Diet enrichment with arachidonic and docosahexaenoic acid during the lactation period attenuates the effects of intrauterine growth restriction from birth to maturity in the guinea pig and improves maternal bone mass

Laura Lynn Burr

School of Dietetics and Human Nutrition
McGill University, Montreal

February 2008

A thesis submitted to McGill University in partial fulfillment of the requirements of the degree of

Masters of Science

Your file Votre référence
ISBN: 978-0-494-51074-2
Our file Notre référence
ISBN: 978-0-494-51074-2

Table of Contents **Page**

Abbreviations used in the text

AA	**Arachidonic acid**
AGA	**Appropriate for gestational age**
ALA	**Alpha-linolenic acid**
C	**Control (diet)**
BA	**Bone area**
BMC	**Bone mineral content**
BMD	**Bone mineral density**
DPD	**Deoxypyridinoline**
DHA	**Docosahexaenoic acid**
DXA	**Dual-energy x-ray absorptiometry**
ELISA	**Enzyme-linked immuosorbent assay**
EPA	**Eicosapentaenoic acid**
GLA	**Gamma-linolenic acid**
IGF-1	**Insulin-like growth factor 1**
IUGR	**Intrauterine growth restriction**
LA	**Linoleic acid**
LC PUFA	**Long-chain polyunsaturated fatty acid**
LP	**Low-protein (diet)**
LS	**Lumbar spine**
NF- κB	**Nuclear factor kappa-B**
OPG	**Osteoprotegerin**
PGE_2	**Prostaglandin E_2**
PPAR	**Peroxisome proliferator activator receptor**
PTH	**Parathyroid hormone**
PUFA	**Polyunsaturated fatty acid**
RANKL	**Receptor activator of NF-κB ligand**
RBC	**Red blood cell**
SGA	**Small for gestational age**
WB	**Whole body**

Acknowledgements

The author wishes to extend sincere thanks to the following individuals for their contributions to this thesis:

- Mr. Gordon Bingham for providing diligent and dedicated animal care.
- Dr. Thomas Brenna from Cornell University who graciously loaned cages for guinea pig housing.
- Dr. Atul Sharma for providing statistical assistance.
- Charles Viau and Karyn Tang for their assistance with animal care.
- Ms. Lise Grant and Ms. Francine Tardiff for administrative assistance.
- Drs. Sarah Kimmins and Grace Egeland, members of the thesis advisory committee, for providing time, advice and suggestions.
- Dr. Hope Weiler, supervisor, for providing advice and guidance.

Preamble to the thesis

This study was designed by both the candidate and thesis supervisor to build on previously made observations by H. Weiler's research team. The study was proposed as a longitudinal animal trial and this thesis represents part of the data collected and analyzed solely by the candidate and was deemed to be sufficient, original scholarship for a Masters thesis by the candidate's supervisory committee. Thus, additional data has been collected to build on the work published in this thesis.

Abstract

Intrauterine growth restriction (IUGR) reduces bone mass by 10-30% and impairs arachidonic (AA) and docosahexaenoic (DHA) acid status in infants. Because AA and DHA enhance neonatal bone mass, the aim of this study was to determine the effects of dietary 0.5% AA and 0.2% DHA (w/w) prior to weaning on bone and growth. 40 guinea pigs were randomized to either a control (C) or low-protein diet (LP) during pregnancy and the C diet or the C diet with AA+DHA during lactation. Measurements included bone mass, metabolism, and strength, and erythrocyte lipid of sows and offspring from birth to 16 wk post-partum. The LP diet induced IUGR, while the AA+DHA increased bone mass by 5-20% in sows and offspring and corrected growth and bone mass in IUGR pups. Thus, AA+DHA provided in lactation rescues the growth trajectory in an IUGR state and is beneficial to maternal and neonatal bone mass.

Résumé

Le retard de croissance intra-utérin (RCIU) réduit de 10 à 30% la masse osseuse et affecte la synthèse endogène des acides arachidonique (ARA) et docosahexanoïque (DHA) chez les nouveaux-nés. Étant donné l'importance de AA et de DHA pour la minéralisation des os au stade précoce de la vie, notre étude visa à déterminer l'effet sur la masse osseuse d'une consommation de 0.5% ARA et de 0.2% DHA (p/p) pendant la période précédant le sevrage.
40 cochons d'inde (truies) enceintes ont reçu au hasard soit le régime témoin ou le régime appauvri en protéine durant la grossesse et soit le régime témoin ou le même régime avec ARA+DHA pendant l'allaitement. La masse osseuse, le métabolisme et la solidité ainsi que les gras insaturés présent dans les globules rouges des truies et de leur progéniture ont été mesuré à la naissance, au 3eme jour, au 21eme jour, à la 8eme semaine et à la 16eme semaine. Le régime appauvri en protéine amène à IUGR, plus particulièrement chez les males, alors que le régime avec ARA+DHA augmente la masse osseuse par 5-20% chez les truies et ses petits et rétablit la croissance et la masse osseuse chez les petits ayant RCIU.

1.0 Chapter One
Introduction

1.1 Rationale

1.2 Objectives

1.3 Hypotheses

1.0 Introduction

1.1 Study Rationale

Intrauterine growth restriction (IUGR) refers to failure of a fetus to reach a genetically determined potential size *in utero* (1). An IUGR infant may be born small-for-gestational age (SGA) with a weight or crown-heel length 2 standard deviations below the mean for gestational age (2). A recent report by the Canadian Institute for Health Information states that due to an 8% increase in cesarean sections, usage of fertility drugs, and higher maternal age, the incidence of babies born SGA is increasing to 1 in every 15 live births (3). Health problems associated with IUGR may be present at birth, though there are many long-term consequences to health, one of which is reduced bone mass (4).

IUGR infants are reported to have 20-60% lower bone area (BA) and mineral content (BMC) at the lumbar spine (LS), humerus, and radius in comparison to healthy infants (5, 6). Whole body (WB) bone mass (BMC and bone mineral density (BMD)) is also 10-36% lower in infants born small than in appropriate for gestational age (AGA) (7, 8). These observations persist at 6 mo of age, when 15% lower WB BA and 26% lower WB BMC were reported in one study (9). During childhood, it is not clear whether or not bone remains compromised. Normal WB, LS, and femoral neck bone mass is reported at 8 y of age in children born SGA but 10% lower spine BMC and 12% higher osteocalcin concentrations in SGA females at 10 y of life is also reported (10, 11). However, it is apparent that in old age, birth weight is positively associated with BA and BMC at the LS, radius, and femoral neck (12, 13). Thus, bone that is compromised as a result of IUGR may predispose an individual to fracture or kyphosis later in life. Early life intervention that promotes recovery of bone size and mass is necessary to reverse the adverse effects of a nutritionally compromised *in utero* environment. Long-chain polyunsaturated fatty acid (LC PUFA) supplementation in the neonatal diet may be one way to achieve healthier bone across the lifespan.

Long-chain polyunsaturated fatty acids, most notably arachidonic (AA) and/or docosahexaenoic (DHA) acid elevate WB BMD and LS BMD by 5-20% in young, aged, and ovariectomized animal models (14-16). Calcium absorption has also been shown to be 20% greater in male weanling rats fed 1.25% (w/w) DHA for 6 wk (17). In addition to calcium absorption, diet enrichment with DHA has positive effects on bone quality by elevating femoral calcium content by 12%, femoral and LS BMD by 8%, and increasing femoral strength (17). In weanling pigs, providing AA and DHA in the diet in a 5:1 ratio for 21 d increased WB, femoral and LS BMD, and WB BMC (18). However, it is presently unknown whether AA and DHA have similar benefits in bone compromised by IUGR. The long-term effects of providing a short-term LC PUFA supplement in neonatal life are also unknown.

Based on the above research AA and DHA are promising candidates for a neonatal nutritional supplement to optimize bone mass, and additionally, have other important roles in neural and visual development (19). Both LC PUFA can be made endogenously and are provided in small amounts in breast milk (20). However, IUGR infants have lower rates of endogenous LC PUFA synthesis (21). In addition, present dietary intakes of AA and DHA in lactating women in North America suggest that the levels of DHA in breast milk is not optimal and has declined in recent years (20). Currently there are no dietary recommendations for AA and DHA consumption in neonatal life (22). Thus, establishing specific AA and DHA requirements to aid in the recovery of low neonatal bone mass in IUGR infants may assist with clarifying the necessary components of the diet in early life.

Lastly, maternal diet enrichment with AA and DHA may positively effect maternal bone mass, as these fatty acids are proven beneficial in post-menopausal women (23). During pregnancy and lactation there is considerable stress placed on maternal bone (24) and the magnitude of bone mineral loss from the adult female skeleton is the greatest during lactation than any other period of life (25).

It is presently unknown how AA and DHA affect female bone mass and turnover during this period of life.

1.2 Study objectives

1.2.1 General objective

The objective of this study was to determine the effects on maternal and offspring bone mass of enriching the lactation diet with AA and DHA in an IUGR animal model. In addition, this study will determine if AA and DHA in the pre-weaning diet affect bone mass solely during the neonatal period or beyond.

1.2.2 Objectives

Objectives 1-3 are to be achieved by using the guinea pig model (both well-nourished (control) and growth restricted offspring).

The objectives of this study are:

1) To enrich the diet of nursing offspring with AA and DHA solely during the lactation period to determine if these fatty acids:
    a) affect body size and composition.
    b) affect bone mass, strength, and bone metabolism.
    c) have effects on the above parameters that persist into early adult life (16 wk of age), or post-supplementation.
2) To measure erythrocyte lipid concentrations in pups to determine the effect of enriching the maternal lactation diet with AA and DHA. The erythrocyte will provide a reliable estimation of the fatty acid concentrations in other tissues (26).
3) To determine if maternal bone mass and bone turnover is affected during and post-lactation, by providing AA and DHA in the lactation diet.

This research is considered hypothesis generating, though the following general hypotheses are stated as:

1.3 Null hypotheses

1) AA and DHA will have no effect on growth, body composition, or bone mass, strength and bone metabolism in both well-nourished (control) and growth restricted offspring.
2) There will be no persisting or lasting effects of enriching the lactation diet with AA and DHA.
3) The addition of AA and DHA to the maternal diet will not be reflected in the erythrocyte fatty acid concentrations of pups.
4) Maternal bone mass will not be affected by the provision of AA and DHA during lactation.

To address the above objectives it is necessary to have a thorough understanding of bone, IUGR and LC PUFA. The following section is a review of the literature.

2.0 Chapter Two

Literature review

2.1 Bone development and assessment

2.2 The pigmented guinea pig model

2.3 Models of IUGR

2.4 Prevalence and causes of IUGR and effects on growth

2.5 Neonatal fatty acid metabolism

2.6 Fatty acids and bone

2.7 Mechanisms of action for LC PUFA in bone

2.0 Literature review

2.1 Bone development and assessment

2.1.1 Bone formation and modeling

During pregnancy, the fetal skeleton develops by two distinct mechanisms, intra-membranous and endochondral ossification (27). The later forms the long bones and involves the development of cartilage that will be replaced by bone and bone marrow. Cartilage formation is driven by the differentiation of mesenchymal cells into chondroblasts (27). These cells become embedded in a cartilaginous matrix and continue to proliferate as chondrocytes. Initially, growth occurs appositionally with continuous differentiation of the mesenchyme into chondroblasts and interstitially by synthesis of a new cartilaginous matrix between chondrocytes, or formation of the growth plate (28). Chondroblasts continue to divide, while a sequence of cartilage resorption, woven bone formation (primary spongiosa), secondary resorption of woven bone, and formation of lamellar bone (secondary spongiosa) occurs. Formation and resorption of the spongiosa is driven by the activity of osteoblasts and osteoclasts respectively. At the end of this process, trabecular bone is formed and will continue to be modeled throughout life by the activity of osteoblasts and osteoclasts (28).

Osteoblasts are bone forming cells that deposit osteoid and mineral and osteoclasts resorb bone. Though osteoblasts and osteoclasts differentiate from distinct precursors (mesenchymal and macrophage cells respectively) the activity of the cells are inextricably linked. Osteoblasts express receptor activator of NF-κB ligand (known as RANKL) that is obligatory for the recruitment and maturation of osteoclast progenitors and the cessation of bone resorption initiates the differentiation of osteoblasts (29). Therefore, both cell types participate in bone modeling (27). Abnormalities in the regulation of bone resorption and formation may result in reduced bone mass (29).

2.1.2 Assessment of bone modeling and quality

The condition of the skeletal system is best assessed by evaluating bone metabolism and bone quality (bone mass and strength) (30). BMC and BMD are clinically relevant as frequent measurements associated with fracture risk in humans (31). Thus, dietary interventions that result in percent changes in BMC and BMD may be clinically relevant when considering osteoporosis and fracture prevention. Bone mass measurement using dual-energy x-ray absorptiometry (DXA) is a non-invasive method to assess bone and DXA is validated for use with animal models, including guinea pigs (32). Quantifying bone strength in addition to bone mass is an important determinant of bone quality and accounts for bone parameters that BMC and BMD do not capture, such as elasticity (33). Performing 3-point bending assessments of the long bones and compression tests of the lumbar vertebrae and femoral neck provide an assessment of both cortical (long bones) and trabecular (vertebrae) rich areas, the 2 types of bone in the body.

2.1.3 Serum markers of bone metabolism

Osteocalcin and deoxypyridinoline (DPD) are markers of bone metabolism commonly used to measure bone formation and resorption respectively and are associated with growth velocity in children (34).

Osteocalcin is the primary non-collagenous protein in the bone matrix and is synthesized exclusively in bone by osteoblasts. It is released in very small amounts into circulation in response to skeletal growth and therefore is considered a specific and sensitive marker of bone formation (35).

Type-one collagen in bone is comprised of a triple helix and the collagen strands are connected by two cross-links, pyridinoline and DPD. During the process of bone resorption, osteoclasts cleave the cross-links in type-one collagen, releasing into circulation free pyridinoline and DPD. Pyridinoline is not specific to bone and is found in other types of collagen but DPD is specific to bone type-one collagen. Therefore, measurement of DPD is considered preferable. Both total

and free forms of DPD are present in serum in low amounts and excreted in the urine in greater quantities (35). Both osetocalcin and DPD have diurnal variation, with highest values in the morning and lowest in the evening (35).

2.1.4 Dual energy x-ray absorptiometry

Dual-energy x-ray absorptiometry (DXA) is the preferred method to evaluate BMC and BMD in both humans and animals. Bone area, lean and body fat mass can also be easily quantified. Because DXA software is now refined for bone research using small-animal models, this removes the need for a very large number of animals in longitudinal trials (36). A DXA uses radiation at two different levels in combination with a detector to measure x-rays absorbed by tissue. During a scan, these x-rays pass through a subject and bone, fat, and muscle tissue stop the progression of x-rays, varying by tissue composition (36). Ultra-high resolution software is used for small animals, which slows the speed of the scanning arm and increases the number of scan lines to produce more accurate measurements (36). The effective dose of radiation to measure bone mass in animals is 0.015 μSv (36-38).

Dual-energy x-ray absorptiometry has been validated for use in the guinea pig. Reported CVs for *in vivo* scans of the guinea pig femur, femoral neck, and tibia are reported to be 0.69, 1.77, and 0.62 respectively for BMD and 2.92, 0.32, and 3.76 respectively for BMC, indicating good precision in this animal model (39). The correlation between *ex vivo* BMC scans and bone ash is reported to be r^2=0.934, indicating that DXA is an accurate measure of bone mass in the guinea pig (39).

2.1.5 Biomechanical strength testing

Bone biomechanics are based on the principles of stress and strain as a function of force. Stress is defined as force per unit of area and is typically applied in a compressive or tensile manner in bone mechanical testing (33). Strain is deformation of the bone due to the load applied. Stress, load, and the deformation

that occurs due to the load (strain) are expressed in a load deformation curve (stress-strain curve). Extrinsic stiffness or rigidity is illustrated in the elastic region of the curve in which the bone may resume its normal shape and functionality if the load is removed. Once the bone has reached the yield stress, or its yield strength, it has sustained permanent damage beyond the elastic region of stress. Ultimate strength is the maximum stress the bone can withstand before fracture and breaking strength is the ultimate force required to fracture the bone (33).

Stress at yield, maximum stress, and stress at break are all important indicators of the overall strength and quality of the bone. Stress or load at yield is a measurement of the elasticity of the bone while maximum stress or load indicates the ultimate load the bone can withstand before fracturing. Stiffness, toughness, and resilience can all be calculated from the load deformation curve and are representative of bone rigidity, work energy necessary before fracture, and fragility respectively. Young's modulus is the slope obtained from the load deformation curve and represents the intrinsic or overall stiffness and rigidity of the bone, independent of the bone size. It is a fairly good indicator of the overall bone quality (33). The 3-point bending test is repeatedly described in the literature (40, 41). Previously, bone strength has not been assessed in young guinea pigs so methods will be adapted for the size of bone.

The multi-coloured guinea pig is not commonly use to study bone health. The following section will explain the rationale behind choosing the guinea pig for this study.

2.2 The pigmented guinea pig model

Despite common classification, genetically, the guinea pig is not a rodent (42). Recently, various guinea pig strains have been used in studies of osteoarthritis, audibility and inner ear functioning, and the effects of the intrauterine environment on gestation and fetal development (43). This model is useful for

studying the later due to the distinct advantage of small litters and fetal maturation before birth (44). Fetal guinea pigs mineralize bones *in utero*, permiting bone mass measurements at birth (45).

Guinea pigs have a 12-14 d oestrus cycle, in which oestrus lasts for 2-4 d. Time of conception is difficult to determine, which is a drawback to using this model. Gestational length ranges between 60-72 d and may be as long as 80 d. Uncomplicated deliveries are generally 20-40 min in duration and litter sizes are typically 1-6 pups (46). Smaller litters of 2-3 pups often lead to complications during delivery due to larger sized offspring. Female offspring are sexually mature and able to conceive by 4 wk of age, while males reach sexual maturity by 6 wk of age (46).

As a small, rather than large mammal to use in research, the guinea pig bone more closely mimics the human than the rat. Alike humans, the guinea pig has an essential vitamin C requirement for the synthesis of collegen within the bone matrix and at birth has actively erythropoietic bone marrow (46). The newborn guinea pig exhibits a WB BMC per kilogram body weight very similar to that of a human infant (46) and also has no tail, which confounds growth rates in rats and mice (47). Furthermore, guinea pigs do not lose bone mass prior to 1 y of age (32), unlike the rodent model, which begins to lose bone mass by 9-12 mo (48, 49). For this study, the pigmented or multi-coloured guinea pig strain will be used because it does not develop spontaneous osteoarthritis (50).

Though no previous study has examined the effects of dietary long LC PUFA on bone mass in the guinea pig, DXA has been validated and used in studies of guinea pigs (32, 39). In addition, viable, low-birth weight guinea pigs have been bred successfully in several studies (43, 51-53). Diets enriched with LC PUFA, specifically fish oil have been fed to guinea pigs with no adverse effects (54, 55).

While this model may seem appropriate for the study of bone in early life, there are drawbacks and limitations to using this model. Many standard assays for measuring markers of bone metabolism and grown hormones are not validated for use in guinea pigs. For example, to the best of our knowledge, there is no documentation of the amino acid sequence for parathyroid hormone in this species, and thus determining a suitable antibody to measure this protein is very difficult. Additionally, this species is highly sensitive to stressors, such as distruptions or changes in feeding schedules or chow and changes in housing conditions (56) (57). Pregnant females may develop abnormal blood glucose metabolism and eclampsia, which often results in litter abortion and maternal death (58). Despite this, the ability to measure bone mass immediately following birth is the primary benefit to using the guinea pig. Also, known stressors, such as single-housing, wirebottom cages, and abrupt diet changes will not be used in this study.

After selection of the guinea pig model, the method in which to induce IUGR came into question. Previously, an energy restricted model was used in this species, though it is the protein restricted model that is known to produce changes in bone mass and metabolism in rats.

2.3 Models of intrauterine growth restriction

Population-based studies and epidemiological research has provided evidence that poor *in utero* nutrition and low birth weight predisposes an individual to hypertension (59), insulin resistance (60), obesity (61), cardiovascular disease (62), and low bone mass (63) with age. Though there are theories as to why this occurs, such as the "thrifty phenotype" hypothesis (64), determining methods to attenuate these sequelea remains ongoing and is made possible by animal models that mimic developmental programming.

To induce IUGR in animal models through diet, energy or dietary protein is restricted during pregnancy and, as the next section addresses, these two diets

produce varying results in offspring. Different animals are used in the literature, including rodents, guinea pigs, pigs, and sheep. Guinea pigs may be emerging as a more appropriate small animal model than rodents, given that guinea pigs achieved a greater degree of developmental maturity *in utero* than rats and mice (46).

2.3.1 Energy vs. protein restriction to induce IUGR

In larger animal models, such as sheep, energy restriction is predominantly used and rodent models use mild (30%), moderate (50%), and severe (70%) energy restriction in pregnancy to produce low birth weight offspring (65-67). However, decreased dietary protein intake is a more common type of nutrient restriction in the literature. While decreasing protein intake by 50% throughout pregnancy typically reduces the birth weight of offspring (68), there are also reports of elevated birth weight (69). There is evidence that both energy and protein restriction result in changes to offspring development, suggestive of intrauterine programming. However, there seems to be minor evidence that low-protein diets are more effective at programming changes in enzyme function and gene expression than energy restricted diets.

