fur, lameness, bloody vaginal discharge or abortion. If vaginal/nasal/eye discharge was observed outside the restraint period, the animal was given 12 hours (without restraint) to see if there was any improvement. If the clinical signs abated, but returned a second time, the animal would be euthanized. If there was no improvement in a period of 12 hours, the University veterinarians were consulted for advice on whether to euthanize the animal.

The pregnant females were weighed daily following each stress session, and then every other day during the other two weeks of the pregnancy. These weight data from stressed animals were compared to weight data of age-matched control animals with the aim of eliminating from the study any that diverged from controls by more than (15%). None of the animals diverged from the controls by more than 15%.

My observations were consistent with those from previous studies (D'mello and Liu, 2006) where almost 100% of animals showed normal exploratory and locomotive behavior after being returned to their home cages after stress induction. However if a rat was injured as a result of immobilization, which was never the case for this study group, it would have been immediately euthanized.

Note that because the research aim was to study the effects of stress on bone health, the proposed experiments necessarily involved placing animals in a stressful situation. However, every attempt was made to minimize the discomfort of the animals. Although animals were purposefully put in stressful conditions, care was taken not to cause them any physical harm and render them unhealthy. Thus, they were placed in the bags in such a way as to maximize their comfort (keeping them upright, for example) and minimize the opportunity for physical harm. They were also monitored very carefully to ensure that their health was maintained throughout these procedures. If an animal met any of the following criteria, it was removed from the study:

limb pain (when an animal could not use a paw normally, or holds its paw against its body, indicating a problem with the limb), unexpectedly large weight loss (greater than 15% compared to controls), lack of grooming or lethargy, abortion, or failure to eat and drink. Three of the confirmed pregnant females had an unexpectedly large weight loss and consequently did not give birth on the expected day. Even though no blood was observed on the floor of the cage, they were assumed to have aborted the pups and eaten them. Consequently, these females were not included in the study.

Over the course of the project, a total of seven rats were removed from the study and euthanized. In addition to the three females removed from the study, as described in the preceding paragraph, another pregnant female observed to not eat and drink on the day she was to give birth was found dead in her cage the following morning. One pregnant female that was one week overdue from the time she was expected to deliver was removed from the study and euthanized. The veterinarians performed an autopsy and she was found to have a fluid-filled uterus. Two pups that were observed to be underweight, 50% lower in weight compared to their littermates were removed from the study and euthanized.

III.c. Control Group

Animal breeding for the control group was done as described above. Animals in this group were not subjected to immobilization stress. They had unlimited access to food and water. As mentioned earlier, only one control group of 7 females was used for the entire study. This was because the animals were bred in a controlled environment, so one control group was enough for the entire experiment. Weight measurements were done daily for the entire gestation period to be able to make comparisons with all the other experimental groups. As discussed below, fecal sample collection was done on gestational days 2, 5, 9, 12, 15, and 18.

IV. FECAL MATTER SAMPLING

In order to confirm that the immobilization was causing stress (and thereby elevating cortisol levels), cortisol levels were measured twice a week for all pregnant dams. On the days that cortisol levels were monitored, the monitoring was done multiple times over the course of those 24 hours. Considering how regularly hormone levels were monitored (multiple times a day, twice a week for 3 weeks), fecal matter sampling was the best, least invasive method. Various studies have examined corticosterone metabolism and excretion (Lepschy et al., 2007; Touma et al., 2003; Touma et al., 2004; Bamberg et al., 2001; Mateo and Cavigelli, 2005). These studies successfully validated the use of enzyme immunoassay to reliably measure fecal corticosterone metabolites in laboratory rats.

Pregnant dams were singly housed on recycled paper bedding, Tekfresh (Harlan Laboratories, Madison Wisconsin, USA). To sample fecal matter, fresh bedding was provided every 4-8 hours (at 8AM, 12PM, 4PM, 8PM, and 12AM). Fecal samples were then taken from the soiled bedding.

Fecal stress hormone metabolite levels peak 7 to 16 hours after the stress (Bamberg et al., 2001). As noted above, pregnant animals were stressed three times during the day, 9AM, 1PM and 5PM every day for seven days. Fecal sampling was done on gestational days 2 and 5 for group 1, gestational days 9 and 12 for group 2, on days 15 and 18 for group 3, and on gestational

days 2, 5, 9, 12, 15, and 18 for the control group (who did not undergo immobilization stress). All fecal samples were stored at -80°C immediately after collection until analysis.

IV.a. Extraction of Fecal Corticosterone Metabolites (CMs)

Each sample was put in an aluminum foil cup and dried in an oven at 60°C for a minimum of 4 hours. The time it took for each sample to dry varied, with some taking as long as 12 hours. Each sample was then crushed and well homogenized with a mortar and pestle. An amount of 0.1g of each sample was placed in a clean test tube and 2ml of 80% methanol added. The samples were then shaken 3 times for 30 seconds each, using a hand vortex. This was followed by centrifugation at a speed of 2500g for 15 minutes. An amount of 0.5 ml of the supernatant was then transferred to a clean vial and evaporated under a gentle stream of nitrogen. These samples were then sent to the University of Veterinary Medicine, Department of Biomedical Sciences, Vienna Austria, where they were assayed by 5α-pregnanae-3β, 11β,21-triol-20-one enzyme immunoassay (EIA), (Touma et al., 2003, Touma et al., 2004, Lepschy et al., 2007).

I performed a comparative analysis using the corticosterone enzyme immunoassay kit (Cayman Chemical Company, Ann Arbor, MI, USA) to check the data from Austria. This assay has been validated for measuring fecal glucocorticoids. It was performed as follows; each sample was put in an aluminum foil cup and dried in an oven at 60°C for a minimum of 4 hours. This is the least amount of time it took for the fecal matter to be dry enough for easy crushing. The sample was then crushed and well homogenized with a mortar and pestle. An amount of 0.2g of each sample was placed in a clean test tube and 2 ml of 90% ethanol added. The samples were then shaken 3 times for 30 seconds each, using a hand vortex. This was followed by centrifugation at a speed of 2500g for 20 minutes. The supernatant was then transferred to a clean vial and evaporated under a gentle stream of nitrogen. The extract was dissolved in 500µl of the EIA buffer and assayed by a corticosterone enzyme immunoassay kit (Cayman Chemical Company, Ann Arbor, MI, USA).

V. MONITORING FOOD INTAKE OF THE PREGNANT DAMS

To determine whether immobilization stress had an effect on the caloric intake of the dams, the amount of food intake during the stress period was compared to the amount of food intake when the dams were not being stressed. This was done by measuring the weight of food placed in the cage at the beginning of a 24-hour cycle and subtracting the weight of the food at the end of the 24 hours every day during the gestation period. The cage floor was checked to ensure any spilled food pellets were collected and measured as well. This procedure did not apply to the offspring.

VI. REARING OF THE OFFSPRING

The offspring were raised to various stages of maturity and then sacrificed to determine the effect of the prenatal stress.

All of the 28 dams gave birth on day 21 or 22 of gestation, as is standard for rats. The litter sizes ranged from 5 to 22. Since a maximum of 8 pups was required per dam, culling was

done for those mothers with >8 pups soon after delivery to standardize the litter size. This helped avoid excessive food competition among the offspring during the first two weeks of their life in which they mainly depend on breastfeeding for food. The pups were kept with their mothers for the first 3 weeks of life, at which point they were weaned (as is standard practice for the care of laboratory rats). At this time, the mothers were sacrificed and weanling rats housed at 4 per cage. As soon as the pups reached 6 weeks of age, they were housed as adults. This was in accordance with the guidelines developed by ACUC.

Pups were raised under normal conditions with standard diets and sacrificed at different ages (one group was sacrificed at birth and the others at 1 month, 2 months, 3 months, and 4 months). In total, there were 4 main pup groups defined by when the mother was stressed, and within these 4 pup groups there were four sub-groups consisting of 11 offspring sacrificed at each stage of development.

Stress period for the pregnant dams	GW 1	GW 2	GW 3	No Stress (Control)	Total
Total number of pregnant dams	7	7	7	7	28
Offspring per dam	8	8	8	8	
Total offspring per group	56	56	56	56	224

Table 2.3 Total number of offspring.

Stress period for the pregnant dams	GW 1	GW 2	GW 3	No stress (Control)	Total
Total number of pregnant dams	7	7	7	7	28
Total offspring per group	56 (~8 per dam)	56(~8 per dam)	56(~8 per dam)	56(~8 per dam)	224
Number of pups to be sacrificed at birth	11	11	11	11	44
Number of pups to be sacrificed at the end of week 4	11	11	11	11	44
Number of pups to be sacrificed at the end of week 8	11	11	11	11	44
Number of pups to be sacrificed at the end of week 12	11	11	11	11	44
Number of pups to be sacrificed at the end of week 16	11	11	11	11	44

Table 2.4 Sacrifice schedule for offspring.

The total number of offspring expected from the 28 dams was 224. A minimum of 11 pups per group for a total of 20 groups was required for the study.

VII. EUTHANASIA

Two different methods of euthanasia were used, depending on the age of the rats.

VII.a. One Day Old Pups

A few cotton balls were soaked with isoflurane and put under a wire mesh in the glass jar. The pups were placed in the jar and the lid closed, making sure they did not touch any of the liquid. When the pups stopped moving, they were removed from the jar and decapitated with a sharp pair of scissors. Extra caution was taken by the researcher to avoid inhaling any of the isoflurane. The procedure was carried out in the necropsy room, which is well ventilated. At the end of the procedure, the wet cotton balls were evaporated dry in the hood in the necropsy room before they were discarded.

VII.b. Four-Weeks and Older Animals

Four week and older animals were euthanized individually in the euthanasia chamber. Carbon dioxide (CO_2) was turned on to as high a flow rate as possible with the chamber tightly closed. The animals remained in the chamber with the gas flowing for 90 seconds. A blood sample was immediately drawn from the animal by cardiac puncture and preserved on ice until it was centrifuged to separate plasma from the blood cells. This was followed by bilateral thoracotomy for rats over 200 grams or cervical dislocation for rats less than 200 grams, as a secondary measure to ensure the animals were dead.

The animals were then dissected to remove the hind limbs by first making a small incision by the ankle and cutting all the way to the hip region. This was followed by dissection around the limb removing the skin and muscles to expose the femoral head. The femoral head was dislocated from the hip socket and the limb separated from the rest of the body. The tibia and femur were then defleshed and immediately put in a vial full of 10% phosphate buffered formalin (Iwaniec et al., 2008). The formalin fixation was performed at 4°C for 24 hours in capped vials and then transferred to 70% ethanol for storage until processing (Iwaniec et al., 2008). All bones in solutions were stored at 4°C.

VIII. BONE HISTOLOGY

VIII.a. Tissue Processing for Histological Analysis

Any traces of muscle and cartilage tissues that remained on the bones at necropsy were removed before sizing them for embedding. The proximal part of the tibia (1 cm from the proximal end) was dehydrated in graduated series of ethanol. The proximal tibia proceeded to embedding, sectioning, staining and histomorphometric analysis as described in detail below. Bone measures were carried out in the growth plate and a region corresponding to secondary spongiosa, 1mm distal to the growth plate (Figure 2.1) (Yao et al., 2010). The measurements

were used to assess the size of the growth plate, trabecular tissue area, trabecular bone area, trabecular bone perimeter, osteoblast surface, osteoid surface, erosion surface and number of osteoclasts.

All of these variables were measured in the proximal tibia because, compared to the femur (the other weight bearing bone of relevance to osteoporosis), the tibia has a relatively flat top that makes it easier to assess the growth plate and the trabecular bone tissue area directly below it. The proximal femur has three divisions, the head, greater trochanter, and lesser trochanter, that make it hard to take simple measurements of the growth plate as well as define the area below it.

VIII.a.i. Dehydration and clearing

The bones were dehydrated in increasing concentrations of ethanol (EtOH) and cleared in solution of 99% methyl methacrylate (MMA) (Sigma-Aldrich M55909-2L) and 100% ethanol mixed in the ratio of 1:1 according to the schedule outlined below:

1. 70% EtOH 2 hours under vacuum (17 in Hg)
2. 80% EtOH 2 hours under vacuum (17 in Hg)
3. 80% EtOH 2 hours under vacuum (17 in Hg)
4. 95% EtOH 2 hours under vacuum (17 in Hg)
5. 95% EtOH 2 hours under vacuum (17 in Hg)
6. 100% EtOH 2 hours under vacuum (17 in Hg)
7. 100% EtOH 2 hours under vacuum (17 in Hg)
8. MMA:100% EtOH 1:1 mix; 2 hours under vacuum (17 in Hg)
9. MMA 2 hours under vacuum (17 in Hg)

VIII.a.ii. Methyl Methacrylate (MMA) Infiltration & Embedding

To be able to obtain thin sections for analysis of the growth plate and the trabecular bone, the mineralized bones were embedded in 99% methyl methacrylate, which is a hard substrate that matches the hardness of calcified bone tissue. Preparation of solutions for infiltration and embedding was done under a fume hood in glass containers. The infiltration media consisted of 96% methyl methacrylate (Sigma-Aldrich M55909-2L) and 4% dibutyl phthalate (Fisher Scientific AC16660-0010). The tissues were infiltrated for 48 hours. The embedding media consisted of 96% methyl methacrylate, 4% dibutyl phthalate and 0.25% perkadox 16 (Fisher Scientific, NC9829102). The embedding was done according to an unpublished protocol obtained from Professor David Burr's laboratory at Indiana University, School of Medicine. For details on the protocol please contact Professor David Burr.

VIII.a.iii. Preparation of Pre-polymerized Base

In order to prevent the specimens from sitting on the bottom of the vials when embedding, a small amount of the embedding media was poured into the plastic vials and allowed to solidify into a base. This base allowed easy orientation of specimens for sectioning and trimming of the block prior to sectioning. The following procedure was used to prepare the

base: four millimeters of the embedding media were poured into polypropylene plastic vials and placed uncapped under a vacuum (17 in Hg) for 2 hours. The vials were then removed from the vacuum, tightly capped and placed in a water bath maintained at 32°C overnight to accelerate polymerization. The following morning the plastic was checked for stage of solidification. Vials that were fully polymerized were removed from the water bath and stored at room temperature until used.

VIII.a.iv. Embedding

Specimens were removed from infiltration media and placed in a vial with the previously polymerized base. The specimens were covered with embedding media (0.25% catalyst) and placed (uncapped) under vacuum (17 in Hg) for 2 hours. Vials were then removed from the vacuum, and the specimen oriented for sectioning. The lids were fastened tightly and placed in a refrigerator overnight. The specimens were then removed from the refrigerator and the orientation and solution volume checked. If necessary, topping with embedding media was done to keep the specimen covered and the lid fastened tightly afterwards. The vials containing specimens in the embedding media were placed in a water bath maintained at 45°C to accelerate polymerization. This took 48 hours on average. Mineralized bone histology requires plastic embedding, which takes a much longer time than paraffin embedding when dealing with demineralized bone.

VIII.a.v. Sectioning

Sectioning was done using a Leica 2165 Microtome (Leica, Heidelberg, Germany) with a tungsten carbide knife, D-profile (Delaware Diamond Knives, Wilmington, DE). The blocks were placed in the microtome using specialized holder and trimmed to a desired anatomical position within the bone. Longitudinal sections, 4-μm thick were obtained. Thin sectioning of bone requires a semi-automated microtome. This is because the sections come out one at a time and is necessary to use both hands to pick them up and lay them on slides. The semi-automated microtome rotates by itself at a click of a button enabling the researcher to catch the slides as they come out. The sections were lifted off the knife with a pair of fine-tipped tweezers and placed on 2% gelatinized slides for adherence. After placing the section on a gelatinized slide, the section was flattened using a fine paintbrush dipped in 70% alcohol. In order to prevent waves/wrinkles when placing sections on slides, the surface of the slide for section placement was saturated with 70% alcohol. The section was then covered with a piece of plastic (Glad© brand sandwich bags) the size of the slide, placed flat in between two sheets of paper towels and flattened out with a small roller. The paper towels absorbed the excess alcohol. The slides were then air-dried. When dry, multiple slides of 3-4 were placed in between two blank slides and clamped with two small C-clamps. The clamped slides were then placed in a drying oven at 53°C overnight. The next morning, the slides were unclamped, separated, and the plastic covers removed from the sections. The best sections with minimal artifacts were then chosen for analysis.

VIII.a.vi. Deplastisizing

Removal of methyl methacrylate and rehydration of mounted specimens was necessary prior to staining in order to provide deeper penetration and greater contrast.

1. Histoclear I 5min
2. Histoclear II 5min
3. Histoclear III 5min
4. Acetone I 5min
5. Acetone II 5min
6. 100% EtOH 2min
7. 95% EtOH 2min
8. 70% EtOH 2min
9. dH_2O 2min
10. dH_2O 2min

VIII.a.vii. Staining: Von Kossa Method with McNeal's Tetrachrome Counterstain

This stain was used because it gives clear details of the cellular structure in bone (Iwaniec et al, 2008). Select section of the proximal epiphysis were stained and used to assess the total area measures of the different chondrocyte zones in the proximal epiphysis (resting, proliferative and hypertrophic) determined based on cell morphology in accordance with previously published methods, (Snow and Keiver, 2007; Howell and Dean, 1992; Miralles-Flores and Delgado-Baeza, 1992). The sections were also used to assess trabecular total tissue area, trabecular bone area, trabecular bone perimeter, the linear extent of osteoblasts and the osteoid matrix as indicators of bone formation, number of osteocytes and the linear extent of eroded bone surface as indicators of bone resorption (Parfitt et al.,1987; Erben et al., 1997).

Note: While Von Kossa is a good stain for mineralized tissue, it turns all mineral tissues black and therefore needs to be counter stained with McNeal's Tetrachrome which is metachromatic.

Reagents:

1. Silver nitrate solution (5%) (Binds to calcium and phosphorus minerals)

Silver Nitrate	5 gm / 25 gm
Distilled water	100 ml / 500m

- This solution was filtered before each use and a dedicated silver funnel was used since silver stains everything black.
- The silver nitrate was then poured into a dedicated staining container and covered.
- The solution was kept in a dark cabinet in light-proof container and was re-used.

2. Sodium Carbonate-Formaldehyde Solution:
   (acts as a developer by turning Silver nitrate black).

Sodium Carbonate	5gm / 30 gm
Formaldehyde	25 ml / 150 ml
Distilled Water	75 ml / 450 ml

This solution was filtered before use.

3. Farmer's Diminisher – a fresh batch (210 ml) was mixed for each run (This is a fixative that prevents silver nitrate from coming out).

Sodium Thiosulfate	20 gm
Potassium Ferricyanide	I gm
Distilled Water	210 ml

- Once the potassium ferricyanide was added, this solution was stable for only about 45 min.
- The sodium thiosuflate was dissolved in the distilled water first, then potassium ferricyanide added.

4. McNeal's Tetrachrome Solution (2%) (Polysciences, Cat. #2783);

McNeal's Tetrachrome*	2.0 gm /10 gm
Double distilled water	100 ml / 500 ml

- The dye was added and slowly brought to boiling point.
- The heat was turned off and the solution allowed to stir overnight.
- The solution was then filtered before use.

Staining procedure

Covering film was removed and sections de-plasticized in acetone (Kacena et al., 2004). This was followed by rehydration through graded series of ethanol (100% EtOH, 95% EtOH, 70% EtOH and distilled water x 2). Staining was carried out as follows:

1. Sections stained in Silver Nitrate solution for 10 minutes in the dark
2. Rinsed in distilled water x 3 for 1 minute each
3. Stained in Sodium Carbonate-Formaldehyde solution for 2 minute
4. Rinsed in distilled water x 2 for 1 minute each (Potassium Ferricyanide to Farmer's added at this point)
5. Stained in Farmer's diminisher for 30 seconds (timing was very critical for this step).
6. Washed in running tap water for 20 minutes
7. Rinsed in distilled water for 1 minute
8. Stained in McNeal's tetrachrome solution for 10 – 15 minutes
9. Rinsed in distilled water x3 for 1 minute each
10. Dehydrated in 1 change of 70% EtOH, 95% EtOH and 100% EtOH; blotted between changes of alcohol

11. Cleared in 2 changes of xylene
12. Coverslipped with Eukitt
13. Allowed to dry under the fume hood overnight.

VIII.b. Bone Histomorphometry

The quantitative changes in bone were determined by histomorphometry. This primarily involves quantitative histology based on stereology that evaluates micro-architecture in two-dimensional sections (Partiff et al., 1987), using semi-automated image analysis software (Bioquant Image Analysis Corporation, Nashville, TN, USA) linked to a microscope and a camera. This technique extracts quantitative information about bone, which is three dimensional, from measurements made on two-dimensional histology sections of the bone. The bone measurements, units and terminology used in histomorphometric analysis were defined and derived according to the Nomenclature Committee of The American Society for Bone and Mineral Research (Partiff et al., 1987). The growth plate measurements were defined and derived according to Howell and Dean (1992). All the techniques used in this section were developed by Bioquant Image Analysis Corporation, Nashville, TN, USA. A total of nine outcome variables were measured from the growth plate and the secondary spongiosa region using the detailed protocol described in the rodent trabecular bone histomorphometry manual by Bioquant Image Analysis Corporation (http://osteo.bioquant.com/home/brightfield-rodent-trabecular). The variables measured were; a) trabecular total tissue area, b) trabecular bone area, c) trabecular bone perimeter, d) osteoblast surface (the linear extent of osteoblasts on the bone surface), e) osteoid surface (the linear extent of osteoid matrix on the bone surface), f) number of osteoclasts, g) erosion surface (the linear extent of bone surface eroded by osteoclasts), h) the thickness of the upper zone of the growth plate (consisting of resting and proliferating chondrocytes), and i) the thickness of the lower zone of the growth plate (consisting of the hypertrophic chodrocytes). The detailed description of how each variable was measured is given below.

The first step to taking the measurements was to mount the slide containing a stained rat proximal tibia section on a microscope stage linked to a camera. An image of the section was captured at a 20X magnification and projected on a computer monitor. All measurements were taken from the computer screen. The computer used had the Bioquant Image Analysis software installed on it.

VIII.b.i. Total Tissue Area

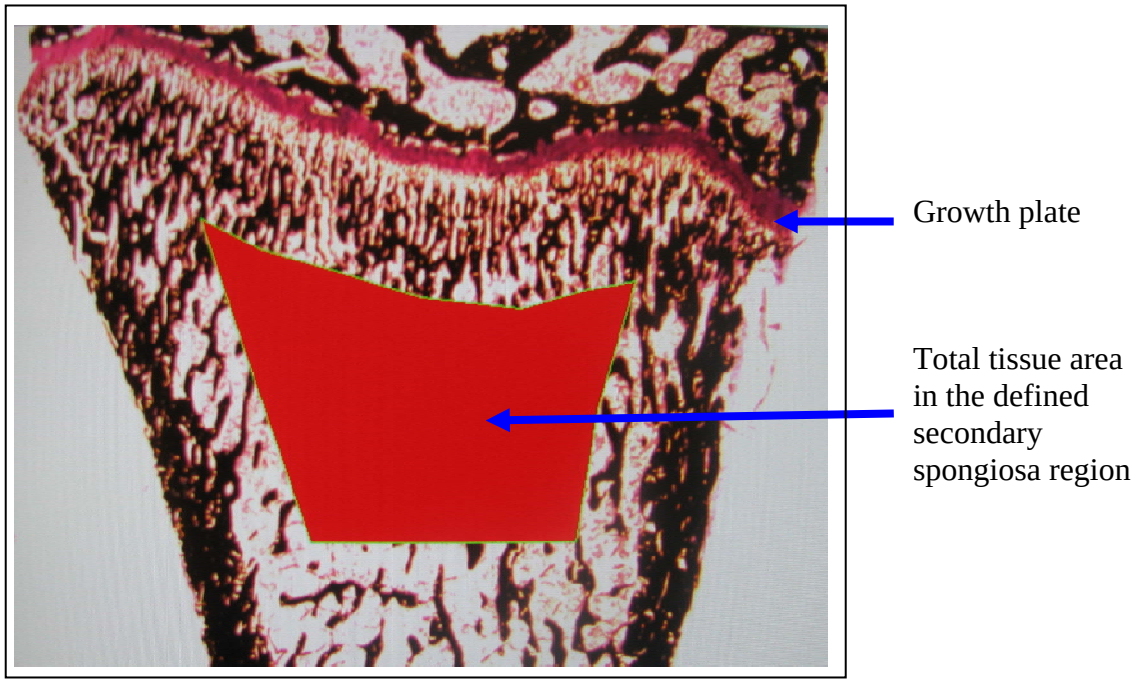

Figure 2.1 Image of a rat proximal tibia section at a magnification of 20X (stained using Von Kossa method with McNeal's tetrachrome counterstain) showing the growth plate and the defined total tissue area in secondary spongiosa region.

Total tissue area is a measure of the area of entire trabecular bone tissue defined (Figure 2.1) (which include the mineralized bone, collagen matrix, empty space and bone cells) in the secondary spongiosa region. It is significant to the overall hypothesis of this study because it assesses whether maternal prenatal stress negatively affects the size of mature trabecular bone tissue hence the bone quality. "Bone quality describes aspects of bone composition and structure that contribute to strength independently of bone mineral density. These include bone turnover, microarchitecture, mineralisation, level of microdamage and the composition of bone matrix and mineral" (Compston, 2006, p. 579). A reduction in the overall size of the trabecular tissue can be interpreted as a compromise in bone quality if accompanied by reduced level of mineralization (Compston, 2006). The tissue area is also used as a proxy for size to determine the male female difference of the tibia in this study. It is within this region that all the other variables except for the growth plate were measured.

The total tissue area was defined by creating a region of interest, the metaphyseal standardized region, which is 1mm from the growth plate and comprises only the secondary spongiosa (Yao et al., 2010; Parfitt et al., 1987; Parfitt, 1988; Bioquant Image Analysis Corporation, Nashville, TN, rodent trabecular bone histomorphomery manual). Defining the tissue area as 1mm below the growth plate eliminates the primary spongiosa, which is mainly composed of newly formed bone that is not well-mineralized. The secondary spongiosa region is more mature, enlarged and well-mineralized bony trabeculae. This makes the secondary spongiosa more informative when assessing the level of mineralization. Since the focus was on

the trabecular bone, the sides of the area were defined as 500μm from the edge of the bone to eliminate the cortical bone. Using the same metric measure ensured consistency in the amount of tissue eliminated from the edge of the bone. To get a flat bottom for the polygon, I used the top right corner as a reference point to measure an area that was at most 3mm from the growth plate. All together, this definition gave me an area of analysis in the secondary spongiosa of the proximal tibia that included trabecular area between 1mm and 3mm distal to the growth plate and excluded the cortex (Yao et al., 2010). Total tissue area is defined as the boxed-in area as shown in Figure 2.1. The defined area is the preferred standard because it has a continuous representation of the mature mineralized trabecular bone tissue. The area above it is largely composed of primary spongiosa with recently formed bone of lower mineralization and the area below it that has a marrow cavity. Therefore any effects on bone cellular functions and mineralization is well captured in the secondary spongiosa region.

To generate the total tissue area number (area of everything covered by the polygon, Figure 2.1, which includes mineralized bone, collagen matrix, empty space and bone cells) the defined region was highlighted and the software automatically calculated the size of the area covered when prompted. The software is programmed to automatically record the number in the output report. For more information on the techniques see rodent trabecular bone histomorphometry manual by Bioquant Image Analysis Corporation (http://osteo.bioquant.com/home/brightfield-rodent-trabecular).

VIII.b.ii. Bone Area

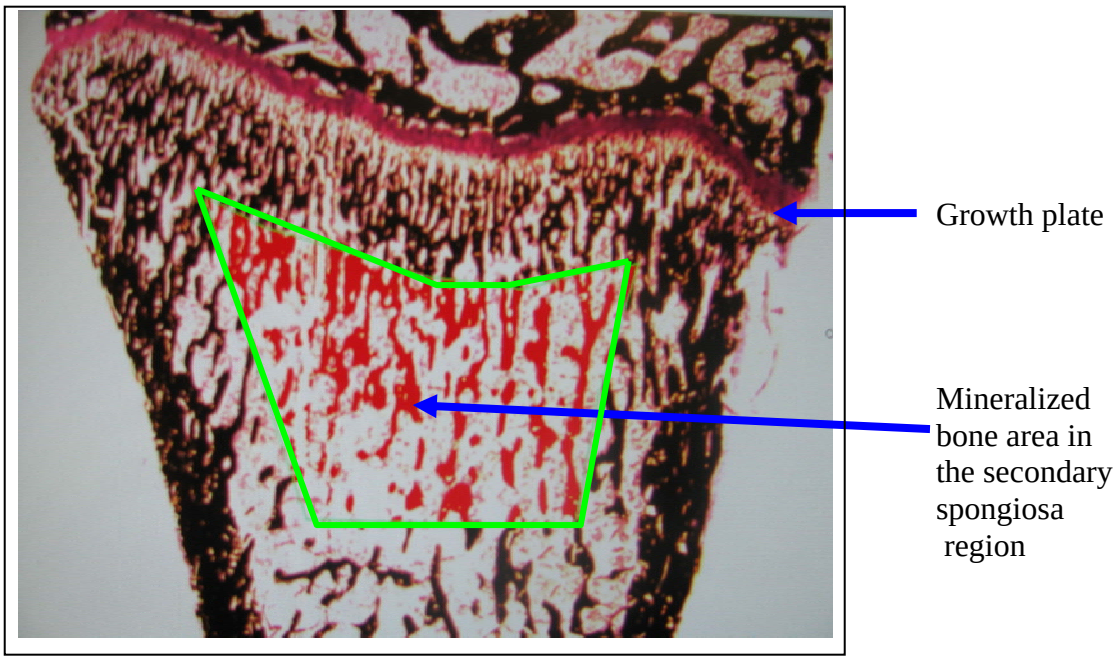

Figure 2.2 Image of a rat proximal tibia section at a magnification of 20X (stained using Von Kossa method with McNeal's tetrachrome counterstain) showing the mineralized bone area (thresholded in red) in the secondary spongiosa region.

The mineralized bone area inside the polygon is thresholded red as shown in Figure 2.2. Bone area is defined as the total mineralized bone area in the secondary spongiosa region covered in red. This is different from the total tissue area as it is a measure of the mineralized area only. However, it is measured in the same area as defined for total tissue area. The level of mineralization is important to the overall hypothesis of the study because minerals make up the inorganic components of bone. This is essential for skeletal functions (structural support, protection of vital organs, mineral storage, production of blood cells and aid in locomotion). If stress has negative effects on the level of mineralization, it negatively affects the bone mineral composition and its functions.

To generate the bone area number (the area inside the polygon covered in red), the area was highlighted and the software automatically calculated the size of the area when prompted. The software is programmed to automatically record the number in the output report. For more information on the techniques see rodent trabecular bone histomorphometry manual by Bioquant Image Analysis Corporation (http://osteo.bioquant.com/home/brightfield-rodent-trabecular).

VIII.b.iii. Bone Perimeter

Bone perimeter is defined as the total perimeter of each of the mineralized area in the secondary spongiosa region (Figure 2.2). It was measured in the same area as defined for total tissue area. The trabecular bone area is threshholded, it appears red in Figure 2.2. The software

is prompted to automatically draw a line around each bone surface area and calculate the total perimeter. The number is automatically recorded in the output report. For more information on the techniques see rodent trabecular bone histomorphometry manual by Bioquant Image Analysis Corporation (http://osteo.bioquant.com/home/brightfield-rodent-trabecular).

VIII.b.iv. Osteoblast Surface

Osteoblast surface refers to the bone surface perimeter covered by osteoblasts. This section assessed the amount, in terms of linear extent along the bone surface in a cross section, of bone forming cells available to secrete the collagen matrix (osteoid) that calcifies to form bone. A higher number indicates an increase in the rate of bone formation (Erben et al., 1997). This is significant to the overall hypothesis of this study because it is a measure of whether maternal prenatal stress reduces the number of osteoblasts, which are essential for bone formation. The linear extent of osteoblasts was measured in the defined area as shown in Figure 2.2.

Below are images showing;

1. An overview window of the defined area showing one example of an internal small square, which is a subsection of the defined tissue area, from which osteoblasts' surface was measured per a single projection (Figure 2.3a). Note that, a high magnification (20X) was required to see and measure the linear extent of osteoblasts. Magnifying the entire defined region made it too big to fit on the computer screen. To be able to measure the linear extent of osteoblasts from the entire defined region, a very small section of the polygon, as shown in the overview image (Figure 2.3a) window, could be projected on the computer screen at one time. The osteoblasts were quantified by measuring the linear extent of cells on the bone surface across the section. This was done by drawing a line along the osteoblasts lining the bone surface and the length of the line recorded. After covering the sub-area of the polygon under the view, the field of view was moved to the next sub-area and the same procedure repeated until the entire polygon was covered. The software calculated the total linear extent of the osteoblasts lining the bone surface in the defined region and gave the final value in the output report. The total value was used in the final analysis. Individual cell count is the most ideal way of assessing the number of osteoblasts but the hundreds of cells present in one tibia section, the younger animals having the most number of cells, makes it difficult to collect the data in that format.
2. A higher magnification image (20X) (Figure 2.3b), of an area equivalent in size to the small internal square in Figure 2.3a showing the osteoblast cells lining the bone surface. This indicates the amount, in terms of linear extent of cells in a cross section, of bone forming cells available to secrete the osteoid that calcifies to form bone. A higher number indicates the bone is forming fast (Erben et al., 1997). For more information on the techniques see rodent trabecular bone histomorphometry manual by Bioquant Image Analysis Corporation (http://osteo.bioquant.com/home/brightfield-rodent-trabecular).

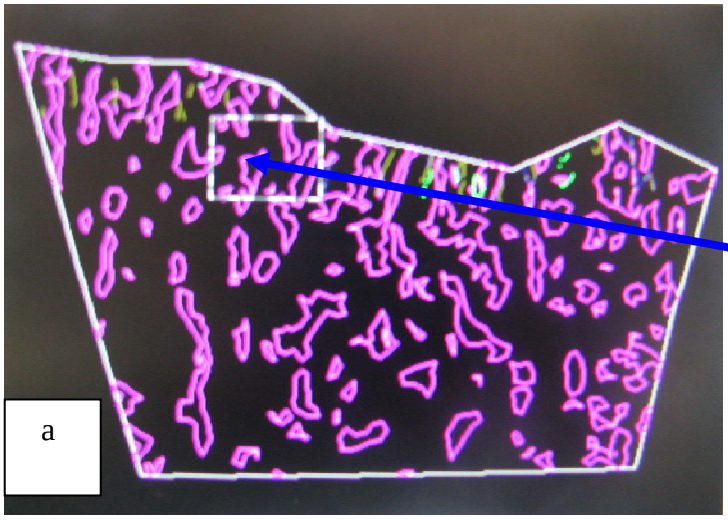

Figure 2.3a An overview window of the defined area showing one example of an internal small square, which is a subsection of the defined tissue area, from which osteoblasts' surface was measured per a single projection. (See Figure 2.2 for example of reference section definition).

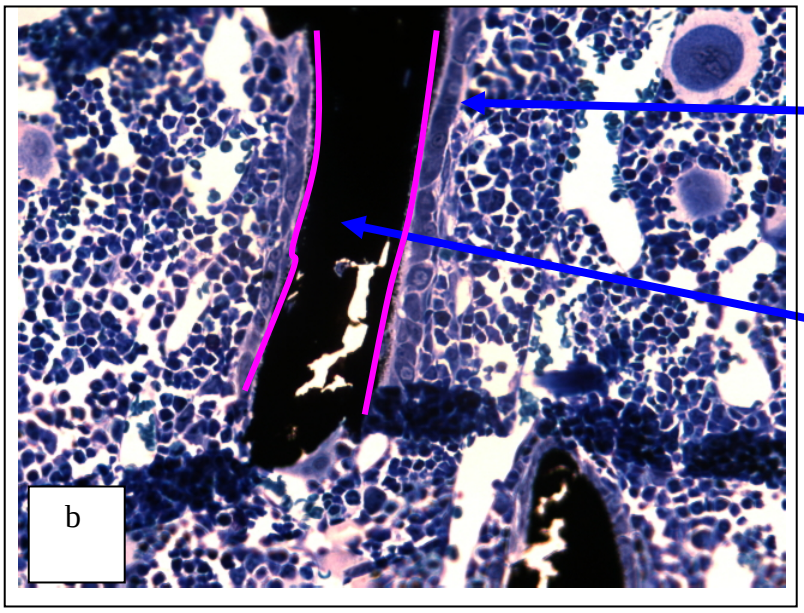

Figure 2.3b A higher magnification image (20X) of an area equivalent in size to the small internal square in Figure 2.3a showing the osteoblast cells lining the bone surface.

VIII.b.v. Osteoid Surface

Osteoid surface refers to the bone surface perimeter covered by osteoid matrix. This measurement assessed the amount of collagen matrix, in terms of linear extent along the bone surface in a cross section, secreted by the osteoblasts. This indicates how active the bone forming cells are and hence the rate at which the bone is forming (Erben et al., 1997). It is significant to the overall hypothesis of this study because it is a measure of whether stress negatively affects the function of osteoblasts by reducing the amount of collagen matrix secreted.

1. The first two steps of this process are identical to the first two steps in the measurement protocol for osteoblast surface, described above.
2. The primary difference for measuring osteoid surface can be seen in Figure 2.4 where the osteoid surface is noted, i.e, a thick layer of collagen matrix secreted and laid down by the osteoblast cells. The amount of collagen matrix secreted by the osteoblasts indicates how active the bone forming cells is and hence the rate at which the bone is forming (Erben et al., 1997). For more information on the techniques see rodent trabecular bone histomorphometry manual by Bioquant Image Analysis Corporation (http://osteo.bioquant.com/home/brightfield-rodent-trabecular).

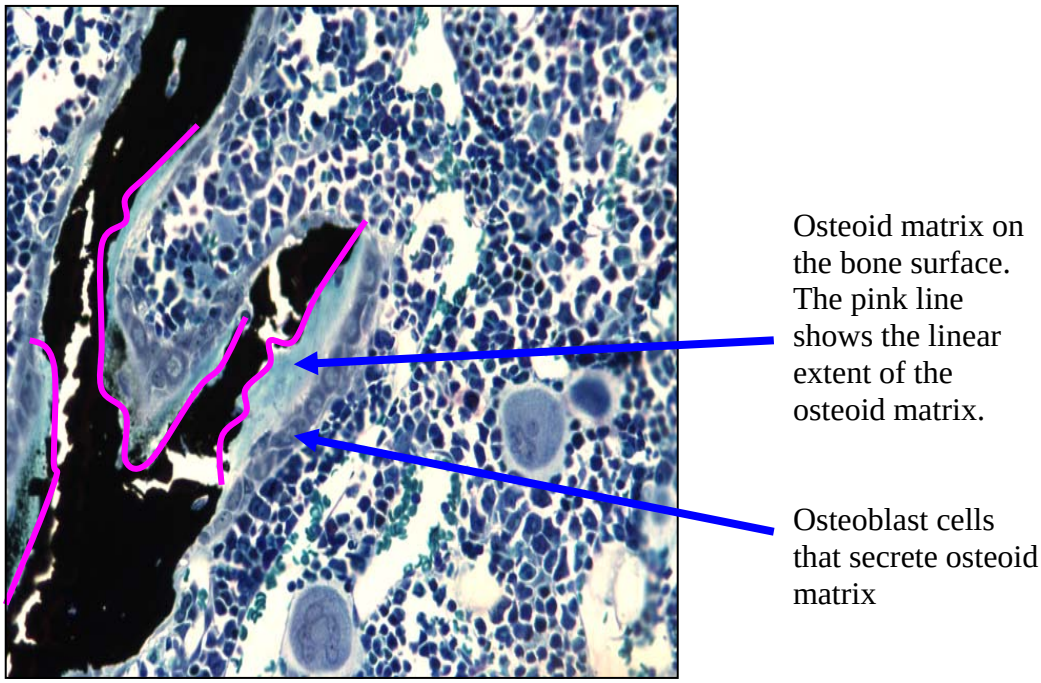

Figure 2.4 A higher magnification image (20X) of an area equivalent in size to the small internal square in Figure 2.3a showing the osteoid surface i.e. a thick layer of collagen matrix secreted and laid down by the osteoblast cells.

VIII.b.vi. Osteoclast Number/Erosion Surface

This measurement assessed the number of bone-resorbing cells available at any particular time point to erode the bone surface. The higher the number of osteoclasts, the higher the bone resorption rate (Erben et al., 1997). This is significant to the overall hypothesis of this study because it is a measure of whether maternal prenatal stress leads to an abnormal increase in the numbers of osteoclasts which results to higher bone resorption.

Figure 2.5 shows a higher magnification image (20X) of an area equivalent in size to the small internal square in Figure 2.3a showing osteoclast cells. The total count indicates the number of bone resorbing cells available at any particular time to erode the bone surface. The higher the number, the higher the resorption rate (Erben et al., 1997). For more information on the techniques see rodent trabecular bone histomorphometry manual by Bioquant Image Analysis Corporation (http://osteo.bioquant.com/home/brightfield-rodent-trabecular).

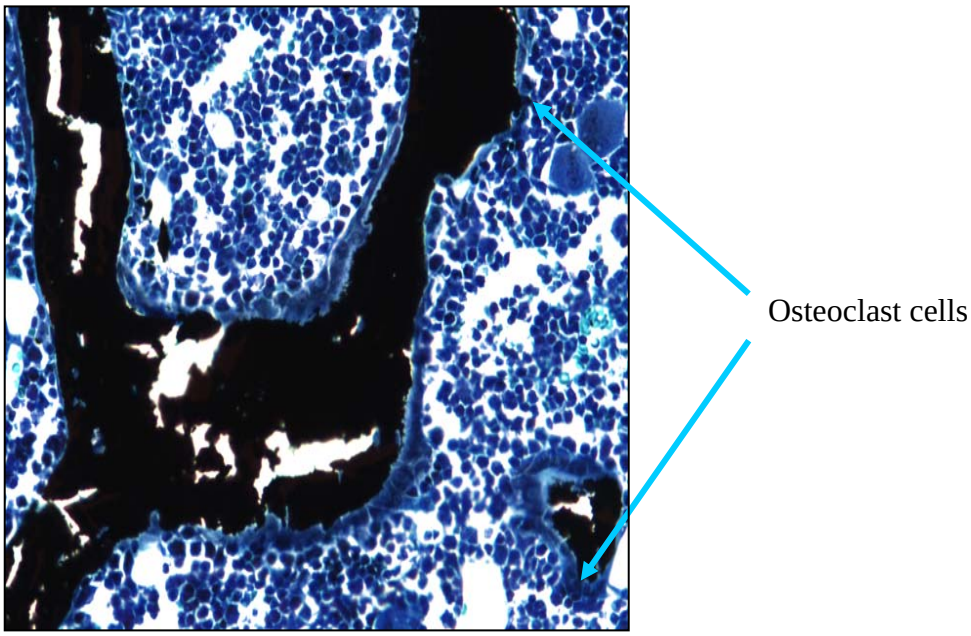

Figure 2.5 A higher magnification image (20X) of an area equivalent in size to the small internal square in Figure 2.3a showing osteoclast cells. The total count indicates the number of bone resorbing cells available at any particular time to erode the bone surface.

VIII.b.vii. Erosion Surface

Erosion surface refers to the bone surface perimeter that is eroded by osteoclasts. This measurement assesses the size of bone surface resorbed by osteoclasts at any time point, which indicates how active the bone resorbing cells are (Erben et al., 1997). This assessment is

significant to the overall hypothesis of this study because it is a measure of whether maternal prenatal stress enhances the function of osteoclasts, which results to higher bone resorption.

Below is a higher magnification image (20X) (Figure 2.6) of an area equivalent in size to the small internal square in Figure 2.3a showing the eroded surface, i.e. a bone surface from which the osteoclast has resorbed the mineralized part. This indicates the size of bone surface resorbed by osteoclasts at one time point, which indicates how active the bone resorbing cells are. The larger the resorbed area the higher the resorption rate (Erben et al., 1997). For more information on the techniques see the rodent trabecular bone histomorphometry manual by Bioquant Image Analysis Corporation (http://osteo.bioquant.com/home/brightfield-rodent-trabecular).

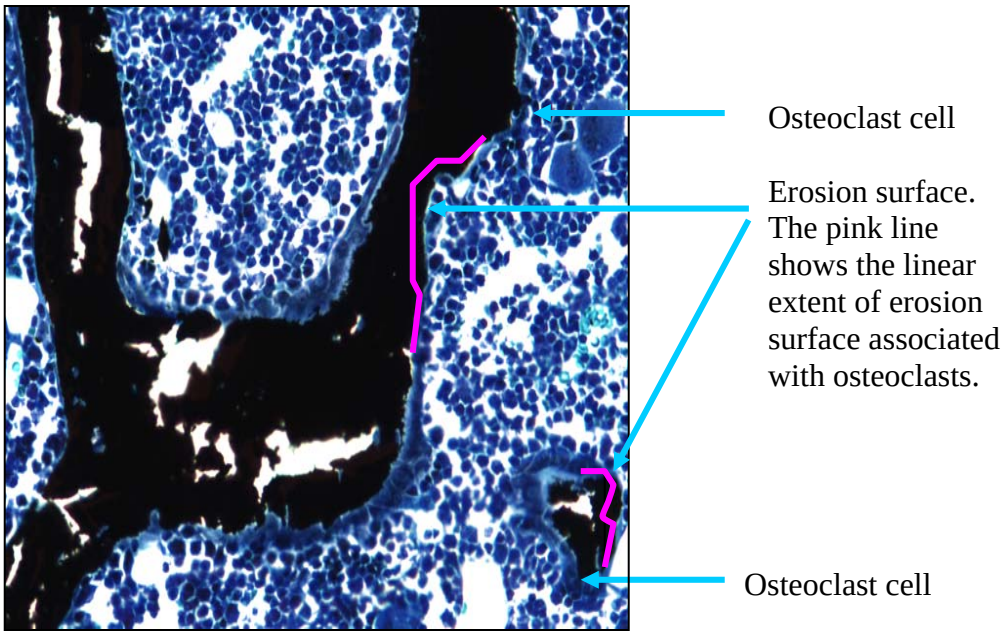

Figure 2.6 A higher magnification image (20X) of an area equivalent in size to the small internal square in Figure 2.3a showing the eroded surface, i.e. a bone surface from which the osteoclast has resorbed the mineralized part.

VIII.b.viii. Resting and Proliferative Chondrocyte Zone of the Growth Plate

The growth plate can be divided into two zones based on the developmental stages of its chondrocytes (Howell and Dean, 1992). The upper zone is composed of chondrocytes that are in the resting and proliferative stages while the lower zone is composed of chondrocytes that are in the hypertrophic stage (Figures 2.7 and 2.8) (Howell and Dean, 1992).

This measurement assessed the size of the area containing the chondrocytes that are in the resting and proliferative stages. It indicates the rate at which cells in the growth plate are forming and differentiating. This is essential to the overall hypothesis of the study because it is a

measure of whether maternal prenatal stress negatively affects formation and differentiation of chondrocytes in the growth plate, which is important to bone growth**.**

Figure 2.9 illustrates how the thickness of the upper zone of the growth plate was measured. The mean length of the lines running across the zone was considered the overall thickness of the upper zone. The end points of these lines were defined by drawing a line at the uppermost boundary of the growth plate containing the resting chondrocytes based on the cell morphology (Howell and Dean, 1992) and another line at the lower boundary of the proliferating chondrocytes. Bioquant software was calibrated to draw random lines at an even distance apart, marking the thickness of the area between the two defined borderlines. The software looked for the maximum distance between any two points. The average length of all the lines was considered the thickness of the zone. The size of the area containing the chondrocytes that are in the resting and proliferative stages indicate the rate at which cells in the growth plate are forming and differentiating (Snow and Keiver, 2007). For more information on the techniques see the rodent trabecular bone histomorphometry manual by Bioquant Image Analysis Corporation (http://osteo.bioquant.com/home/brightfield-rodent-trabecular).

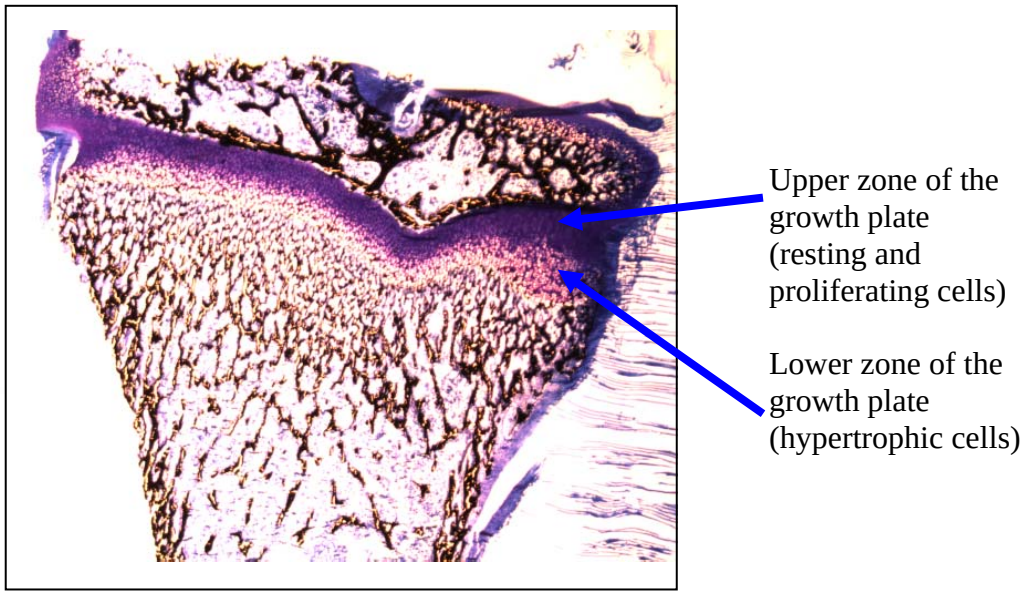

Figure 2.7 Image of a rat proximal tibia section (stained using Von Kossa method with McNeal's tetrachrome counterstain) showing the upper zone of the growth plate containing resting and proliferative cells and the lower zone of the growth plate containing the hypertrophic cells right below.

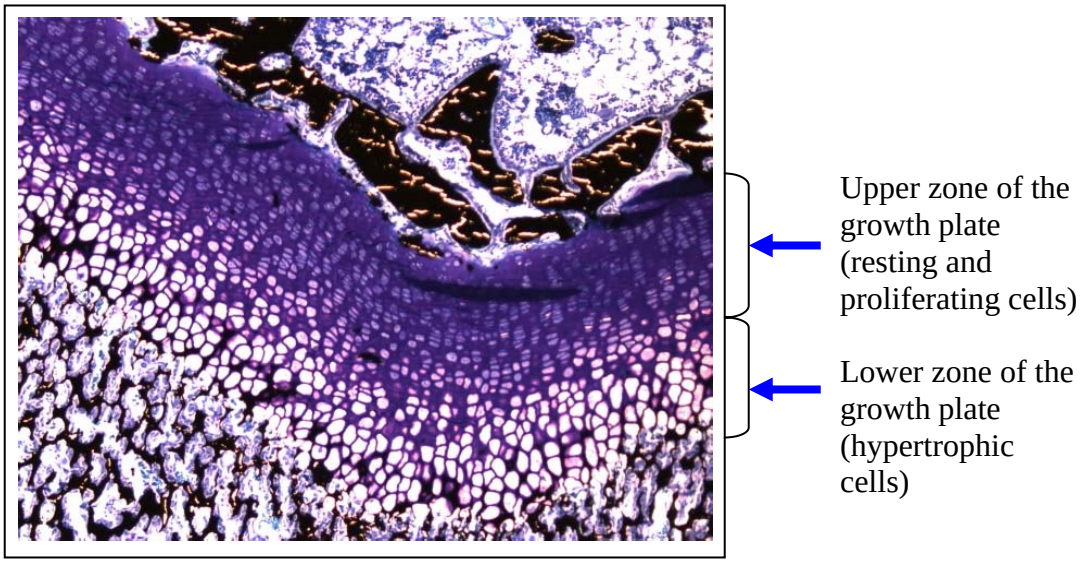

Figure 2.8 Close-up image of the stained rat growth plate at a magnification of 20X showing the upper zone stained and the lower zone.

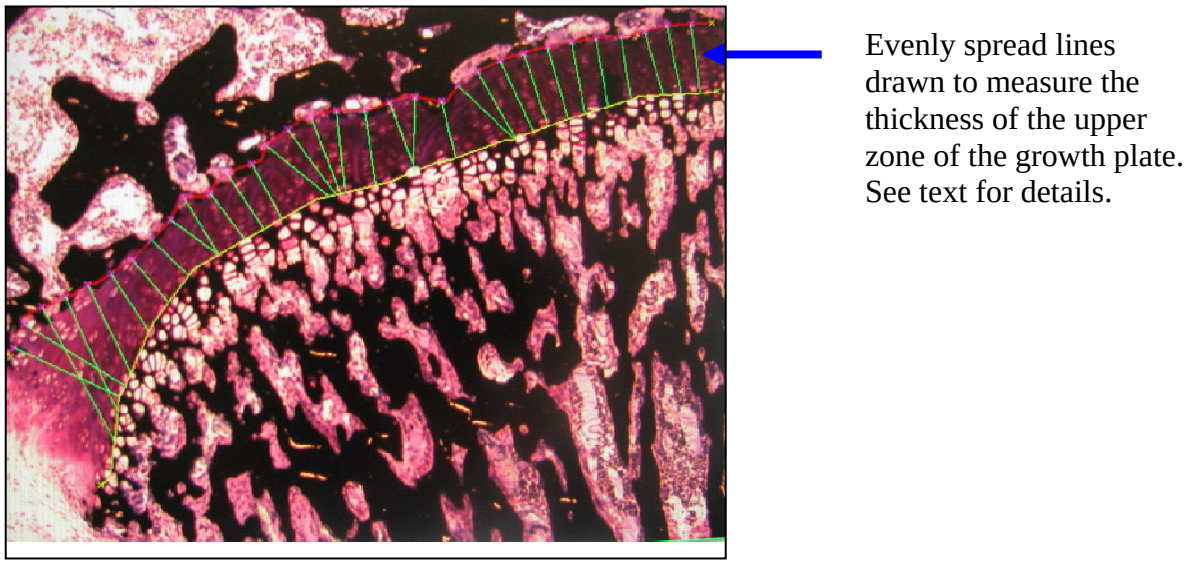

Figure 2.9 Image of a section of rat growth plate at a magnification of 10X showing how the thickness of the upper zone (resting and proliferating cells) was measured.

VIII.b.ix. Hypertrophic Chondrocyte Zone of the Growth Plate

This measurement assessed the size of the area containing chondrocytes that are in the hypertrophic stage. This stage follows the proliferative stage described above and indicates the rate at which the cells in the growth plate are maturing. This is essential to the overall

hypothesis of the study because it is a measure of whether maternal prenatal stress negatively affects maturation of chondrocytes in the growth plate, which is important to bone growth**.**

Figure 2.10 illustrates how the thickness of the lower zone of the growth plate was measured. The mean length of the lines running across the zone was considered the overall thickness of the lower zone. The thickness of the growth plate was measured at a magnification of 10X. The size of the hypertrophic zone was quantified by drawing a line at the upper boundary of the growth plate area containing the hypertrophic chondrocytes based on the cell morphology (Howell and Dean, 1992) and another line at the lower boundary of the hypertrophic chondrocytes. Bioquant software was calibrated to draw random lines at an even distance apart, marking the thickness of the area between the two defined borderlines. The software looked for the maximum distance between any two points. The average length of all the lines was considered the thickness of the zone. The size of the area containing chondrocytes that are in the hypertrophic zone indicate the rate at which the cells in the growth plate are maturing (Snow and Keiver, 2007).

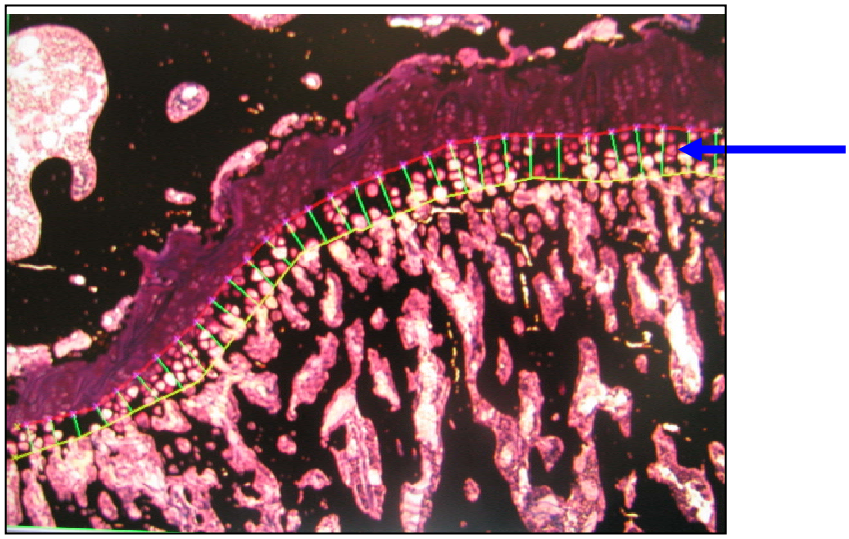

Figure 2.10 Image of a section of rat growth plate at a magnification of 10X showing how the thickness of the lower zone (hypertrophic cells) was measured.

IX. SUMMARY OF THE PROTOCOLS

The study design of this project was such that the first part was a timed experiment. This required extreme attention to detail to ensure stress was induced to the mothers at the right time and the offspring euthanized at the right time. Training on animal breeding, stress induction, fecal sampling, food intake monitoring, offspring rearing and euthanasia was received from the laboratories of Professor Daniela Kaufer in the Department of Integrative Biology and Professor Darlene Francis in the Department of Psychology, University of California Berkeley as well as the Office of Laboratory Animal Care.

Tissue processing for histology and the histology training was received in Prof. David Burr's laboratory at Indiana University School of Medicine. Tissue sectioning, staining and histomorphometry were performed at the University of California Davis in the laboratory of Drs. Nancy Lane and Wei Yao.

X. STATISTICAL ANALYSIS

The mean and standard deviation was calculated for all outcome variables. Statistical differences between the stressed groups and control group were analyzed using t-test, F-test and Tukey-Kramer Honestly Significant Difference test (JMP. 2008. Version 8. SAS Institute Inc., Cary, NC). Linear regression analysis was performed to establish the relationship between stress *in utero* and indicators of bone development in offspring born to stressed mothers (StataCoRP. 2009. Stata; Release 11. Statistical software. College Station, TX: StataCorp LP). *P* values equal to or less than 0.05 were considered significant.

CHAPTER 3: RESULTS FROM LIVE ANIMAL DATA

In this Chapter, results on the data collected from live animals are reported. These are; a) maternal weight gained during pregnancy, b) litter size, c) cortisol (stress) hormone level in dams during gestation period, d) food intake by the mothers during the gestation period, and e) offspring weight gain.

I. MATERNAL WEIGHT GAIN DURING PREGNANCY

I.a. Overview

Results on the weight gained by mothers from the first to the last day of pregnancy are reported in this section. The results showed that pregnant dams that were stressed during weeks 1 and 2 of their pregnancies gained significantly less weight over the duration of the pregnancy than did those stressed in week 3 or in the control group.

I.b. Maternal Weight Gain From Gestation Day 1 to Day 21

The Analysis of Variance (ANOVA) on the means of the amount of weight gained by each maternal group, controls, mothers stressed in gestation week 1 (GW1), mothers stressed in gestation week 2 (GW2) and mothers stressed in gestation week 3 (GW3) showed a significant F-test ($p < 0.0001$) (Table 3.1). This means overall there was at least one significantly different pair of means (Figures 3.1 and 3.2).

Sources of variation in the data	Degrees of Freedom (DF)	Sum of Squares	Mean square	F Ratio	Prob> F
Group	3	246451.9	82150.6	26.5122	< .0001*
Error	483	1496620.8	3098.6		
Total variation	486	1743072.7			

Table 3.1 Analysis of variance of weight by maternal group.

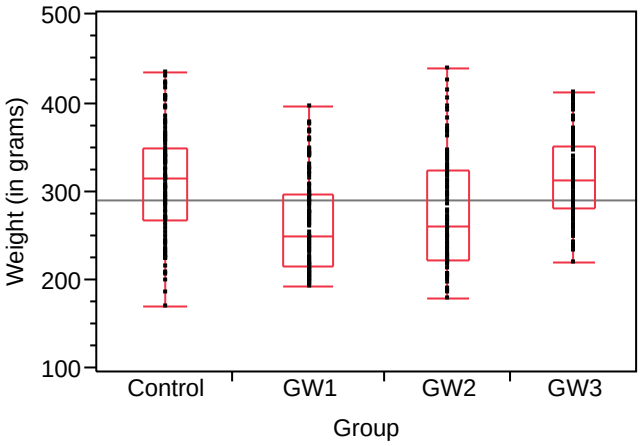

Figure 3.1 Quantile plot showing the shape and symmetry of data for maternal weight gain from day 1 to day 21 (gestation period). Weight was measured in grams. Control = control group, GW1 = mothers stressed in gestation week 1, GW2 = mothers stressed in gestation week 2, GW3 = mothers stressed in gestation week 3. The horizontal line inside the quantile box plot represents the median (50th quantile). Half the values are at or below 50th quantile and half are above. The top and bottom of the box represent the 25th and 75th quantiles. Lines (called whiskers) extend from both ends of the box to the outer-most data point that falls within 1.5 times the range from the 25th to the 75th quantile.