Despite this, one drawback to using the low-protein diet is the discrepancy in the compositions used in the literature, specifically in proportions and types of macronutrients. For example, the diet used by Langley and colleagues (68) contains twice the amount of lipid (corn oil) as that used by Snoeck and colleagues (soy oil) and due to the type of oil chosen, the later using soy provides about triple the amount of n-3 essential fatty acids (70). As both high and low intakes of polyunsaturated fatty acids (PUFA) during pregnancy affect health outcomes in offspring (71-73), the lipid content of the diet is an important variable. Another important distinction between diets is the use of starch or simple sugar to replace the energy lost from the reduction in dietary protein. Where Langley and colleagues (68) use a complex carbohydrate, Snoeck and

colleagues (70) use simple sugars, which may be partially responsible for insulin resistance and pancreatic dysfunction in offspring.

Programming outcomes are observed in the liver and pancreas. Pancreatic structure and function are modified by energy and protein restriction (74). Using a low-protein diet in pregnancy, the expression of hepatic enzymes (75) are altered by protein restriction during pregnancy (76). More importantly, pancreatic genes involved in mitochondrial respiration and glucagon metabolism are down-regulated in these offspring (77, 78), which has implications for the later development of impaired glucose tolerance and type-2 diabetes mellitus.

In the guinea pig species, energy restriction by 30% results in impaired glucose tolerance in male, but not female offspring (51). Reduced insulin production also occurs in energy restricted rat pups at weaning, with further decrease with age (67, 79). Maternal protein restriction appears to result in greater loss of pancreatic function with age and premature onset of insulin resistance and impaired glucose tolerance (80, 81).

Blood pressure also may be altered by nutrition in pregnancy. Both 30% and 50% of a normal energy intake resulted in elevated systolic and mean arterial blood pressure in rodents (65, 82). Using the guinea pig species, Kind and colleagues demonstrate that feeding sows 70% of a normal energy intake increases systolic blood pressure in male offspring, but not females (53). The reduced protein diet also results in elevation in offspring blood pressure (79). A low-protein intake throughout pregnancy is reported to elevate systolic blood pressure (68, 83) and pre-pregnancy protein restriction exacerbates this effect (84).

2.3.2 Body composition, bone mass and models of IUGR

There is marginally more evidence that energy restriction programs body composition to a greater degree than low-protein diets. Energy restricted male guinea pigs have greater retroperitoneal fat pad mass (51), which is supported by

evidence from rodent studies in which both male and female rats had elevated retroperitoneal and gonadal fat mass (85, 86). In addition, male offspring of calorie restricted guinea pigs demonstrate elevated total serum cholesterol (52). There is little evidence of changes in fat pad mass in offspring from protein restricted rat dams. Decrease adult fat pad mass is reported in one study that fed 8% protein during pregnancy (80), though as reported later, reduced protein exposure *in utero* alters expression of enzymes involved in *de novo* lipogenesis that may predispose offspring to fat accumulation with age (87).

Low bone mass at birth (88) and with age are outcomes of developmental programming (88, 89). Though there is limited research in this area, primarily a 50% reduction in dietary protein intake during pregnancy has been used to induce low bone mass in neonatal animal models (89). At approximately 78 to 88 wk of life, BA and BMC were lower in rats born to protein restricted dams during pregnancy than controls (88) and is additional evidence that *in utero* protein restriction elevates bone turnover in rats and in cultured rodent osteoblasts (89).

IUGR has been induced by ligation of uterine arteries in rat dams for the study of bone. Using this method WB BMC did not significantly differ from controls at approximately 45 d of age, though this was reduced at 6 mo in male rats (90). Interestingly, while protein deficiency post-weaning reduces bone mass and increases risk of fracture, dietary energy restriction post-weaning does not affect bone strength or mass in male rodents (91). Thus, there appears to be a compulsory requirement for protein in bone development and re-modeling.

Given the above literature, the most suitable diet to induce IUGR with proven effects on bone mass is protein restriction during pregnancy. It is also understandable that given that bone development is dependant on available protein for bone matrix synthesis, mineralization and modeling that a deficiency in protein will result in under-mineralized, weaker bones and overall growth retardation. However, this model can not entirely mimic IUGR in human

neonates because this phenomenon is multi-factorial in origin and its outcomes are multi-faceted.

2.4 Prevalence and causes of IUGR and effects on growth

An SGA neonate is most commonly defined as having a birth weight or length that is at least 2 standard deviations below the mean for gestational age (92). Despite advances in pre- and perinatal care, the prevalence of SGA births has not decreased in 30 y. In Canada, the proportion of preterm births, including SGA births, increased from 6.4% in 1981 to 7.1% in 1996 (93). Presently 10% of live births are affected by IUGR in North America (94). In developing countries, the health risks associated with SGA births are of greater concern, as it is estimated that over 25% of neonates display the weight and length measurements that are characteristic of SGA infants (95). Possible maternal causes of IUGR include chronic or pregnancy-associated hypertension or diabetes, dietary protein or caloric deficiencies, smoking, substance abuse, nulliparity, and low maternal weight gain during pregnancy (93).

Annually, more than 13 million dollars are spent on the healthcare of SGA neonates in Canada (93). However, it is important to consider that SGA infants not only require greater medical attention in infancy and childhood than appropriate for gestational age (AGA) infants, but place a greater burden on the healthcare system in older age due to a greater risk of developing chronic disease (96, 97). Thus, a potential dietary intervention to lessen the disease risk and health burden of SGA neonates would be economical and beneficial to the Canadian healthcare system.

2.4.2 IUGR, growth in young life, and body composition

Eighty-seven percent of SGA infants experience "catch-up" growth during the first few months of life. However, bone mineralization is one parameter of health that remains compromised throughout life (96, 98). In addition, those infants that display rapid catch-up growth are at the greatest risk for health problems later in

life (99, 100). During infancy, body weight and length are strong predictors of WB BMC, which increases by approximately 400% during this period of life (96, 101). Therefore, it is evident that SGA neonates with reduced birth weight or length may have a lower BMC than infants born AGA.

Small for gestational age neonates are 7 times more likely to be of short stature in adulthood than neonates that are AGA (102). Birth length, and to some extent birth weight, are related to post-natal catch-up growth and height is an important factor with regard to bone mass. Infants in the lowest percentile for length at birth remain short in adolescence and adulthood. Children who are shorter display a younger bone age, slower skeletal maturation, and earlier onset of puberty that may impact hormonal regulation of bone mass (103).

Reduced body weight and alterations in body composition may impact bone modeling in neonates born SGA (8). Body weight of SGA babies matches or exceeds that of AGA children by 5 y of age (104). This catch-up growth appears to be sex-dependent. SGA males gain weight more rapidly than females in infancy, though the same proportion of males and females achieve normal weight by 4 y of age, suggesting females gain weight at a slower, steadier rate (104). However at 20 y of age, a period of life where attainment of peak bone mass is of concern, SGA males weigh less and are shorter than their AGA counterparts. For females, there appears to be a lesser difference between the weight and height of individuals born AGA and SGA by this age (105). Thus, males may be at a greater risk for poor long-term bone quality than females given that body weight is significantly correlated to bone mass in middle to late life. However, alterations in the body composition of SGA individuals also impact bone.

Lean mass is positively associated with bone mass throughout the lifespan, but especially during childhood (106). *In utero* programming of the endocrine system impacts fat and muscle distribution in the body. IUGR is associated with reduced muscularity and higher body fat percentages in children and adults whereas lean

mass has been found to positively correlate with birth weight (107). Differences in weight between AGA and SGA infants is primarily due to a greater attainment of lean mass in AGA infants and a higher body fat percentage in those born SGA (108). Thus, SGA children have reduced muscularity and increased fat mass throughout childhood. This may be detrimental to bone and have a negative impact on fracture risk as fat mass is negatively correlated with BMC and positively associated with fractures in childhood (109-111).

2.4.3 IUGR and bone mass

At birth, body weight and length are highly predictive of whole BA (112, 113) and BA is reduced in light, short infants because of smaller body size. Compared to neonates of normal weight at birth, SGA infants have shorter long bones accompanied by smaller cortical areas and diaphyseal diameters at the femur, tibia, and humerus (114). Reduced bone size is concurrent with low bone mass in SGA neonates.

A 67% and 40%, lower BMC is reported at the humerus and radius respectively of newborn SGA neonates than those born AGA, while reduced BMC and BMD are observed at the lumbar spine (6, 115, 116). Animal models of the SGA infant also have low WB bone mass in very early life (117, 118). Small body size is one cause of low bone mass, as BMC has a high degree of correlation with BA and body length and weight at birth (9). However, despite achievement of appropriate body size with age in some SGA infants, reduced bone mass persists into childhood and older age (119) (12). Thus, it is proposed that small body size at birth is not the only cause of low bone mass in individuals born SGA.

Human infants born small have reduced epiphyseal development in the first 5 d of life than babies of normal weight and length (120). Similarly, the SGA rodent model exhibits wider tibial epiphyseal growth plates than AGA rodents late in life, suggesting that long bone growth in early life is delayed (88). In cell culture, mesenchymal cells harvested from SGA rodents at 8 wk of life exhibit lower

osteoblast differentiation than AGA controls (89). This is a possible cause for the observed abnormal epiphyseal growth in SGA humans and rodents. However, mesenchymal cells obtained at 16 wk of life from SGA rats demonstrate accelerated rates of osteoblast differentiation. This suggests that rates of bone remodeling increase with age in those born SGA (89). Moreover, accelerated bone turnover may remain elevated throughout life (98), possibly predisposing babies born SGA to low bone mass with age.

Pre-term infants have a high rate of bone turnover as indicated by a steady increase in urinary DPD excretion during the first month of life, accompanied by serum osteocalcin concentrations exceeding that of term infants (121). SGA males at 20 y of life have 58% and 56% higher serum alkaline phosphatase and osteocalcin respectively than AGA males. This is in addition to a urinary DPD excretion that is 20% greater than that of control individuals. Elevated DPD and osteocalcin values suggest that there is a greater rate of bone remodeling amongst those born SGA in comparison to AGA controls (98).

Many epidemiological studies associate being small at birth and 1 y of age with low BMC and BMD in men and women in the 7^{th} and 8^{th} decade of life (122). However, the only study that assessed osteocalcin and bone resorption did not find a significant association between weight at birth and either marker of bone turnover, though both were negatively associated with BMD at the LS and femoral neck (4). The evidence that BA and BMC are more frequently associated with size at birth than BMD (12, 107, 123) is significant because BMC is affected by bone growth while BMD corrects for skeletal size, suggesting individuals born small may have life long impaired bone mineralization. Numerous reports that weight at 1 y is more strongly associated with bone mass in later life (107, 124, 125) suggests that the trajectory of bone modeling may be set within the first year of life. Thus, without intervention, low birth weight and being small at 1 y appears to result in low bone mass in later life

In trabecular rich areas of bone that are sensitive to bone turnover, such as the LS or femoral neck, low bone mass is concerning as it may pre-dispose an individual to kyphosis or hip fracture with age. With an aging population, osteoporotic fractures will become a greater contributor to health care expenses as well as to morbidity and mortality rates amongst Canadians (126, 127). While there are various nutrition and exercise regimens suggested to improve life long bone health for infants born small, diet enrichment with LC PUFA during lactation has relatively new potential to normalize the observed sequelea of increased bone turnover and decreased bone mass with age.

2.4.4 IUGR, bone mass, and diet

Diet has a significant impact on bone mass throughout life. Therefore, early life dietary intake may affect bone mass in SGA neonates. Breast feeding and formula feeding has not been observed to correlate with BMC or BMD in adulthood (4). In addition, no effects on bone mass were observed from feeding formula enriched with LC PUFA to infants born prematurely at 12 months of age (128).

Currently, selected infant formulas are supplemented with the LC PUFA arachidonic acid (AA) and docosahexaenoic acid (DHA), based on evidence that these fatty acids aid neurological and visual development (129). Research has concluded that dietary AA and DHA are beneficial to growth in AGA infants and to bone mass in healthy, growing piglets (18). It has also been reported that SGA infants have low amounts of stored LC PUFA and have a reduced ability to synthesize LC PUFA (21, 130). Therefore, it is hypothesized that the growth of SGA infants would benefit from AA and DHA supplementation. However, at present, research evidence remains unsubstantiated to recommend LC PUFA supplementation in SGA infants to improve growth or bone health.

2.5 Neonatal fatty acid metabolism

Long-chain polyunsaturated fatty acids (LC PUFA) are n-6 and n-3 fatty acids resulting from the elongation and desaturation of linoleic (LA; n-6) and α-linolenic acid (ALA; n-3). LA and ALA are essential fatty acids that must be obtained from placental transfer for the fetus and breast milk or formula diet after birth. AA (n-6) and DHA (n-3) are LC PUFA derivatives of LA and ALA respectively that can be synthesized in the body and are critical for a developing fetus and during the first y of life for normal brain and retina function.

Arachidonic acid (AA) and docosahexaenoic acid (DHA) are acquired from maternal supply throughout pregnancy, though placental transfer is highest during the 3rd trimester (131). LC PUFA are proportionally higher in fetal than maternal plasma (19, 132) and are selectively transported to the fetal compartment (133), likely as a physiological response to the importance of these nutrients in neural and visual development. DHA is preferentially stored over AA (134). IUGR reduces the fetal:maternal ratio of circulating AA and DHA, indicating SGA neonates have reduced LC PUFA status at birth (130). In addition, the percentage of AA in maternal and umbilical cord red blood cells (RBC) is positively correlated with WB BMC at term in healthy infants (135). This suggests that impaired AA status in SGA infants could contribute to reduced bone mass at birth.

By the 2nd trimester the fetus is capable of synthesizing AA and DHA from precursory n-6 and n-3 fatty acids obtained from the mother (136). Regardless of birth size, infants can endogenously synthesize LC PUFA (137), though the rate of synthesis of AA and DHA is believed to be lower for SGA neonates in comparison to AGA neonates (130).

The most recent report of breast milk LC PUFA content for North American women states a mean percentage (± SD) of 0.4 ± 0.03 AA and 0.25 ± 0.08 DHA, or an approximate 2:1 ratio of AA:DHA (20). Of this, the amount of AA and

DHA actually absorbed by an SGA infant is about 80% of each fatty acid (138). However, the amount of AA and DHA present in the breast milk of women is rapidly declining in association with reduced consumption of these fatty acids in the diet (139, 140).

2.6 Fatty acids and bone

As one of the major macronutrients in the diet, and one that is often over consumed (141), dietary fat has the potential to affect health in numerous ways. Osteoporosis is not typically attributed to dietary fat consumption, but there is increasing evidence that long-chain lipids have the potential to optimize bone mass (134). Fat deficient animals develop severe osteoporosis (134), and thus it is recognized that fatty acids likely play an essential role in bone metabolism. While high- and low fat diets are both detrimental to bone quality (142) LC PUFA elevate WB BMD and LS BMD by 5-20% in young, aged, and ovariectomized animal models (14-16). Receptors for long-chain lipids are present in bone tissue and LC PUFA may also affect bone via mechanisms for calcium absorption and by altering metabolic and growth hormones (143, 144). With a growing body of supporting evidence, PUFA and LC PUFA may soon be promoted for their role in bone development.

2.6.1 Epidemiological evidence for the importance of PUFA and LC PUFA in bone health

Limited epidemiological research provides evidence that there is an association between PUFA intakes, bone mass and bone quality. In men and women, consuming lower amounts of n-6 fatty acids and more n-3 fatty acids is associated with higher total hip BMD (145). Terano and colleagues (146) compared women in an urban city to those from a fishing community and reported that BMD at the radius was greater in women from the community consuming higher amounts of fish. These women had higher circulating levels of DHA. Conversely, PUFA intake was correlated with lower femoral neck bone mass in menopausal women, but only in those with low calcium consumption, highlighting the association

between PUFA and calcium. Calcium absorption was shown to be 30% higher with 4% of dietary lipid as tuna oil (23, 134). Also in children, the g/d of LC PUFA consumed was associated, independently of calcium intake, with greater gains in BMD during one year (147). An older investigation of Greek men and women age 25-69 that routinely consume high levels of monounsaturated lipids and PUFA found a strong correlation between PUFA intake and distal radius BMD (148).

2.6.2 The effects of dietary PUFA on bone mass

Flaxseed is rich in ALA and is a common dietary source of this PUFA. Ground flax, when provided as 5% or 10% (w/w) reduces bone resorption in female weanling rats (149). When male and female rats were fed from birth 10% (w/w) purified ground flax, there is a detrimental effect on bone strength after 50 d of feeding, evident by a 10% decrease in femoral ultimate bending stress but normal femoral BMC and BMD. The negative effects on biomechanical properties were transient, as after 132 d of feeding there were no positive or adverse effects of the high flax diet (40, 41). Further studies in weanling mice indicate 10% flax oil has no effect on bone density or femoral strength (150). Similarly, PUFA do not change serum osteocalcin or bone mass in piglets fed formula rich in flaxseed oil (approximately 3% ALA) from birth until weaning. Serum DPD was reduced by 15% with the high ALA diet, though this was not statistically significant (151). These observations in animals are mirrored in post-menopausal women.

In menopausal women, year long diet enrichment with flaxseed (to provide 9.12 g/d of ALA) had no effect on femoral neck BMD, and while there was a trend towards greater BMD loss at the lumbar spine with flax consumption, this was not significant (152). A similar study providing 40 g flaxseed/d (or 9 g/d ALA) for 3 mo report no significant effects of flax on serum DPD, though there was a 3% increase in this marker between baseline and the end of the trial (153). In men and women, diets rich in walnut and flax, providing 1.6:1 or 3.5:1 LA:ALA ratios significantly reduced bone resorption by 15% and 11% respectively in

comparison to a control group consuming a 9:1 LA:ALA ratio daily. Bone resorption was greater by 5% in the group consuming a 3.5:1 ratio in comparison to the other diet groups, which suggests that there is an optimal ratio that affects bone mass (154).

Feeding n-6 and n-3 lipids in low and high ratios confirm that the ratio of LA:ALA significantly affects bone mass, and that LC PUFA have a greater capacity to affect bone growth and metabolism than PUFA. Linoleic acid rich diets fed to rats in comparison to a diet with LA and DHA, or rich in both ALA and DHA. Diets rich in LA resulted in higher prostaglandin E_2 (PGE_2) production in the femur deemed to be a negative effect on bone formation rate. However, the rats that received high amounts of ALA and DHA had lower PGE_2 production. There seemed to be no effect of these diets on markers of bone resorption and a lower n-6:n-3 ratio in bone was associated with greater bone formation rates (155). A similar study that fed 21 d old chicks diets with varying LA:ALA ratios in contrast to a diet high in DHA reports higher tibia cortical bone area in those that received the DHA diet. However, other groups fed LA:ALA in 17:1 and 11:1 ratios did not differ from the DHA group. In addition, the LA:ALA 11:1 group had the highest insulin like growth factor-1 (IGF-1) at the end of the growth phase (156), which is positively associated with bone mass (157).

These studies propose that PUFA that are of n-3 in origin, specifically DHA, are most supportive of optimal bone formation, but there is also evidence that the appropriate n-6:n-3 ratio may be equally critical. Due to the various dietary n-6:n-3 ratios that are used in these studies it is difficult to conclude that DHA alone is truly the optimal PUFA for bone health. One proposed mechanism through which DHA improves bone quality is through reduced PGE_2 production. However, Lucia and colleagues treated piglets with dietary AA and DHA (0.8:0.1 ratio) and PGE_2 separately and together, reporting that the separate treatments improved bone mass, but the combined treatment was detrimental, illustrating the

mechanism by which PUFA improve bone may not be through PGE_2 metabolism (158).

When shorter chain PUFA are provided in the diet, they may be used to synthesize numerous and diverse prostaglandins, used for storage, and in some cases, for energy production. In addition, the elongation of LA and ALA to AA, eicosapentaenoic acid (EPA), and DHA are dependant on enzymatic processes. Therefore, providing the LC PUFA directly in the diet without relying on endogenous conversion of PUFA to longer chain derivatives likely provide greater benefits to bone mass.

2.6.3 The effects of LC PUFA on bone mass

Feeding n-3, LC PUFA alone through diets enriched in fish oil was tested in young and old animal models. In weanling male and female rats, femur and lumbar vertebrae size, mineral content and density were not affected after 5 wk supplementation with 6.4 mg/d DHA. Peak load of the 5th lumbar vertebra was reduced by 15% in fish oil fed female rats, which is unfavorable (159). Urinary calcium excretion was not affected by this diet, but calcium absorption was almost 20% greater in male weanling rats fed 1.25% (w/w) DHA for 6 wk in a study by Kruger and colleagues (2005). In addition to calcium absorption, diet enrichment with DHA had positive effects on bone quality by elevating femoral calcium content, femoral and lumbar vertebra BMD by 12%, 8%, and 7% respectively and there was a 5% increase in maximum femoral load (17).

These observations persist in bone that is compromised in ovariectomized models. Ovariectomized female mice that received approximately 0.5% (w/w) DHA daily were protected against bone loss as measured by DXA at the lumbar spine, and trabecular bone mass was maintained (16). Conversely, in ovariectomized rats fed 0.1 g/kg body wt/d EPA or 1.0 g/kg body wt/d EPA the higher dose lowered femoral BMD while the lower dose had no protective effects on BMD or serum markers of bone resorption (160). In old (12 mo) male rats feeding approximately

15.3% (w/w dietary lipid) DHA for 5 wk appears to protect against femur mineral losses, though this can not be stated with confidence, as this diet had almost 30% more vitamin D than other diet treatment groups (15).

Evidently, diet enrichment with fish oil alone may provide some protection against age and hormone-related bone loss. However, in a growing model of bone, DHA alone is insufficient to optimize bone mass accretion. A recent study shows that in young adults (20-40 y), a diet enriched with DHA fatty acids (from salmon oil) increased bone resorption and decreased osteocalcin concentrations, suggesting that DHA alone unfavorably alters bone turnover (161). In rats, illustrate, Claassen and colleagues (1995) fed gamma-linoleic acid (GLA) and EPA in 3:1, 1:1 and 1:3 ratios. After 6 wk, the 1:3 and 1:1 diets resulted in a 24% and 15% decrease respectively in femoral calcium concentrations whereas the 3:1 GLA:EPA diet had no effect. This diet also had the most favorable effect on calcium balance with respect to fecal and urinary excretion and bone resorption markers, though femur size did not differ between groups (162, 163). Weiler and colleagues (2000) provided additional evidence that both n-6 and n-3 are important to growing bone by feeding weanling piglets a 5:1 ratio of n-6:n-3 fatty acids and a treatment group the same ratio of AA:DHA. Most significantly, WB, femoral, lumbar vertebrae BMD, and whole body BMC were significantly greater in the group fed AA:DHA. Moreover, this was the first study to report elevated BMD as measured by DXA after a short-term supplement with LC PUFA (18). However the prior work by Claassen was not reproduced in piglets fed AA and DHA in a 5:1 ratio in various doses. Calcium absorption and urinary calcium excretion were both unaffected by all diets containing AA and DHA, though there was a small (2%) but significant increase in femoral calcium concentration in piglets fed 1.0:0.2 g/d (w/w) AA:DHA (14).

Determining the optimal ratio of AA and DHA in the neonatal diet for bone is equally important as determining the optimal type of PUFA for bone health. It is evident that total reduction in the n-6:n-3 ratio from very high to very low has

some positive effect on alkaline phosphatase but is limited in potential to improve bone formation and increase bone mass (155). Feeding 3:1 and 4:1 ratios of GLA:EPA to young rats increases bone calcium and calcium balance, whereas lower ratios of 2:1 and 1:1 have a lesser effect (163). However ratios of 5:1 and 9:1 AA:DHA improve bone mass in piglets (151). To further support these observations Blanaru and colleagues (2004) report the greatest effect of LC PUFA on whole body BMC and BMD by feeding 0.6:0.1 and 0.75:0.1 AA:DHA (164). A 5:1 ratio (AA:DHA) in varying amounts did not provide additional benefits to bone mass or metabolism, confirming that it is the 5:1 ratio of AA:DHA that is most optimal with regard to bone health in young, healthy growing animal models (14).

2.6.4 LC PUFA and bone mass in SGA infants

For both AGA and SGA neonatal piglets (14, 18, 117) and rodents (118, 165), dietary enrichment with a ratio of AA:DHA (such as 6:1 or 2.5:1) that provides slightly more AA than human milk during the suckling period increases bone mass, or prevents lower bone mass in the SGA offspring. In addition, tissue AA status is associated with improvements to bone, unlike prior reports that n-3 LC PUFA, such as DHA, increase long bone strength and mineralization (166, 167), increase bone formation (144), and reduce bone resorption (16).

Studies suggest that consumption of LC PUFA from breast milk slows body growth (168) and bone mineralization (169) within the first y of life in preterm infants, but will result in higher or uncompromised body size and bone mass long-term. When infants receive breast milk or formula enriched with calcium, phosphorus, and vitamin D, bone mineralization rates are elevated in a relatively short period of time (4-6 mo of age). However, in the long term (1-5 y of age) feeding un-supplemented breast milk to preterm infants results in higher BMC (170-172). This signifies that nutrients other than the minerals present in breast milk and not in standard formula are benefiting long-term bone mineralization. These formula-fed infants, despite receiving a diet higher in essential minerals in

early life, did not receive AA and DHA at a level equivalent to that in breast milk, suggesting the LC PUFA could be required for optimizing growth.

As previously noted, both the human and animal SGA neonate appears to have an accelerated rate of bone turnover in the first y of life. However, in studies using piglets of normal birth weight (173) and preliminary studies using SGA piglets and rodents in our laboratory (117, 118), enriching the suckling diet with a ratio of AA:DHA very similar to that in breast milk appears to normalize or slow bone formation. Biochemical markers indicate that bone resorption and turnover rates were lower in SGA piglets and rats that received AA and DHA. By 16 d of life, piglets that received LC PUFA had elevated LS BA, BMC, and BMD, while female rats at 63 d of life displayed compromised LS bone mass and WB BMD accompanied by elevated osteocalcin levels if LC PUFA was not given. Thus, it is hypothesized that osteoblast differentiation was reduced, or normalized, by dietary LC PUFA. While these trials in SGA animal models suggest that later life bone mass is uncompromised by providing AA and DHA in very early life, why this occurs is unknown. Hypotheses include alterations in lean body mass and muscularity, up-regulation of calcium metabolism modulation of PGE_2, cytokine and hormone production, and involvement of peroxisome proliferator activator receptor-gamma (PPARγ).

In the following section, these possible mechanisms through which LC PUFA elevate bone mass in the IUGR neonate will be briefly discussed.

2.7 Mechanisms of action for LC PUFA in bone

From the time that LC PUFA were known to affect bone health, research has attempted to ascertain the mechanisms responsible. There are currently several possible explanations, including the involvement of peroxisome proliferator activator receptors (PPAR), leptin, prostaglandins, the receptor activator of NF-κB (RANK), calcitriol and parathyroid hormone (PTH).

2.7.1 PPAR activation and bone mass

The PPAR family (α, γ, or δ) are gene transcription factors (174) and as such, changing PPAR expression may have permanent or life long effects on bone mass (174). Fatty acids and their metabolites are ligands for PPARγ-1 and PPARγ-2 (175). Direct addition of AA and DHA to an osteoblast cell culture elevates PARRγ-2 expression in these cells (176) and reduces differentiation (176, 177). Therefore, it is apparent that *in vitro* LC PUFA may be regulators of bone mineralization as mediators of cell differentiation and activity of osteoblasts. Very small concentrations of fatty acids are capable of activating a PPARγ, the subtype found in bone tissue (178). Therefore, minor increases in tissue AA and DHA status from diet may have the capacity to regulate PPARγ. Dietary AA, DHA or a combination of both fed to mice increased PPARα activation in the liver (175), which shows PPARα is responsive to diet interventions (176).

Bone marrow contains a diverse array of cell types, including osteoblasts, hematopoietic stem cells, and adipocytes. PPARγ drives the conversion of osteoblast-type cells to adipocytes in differentiating mesenchymal cells (175). Age-related bone loss is associated with a higher proportion of adipocytes present in the bone matrix and studies demonstrate that PPARγ activation (179) and expression (180) impair osteoblast differentiation and bone formation, and mature adipocytes inhibit osteoblast activity (177). Activation of PPARγ-2 most often results in terminal differentiation of adipocytes, production of lipoprotein lipase in bone, and suppression of Runx2/Cbfa1 signaling (181). Activation of PPARγ-1 is reported to reduce osteocalcin protein expression and overall bone formation, but does not impair the Runx2/Cbfa1 pathway. Thus, PPARγ-1 signaling produces adipocyte-like cells that may be converted to osteoblasts by the Runx2/Cbfa1 pathway if provided with an osteogenic stimulus (181). Both forms of PPARγ will inhibit bone formation, as seen in PPARγ$^{hyp/hyp}$ mice that do not express PPARγ-1 or -2 and have increased trabecular thickness in the LS and femur (182). However, ligands play a critical role in determining the extent of PPARγ activation in bone marrow mesenchymal cells. One ligand that increases

expression of PPARγ-1 is derived from AA (183) and stimulates adipocyte production (184). It is also known that ligands structurally most alike AA, and those derived from LA do not cause activation of PPARγ-2 that results in terminal differentiation of mesenchymal cells to adipocytes. Rather, these ligands inhibit mineralization and osteocalcin protein expression, but cause no change in Runx2/Cbfa1 expression (184). While it is known that AA increases expression of PPARγ-2, it is unknown if AA acts as a ligand to terminally differentiate mesenchymal cells to adipocytes.

In theory, activation of PPARγ-2 by ligands structurally alike AA would result in slower bone mineralization and reduced osteocalcin secretion in serum, but would not cause permanent differentiation of mesenchymal cells into adipocytes. In addition, if terminally differentiated adipocytes were not produced, no permanent negative effects of lipid accumulation in bone should be observed. This may be the mechanism responsible for previous observations of slower bone mineralization in AA supplemented AGA piglets (173) and SGA rodents (118). Therefore, it may be that AA, by activation of PPARγ slows osteoblast differentiation in very early life such that the trajectory for bone mass is reset for the remainder of life.

2.7.2 Leptin suppression and bone mass

Leptin is a fat and energy regulating hormone (185) It is secreted by white adipose tissue and predominantly acts as a satiety signal in the ventromedial nucleus of the hypothalamus (86). Bone formation is inhibited by serum leptin concentrations due to the regulatory effect of leptin on several growth hormones, as well as the binding of leptin to high affinity receptors in bone (186). Leptin deficient mice have elevated serum osteocalcin and higher bone mass (187). Simple experimentation with serum leptin concentrations demonstrate that bone is highly sensitive to serum leptin (188).

There is evidence that leptin signaling has a dominate role over other hormonal regulation in bone. Leptin deficient rats have elevated bone mass despite elevated bone resorption due to hypogonadism (188). In starved mice with lower growth and sex hormone secretion, leptin prevents decreases in serum osteocalcin (187). Furthermore, in calorie restricted mice that have 5% and 70% reductions in tibia length and IGF-I respectively, leptin injections restore tibia bone area, but further reduce IGF-I by 10% (189). Thus, the role of leptin in bone maintenance and mineral accretion may supersede that of other bone mediating hormones. Understandably, suppression of leptin secretion from adipose tissue may be of potential benefit to improve compromised bone mass. LC PUFA, with the exception of EPA, are suppressors of leptin secretion and mRNA in adipose tissue.

Arachidonic acid suppresses basal and insulin stimulated leptin secretion by 15-20% in cultured rat adipocytes (190) while similar observations were made in bovine adipose stromal cells treated with a mixture of AA and DHA (191). Pigs provided with 5.8% of diet as fish oil for 21 d have a 50% reduction in leptin mRNA in dorsal adipose tissue, though no change in serum leptin was observed (192), possibly due to the short length of the feeding trial. Conversely, EPA increases basal leptin mRNA expression in rodent adipocytes (193). In children, levels of AA in plasma lipids are inversely correlated with serum leptin, suggesting the suppressive action of AA observed in cell culture persist *in vivo* (194). Thus, inhibition of leptin by AA may also promote bone formation in neonates.

2.7.3 Prostaglandins and bone mass

Prostaglandin E_2 (PGE_2) is the prostaglandin synthesized from AA. The level of AA in phospholipid membranes can be modulated by the amount of AA and precursors of AA in the diet and alter concentrations in bone (144) and cartilage (195). Consequently, it has been shown that PGE_2 release from tissue, including bone, can be modulated by the level of AA in the diet.

Prostaglandins appear to have a diverse and complicated role in bone modeling. Though PGE_1 is present in the human skeleton (196, 197) PGE_2 is the most abundant eicosanoid in bone, mediates biomechanical bone strength (198), and stimulates bone turnover (199). PGE_2 mediates growth factors and hormones, such as IGF-I (200), and calcitriol (201), and stimulates collagen (202) and alkaline-phosphatase (203). Cytokines also stimulate the synthesis and release of PGE_2 in bone (204, 205) (206-208). Thus, PGE_2 expression impacts the bone resorption mediators, but also helps regulate factors involved in bone formation. For this reason, it appears that both high and low concentrations of PGE_2 are detrimental to bone.

There are numerous studies that detail the beneficial actions of systemically administered PGE_2 in bone. In brief, increased bone strength (209), proliferation of osteoblasts (210), cancellous bone formation (211-214), and the prevention of bone loss due to aging (215-218) and ovariectomy (219, 220) are observed in animal models and tissue cultures. Other research implicates a negative effect of PGE_2 on bone. Associations between dietary n-3 PUFA intake, reduced *ex vivo* PGE_2 production in bone, increased IGF-I synthesis, and elevated tibia bone formation supports the hypothesis that PGE_2 may have detrimental effects at higher concentrations (156). Similarly, DHA has resulted in slowed tooth movement (221), and lowered *ex vivo* PGE_2 biosynthesis in bone (222). Elevated PGE_2 reduced osteocalcin production in piglets (151) and dietary AA was positively associated with urinary markers of bone resorption (164). *In vitro*, several studies show an increase in bone formation markers and up regulation of osteoblast activity with reduced PGE_2 production in bone (223, 224).

Still other animal studies find no correlation between dietary LC PUFA, elevated bone mass, and the PGE_2 present in bone (158). For example, changes in bone mass were observed by feeding an AA:DHA ratio of 5:1, but no differences *in ex vivo* release of PGE_2 from the tibia were observed (14). This suggests

independent effects of LC PUFA and PGE_2 on bone mass. Thus, it is apparent that the modulation of PGE_2 biosynthesis only in-part affects bone mineral homeostasis and can not be the sole mechanism through which LC PUFA improve bone mass.

2.7.4 RANKL, RANK and bone mass

Receptor activator of NF-κB ligand (RANKL) is expressed on the surface of osteoblasts and binds to receptor activator of NF-κB (RANK). The RANK is a transmembrane receptor located on the surface of immature osteoclasts. The interaction between RANKL and RANK initiates gene sequencing that results in the differentiation and maturation of the macrophage precursor to an active osteoclast (225). Receptor activator of NF-κB ligand gene expression is up-regulated by calcitriol and parathyroid hormone, glucocorticoids, and PGE_2, which stimulate bone resorption (226). Receptor activator of NF-κB ligand selectively increases COX-2 expression in vitro, elevating synthesis of PGE_2 (227). *In vitro*, T cells produce RANKL and increase synthesis of osteoclasts and chronic hyper-activation of T cells may lead to continual over-expression of RANKL (225). It is possible that over-expression of RANKL elevates the rate of bone turnover in SGA neonates resulting in reduced bone mass with age. Abnormal regulation of T cell synthesis is observed in preterm infants (228, 229) may increase RANKL on the surface of osteoblasts. Dietary fish oils (EPA and DHA) normalize T cell proliferation in the offspring of diabetic rats, and down-regulate elevation of RANKL expression in T cells using an ovariectomized mouse model (16).

2.7.5 Insulin-like growth factors (IGF)

IGF are essential factors for bone growth expressed by actively proliferating osteoblasts that are involved in bone remodeling and type-I collagen synthesis (230). There are 2 isoforms that are produced locally in bone, IGF-I and IGF-II. Though IGF-II is produced more abundantly, IGF-I is more closely regulated by

GH (156). The actions of IGF at the cellular level are mediated by cell membrane receptors and IGF binding proteins-1 to -6 (IGFBP).

IGF-I is produced locally in bone, or may be derived from circulating concentrations (231). Regardless of origin, IGF-I is a potent stimulator of both bone formation and turnover, especially longitudinal bone growth (232, 233). Circulating levels of IGF-I are associated with biochemical indicators of bone resorption and formation, suggesting that activity of both osteoblasts and osteoclasts is mediated by IGF-I (234).

PGE_2 increases both transcription of IGF-I and IGFBP-3 (235) and the level of IGF-I in bone tissue of rodents (200). Therefore, higher levels of endogenous PGE_2 synthesis in bone may result in elevated rates of bone turnover and if prolonged, reduced bone mass. Furthermore, plasma and liver IGF-I and *ex vivo* PGE_2 synthesis in bone are reported to be reduced in chicks fed soybean oil in comparison to those fed menhaden (144).

2.7.6 Calcitriol and parathyroid hormone (PTH)

Calcitriol (1, 25-dihydroxyvitamin D3), the biologically active form of vitamin D, will only be briefly mentioned as a regulatory hormone in calcium metabolism in conjunction with PTH. PTH stimulates formation of active vitamin D and serves to regulate calcium and phosphorus excretion in the kidney. Calcitriol will induce the release of calcium from the bone matrix into the blood stream when dietary calcium intake is insufficient to meet cellular requirements. Bone mineralization is also enhanced by increased calcium absorption and reduced renal excretion (236). Generally, calcitriol mediated bone formation and resorption seems independent of LC PUFA metabolism. However, has been observed that calcitriol-dependent calcium absorption is affected by the composition and fluidity of intestinal membranes and that intestinal calcium absorption is enhanced by dietary intake of n-3 LC PUFA consumption in rats (157).

3.0 Chapter Three

Paper One

Early life diet enrichment with arachidonic and docosahexaenoic acid attenuates effects of fetal growth restriction on growth and bone mass from birth to maturity in pigmented guinea pigs

3.1 Introduction to Paper One

This paper reports the results of the diet interventions in the pups from birth to sexual maturity at 16 wk of age.

The manuscript was prepared to submit to Prostaglandins, Leukotrienes, and Essential Fatty Acids.

This thesis was referenced as a whole and references are found on **page 105**.

Please note:

The introduction to this paper overlaps with information written in the literature review of the thesis to ensure this paper can stand alone as a manuscript to submit to a journal.

H. Weiler is a co-author for this paper, whose contributions are detailed in the letter of co-authorship in **Appendix C, page 139.**

3.2 Abstract

Intrauterine growth restriction (IUGR) may result in a neonate born with 10-30% lower whole body bone mineral content (BMC) and density (BMD) than a healthy fetus. IUGR neonates also have impaired arachidonic (AA) and docosahexaenoic acid (DHA) status. Because AA and DHA are important nutrients for optimal early life bone mineralization, the aim of this longitudinal study was to determine if providing 0.5% and 0.2% of dietary lipid as AA and DHA respectively would attenuate the effects of IUGR on bone and body composition in an animal model. 40 pigmented female guinea pigs were mated at 12 wk of age and were randomized to receive either a control (C) or 50% reduced protein diet (LP) during pregnancy and the C diet or the same diet with AA+DHA during lactation. Measurements included body composition and bone mass, serum markers of bone metabolism, bone strength, and erythrocyte fatty acid analysis. Results indicated that the LP diet induced IUGR as evidenced by reduced body length and bone mass in pups. The LP had a more detrimental effect on bone mass in males than females, and the AA+DHA diet served to improve body length and bone mass in all LP-pups. Overall AA+DHA elevated lumbar spine BMC by 5% regardless of age, gender, or gestational diet. Interestingly, the AA+DHA diet had a more significant effect on bone mass at wk 16 than at any other time point. This is the first study to show a programming effect of enriching the lactation diet with AA and DHA and will be continued to identify if these observations are transient or persist at an older age.

3.3 Introduction

In North America, intrauterine growth restriction (IUGR) affects 10% of live births (94). This percentage is predicted to increase due to an older mean age of women at time of conception, greater use of fertility drugs and cesarean section deliveries (3). An IUGR fetus fails to reach a genetically determined potential size, which has chronic consequences for the health and growth of the infant, including compromised bone mass at birth and beyond (6, 98).

Evidence that birth weight is associated with bone mass is provided by epidemiological studies that positively correlate birth weight and weight at 1 y of age with both bone area (BA) and mineral content (BMC) in the 7^{th} and 8^{th} decade of life (125). The strength of this association may also differ by gender. Birth weight is more strongly associated with whole body (WB) BMC in women than in men ($r=0.31$ in men and $r=0.45$ in women) (107), which was also observed for BA of the lumbar spine (LS; $r=0.12$ vs. $r=0.18$) and femur ($r=0.17$ vs. $r=0.26$) (12). However, when weight at 1 year of age is correlated with BMC at the LS, this relationship is stronger in men than in women ($r=0.17$ vs. $r=0.13$) (12). These studies suggest that long-term, females may require an intervention to reverse the effects of IUGR, while in males, early catch-up growth is important for optimal bone mass.

Being born IUGR results in low bone mass and/or accelerated bone turnover immediately post-partum and during childhood and young adulthood (6, 108, 237). IUGR infants at birth are reported to have 20-60% lower BMC at the humerus, radius, and LS. WB BMC and mineral density (BMD) are also 10-36% lower (8) and reduced WB BA and BMC persist at 6 mo of age (9). While small bones are predictable for low birth weight babies, there is evidence that abnormalities in bone remodeling persist in young adulthood despite achievement of normal body size. At 20 y of age, a pivotal time for attainment of peak bone mass, IUGR males have 20% higher serum osteocalcin concentrations than those born a healthy weight (98), suggesting a higher rate of bone turnover. To

optimize peak bone mass achievement and inhibit incidence of osteoporosis, reversing the effects on bone of a nutritionally compromised *in utero* environment in early life is important. Long-chain polyunsaturated fatty acids (LC PUFA) are a component of the neonatal diet that has the potential to reset the trajectory of bone mass accretion across the lifespan.