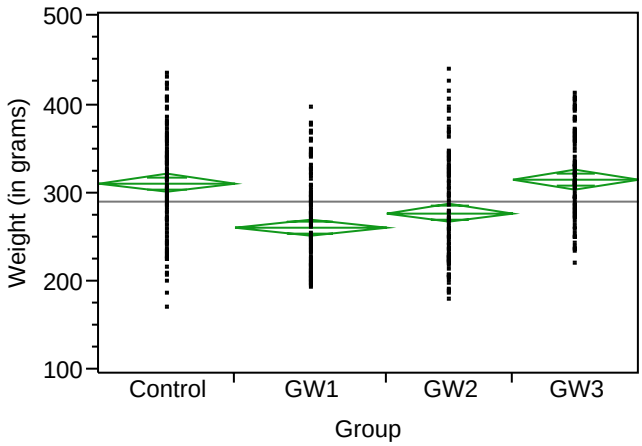

Figure 3.2 One-way ANOVA of weight by maternal group. Means diamonds are a picture of the ANOVA. If there is a statistical difference between one or more pair of means, the ANOVA shows a significant F-test. The side points of each diamond are connected by a horizontal line at the mean of each group. The top and bottom diamond points are the upper and lower 95% confidence points of each group. The lines that

slice the top and bottom of the diamonds are called overlap marks. If there is horizontal separation between the top overlap of one group and the bottom overlap of another, the means of those two groups could be significantly different.

To identify the means that differed, a Tukey-Kramer Honestly Significant Difference (HSD) test was performed. This test statistically compares each pair of means (JMP 8 Statistical Discovery. Cary, NC: SAS Institute Inc). The test showed the means of the control and GW3 were not significantly different from each other. The means of GW1 and GW2 groups were also not significantly different from each other (Figure 3.3). However the means of the controls and GW3 were significantly different from the means of the GW1 and GW2 groups ($p < 0.0001$) (Tables 3.2 and 3.3).

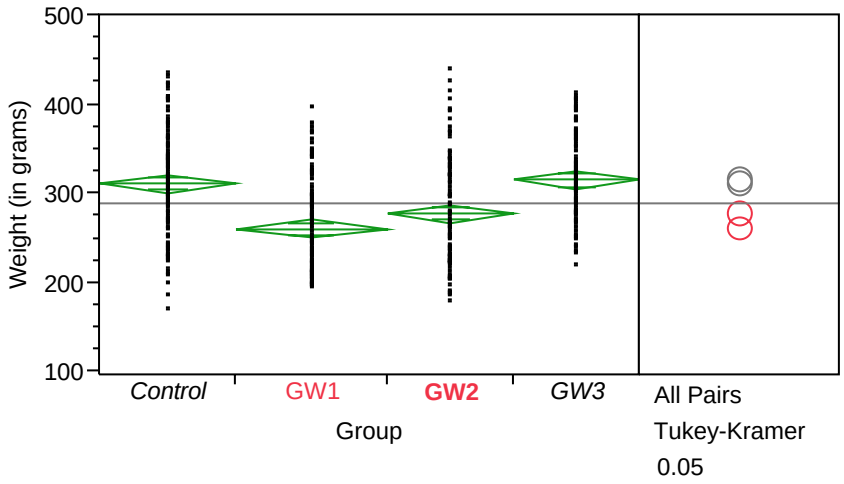

Figure 3.3 Comparison for all pairs of means of maternal gestational weight gain using Tukey-Kramer (HSD). The Tukey-Kramer (HSD) adds comparison circles to the plot and statistically compares each pair of means. Comparison circles are a visualization of multiple comparison tests. There is a circle for each group with a horizontal diameter that aligns with its group mean. Circles with the same color are not statistically different while those with different colors represent groups with statistically different means.

	Control	GW1	GW2	GW3
Control	-18.37	30.56	15.12	-14.75
GW1	30.56	-17.34	-3.58	34.18
GW2	15.12	-3.58	-19.01	18.75
GW3	-14.75	34.18	18.75	-19.01

Table 3.2 Tukey-Kramer HSD test results. Positive values show pairs of means that are significantly different.

Group	Letter	Mean
Control	A	311.46
GW1	B	263.03
GW2	B	277.64
GW3	A	315.40

Table 3.3 Alternative representation of the Tukey-Kramer HSD test results by assigning a letter to the groups. Means that have the same letter are not significantly different.

II. LITTER SIZE

Results of the number of pups born per maternal group are reported in this section. The results showed that the number of pups born to females stressed in weeks 1 and 2 was greater on average than for the controls, or those stressed in week 3 (Table 3.4). However, there was much more variation in the number of pups born in the control group and GW3 and much less variation in the number of pups born in GW1 and GW2 (Table 3.4).

Group	No of mothers	Mean	Standard deviation	Range	Minimum # of pups born	Maximum # of pups born
Control	7	11.14	3.98	9	6	15
GWI	7	13.14	1.68	4	11	15
GW2	7	13.86	1.77	5	11	16
GW3	7	12.29	5.82	16	5	21
Total	28	12.61	3.67			

Table 3.4 Mean and standard deviations of the number of pups born in each group.

II.a. Maternal Weight Gain During Pregnancy and the Number of Pups Born

There was a positive correlation between maternal weight gained during pregnancy and the number of pups born ($p = 0.0054$) (Figures 3.4 and 3.5).

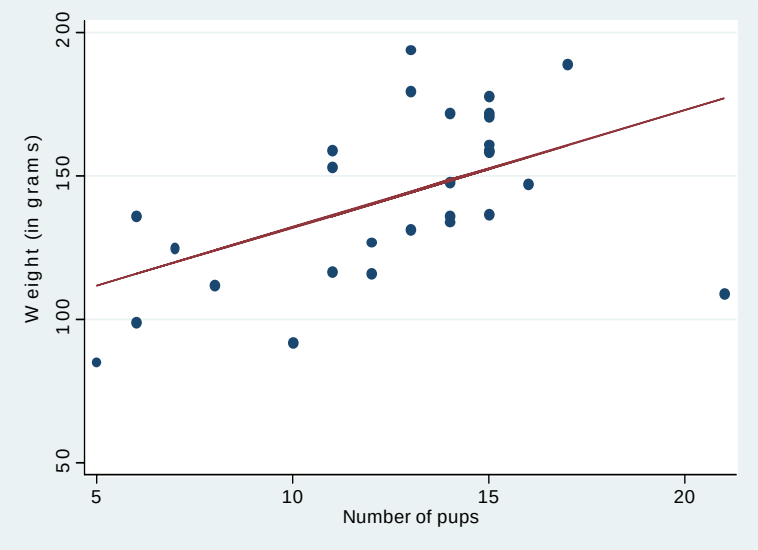

Figure 3.4 Correlation between the number of pups born and weight (in grams) gained by the mother during pregnancy.

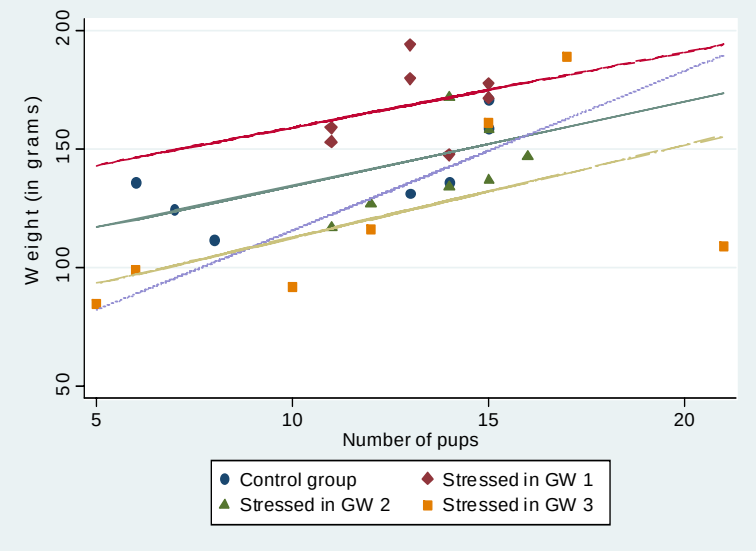

Figure 3.5 Correlation between the number of pups born and weight (in grams) gained by the mother during pregnancy for each group (C = control group, 1 = mothers stressed in gestation week 1, 2 = mothers stressed in gestation week 2, 3 = mothers stressed in gestation week 3).

II.b. Summary

The mean weight gain in the controls was similar to that in GW3, whereas the mean weight gain in GW1 was similar to that in GW2. There was higher weight gain in the mothers that bore a higher number of pups. There was greater variation in the number of pups born in control and GW3 groups compared to GW1 and GW3.

III. CORTISOL (STRESS) HORMONE LEVEL IN DAMS DURING THE GESTATION PERIOD

III.a. Overview

Results on the level of cortisol hormone in pregnant mothers are reported in this section. Cortisol hormone levels are a surrogate measure of stress (Touma et al., 2003, Touma et al., 2004, Lepschy et al., 2007). In this dissertaion, cortisol hormone was quantified as micrograms per gram of feces (μg/g) (Touma et al., 2003, Touma et al., 2004, Lepschy et al., 2007). Refer to Chapter Two: Materials and Methods, for how the cortisol hormone level was extracted and measured.

The mean cortisol hormone levels in controls were consistently lower than those of the stressed animals. The difference varied depending on the time of pregnancy. The levels of cortisol hormone were also lower in the stressed animals prior to the induction of stress and increased immediately after induction of stress.

III.b. Maternal cortisol hormone levels

The mean cortisol hormone levels in the mothers stressed during their first week of pregnancy (GW1) were significantly higher compared to those of control mothers in their first week of pregnancy (GW1 = 3.72μg/g, controls = 1.66μg/g, difference = 2.06μg/g; $p = 0.0007$) (Figure 3.6).

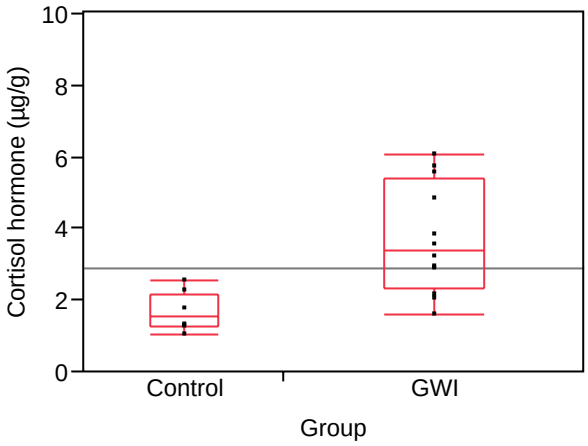

Figure 3.6 Cortisol hormone levels in control group (Control) and the group of mother stressed in gestation week 1 (GW1) during the first week of pregnancy. The hormone levels in this figure were measured for the first week of pregnancy and quantified as micrograms per gram of feces (µg/g).

The mean cortisol hormone levels in the mothers stressed during their second week of pregnancy (GW2) were also significantly higher compared to those of control mothers in their second week of pregnancy (GW2 = 3.34µg/g, controls = 1.73µg/g, difference = 1.62µg/g; p = 0.0205) (Figure 3.7). However, the cortisol hormone level difference between the controls in week two and GW2 (1.62µg/g) was less compared to the hormone level difference between the controls in week one and GW1 (2.06µg/g).

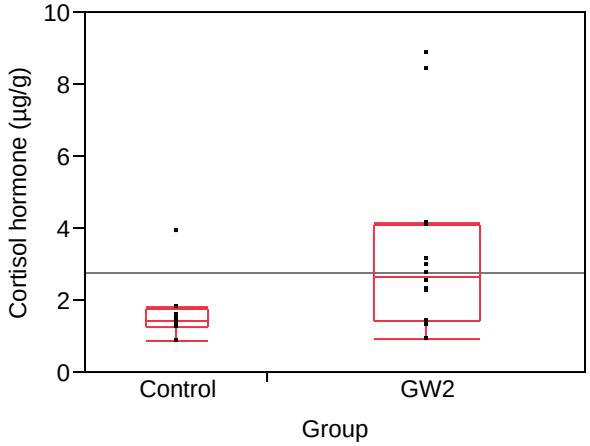

Figure 3.7 Cortisol hormone levels in control group (Control) and the group of mother stressed in gestation week 2 (GW2). The hormone levels in this figure were measured

for the second week of pregnancy and quantified as micrograms per gram of feces (μg/g).

The mean cortisol hormone levels in the mothers stressed during their third week of pregnancy (GW3) were significantly higher compared to those of control mothers in their third week of pregnancy (GW3 = 3.36μg/g, controls = 2.38μg/g, difference = 0.98μg/g; $p = 0.017$) (Figure 3.8). However, the difference in cortisol hormone level between controls and GW3 (0.98μg/g) was the least compared to the difference between controls and GW1 (2.06μg/g) and the difference between controls and GW2 (1.62μg/g).

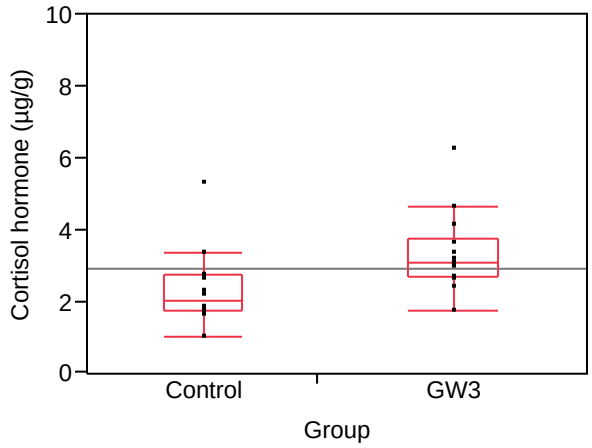

Figure 3.8 Cortisol hormone levels in the control group (Control) and the group of mother stressed in gestation week 3 (GW3). The hormone levels in this figure were measured for the third week of pregnancy and quantified as micrograms per gram of feces (μg/g).

To further explore the apparent declining levels of difference in cortisol hormone levels between controls and stressed animals as the pregnancy advanced, the hormone levels for controls were plotted independently and found to increase significantly with the progression of the pregnancy ($P = 0.0344$) (Figure 3.9). However results from a pair wise comparison of mean cortisol level in controls during the first week (ControlWk1 = 1.66μg/g), second week (ControlWk2 = 1.73μg/g), and third week (ControlWk3 = 2.66μg/g) of gestation using the Tukey-Kramer HSD test was not significant ($p = 0.0960$) (Figures 3.10 and 3.11, Table 3.5).

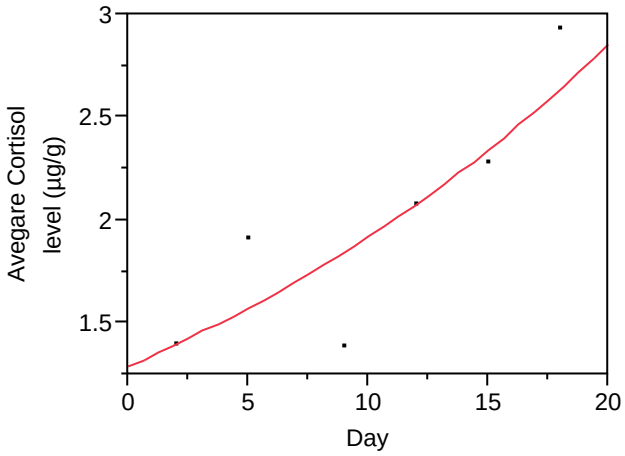

Figure 3.9 Cortisol hormone levels in control group (Control). The hormone levels ploted in this figure were measured from day 1 to 20 of gestation and quantified as micrograms per gram of feces (µg/g).

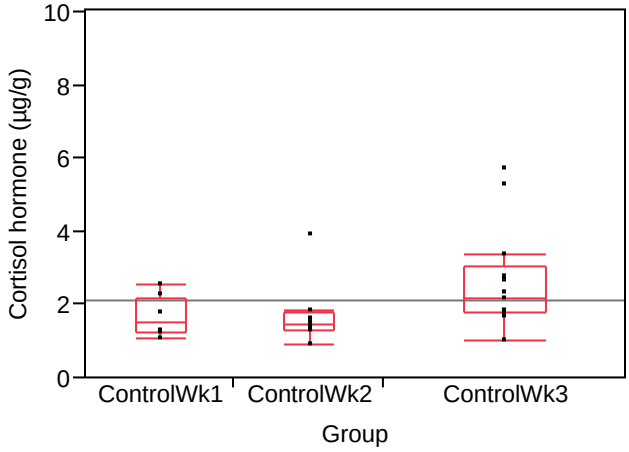

Figure 3.10 Quantile plot showing the shape and symmetry of cortisol hormone data in controls during the first (ControlWk1), second (ControlWk2), and third (ControlWk3) week of gestation. Cortisol hormone is quantified as micrograms per gram of feces (µg/g).

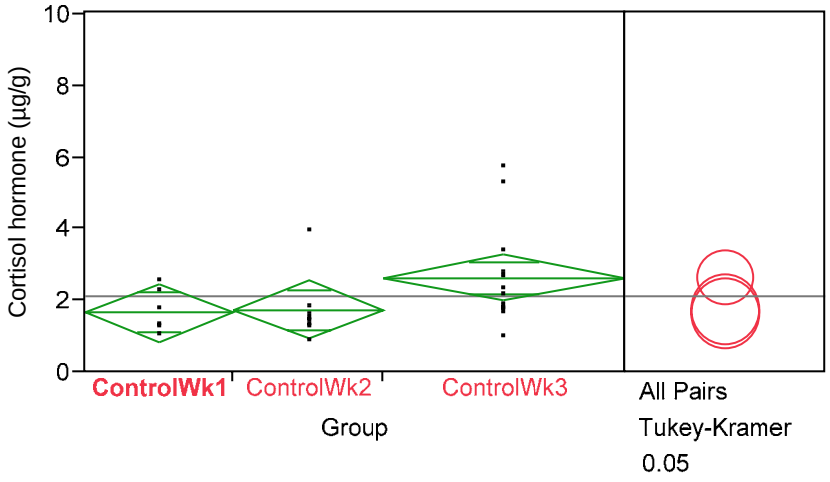

Figure 3.11 Comparison of all pairs of means of cortisol hormone level in controls during the first (ControlWk1), second (ControlWk2), and third (ControlWk3) week of gestation using Tukey-Kramer (HSD). Cortisol hormone is quantified as micrograms per gram of feces (µg/g).

Sources of variation in the data	Degrees of Freedom (DF)	Sum of Squares	Mean square	F Ratio	Prob> F
Group	2	6.36	3.18	2.57	0.0960
Error	26	32.22	1.24		
Total variation	28	38.58			

Table 3.5 Analysis of variance of mean cortisol hormone levels in the control group during gestation week 1, gestation week 2 and gestation week 3.

A similar trend was also observed when cortisol hormone levels in stressed groups were compared. Results from a pair wise comparison of the mean cortisol levels in stressed groups, GW1 = 3.72µg/g, GW2 = 3.35µg/g and GW3 = 3.36µg/g were not significant (p = 0.8409) (Figures 3.12 and 3.13, Table 3.6).

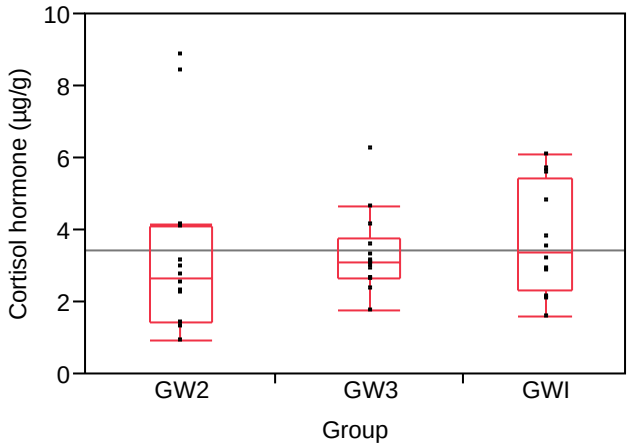

Figure 3.12 Quantile plot showing the shape and symmetry of cortisol hormone data in GW1, GW2 and GW3. Cortisol hormone is quantified as micrograms per gram of feces (µg/g).

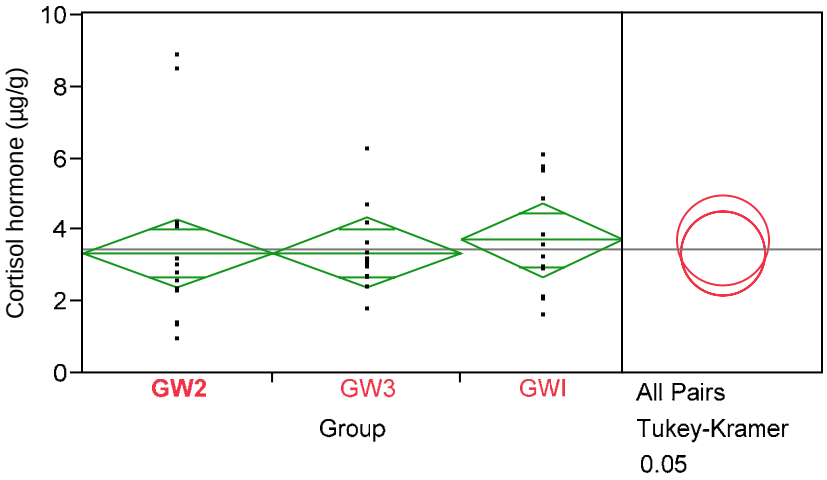

Figure 3.13 Comparison of all pairs of means of cortisol hormone levels (micrograms per gram of feces (µg/g) in GW1, GW2 and GW3 using Tukey-Kramer (HSD). Cortisol hormone is quantified as micrograms per gram of feces (µg/g).

Sources of variation in the data	Degrees of Freedom (DF)	Sum of Squares	Mean square	F Ratio	Prob> F
Group	2	1.13	0.56	0.17	0.8409
Error	37	199.68	3.23		
Total variation	39	120.81			

Table 3.6 Analysis of variance of mean cortisol hormone levels in stressed groups GW1, GW2, GW3.

The increase in cortisol hormone over the course of the pregnancy does not represent a significant pattern. Therefore, the cortisol levels measured for all three weeks of the control group were combined as one group for the next analysis. Also, since the stressed groups were not significantly different from each other, all three groups were combined as one for the next analysis.

T- test results for the combined means for the control and stressed groups showed a highly significant difference (controls = 2.12µg/g, GW = 3.46µg/g, difference = 1.35 µg/g, p = 0.0003) (Figure 3.14).

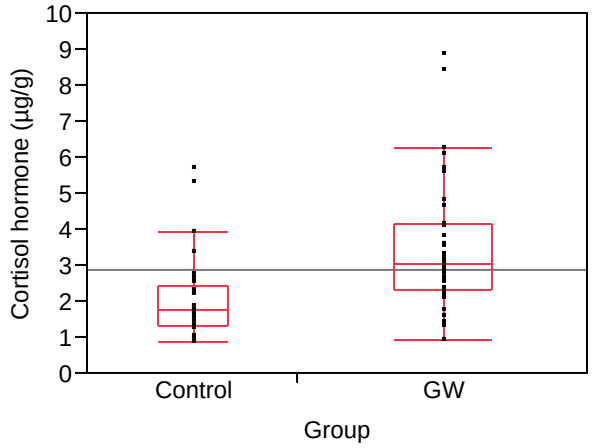

Figure 3.14 Comparison of means of cortisol hormone level in all controls and all stressed mothers during pregnancy. Cortisol hormone is quantified as micrograms per gram of feces (µg/g).

The cortisol hormone levels in the basal samples collected from GW2 and GW3 were lower compared to the other two time points when the animal was under stress. This was expected (Figures 3.15 and 3.16).

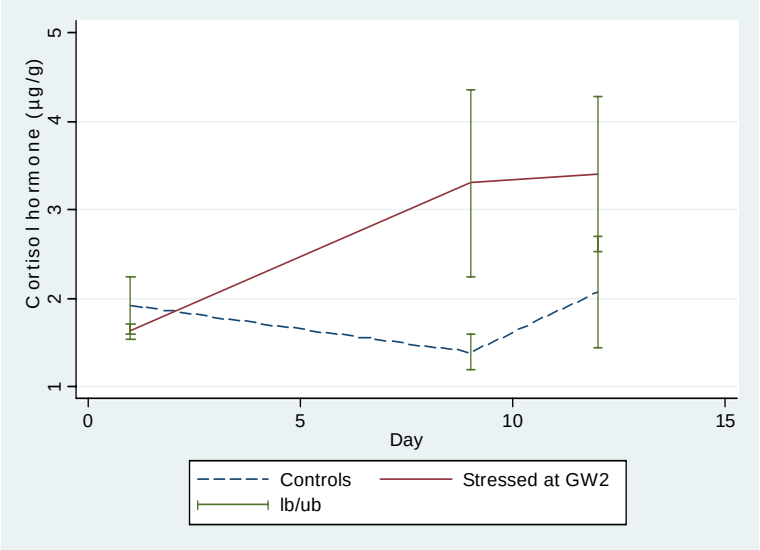

Figure 3.15 Comparison of cortisol hormone level at baseline and stress days in GW2. The dashed line represents the controls (0) while the solid line represents GW2 (2). lb/ub stands for the lower boundary (lb) and upper boundary (ub) at 95% confidence interval.

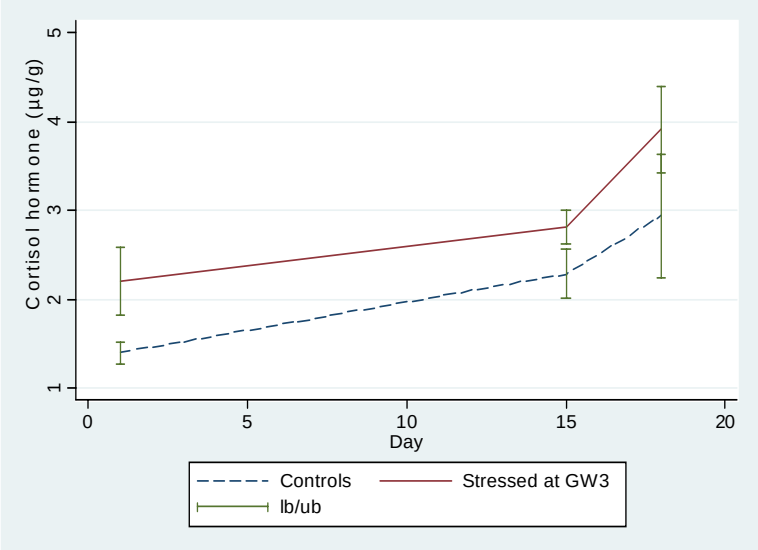

Figure 3.16 Comparison of cortisol hormone level at baseline and stress days in GW3. The dashed line represents the controls (0) while the solid line represents GW3 (3). lb/ub stands for the lower boundary (lb) and upper boundary (ub) at 95% confidence interval.

IV. FOOD INTAKE BY THE MOTHERS DURING THE GESTATION PERIOD

Results on the amount of food consumed by the mothers during pregnancy are reported in this section. The stressed animals consumed 3 grams (12.5%) less food per day compared to the controls ($p < 0.005$) (Figure 3.17). On the day before delivery all of the animals increased their food intake, almost doubling their norm.

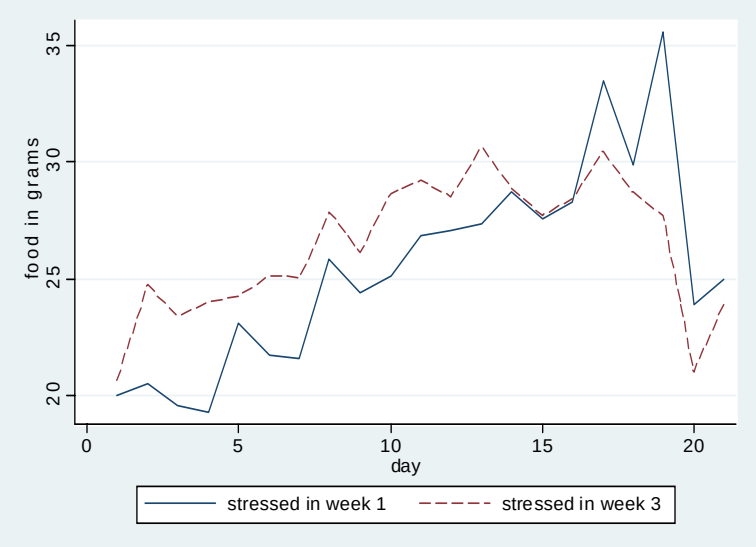

Figure 3.17 Plot of daily food consumption for dams in GW1 (solid line) and GW3 (dashed line) groups. Note that GW1 animals ate less during their week of stress and the following week than did animals that had not yet been stressed. The reverse happened in week 3. As soon as the animals stressed in week 3 started their stress regimen, their food consumption dropped compared to the GW1. At this point, it was 8 days after the GW1 completed their stress regimen.

V. OFFSPRING WEIGHT GAIN

The average weight (in grams) of offspring for each group at the time of termination is reported in this section. Both male and female offspring born to mothers stressed in GW3 were heavier compared to all the other groups (Figures 3.18 and 3.19). However, pair-wise comparison of the means in weight for each group using the Tuker-Kramer HSD test showed no statistically significant difference for males ($p = 0.33$) or females ($p = 0.9$) (Figures 3.20 and 3.21).

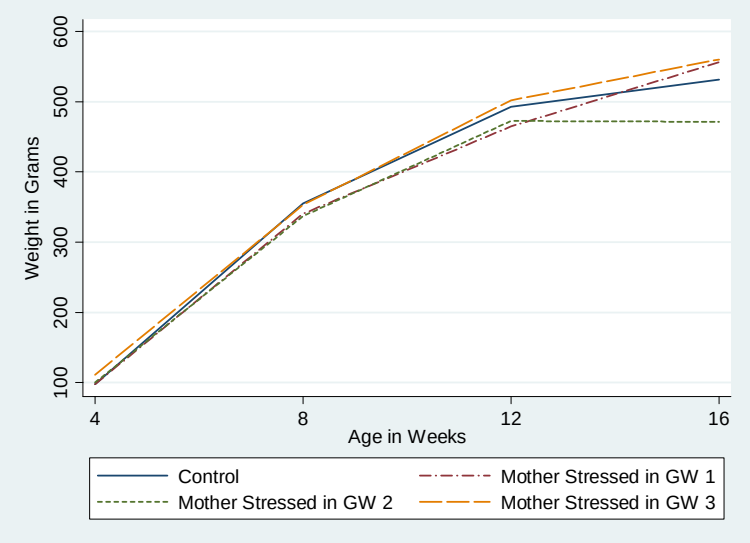

Figure 3.18 Mean weight (in grams) of male offspring at age 4, 8, 12 and 16 weeks.