Long-chain fatty acids are postulated to promote optimal bone health, especially during growth. Feeding docosahexaenoic acid (DHA) with or without arachidonic acid (AA) enhances calcium absorption by 20%, elevates WB BMC and BMD by 5% and femoral and LS BMD by 8-12% in weanling rats and piglets (18, 157, 238). It is presently unknown whether AA and DHA have similar benefits in human bone compromised by IUGR. There is evidence that providing formula with 0.2% (w/w) DHA to preterm infants reduces body weight and length by 4-10% in males (239). When combined with AA, DHA enhances total lean body mass by 6% and reduces fat mass by 20% in both males and females (128), suggesting these LC PUFA are mediators of growth and body composition for IUGR infants.

AA and DHA are promising candidates to improve bone mass in IUGR neonates. While they can be made endogenously and are obtained through breast milk, IUGR infants have lower rates of endogenous LC PUFA synthesis (21, 240). AA tissue concentrations are positively correlated with bone (135) and thus it is postulated that inadequate endogenous synthesis and stores of AA and DHA may exacerbate the long-term sequelea of IUGR on bone mass.

There is consensus that lactating women should consume 200 mg or 0.2% of dietary fat as DHA for optimal health of the infant (241). It is thought that dietary intake of AA is adequate for lactating women, necessitating only a DHA recommendation (241). To establish the benefits of an LC PUFA supplement on peak bone mass in humans would require 25 to 40 y of study. Such question is suitable to address in an animal model of IUGR that takes only months to reach

peak bone mass. For animals however, a source of AA is uncommon, necessitating supplementation of both AA and DHA to replicate the most current recommendations for lactating mothers. Given this, the objective of the study was to determine the effects of a maternal lactation diet containing 0.5% of AA and 0.2% of DHA on bone mass and metabolism, body composition, and tissue fatty acids from birth until 16 wk of age in IUGR offspring.

3.4 Materials and methods

Animals and diet

The pigmented guinea pig was the animal model selected for this study. The guinea pig fetus mineralizes *in utero*, enabling assessment of bone mass immediately following birth. In comparison to other small animal models, guinea pigs have a long gestation (70-80 d), ensuring lengthy exposure to the intrauterine environment (46). Additionally, guinea pigs have similar BMC g/kg body weight to infants, making the guinea pig a suitable model of bone in the human infant.

Ethical approval for this study was obtained from the Macdonald Campus Facility Animal Care Committee of McGill University. All procedures followed the Canadian Council on Animal Care guidelines (242). Female guinea pig sows (n=40) and males boars (n=10) were purchased from Elm Hill Laboratories (Chelmsford, MA, USA) at 4 wk of age and acclimatized for 8 wk. Prior to mating, sows were randomized to receive one of two pregnancy diets, a purified control (C) or low-protein (LP) diet. They were also randomized *a priori* to one of two lactation diets, the C diet or the same diet enriched with LC PUFA (AA+DHA). Sows were randomized to a male boar and mated at 12 wk of age. The pregnancy diet (diet during gestation) was provided at the onset of mating. Following confirmation of conception, sows were separated from the boar and housed individually until delivery, at which time the lactation diet was immediately provided. Sows were maintained on the lactation diet for 21 d. At weaning, pups were fed a C diet (Harlan Teklad 2041, Madison, WI, USA) which was nutritionally identical to the C diet for sows, but was not purified. This was to maintain health over the long term. Pups were housed in same sex pairs until 16 wk post-partum. Rooms were maintained on a 12 h light-dark cycle at 22-24°C.

The purified diets were purchased from Purina Testdiet (Richmond, IN, USA). The C diet was suited for growth and reproduction following AIN-93G specifications (243). The LP diet was balanced for energy and mineral content,

but contained half the protein than the C diet (**Table 1**). The AA+DHA diet was also balanced for energy and mineral content. The LC PUFA sources were ARASCO® and DHASCO® (Martek Biosciences, Columbia, MD, USA). AA and DHA was provided in a 2.5:1 ratio per kg of diet, or as 0.5% and 0.2% of dietary lipid respectively (**Table 1**). All diets were apple flavored to prevent the sows from perceiving changes in diet, which is known to cause stress in this species (57). Investigators were blinded to the identity of diet groups through letter coding. All test diet was kept frozen to preserve the LC PUFA and sows were provided with fresh diet every 24 hr to ensure minimal oxidation of the LC PUFA.

Sampling

Pup body weight was measured weekly for the entire study duration. At time of DXA, in an anaesthetized state, body length was measured from nose to rump. Food disappearance per cage was monitored thrice weekly for sows during pregnancy and during lactation.

When a litter had more than 2 pups, the pups were randomized to the study endpoints of d 3, d 21 or to 16 wk. Pups randomized to d 3 or d 21 were removed at those ages to provide for bone strength assessment. Where feasible, 1 male and 1 female from each litter remained in the study. Pups removed at d 3 and d 21 were anaesthetized with isoflurane (Aerrane, Baxter, Deerfield, IL, USA) prior to cardiac puncture. The femurs, tibias, and lumbar vertebra (LS) were removed, cleaned of soft tissue, wrapped in 0.9% saline soaked gauze and stored at -20°C for analysis.

In the pups studied to wk 16, blood samples were taken from the saphenous vein at d 3, d 21, 8 wk, and 16 wk post-partum. Serum and plasma (lithium heparin) were obtained after centrifugation (1800 G for 20 min) and stored at -80°C. Red blood cells (RBC) from the plasma sample were then rinsed with 0.9% saline to

remove additional plasma, flushed with nitrogen, and stored at -80°C for a maximum of 28 d before analysis for fatty acid content.

Bone quality assessment

In an anesthetized state, bone mass was measured for the WB, LS, femur, and tibia using DXA (small animal software package; 4500A, Hologic Inc., Bedford, MA, USA) at d 3, d 21, 8 wk, and 16 wk post-partum. Body composition was also assessed by DXA. All scans were analyzed by the same investigator (LB). Average CVs were 2.5, 3.0, and 5.7 for replicate BMC scans of the whole body, regional right femur, and scan analysis of the right femur respectively.

Pups removed at d 3 and d 21 were also scanned using DXA in an identical manner as above prior to necropsy. Excised femurs and tibias were measured for length, diaphysis width, head width (femur only), neck width and height (femur only), and the width of the distal diaphysis (knee) using digital calipers (Control Company, Friendswood, TX, USA). Biomechanical tests were performed using an Instron 5544 (Instron, Canton, MA, USA). Bone strength was only assessed in a sub-sample of pups, genders pooled, terminated at d 3 and d 21. For all bone strength measurements n=9 for d 3 (5 LP, 4 C) and n=24 for d 21 (6 per each combined pregnancy x lactation diet group). Prior to all tests, bones samples were warmed and hydrated for 10-15 min in 37°C 0.9% saline. For the long bones, the middle of the diaphysis was measured and marked and the bone placed between 2 supports spanning 12 mm. The anvil was aligned at the middle of the diaphysis and a load applied (0.05 mm/min) until a fracture in the long bone was detected. For femoral neck strength testing, the proximal end of the femur was mounted in resin (Coltene Whaledent, Cuyahoga Falls, OH, USA) and a load was applied (0.05 mm/min) to the femoral head until fracture of the neck. For these tests, the head remained intact and there was no visible evidence of compression. LS strength was assessed by removing the cortical processes from the vertebral bodies and isolating the 4th and 5th vertebrae. The 5th vertebra was mounted in resin (as above) to provide stability during the compression test and load was

applied (0.05 mm/min) until full compression of the 4th vertebral body. All results were obtained from the load deformation curve and calculated using Bluehill software (Version 2.0, Instron, Canton, MA, USA).

Biochemistry

Plasma osteocalcin was measured in duplicate using an ELISA (MetraOsteocalcin, Quidel, San Diego, CA, USA). This assay is validated for measuring osteocalcin in guinea pigs (244). Serum total deoxypyridinoline (DPD) was also measured with an ELISA (MetraDPD, Quidel, San Diego, CA, USA) and inter-assay CVs were 3.2 and 3.0 for each ELISA respectively. This assay is known to have cross-reactivity with guinea pig DPD according to manufacture specifications.

Fatty acids from RBC were extracted using a modified Folch technique and methylated with methanolic HCl for 1 h as previously described (151). Methyl esters were identified using gas chromatography (Varian, CP 3800, Varian Inc, Cary, NC, USA). For each sample, 1µl was injected in splitless mode at 200°C, with a column run time of 47 minutes and final oven temperature of 350°C.

Statistical analysis

The sample size estimate of n=80 pups (n=20 per group) was based on the ability to detect a 5% difference in femur and whole body BMC with a power of 0.80 and alpha of 0.05. It was assumed sows would deliver a minimum of 2 pups per litter, hence a sample of n=40 sows. A factorial ANOVA was performed to determine differences between diet groups for sows. For pups, a mixed model ANOVA was used with fixed effects of time (d 3, d 21, wk 8, wk 16), gender (male, female), and pregnancy diet (control, LP), and lactation diet (control, AA+DHA) and the random effect of the error associated with all 4 fixed effects. The litter size was tested in the model as a covariate, but found to have a negligible affect on the fit of the model and was therefore not included. Data was tested for normality using a Shapiro-Wilk test and residuals were plotted for

random distribution. Post-hoc tests were adjusted using Bonferroni's correction. All statistics were analyzed using SAS, Version 9.1 (Cary, NC, USA) and $P<0.05$ was considered significant. Results presented are mean ± SD (text, tables) or SEM (figures).

3.5 Results

Pregnancy and delivery

Thirty-two sows conceived and delivered healthy pups. Of those that did not, 2 sows died prior to conception from causes unrelated to the study, 3 died at/prior to d 7 post-partum from delivery complications and 3 sows either did not conceive or delivered stillborn litters and were excluded from the study. Average daily food intake in the last 3 wk of pregnancy (approximately the 3rd trimester) did not differ between groups (C: 42.2 ± 20.7 vs. LP: 37.3 ± 17.1 g/d, P=0.4) nor did average weekly weight gain during this time (C: 64.3 ± 31.0 vs. LP: 57.9 ± 22.2 g/wk, P=0.5). The effect of the AA+DHA diet on the sows is reported in **Paper 2.**

The number of live-born pups per litter that survived beyond 48 h was significantly lower in sows fed the C diet than those fed the LP diet (2.2 ± 0.9 vs. 3.5 ± 1.1 pups, P=0.02). Following birth, 2 pups died within 48 h of delivery and were not included in the data analysis. All pups that survived 48 h appeared healthy for the study duration and a total of 56 pups were followed to study completion.

Body weight and length

Main effects of the pregnancy (LP) diet (P<0.0001) and time (P<0.0001) were observed, and an interaction between these variables (P=0.05) showed the effect of the LP diet was not present at d 3, but caused lower weights thereafter (**Table 2a**). The LP diet did not interact with gender for body weight. A main effect of the lactation diet was not observed (P=0.79) and did not interact with the LP diet (P=0.09), time (P=0.17) or gender (P=0.85). A main effect of gender (P=0.002) was observed in addition to time as stated above, but an interaction between these (P<0.001) showed mean body weight was the same in males and females at d 3 and d 21, but thereafter was greater in males than females (**Table 3a**).

For length, main effects of the LP diet (P<0.0001), lactation diet (P=0.23), time (P<0.0001) and gender (P=0.52) were superseded by a 4-way interaction (P=0.0006, **Figure 1**). This showed that the LP diet resulted in reduced body length at d 3 in both males and females, but by d 21 the males continued to be shorter unless they received the AA+DHA diet that caused recovery of length at d 21 and wk 16. For females, those in the LP diet group showed temporary catch-up growth at d 21 regardless of diet during lactation. Additionally the interaction showed that in females, the AA+DHA supplement resulted in reduced length for control pups at 8 and 16 wk, while it enhanced length in the LP pups at wk 8 and 16. Body length also increased from d 3 to wk 16, but did not differ overall (P=0.05) between males and females (**Table 3**).

Body composition

The pregnancy diet had a main effect on lean body mass (P<0.0001) and an interaction with time (P<0.027) showed that the LP group had similar lean mass as the C group at d 3, but thereafter had lower values (**Table 2a**). The lactation diet did not have a main effect on lean mass (P=0.84) or interaction effects with pregnancy diet (P=0.06), time (P=0.27) or gender (P=0.68). Lean mass also increased as a function of time (P<0.0001) and gender (P<0.001), but an interaction (P<0.001) showed that increments were observed at each time-point while the effects of gender were not observed until 8 wk of age (**Table 3a**). No other interactions were observed for lean body mass.

The pregnancy (P=0.54) and lactation (P=0.87) diets did not have a main effect on body fat (%), although an interaction between the pregnancy diet and time (P<0.04) revealed that values were lower in the LP group at d 3, not different at d 21 and wk 8 and eventually higher than the C group by wk 16 (**Table 2a**). Pregnancy (P=0.23) and lactation (P=0.42) diets did not interact with gender. Main effects of gender (P=0.0001) and time (P<0.0001) interacted (P<0.0001) such that fat (%) did not differ between males and female until wk 8 and wk 16

and that values did not significantly increase between d 3 and d 21, but did so thereafter (**Table 3a**).

Whole body (WB) bone mass

There was a main effect of time ($P<0.0001$) and the pregnancy diet ($P<0.0001$) on WB BA and an interaction between time and the pregnancy diet indicated there was transient catch-up in the LP group at wk 8 (**Table 2a**). There were no main ($P=0.84$) or interaction effects with time ($P=0.21$), gender (0.81) or the pregnancy diet ($P=0.15$) for the effect of the lactation diet on WB BA. Gender ($P=0.0003$) had a main effect on WB BA and in an interaction with time, genders differed only at wk 8 of life (**Table 3a**).

Like WB BA, the LP diet reduced WB BMC at all time points except wk 8 (**Table 2a**) and there was no interaction between the LP diet and gender ($P=0.17$). There were no main ($P=0.95$) or interaction effects of the lactation diet with time ($P=0.51$), gender ($P=0.48$) or the pregnancy diet ($P=0.36$) on WB BMC prior to adjustment for body weight. Overall, males had higher WB BMC than females (**Table 3a**).

After adjustment for body weight, the C and LP pups did not differ overall in WB BMC ($P=0.93$). There was an interaction between the pregnancy and lactation diets, time, and gender. In males, the LP pups that received the AA+DHA through the sows' milk during lactation had higher adjusted WB BMC than the LP pups that received the C diet but this difference was not observed in females (**Figure 2**).

WB BMD was reduced by the LP diet ($P=0.005$, **Table 2a**) and overall, was higher in males than females (**Table 2a**). Further interactions between time, gender and the pregnancy diet showed that the effect of the LP diet was gender-specific, whereby at d 21 WB BMD was reduced in LP males (C: 0.156 ± 0.007 vs. LP: 0.142 ± 0.008 g/cm^2, $P=0.003$), but not females (0.148 ± 0.007 vs. $0.146 \pm$

0.008 g/cm^2, P=0.48). This finding was observed at d 21 post-partum only. There were no main (P=0.12) or interaction effects of the lactation diet on WB BMD.

Regional bone mass measurements

Femur

There was a main effect (P<0.0001) of the pregnancy diet on femur BA (**Table 2b**) and an interaction with gender (P=0.008) showing LP females had only a 5% lower femur BA (1.0 ± 0.4 cm^2) than C females (1.1 ± 0.4 cm^2), which was not statistically significant (P=0.28) whereas LP males had 14% lower (P<0.0001) F BA (0.9 ± 0.4 cm^2) than C males (1.1 ± 0.4 cm^2). Therefore, the LP diet appeared to have a greater detriment in males than females. There was no main effect of the lactation diet on femur BA but there was an interaction between gender, time, and the lactation diet (P=0.05). At wk 8, females had lower femur BA if were nursed by sows fed the AA+DHA diet (1.4 ± 0.1 vs. 1.3 ± 0.1 cm^2, P=0.05) but boys tended to be higher with AA+DHA enrichment (1.3 ± 0.17 vs. 1.4 ± 0.15 cm2, P=0.1). There was no main effect of gender for femur BA (**Table 3b**).

There was a main effect of the pregnancy diet on femur BMC (P=0.0002), but not femur BMD (P=0.09) and the pregnancy diet did not interact with time for both measurements (P>0.05, **Table 2b**). In an interaction with gender (P=0.008), the LP diet had a greater effect on femur BMC in males, whereby male LP pups had 16% lower femur BMC than male C pups (C: 0.40 ± 0.31 g vs. LP: 0.33 ± 0.29 g, P<0.0001), while female LP pups were only 9% lower than female C pups (C: 0.36 ± 0.26 vs. LP: 0.33 ± 0.26 g, P=0.18). The lactation diet had no effect on femur BMC but for femur BMD the LP pups that received the AA+DHA did not differ from C pups while LP pups that did not receive AA+DHA remained compromised (**Figure 3**). Unlike femur BA, femur BMC was higher in males than females (P<0.0001), as was femur BMD (P<0.0001). Further interactions indicated that genders differed at wk 8 and 16 only for femur BMC, and at all time points except d 3 for femur BMD (**Table 3b**).

Tibia

The LP diet reduced all tibia bone mass measurements (**Table 2b**). There were no interactions between the pregnancy diet and time (P=0.46), gender (P=0.76) or lactation diet (P=0.73). For tibia BMD there was a significant 3-way interaction (P=0.003) between time, gender, and pregnancy diet, whereby LP males were significantly lower than C males at d 3 (P=0.0003) and d 21 (P=0.004), but C females did not differ that LP females at any time point prior to wk 16 (P=0.001, **Figure 4a**). There were also no main (P=0.14) or interaction effects of the lactation diet on tibia BA or BMC. However, for tibia BMD there was a significant interaction between the lactation diet and gender (P=0.02) that was superseded by a 3-way interaction between time, gender, and the lactation diet. This interaction indicated that groups did not differ prior to wk 16, at which time the C females had significantly lower tibia BMD than females that received the AA+DHA diet, but males did not differ (P=0.001, **Figure 4b**). There was a main effect of gender for all tibia bone mass measurements (P<0.0001) and in an interaction with time, males and females differed only at 16 wk of life for tibia BA (**Table 3b**), and at 8 wk and 16 wk for tibia BMC (**Table 3b**). Tibia BMD did not differ between genders (**Table 3b**).

Lumbar spine

Lumbar spine (LS) bone mass was reduced by the LP diet (P<0.001) and interacted with time for BMC and BMD, whereby groups differed at wk 8 and wk 16 but not before (**Table 2b**). For LS BA, there was a greater detriment of the LP diet observed in males, whereby C males had 10% higher BA than LP males (1.5 ± 0.5 vs. 1.3 ± 0.6 cm^2, P=0.01), and C females had 7% higher BA than LP females (1.4 ± 0.5 vs. 1.3 ± 0.5 cm^2, P=0.05). There was no main effect of the lactation diet (P=0.14), but there was a 2-way interaction between the lactation diet and time (P=0.01), which was superseded by a 3-way interaction between time, and both pregnancy and lactation diet (P=0.04). This indicated that the C pups fed the AA+DHA during lactation had higher LS BA at wk 8 than the C

pups fed the C diet during lactation while no effects of the AA+DHA diet were observed in the LP pups at this time. At wk 16 this observation persisted in the C pups and AA+DHA fed LP pups did not differ significantly from C pups (**Figure 5**). A main effect of the lactation diet was observed for LS BMC (P=0.05), whereby all AA+DHA fed pups had 4% higher BMC than C pups (C: 0.29 ± 0.18 vs. AA+DHA: 0.30 ± 0.19 g). With a significant interaction with time (P=0.002), the AA+DHA diet elevated LS BMC at wk 16 of life (C: 0.6 ± 0.1 vs. AA+DHA: 0.7 ± 0.1 g) but were no significant increases at previous time points. There were no main (P=0.64) or interaction effects of the lactation diet on LS BMD. For all LS bone mass measurements, males had greater bone mass than females and for BMC and BMD, gender interacted with time so that at wk 8 and wk 16 males and females were significantly different (P<0.001, **Table 3b**).

Biochemistry

Blood markers of bone metabolism decreased over time (data not shown). Osteocalcin was 60% lower at wk 16 than d 3 and similarly, DPD was 50% lower a wk 16 than d 3. There was no main effect of the pregnancy diet (P=0.24) but in an interaction with time the LP diet reduced osteocalcin at d 3 only (C: 53.1 ± 15.7 vs. LP: 44.6 ± 11.6 nmol/L, P=0.01) but did not differ at other time points. There was no main effect of the lactation diet (P=0.57) but in an interaction with time, the AA+DHA pups had lower osteocalcin than C pups at d 3 only (52.9 ± 14.4 vs. 44.7 ± 13.2 nmol/L, P=0.005). There was also a 2-way interaction between the lactation and pregnancy diets. For the pups given the C diet *in utero*, the AA+DHA diet significantly lowered osteocalcin concentrations to values that did not differ from the LP pups (**Figure 6**). There was a main effect of gender on osteocalcin to show males had higher concentrations than females (30.7 ± 13.7 vs. 34.4 ± 15.1 nmol/L, P=0.03).

For DPD, there was no main effect of the pregnancy diet (P=0.12) but there was a significant interaction with time (P=0.004) to show that DPD was highest in C pups at d 3 (C: 12.1 ± 2.9 vs. LP: 10.6 ± 3.3 nmol/L) and at all other times C and

LP pups did not differ in this bone resorption marker. There was no main effect of the lactation diet (P=0.24) or gender (P=0.89), but in an interaction between the lactation diet and gender (P=0.03) the AA+DHA diet reduced serum DPD by 20% in females (C: 6.82 ± 3.55 vs. AA+DHA: 6.30 ± 2.01 nmol/L, P=0.01), but there was no difference in males (C: 6.60 ± 2.6 vs. AA+DHA: 6.49 ± 3.01 nmol/L, P=0.07).

RBC AA and DHA concentrations both differed with time, with levels steadily increasing from d 3 to wk 8, but at wk 16 concentrations were no different from those at wk 8 (data not shown). There were no main or interaction effects of the LP diet on RBC AA and DHA (data not shown). For the lactation diet effects, there was a trend towards elevated RBC AA in all pups that received the AA+DHA diet (C: 10.6 ± 2.6 vs. AA+DHA: 11.0 ±2.1 g/100g, P=0.07). Overall, RBC DHA concentrations were 30% higher in the AA+DHA fed pups (C: 0.364 ± 0.175 vs. AA+DHA: 0.508 ± 0.155 g/100g, P<0.0001). Furthermore, pups fed AA+DHA had elevated RBC DHA at d 21, but also at wk 16 (**Figure 7**). There was a significant difference between males and females in RBC AA (males: 10.6 ± 2.5 vs. females: 11.2 ± 2.3 g/100g, P=0.02), but not DHA (0.443 ± 0.150 vs. 0.439 ± 0.201 g/100g, P=0.09). Both AA and DHA were elevated by 40% and 80% respectively in sow milk for those sows fed the AA+DHA diet and this is reported in greater detail in **Paper 2**.

Bone biomechanics

Only main effects of diets were tested owing to lack of repeated measures and inability to test for gender effects in the smaller sub-sample.

Femur

The LP diet did not alter femur wet weight, length, or any morphometric measurements at d 3 or d 21 (data not shown). The lactation diet group was not different that controls at d 3 for any morphometric measurements (data not shown). At d 21 the AA+DHA diet group had reduced femur wet weight (C:

0.349 ± 0.060 vs. AA+DHA: 0.267 ± 0.070 g, P=0.03) and length (24.0 ± 1.4 vs. 22.1 ± 1.5 mm, P=0.04) but no other measurements were affected (data not shown). The LP diet affected Young's modulus, load and extension at break, and ultimate stress (**Table 4**). There was an interaction between the pregnancy and lactation diets at d 21 for the femur whereby C pups that received the AA+DHA diet during lactation had a significantly lower load at break than other groups (**Figure 8a**).

Tibia

There was no effect of the LP diet on tibia measurements or wet weight (data not shown). The lactation diet group was not different than controls at d 3 (data not shown) but the AA+DHA diet decreased diaphysis width (2.00 ± 0.20 vs. 1.81 ± 0.23 mm, P=0.01) and there were no interaction effects of the diets (data not shown). The LP diet reduced strain and extension at break, but elevated Young's modulus, maximum stress, and load at break (**Table 4**).

4th Vertebral body

The height of the 4th vertebral body was unaffected by diet (**Table 4**). Young's modulus for the stress vs. strain curve was reduced by the LP diet (**Table 4**) but this did not reach significance. Maximum compressive load and stress were also reduced by the LP diet (**Table 4**). There were no effects of the lactation diet (data not shown).

Femoral neck

There was no effect of diet on neck size (**Table 4**). There was no main effect of the pregnancy diet (**Table 4**). The AA+DHA fed pups at d 21 had higher maximum strain (C: 0.016 ± 0.005 vs. AA+DHA: 0.030 ± 0.011 kgf, P=0.008) and Young's modulus (C: 31.4 ± 12.0 vs. AA+DHA: 43.8 ± 20.2 MPa, P=0.03) regardless of pregnancy diet. An interaction between the diets for Young's modulus was observed in that the C pups that received the AA+DHA diet had the highest modulus (**Figure 8b**).

3.6 Discussion

The AA and DHA content of the early-life diet is now recognized as a factor that affects optimal bone development. Fish oil fed to young chicks enhances tibia bone mass (156), but may reduce vertebral bone strength in female rats (159). Recent research young adults shows that DHA enriched diets reduce bone formation, elevate bone resorption and serum 25-OH-vitamin D (161). Studies providing both AA and DHA to suckling piglets have identified that these lipids when given together, elevate whole body and regional bone mass. Because these LC PUFA can change tissue lipid profiles, well designed animal trials advise that the optimal ratio of dietary AA:DHA is 5:1 (14, 164). It is also clear that in human neonates, both AA and DHA are required for optimal growth (239). This study is the first to examine the effect of LC PUFA on IUGR compromised bone mass. Because recommendations are now emerging for lactating women to consume 0.2% of dietary fat as DHA (241) investigation is needed to determine if AA and DHA have prolonged effects on bone mass.

Intrauterine growth restriction was achieved using the LP diet. Pups were shorter at birth and beyond and body weight and bone mass were reduced across 16 wk of life. Body weight did not significantly differ between groups at birth, suggesting that the pups were not born prematurely. Lower lean body mass accompanied by 5-8% greater body fat percentage was also observed in the IUGR pups, which is consistent with previous observations in growth restricted fetal guinea pigs (43) and in infants and children born small (100). At 16 wk, or 112 d of age, the LP diet had a more detrimental effect on BA in males than females. Similar gender differences were observed by Kind and colleagues studying the effect of *in utero* programming in the multi-coloured guinea pig using a calorie restricted model. In this model, there was an effect of growth restriction on systolic blood pressure (53) and glucose tolerance (51) in males, but not females.

The lactation diet intervention affected both healthy and compromised bone mass. There was an overall outcome of the AA+DHA diet to elevate LS BMC by 5% overall and by 8% at wk 16 of life and tibia BMD by 10% in both genders. It is significant that BMC was elevated in these regions by the diet intervention, as the LS is a frequent site of mineral loss, fracture and compressive deformities in children with poor bone quality (245).

This is the first known report of positive outcomes in bone mass following a diet intervention during the suckling period only. This is evidence that the fatty acid composition of maternal milk has an effect on bone development from birth to sexual maturity. Prior investigations providing AA and DHA in formula to healthy early-weaned pigs (for 15 d) readily observed positive outcomes in bone post-supplementation (158, 164). This study observed immediate positive effects of the AA+DHA diet on body length, femur bone mass and bone formation, but also observed further benefits to tibia BMD and LS bone mass emerge at wk 16 of life. This is evidence that pre-weaning diet supplementation with AA and DHA has sustained benefits after the supplement is withdrawn. This is of interest given that further benefits of AA and DHA became apparent at a time post-puberty when the achievement of peak bone mass is of concern (246).

Not only did the AA+DHA diet positively affect uncompromised bone mass, but also body size and bone that was compromised by IUGR. Body length was lower in IUGR pups not receiving AA+DHA, while those that received the AA+DHA were not different from the controls. Body size is an important predictor of BMD in children and adolescents and may have increased in the LP pups as a result of improved growth. AA and DHA are known to elevate lean mass in pre-term infants (128) and similarly, this study reports catch-up growth in body length. Because short stature may be associated with morbidity in young life (247) this may be a clinically relevant finding. Furthermore, IUGR infants often have rapid gain in weight but height does not follow the same pattern, which contributes to a predisposition to obesity later in life (248). This study suggests that AA and

DHA may assist with achieving normal linear growth, which may help reduce morbidities and mortality amongst the IUGR population.

The AA and DHA had a moderate affect on bone metabolism. The LC PUFA diet reduced serum DPD notably (20%) in females, which may have contributed to higher tibia BMD. Furthermore, in control pups the AA+DHA diet reduced plasma osteocalcin, which suggests overall reduced bone turnover. In LP pups, the opposite effect was observed. Osteocalcin was elevated in these pups to a mean value not significantly different from controls, suggesting the AA+DHA diet normalized bone formation in IUGR pups. This observation is highly relevant in the IUGR neonate, as IUGR rat pups are reported to have abnormal bone modeling (89). Whole body BMC, femur BMD, and LS BA were increased by 5%, 2%, and 4% respectively in the LP pups that received the AA and DHA. Similar to findings that AA and DHA elevates whole body bone mass in healthy piglets (18) this study now shows similar benefits to bone compromised by IUGR and a possible resetting of the trajectory of bone mass. Though the positive findings in our study should not be overlooked, we report lower magnitudes of change than previous research. In rodents, DHA elevated femur and LS BMD by 7-8% (17), and in piglets, AA alone or a 0.6:0.1 ratio AA:DHA elevated WB and femur bone mass by 15-20% (18, 164). In agreement with our findings, Groh-Wargo and colleagues report that AA and DHA (2:1) fed to preterm neonates had no effect on WB BMC or BMD (128). This study may have found greater magnitude of change had we met the desired sample size (n=20 pups per group) or had less variability between animals. It is difficult to determine whether the small magnitude of change is due to the IUGR model, or from the amount and ratio of the fatty acid supplement.

In all pups, the AA and DHA was well incorporated into tissues by elevating RBC DHA by 56% and 20% at d 21 and wk 16 respectively. It is evident that there was preferential incorporation of DHA into the RBC, which is known to occur in infant metabolism (140, 239). Whether or not there would have been a greater

effect of the supplement observed in bone if the RBC DHA concentrations were lower is unknown. As previously stated, supplementing DHA alone and up-regulating the elongation of n-3 fatty acids is detrimental to growth in infants (239), to bone mass in young adults (161), and impairs bone strength in young mice (41, 159). Conversely, the improvements in body length and bone mass observed at wk 16, post-supplementation, may have been resulting from the higher DHA concentration in the RBC. In older male rats, ovariectomized rats, and post-menopausal women, DHA and other long-chain n-3 fatty acids elevate bone mass (15, 23, 134). Additionally, epidemiological studies associated high consumption of fish oils with higher radial BMD in women, and overall a greater intake of n-3 fatty acids is associate with greater total hip BMD in older men and women (146). Why the elevation at wk 16 occurred is unclear, as RBC fatty acid profiles should represent the last 3-4 wk of fatty acid consumption in the diet. Perhaps the supplement altered the production of enzymes responsible for endogenous elongation of AA and DHA precursors, so these pups had the propensity towards higher circulating DHA and lower AA concentrations post-weaning. While it would be optimal to measure the fatty acid concentrations in other tissues, RBC fatty acid profiles are a fairly good estimation of the concentrations in other tissues (26).

Measurements of bone strength suggest that the LC PUFA was detrimental to bone quality by reducing the load required to fracture the long bones. Previous studies report a negative, but transient, effect on femur strength and strength of the 5th vertebral body following supplementation with fish oil in young male and female mice (41, 159). In the present study, the vertebral body strength was improved by AA and DHA (assessed by Young's modulus) in the control pups. Alike the findings for bone mass, perhaps in this study the amount of DHA in the diet was too high to observe optimal benefits to bone. Furthermore, the bone strength results in this study must be interpreted with caution because of the small sample size.

The guinea pig was a good model in which to observe the emergence of gender differences at sexual maturity. Both males and females were considered sexually mature by 12 wk of life, though females can be bred as early as 4 wk of age, maturing earlier than males. The results from this study suggest that the LP and AA+DHA diet affected genders differently. Males appeared to be compromised by the LP diet earlier in life (d 21) and catch-up with or without intervention later in life (wk 16). Conversely, females appeared to be uncompromised early in life (d 21), but compromised at an older age (wk 16). For whole body BMC, the males appeared to require the LC PUFA intervention to achieve an uncompromised BMC relative to controls, but in females, this was not seen. These observations may have been a result of the timing of sexual maturity. Estrogen production in females begins to elevate around d 21 of life and continues to increase throughout sexual maturity, whereas males likely experienced elevated sex hormones later, by wk 8 post-partum (49). Perhaps the increase in female sex hormones served to rescue bone mass and growth in the IUGR pups so that the AA+DHA supplement was not needed at this time. Males may require the intervention due to later production of testosterone, known to benefit bone mass.

In conclusion, this study supports the growing body of literature that identifies LC PUFA as essential nutrients for optimal neonatal bone mass and indicates that the benefits to bone persist across the lifespan. Recently, there is consensus that lactating women should consume 0.2% of dietary fat as DHA (241), and this study suggests that when consumed with 0.5% of dietary fat as AA, there are benefits to growth and bone mass that persist into post-pubertal age. Most importantly, AA and DHA appear to rescue the trajectory of growth, femur bone mass, and bone mineralization in neonates born following *in utero* growth restriction (241). While the multi-coloured guinea pig is an appropriate model in which to study the effects of *in utero* growth restriction on bone and early life intervention, future clinical studies in neonates are essential to confirm the findings in this study.

3.7 Acknowledgements

Funding for this research was provided by the Natural Sciences and Engineering Research Council of Canada (NSERC) and funding for DXA equipment provided by the Canada Foundation for Innovation and Canada Research Chairs Program. L. Burr received a Canada Graduate Scholarship from NSERC. The authors would like to thank Mr. Gordon Bingham for assistance with animal care and Dr. Thomas Brenna of Cornell University for supplying cages for animal housing, as well as Dr. Atul Sharma for providing statistical assistance. ARASCO® and DHASCO® were graciously donated by Martek Biosciences.

3.8 Tables and figures for Paper One

Table 1: Diet composition

Ingredient[1]	Control	Low-protein	AA+DHA
Soy protein	161.0	80.5	161.0
Casein	53.0	26.5	53.0
L-Cystine	0.5	0.5	0.5
L-Methionine	3.0	3.0	3.0
Corn starch	217.9	325	217.9
Cellulose	140.0	140.0	140.0
Sucrose	290.0	290.0	290.0
Soybean oil	60.0	60.0	60.0
[2]ARASCO® oil (38-44% AA)	None	None	0.8205
DHASCO® oil (40-45% DHA)	None	None	0.3283
Ascorbic acid, coated (97.5%)	2.3	2.3	2.3
Vitamin Mix	10.0	10.0	10.0
Potassium acetate	20	20	20
Calcium phosphate	14.8	14.8	14.8
Calcium carbonate	12.1	12.1	12.1
Potassium citrate	7.0	7.0	7.0
Magnesium oxide	5.0	5.0	5.0
Sodium chloride	3.0	3.0	3.0
Ferric citrate	0.6	0.6	0.6
Manganese sulfate	0.3	0.3	0.3
Zinc carbonate	0.11	0.11	0.11
Cupric sulfate	0.03	0.03	0.03
Chromium potassium sulfate	0.01	0.01	0.01
Potassium iodate	0.01	0.01	0.01
Sodium selenite	0.0004	0.0004	0.0004
tert-butylhydroquinone	0.012	0.012	0.012

[1]Ingredients g/kg of diet. [2] 0.3118 g of AA or 0.5% of dietary fat and 0.1313 g of DHA or 0.2% of dietary fat. Thus a ratio of approximately 2.5:1 AA:DHA

Table 2a: The effect of the pregnancy diet on body composition and whole body bone mass over time.

Measurement[1]	Diet	Day 3	Day 21	Wk 8	Wk 16	P value		
						Time	Diet	T x D
Body weight (g)	Control[2]	110±17[a]	303±39[b]	616±69[d]	911±98[f]	<0.0001	<0.0001	0.05
	LP	89±16[a]	267±31[c]	576±109[e]	840±116[g]			
Body length (cm)	Control	17.8±0.63[a]	23.3±1.4[b]	29.5±2.2[c]	33.4±1.4[e]	<0.0001	<0.0001	<0.0001
	LP	12.7±1.6[a]	22.9±1.9[b]	28.0±1.5[d]	31.8±2.1[f]			
Lean body mass (g)	Control	93.1±13.9[a]	263±31[b]	532±58[d]	743±88[f]	<0.0001	<0.0001	0.02
	LP	77.5±14.1[a]	229±25[c]	492±87[e]	682±87[g]			
Body fat (%)	Control	15.5±3.4[a]	13.0±2.2[b]	13.5±2.1[b]	18.4±3.9[c]	<0.0001	<0.0001	0.01
	LP	14.3±3.0[b]	14.1±2.1[b]	14.3±3.5[b]	19.0±4.4[d]			
WB BA[3] (cm^2)	Control	27.9±3.6[a]	49.5±4.1[c]	78.0±6.6[e]	102.8±8.5[f]	<0.0001	<0.0001	<0.0001
	LP	23.1±4.1[b]	44.5±3.6[d]	76.0±8.5[e]	96.3±8.1[g]			
WB BMC (g)	Control	3.41±0.60[a]	7.61±0.97[c]	15.40±1.94[e]	23.78±2.72[f]	<0.0001	NS	0.02
	LP	2.67±0.61[b]	6.44±0.75[d]	15.08± 2.62[e]	21.83±2.80[g]			
WB BMD (g/cm^2)	Control	0.121±0.009[a]	0.153±0.008[c]	0.197±0.010[e]	0.231±0.012[f]	<0.0001	NS	0.0006
	LP	0.114±0.010[b]	0.144±0.008[d]	0.195±0.013[e]	0.227±0.015[f]			

[1]Values reported are mean ± SD. Different letters represent a significant difference between time points and pregnancy diet groups. [2]Sample size: n=26 Control, n=24 LP. [3]Abbreviations: whole body (WB), bone area (BA), bone mineral content (BMC), bone mineral density (BMD), time by diet interaction (T x D), not significant (NS).

Table 2b: The effect of the pregnancy diet on regional mass over time.

Measurement[1]	Pregnancy diet	Day 3	Day 21	Wk 8	Wk 16	P value	
						Time	Pregnancy diet
Femur BA[2] (cm^2)	Control[3]	0.7 ± 0.1^{a}	0.9 ± 0.15^{c}	1.4 ± 0.1^{e}	1.7 ± 0.1^{g}	<0.0001	<0.0001
	LP	0.6 ± 0.1^{b}	0.8 ± 0.1^{d}	1.4 ± 0.2^{f}	1.6 ± 0.13^{h}		
Femur BMC (g)	Control	0.09 ± 0.02^{a}	0.28 ± 0.08^{c}	0.57 ± 0.09^{e}	0.86 ± 0.10^{g}	<0.0001	0.0004
	LP	0.06 ± 0.02^{b}	0.22 ± 0.03^{d}	0.54 ± 0.11^{f}	0.80 ± 0.11^{h}		
Femur BMD (g/cm^2)	Control	0.127 ± 0.027^{a}	0.289 ± 0.059^{b}	0.408 ± 0.052^{c}	0.502 ± 0.049^{d}	<0.0001	NS
	LP	0.105 ± 0.025^{a}	0.272 ± 0.042^{b}	0.398 ± 0.069^{c}	0.499 ± 0.067^{d}		
Tibia BA (cm^2)	Control	0.9 ± 0.1^{a}	1.3 ± 0.1^{c}	1.8 ± 0.2^{e}	2.1 ± 0.2^{g}	<0.0001	<0.0001
	LP	0.7 ± 0.1^{b}	1.2 ± 0.1^{d}	1.7 ± 0.2^{f}	2.0 ± 0.7^{h}		
Tibia BMC (g)	Control	0.08 ± 0.02^{a}	0.20 ± 0.04^{c}	0.40 ± 0.06^{e}	0.61 ± 0.07^{g}	<0.0001	0.0002
	LP	0.05 ± 0.01^{b}	0.16 ± 0.02^{d}	0.37 ± 0.07^{f}	0.57 ± 0.08^{h}		
Tibia BMD (g/cm^2)	Control	0.089 ± 0.017^{a}	0.147 ± 0.022^{c}	0.219 ± 0.014^{e}	0.297 ± 0.062^{g}	<0.0001	0.001
	LP	0.073 ± 0.017^{b}	0.131 ± 0.012^{d}	0.216 ± 0.024^{f}	0.285 ± 0.026^{h}		
LS BA (cm^2)	Control	0.8 ± 0.1^{a}	1.4 ± 0.1^{c}	1.9 ± 0.1^{e}	2.3 ± 0.2^{g}	<0.0001	<0.0001
	LP	0.7 ± 0.1^{b}	1.2 ± 0.1^{d}	1.8 ± 0.1^{f}	2.1 ± 0.2^{h}		
LS BMC[4] (g)	Control	0.15 ± 0.04^{a}	0.21 ± 0.04^{b}	0.41 ± 0.04^{c}	0.64 ± 0.10^{e}	<0.0001	<0.0001
	LP	0.11 ± 0.02^{a}	0.18 ± 0.03^{b}	0.39 ± 0.07^{d}	0.59 ± 0.07^{f}		
LS BMD (g/cm^2)	Control	0.184 ± 0.019^{a}	0.150 ± 0.015^{b}	0.215 ± 0.020^{d}	0.280 ± 0.025^{e}	<0.0001	0.002
	LP	0.170 ± 0.022^{a}	0.143 ± 0.011^{b}	0.213 ± 0.017^{c}	0.265 ± 0.019^{f}		

[1]Values reported are mean ± SD. Different letters represent a significant difference between time points and pregnancy diet groups. [2]Abbreviations: lumbar spine (LS), bone area (BA), bone mineral content (BMC), bone mineral density (BMD), not significant (NS). [3]Sample size: n=26 Control, n=30 LP. [4]LS BMC and BMD had a significant interaction between the pregnancy diet and time, P<0.004. Differences between groups are identified by different letters.