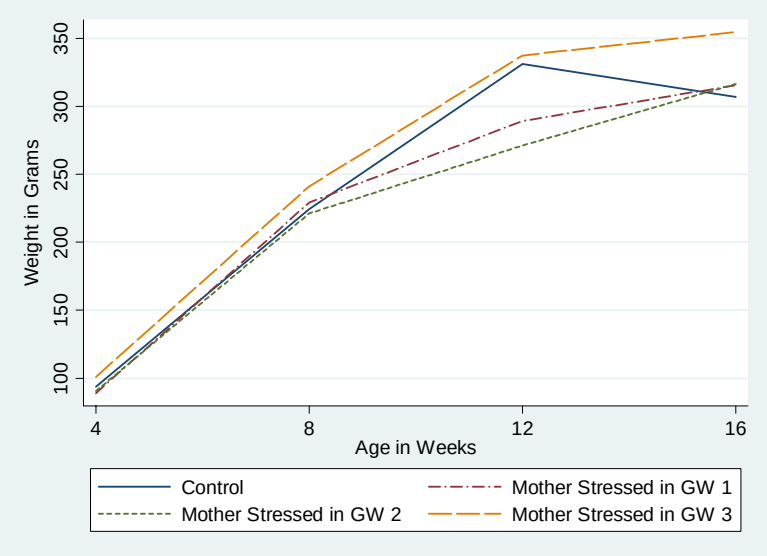

Figure 3.19 Mean weight (in grams) of female offspring at age 4, 8, 12 and 16 weeks.

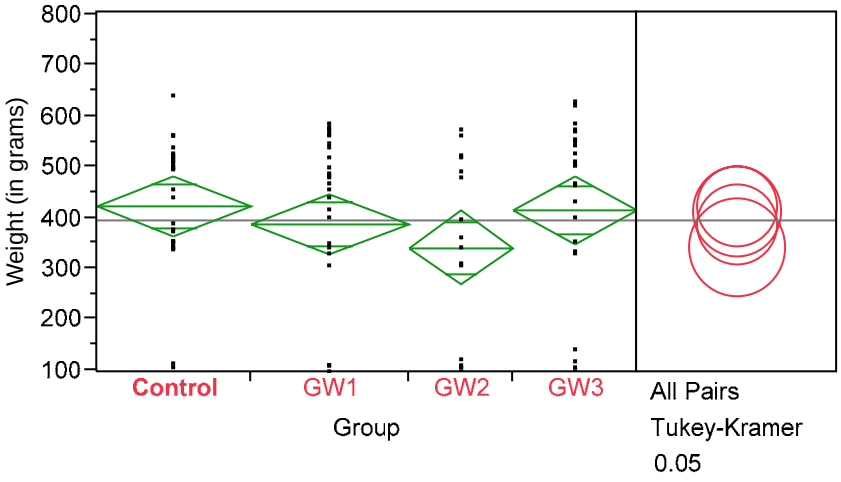

Figure 3.20 Comparison for all pairs of means in male offspring using Tukey-Kramer (HSD).

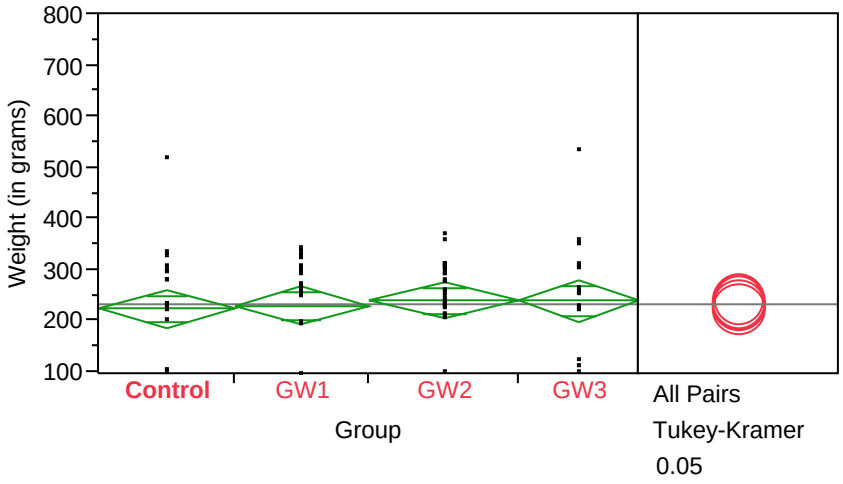

Figure 3.21 Comparison for all pairs of means in female offspring using Tukey-Kramer (HSD).

VI. SUMMARY

This Chapter reported results for five different aspects of this research; a) maternal weight gain, b) litter size, c) level of cortisol hormone in pregnant mothers, d) amount of food consumed by the mothers during pregnancy, e) average weight of offspring for each group at the time they were sacrificed.

The pregnant dams that were stressed during weeks 1 and 2 of their pregnancies gained significantly less weight over the duration of the pregnancy than did those stressed in week 3 or in the control group. The results show that the number of pups born to females stressed in weeks 1 and 2 was greater on average than for the controls, or those stressed in week 3. There was much more variation in the number of pups born in the control group and GW3 and much less variation in the number of pups born in GW1 and GW2 groups. The mean cortisol hormone levels in controls were consistently lower than those of stressed animals. The difference varied depending on the time of pregnancy. The levels of cortisol hormone were also lower in the stressed animals prior to the induction of stress and increased immediately after induction of stress. The stressed animals consumed 3 grams (12.5%) less food per day compared to the controls. On the day before delivery all the animals increased their food intake, almost doubling their norm.

Both male and female offspring born to mothers stressed in GW3 were heavier compared to all the other groups. After sacrificing the offspring, which was done at 4, 8, 12 and 16 weeks old, their tibias were removed at necropsy and processed for histology as described in *Chapter 2: Material and Methods*. In the next chapter, results from the histological analysis of the proximal tibia are reported.

CHAPTER 4: RESULTS OF THE HISTOLOGICAL ANALYSIS OF THE OFFSPRING'S TIBIA

The quantitative parameters of bone were determined by histomorphometry. This primarily involves quantitative histology based on stereology that evaluates micro-architecture in two-dimensional sections, using semi-automated image analysis (Bioquant Image Analysis Corporation, Nashville, TN, USA) linked to a microscope. The variables measured are indicators for bone development, and are listed in Table 4.1.

The bone measurements, units and terminology used in histomorphometric analysis were defined and derived according to the Nomenclature Committee of The American Society for Bone and Mineral Research (Partiff et al., 1987). The growth plate measurements were defined and derived according to Howell and Dean (1992). All the techniques used to derive the results reported in this section were developed by Bioquant Image Analysis Corporation, Nashville, TN, USA. A total of nine outcome variables (Table 4.1) were measured from the growth plate and the secondary spongiosa region using the detailed protocol described in *Chapter 2: Materials and Methods*.

The results reported in this chapter focus on the comparison between offspring of the control group and offspring of mothers stressed in gestation week 3 (GW3). The decision to initially focus on GW3 offspring was informed by the fact that this is the week during which rats' bones mineralize. I therefore hypothesize that maternal stress during this stage of pregnancy will have the greatest impact on the quality of the bone of the offspring *(Chapter 1: Introduction).*

Data collection for the histomorphometry phase of the project was very time-intensive. As such, the other experimental groups will be studied at a later date and the results reported elsewhere.

Outcome Variables Measured	Feature Indicated
Total tissue area (mm^2)	Area of entire trabecular bone tissue defined (which include the mineralized bone, collagen matrix, empty space and bone cells). This is significant to the overall hypothesis of this study because it assesses whether maternal prenatal stress negatively affects the size of mature trabecular bone tissue. The total tissue area is also used as a proxy for size of the tibia in this study.
Bone area (mm^2)	Area of the mineralized bone. Bone minerals make up the inorganic component of bone which is essential for skeletal functions.
Bone perimeter (mm)	Perimeter of the mineralized bone. This is an alternative way of assessing size of the bone mineral part.
Osteoblast surface (mm)	The amount of bone forming cells available to secrete the collagen matrix (osteoid) that calcifies to form bone. A higher number indicates an increase in the rate of bone formation (Erben et al., 1997).
Osteoid surface (mm)	The amount of collagen matrix secreted by osteoblasts. This indicates how active the bone forming cells are and hence the rate at which the bone is forming (Erben et al., 1997).
Number of osteoclasts per erosion surface (OC/mm)	The number of bone-resorbing cells available at this particular time point to erode the bone surface. The higher the number, the higher the resorption rate (Erben et al., 1997).
Erosion surface (mm)	The size of bone surface resorbed by osteoclasts at a time point that indicates the activity level of the bone resorbing cells (Erben et al., 1997).
Proliferative zone of the growth plate (mcm)	The size of the area containing the chondrocytes that are in the resting and proliferative stages that indicate the rate at which cells in the growth plate are forming and differentiating (Snow and Keiver, 2007).
Hypertrophic zone of the growth plate (mcm)	The size of the area containing chondrocytes that are in the hypertrophic stage. This stage follows the proliferative stage described above and indicates the rate at which the cells in the growth plate are maturing (Snow and Keiver, 2007).

Table 4.1 Variables measured and their definitions.

All of these variables were measured in the proximal tibia because, compared to the femur (the other weight bearing bone of relevance to osteoporosis), the tibia has a relatively flat

top that makes it easier to assess the growth plate and the trabecular bone area directly below it. The proximal femur has three divisions (the head, greater trochanter, and lesser trochanter) that make it hard to take simple measurements of the growth plate, or to define the area below it (*Chapter 2: Materials and Methods*). The nine variables described above were measured from the growth plate and the secondary spongiosa region, which is 1mm below the growth plate and mainly compost of mature, enlarged and well-mineralized bony trabeculae (Yao et al., 2010).

In the first step of the analysis I used a two-sample t-test to look at male and female differences in the total tissue area of the offspring at the ages of 4, 8, 12 and 16 weeks. Total tissue area is a measure of the area of entire trabecular bone tissue defined (which includes the mineralized bone, collagen matrix, empty space and bone cells) in the secondary spongiosa region (see Figure 2.1). A reduction in the overall size of the trabecular tissue can be interpreted as a compromise in bone quality if accompanied by reduced level of mineralization (Compston, 2006). The total tissue area is also used as a proxy for size of the tibia in this study. Since all the other trabecular variables assesed are measured from the defined total tissue area region, it is the most all-encompassing variable for defining the male:female difference. However one caveat to this is that if one of the variables within the defined total tissue area region is different for males and females, this difference is not reflected in the total tissue area measurement. For example, if the males have more osteoblast surface relative to osteoid surface (as defined below) compared to females, this difference will not be captured in the total tissue area measurement, hence the separate analysis presented for each variable.

Note that this first analysis was mainly designed to test whether there was a size difference between the males and females. This was important for determining whether to control for sex or not in the subsequent analysis. If the males differ from females in size, it is important to control for sex in order to isolate the stress effect.

I. TOTAL TISSUE AREA

Total tissue area is a measure of the area of entire trabecular bone tissue defined (which include the mineralized bone, collagen matrix, empty space and bone cells) in the secondary spongiosa region (see Figure 2.1, page 37 for visual representation).

The combined averages for all the offspring born to GW3 mothers and those born to control mothers indicate that the total tissue area in the secondary spongiosa region in males is significantly larger compared to the females at age 8, 12 and 16 weeks ($p < 0.05$) (Table 4.2).

Age in weeks	4	8	12	16
Difference	0.07 (n=12)	2.12*** (n=21)	2.18*** (n=20)	2.35*** (n=26)

Table 4.2 Difference in averages of tissue volume between male and females pups from both GW3 mothers and control mothers. *** = $p < 0.001$.

In the next step of analysis I separated GW3 offspring from control offspring and used a two sample t-test to look at male and female differences in the total tissue area of the offspring at each age, i.e. 4, 8, 12 and 16 weeks. The averages for the pups born to GW3 mothers and those

born to control mothers indicate that for both groups the males are significantly larger than the females at age 8, 12 and 16 weeks ($p < 0.05$) (Table 4.3).

Age in weeks	4	8	12	16
Difference in GW3 offspring	0.46 (n=7)	2.05*** (n=13)	2.89*** (n=8)	2.65* (n=8)
Difference in control offspring	0.41 (n=5)	2.34* (n=8)	1.61* (n=12)	2.19** (n=18)

Table 4.3 Difference in averages of total tissue area between male and females pups born to GW3 mothers and those born to control mothers analyzed separately. Key to the significance levels: * = $p < 0.05$; ** = $p < 0.01$; *** = $p < 0.001$.

From the above results it is evident that total tissue area varies by sex, regardless of whether it was born to a GW3 mother or a control mother. Therefore, taking the larger size of the males into account, I used a statistical model that removes the male and female difference so I could isolate the stress effect. Also, since I did not have an equal number of male and female pups from the GW3 mothers and control mothers, I needed to remove the size difference between males and females to be able to compare GW3 and control offspring.

To accomplish this, I used a linear regression model, which allowed the difference in mean outcome between GW3 offspring and control offspring to vary with pups' age. I used a Wald test to check for how *in utero* stress during week three of gestation affected bone development at different ages postnatal (StataCoRP. 2009. Stata; Release 11. Statistical software. College Station, TX: StataCorp LP).

Equation 4.1

$$yi = \beta_1 X_{i1} + \beta_2 X_{i2} + \beta_3 X_{i3} + \beta_4 X_{i4} + \beta_5 X_{i5} + \beta_6 X_{i6} + \beta_7 X_{i7} + \beta_8 X_{i8} + \varepsilon i$$

$$yi = \beta_{1sex} + \beta_{2GW3\ offspring,} + \beta_{3\ age\ 8\ weeks} + \beta_{4\ age\ 12\ weeks} + \beta_{5\ age\ 16\ weeks} + \beta_{6\ GW3\ offspring,\ 8\text{-}weeks\ old} + \beta_{7\ GW3\ offspring,\ 12\text{-}weeks\ old} + \beta_{8\ GW3\ offspring,\ 16\text{-}weeks\ old} + \varepsilon i$$

In the equation, (yi) is the outcome variable as shown in Table 4.1 and the predictor variables are as follows, sex of the pups (X_1), whether the pups were born to GW3 mothers (X_2), age of the pups (X_3, X_4, X_5), whether the prenatal stress effect was different at different ages (X_6, X_7, X_8), and the error term (εi).

If the difference in mean outcome for GW3 offspring and control offspring did not vary with pups' age ($p > 0.10$), I estimated stress effect with a model that controlled for pups' sex and age.

Equation 4.2

$$yi = \beta_1 X_{i1} + \beta_2 X_{i2} + \beta_3 X_{i3} + \varepsilon i$$

$$yi = \beta_{1sex} + \beta_{2\ GW3\ offspring,} + \beta_{3\ age\ in\ weeks} + \varepsilon i$$

All models adjusted standard errors for correlation between pups from the same litter using the Huber-White Sandwich estimator.

These equations were developed based on the explanation of how to analyze correlated data (http://www.ats.ucla.edu/stat/stata/library/cpsu.htm) using the statistical program, Stata 11 (StataCoRP. 2009. Stata; Release 11. Statistical software. College Station, TX: StataCorp LP). This program has the option of using clustered robust standard errors, which correct the standard errors to account for the intraclass correlation. In the end, this analysis yields results with more robust or larger standard errors compared to an analysis done with the same data assuming there was no intraclass correlation. My study design was such that in each group I had pups from the same litter, which are correlated. To correctly analyze the data I had to take into account the correlation. If not, the standard errors of the estimates would be underestimated rendering the significance tests invalid. This happens because in an analysis, the standard errors reported assume that each observation in the data set is independent.

The consequence of this type of study design and analysis is that the larger the intraclass correlations, the less unique the information contained by each observation. Also with clustering, the effective sample size is diminished

Presented below are further results from the total tissue area analysis. Refer to Figure 2.1 for definition.

Age in weeks	Control	Offspring born of GW3	Combined mean	Estimated difference adjusted for sex	*p* value
4	4.55 (n=5)	4.36 (n=7)	4.43	-0.18	0.788
8	6.27 (n=8)	6.42 (n=13)	6.37	0.40	0.300
12	7.03 (n=12)	6.61 (n=8)	6.86	0.14	0.733
16	6.77 (n=18)	7.28 (n=8)	6.92	0.22	0.733
Total	6.49	6.25	6.38	0.58	

Table 4.4 Means of Total Tissue Area (mm squared).

Table 4.4 shows the results of the tabulated mean for each group at different ages, the combined mean, the estimated difference between the controls and GW3 offspring adjusted for sex and the *p* value. The estimated difference is calculated using the regression analysis, described in equation 4.2 above. The reported *p* value is from the regression analysis which takes into account that pups from the same litter are correlated as noted above. Due to these correlations, the assumption is that I did not have independent observations and therefore could not use a likelihood ratio test.

Controlling for sex, there was no evidence of a difference in mean total tissue area for GW3 offspring as compared to control offspring at ages 4, 8, 12 and 16 weeks. The following is a graphical presentation of the means in Table 4.4.

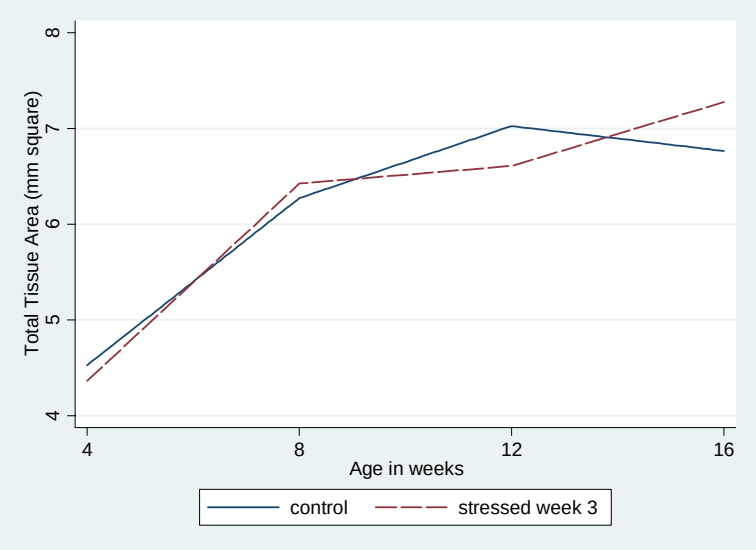

Figure 4.1 Graph showing the change in total tissue area with age in GW3 offspring (dashed line) and control offspring (solid line).

The second linear regression analysis (using equation 4.1) that examines how *in utero* stress during week three of gestation affected bone development at different ages postnatal failed to demonstrate the presence of a difference in the way maternal prenatal stress affects total tissue area at different developmental stages, for GW3 offspring compared to the control offspring at age 4, 8, 12 and 16 weeks (p = 0.7200). In other words, the pattern of bone development in GW3 offspring and control offspring does not vary significantly.

II. BONE AREA

Bone area is defined as the total mineralized bone area in the secondary spongiosa region (see Figure 2.2, page 39 for visual representation). This is different from the total tissue area as it is a measure of the mineralized bone tissue only.

Age in weeks	Control	Offspring born of GW3	Combined mean	Estimated difference adjusted for sex	*p* value
4	1.06 (n=5)	1.25 (n=7)	1.17	0.11	0.664
8	1.40 (n=8)	2.16 (n=13)	1.87	0.82***	0.001
12	1.27 (n=12)	1.61 (n=8)	1.40	0.30	0.16
16	1.39 (n=18)	1.53 (n=8)	1.43	0.17	0.58
Total	1.32	1.72	1.50	1.4	

Table 4.5 Means of Bone Area (mm square).

Table 4.5 shows the results of the tabulated mean for each group at different ages, the combined mean, the estimated difference between the controls and GW3 offspring adjusted for sex and the *p* value. The estimated difference is calculated using the regression analysis, described in equation 4.2, above. The reported *p* value is from the regression analysis which takes into account that pups from the same litter are correlated as noted above.

Mean bone area is significantly higher in the GW3 offspring compared to the control offspring at the age of 8 weeks ($p = 0.001$). The following is a graphical presentation of the means in Table 4.5.

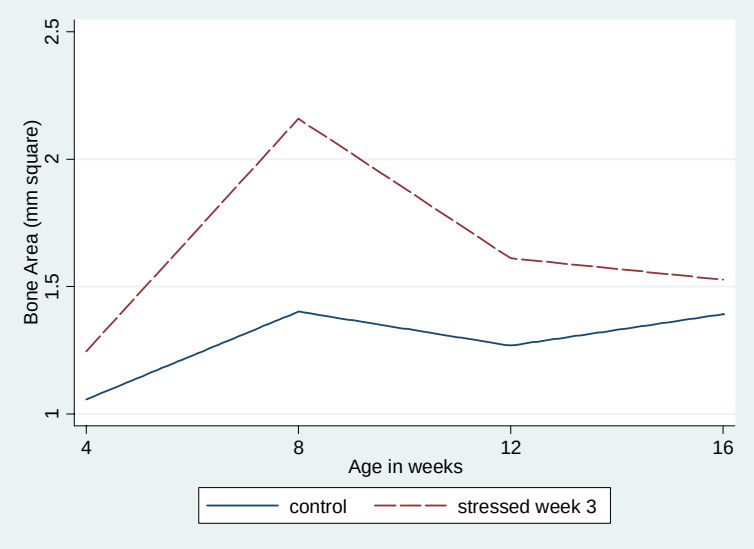

Figure 4.2 Graph showing the change in bone area with age in GW3 offspring (dashed line) and control offspring (solid line).

The second linear regression analysis (using equation 4.1), that examines how *in utero* stress during week three of gestation affected bone development at different ages postnatal, failed to demonstrate the presence of any difference in the way maternal prenatal stress affects bone area at different developmental stages, for GW3 offspring compared to the control offspring at ages 4, 8, 12 and 16 weeks ($p = 0.3301$). Even though the bone area is significantly higher in GW3 offspring at the age of 8 weeks, the overall pattern of bone development in GW3 offspring and control offspring does not vary significantly. However the graph emphasizes the small difference. Since all siblings (animals born to the same mother) were clustered at each age group in the analysis, the effective sample size was diminished. Small sample sizes give results that lack precision.

II.a. Ratio of Bone Area to Total Tissue Area

From the definitions of total tissue area (Figure 2.1) and bone area (Figure 2.2), bone area is a correlated variable with tissue volume. A regression analysis on the ratio of bone area to total tissue area showed no significant difference in the way maternal prenatal stress affects the

ratio at different developmental stages, for GW3 offspring compared to the control offspring at ages 4, 8, 12 and 16 weeks (p = 0.4048).

III. BONE PERIMETER

Bone perimeter was measured in the same area as defined for total tissue area (see Figure 2.2, page 39 for visual representation). The trabecular bone area is thresholded, it appears red in Figure 2.2.

Age in weeks	Control	Offspring born of GW3	Combined mean	Estimated difference adjusted for sex	*p* value
4	41.39 (n=5)	38.62 (n=7)	39.77	-3.77	0.714
8	54.18 (n=8)	60.74 (n=13)	58.24	7.40	0.288
12	47.27 (n=12)	44.43 (n=8)	46.14	-2.51	0.779
16	44.53 (n=18)	47.68 (n=8)	45.50	5.02	0.542
Total	46.73	49.91	48.18	6.14	

Table 4.6 Means of Bone Perimeter (mm).

Table 4.6 shows the results of the mean for each group at different ages, the combined mean, the estimated difference between the controls and GW3 offspring adjusted for sex and the *p* value. The estimated difference is calculated using the regression analysis, described in equation 4.2, above. The reported *p* value is from the regression analysis which takes into account that pups from the same litter are correlated as noted above.

Controlling for sex, there was no evidence of a difference in mean bone perimeter for GW3 offspring as compared to control offspring at age 4, 8, 12 and 16 weeks. The following is a graphical presentation of the means in Table 4.6.

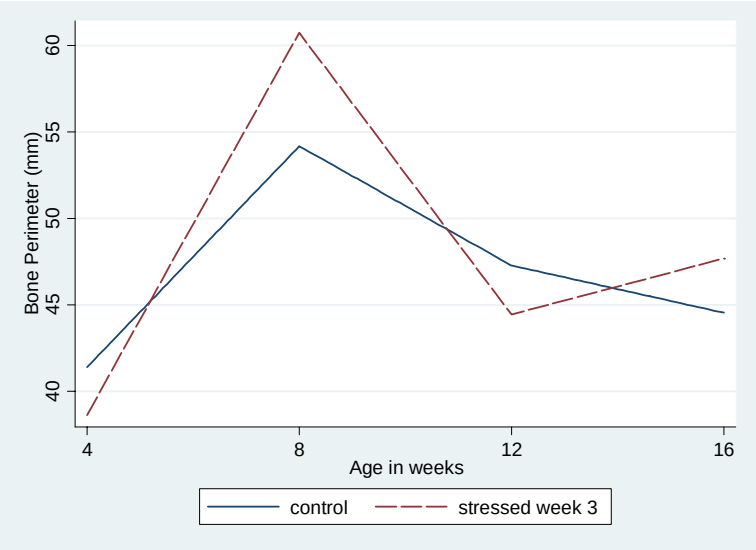

Figure 4.3 Graph showing the change in bone perimeter with age in GW3 offspring (dashed line) and control offspring (solid line).

The second linear regression analysis (using equation 4.1) that examines how *in utero* stress during week three of gestation affected bone development at different ages postnatal failed to demonstrate the presence of a difference in the way maternal prenatal stress affects bone perimeter at different developmental stages, for GW3 offspring compared to the control offspring at age 4, 8, 12 and 16 weeks ($p = 0.7758$). The pattern of bone development in GW3 offspring and control offspring does not vary significantly.

III.a. Ratio of Bone Perimeter to Total Tissue Area

From the definitions of total tissue area (Figure 2.1) and bone perimeter (Figure 2.2), bone perimeter is a correlated variable with total tissue area. A regression analysis on the ratio of bone perimeter to total tissue area showed no significant difference in the way maternal prenatal stress affects the ratio at different developmental stages, for GW3 offspring compared to the control offspring at ages 4, 8, 12 and 16 weeks ($p = 0.0563$).

IV. OSTEOBLAST SURFACE

Osteoblast surface refers to the bone surface perimeter covered by osteoblasts (see Figure 2.3b, page 41 for visual representation). This measurement assessed the amount, in terms of linear extent along the bone surface in a cross section, of bone forming cells available to secrete the collagen matrix (osteoid) that calcifies to form bone. The osteoblasts were quantified by measuring the linear extent of cells on the bone surface across the section. This was done by drawing a line along the osteoblasts lining the bone surface and the length of the line recorded. A higher total number indicates the bone is forming fast (Erben et al., 1997). This is significant

to the overall hypothesis of this study because it is a measure of whether maternal prenatal stress reduces the number of osteoblasts, which are essential for bone formation.

Age in weeks	Control	Offspring born of GW3	Combined mean	Estimated difference adjusted for sex	*p* value
4	9.59 (n=5)	8.88 (n=7)	9.18	-1.48	0.610
8	11.31 (n=8)	16.80 (n=13)	14.71	5.91	0.144
12	12.14 (n=12)	21.66 (n=8)	15.95	11.60*	0.040
16	10.28 (n=18)	14.92 (n=8)	11.71	3.62	0.255
Total	10.91 (n=43)	15.92 (n=36)	13.20	19.65	

Table 4.7 Means of Osteoblast Surface (mm).

The table shows the results of the mean for each group at different ages, the combined mean, the estimated difference between the controls, and GW3 offspring adjusted for sex and the *p* value. The estimated difference is calculated using the regression analysis, described in equation 4.2, above. The reported *p* value is from the regression analysis which takes into account that pups from the same litter are correlated as noted previously.

There is a significant difference in mean osteoblast surface in GW3 offspring compared to the control offspring at the age of 12 weeks (*p* value = 0.040). The GW3 offspring have a significantly higher mean in linear extent of osteoblasts. This is contrary to what I expected, that the control offspring would have a higher linear extent of osteoblast cells, as the actual results demonstrate that the offspring stressed *in utero* have a higher bone formation rate compared to the controls at 12 weeks postnatal. However it is consistent with results on weight gain in *Chapter 3: Results from Live Animal Data*, where it was shown that the offspring from GW3 mothers are heavier in body weight compared to offspring from control mothers. The following is a graphical presentation of the means in Table 4.7.

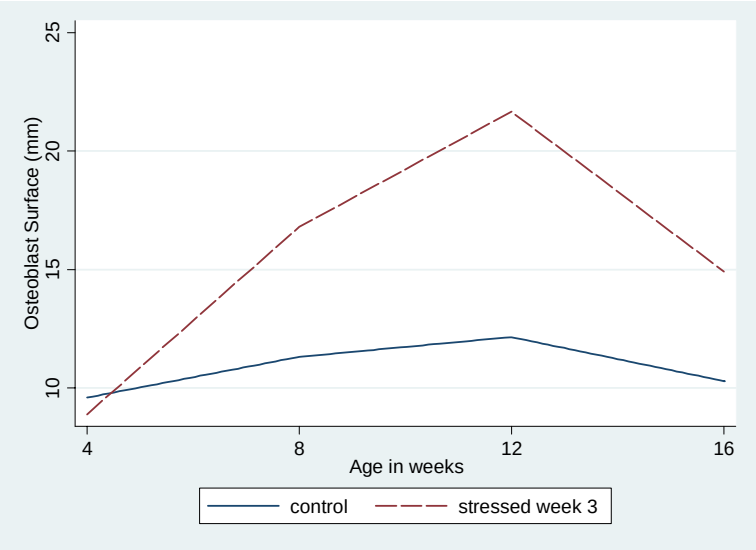

Figure 4.4 Graph showing the change in osteoblast surface with age in GW3 offspring (dashed line) and control offspring (solid line).

The second linear regression analysis (using equation 4.1) that examines how *in utero* stress during week three of gestation affected bone development at different ages postnatal demonstrates that there is a significant difference in the way maternal prenatal stress affects osteoblast surface at different developmental stages, for GW3 offspring compared to the control offspring at age 4, 8, 12 and 16 weeks $p = 0.0333$). The patterns of bone formation in GW3 offspring and control offspring vary significantly.

IV.a. Ratio of Osteoblast Surface to Bone Perimeter

From the definitions of bone perimeter (Figure 2.2) and osteoblast surface (Figure 2.3b), osteoblast surface is correlated with bone perimeter. A regression analysis on the ratio of osteoblast surface to bone perimeter showed a significant difference in the way maternal prenatal stress affects the ratio at different developmental stages for GW3 offspring compared to the control offspring at ages 4, 8, 12 and 16 weeks ($p = 0.0415$). Note that this is the only ratio that was found to be significant.