Table 3a: Gender differences in body composition and whole body bone mass over time.

Measurement[1]	Gender	Day 3	Day 21	Wk 8	Wk 16	P value Time	P value Gender	P value Interaction
Body weight (g)	Male[2]	99 ±21[a]	285±40[b]	620±99[c]	921±122[e]	<0.0001	0.002	<0.0001
	Female	100±18[a]	285±40[b]	565±77[d]	826±75[f]			
Body length (cm)	Male	14.9±3.0[a]	22.8±1.6[b]	29.6±2.2[c]	33.3±1.8[d]	<0.0001	NS	NS
	Female	15.4±2.7[a]	23.4±1.6[b]	28.0±1.5[c]	31.7±1.7[d]			
Lean mass (g)	Male	83.7±15.1[a]	247±33[b]	540±75[c]	763±82[e]	<0.0001	<0.0001	<0.0001
	Female	85.7±16.9[a]	245±31[b]	476±60[d]	657±66[f]			
Body fat (%)	Male	14.4±2.9[a]	13.1±2.1[a]	12.5±2.1[a]	16.8±4.2[c]	<0.0001	<0.0001	<0.0001
	Female	15.3±3.5[a]	14.0±2.2[a]	15.5±3.6[b]	20.5±3.1[d]			
WB BA[3] (cm^2)	Male	23.1±4.1[a]	47.5±5.0[b]	79.3±8.2[c]	102.7±8.2[e]	<0.0001	0.003	<0.0001
	Female	27.9±3.6[a]	46.6±4.1[b]	74.2±5.9[d]	95.6 ±5.7[f]			
WB BMC (g)	Male	3.03±0.77[a]	7.15±1.17[b]	16.04±2.49[d]	24.41±2.78[f]	<0.0001	<0.0001	<0.0001
	Female	3.02±0.60[a]	6.89±0.85[c]	14.26±1.77[e]	21.03±1.68[g]			
WB BMD (g/cm^2)	Male	0.117±0.008[a]	0.150±0.010[b]	0.200±0.010[c]	0.237±0.011[e]	<0.0001	<0.0001	<0.0001
	Female	0.118±0.010[a]	0.147±0.007[b]	0.190±0.011[d]	0.222±0.010[f]			

[1]Values reported are mean ± SD. Different letters represent a significant difference between time points and genders.
[2]Sample size: n=33 male, n=23 female. [3]Abbreviations: whole body (WB), bone area (BA), bone mineral content (BMC), bone mineral density (BMD), not significant (NS).

Table 3b: Gender differences in regional bone mass over time

Measurement[1]	Gender	Day 3	Day 21	Wk 8	Wk 16	P value Time	P value Gender	P value Interaction
Femur BA[2]	Male[3]	0.6±0.1[a]	0.9±0.1[b]	1.4±0.2[c]	1.7±0.2[d]	<0.0001	NS	NS
	Female	0.7±0.1[a]	0.9±0.1[b]	1.4±0.1[c]	1.7±0.1[d]			
Femur BMC (g)	Male	0.07±0.02[a]	0.26±0.08[b]	0.58±0.11[d]	0.89±0.09[f]	<0.0001	<0.0001	<0.0001
	Female	0.08±0.03[a]	0.24±0.05[c]	0.52±0.09[e]	0.77±0.09[g]			
Femur BMD (g/cm^2)	Male	0.117±0.031[a]	0.293±0.052[b]	0.421±0.065[d]	0.534±0.040[f]	<0.0001	<0.0001	<0.0001
	Female	0.115±0.022[a]	0.266±0.049[c]	0.383±0.050[e]	0.463±0.053[g]			
Tibia BA (cm^2)	Male	0.8±0.1[a]	1.3±0.1[b]	1.8±0.2[c]	2.1±0.2[d]	<0.0001	<0.0001	0.01
	Female	0.8±0.1[a]	1.2±0.1[b]	1.7±0.2[c]	2.1±0.1[e]			
Tibia BMC (g)	Male	0.06±0.02[a]	0.18±0.04[b]	0.39±0.07[c]	0.63±0.08[e]	<0.0001	<0.0001	<0.0001
	Female	0.06±0.02[a]	0.17±0.03[b]	0.37±0.06[d]	0.55±0.05[f]			
Tibia BMD (g/cm^2)	Male	0.082±0.021[a]	0.141±0.021[b]	0.221±0.021[c]	0.295±0.023[d]	<0.0001	NS	NS
	Female	0.080±0.017[a]	0.137±0.017[b]	0.213±0.019[c]	0.286±0.066[d]			
LS BA (cm^2)	Male	0.7±0.1[a]	1.3±0.1[b]	1.9±0.2[c]	2.3±0.2[d]	<0.0001	NS	0.02
	Female	0.7±0.1[a]	1.3±0.1[b]	1.8±0.1[c]	2.1±0.2[e]			
LS BMC (g)	Male	0.14±0.04[a]	0.20±0.03[b]	0.38±0.04[d]	0.65±0.10[e]	<0.0001	<0.0001	<0.0001
	Female	0.13±0.02[a]	0.19±0.03[b]	0.42±0.06[c]	0.57±0.06[f]			
LS BMD (g/cm^2)	Male	0.177±0.019[a]	0.150±0.015[b]	0.222±0.020[d]	0.287±0.025[e]	<0.0001	0.002	0.004
	Female	0.179±0.022[a]	0.145±0.011[b]	0.204±0.017[c]	0.265±0.019[f]			

[1]Values reported are mean ± SD. Different letters represent a significant difference between time points and genders.
[2]Abbreviations: lumbar spine (LS), bone area (BA), bone mineral content (BMC), bone mineral density (BMD), not significant (NS). [3]Sample size: n=33 male, n=23 female.

Table 4: The effect of the pregnancy diet on measurements of bone strength

Bone strength measurement[1]	Control[2]	Low protein	P-value
Femur			
Length	32.4±0.7	30.5±1.6	NS
Young's modulus (MPa)	766±195	1246±259	0.02
Load at break (kgf)	1.67±0.31	2.01±0.32	NS
Extension at break (mm)	1.36±0.82	0.760±0.056	NS
Ultimate stress (MPa)	29.5±10.1	41.8±15.9	NS
Ultimate strain (mm/mm)	0.041±0.008	0.049±0.008	NS
Tibia			
Length	27.2±4.5	26.0±4.3	NS
Young's modulus (MPa)	1949±302	3579±521	0.01
Load at break (kgf)	1.96±0.34	2.46±0.56	0.004
Extension at break (mm)	2.53±0.42	1.41±0.23	0.03
Ultimate stress (MPa)	63±11	117±20	0.02
Ultimate strain (mm/mm)	0.080±0.005	0.032±0.002	0.02
4th vertebral body			
Height	7.00±0.71	7.01±0.33	NS
Young's modulus (MPa)	60.1±12.6	40.0±9.0	NS
Maximum compressive load (kgf)	16.0±0.9	5.13±5.01	0.009
Maximum compressive extension (mm)	5.21±2.68	3.01±1.26	NS
Ultimate stress (MPa)	1.95±1.6	0.602±0.423	0.03
Ultimate strain (mm/mm)	0.056±0.027	0.037±0.014	NS
Femoral neck			
Height	1.53±0.51	1.42±0.67	NS
Young's modulus (MPa)	42.2±14.6	32.9±11.1	NS
Maximum compressive load (kgf)	2.04±0.88	1.49±0.64	NS
Maximum compressive extension (mm)	0.689±0.009	0.483±0.005	NS
Ultimate stress (MPa)	0.255±0.021	0.186±0.009	NS
Ultimate strain (mm/mm)	0.029±0.009	0.017±0.006	NS

[1]Results presented are mean ± SD. [2]Sample sizes: n=15 control, n=17 low protein.

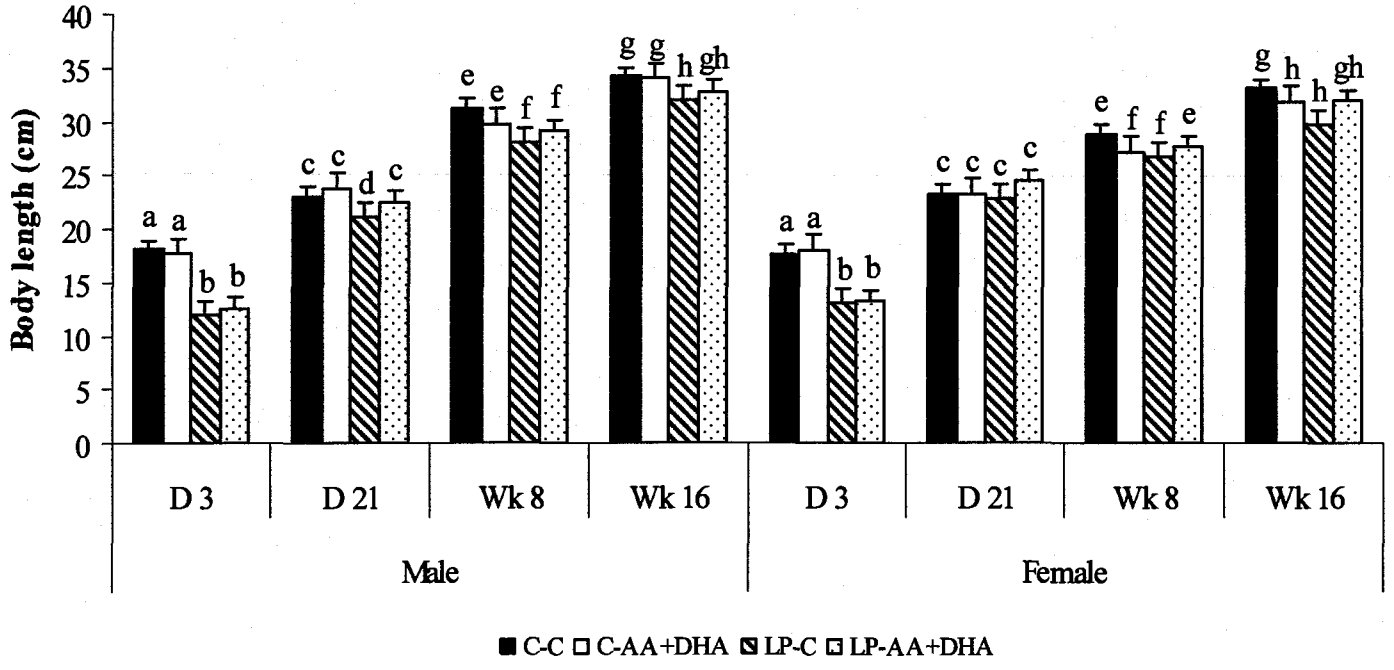

Figure 1: Gender differences in the effect of the pregnancy and lactation diets on body length over time. Values are mean ± SEM, P=0.0006 for the 4-way interaction. Different letters indicate a significant difference between groups (P<0.05). Diet groups (pregnancy-lactation) are represented as the following: solid black bars are control-control (male n=8; female n=6); solid white bars are control-AA+DHA (male n=9; female n=5); stripped bars are low-protein-control (male n=7; female n=5); black and white dotted bars are low-protein-AA+DHA (male n=9; female n=7). Abbreviations: C=Control, AA+DHA=AA and DHA diet, LP=low protein

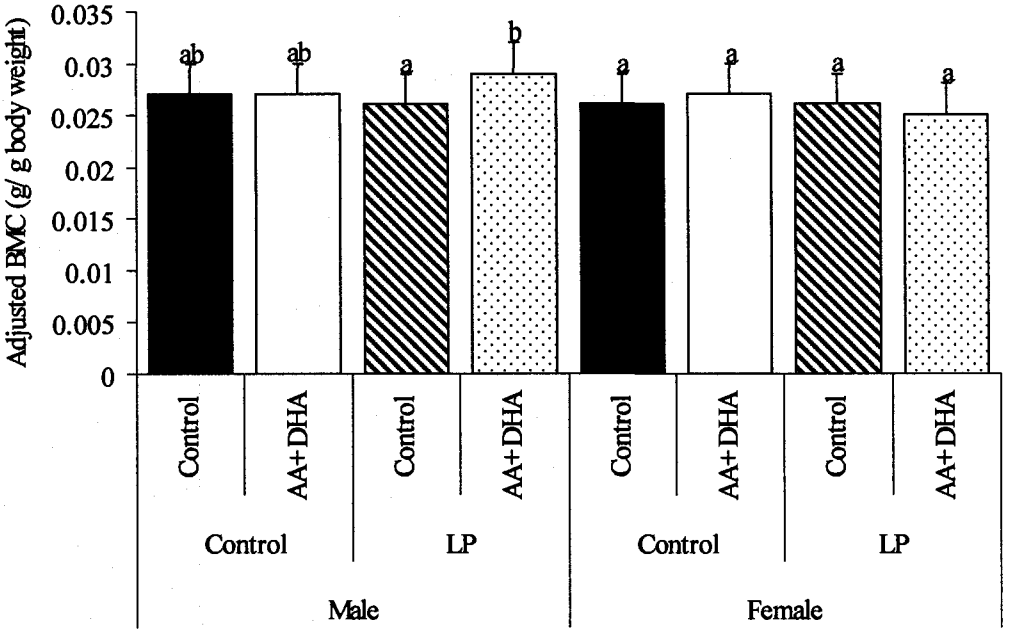

Figure 2: Gender differences in the combined effect of pregnancy and lactation diets on whole body bone mineral content (BMC) adjusted for body weight. Values are mean ± SEM, P=0.03 for the interaction. Different letters indicate a significant difference between groups (P<0.05). Diet groups (pregnancy-lactation) are represented as the following: solid black bars are control-control (male n=8; female n=6); solid white bars are control-AA+DHA (male n=9; female n=5); stripped bars are low-protein-control (male n=7; female n=5); black and white dotted bars are low-protein-AA+DHA (male n=9; female n=7).

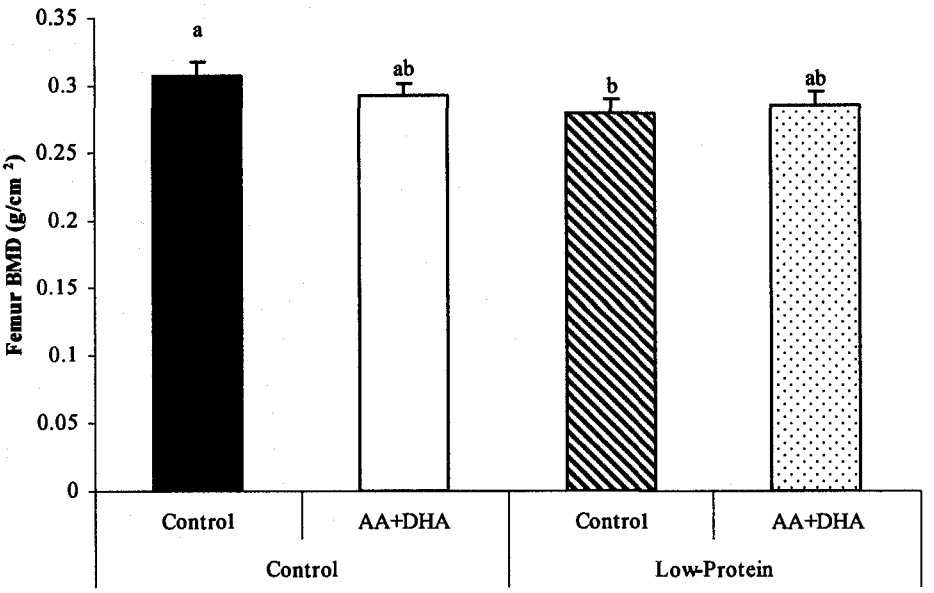

Figure 3: The combined effect of the pregnancy and lactation diets on femur BMD. Values are mean ± SEM, P=0.04 for the interaction. Different letters indicate a significant difference between groups (P<0.05). Diet groups (pregnancy-lactation) are represented as the following: solid black bars are control-control (n=14); solid white bars are control-AA+DHA (n=14); stripped bars are low-protein-control (n=12); black and white dotted bars are low-protein-AA+DHA (n=16).

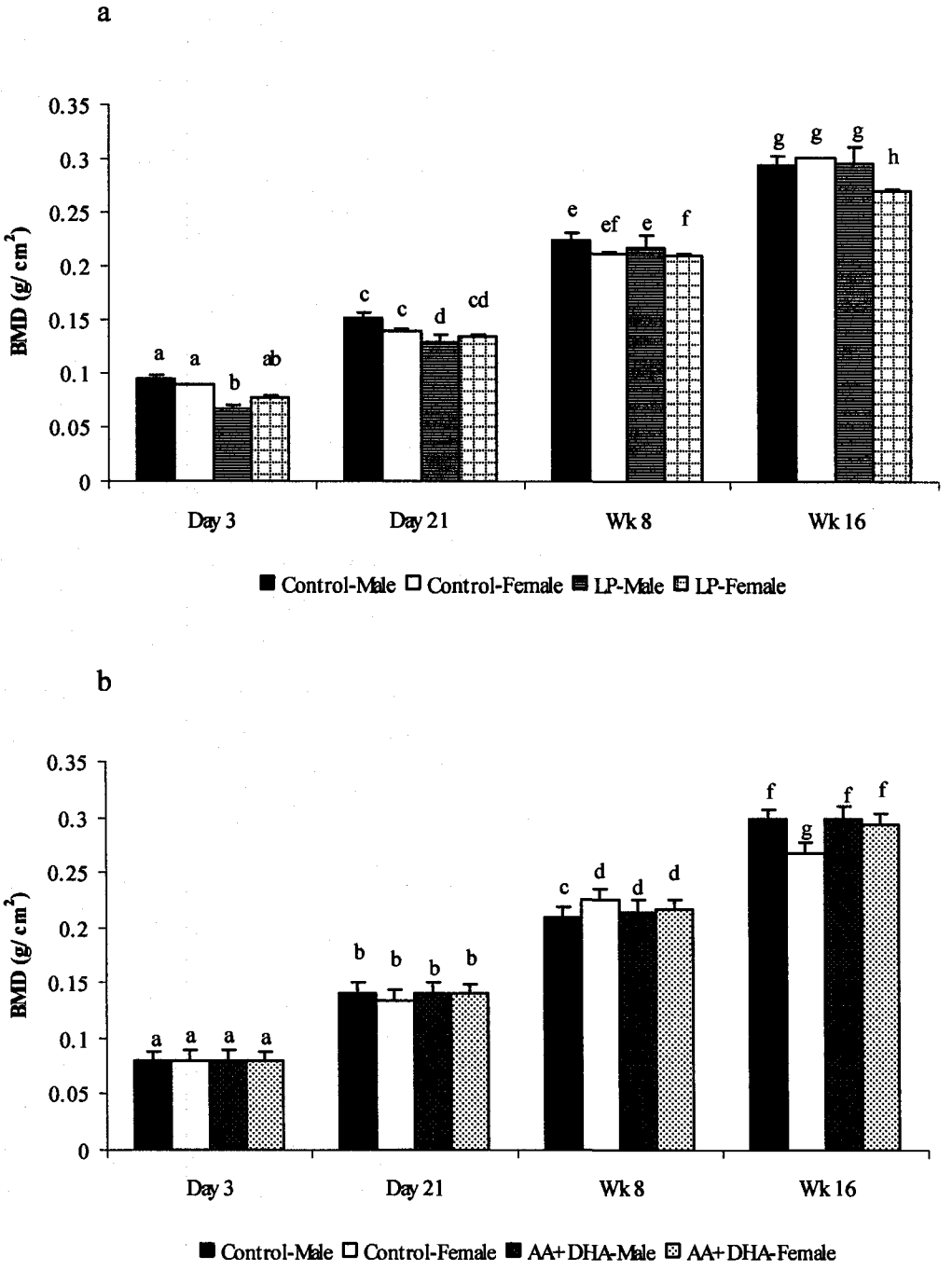

Figure 4: Gender differences in the effect of the pregnancy diet (a) or lactation diet (b) on tibia bone mineral density (BMD). Values are mean ± SEM, P=0.003 (panel a) P=0.01 (panel b) for the interaction. Different letters indicate a significant difference between groups (P<0.05). Abbreviations: AA+DHA=AA and DHA diet, LP=low protein

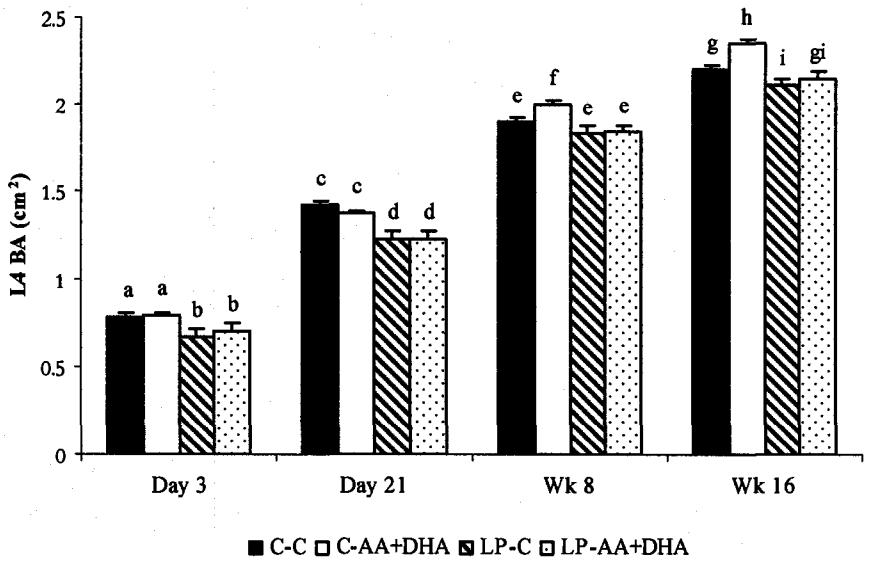

Figure 5: The effects of the pregnancy and lactation diets on lumbar spine bone area (BA) over time. Values are mean ± SEM, P=0.04 for the interaction. Different letters indicate a significant difference between groups (P<0.05). Diet groups (pregnancy-lactation) are represented as the following: solid black bars are control-control (n=14); solid white bars are control-AA+DHA (n=14); stripped bars are low-protein-control (n=12); black and white dotted bars are low-protein-AA+DHA (n=16). Abbreviations: C=Control, AA+DHA=AA and DHA diet, LP=low protein.