V. OSTEOID SURFACE

Osteoid surface refers to the bone surface perimeter covered by osteoid matrix (see Figure 2.4, page 42 for visual representation). This measurement assessed the amount of collagen matrix in terms of linear extent along the bone surface in a cross section secreted by the osteoblasts. The osteoid surface was quantified by measuring the linear extent of the matrix on the bone surface across the section. This was done by drawing a line along the osteoid matrix lining the bone surface and the length of the line recorded. This indicates how active the bone forming cells are and hence the rate at which the bone is forming (Erben et al., 1997). The

higher the total number, the more active the cells are. It is significant to the overall hypothesis of this study because it is a measure of whether stress negatively affects the function of osteoblasts by reducing the amount of collagen matrix secreted.

Age in weeks	Control	Offspring born of GW3	Combined mean	Estimated difference adjusted for sex	*p* value
4	0.42 (n=5)	2.18 (n=7)	4.14	1.44	0.093
8	1.92 (n=8)	4.98 (n=13)	3.81	3.15*	0.046
12	0.99 (n=12)	5.50 (n=8)	2.80	4.94*	0.022
16	1.90 (n=18)	3.12 (n=8)	2.28	0.98	0.446
Total	1.48	4.14	2.69	10.51	

Table 4.8 Means of Osteoid Surface (mm).

The table shows the results of the mean for each group at different ages, the combined mean, the estimated difference between the controls, and the GW3 offspring adjusted for sex and the *p* value. The estimated difference is calculated using the regression analysis, described in equation 4.2, above. The reported *p* value is from the regression analysis, taking into account that pups from the same litter are correlated as noted above.

The mean osteoid surface is significantly higher in GW3 offspring compared to the control offspring at the ages of 8 weeks ($p = 0.046$) and 12 weeks ($p = 0.022$). The following is a graphical presentation of the means in Table 4.8.

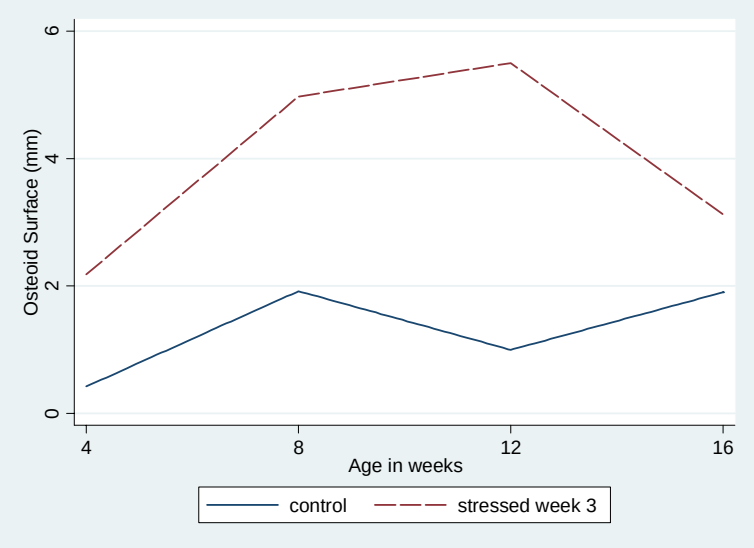

Figure 4.5 Graph showing the change in osteoid surface with age in GW3 offspring (dashed line) and control offspring (solid line).

The second linear regression analysis (using equation 4.1) that examines how *in utero* stress during week three of gestation affected bone development at different ages postnatal failed to demonstrate the presence of any difference in the way maternal prenatal stress affects osteoid surface at different developmental stages, for GW3 offspring compared to the control offspring at age 4, 8, 12 and 16 weeks ($p = 0.1006$). Even though the bone volume is significantly higher in GW3 offspring at the age of 8 weeks and 12 weeks, the overall pattern of bone development in GW3 offspring and control offspring does not vary significantly. However the graph emphasizes the small difference. Since all siblings (animals born to the same mother) were clustered at each age group in the analysis, the effective sample size was diminished. Small sample sizes give results that lack precision.

V.a. Ratio of Osteoid Surface to Bone Perimeter

From the definitions of bone perimeter (Figure 2.2) and osteoid surface (Figure 2.4), osteoid surface is a correlated variable with bone perimeter. A regression analysis on the ratio of osteoid surface to bone perimeter showed no significant difference in the way maternal prenatal stress affects the ratio at different developmental stages, for GW3 offspring compared to the control offspring at ages 4, 8, 12 and 16 weeks ($p = 0.1399$).

VI. OSTEOCLAST NUMBER/EROSION SURFACE

This measurement assessed the number of bone-resorbing cells available at any particular time point to erode the bone surface (see Figure 2.5, page 43 for visual representation). The osteoclasts were quantified by individual cell count. The higher the number of osteoclasts, the higher the bone resorption rate (Erben et al., 1997). This is significant to the overall hypothesis of this study because it is a measure of whether maternal prenatal stress leads to an abnormal increase in the numbers of osteoclasts which results to higher bone resorption.

Age in weeks	Control	Offspring born of GW3	Combined mean	Estimated difference adjusted for sex	*p* value
4	45.18 (n=5)	245.72 (n=7)	162.163	233.31	0.077
8	95.32 (n=8)	120.99 (n=13)	111.21	26.80	0.520
12	89.26 (n=12)	102.95 (n=8)	94.74	-29.07	0.735
16	74.18 (n=18)	66.57 (n=8)	71.84	-9.05	0.829
Total	78.95	129.14	101.82	221.99	

Table 4.9 Means of Osteoclast number/Erosion surface (OC/mm).

The table shows the results of the tabulated mean for each group at different ages, the combined mean, and the estimated difference between the controls and GW3 offspring adjusted for sex and the *p* value. The estimated difference is calculated using the regression analysis,

described in equation 4.2, above. The reported *p* value is from the regression analysis which takes into account that pups from the same litter are correlated as noted above.

Controlling for sex, there was no evidence of a difference in the number of osteoclasts per erosion surface for GW3 offspring compared to the control offspring at age of 4, 8, 12 and 16 weeks. The following is a graphical presentation of the means in Table 4.6.

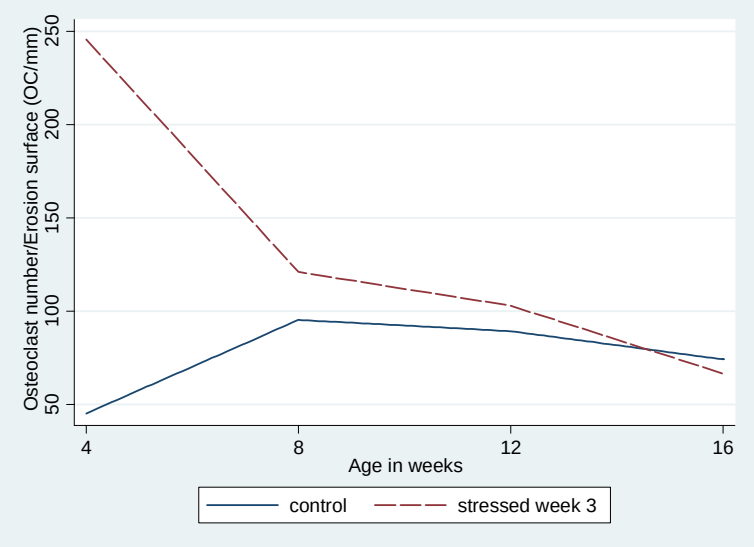

Figure 4.6 Graph showing the number of osteoclasts with age in GW3 offspring (dashed line) and control offspring (solid line).

The second linear regression analysis (using equation 4.1) examines how *in utero* stress during week three of gestation affected bone development at different ages postnatal and failed to demonstrate the presence of any difference in the way maternal prenatal stress affects the number of osteoclasts per erosion surface at different developmental stages, for GW3 offspring compared to the control offspring at the ages of 4, 8, 12 and 16 weeks ($p = 0.1544$). The pattern of bone development in GW3 offspring and control offspring does not vary significantly. However the graph emphasizes the small difference. Since all siblings (animals born to the same mother) were clustered at each age group in the analysis, the effective sample size was diminished. Small sample sizes give results that lack precision.

VI.a. Ratio of Osteoclast Number to Bone Perimeter

From the definitions of bone perimeter (Figure 2.2) and osteoclast number (Figure 2.5), osteoclast number is a correlated variable with bone perimeter. A regression analysis on the ratio of osteoclast number to bone perimeter showed no significant difference in the way maternal prenatal stress affects the ratio at different developmental stages, for GW3 offspring compared to the control offspring at ages 4, 8, 12 and 16 weeks ($p = 0.4288$).

VII. EROSION SURFACE

Erosion surface refers to bone surface perimeter eroded by osteoclasts (see Figure 2.6, page 44 for visual representation). This measurement assesed the size of bone surface resorbed by osteoclasts at the time of sacrifice, which indicates how active the bone resorbing cells were (Erben et al., 1997). The erosion surface was quantified by measuring the linear extent of the bone surface eroded by osteoclasts. This was done by drawing a line along the eroded bone surface and the length of the line recorded. A higher total number indicates the bone is forming fast (Erben et al., 1997). This assessment is significant to the overall hypothesis of this study because it is a measure of whether maternal prenatal stress enhances the function of osteoclasts, which results to higher bone resorption.

Age in weeks	Control	Offspring born of GW3	Combined mean	Estimated difference adjusted for sex	*p* value
4	1.46 (n=5)	0.92 (n=7)	1.14	-0.64	0.074
8	0.94 (n=8)	0.81 (n=13)	0.86	-0.07	0.597
12	1.31(n=12)	0.87 (n=8)	1.13	-0.33	0.359
16	0.98 (n=18)	0.72 (n=8)	0.90	-0.22	0.329
Total	1.12	0.83	0.99	-1.26	

Table 4.10 Means of Erosion Surface (mm).

The table shows the results of the tabulated mean for each group at different ages, the combined mean, the estimated difference between the controls and GW3 offspring adjusted for sex and the *p* value. The estimated difference is calculated using the regression analysis, described in equation 4.2, above. The reported *p* value is from the regression analysis which takes into account that pups from the same litter are correlated as noted above.

Controlling for sex, there was no evidence of a difference in mean bone surface for GW3 offspring as compared to control offspring at age 4, 8, 12 and 16 weeks. The following is a graphical presentation of the means in Table 4.9.

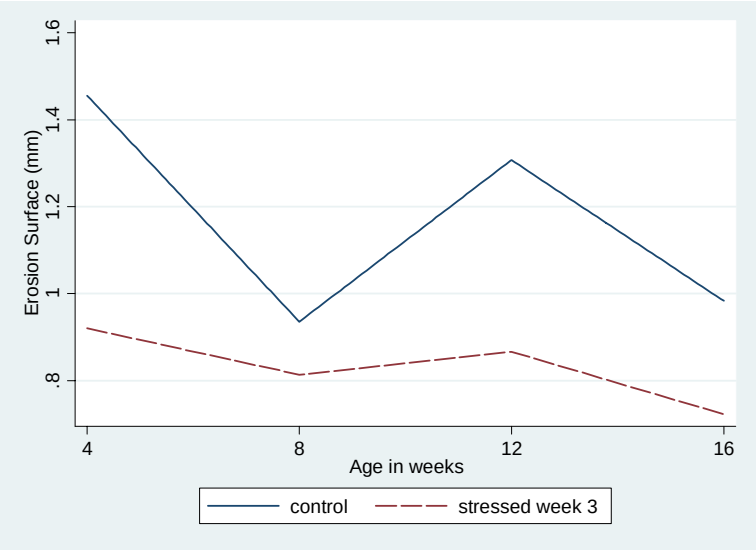

Figure 4.7 Graph showing the change in erosion surface with age in GW3 offspring (dashed line) and control offspring (solid line).

The second linear regression analysis (using equation 4.1) that examines how *in utero* stress during week three of gestation affected bone development at different ages postnatal failed to demonstrate the presence of any difference in the way maternal prenatal stress affects erosion surface for GW3 offspring compared to the control offspring at age 4, 8, 12 and 16 weeks (p = 0.4142). The pattern of bone development in GW3 offspring and control offspring does not vary significantly. However the graph emphasizes the small difference. Since all siblings (animals born to the same mother) were clustered at each age group in the analysis, the effective sample size was diminished. Small sample sizes give results that lack precision.

VII.a. Ratio of Erosion Surface to Bone Perimeter

From the definitions of bone perimeter (Figure 2.2) and erosion surface (Figure 2.6), erosion surface is a correlated variable with bone perimeter. A regression analysis on the ratio of erosion surface to bone perimeter showed no significant difference in the way maternal prenatal stress affects the ratio at different developmental stages, for GW3 offspring compared to the control offspring at ages 4, 8, 12 and 16 weeks (p = 0.4288).

VIII. RESTING AND PROLIFERATIVE CHONDROCYTE ZONE OF THE GROWTH PLATE

The growth plate is the center for bone growth (Howell and Dean, 1992). The measurements presented in this section assessed the size of the area containing the growth plate chondrocytes that are in the resting and proliferative stages (see Figures 2.8 and 2.9, page 46 for visual representation). This measurement indicates the rate at which cells in the growth plate are forming and differentiating. The size of the zone was quantified by drawing a line at the

uppermost boundary of the growth plate containing the resting chondrocytes based on the cell morphology (Howell and Dean, 1992) and another line at the lower boundary of the proliferating chondrocytes. Bioquant software was calibrated to draw random lines at an even distance apart, marking the thickness of the area between the two defined borderlines. The software looked for the maximum distance between any two points. The average length of all the lines was considered the thickness of the zone. The size of the area containing the chondrocytes that are in the resting and proliferative stages indicate the rate at which cells in the growth plate are forming and differentiating (Snow and Keiver, 2007). This is essential to the overall hypothesis of the study because it is a measure of whether maternal prenatal stress negatively affects formation and differentiation of chondrocytes in the growth plate, which is important to bone growth**.** See *Chapter 2: Materials and Methods*.for details of how the measurement was collected.

Age in weeks	Control	Offspring born of GW3	Combined mean	Estimated difference adjusted for sex	*p* value
4	302.83 (n=5)	210.29 (n=7)	248.85	-92.78*	0.023
8	195.73 (n=8)	123.56 (n=13)	151.05	-63.97	0.063
12	166.01 (n=12)	150.84 (n=8)	159.94	-8.67	0.485
16	130.73 (n=18)	123.35 (n=8)	128.46	-10.93	0.305
Total	172.68	146.44	128.46	176.35	

Table 4.11 Means of Proliferative Zone (mcm) (a thousand circular mils).

Table 4.11 shows the results of the tabulated mean for each group at different ages, the combined mean, the estimated difference between the controls and GW3 offspring adjusted for sex and the *p* value. The estimated difference is calculated using the regression analysis, described in equation 4.2, above. The reported *p* value is from the regression analysis which takes into account that pups from the same litter are correlated as noted above.

There is a significant difference in mean thickness of the proliferative zone of the growth plate in GW3 offspring compared to the control offspring at the age of 4 weeks ($p = 0.023$), a time when the proliferative zone of the growth plate in the control animals is significantly thicker. The following is a graphical presentation of the means in Table 4.11.

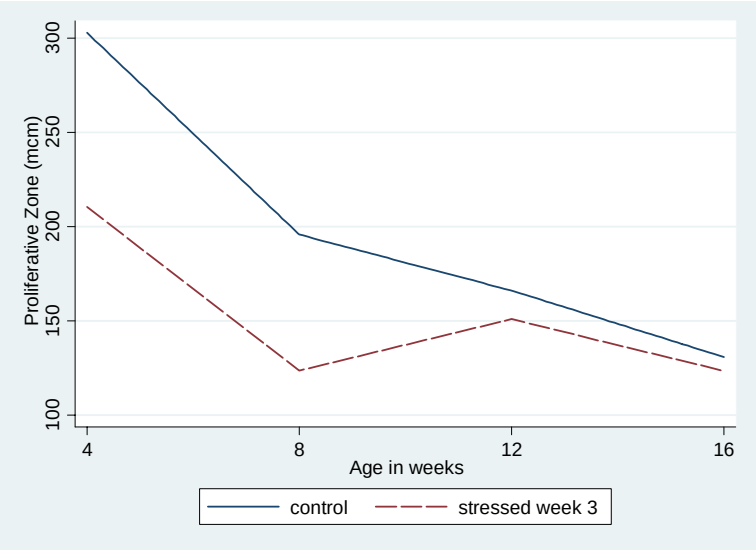

Figure 4.8 Graph showing change in the upper zone of the growth plate (containing resting and proliferative cells) with age in GW3 offspring (dashed line) and control offspring (solid line).

The second linear regression analysis (using equation 4.1), that examines how *in utero* stress during week three of gestation affected bone development at different ages postnatal, demonstrates that there is a significant difference in the way maternal prenatal stress affects the size of the proliferative zone of the growth plate for GW3 offspring compared to the control offspring at the ages of 4, 8, 12 and 16 weeks ($p = 0.0141$). The patterns of growth in the upper zone of the epiphyseal plate vary significantly between GW3 offspring and control offspring.

IX. HYPERTROPHIC CHONDROCYTE ZONE OF THE GROWTH PLATE

This measurement assessed the size of the area containing chondrocytes that are in the hypertrophic stage (see Figure 2.8 on page 46 and Figure 2.10 on page 47 for visual representation). This stage follows the proliferative stage described above (in Section VIII) and indicates the rate at which the cells in the growth plate are maturing. The size of the hypertrophic zone was quantified by drawing a line at the upper boundary of the growth plate area containing the hypertrophic chondrocytes based on the cell morphology (Howell and Dean, 1992) and another line at the lower boundary of the hypertrophic chondrocytes. Bioquant software was calibrated to draw random lines at an even distance apart, marking the thickness of the area between the two defined borderlines. The software looked for the maximum distance between any two points. The average length of all the lines was considered the thickness of the zone. The size of the area containing chondrocytes that are in the hypertrophic zone indicate the rate at which the cells in the growth plate are maturing (Snow and Keiver, 2007). This is essential to the overall hypothesis of the study because it is a measure of whether maternal prenatal stress negatively affects maturation of chondrocytes in the growth plate, which is

important to bone growth**.** See *Chapter 2: Materials and Methods* for more detail and figures on how this trait was measured.

Age in weeks	Control	Offspring born of GW3	Combined mean	Estimated difference adjusted for sex	*P* value
4	179.64 (n=5)	126.15 (n=7)	148.43	-48.66	0.244
8	88.81 (n=8)	70.40 (n=13)	77.41	-15.06	0.405
12	75.44 (n=12)	75.02 (n=8)	75.27	1.78	0.735
16	62.14 (n=18)	62.62 (n=8)	62.29	-0.46	0.890
Total	84.47	80.54	82.68	-62.4	

Table 4.12 Means of Hypertrophic Zone (mcm) (a thousand circular mils).

The table shows the results of the tabulated mean for each group at different ages, the combined mean, the estimated difference between the controls and GW3 offspring adjusted for sex and the *p* value. The estimated difference is calculated using the regression analysis, described in equation 4.2, above. The reported *p* value is from the regression analysis which takes into account that pups from the same litter are correlated as noted above.

Controlling for sex, there was no evidence of a difference in the thickness of the hypertrophic zone of the growth plate for GW3 offspring as compared to control offspring at age of 4, 8, 12 and 16 weeks. The following is a graphical presentation of the means in Table 4.12.

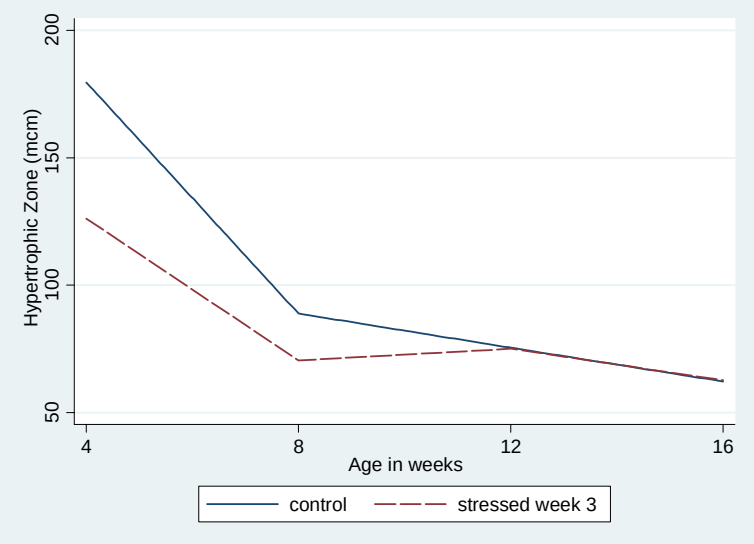

Figure 4.9 Graph showing change in the lower zone of the growth plate (hypertrophic cells) with age in GW3 offspring (dashed line) and control offspring (solid line).

The second linear regression analysis (using equation 4.1) that examines how *in utero* stress during week three of gestation affected bone development at different ages postnatal failed to demonstrate the presence of a difference in the way maternal prenatal stress affects the thickness of the hypertrophic zone of the growth plate for GW3 offspring compared to the control offspring at the ages of 4, 8, 12 and 16 weeks ($p = 0.3149$). The pattern of growth in the lower zone of the epiphyseal plate does not vary significantly between GW3 offspring and control offspring.

X. COMPLICATIONS OF THE ANALYSIS

One major limitation of this analysis was that small sample size ultimately compromised the statistical power. This came about as a result of several factors that were not accounted for in the study design. The first was that to obtain 11 pups from 7 different mothers meant that some pups would be drawn from the same mother. Pups from the same litter are correlated and so I had to employ a statistical analysis that accounts for this correlation, which diminished effective sample size. The larger the intraclass correlations, the less unique the information contained by each observation. The Animal Care and Use Committee is very strict about using the smallest number of animals possible for a study, understandably. However, I was a bit too overly optimistic about how small a sample I could work with statistically.

The second factor complicating my statistics was that having raised the exact number of pups required for the analysis meant that I did not have any buffer. This became problematic at the last stage during the histology analysis, when I realized that even though all the bones were fixed in the same way soon after extracting them from the animals at necropsy (by putting them in vial full of 10% phosphate buffered formalin at 4°C for 24 hours as described in the methods chapter), the cellular structure in some of the bones was not well preserved, making it impossible to study. This further reduced the sample size because I could not go back and refix the bones.

Therefore, even though I had initially planned to have large enough sample sizes for my analysis, these anticipated and unforeseen problems compromised the power of my study.

XI. SUMMARY

This chapter reports on how pups born to mothers stressed in gestation week 3 differ in bone development from those born to control mothers. Histology slides were prepared from the same anatomical region in the proximal tibia from each animal, and a suite of parameters representative of bone development were measured from the sections. Bone histomorphometry was performed using semi-automated image analysis (Bioquant Image Analysis Corporation, Nashville, TN, USA) linked to a microscope.

In all groups male pups had larger bones compared to females starting at the age of 8 weeks. However, there was no significant difference in tissue volume and the perimeter of the bone surface area between the GW3 offspring and control offspring. The GW3 offspring had a higher bone formation rate as indicated by their higher bone volume at the age of 8 weeks, a higher number of osteoblasts (the bone forming cells) at the age of 12 weeks, and a bigger area of the osteoid surface at the age of 8 and 12 weeks, which is the collagen matrix laid down by osteoblasts that eventually gets mineralized to form the bone. There was no significant difference in bone resorption rate between the two groups.

The control offspring had a much bigger growth plate at the age of 4 and 8 weeks, indicating a more active growth face at an early age compared to the experimental group. The width of the growth plate reduced drastically as the animals advance in age, which was expected.

CHAPTER 5: DISCUSSION

Osteoporosis is one of the most important health conditions affecting aging humans (≥ 60 years in age), particularly women in North America and Europe (Jordan and Cooper, 2002). One of the risk factors for osteoporosis is not attaining maximum peak bone mass density for one's genetic potential. This risk factor is ascribed to poor environmental conditions and is irreversible (Cooper et al., 2009).

Psychological stress has been demonstrated to predispose pre-menopausal women to osteoporosis (Eskandari et al., 2007; Cizza et al., 2001). Since environmental factors acting in prenatal life are known to influence adult health (Gluckman et al., 2005), I hypothesized that psychological stress during pregnancy, and the resultant high levels of cortisol would affect bone formation in the fetus. This affect could compromise early bone development in the fetus and diminish the potential for attaining peak bone mass density in young adults, and therefore be associated with a higher risk of osteoporosis later in life.

To test this hypothesis I induced immobilization stress to pregnant Wistar rats at different gestational stages. Group 1 mothers were stressed during gestation week 1, Group 2 during gestation week 2, Group 3 during gestation week 3, and the control group mothers were not stressed at all. During gestation I monitored the dams' cortisol hormone levels (through fecal sampling), food intake, and maternal weight gain. After birth the pups were raised in a stress-free environment with *ad libitum* access to food and water. Different sets of pups were euthanized at 4, 8, 12 and 16 weeks of age and histological analysis of their proximal tibias performed.

The detailed results from this study are presented in earlier chapters. Here, I provide a summary of the results and the implications of these findings to our understanding of how prenatal stress affects bone health.

I. MATERNAL WEIGHT GAIN AND LITTER SIZE

During gestation a difference was observed in the pattern of weight gain among the dams in the study. The pregnant dams that were stressed during gestation week 1 and 2 gained significantly less weight compared to those stressed in either week 3 or the controls. This was consistent with previous studies in which prenatal stress was induced by glucocorticoid injection (O'Regan et al., 2008) and by immobilization (D'mello and Liu, 2006). This may correlate with my finding that the stressed dams also had lower food intake compared to the controls. In addition, stress induction resulted in significantly higher levels of cortisol hormone in stressed dams compared to the controls. This was also consistent with results from a previous study that used the same protocol (D'mello and Liu, 2006).

As pregnancy advanced, there was a general trend of incremental weight increase in the female due to fetal growth. However there was a wide range of variation in the amount of weight gained in my study that was likely dependent on the number of fetuses conceived, caloric intake and the cortisol levels in the dams due to stress. I will explore these factors in more detail below.

I.a. Number of Pups Born

I found a positive correlation between maternal weight gained during pregnancy and the number of pups born, as expected. However, in contrast to this overall trend, the animals stressed in gestation week 1 and gestation week 2 had the highest average number of pups but gained the least amount of weight on average. This demonstrates that the pups born from these two groups were smaller in weight and size. There was no difference in the average amount of weight gained by the mothers stressed in gestation week 1 and 2. Since cortisol hormone levels in these two groups were significantly higher than those of the controls the impact of stress to the mother seems to have affected fetal development at this early stage, causing them to be smaller. This result appears to be consistent with a previous study in hamsters in which social stress during early pregnancy resulted in dams giving birth to male pups that were smaller in size compared to the controls (Pratt and Lisk, 1989).

By injecting pregnant rats with cortisol hormone Megías and colleagues (1983) noted a slight reduction in litter size and individual pup weight in hormone treated dams. In human studies, maternal cortisol is known to cross the placenta and likely influence fetal development (Glover et al., 1999, Gitau et al., 1998). Elevated fetal cortisol levels, such as evident in fetuses of depressed mothers, result in delayed prenatal growth, prematurity, and low birth weight (Field et al., 2006). Even though I did not collect birth weights in my study, the weight data from the mothers during gestation showed that mothers with significantly higher cortisol levels (GW1 and GW2) had the lowest weight gain on average at the end of their gestation period. These same groups that gained the least amount of weight had the largest average number of pups. I can therefore infer that pups born to GW1 and GW2 mothers had lower birth weight compared to the controls. The potential underlying mechanism, as we know from other studies cited above, is that prolonged exposure of the fetus to cortisol can negatively affect fetal growth and organ development. Excess cortisol alters the normal functioning of hypothalamo-pituitary-adrenal (HPA) axis, which may last for a lifetime (Kapoor et al., 2006).

My conclusion about offspring birth weight is mainly inferential based on maternal weight gain because I did not collect the offspring birth weight, and neither did I determine sex ratios before culling the pups. Because my study focused on the effect of prenatal stress on bone development, I tried to ensure that once the pups were born, the mothers and the pups were in a stress-free environment. Handling the pups, especially so soon after birth, increases the anxiety (stress) level in the mothers as they try to protect their newborns. This would have been a confounding factor to the study of prenatal stress. Furthermore, handling the pups particularly at this stage, would have been stressful to them as well, which I wanted to avoid. In hindsight, given the potentially significant differences in offspring sex ratio and birth weight information I should have developed a non-stressful means by which these data might have been collected. If the project is ever repeated more data at birth will be recorded to help explore some of the points raised by this initial study.

I.b. Caloric Intake

Even though there was a general trend of increase in food intake for the pregnant dams, the animals undergoing stress consumed significantly less food compared to the controls. In the first week of gestation, the animals that were being stressed ate 3 grams (12.5% on average) less

per day compared to the controls. Previous studies in which pregnant rats were injected with cortisol daily also showed that there was significant reduction in daily food intake (Megías et al., 1983). High levels of glucocorticoids have been shown to have a catabolic effect, which increases levels of amino acids, glucose, and lipids in plasma. When the levels of these food molecules are high in plasma, the hunger signal in the brain is turned off (Delaere et al., 2010, Valassi et al., 2008).

Pregnancy alone leads to increased food intake in rats (López-Frías et al., 1985) to meet the increasing nutritional needs of the growing fetuses.

> *"Nitrogen metabolism in rats during pregnancy is thought to follow a biphasic course…The first fourteen days are mainly dominated by an anabolic phase characterized by marked decrease in amino acid catabolism. During this phase, the dam builds up a reserve of protein. This catabolic phase occurs regardless of the protein intake of the dam, and is under hormonal control. Rapid growth of the fetus takes place during the last week of gestation. This is when the protein reserves are withdrawn and utilized" (Naismith and Morgan, 1976; p. 565).*

The hypothalamus controls food intake (Delaere et al., 2010, Valassi et al., 2008). The body's nutritional status is sensed and interpreted by the hypothalamus through signals transmitted by the peripheral and central nervous systems (Valassi et al., 2008). If the body is in a deficient nutritional and energy status, the hunger signal is turned on, which leads to the desire to feed. On the other hand, if there are enough food molecules circulating in plasma ready to be utilized by body cells, the hunger signal is turned off.

The catabolic effect of glucocorticoids, as induced by the high cortisol level in stressed dams in my study, is assumed to have resulted in high circulating levels of plasma amino acids, glucose and lipids in plasma (Delaere et al., 2010, Valassi et al., 2008). As a result, the animals' food intake reduced.

The other possible contributing factor to the reduced food intake in the stressed dams is that during the stress regimen they spent 1 hour and 15 minutes daily without access to food and water (when they were restricted in a decapicone bag). The animals resumed feeding normally soon after they were returned to their home cage.

On the day before delivery all of the animals increased their food intake, almost doubling their norm. This could be a normal process triggered by the hypothalamus in preparation for delivery.

I.c. Cortisol Levels in the Dams and the Impact on Pups

The dams in the stressed study groups had an elevated level of cortisol hormone compared to controls, as expected. Given that cortisol crosses the placenta (Glover et al., 1999, Gitau et al., 1998), it is plausible that it will alter the *in utero* environmental condition of the fetuses.