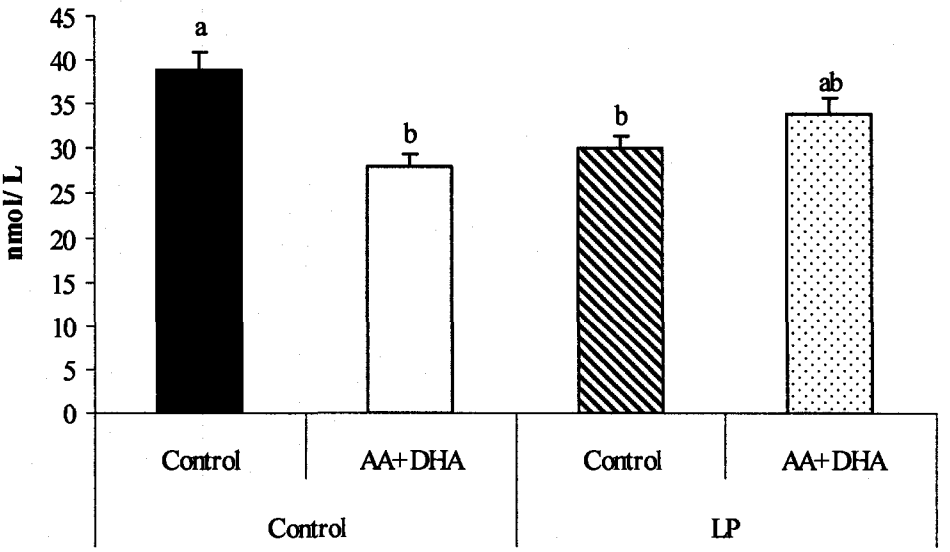

Figure 6: The effects of the pregnancy and lactation diets on osteocalcin. Values are mean ± SEM, P=0.03 for the interaction. Different letters indicate a significant difference between groups (P<0.05). Diet groups (pregnancy-lactation) are represented as the following: solid black bars are control-control (n=14); solid white bars are control-AA+DHA (n=14); stripped bars are low-protein-control (n=12); black and white dotted bars are low-protein-AA+DHA (n=16).

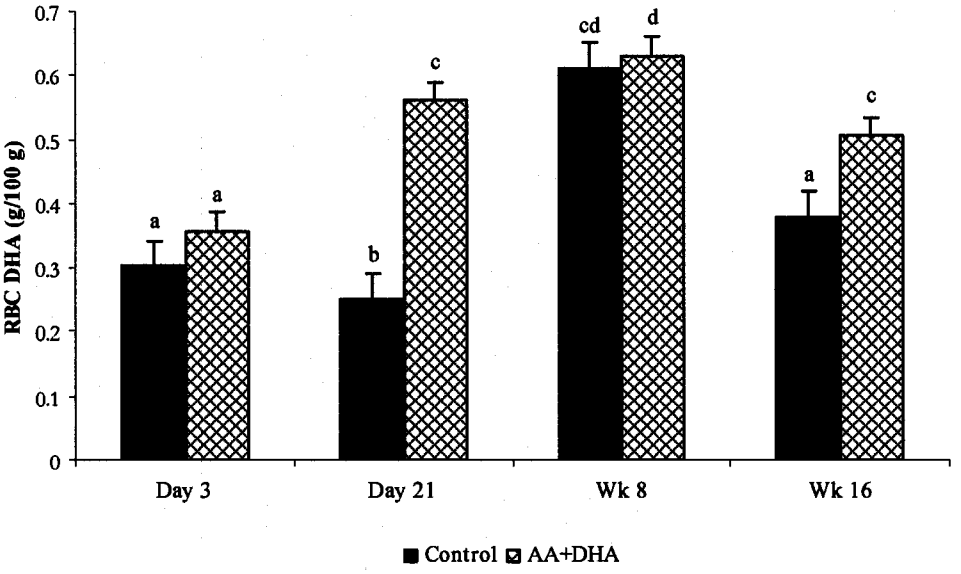

Figure 7: The effect of the lactation diet on red blood cell (RBC) docosahexaenoic acid (DHA) over time. Values are mean ± SEM g/100 g fatty acids, P=0.002 for the interaction. Different letters represent a significant difference between groups (P<0.01). Solid dark bars represent control pups (n=27). Light bars represent AA+DHA pups (n=26).

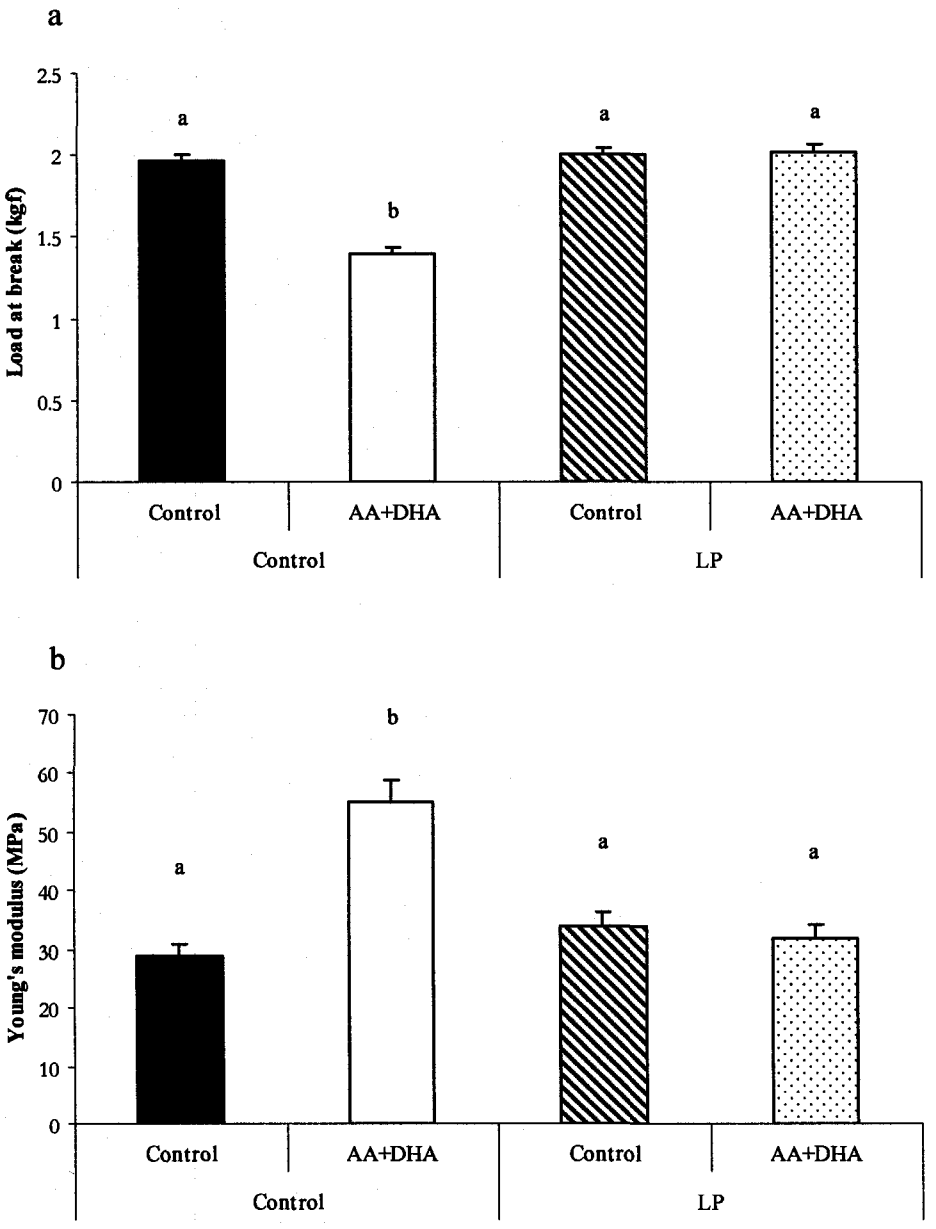

Figure 8: The combined effect of the pregnancy and lactation diets on femur load at break (a) or on Young's modulus for femoral neck compression test (b). Values are mean ± SEM, P=0.01 (panel a), P=0.02 (panel b) for the interaction. Different letters indicate a significant difference between groups (P<0.05). Diet groups (pregnancy-lactation) are represented as the following: solid black bars are control-control (n=6); solid white bars are control-AA+DHA (n=6); solid gray bars are low-protein-control (n=6); black and white checked bars are low-protein-AA+DHA (n=6).

3.9 Preface to Paper 2

During the design of the study reported in Paper 1, there was opportunity to research the effect of AA and DHA on maternal bone mass during lactation. To the best of the author's knowledge, this is novel experimentation that has not been reported in the literature. Furthermore, it was important to understand the effect of the AA and DHA supplement on maternal bone mass to eliminate the possibility of a detrimental effect. The following chapter will describe the study as it pertains to the maternal bone mass. The addition of this paper complements the findings in Paper 1 to more thoroughly draw conclusions from this research.

4.0 Chapter Four

Paper Two

Diet enrichment with arachidonic and docosahexaenoic acid during lactation improves bone mineral content and density in pigmented guinea pigs

4.1 Introduction to Paper Two

This paper reports the data collected and analyzed from the guinea pig sows. It contains data related to feed intake and weight gain during pregnancy, body composition and bone mass measurements, and biochemistry.

The manuscript was prepared to submit to the British Journal of Nutrition.

The thesis is referenced as a whole and references are found on **page 105.**

Please note:

H. Weiler is a co-author for this paper, whose contributions are detailed in the letter of co-authorship in **Appendix C, page 139.**

4.2 Abstract

The magnitude of bone mineral loss from the adult female skeleton is greatest during lactation. Bone recovery is necessary for the preservation of bone mass throughout adulthood until menopausal bone resorption commences. Arachidonic (AA) and docosahexaenoic acid (DHA) are shown to increase femoral neck bone mineral density (BMD) by 2% and prevent bone loss at the lumbar spine in post-menopausal women. In a similar state of hormone-driven bone resorption, this study aimed to determine whether moderate diet enrichment with AA and DHA would affect bone mass during lactation and post-weaning. 40 guinea pig sows were randomized to either a control diet or the same diet with 0.5% and 0.2% of lipid as AA and DHA respectively during lactation. Following delivery of a healthy litter, the lactation diet was provided for 21 d prior to weaning, at which time all sows were fed the control diet until 8 wk post-partum. Bone mass was assessed by dual-energy x-ray absorptiometry and blood collected prior to mating, and at d 3, d 21, and 8 wk post-partum. Results indicate that the diet intervention had no effect on whole body bone mass or composition. Tibia and lumbar spine bone mass were positively affected with 3-11% increased bone mineral content and 5-7% increased density. In sows fed the fatty acids, AA and DHA were 30% and 80% greater respectively in milk lipid and DHA was 22% greater in red blood cells. In summary, this study shows for the first time that a lactation diet enriched with AA and DHA increases regional BMD in females. Fatty acids were effectively incorporated into sow milk and tissues. As LC PUFA are encouraged in the diet of lactating women for the health of the neonate, this study indicates there is additional benefit to maternal bone mass.

4.3 Introduction

The rate of bone loss during lactation is greater than the annual rate of mineral loss in post-menopausal women (25). During lactation, minerals from the maternal skeleton are released into circulation for production of breast milk (249) This may result in a 4% decrease in maternal total body bone mineral density (BMD) and a 5-7% decrease in lumbar spine and hip bone mineral content (BMC) and BMD (250-252).

Following lactation, there is a rapid transition from bone catabolism to anabolism (131). Osteoblast progenitor cell proliferation and high rates of osteoblast activity are noted on the bone surface of rodents (253). In humans, osteocalcin becomes elevated at this time (24). A proportion of the bone mass lost during lactation is recovered, though bone mass may be lower following lactation than prior to conception for women that have yet to attain peak bone mass (254). Long-chain, polyunsaturated fatty acids (LC PUFA) may be an advantageous supplement as they are known to enhance calcium absorption and improve bone mass in women (134).

Dietary long-chain, polyunsaturated fatty acid (LC PUFA) consumption has not been investigated for potential benefit to maternal bone recovery. LC PUFA, most notably arachidonic (AA) and docosahexaenoic (DHA) acid elevate whole body BMD and LS BMD by 5-20% in young, aged, and ovariectomized animal models (14-16). Feeding 8 mo old ovariectomized mice 0.5% (w/w) DHA daily protected against BMD loss at the lumbar spine as measured by dual-energy x-ray absorptiometry (DXA) and maintained lumbar spine trabecular bone mass (16). In post-menopausal women, providing an 18 mo diet supplement of LC PUFA in a 10:1 n-6:n-3 ratio increased femoral neck BMD by 1.5% and protected against LS bone loss, while women not receiving LC PUFA lost 2-3.5% of BMD in these areas (23). Given these positive outcomes of low-dose LC PUFA supplementation in a hormone-driven state of high bone resorption, enriching the

maternal diet with AA and DHA during lactation may slow mineral loss and promote bone mass recovery.

Canada's present public health policy regarding nutrient intake during early neonatal life states the optimal amount of fatty acids in breast milk is an unresolved issue in the composition of the infant diet (255). Despite the lack of public policy, there is now consensus in the literature that lactating women should consume 0.2% of dietary fat as DHA (241). Long-chain fatty acids are critical nutrients for neonatal neural and visual development (256). The most recent report of LC PUFA content in the breast milk of North American women states a mean of 0.4 ± 0.03% AA and 0.25 ± 0.08% DHA, or an approximate 2:1 ratio of AA:DHA (20). However, the amount of AA and DHA present in the breast milk of Canadian women is decreasing in association with the decline in dietary intake of these fatty acids, especially DHA (139, 140).

Given the impact of maternal LC PUFA status during lactation on neonatal development and evidence that LC PUFA may protect against BMD loss, the aim of this study was to determine if diet enrichment with 0.5% and 0.2% of dietary lipid (w/w) as AA and DHA respectively improve maternal bone mass during and following lactation in an animal model. The pigmented (multi-coloured) guinea pig was chosen for this study for its relatively long gestation (62-70 d) and small litter size (1-5), making this model representative the reproductive demands placed on pregnant and lactating women due to fetal and neonatal bone mineral accrual.

4.4 Materials and methods

Animals and diet

Ethical approval was obtained from the Macdonald Campus Facility Animal Care Committee of McGill University in accordance with the Canadian Council on Animal Care guidelines (242). Female guinea pig sows (n=40) and male boars were purchased from Elm Hill Laboratories (Chelmsford, MA, USA) at 4 wk of age and acclimatized for 8 wk. Prior to mating, guinea pigs were randomly assigned to a control (C) or LC PUFA enriched (AA+DHA) diet during lactation. Sows were mated at 12 wk of age after random assignment to a male boar. Following confirmation of conception, sows were separated from the boar and housed individually until delivery, at which time the lactation diet was immediately provided. Sows were maintained on the lactation diet for 21 d and upon weaning, were housed in pairs and fed a control diet (Harlan Teklad 2041, Madison, WI, USA) until 8 wk post-partum. Rooms were maintained on a 12 h light-dark cycle at 22-24°C.

Diets were purchased from Purina Testdiet (Richmond, IN, USA) and formulated to our specifications. The LC PUFA sources were ARASCO® and DHASCO® (Martek Biosciences, Columbia, MD, USA) and fed as 0.5% and 0.2% of dietary lipid respectively. The LC PUFA diet was fed every 24 h and uneaten food discarded. This diet was stored at -20°C to prevent oxidation of the fatty acids. Both diets have been previously detailed in **Paper one**. Investigators responsible for providing diet and performing data collection and analysis were blinded to the treatment groups.

Sampling and measurements

Body weight was measured weekly for the entire study duration and at time of DXA. Food disappearance per cage was monitored tri-weekly during pregnancy and during lactation but not following weaning.

Blood samples were taken from the saphenous vein prior to mating (baseline), d 3, d 21, and 8 wk post-partum, spun at 2000 G for 20 min, and serum and plasma stored at -80°C. Red blood cells (RBC) were rinsed with 0.9% saline to remove additional plasma, flushed with nitrogen, and stored at -80°C for a maximum of 28 d before analysis.

Bone mass was measured under anesthetic for the whole body, lumbar spine, femur, and tibia using DXA (4500A, Hologic Inc., Bedford, MA, USA) with the small animal software package at (baseline), d 3, d 21, and 8 wk post-partum. Body composition was also assessed by DXA. All scans were performed and analyzed by the same operator. Average CVs were 2.5, 3.0, and 5.7 for replicate whole body scans, regional scans, and scan analysis respectively.

Milk samples were collected on d 7 post-partum, which is a peak day of milk production. Sows were removed from pups and an intra-peritoneal injection of 0.3 ml oxytocin was administered 30 min prior to sampling. Milk was manually expressed under anesthetic. Samples were flushed with nitrogen and stored at -80°C prior to analysis for fatty acid content.

Biochemistry

Plasma osteocalcin was measured in duplicate using an ELISA (MetraOsteocalcin, Quidel, San Diego, CA, USA) known to cross-react with the guinea pig (244). Serum deoxypyridinoline (DPD) was also measured with an ELISA (MetraDPD, Quidel, San Diego, CA, USA) that cross-reacts with the guinea pig (as per manufacturer's specifications) and inter-assay CVs were 3.2 and 3.0 for each ELISA respectively.

Fatty acids from milk and RBC were extracted using a modified Folch technique and methylated with methanolic HCl for 1 h as previously described (151). Methyl esters were identified using gas chromatography (Varian, CP 3800, Varian Inc, Cary, NC, USA).

Statistical analysis

A factorial ANOVA was performed to determine differences between the main effects of time (baseline, d 3, d 21, wk 8) and diet (control, AA+DHA). Post-hoc interactions between time and diet were adjusted using Bonferroni's correction. Data was tested for normality using a Shapiro-Wilk test and residuals were plotted for random distribution. All statistics were analyzed using SAS, Version 9.1 (Cary, NC, USA) and $P<0.05$ was considered significant. Results presented are mean ± SD (text, tables) or SEM (figures).

(Please note: This study was initially designed to analyze the offspring of the sows. Thus, sample size for the study was determined based on outcomes measured in the sows' pups. However, calculating a sample size based on the data collected, a sample size of n=12 sows per group would be sufficient to detect a 5% difference in whole body BMC between diet groups with a power of 0.8 and alpha of 0.05.)

4.5 Results

Conception and delivery

Thirty-two sows successfully conceived, delivered and nursed healthy litters until weaning and are included in the analysis. Of the sows that did not, 2 died prior to conception from causes unrelated to the study, 3 died at or prior to d 7 post-partum from delivery complications/sepsis and 3 sows either did not conceive or delivered stillborn litters twice and therefore were not mated again.

Feed intake and weight gain

Average daily food intake in the last 3 wk of gestation (approximately the 3rd trimester) did not differ between groups (C: 42.2 ± 20.7 vs. AA+DHA: 37.3 ± 17.1 g/d, P=0.41) nor did average weekly weight gain during this time (34.3 ± 31.0 vs. 27.9 ± 22.2 g/wk, P=0.5). Intake of the lactation diet did not significantly differ between groups (30.2 ± 15.3 vs. 35.3 ± 18.9 g/d, P=0.23).