The mean cortisol hormone levels in the stressed mothers were significantly higher compared to those of controls. However, the average difference between the stressed groups and the controls were not consistent from week to week but rather reduced through time of gestation. The animals stressed in gestation week 1 had the significantly highest cortisol hormone level compared to controls (GW1 = 3.72μg/g, controls = 1.66μg/g, difference = 2.06μg/g), followed

by those stressed in gestation week 2 (GW2 = 3.34μg/g, controls = 1.73μg/g, difference = 1.62μg/g) and those stressed in gestation week 3 (GW3 = 3.36μg/g, controls = 2.38μg/g, difference = 0.98μg/g). Stressing the animals in gestation week 1 more significantly raised their cortisol hormone levels relative to the controls, but the difference declined during pregnancy. This result raises the possibility that pregnancy, by itself, has an effect on cortisol hormone level. Reviewing the changes in cortisol hormone level over pregnancies only in the control group revealed that the cortisol level does increase throughout gestation.

Cortisol concentration has been demonstrated to increase significantly during pregnancy in humans (Damjanovic et al., 2009; Noguchi, 1988; Mastorakos and Ilias, 2003). This can be attributed to "production of corticotropin-releasing hormone (CRH) in the placenta, decidua, and fetal membranes" (Mastorakos and Ilias, 2003; p. 139), enlargement of maternal pituitary gland (Taylor, 2001), and increased secretion of corticotropin by the pituitary gland (Magiakou et al., 1996). An increase in estrogen hormone, which is necessary to support pregnancy and inhibit ovulation (Jones and Lopez, 2006), also results in increased plasma cortisol. This is because the elevated estrogen levels reduce cortisol catabolism by the liver (Mastorakos and Ilias, 2003). These series of events during pregnancy lead to a steady rise in cortisol hormone that peaks in the third trimester (Mastorakos and Ilias, 2003). The increase in placental glucocorticoids is essential for maturation of the fetal organ system, including the liver, lung, adrenal gland, and brain, in preparation for survival outside the uterine environment (Challis et al., 2001). It also influences the timing and onset of delivery (Challis et al., 2001).

This pattern explains the decline in variation of hormone level between the stressed groups and the control group of animals in my study. In gestation week one, the difference in cortisol hormone level was much larger because the controls were at the very initial stage of pregnancy. In the control animals at this stage, the cortisol hormone level had not been altered much by pregnancy. During gestation week two, the difference between cortisol hormone level in controls and stressed animals was still significant, but not by as much as in week one. At this stage in the controls, the maternal physiological changes due to the pregnancy were having a greater affect on cortisol levels, causing them to rise. In rat gestation week three, the last third period of pregnancy, the cortisol hormone level was even higher in the controls. However, the stressed animals still had a significantly higher level of the stress hormone in comparison. The cortisol hormone level in the stressed pregnant dams was both due to the pregnancy and to the induced stress. "Thus, as in humans, normal rat pregnancy is a transient, but physiologic period of relative hypercortisolism" (Mastorakos and Ilias, 2003; p. 140).

Experimental studies demonstrate that the reproductive experience (breeding, pregnancy, parturition, and lactation) in rats leads to elevated levels of corticosterone hormone (Pawluski et al., 2009; Rima et al., 2009; Macbeth and Luine, 2010). Just as in humans (Grajeda and Perez-Escamilla, 2002), these studies found primiparous females to have the highest elevation in corticosterone hormone compared to multiparous females. This implies that bearing offspring for the first time is associated with higher levels of corticosterone hormone compared to the subsequent pregnancies in both rodents and humans (Pawluski et al., 2009; Grajeda and Perez-Escamilla, 2002).

Other studies have shown that pregnant rats exposed to both emotional and physical stress (Neumann et al., 1998) were found to express a progressively reduced response to the stressors during the last week of gestation and during lactation. "This could be due to a reduced synthesis of basal corticotropin releasing hormone (CRH) by the paraventricular nuclei in the

hypothalamus, reduced binding of CRH in the adenohypophysis, and reduced CRH stimulated corticotropin secretion" (Neumann, 2001; p. 147). There is general trend of reduced activity in the hypothalamic-pituitary-adrenal (HPA) axis, which may serve a protective role against the negative effects of stress for both the mother and offspring (Macbeth and Luine, 2010).

A slight increase in cortisol levels during gestation is necessary for proper maturation of the organ system in the fetus before birth, as well as to the timing and onset of parturition (Challis et al., 2001). However, excess cortisol (that may result from abnormal circumstances such as those induced on rats in this study), has deleterious effects. Such circumstances in humans include disease, under-nutrition and depression (Murray and Lopez, 1997; Kajantie, 2006). For lack of a better model to help us understand the long-term effects of stress that goes beyond the normal pregnancy challenge in humans, laboratory animals like rats, sheep, mice and non-human primates have been subjected to conditions that intentionally raise their cortisol hormone levels during gestation. These include restraint, immobilization, forced swimming, forced treadmill exercise, noise stress, nutrient deficiency, electric shock and administration of CRH (reviewed in Lelovas et al., 2008).

Low birth weight in the offspring is one feature that has been shown to manifest across all types of stress in humans and animals (O'Regan et al., 2010; Cottrell and Seckl, 2009; De Blasio et al., 2007; Schneider et al., 1999). Such offspring tend to have a compensatory mechanism when born, which results in rapid weight gain (Cottrel and Seckl, 2009). In a resource-rich environment, this catch-up growth mechanism leads to metabolic diseases like hypertension, insulin resistance, obesity, cardiovascular disease and Type II diabetes (Gluckman et al., 2005).

In this section I have made the case that the elevated cortisol levels seen in the stressed pregnant dams in my study could cause detrimental effects to the pups. The next section explores data from the pups in order to explore this possibility, and as such, test the overarching hypothesis of my thesis.

II. OFFSPRING WEIGHT GAIN

Comparing offspring from all four groups of this study (i.e., born to mothers stressed in gestation week 1 (GW1), born to mothers stressed in gestation week 2 (GW2), born to mothers stressed in gestation week 3 (GW3), and born to controls), there was a slight difference in the pattern of average weight gain with the GW3 offspring being highest. These differences were not statistically significant but may have a large effect on bone development as discussed below. As already noted by other researchers (e.g., Gluckman et al., 2005), offspring exposed to stressful environmental conditions *in utero* tend to have a compensatory mechanism that helps them thrive. My study supports this observation, given that GW3 offspring tend to be heavier at all developmental stages studied compared to the control offspring. The results are also consistent with those from Vickers and colleagues (2000). Their study found that offspring from mothers that were underfed during gestation had a significantly higher food intake compared to controls beginning at an early postnatal age, and their food intake increased as they grew older. Such a phenomenon eventually leads to obesity in adulthood. "The fetus adjusts its biology in response to signals from its undernourished mother, allowing it to survive until birth, but predisposing it to the adverse consequences of such programmed thriftiness in adulthood"

(Gluckman, 2009; p. 201). This happens when there is a mismatch between the initial anticipation-adaptive response and the actual environment encountered after birth.

Because my study mainly focused on the effect of prenatal stress on bone development of the offspring, I hypothesized that one of the long-term detrimental effects that exposure to high cortisol level *in utero* will have on the offspring is hindered bone development, a risk factor for osteoporosis in human. Therefore the following part of the discussion focuses on the results obtained from analysis of pup weight gain and bone developmental pattern.

III. PATTERN OF BONE DEVELOPMENT IN OFFSPRING

This part of the discussion is based on results reported in Chapter Four. These focused on the comparison of bone development between offspring of the control group and offspring of mothers stressed during gestation week 3 (GW3). I decided to first analyze GW3 offspring because this is the week during which rats' bones initially mineralize. The histomorphometry phase of the project was very time-intensive. As such, the two other experimental groups will be studied at a later date and the results reported elsewhere.

Rats are sexually dimorphic animals with males being larger than females. In my study, this difference was reflected in total tissue area of trabecular bone in the proximal tibia. The males had a significantly larger total tissue area compared to the females from the age of 8 weeks in both GW3 and control offspring.

The novel observation of this study was that offspring born to stressed mothers (GW3) had a higher bone area and a potentially higher bone formation rate indicated by a higher number of osteoblasts (bone forming cells) at the age of 12 weeks, and a larger area of the osteoid surface (collagen matrix laid down by osteoblasts that eventually get mineralized to form the bone) at the ages of 8 and 12 weeks. Even though this was contrary to expectation, it implies that these pups were growing faster than the controls (considering the fact that GW3 offspring were heavier on average compared to all the other groups), which was also reflected in their bone development. This difference manifested by the age of 8 weeks.

Previous studies have examined the effects of corticosteroids on growing bones of rat and mice by injecting pregnant mothers with cortisone acetate during the second and third week of gestation (Ornoy, 1971a, b; Ornoy and Horowitz, 1972; Atkin and Ornoy, 1981; Atkin et al., 1984). In each of these studies the experimental newborns had shorter and smaller long bones, thinner metaphyseal and diaphyseal trabeculae, and abnormal calcification compared to the controls. All these differences were obvious during the first week after birth but by day 30 postnatal no morphological differences could be detected between the groups exposed to high cortisol level *in utero* and control groups.

In my study, rather than injecting corticosteroids into the pregnant rats, I employed a method that could prompt the body to produce higher levels of cortisol hormone itself, and therefore mimic the more natural way the mother's body deals with stressful conditions during pregnancy. I also studied the offspring for a longer time postnatally. This allowed for an analysis of bone growth after the apparent catch-up growth period ended, 30 days postnatal as suggested by the previous studies (Ornoy, 1971a, b; Ornoy and Horowitz, 1972; Atkin and Ornoy, 1981; Atkin et al., 1984). As such, my analysis was done on pups that were 4 to 16 weeks old. Male rats are sexually mature at 12 weeks (Nassr et al., 2010) and classified as adults

at 6 months. Female rats on the other hand are sexually mature at 5-6 weeks (Lewis et al., 2002) and classified as adults at 5-6 moths.

Results from this dissertation suggest that given similar environmental conditions in postnatal life, offspring born to mothers stressed in gestation week three (GW3) and those born to control mothers followed different trajectories in postnatal development. The GW3 offspring developed faster in terms of general body growth and bone growth compared to the control offspring.

Since the GW3 mothers reduced food intake compared to the controls, nutrient restriction and exposure to high cortisol levels likely contributed to the observed outcomes in the GW3 offspring. Having experienced a poor environment *in utero* the GW3 offspring may have undergone changes in their physiology and metabolism that would enable them to thrive in similar conditions during their postnatal life. An example of such a programming shift could be a more efficient metabolism in order to maximize on the scarce nutritional resources available to them. Such changes during this crucial developmental period have been demonstrated to be permanent and perhaps provide an evolutionary advantage (Cooper et al., 2009). However, if the offspring is born in an environment that is not as stressful as that experienced *in utero,* they encounter a mismatch from what their metabolism was programmed for. This may predispose them to metabolic disorders like diabetes at a later stage in life (Gluckman et al., 2005). This phenomenon is currently known as the *Thrifty Phenotype Hypotheses* and was first proposed by Hales and Barker in 1992 in regard to Type II diabetes. Following a number of studies, they proposed that poor nutrition *in utero* and during early life of an infant negatively affects the function of Beta cells of the islet of Langerhans, leading to impaired glucose tolerance (Hales and Barker, 1992). This is supposed to be an adaptation in preparation for a poor nutritional environment in postnatal life, but instead it predisposes the individual to development of Type II diabetes later in life if the poor conditions are not realized (Hales and Barker, 1992).

The GW3 offspring in my study were raised in similar conditions to those of the control offspring. Both groups had unlimited access to food and water, and were only handled twice a week when the cages were cleaned and food added to their feeding trays. This was done to minimize the stress from handling as much as possible. Therefore, in the relatively stress free and nutrient sufficient environment, the GW3 offspring thrived and eventually outperformed the control offspring. They ended up having a higher average body weight, higher bone formation rate, and higher bone area. There was no difference in bone resorption rate between GW3 offspring and control offspring.

One of the limitations of my study design was that given the need to minimize the number of times the offspring were handled in order to avoid stressing them, I did not monitor their daily food intake. This would have required measuring the amount of food-consumed daily by opening the cage, measuring the food and putting it back. Additionally, since the animals were housed in groups (and later, in pairs), I would have needed to keep them in isolation to properly monitor food in-take, creating isolation stress that would compromise their postnatal development. Even without the data on offspring food intake, my assumption is that since the GW3 offspring weighed more on average, they must have been eating more.

In humans, exposure to high cortisol levels *in utero* has been demonstrated to persist in the offspring during postnatal life (De Bruijin et al., 2009). In order to determine whether or not this same phenomenon occurs in rats, I need to test whether the rat offspring born to stressed mothers in my study had persistent high levels of cortisol hormone during postnatal life.

However, this would have required extra handling of the offspring to collect fecal samples for the hormone analysis, which I tried to avoid as much as possible.

In addition to bone mineralization rate, I was also interested in understanding how the linear growth rate of bone differed between GW3 offspring and control offspring. I did this by assessing the size of the growth plate. The upper zone of the growth plate, consisting of the resting and the proliferation cells, was significantly thicker in the control offspring at the age of 4 weeks compared to the GW3 offspring. The difference disappeared as soon as the animals reached the age of 8 weeks. The thickness of the growth plate reduces drastically as the animals advance in age, which is expected because it eventually closes, marking the end of the appendicular growth period. My results suggest that the control animals had a more active linear growth phase at the younger stage compared to experimental animals. In turn, this may indicate that *in utero* exposure to cortisol had a significant negative effect on the linear growth of the bone. Previous studies have suggested that corticosteroids inhibit linear growth of bone due to disturbance in the growth plate (Follis, 1951) and disturbance in the formation of the collagen matrix (Jee et al., 1970; Peck et al., 1967; Storey, 1960; Sissons and Hadfield, 1955), the latter potentially mediated through reduction of osteoblastic activity. Therefore, in my study, the results demonstrate that prenatal stress significantly affected the growth plate of the offspring. Further investigation is required to understand how this may negatively affect adult skeletal size. The lower zone of the growth plate, which consists of hypertrophic cells, did not differ between the two groups.

IV. CONCLUSIONS FROM THIS STUDY

In conclusion, this research demonstrated that the experience of stress during pregnancy in rats has negative consequences for both the mother and the offspring. The caloric intake in the mother is reduced, potentially due to the excess cortisol that alters the hypothalamic control of food intake. Whether or not this reduced food intake results in nutrient restriction remains to be determined, but it does leave the possibility that the offspring may have been nutrient-restricted *in utero*. We do know from my data that the offspring were at least exposed to high levels of cortisol. Whether both nutrient restriction and high cortisol levels of exposure, or just the cortisol exposure affected the offspring, these animals appear to be born with an altered metabolism that results in faster growth and higher weight gain compared to controls. The positive effect of this fast growth is that the offspring stressed *in utero* ended up with a higher bone area compared to the control.

The only analysis that showed that the control offspring did better than GW3 offspring was that of the growth plate. At age 4 weeks, control offspring had a significantly thicker growth plate compared to GW3 offspring. Therefore, the negative effect of prenatal stress was evident in the upper zone of the growth plate even at the age of 4 weeks, when the seemingly catch-up growth is expected to have occurred in all aspects of bone development measured. This seems to be the most sensitive part of bone development in regards to prenatal cortisol exposure, but this effect is lost by the time the rats are 12 weeks old.

V. IMPLICATIONS FOR STUDY OF OSTEOPOROSIS AND OTHER METABOLIC DISEASES IN HUMANS

In terms of most of the bone developmental parameters I studied, elevated cortisol levels in pregnant dams appear not to have a negative effect. "This may be because of the mother's body's mechanisms that dampen the effect of the hypothalamic-pituitary-adrenal HPA axis" (Macbeth and Luine, 2010; p. 453), but this remains to be explored for other elements of the offspring's physiology. Clearly, since the upper zone of the growth plate was shortened in the GW3 pups, and that they did have elevated growth rates in this study, GW3 pups did not have an identical physiology to that of the controls. Something is different about them.

The second observation relates to human evolution. The offspring born of stressed mothers (GW3) had higher bone areas and higher body weights compared to the controls. The offspring stressed *in utero* seem to have had a compensatory mechanism that lead to faster postnatal growth and weight gain. Unfortunately, such rapid weight gain predisposes to obesity and other metabolic diseases in adulthood in rats (Rose and Desai, 2005). This is also observed in modern human populations (Gluckman and Hanson, 2007; Gluckman et al., 2005).

Modern human populations, particularly in developed countries, have transitioned from a lifestyle of foraging or subsistence farming (with their diverse diet of non-processed foods and plentiful physical activity) to a lifestyle with a diet of energy-dense processed foods and low levels of physical activity. Given our evolutionary history, the metabolism of the current human populations seems to have evolved in an environment with minimally sufficient amount of nutrients. Therefore there is a mismatch between what our metabolism was selected for and the current nutritional and physical activity environment of growing numbers of humans.

A contemporary example of this phenomenon is the Dutch population born during the famine experienced between November 1944 and May 1945 due to World War II (Kyle and Pichard, 2006). The fetuses that experienced nutritional deprivation *in utero* due to maternal under-nutrition in the first and second trimesters were born with normal birth weight, but ended up with various health conditions as adults. These included obesity, increased risk of developing breast cancer, increased glucose intolerance, increased stress sensitivity, and increased coronary artery heart disease (Painter et al., 2005; Painter et al., 2006a, b; Roseboom et al., 2006). On the other hand, those that experienced *in utero* nutritional deprivation due to maternal under-nutrition in the third trimester had a lower birth weight and height, increased cardiovascular disease, increased hypertension, and increased Type II diabetes (Roseboom et al., 2001).

The human fetus seems to adjust its metabolism in response to the availability of nutrients from the mother. Therefore in the case of under-nutrition, the fetus is programmed to metabolize more efficiently in order to benefit from the scarce available nutritional resources. This allows it to survive until birth. If the same conditions prevail after birth, this phenotype serves to the newborn's advantage. "However, if the fetus is born in a nutrient sufficient environment, this phenotype unfortunately predisposes the baby to obesity due to its propensity to store fat in a nutrient sufficient environment" (Gluckman and Hanson, 2007; p. 466). The low birth weight in humans followed by catch-up growth also results in an increased risk for developing insulin resistance leading to Type II diabetes (Gluckman and Hanson, 2007; Gluckman et al., 2005). The advantage to rapid increase in body weight is that it leads to high muscle weight and bone mass, and possibly increased estrogen levels. These are protective against osteoporosis in adults.

The patterns described above for human prenatal stress does not fit with the results from my study on rats. Therefore, while it is evident that stress during gestation does have an impact on rat postnatal health, much remains to be explored about the possible differences between how rats and humans respond to this experience.

VI. FUTURE DIRECTIONS

The results from this research were contrary to my expectations. I had anticipated that maternal stress during gestational week 3 would result in compromised bone structure in the offspring. In contrast, I found that the offspring from GW3-stressed mothers had faster bone mineralization. This raises questions requiring follow-up study.

My next goal is to analyze the bones from the GW1 offspring and GW2 offspring in order to determine how stress in these earlier weeks compares or contrasts with the results from GW3. My prediction is that given the higher cortisol levels in GW1 and GW2 mothers, coupled with the least average weight gain during pregnancy, the bone from their offspring will be poorly developed compared to the controls and GW3 offspring.

I am also interested in performing Micro-CT scanning and mechanical testing on the bones to find out whether there is a difference in the microstructure and mechanical strength of the different groups. Also, since I have a blood sample from each animal, it would be interesting to analyze the cortisol level in the offspring and compare this to their mothers, as well as to perform an analysis of the biochemical markers of bone turnover. This will give us a more holistic understanding of how maternal stress at different gestational stages affects bone development and in turn, a better understanding of how the timing of stress during human pregnancy may affect the long-term health of offspring.

REFERENCES

Adachi T, Kameo Y, Hojo M. Trabecular bone remodelling simulation considering osteocytic response to fluid-induced shear stress. Philos Transact A Math Phys Eng Sci. 2010;368:2669-2682.

Adams JE. Quantitative computed tomography. Eur J Radiol. 2009;71:415-424.

Ahrens M, Ankenbauer T, Schröder D, Hollnagel A, Mayer H, Gross G. Expression of human bone morphogenetic proteins-2 or -4 in murine mesenchymal progenitor C3H10T1/2 cells induces differentiation into distinct mesenchymal cell lineages. DNA Cell Biol. 1993;12:871-880.

Aitken JM, Armstrong E, Anderson JB. Osteoporosis after oophorectomy in the mature female rat and the effect of oestrogen and-or progestogen replacement therapy in its prevention. J Endocrinol. 1972;55:79-87.

Albanese CV, Rendine M, De Palma F, Impagliazzo A, Falez F, Postacchini F, Villani C, Passariello R, Santori FS. Bone remodelling in THA: A comparative DXA scan study between conventional implants and a new stemless femoral component. A preliminary report. Hip Int. 2006;16 (Suppl 3):S9-S15.

Anderson PH, Sawyer RK, Moore AJ, May BK, O'Loughlin PD, Morris HA. Vitamin D depletion induces RANKL-mediated osteoclastogenesis and bone loss in a rodent model. J Bone Miner Res. 2008;23:1789-1797.

Atkin I, Ornoy A. Transplacental effects of cortisone acetate on calcification and ossification of long bones in mice. Metab Bone Dis Relat Res. 1981;3:199-207.

Atkin I, Ornoy A, Soskolne WA. Comparison of the effects of cortisone acetate on calcification, ossification, and resorption of fetal mice long bones--in vivo and in vitro studies. Adv Exp Med Biol. 1984;171:137-148.

Bagi CM, Mecham M, Weiss J, Miller SC. Comparative morphometric changes in rat cortical bone following ovariectomy and/or immobilization. Bone. 1993;14:877-883.

Ballock RT, O'Keefe RJ. The biology of the growth plate. J Bone Joint Surg Am. 2003;85:715-726.

Bamberg E, Palme R, Meingassner JG. Excretion of corticosteroid metabolites in urine and faeces of rats. Lab Anim. 2001;35:307-314.

Barker DJ. The intrauterine origins of cardiovascular disease. Acta Paediatr Suppl. 1993;82 Suppl 391:93-99.

Barker DJ. Fetal origins of coronary heart disease. BMJ. 1995a;311:171-174.

Barker DJ. The fetal and infant origins of disease. Eur J Clin Invest. 1995b;25:457-463.

Barker DJ. Intrauterine programming of adult disease. Mol Med Today. 1995c;1:418-423.

Barker DJ. The Welcome Foundation Lecture, 1994. The fetal origins of adult disease. Proc Biol Sci. 1995d; 262:37-43.

Barker DJ, Godfrey KM, Fall C, Osmond C, Winter PD, Shaheen SO. Relation of birth weight and childhood respiratory infection to adult lung function and death from chronic obstructive airways disease. BMJ. 1991;303:671-675.

Barker DJ, Osmond C, Golding J, Kuh D, Wadsworth ME. Growth in utero, blood pressure in childhood and adult life, and mortality from cardiovascular disease. BMJ. 1989a;298:564-567.

Barker DJ, Winter PD, Osmond C, Margetts B, Simmonds SJ. Weight in infancy and death from ischaemic heart disease. Lancet. 1989b;2:577-580.
Becker DJ, Kilgore ML, Morrisey MA. The societal burden of osteoporosis. Curr Rheumatol Rep. 2010;12:186-191.
Bellinger DL, Lubahn C, Lorton D. Maternal and early life stress effects on immune function: relevance to immunotoxicology. J Immunotoxicol. 2008;5:419-444.
Bergman K, Glover V, Sarkar P, Abbott DH, O'Connor TG. In utero cortisol and testosterone exposure and fear reactivity in infancy. Horm Behav. 2010;57:306-312.
Berkowitz GS, Wolff MS, Janevic TM, Holzman IR, Yehuda R, Landrigan PJ.The World Trade Center disaster and intrauterine growth restriction. JAMA. 2003;290:595-596.
Bikle DD, Genant HK, Cann C, Recker RR, Halloran BP, Strewler GJ. Bone disease in alcohol abuse. Ann Intern Med. 1985;103:42-48.
Bioquant Image Analysis Corporation. 5611 Ohio Avenue, Nashville, TN. Bone histomorphomery manual (http://osteo.bioquant.com/home/brightfield-rodent-trabecular).
Black LJ, Sato M, Rowley ER, Magee DE, Bekele A, Williams DC, Cullinan GJ, Bendele R, Kauffman RF, Bensch WR, et al. Raloxifene (LY139481 HCI) prevents bone loss and reduces serum cholesterol without causing uterine hypertrophy in ovariectomized rats. J Clin Invest. 1994;93:63-69.
Blazkova S, Vytrisalova M, Palicka V, Stepan J, Byma S, Kubena AA, Hala T, Vlcek J. Osteoporosis risk assessment and management in primary care: focus on quantity and quality. J Eval Clin Pract. 2010 Jun 10. [Epub ahead of print].
Bohatyrewicz A. [The effect of sodium fluoride on selected biochemical markers of bone turnover in ovariectomized rats] Pol Merkur Lekarski. 1998;5:192-194.
Böhme K, Conscience-Egli M, Tschan T, Winterhalter KH, Bruckner P. Induction of proliferation or hypertrophy of chondrocytes in serum-free culture: the role of insulin-like growth factor-I, insulin, or thyroxine. J Cell Biol. 1992;116:1035-1042.
Börjesson A, Lagerquist M, Liu C, Shao R, Windahl S, Karlsson C, Sjögren K, Movérare-Skrtic S, Antal M, Krust A, Mohan S, Chambon P, Sävendahl L, Ohlsson C. The role of estrogen receptor-alpha in growth plate cartilage for longitudinal bone growth. J Bone Miner Res. 2010 Jun 18. [Epub ahead of print].
Boufous S, Finch CF, Lord SR. Incidence of hip fracture in New South Wales: are our efforts having an effect? Med J Aust. 2004;180:623-626.
Boyd SK, Moser S, Kuhn M, Klinck RJ, Krauze PL, Müller R, Gasser JA. Evaluation of three-dimensional image registration methodologies for in vivo micro-computed tomography. Ann Biomed Eng. 2006;34:1587-1599.
Boyle WJ, Simonet WS, Lacey DL. Osteoclast differentiation and activation. Nature. 2003;423:337-342.
Braunwald E, Fauci AS, Kasper DL, Hauser SL, Longo DL, Jameson JL. Harrison's Principles of Internal Medicine. 15th ed. New York: McGraw-Hill; 2001.
Breen SA, Loveday BE, Millest AJ, Waterton JC. Stimulation and inhibition of bone formation: use of peripheral quantitative computed tomography in the mouse in vivo. Lab Anim. 1998;32:467-476.
Buitelaar JK, Huizink AC, Mulder EJ, de Medina PG, Visser GH. Prenatal stress and cognitive development and temperament in infants. Neurobiol Aging. 2003;24 (Suppl 1):S53-60; discussion S67-S68.

Burch WM, Lebovitz HE. Triiodothyronine stimulates maturation of porcine growth-plate cartilage in vitro. J Clin Invest. 1982;70:496-504.
Burstein AH, Reilly DT, Martens M. Aging of bone tissue: mechanical properties. J Bone Joint Surg Am. 1976;58:82-86.
Bygren LO, Edvinsson S, Broström G. Change in food availability during pregnancy: Is it related to adult sudden death from cerebro- and cardiovascular disease in offspring? Am J Hum Biol. 2000;12:447-453.
Cameron DA. The fine structure of osteoblasts in the metaphysis of the tibia of the young rat. J Biophys Biochem Cytol. 1961;9:583-595.
Campbell GM, Ominsky MS, Boyd SK. Bone quality is partially recovered after the discontinuation of RANKL administration in rats by increased bone mass on existing trabeculae: an in vivo micro-CT study. Osteoporos Int. 2010 May 18. [Epub ahead of print].
Canalis E. Mechanisms of glucocorticoid action in bone. Curr Osteoporos Rep. 2005;3:98-102.
Canalis E. Update in new anabolic therapies for osteoporosis. J Clin Endocrinol Metab. 2010;95:1496-1504.
Canalis E, Bilezikian JP, Angeli A, Giustina A. Perspectives on glucocorticoid-induced osteoporosis. Bone. 2004;34:593-598.
Canalis E, Delany AM. Mechanisms of glucocorticoid action in bone. Ann N Y Acad Sci. 2002;966:73-81.
Carter DR, Beaupre GS. In: Skeletal function and form; mechanobiology of skeletal development, aging and regeneration. New York: Cambridge University Press; 2001.
Challis JR, Sloboda D, Matthews SG, Holloway A, Alfaidy N, Patel FA, Whittle W, Fraser M, Moss TJ, Newnham J. The fetal placental hypothalamic-pituitary-adrenal (HPA) axis, parturition and post natal health. Mol Cell Endocrinol. 2001;18:135-144.
Charles River Laboratories International, Inc. 251 Ballardvale Street, Wilmington, MA (http://www.criver.com/SiteCollectionDocuments/rm_rm_d_pregnant_rodent.pdf).
Cizza G, Ravn P, Chrousos GP, Gold PW. Depression: a major, unrecognized risk factor for osteoporosis? Trends Endocrinol Metab. 2001;12:198-203.
Clarke AS, Schneider ML. Prenatal stress has long-term effects on behavioral responses to stress in juvenile rhesus monkeys. Dev Psychobiol. 1993;26:293-30.
Coe CL, Crispen HR. Social stress in pregnant squirrel monkeys (Saimiri boliviensis peruviensis) differentially affects placental transfer of maternal antibody to male and female infants. Health Psychol. 2000;19:554-559.
Collard M, Wood B. Hominin homoiology: an assessment of the impact of phenotypic plasticity on phylogenetic analyses of humans and their fossil relatives. J Hum Evol. 2007;52:573-584.
Compston J. Bone quality: what is it and how is it measured? Arq Bras Endocrinol Metabol. 2006;50:579-585.
Compston JE. Sex steroids and bone. Physiol Rev. 2001;81:419-447.
Cooper C, Campion G, Melton LJ 3rd. Hip fractures in the elderly: a world-wide projection. Osteoporos Int. 1992;2:285-289.
Cooper C, Harvey N, Cole Z, Hanson M, Dennison E. Developmental origins of osteoporosis: the role of maternal nutrition. Adv Exp Med Biol. 2009;646:31-39.
Cooper C, Westlake S, Harvey N, Javaid K, Dennison E, Hanson M. Developmental origins of osteoporotic fracture. Osteoporos Int. 2006;17:337-347.
Cottrell EC, Seckl JR. Prenatal stress, glucocorticoids and the programming of adult disease.

Front Behav Neurosci. 2009;3:19.
Cummings SR. Evaluating the benefits and risks of postmenopausal hormone therapy. Am J Med. 1991;9:14-18.
Cutler GB Jr. The role of estrogen in bone growth and maturation during childhood and adolescence. J Steroid Biochem Mol Biol. 1997;61:141-144.
Damjanovic SS, Stojic RV, Lalic NM, Jotic AZ, Macut DP, Ognjanovic SI, Petakov MS, Popovic BM. Relationship between basal metabolic rate and cortisol secretion throughout pregnancy. Endocrine. 2009;35:262-268.
Davis EP, Glynn LM, Schetter CD, Hobel C, Chicz-Demet A, Sandman CA. Prenatal exposure to maternal depression and cortisol influences infant temperament. J Am Acad Child Adolesc Psychiatry. 2007;46:737-46.
De Blasio MJ, Dodic M, Jefferies AJ, Moritz KM, Wintour EM, Owens JA. Maternal exposure to dexamethasone or cortisol in early pregnancy differentially alters insulin secretion and glucose homeostasis in adult male sheep offspring. Am J Physiol Endocrinol Metab. 2007;293:75-82.
De Bruijn AT, van Bakel HJ, Wijnen H, Pop VJ, van Baar AL. Prenatal maternal emotional complaints are associated with cortisol responses in toddler and preschool aged girls. Dev Psychobiol. 2009;51:553-563.
De Kloet ER, Joëls M, Holsboer F. Stress and the brain: from adaptation to disease. Nat Rev Neurosci. 2005;6:463-475.
Delaere F, Magnan C, Mithieux G. Hypothalamic integration of portal glucose signals and control of food intake and insulin sensitivity. Diabetes Metab. 2010 Jun 17. [Epub ahead of print].
Delany AM, Durant D, Canalis E. Glucocorticoid suppression of IGF I transcription in osteoblasts. Mol Endocrinol. 2001;15:1781-1789.
De Los Rios P, Hill DJ. Expression and release of insulin-like growth factor binding proteins in isolated epiphyseal growth plate chondrocytes from the ovine fetus. J Cell Physiol. 2000;183:172-181.
Deminière JM, Piazza PV, Guegan G, Abrous N, Maccari S, Le Moal M, Simon H. Increased locomotor response to novelty and propensity to intravenous amphetamine self-administration in adult offspring of stressed mothers. Brain Res. 1992;586:135-139.
Dennison E, Cole Z, Cooper C. Diagnosis and epidemiology of osteoporosis. Curr Opin Rheumatol. 2005;17:456-461.
Dennison E, Fall C, Cooper C, Barker D. Prenatal factors influencing long-term outcome. Horm Res. 1997; 48 (Suppl 1):S25-S29.
DeSantis DT, Schmaltz LW. The mother-litter relationship in developmental rat studies: cannibalism vs caring. Dev Psychobiol. 1984;17:255-262.
De Weerth C, van Hees Y, Buitelaar JK. Prenatal maternal cortisol levels and infant behavior during the first 5 months. Early Hum Dev. 2003;74:139-51.
D'mello AP, Liu Y. Effects of maternal immobilization stress on birth weight and glucose homeostasis in the offspring. Psychoneuroendocrinology. 2006;31:395-406.
Doğan M, Derman O, Oksüz-Kanbur N, Akgül S, Kutluk T. The effects of nutrition and physical activity on bone development in male adolescents. Turk J Pediatr. 2009;51:545-550.
Dole N, Savitz DA, Hertz-Picciotto I, Siega-Riz AM, McMahon MJ, Buekens P. Maternal stress and preterm birth. Am J Epidemiol. 2003;157:14-24.

Ducy P. Cbfa1: a molecular switch in osteoblast biology. Dev Dyn. 2000;219:461-471.
Ducy P, Schinke T, Karsenty G. The osteoblast: a sophisticated fibroblast under central surveillance. Science. 2000;289:1501-1504.
Emtage JS, Lawson EM, Kodicek E. The response of the small intestine to vitamin D. Correlation between calcium-binding-protein production and increased calcium absorption. Biochem J. 1974;144:339-346.
Engelke K, Adams JE, Armbrecht G, Augat P, Bogado CE, Bouxsein ML, Felsenberg D, Ito M, Prevrhal S, Hans DB, Lewiecki EM. Clinical use of quantitative computed tomography and peripheral quantitative computed tomography in the management of osteoporosis in adults: the 2007 ISCD Official Positions. J Clin Densitom. 2008 Jan-Mar;11(1):123-62.

Ensrud KE, Cauley J, Lipschutz R, Cummings SR. Weight change and fractures in older women. Study of Osteoporotic Fractures Research Group. Arch Intern Med. 1997;157:857-863.
Erben RG. Trabecular and endocortical bone surfaces in the rat: modeling or remodeling? Anat Rec. 1996;246:39-46.
Erben RG, Scutt AM, Miao D, Kollenkirchen U, Haberey M. Short-term treatment of rats with high dose 1,25-dihydroxyvitamin D3 stimulates bone formation and increases the number of osteoblast precursor cells in bone marrow. Endocrinology. 1997;138:4629-4635.
Eskandari F, Martinez PE, Torvik S, Phillips TM, Sternberg EM, Mistry S, Ronsaville D, Wesley R, Toomey C, Sebring NG, Reynolds JC, Blackman MR, Calis KA, Gold PW, Cizza G; Premenopausal, Osteoporosis Women, Alendronate, Depression (POWER) Study Group. Low bone mass in premenopausal women with depression. Arch Intern Med. 2007;167:2329-2336.
Fazzalari NL. Bone remodeling: A review of the bone microenvironment perspective for fragility fracture (osteoporosis) of the hip. Semin Cell Dev Biol. 2008;19:467-472.
Ferretti M, Bertoni L, Cavani F, Zavatti M, Resca E, Carnevale G, Benelli A, Zanoli P, Palumbo C. Influence of ferutinin on bone metabolism in ovariectomized rats. II: role in recovering osteoporosis. J Anat. 2010;217:48-56.
Field T, Diego M. Cortisol: The Culprit Prenatal Stress Variable. Int J Neurosci. 2008;118:1181-1205.
Field T, Diego M, Hernandez-Reif M. Prenatal depression effects on the fetus and newborn: a review. Infant Behav Dev. 2006;29:445-55.
Finsen V, Johnsen LG, Tranø G, Hansen B, Sneve KS. Hip fracture incidence in central norway: a followup study. Clin Orthop Relat Res. 2004;419:173-178.
Follis RH Jr. Effect of cortisone on growing bones of the rat. Proc Soc Exp Biol Med. 1951;76:722-724.
Fonseca ES, Massoco CO, Palermo-Neto J. Effects of prenatal stress on stress-induced changes in behavior and macrophage activity of mice. Physiol Behav. 2002;77:205-215.
Frenkel B, Hong A, Baniwal SK, Coetzee GA, Ohlsson C, Khalid O, Gabet Y. Regulation of adult bone turnover by sex steroids. J Cell Physiol. 2010;224:305-310.
Frost HM. Skeletal structural adaptations to mechanical usage (SATMU): 1. Redefining Wolff's law: the bone modeling problem. Anat Rec. 1990;226:403-413.
Frost HM. The role of changes in mechanical usage set points in the pathogenesis of osteoporosis. J Bone Miner Res. 1992;7:253-261.
Frost HM, Stratmeyer ME. In-vivo effects of diagnostic ultrasound. Lancet. 1977;1:999.

Fusby JS, Kassmeier MD, Palmer VL, Perry GA, Anderson DK, Hackfort BT, Alvarez GK, Cullen DM, Akhter MP, Swanson PC. Cigarette smoke-induced effects on bone marrow B-cell subsets and CD4(+):CD8(+) T-cell ratios are reversed by smoking cessation: Influence of bone mass on immune cell response to and recovery from smoke exposure. Inhal Toxicol. 2010 May 20. [Epub ahead of print].

Gallagher A, Chambers TJ, Tobias JH. The estrogen antagonist ICI 182,780 reduces cancellous bone volume in female rats. Endocrinology. 1993;133:2787-2791.

Gallagher JC, Riggs BL, Eisman J, Hamstra A, Arnaud SB, DeLuca HF. Intestinal calcium absorption and serum vitamin D metabolites in normal subjects and osteoporotic patients: effect of age and dietary calcium. J Clin Invest. 1979;64:729-736.

Gasser JA, Green JR, Shen V, Ingold P, Rebmann A, Bhatnagar AS, Evans DB. A single intravenous administration of zoledronic acid prevents the bone loss and mechanical compromise induced by aromatase inhibition in rats. Bone. 2006;39:787-795.

Geiser M, Trueta J. Muscle action, bone rarefaction and bone formation; an experimental study. J Bone Joint Surg Br. 1958;40:282-311.

Genant HK, Jiang Y. Advanced imaging assessment of bone quality. Ann N Y Acad Sci. 2006;1068:410-428.

Gibbs RA, Weinstock GM, Metzker ML, Muzny DM, Sodergren EJ, Scherer S, Scott G, Steffen D, Worley KC, Burch PE, Okwuonu G, Hines S, Lewis L, DeRamo C, Delgado O, Dugan-Rocha S, Miner G, Morgan M, Hawes A, Gill R, Celera, Holt RA, Adams MD, Amanatides PG, Baden-Tillson H, Barnstead M, Chin S, Evans CA, Ferriera S, Fosler C, Glodek A, Gu Z, Jennings D, Kraft CL, Nguyen T, Pfannkoch CM, Sitter C, Sutton GG, Venter JC, Woodage T, Smith D, Lee HM, Gustafson E, Cahill P, Kana A, Doucette-Stamm L, Weinstock K, Fechtel K, Weiss RB, Dunn DM, Green ED, Blakesley RW, Bouffard GG, De Jong PJ, Osoegawa K, Zhu B, Marra M, Schein J, Bosdet I, Fjell C, Jones S, Krzywinski M, Mathewson C, Siddiqui A, Wye N, McPherson J, Zhao S, Fraser CM, Shetty J, Shatsman S, Geer K, Chen Y, Abramzon S, Nierman WC, Havlak PH, Chen R, Durbin KJ, Egan A, Ren Y, Song XZ, Li B, Liu Y, Qin X, Cawley S, Worley KC, Cooney AJ, D'Souza LM, Martin K, Wu JQ, Gonzalez-Garay ML, Jackson AR, Kalafus KJ, McLeod MP, Milosavljevic A, Virk D, Volkov A, Wheeler DA, Zhang Z, Bailey JA, Eichler EE, Tuzun E, Birney E, Mongin E, Ureta-Vidal A, Woodwark C, Zdobnov E, Bork P, Suyama M, Torrents D, Alexandersson M, Trask BJ, Young JM, Huang H, Wang H, Xing H, Daniels S, Gietzen D, Schmidt J, Stevens K, Vitt U, Wingrove J, Camara F, Mar Albà M, Abril JF, Guigo R, Smit A, Dubchak I, Rubin EM, Couronne O, Poliakov A, Hübner N, Ganten D, Goesele C, Hummel O, Kreitler T, Lee YA, Monti J, Schulz H, Zimdahl H, Himmelbauer H, Lehrach H, Jacob HJ, Bromberg S, Gullings-Handley J, Jensen-Seaman MI, Kwitek AE, Lazar J, Pasko D, Tonellato PJ, Twigger S, Ponting CP, Duarte JM, Rice S, Goodstadt L, Beatson SA, Emes RD, Winter EE, Webber C, Brandt P, Nyakatura G, Adetobi M, Chiaromonte F, Elnitski L, Eswara P, Hardison RC, Hou M, Kolbe D, Makova K, Miller W, Nekrutenko A, Riemer C, Schwartz S, Taylor J, Yang S, Zhang Y, Lindpaintner K, Andrews TD, Caccamo M, Clamp M, Clarke L, Curwen V, Durbin R, Eyras E, Searle SM, Cooper GM, Batzoglou S, Brudno M, Sidow A, Stone EA, Venter JC, Payseur BA, Bourque G, López-Otín C, Puente XS, Chakrabarti K, Chatterji S, Dewey C, Pachter L, Bray N, Yap VB, Caspi A, Tesler G, Pevzner PA, Haussler D, Roskin KM, Baertsch R, Clawson H, Furey TS, Hinrichs AS, Karolchik D, Kent WJ, Rosenbloom KR, Trumbower H, Weirauch M, Cooper DN, Stenson PD, Ma B, Brent M, Arumugam M,

Shteynberg D, Copley RR, Taylor MS, Riethman H, Mudunuri U, Peterson J, Guyer M, Felsenfeld A, Old S, Mockrin S, Collins F; Rat Genome Sequencing Project Consortium. Genome sequence of the Brown Norway rat yields insights into mammalian evolution. Nature. 2004;428:493-521.
Gitau R, Cameron A, Fisk NM, Glover V. Fetal exposure to maternal cortisol. Lancet. 1998;352:707-708.
Giustina A, Wehrenberg WB. The role of glucocorticoids in the regulation of Growth Hormone secretion: mechanisms and clinical significance. Trends Endocrinol Metab. 1992;3:306-311.
Glover V, Teixeira J, Gitau R, Fisk NM. Mechanisms by which maternal mood in pregnancy may affect the fetus. Contemporary Reviews in Obstetrics and Gynecology. 1999;11:155-160.
Gluckman PD, Beedle A, Hanson M. Principles of evolutionary medicine. New York : Oxford University Press; 2009.
Gluckman PD, Hanson MA. Developmental plasticity and human disease: research directions. J Intern Med. 2007;261:461-471.
Gluckman PD, Hanson MA, Pinal C. The developmental origins of adult disease. Matern Child Nutr. 2005;1:130-141.
Glynn LM, Wadhwa PD, Dunkel-Schetter C, Chicz-Demet A, Sandman CA. When stress happens matters: effects of earthquake timing on stress responsivity in pregnancy. Am J Obstet Gynecol. 2001;184:637-642.
Godoy R, Tanner S, Reyes-García V, Leonard WR, McDade TW, Vento M, Broesch J, Fitzpatrick IC, Giovannini P, Huanca T; Naveen Jha Bolivian TAPS Study Team. The effect of rainfall during gestation and early childhood on adult height in a foraging and horticultural society of the Bolivian Amazon. Am J Hum Biol. 2008;20:23-34.
Gold DT. The clinical impact of vertebral fractures: quality of life in women with osteoporosis. Bone. 1996;18(Suppl 3):S185-S189.
Goldring MB, Tsuchimochi K, Ijiri K. The control of chondrogenesis. J Cell Biochem. 2006;97:33-44.
Goulding A, Gold E. A new way to induce oestrogen-deficiency osteopaenia in the rat: comparison of the effects of surgical ovariectomy and administration of the LHRH agonist buserelin on bone resorption and composition. J Endocrinol. 1989;121:293-298.
Götz AA, Stefanski V. Psychosocial maternal stress during pregnancy affects serum corticosterone, blood immune parameters and anxiety behaviour in adult male rat offspring. Physiol Behav. 2007;90:108-115.
Grados F, Marcelli C, Dargent-Molina P, Roux C, Vergnol JF, Meunier PJ, Fardellone P. Prevalence of vertebral fractures in French women older than 75 years from the EPIDOS study. Bone. 2004;34:362-367.
Grajeda R, Pérez-Escamilla R. Stress during labor and delivery is associated with delayed onset of lactation among urban Guatemalan women. J Nutr. 2002;132:3055-3060.
Griffith JF, Wang YX, Zhou H, Kwong WH, Wong WT, Sun YL, Huang Y, Yeung DK, Qin L, Ahuja AT. Reduced bone perfusion in osteoporosis: likely causes in an ovariectomy rat model. Radiology. 2010;254:739-746.
Grimm VE. Effect of teratogenic exposure on the developing brain: research strategies and possible mechanisms. Dev Pharmacol Ther. 1987;10:328-345.
Hadley ME, Levine JE. 6th ed. Upper Saddle River, NJ: Pearson Prentice Hall; 2007.
Hales CN, Barker DJ, Clark PM, Cox LJ, Fall C, Osmond C, Winter PD. Fetal and infant growth

and impaired glucose tolerance at age 64. BMJ. 1991;303:1019-1022.
Hales CN, Barker DJ. Type 2 (non-insulin-dependent) diabetes mellitus: The thrifty phenotype hypothesis. Diabetologia. 1992;35:595-601.
Hans D, Krieg MA. Quantitative ultrasound for the detection and management of osteoporosis. Salud Publica Mex. 2009;51(Suppl 1):S25-S37.
Havill LM. Heritability of lumbar trabecular bone mechanical properties in baboons. Musculoskelet Neuronal Interact. 2007;7:316-317.
Havill LM, Mahaney MC, L Binkley T, Specker BL. Effects of genes, sex, age, and activity on BMC, bone size, and areal and volumetric BMD. J Bone Miner Res. 2007;22:737-746.
Hildreth BE 3rd, Werbeck JL, Thudi NK, Deng X, Rosol TJ, Toribio RE. PTHrP 1-141 and 1-86 increase in vitro bone formation. J Surg Res. 2010;162:9-17.
Hoekman K, Löwik CW, vd Ruit M, Bijvoet OL, Verheijen JH, Papapoulos SE. Regulation of the production of plasminogen activators by bone resorption enhancing and inhibiting factors in three types of osteoblast-like cells. Bone Miner. 1991;14:189-204.
Hornby SB, Evans GP, Hornby SL, Pataki A, Glatt M, Green JR. Long-term zoledronic acid treatment increases bone structure and mechanical strength of long bones of ovariectomized adult rats. Calcif Tissue Int. 2003;72:519-527.
Hosking D, Alonso CG, Brandi ML. Management of osteoporosis with PTH: treatment and prescription patterns in Europe. Curr Med Res Opin. 2009;25:263-270.
Howell DS, Dean DD. The biology, chemistry and biochemistry of the mammalian growth plate. In: Coe FL, Favis MJ, editors. Disorders of bone and mineral metabolism. New York: Raven Press; 1992. p. 313-353.
Huizink AC, Robles de Medina PG, Mulder EJ, Visser GH, Buitelaar JK. Stress during pregnancy is associated with developmental outcome in infancy. J Child Psychol Psychiatry. 2003;44:810-818.
Ilias I, Alesci S, Gold PW, Chrousos GP. Depression and osteoporosis in men: association or casual link? Hormones (Athens). 2006;5:9-16.
Iwaniec UT, Wronski TJ, Turner RT. Histological analysis of bone. Methods Mol Biol. 2008;447:325-341.
Jacobs HS. Hormone replacement therapy and breast cancer. Endocr Relat Cancer. 2000;7:53-61.
Jameson LJ. Harrison's Endocrinology. Volume 323. New York: McGraw-Hill Medical Pub; 2006.
Jee WS, Frost HM. Skeletal adaptations during growth. Triangle. 1992;31:77-88.
Jee WS, Park HZ, Roberts WE, Kenner GH. Corticosteroid and bone. Am J Anat. 1970;129:477-479.
Jee WS, Yao W. Overview: animal models of osteopenia and osteoporosis. J Musculoskelet Neuronal Interact. 2001;1:193-207.
Jiang Y, Zhao J, Liao EY, Dai RC, Wu XP, Genant HK. Application of micro-CT assessment of 3-D bone microstructure in preclinical and clinical studies. J Bone Miner Metab. 2005;23:S122-S131.
JMP, Version 8. SAS Institute Inc., Cary, NC, 2008.
Jones RE, Lopez KH. Human reproductive biology. Burlington, MA : Elsevier Academic Press; 2006.
Jordan KM, Cooper C. Epidemiology of osteoporosis. Best Pract Res Clin Rheumatol.

2002;16:795-806.
Jørkov ML, Heinemeier J, Lynnerup N. The petrous bone--a new sampling site for identifying early dietary patterns in stable isotopic studies. Am J Phys Anthropol. 2009;138:199-209.
Judd HL, Meldrum DR, Deftos LJ, Henderson BE. Estrogen replacement therapy: indications and complications. Ann Intern Med. 1983;98:195-205.
Juul A. The effects of oestrogens on linear bone growth. Hum Reprod Update. 2001;7:303-313.
Kajantie E. Fetal origins of stress-related adult disease. Ann N Y Acad Sci. 2006;1083:11-27.
Kalkwarf HJ, Laor T, Bean JA. Fracture risk in children with a forearm injury is associated with volumetric bone density and cortical area (by peripheral QCT) and areal bone density (by DXA). Osteoporos Int. 2010 Jun 23. [Epub ahead of print].
Kalu DN. Evaluation of the pathogenesis of skeletal changes in ovariectomized rats. Endocrinology. 1984;115:507-512.
Kammerer CM, Dualan AA, Samollow PB, Périssé AR, Bauer RL, MacCluer JW, O'Leary DH, Mitchell BD. Bone mineral density, carotid artery intimal medial thickness, and the vitamin D receptor BsmI polymorphism in Mexican American women. Calcif Tissue Int. 2004;75:292-298.
Kanis JA. Assessment of fracture risk and its application to screening for postmenopausal osteoporosis: synopsis of a WHO report. WHO Study Group. Osteoporos Int. 1994;4:368-381.
Kanis JA. Diagnosis of osteoporosis and assessment of fracture risk. Lancet. 2002;359:1929-1936.
Kanis JA, Borgstrom F, De Laet C, Johansson H, Johnell O, Jonsson B, Oden A, Zethraeus N, Pfleger B, Khaltaev N. Assessment of fracture risk. Osteoporos Int. 2005;16:581-589.
Kanis JA, Melton LJ 3rd, Christiansen C, Johnston CC, Khaltaev N. The diagnosis of osteoporosis. J Bone Miner Res. 1994;9:1137-1141.
Kapoor A, Dunn E, Kostaki A, Andrews MH, Matthews SG. Fetal programming of hypothalamo-pituitary-adrenal function: prenatal stress and glucocorticoids. J Physiol. 2006;572:31-44.
Kapoor A, Kostaki A, Janus C, Matthews SG. The effects of prenatal stress on learning in adult offspring is dependent on the timing of the stressor. Behav Brain Res. 2009;197:144-149.
Karasik D, Cupples LA, Hannan MT, Kiel DP. Age, gender, and body mass effects on quantitative trait loci for bone mineral density: the Framingham Study. Bone. 2003;33:308-316.
Kavuncu V, Sahin S, Baydas G, Ilhan N, Ozercan I, Yasar A, Pekkutucu I, Ilhan N, Ozercan R. A comparison of estrogen and two different doses of calcitonin in ovariectomized rats. Yonsei Med J. 2003;44:508-516.
Kay G, Tarcic N, Poltyrev T, Weinstock M. Prenatal stress depresses immune function in rats. Physiol Behav. 1998;63:397-402.
Kayanja MM, Ferrara LA, Lieberman IH. Distribution of anterior cortical shear strain after a thoracic wedge compression fracture. Spine J. 2004;4:76-87.
Kemper HC, Twisk JW, van Mechelen W, Post GB, Roos JC, Lips P. A fifteen-year longitudinal study in young adults on the relation of physical activity and fitness with the development of the bone mass: The Amsterdam Growth And Health Longitudinal Study. Bone. 2000;27:847-853.
Khoo BC, Brown K, Cann C, Zhu K, Henzell S, Low V, Gustafsson S, Price RI, Prince RL.

Comparison of QCT-derived and DXA-derived areal bone mineral density and T scores. Osteoporos Int. 2009;20:1539-1545.
Kim MJ, Shim MS, Kim MK, Lee Y, Shin YG, Chung CH, Kwon SO. Effect of chronic alcohol ingestion on bone mineral density in males without liver cirrhosis. Korean J Intern Med. 2003;18:174-180.
Klein-Nulend J, Nijweide PJ, Burger EH. Osteocyte and bone structure. Curr Osteoporos Rep. 2003;1:5-10.
Klein SL, Rager DR. Prenatal stress alters immune function in the offspring of rats. Dev Psychobiol. 1995;28:321-336.
Koshihara M, Masuyama R, Uehara M, Suzuki K. Effect of dietary calcium: Phosphorus ratio on bone mineralization and intestinal calcium absorption in ovariectomized rats. Biofactors. 2004;22:39-42.
Koshihara M, Masuyama R, Uehara M, Suzuki K. Reduction in dietary calcium/phosphorus ratio reduces bone mass and strength in ovariectomized rats enhancing bone turnover. Biosci Biotechnol Biochem. 2005;69:1970-1973.
Krieg MA, Barkmann R, Gonnelli S, Stewart A, Bauer DC, Del Rio Barquero L, Kaufman JJ, Lorenc R, Miller PD, Olszynski WP, Poiana C, Schott AM, Lewiecki EM, Hans D. Quantitative ultrasound in the management of osteoporosis: the 2007 ISCD Official Positions. J Clin Densitom. 2008;11:163-187.
Kyle UG, Pichard C. The Dutch Famine of 1944-1945: a pathophysiological model of long-term consequences of wasting disease. Curr Opin Clin Nutr Metab Care. 2006;9:388-394.
Laitinen K, Lamberg-Allardt C, Tunninen R, Karonen SL, Ylikahri R, Välimäki M. Effects of 3 weeks' moderate alcohol intake on bone and mineral metabolism in normal men. Bone Miner. 1991;13:139-151.
Lane NE, Kumer J, Yao W, Breunig T, Wronski T, Modin G, Kinney JH. Basic fibroblast growth factor forms new trabeculae that physically connect with pre-existing trabeculae, and this new bone is maintained with an anti-resorptive agent and enhanced with an anabolic agent in an osteopenic rat model. Osteoporos Int. 2003;14:374-382.
Lane NE, Yao W. Glucocorticoid-induced bone fragility. Ann N Y Acad Sci. 2010;1192:81-83.
Lanes R, Boguszewski CL, Calzada R, Cassorla F, Fideleff H, Boquete H. Growth hormone deficiency: transition from adolescence to adulthood. Highlights from a Latin-American Serono Symposia International Foundation Conference. J Pediatr Endocrinol Metab. 2010;23:225-233.
Lanfranco F, Zirilli L, Baldi M, Pignatti E, Corneli G, Ghigo E, Aimaretti G, Carani C, Rochira V. A novel mutation in the human aromatase gene: insights on the relationship among serum estradiol, longitudinal growth and bone mineral density in an adult man under estrogen replacement treatment. Bone. 2008;43:628-635.
Langley SC, Jackson AA. Increased systolic blood pressure in adult rats induced by fetal exposure to maternal low protein diets. Clin Sci (Lond). 1994;86:217-222.
Lazinski MJ, Shea AK, Steiner M. Effects of maternal prenatal stress on offspring development: a commentary. Arch Womens Ment Health. 2008;11:363-375.
Lelovas PP, Xanthos TT, Thoma SE, Lyritis GP, Dontas IA. The laboratory rat as an animal model for osteoporosis research. Comp Med. 2008;58:424-430.
Lepschy M, Touma C, Hruby R, Palme R. Non-invasive measurement of adrenocortical activity in male and female rats. Lab Anim. 2007;41:372-387.

Leung E, Tasker SL, Atkinson L, Vaillancourt T, Schulkin J, Schmidt LA. Perceived maternal stress during pregnancy and its relation to infant stress reactivity at 2 days and 10 months of postnatal life. Clin Pediatr (Phila). 2010;49:158-165.
Lewiecki EM. Bone densitometry and vertebral fracture assessment. Curr Osteoporos Rep. 2010;8:123-130.
Lewis EM, Barnett JF Jr, Freshwater L, Hoberman AM, Christian MS. Sexual maturation data for Crl Sprague-Dawley rats: criteria and confounding factors. Drug Chem Toxicol. 2002;25:437-458.
Li J, Sarosi I, Yan XQ, Morony S, Capparelli C, Tan HL, McCabe S, Elliott R, Scully S, Van G, Kaufman S, Juan SC, Sun Y, Tarpley J, Martin L, Christensen K, McCabe J, Kostenuik P, Hsu H, Fletcher F, Dunstan CR, Lacey DL, Boyle WJ. RANK is the intrinsic hematopoietic cell surface receptor that controls osteoclastogenesis and regulation of bone mass and calcium metabolism. Proc Natl Acad Sci U S A. 2000;97:1566-1571.
Lindgren JU, Lindholm TS. Effect of 1-alpha-hydroxyvitamin D3 on osteoporosis in rats induced by oophorectomy. Calcif Tissue Int. 1979;27:161-164.
Lindgren U, DeLuca HF. Role of parathyroid hormone and 1,25-dihydroxyvitamin D3 in the development of osteopenia in oophorectomized rats. Calcif Tissue Int. 1982;34:510-514.
Liu Y, Porta A, Peng X, Gengaro K, Cunningham EB, Li H, Dominguez LA, Bellido T, Christakos S. Prevention of glucocorticoid-induced apoptosis in osteocytes and osteoblasts by calbindin-D28k. J Bone Miner Res. 2004;19:479-490.
Llorente E, Brito ML, Machado P, González MC. Effect of prenatal stress on the hormonal response to acute and chronic stress and on immune parameters in the offspring. J Physiol Biochem. 2002;58:143-149.
Lloyd T, Rollings N, Andon MB, Demers LM, Eggli DF, Kieselhorst K, Kulin H, Landis JR, Martel JK, Orr G, et al. Determinants of bone density in young women. I. Relationships among pubertal development, total body bone mass, and total body bone density in premenarchal females. J Clin Endocrinol Metab. 1992;75:383-387.
López Frías M, Llopis J, Montellano MA, Urbano G. Influence of gestation and hydrocortisone-acetate treatment on the nutritive utilization of protein. Nahrung. 1985;29:11-18.
Lumey LH. Decreased birthweights in infants after maternal in utero exposure to the Dutch famine of 1944-1945. Paediatr Perinat Epidemiol. 1992;6:240-253.
Luppen CA, Smith E, Spevak L, Boskey AL, Frenkel B. Bone morphogenetic protein-2 restores mineralization in glucocorticoid-inhibited MC3T3-E1 osteoblast cultures. J Bone Miner Res. 2003;18:1186-1197.
Macbeth AH, Luine VN. Changes in anxiety and cognition due to reproductive experience: a review of data from rodent and human mothers. Neurosci Biobehav Rev. 2010;34:452-467.
MacGillivray MH, Morishima A, Conte F, Grumbach M, Smith EP. Pediatric endocrinology update: an overview. The essential roles of estrogens in pubertal growth, epiphyseal fusion and bone turnover: lessons from mutations in the genes for aromatase and the estrogen receptor. Horm Res. 1998;49(Suppl 1):S2-S8.
Magiakou MA, Mastorakos G, Rabin D, Margioris AN, Dubbert B, Calogero AE, Tsigos C, Munson PJ, Chrousos GP. The maternal hypothalamic-pituitary-adrenal axis in the third trimester of human pregnancy. Clin Endocrinol (Oxf). 1996;44:419-428.

Malerba M, Bossoni S, Radaeli A, Mori E, Bonadonna S, Giustina A, Tantucci C. Growth hormone response to growth hormone-releasing hormone is reduced in adult asthmatic patients receiving long-term inhaled corticosteroid treatment. Chest. 2005;127:515-521.
Malik P, Gasser RW, Kemmler G, Moncayo R, Finkenstedt G, Kurz M, Fleischhacker WW. Low bone mineral density and impaired bone metabolism in young alcoholic patients without liver cirrhosis: a cross-sectional study. Alcohol Clin Exp Res. 2009;33:375-381.
Manelli F, Giustina A. Glucocorticoid-induced osteoporosis. Trends Endocrinol Metab. 2000;11:79-85.
Manolagas SC, Kousteni S, Jilka RL. Sex steroids and bone. Recent Prog Horm Res. 2002;57:385-409.
Marie PJ, Garba MT, Hott M, Miravet L. Effect of low doses of stable strontium on bone metabolism in rats. Miner Electrolyte Metab. 1985;11:5-13.
Marieb EN, Mallatt J, Wilhelm PB. Human Anatomy. San Francisco: Pearson/Benjamin Cummings; 2005.
Martin B. Aging and strength of bone as a structural material. Calcif Tissue Int. 1993;53 (Suppl 1):S39-S40.
Martin RB, Burr DB, Schaffler MB. Effects of age and sex on the amount and distribution of mineral in Eskimo tibiae. Am J Phys Anthropol. 1985;67:371-380.
Martin RB, Atkinson PJ. Age and sex-related changes in the structure and strength of the human femoral shaft. J Biomech. 1977;10:223-231.
Mastorakos G, Ilias I. Maternal and fetal hypothalamic-pituitary-adrenal axes during pregnancy and postpartum. Ann N Y Acad Sci. 2003;997:136-149.
Mateo JM, Cavigelli SA. A validation of extraction methods for noninvasive sampling of glucocorticoids in free-living ground squirrels. Physiol Biochem Zool. 2005;78:1069-1084.
Mazziotti G, Angeli A, Bilezikian JP, Canalis E, Giustina A. Glucocorticoid-induced osteoporosis: an update. Trends Endocrinol Metab. 2006 ;17:144-149.
McClung M. Use of highly potent bisphosphonates in the treatment of osteoporosis. Curr Osteoporos Rep. 2003;1:116-122.
McClung M. Bisphosphonates. Arq Bras Endocrinol Metabol. 2006 ;50:735-44.
Megías MV, López Frías M, Llopis J, Montellano MA, Urbano G. Effect of cortisol on some nutritional and biochemical parameters in pregnant rats and their foetuses. Comp Biochem Physiol A Comp Physiol. 1983;75:615-617.
Memon A, Pospula WM, Tantawy AY, Abdul-Ghafar S, Suresh A, Al-Rowaih A. Incidence of hip fracture in Kuwait. Int J Epidemiol. 1998;27:860-865.
Miralles-Flores C, Delgado-Baeza E. Histomorphometric analysis of the epiphyseal growth plate in rats after prenatal alcohol exposure. J Orthop Res. 1992;10:325-336.
Morton NE. The inheritance of human birth weight. Ann Hum Genet. 1955;20:125-134.
Mosekilde L, Mosekilde L. Normal vertebral body size and compressive strength: relations to age and to vertebral and iliac trabecular bone compressive strength. Bone. 1986;7:207-212.
Mouse Genome Sequencing Consortium, Waterston RH, Lindblad-Toh K, Birney E, Rogers J, Abril JF, Agarwal P, Agarwala R, Ainscough R, Alexandersson M, An P, Antonarakis SE, Attwood J, Baertsch R, Bailey J, Barlow K, Beck S, Berry E, Birren B, Bloom T, Bork P, Botcherby M, Bray N, Brent MR, Brown DG, Brown SD, Bult C, Burton J, Butler J, Campbell RD, Carninci P, Cawley S, Chiaromonte F, Chinwalla AT, Church DM, Clamp M, Clee C, Collins FS, Cook LL, Copley RR, Coulson A, Couronne O, Cuff J, Curwen V, Cutts

T, Daly M, David R, Davies J, Delehaunty KD, Deri J, Dermitzakis ET, Dewey C, Dickens NJ, Diekhans M, Dodge S, Dubchak I, Dunn DM, Eddy SR, Elnitski L, Emes RD, Eswara P, Eyras E, Felsenfeld A, Fewell GA, Flicek P, Foley K, Frankel WN, Fulton LA, Fulton RS, Furey TS, Gage D, Gibbs RA, Glusman G, Gnerre S, Goldman N, Goodstadt L, Grafham D, Graves TA, Green ED, Gregory S, Guigó R, Guyer M, Hardison RC, Haussler D, Hayashizaki Y, Hillier LW, Hinrichs A, Hlavina W, Holzer T, Hsu F, Hua A, Hubbard T, Hunt A, Jackson I, Jaffe DB, Johnson LS, Jones M, Jones TA, Joy A, Kamal M, Karlsson EK, Karolchik D, Kasprzyk A, Kawai J, Keibler E, Kells C, Kent WJ, Kirby A, Kolbe DL, Korf I, Kucherlapati RS, Kulbokas EJ, Kulp D, Landers T, Leger JP, Leonard S, Letunic I, Levine R, Li J, Li M, Lloyd C, Lucas S, Ma B, Maglott DR, Mardis ER, Matthews L, Mauceli E, Mayer JH, McCarthy M, McCombie WR, McLaren S, McLay K, McPherson JD, Meldrim J, Meredith B, Mesirov JP, Miller W, Miner TL, Mongin E, Montgomery KT, Morgan M, Mott R, Mullikin JC, Muzny DM, Nash WE, Nelson JO, Nhan MN, Nicol R, Ning Z, Nusbaum C, O'Connor MJ, Okazaki Y, Oliver K, Overton-Larty E, Pachter L, Parra G, Pepin KH, Peterson J, Pevzner P, Plumb R, Pohl CS, Poliakov A, Ponce TC, Ponting CP, Potter S, Quail M, Reymond A, Roe BA, Roskin KM, Rubin EM, Rust AG, Santos R, Sapojnikov V, Schultz B, Schultz J, Schwartz MS, Schwartz S, Scott C, Seaman S, Searle S, Sharpe T, Sheridan A, Shownkeen R, Sims S, Singer JB, Slater G, Smit A, Smith DR, Spencer B, Stabenau A, Stange-Thomann N, Sugnet C, Suyama M, Tesler G, Thompson J, Torrents D, Trevaskis E, Tromp J, Ucla C, Ureta-Vidal A, Vinson JP, Von Niederhausern AC, Wade CM, Wall M, Weber RJ, Weiss RB, Wendl MC, West AP, Wetterstrand K, Wheeler R, Whelan S, Wierzbowski J, Willey D, Williams S, Wilson RK, Winter E, Worley KC, Wyman D, Yang S, Yang SP, Zdobnov EM, Zody MC, Lander ES. Initial sequencing and comparative analysis of the mouse genome. Nature. 2002;420:520-562.

Murray CJ, Lopez AD. Global mortality, disability, and the contribution of risk factors: Global Burden of Disease Study. Lancet. 1997;349:1436-1442.

Naismith DJ, Morgan BL. The biphasic nature of protein metabolism during pregnancy in the rat. Br J Nutr. 1976;36:563-566.

Nakahama KI. Cellular communications in bone homeostasis and repair. Cell Mol Life Sci. 2010 Aug 8. [Epub ahead of print].

Nassr AC, Arena AC, Toledo FC, Bissacot DZ, Fernandez CD, Spinardi-Barbisan AL, Pires PW, Kempinas WG. Effects of gestational and lactational fenvalerate exposure on immune and reproductive systems of male rats. J Toxicol Environ Health A. 2010;73:952-964.

Nelson HD, Humphrey LL, Nygren P, Teutsch SM, Allan JD. Postmenopausal hormone replacement therapy: scientific review. JAMA. 2002;288:872-881.

Nepomnaschy PA, Welch KB, McConnell DS, Low BS, Strassmann BI, England BG. Cortisol levels and very early pregnancy loss in humans. Proc Natl Acad Sci U S A. 2006;103:3938-3942.

Neumann ID. Alterations in behavioral and neuroendocrine stress coping strategies in pregnant, parturient and lactating rats. Prog Brain Res. 2001;133:143-152.

Neumann ID, Johnstone HA, Hatzinger M, Liebsch G, Shipston M, Russell JA, Landgraf R, Douglas AJ. Attenuated neuroendocrine responses to emotional and physical stressors in pregnant rats involve adenohypophysial changes. J Physiol. 1998;508:289-300.

Njeh CF, Samat SB, Nightingale A, McNeil EA, Boivin CM. Radiation dose and in vitro precision in paediatric bone mineral density measurement using dual X-ray absorptiometry. Br J Radiol. 1997;70:719-727.
Noguchi M. [Changes in human serum corticosterone and cortisol during pregnancy, labor and delivery]. Nippon Sanka Fujinka Gakkai Zasshi. 1988;40:14-20.
North American Menopause Society. Management of osteoporosis in postmenopausal women: 2006 position statement of The North American Menopause Society. Menopause. 2006;13:340-367; quiz 368-369.
North American Menopause Society. Management of osteoporosis in postmenopausal women: 2010 position statement of The North American Menopause Society. Menopause. 2010;17:25-54; quiz 55-56.
Obel C, Hedegaard M, Henriksen TB, Secher NJ, Olsen J, Levine S. Stress and salivary cortisol during pregnancy. Psychoneuroendocrinology. 2005;30:647-656.
O'Brien CA, Jia D, Plotkin LI, Bellido T, Powers CC, Stewart SA, Manolagas SC, Weinstein RS. Glucocorticoids act directly on osteoblasts and osteocytes to induce their apoptosis and reduce bone formation and strength. Endocrinology. 2004;145:1835-1841.
Ogden JA. Radiology of postnatal skeletal development. IX. Proximal tibia and fibula. Skeletal Radiol. 1984;11:169-177.
Ogden JA, McCarthy SM. Radiology of postnatal skeletal development. VIII. Distal tibia and fibula. Skeletal Radiol. 1983;10:209-220.
Ohnaka K, Tanabe M, Kawate H, Nawata H, Takayanagi R. Glucocorticoid suppresses the canonical Wnt signal in cultured human osteoblasts. Biochem Biophys Res Commun. 2005;329:177-181.
Okumura H, Yamamuro T, Kasai R, Hayashi T, Tada K, Nishii Y. Effect of 1 alpha-hydroxyvitamin D3 on osteoporosis induced by immobilization combined with ovariectomy in rats. Bone. 1987;8:351-355.
Ondrak KS, Morgan DW. Physical activity, calcium intake and bone health in children and adolescents. Sports Med. 2007;37:587-600.
O'Regan D, Kenyon CJ, Seckl JR, Holmes MC. Prenatal dexamethasone 'programmes' hypotension, but stress-induced hypertension in adult offspring. J Endocrinol. 2008;196:343-352.
O'Regan D, Kenyon CJ, Seckl JR, Holmes MC. Environmental disturbance confounds prenatal glucocorticoid programming experiments in Wistar rats. Lab Anim. 2010;44:199-205.
Ornoy A. The effects of maternal hypercortisonism and hypervitaminosis D2 on fetal osteogenesis and ossification in rats. Teratology. 1971a;4:383-394.
Ornoy A. Transplacental effects of cortisone and vitamin D 2 on the osteogenesis and ossification of fetal long bones in rats. Isr J Med Sci. 1971b;7:540-543.
Ornoy A, Horowitz A. Postnatal effects of maternal hypercortisonism on skeletal development in newborn rats. Teratology. 1972;6:153-158.
Osmond C, Barker DJ, Winter PD, Fall CH, Simmonds SJ. Early growth and death from cardiovascular disease in women. BMJ. 1993;307:1519-1524.
Owsley DW, Jantz RL. Long bone lengths and gestational age distributions of post-contact period Arikara Indian perinatal infant skeletons. Am J Phys Anthropol. 1985;68:321-328.
Painter RC, De Rooij SR, Bossuyt PM, Osmond C, Barker DJ, Bleker OP, Roseboom TJ. A possible link between prenatal exposure to famine and breast cancer: a preliminary study. Am

J Hum Biol. 2006a;18:853-856.
Painter RC, de Rooij SR, Bossuyt PM, Phillips DI, Osmond C, Barker DJ, Bleker OP, Roseboom TJ. Blood pressure response to psychological stressors in adults after prenatal exposure to the Dutch famine. J Hypertens. 2006b;24:1771-1778.
Painter RC, Roseboom TJ, Bleker OP. Prenatal exposure to the Dutch famine and disease in later life: an overview. Reprod Toxicol. 2005;20:345-352.
Palermo Neto J, Massoco CO, Fávare RC. Effects of maternal stress on anxiety levels, macrophage activity, and Ehrlich tumor growth. Neurotoxicol Teratol. 2001;23:497-507.
Palumbo C, Ferretti M, Bertoni L, Cavani F, Resca E, Casolari B, Carnevale G, Zavatti M, Montanari C, Benelli A, Zanoli P. Influence of ferutinin on bone metabolism in ovariectomized rats. I: role in preventing osteoporosis. J Bone Miner Metab. 2009;27:538-545.
Parfitt AM. Age-related structural changes in trabecular and cortical bone: cellular mechanisms and biomechanical consequences. Calcif Tissue Int. 1984;36(Suppl 1):S123-S128.
Parfitt AM. The actions of parathyroid hormone on bone: relation to bone remodeling and turnover, calcium homeostasis, and metabolic bone diseases. II. PTH and bone cells: bone turnover and plasma calcium regulation. Metabolism. 1976;25:909-955.
Parfitt AM. Bone histomorphometry: standardization of nomenclature, symbols and units. Summary of proposed system. Bone Miner. 1988;4:1-5.
Parfitt AM. Osteonal and hemi-osteonal remodeling: the spatial and temporal framework for signal traffic in adult human bone. J Cell Biochem. 1994;55:273-286.
Parfitt AM. Bone remodeling, normal and abnormal: a biological basis for the understanding of cancer-related bone disease and its treatment. Can J Oncol. 1995;5(Suppl 1):S1-S10.
Parfitt AM, Drezner MK, Glorieux FH, Kanis JA, Malluche H, Meunier PJ, Ott SM, Recker RR. Bone histomorphometry: standardization of nomenclature, symbols, and units. Report of the ASBMR Histomorphometry Nomenclature Committee. J Bone Miner Res. 1987;2:595-610.
Pawluski JL, Charlier TD, Lieblich SE, Hammond GL, Galea LA. Reproductive experience alters corticosterone and CBG levels in the rat dam. Physiol Behav. 2009;96:108-114.
Pearson OM, Lieberman DE. The aging of Wolff's "law": ontogeny and responses to mechanical loading in cortical bone. Am J Phys Anthropol. 2004;(Suppl 39):S63-S99.
Peck WA. The effects of glucocorticoids on bone cell metabolism and function. Adv Exp Med Biol. 1984;171:111-119.
Peck WA, Brandt J, Miller I. Hydrocortisone-induced inhibition of protein synthesis and uridine incorporation in isolated bone cells in vitro. Proc Natl Acad Sci U S A. 1967;57:1599-1606.
Petersen MM, Nielsen PK, Lewin E, Olgaard K. Dual energy X-ray absorptiometry in small rats with low bone mineral density. Calcif Tissue Int. 2000;67:455-459.
Pinheiro MM, Ciconelli RM, Martini LA, Ferraz MB. Clinical risk factors for osteoporotic fractures in Brazilian women and men: the Brazilian Osteoporosis Study (BRAZOS). Osteoporos Int. 2009;20:399-408.
Pinheiro MM, dos Reis Neto ET, Machado FS, Omura F, Yang JH, Szejnfeld J, Szejnfeld VL. Risk factors for osteoporotic fractures and low bone density in pre and postmenopausal women. Rev Saude Publica. 2010;44:479-85.
Pratt NC, Lisk RD. Effects of social stress during early pregnancy on litter size and sex ratio in the golden hamster (Mesocricetus auratus). J Reprod Fertil. 1989;87:763-769.
Ralston SH. Genetic determinants of osteoporosis. Curr Opin Rheumatol. 2005;17:475-479.

Richards CD, Langdon C, Deschamps P, Pennica D, Shaughnessy SG. Stimulation of osteoclast differentiation in vitro by mouse oncostatin M, leukaemia inhibitory factor, cardiotrophin-1 and interleukin 6: synergy with dexamethasone. Cytokine. 2000;12:613-621.
Rima BN, Bardi M, Friedenberg JM, Christon LM, Karelina KE, Lambert KG, Kinsley CH. Reproductive experience and the response of female Sprague-Dawley rats to fear and stress. Comp Med. 2009;59:437-443.
Roseboom T, de Rooij S, Painter R. The Dutch famine and its long-term consequences for adult health. Early Hum Dev. 2006;82:485-491.
Roseboom TJ, van der Meulen JH, Ravelli AC, Osmond C, Barker DJ, Bleker OP. Effects of prenatal exposure to the Dutch famine on adult disease in later life: an overview. Mol Cell Endocrinol. 2001;185:93-98.
Ross MG, Desai M. Gestational programming: population survival effects of drought and famine during pregnancy. Am J Physiol Regul Integr Comp Physiol. 2005 Jan;288(1):R25-R33.
Rubin J, Ackert-Bicknell CL, Zhu L, Fan X, Murphy TC, Nanes MS, Marcus R, Holloway L, Beamer WG, Rosen CJ. IGF-I regulates osteoprotegerin (OPG) and receptor activator of nuclear factor-kappaB ligand in vitro and OPG in vivo. J Clin Endocrinol Metab. 2002;87:4273-4279.
Sampson HW, Perks N, Champney TH, DeFee B 2nd. Alcohol consumption inhibits bone growth and development in young actively growing rats. Alcohol Clin Exp Res. 1996;20:1375-1384.
Sapolsky RM. The influence of social hierarchy on primate health. Science. 2005;308:648-652.
Sarkar P, Bergman K, O'Connor TG, Glover V. Maternal antenatal anxiety and amniotic fluid cortisol and testosterone: possible implications for foetal programming. J Neuroendocrinol. 2008;20:489-496.
Saville PD. Changes in bone mass with age and alcoholism. J Bone Joint Surg Am. 1965;47:492-499.
Scanes CG, Lazarus D, Bowen S, Buonomo FC, Gilbreath RL. Postnatal changes in circulating concentrations of growth hormone, somatomedin C and thyroid hormones in pigs. Domest Anim Endocrinol. 1987;4:253-257.
Schneider ML, Roughton EC, Koehler AJ, Lubach GR. Growth and development following prenatal stress exposure in primates: an examination of ontogenetic vulnerability. Child Dev. 1999;70:263-74.
Schuit SC, de Jong FH, Stolk L, Koek WN, van Meurs JB, Schoofs MW, Zillikens MC, Hofman A, van Leeuwen JP, Pols HA, Uitterlinden AG. Estrogen receptor alpha gene polymorphisms are associated with estradiol levels in postmenopausal women. Eur J Endocrinol. 2005;153:327-334.
Seibel MJ. Molecular markers of bone turnover: biochemical, technical and analytical aspects. Osteoporos Int. 2000;11(Suppl 6):S18-S29.
Shalet SM, Rosenfeld RG. Growth hormone replacement therapy during transition of patients with childhood-onset growth hormone deficiency into adulthood: what are the issues? Growth Horm IGF Res. 1998;8(Suppl B):S177-S184.
Shen V, Birchman R, Liang XG, Wu DD, Lindsay R, Dempster DW. Prednisolone alone, or in combination with estrogen or dietary calcium deficiency or immobilization, inhibits bone formation but does not induce bone loss in mature rats. Bone. 1997;21:345-351.
Shen V, Birchman R, Xu R, Lindsay R, Dempster DW. Short-term changes in

histomorphometric and biochemical turnover markers and bone mineral density in estrogen-and/or dietary calcium-deficient rats. Bone. 1995;16:149-156.
Silberstein SD. Menstrual migraine. J Womens Health Gend Based Med. 1999;8:919-931.
Sissons HA, Hadfield GJ. The influence of cortisone on the structure and growth of bone. J Anat. 1955;89:69-78.
Smith E, Frenkel B. Glucocorticoids inhibit the transcriptional activity of LEF/TCF in differentiating osteoblasts in a glycogen synthase kinase-3beta-dependent and -independent manner. J Biol Chem. 2005;280:2388-2394.
Snow ME, Keiver K. Prenatal ethanol exposure disrupts the histological stages of fetal bone development. Bone. 2007;41:181-187.
Sobrian SK, Vaughn VT, Bloch EF, Burton LE. Influence of prenatal maternal stress on the immunocompetence of the offspring. Pharmacol Biochem Behav. 1992;43:537-547.
Søgaard CH, Wronski TJ, McOsker JE, Mosekilde L. The positive effect of parathyroid hormone on femoral neck bone strength in ovariectomized rats is more pronounced than that of estrogen or bisphosphonates. Endocrinology. 1994;134:650-657.
Spelsberg TC, Subramaniam M, Riggs BL, Khosla S. The actions and interactions of sex steroids and growth factors/cytokines on the skeleton. Mol Endocrinol. 1999;13:819-828.
StataCoRP. 2009. Stata; Release 11. Statistical software. College Station, TX: StataCorp LP.
Steinberg ME, Trueta J. Effects of activity on bone growth and development in the rat. Clin Orthop Relat Res. 1981;156:52-60.
Stöhr T, Schulte Wermeling D, Szuran T, Pliska V, Domeney A, Welzl H, Weiner I, Feldon J. Differential effects of prenatal stress in two inbred strains of rats. Pharmacol Biochem Behav. 1998;59:799-805.
Storey E. Bone changes associated with cortisone administration in the rat. Effect of variations in dietary calcium and phosporus. Br J Exp Pathol. 1960;41:207-213.
Styrkarsdottir U, Cazier JB, Kong A, Rolfsson O, Larsen H, Bjarnadottir E, Johannsdottir VD, Sigurdardottir MS, Bagger Y, Christiansen C, Reynisdottir I, Grant SF, Jonasson K, Frigge ML, Gulcher JR, Sigurdsson G, Stefansson K. Linkage of osteoporosis to chromosome 20p12 and association to BMP2. PLoS Biol. 2003;1:351-360.
Sun LW, Fan YB, Li DY, Zhao F, Xie T, Yang X, Gu ZT. Evaluation of the mechanical properties of rat bone under simulated microgravity using nanoindentation. Acta Biomater. 2009;5:3506-3511.
Sutter EG, Mears SC, Belkoff SM. A biomechanical evaluation of femoroplasty under simulated fall conditions. J Orthop Trauma. 2010;24:95-99.
Tägil M, McDonald MM, Morse A, Peacock L, Mikulec K, Amanat N, Godfrey C, Little DGIntermittent PTH((1-34)) does not increase union rates in open rat femoral fractures and exhibits attenuated anabolic effects compared to closed fractures. Bone. 2010;46:852-859.
Talge NM, Neal C, Glover V; Early Stress, Translational Research and Prevention Science Network: Fetal and Neonatal Experience on Child and Adolescent Mental Health. Antenatal maternal stress and long-term effects on child neurodevelopment: how and why? J Child Psychol Psychiatry. 2007;48:245-261.
Tami AE, Leitner MM, Baucke MG, Mueller TL, van Lenthe GH, Müller R, Ito K. Hydroxyapatite particles maintain peri-implant bone mantle during osseointegration in osteoporotic bone. Bone. 2009;45:1117-1124.
Taylor RN. The endocrinology of pregnancy. In: Greenspan FS, Baxter JD, editors. Basic and

Clinical Endocrinology. Prentice Hall. Hart-ford, CT; 2001. p. 575–602.
Thompson DD, Rodan GA. Indomethacin inhibition of tenotomy-induced bone resorption in rats. J Bone Miner Res. 1988;3:409-414.
Thornberry NA, Lazebnik Y. Caspases: enemies within. Science. 1998 Aug 28;281:1312-1316.
Touma C, Palme R, Sachser N. Analyzing corticosterone metabolites in fecal samples of mice: a noninvasive technique to monitor stress hormones. Horm Behav. 2004;45:10-22.
Touma C, Sachser N, Möstl E, Palme R. Effects of sex and time of day on metabolism and excretion of corticosterone in urine and feces of mice. Gen Comp Endocrinol. 2003;130:267-278.
Tsujio M, Mizorogi T, Kitamura I, Maeda Y, Nishijima K, Kuwahara S, Ohno T, Niida S, Nagaya M, Saito R, Tanaka S. Bone mineral analysis through dual energy X-ray absorptiometry in laboratory animals. J Vet Med Sci. 2009;71:1493-1497.
Tuchscherer M, Kanitz E, Otten W, Tuchscherer A. Effects of prenatal stress on cellular and humoral immune responses in neonatal pigs. Vet Immunol Immunopathol. 2002;86:195-203.
Turner RT, Aloia RC, Segel LD, Hannon KS, Bell NH. Chronic alcohol treatment results in disturbed vitamin D metabolism and skeletal abnormalities in rats. Alcohol Clin Exp Res. 1988;12:159-162.
Turner RT, Maran A, Lotinun S, Hefferan T, Evans GL, Zhang M, Sibonga JD. Animal models for osteoporosis. Rev Endocr Metab Disord. 2001;2:117-127.
Turner RT, Vandersteenhoven JJ, Bell NH. The effects of ovariectomy and 17 beta-estradiol on cortical bone histomorphometry in growing rats. J Bone Miner Res. 1987;2:115-122.
University of California, Los Angeles academic technology services, statistical computing available at http://www.ats.ucla.edu/stat/stata/library/cpsu.htm.
Valassi E, Scacchi M, Cavagnini F. Neuroendocrine control of food intake. Nutr Metab Cardiovasc Dis. 2008;18:158-168.
Vanderschueren D, Jans I, van Herck E, Moermans K, Verhaeghe J, Bouillon R. Time-related increase of biochemical markers of bone turnover in androgen-deficient male rats. Bone Miner. 1994;26:123-31.
Vestergaard P, Jorgensen NR, Schwarz P, Mosekilde L. Effects of treatment with fluoride on bone mineral density and fracture risk--a meta-analysis. Osteoporos Int. 2008;19:257-268.
Vickers MH, Breier BH, Cutfield WS, Hofman PL, Gluckman PD. Fetal origins of hyperphagia, obesity, and hypertension and postnatal amplification by hypercaloric nutrition. Am J Physiol Endocrinol Metab. 2000;279:83-87.
Vyas A, Mitra R, Shankaranarayana Rao BS, Chattarji S. Chronic stress induces contrasting patterns of dendritic remodeling in hippocampal and amygdaloid neurons. J Neurosci. 2002;22:6810-6818.
Wadhwa PD. Psychoneuroendocrine processes in human pregnancy influence fetal development and health. Psychoneuroendocrinology. 2005;30:724-743.
Wakshlak A, Weinstock M. Neonatal handling reverses behavioral abnormalities induced in rats by prenatal stress. Physiol Behav. 1990;48:289-292.
Walker-Bone K, Walter G, Cooper C. Recent developments in the epidemiology of osteoporosis. Curr Opin Rheumatol. 2002;14:411-415.
Walker MD, Novotny R, Bilezikian JP, Weaver CM. Race and diet interactions in the acquisition, maintenance, and loss of bone. J Nutr. 2008;138:S1256-S1260.
Watts NB. Bone quality: getting closer to a definition. J Bone Miner Res. 2002;17:1148-1150.

Weinstein RS, Jilka RL, Parfitt AM, Manolagas SC. Inhibition of osteoblastogenesis and promotion of apoptosis of osteoblasts and osteocytes by glucocorticoids. Potential mechanisms of their deleterious effects on bone. J Clin Invest. 1998;102:274-282.
Weinstock M. Gender differences in the effects of prenatal stress on brain development and behaviour. Neurochem Res. 2007;32:1730-1740.
Weinstock M, Fride E, Hertzberg R. Prenatal stress effects on functional development of the offspring. Prog Brain Res. 1988;73:319-331.
Welten DC, Kemper HC, Post GB, Van Mechelen W, Twisk J, Lips P, Teule GJ. Weight-bearing activity during youth is a more important factor for peak bone mass than calcium intake. J Bone Miner Res. 1994;9:1089-1096.
Wengreen HJ, Munger RG, West NA, Cutler DR, Corcoran CD, Zhang J, Sassano NE. Dietary protein intake and risk of osteoporotic hip fracture in elderly residents of Utah. J Bone Miner Res. 2004;19:537-545.
White JR, Wilsman NJ, Leiferman EM, Noonan KJ. Histomorphometric analysis of an adolescent distal tibial physis prior to growth plate closure. J Child Orthop. 2008;2:315-319.
White TD. Human Osteology. 2nd ed. San Diego: Academic Press; 2000.
Wong PK, Christie JJ, Wark JD. The effects of smoking on bone health. Clin Sci (Lond). 2007;113:233-241.
Wronski TJ, Cintrón M, Dann LM. Temporal relationship between bone loss and increased bone turnover in ovariectomized rats. Calcif Tissue Int. 1988;43:179-183.
Wronski TJ, Dann LM, Horner SL. Time course of vertebral osteopenia in ovariectomized rats. Bone. 1989a;10:295-301.
Wronski TJ, Dann LM, Scott KS, Cintrón M. Long-term effects of ovariectomy and aging on the rat skeleton. Calcif Tissue Int. 1989b;45:360-366.
Yamaguchi A, Komori T, Suda T. Regulation of osteoblast differentiation mediated by bone morphogenetic proteins, hedgehogs, and Cbfa1. Endocr Rev. 2000;21:393-411.
Yan L, Zhou B, Prentice A, Wang X, Golden MH. Epidemiological study of hip fracture in Shenyang, People's Republic of China. Bone. 1999;24:151-155.
Yao W, Cheng Z, Pham A, Busse C, Zimmermann EA, Ritchie RO, Lane NE. Glucocorticoid-induced bone loss in mice can be reversed by the actions of parathyroid hormone and risedronate on different pathways for bone formation and mineralization. Arthritis Rheum. 2008;58:3485-3497.
Yao W, Cheng Z, Shahnazari M, Dai W, Johnson ML, Lane NE. Overexpression of secreted frizzled-related protein 1 inhibits bone formation and attenuates parathyroid hormone bone anabolic effects. J Bone Miner Res. 2010;25:190-199.
Yao W, Hadi T, Jiang Y, Lotz J, Wronski TJ, Lane NE. Basic fibroblast growth factor improves trabecular bone connectivity and bone strength in the lumbar vertebral body of osteopenic rats. Osteoporos Int. 2005;16:1939-1947.
Yirmiya R, Goshen I, Bajayo A, Kreisel T, Feldman S, Tam J, Trembovler V, Csernus V, Shohami E, Bab I. Depression induces bone loss through stimulation of the sympathetic nervous system. Proc Natl Acad Sci U S A. 2006;103:16876-16881.
Zeng QQ, Jee WS, Bigornia AE, King JG Jr, D'Souza SM, Li XJ, Ma YF, Wechter WJ. Time responses of cancellous and cortical bones to sciatic neurectomy in growing female rats. Bone. 1996;19:13-21.
Zorbas YG, Yaroshenko YY, Andreyev VG, Kuznetsov NK. Bone tissue changes in rats during

prolonged restriction of motor activity. Physiol Chem Phys Med NMR. 1998;30:219-228.