Body weight and composition

There was no main effect of diet on body weight (P=0.92), body fat mass (P=0.65), body fat percentage (P=0.16) or lean body mass (P=0.10). Body weight, fat mass and lean mass increased significantly over time (P<0.0001), but did not change between d 3 and d 21 post-partum (**Table 1**), with the exception of body fat percentage, which was lowest at d 21 than any other time point.

Whole body bone mass

There was no main effect of diet on whole body bone area (BA, P=0.06), bone mineral content (BMC, P=0.28), or bone mineral density (BMD, P=0.55). All three measurements increase with time at the whole body level (**Table 2**). Diet did not interact with time (P=0.8). There was a steady rate of growth was noted between 12-22 wk of life, or from conception to parturition, with peak BMC and growth at this time, and a plateau from 22-33 wk of life.

Regional bone mass

There was no effect of diet on femur BA (P=0.28), BMC (P=0.51), or BMD (P=0.17). Diet had a main effect on tibia and LS bone mass. The AA+DHA diet reduced tibia BA (P=0.04), but did not affect BA of the LS (P=0.40, **Figure 1**). In both regions, BMC was increased by the AA+DHA diet, but this only reached significance in the LS (P=0.04, **Figure 2**). Lastly, BMD was greater in the tibia and LS in the sows fed the AA+DHA diet (P<0.05, **Figure 3**). There were no interaction effects between time and the AA+DHA diet for regional bone mass measurements (P>0.05).

Time had a significant effect on all regional bone mass measurements (P<0.009) with the exception of femur BMD (P=0.73, **Table 2**). Femur, tibia, and LS BA followed the same trend as whole body BA for growth, with noticeable growth between conception and delivery. Following delivery there was a plateau in BA acquisition, with the exception of lumbar spine BA which increased significantly between weaning (d 21) and wk 8 post-partum (**Table 2**). Femur and tibia BMC and BMD were significantly reduced at d 21 in comparison to wk 8 post-partum and also reduced from d 3 post-partum (**Table 2**). However, there seemed to be sparing of mineral at the LS, as this region showed no change between d 3 and d 21 post-partum (**Table 2**).

Biochemistry

Diet had no effect on osteocalcin (P=0.15). There was a significant effect of time (P=0.02) as serum osteocalcin was higher at baseline (18.3 ± 4.0 nmol/L) and d 21 post-partum (17.7 ± 5.5 nmol/L) than at d 3 (10.0 ± 3.1 nmol/L) and wk 8 (14.2 ± 3.8 nmol/L, P=0.002). DPD was also not affected by diet (P=0.13). There was a significant effect of time (P<0.001) as DPD was significantly higher at d 3 post-partum (7.01 ± 2.25 nmol/L) than any other time points.

Milk fatty acid analysis confirmed the incorporation of the LC PUFA into sow milk as both AA (0.184 ± 0.02 vs. 0.284 ± 0.04 g/100 g fatty acid) and DHA

(0.013 ± 0.005 vs. 0.071 ± 0.007 g/100 g fatty acid) were significantly ($P<0.001$) elevated in milk of the guinea pigs fed the AA+DHA diet.

There was a main effect of the AA+DHA diet to increase RBC DHA (0.511 ± 0.150 vs. 0.655 ± 0.128 g/100 g fatty acid, $P<0.001$), but not RBC AA to a significant level (11.9 ± 1.7 vs 12.5 ± 1.6 g/100 g fatty acid, $P=0.1$). Lastly, in an interaction with time, RBC DHA was highest overall in the AA+DHA group at d 21 (0.383 ± 0.073 g/100 g fatty acid vs. 0.674 ± 0.095 g/100 g fatty acid, $P=0.001$).

4.6 Discussion

Repletion of bone loss following pregnancy and lactation is important to maintain bone quality and density throughout reproductive life (249). Conservation of maternal BMC and BMD during lactation translates to less recovery of bone mass post-weaning (257) This study has shown that a modest maternal diet supplement with AA and DHA provided exclusively during lactation elevates tibia and lumbar spine BMC and BMD in the pigmented guinea pig model.

Previous research studying ovariectomized mice and post-menopausal women indicates DHA elevates LS and femur BMD by 2-4% (23). In a similar state of hormone-driven bone resorption in the lactating guinea pig, 0.5% AA and 0.2% DHA (w/w) in the maternal diet increased tibia and LS BMC by 3% and 11% respectively, and BMD by 7% and 5% respectively. Given that BMC and BMD loss from the lumbar spine in lactating women ranges between 5-7% of pre-lactation bone mass (250) the magnitude by which the AA and DHA supplement in this study elevated regional bone mass is significant.

Change in body weight and composition over time indicated that there was a 15-20% increase in body weight and lean mass over the duration of pregnancy, but that fat mass and body fat percentage decreased by 20% and 27% respectively. Between delivery and the end of lactation there was a 9% decrease in body weight, 15-20% decrease in fat mass and body fat percentage, but only a 3% decrease in lean mass, indicating a sparing of lean mass and utilization of fat mass to compensate for additional energy requirements during lactation.

Bone mass was attained during gestation. Whole body and femur BA, BMC and BMD increased by 12-14%, 15-18%, and 5% respectively over this time, indicating a positive mineral balance in these areas during gestation. However, the tibia did not follow the same trend. While BA increased by 13%, BMD decreased by 10% in the tibia. Thus, this region appeared to be growing during gestation but BMD was compromised. Importantly, AA and DHA elevated and

BMD in this region when fed during lactation and therefore may be of benefit during pregnancy.

During lactation, whole body BA remained constant, and BMC and BMD showed moderate (2%) increases. Similarly lumbar spine BA remained constant and there was a 4% increase in both BMC and BMD. Thus, whole body and lumbar spine mineral content was preserved. However, the femur and tibia both displayed 2-4% increases in BA, but a 2% decrease in both BMC and BMD. Areas rich in trabecular bone, such as the lumbar spine, are believed to be the first and frequent sites of bone resorption in pregnancy, due to the porous, woven structure of this bone (258). However, these results indicate that in this model, it may have been the endocortical surfaces of the long bones resorbed to accommodate the increased demand for mineral in the body. Moreover, because there was continued tibia and femur growth, there was likely intra- and endocortical resorption to continue this growth. Due the decrease in BMC and BMD it appears that the periosteal apposition during lactation was insufficient at maintaining bone integrity, similar to the observations made for lumbar spine and tibia bone mass during pregnancy. Concurrently, serum markers of bone metabolism indicate the sows were in a state of bone catabolism at the onset of lactation, but anabolism by weaning.

Osteocalcin concentrations were high at baseline, which is understandable given the sows were still in a phase of growth, and at d 21 post-partum. Forty-five percent higher osteocalcin concentrations at d 21 indicate bone was in a high state of turnover, possibly to compensate for mineral lost between d 3 and d 21 post-partum. DPD was highest at d 3, declining by d 21 post-partum and remaining unchanged at wk 8. It is not surprising that DPD was lower at d 21 because milk production is considerably reduced by this time in the sow (49), and thus the stress on bone placed by milk production has subsided. Peak lactation occurs between d 4 and d 7 post-partum (46) and thus it would be expected that at d 3, bone resorption would be very high, and gradually decline across the course of

lactation. Therefore, at d 21 it seems augmented formation compensated for mineral loss during lactation, which served to elevated whole body and regional BMC and BMD at wk 8 post-partum. Both the femur and tibia that lost mineral between d 3 and d 21, had gained 10-15% BMC and 5-13% BMD by wk 8 post-partum.

The diet was successful at increasing both AA and DHA in sow milk by 36% and 82% respectively, demonstrating the preferential incorporation of DHA into the milk despite the 2.5:1 ratio of AA:DHA in the diet. In lactating women, dietary fish oil also elevates DHA in breast milk and is reflected in the plasma phospholipid concentrations (259), as was the AA+DHA diet reflected in the RBC fatty acid concentrations in the present study.

In conclusion, providing dietary supplementation for 21 d with AA and DHA effectively elevated tibia and lumbar spine BMD and moderately improved BMC in these regions. Given that these regions are more susceptible to mineral and density loss during gestation, this supplement may help recover bone following pregnancy. AA and DHA are recognized as essential nutrients during fetal and neonatal development (139) and this study demonstrates that these fatty acids also support maternal bone maintenance.

4.7 Acknowledgements

Funding for this research was provided by the Natural Sciences and Engineering Research Council of Canada (NSERC) and funding for the DXA equipment was provided by the Canada Foundation for Innovation and Canada Research Chairs Program. L. Burr received a Canada Graduate Scholarship from NSERC.

The authors would like to thank Mr. Gordon Bingham for assistance with animal care and Dr. Thomas Brenna of Cornell University for supplying cages for animal housing, as well as Dr. Atul Sharma for providing statistical assistance. The ARASCO® and DHASCO® were graciously donated by Martek Biosciences.

4.8 Tables and figures for Paper Two

Table 1: Changes over time in body composition.

Measurement[1]	Baseline[2]	Day 3	Day 21	Wk 8	P-value
Body weight (g)	753 ± 15^{a}	839 ± 15^{b}	769 ± 26^{ab}	973 ± 18^{c}	<0.0001
Whole body fat mass (g)	133 ± 6^{a}	108 ± 6^{b}	87 ± 5^{b}	162 ± 9^{c}	<0.0001
Body fat (%)	17.4 ± 0.5^{a}	12.8 ± 0.5^{b}	10.9 ± 0.5^{c}	17.1 ± 0.5^{a}	<0.0001
Whole body lean mass (g)	620 ± 11^{a}	728 ± 11^{b}	710 ± 11^{b}	804 ± 13^{c}	<0.0001

[1]Measurements are reported as mean ± SEM, $P<0.05$. Different letters indicate a significant effect of time, n=32/time point. [2]Baseline is at 12 wk of age (pre-pregnancy).

Table 2: Changes over time in whole body and femoral bone mass.

Measurement[1]	Baseline	Day 3	Day 21	Wk 8	P-value
Whole body BA (cm^2)	90.7 ± 1.0^a	102.3 ± 1.1^b	102.9 ± 1.0^b	110.4 ± 1.1^c	<0.0001
Whole body BMC (g)	20.26 ± 0.57^a	23.87 ± 0.32^b	24.21 ± 0.34^b	26.91 ± 0.47^c	<0.0001
Whole body BMD (g/cm^2)	0.221 ± 0.002^a	0.233 ± 0.002^b	0.236 ± 0.002^{bc}	0.243 ± 0.001^c	<0.0001
Femur BA (cm^2)	1.6 ± 0.1^a	1.9 ± 0.1^b	1.9 ± 0.1^b	1.9 ± 0.1^b	<0.0001
Femur BMC (g)	0.71 ± 0.02^a	0.86 ± 0.01^b	0.85 ± 0.03^{bc}	0.95 ± 0.02^b	<0.0001
Femur BMD (g/cm^2)	0.443 ± 0.016^a	0.460 ± 0.011^a	0.456 ± 0.012^a	0.484 ± 0.009^a	NS
Tibia BA (cm^2)	1.8 ± 0.1^a	2.1 ± 0.1^b	2.1 ± 0.1^b	2.1 ± 0.1^b	<0.0001
Tibia BMC (g)	0.55 ± 0.02^a	0.57 ± 0.01^b	0.57 ± 0.01^b	0.65 ± 0.01^b	<0.0001
Tibia BMD (g/cm^2)	0.300 ± 0.015^a	0.273 ± 0.004^b	0.271 ± 0.004^b	0.308 ± 0.007^a	0.009
LS BA (cm^2)	1.9 ± 0.1^a	2.3 ± 0.1^b	2.3 ± 0.1^b	2.4 ± 0.1^c	<0.0001
LS BMC (g)	0.52 ± 0.02^a	0.61 ± 0.01^b	0.63 ± 0.06^b	0.71 ± 0.01^c	<0.0001
LS BMD (g/cm^2)	0.264 ± 0.004^a	0.266 ± 0.004^a	0.275 ± 0.005^a	0.296 ± 0.005^b	<0.0001

[1]Measurements are reported as mean ± SEM. Different letters indicate a significant effect of time, $P<0.05$, n=32/group.

Abbreviations: bone area (BA), bone mineral content (BMC), bone mineral density (BMD), lumbar spine (LS)

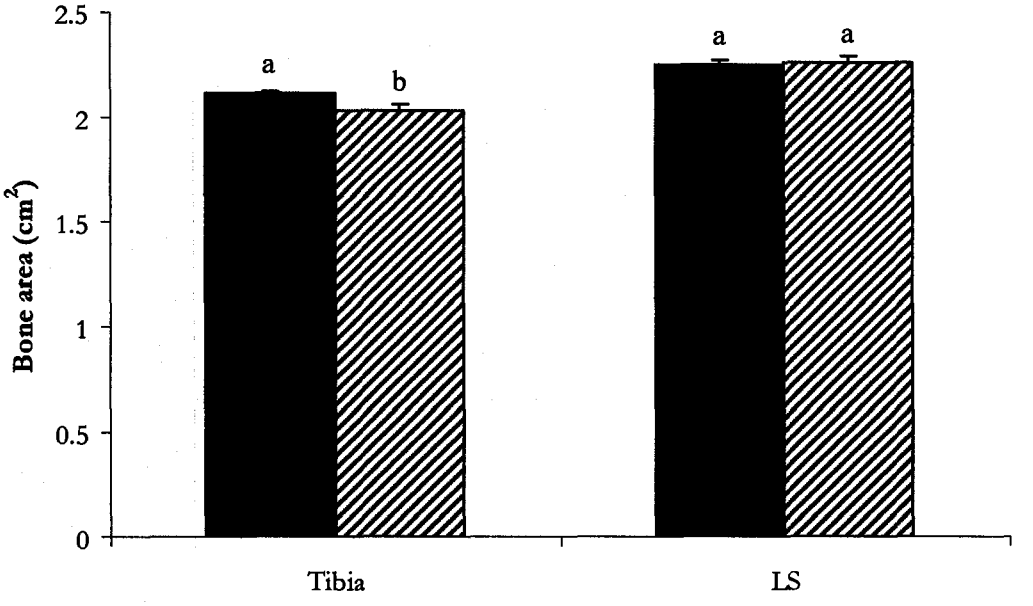

Figure 1: The main effect the lactation diet on tibia and lumbar spine (LS) bone area. Values are means ± SEM, $P<0.05$. The control group (n=17) is identified by a solid bar and the AA + DHA group (n=15) is identified by a patterned bar. Significant difference between groups is indicated by different letters.

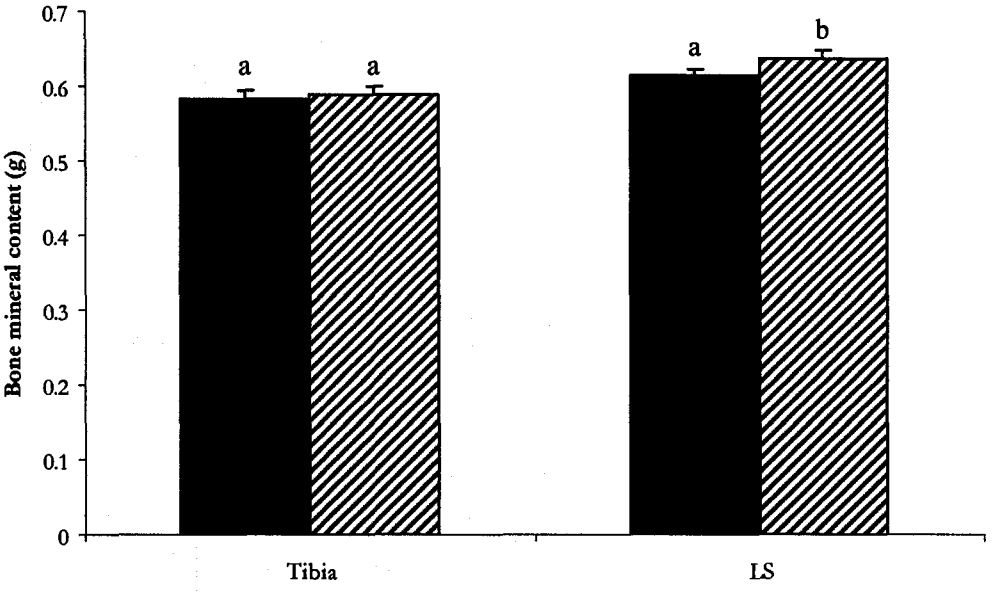

Figure 2: The main effect the lactation diet on tibia and lumbar spine (LS) bone mineral content. Values are means ± SEM, $P<0.05$. The control group (n=17) is identified by a solid bar and the AA + DHA group (n=15) is identified by a patterned bar. Significant difference between groups is indicated by different letters.

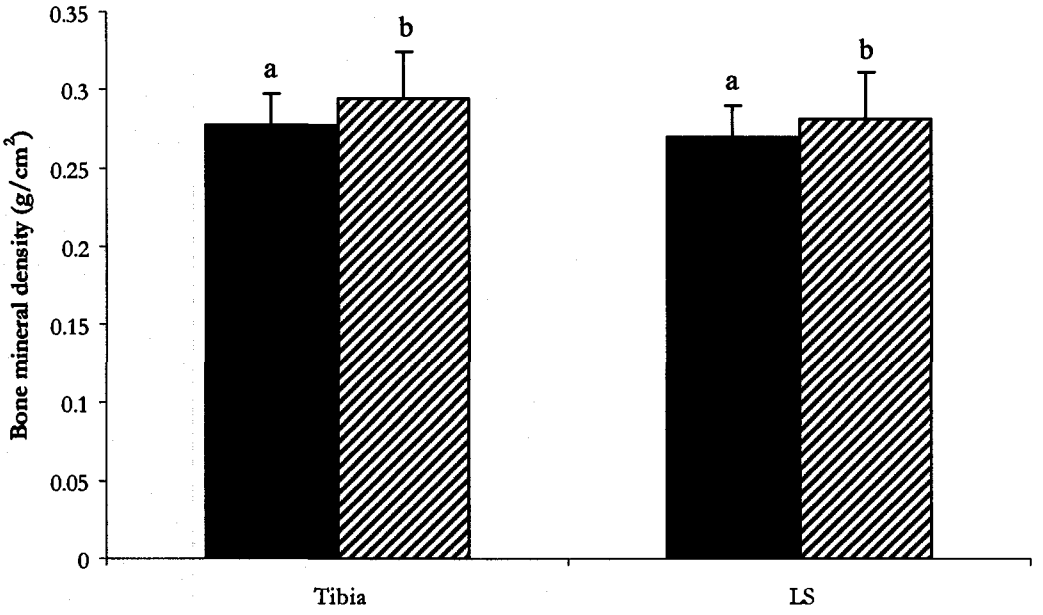

Figure 3: The main effect the lactation diet on tibia and lumbar spine (LS) bone mineral density. Values are means ± SEM, $P<0.05$. The control group (n=17) is identified by a solid bar and the AA + DHA group (n=15) is identified by a patterned bar. Significant difference between groups is indicated by different letters.

5.0 Chapter Five

Conclusions and future directions

5.1 Conclusions

5.2 Strengths and limitations

5.3 Future directions

5.1 Conclusions

The objectives of this study were to determine the effect a lactation diet enriched with AA and DHA on maternal and offspring bone mass and body composition in healthy and growth restricted guinea pigs. Furthermore, the effects of this diet from birth to sexual maturity were to be evaluated. The culmination of the animal trial showed that providing 0.5% and 0.2% of dietary lipid as AA and DHA respectively to lactating guinea pigs modestly improved both maternal and pup bone mass and increased length in the IUGR pups. This study confirms that there is fetal programming of bone mass, as IUGR reduced bone mass from birth until wk 16 of life. However, there is also indication that the effects of IUGR on body length, whole body BMC, and regional bone mass can be reversed with additional AA and DHA in the diet prior to weaning.

5.1.1 The animal model

The guinea pig was selected on the basis that it was a) a small animal b) would permit valid bone mass assessment within the first 48 h of life (39) and c) relative to other small animals, has a long gestational length. This model is not commonly used in bone research, and this was the first guinea pig study conducted in our lab. Therefore, further strengths and weaknesses of the model were identified throughout the animal trial.

The sows adapted well to gradual changes in diet. It is well established that guinea pig are fastidious in food choice and will resist abrupt change in diet composition (260). For this study, 2 control diets were used: one purified and another made with natural ingredients and roughage (**See Paper 1**). Both diets were nutritionally identical. To encourage transition to the purified diet, the 2 control diets were mixed prior to mating. The purified diets were also apple-flavored so the sows would not perceive a diet change after delivery. The flavoring also served to mask any taste of the LC PUFA that might be detected. This method seemed to successfully encourage adequate intake of diet. The energy requirements of guinea pigs (600 g) are stated to be 136 kcal/kg/d of

energy (excluding fiber that is not metabolized for energy) (260). There are no requirements indicated for a pregnant animal. The purified diets contained 3.3 kcal/g and based on mean intake of diet (g/d) the sows consumed 160 kcal/kg/d during pregnancy. Specifically the LP sows consumed 157 kcal/kg/d, which indicates energy-restriction should not be considered a factor in this IUGR model. It is understandable the pregnant sows would consume above the recommendations, especially since weight prior to mating was >600 g and continued to increase over pregnancy. During lactation sows ate an average of 126 kcal/kg/d, which is below the given requirements. This may have occurred due to stress from delivery or multiple procedures, such as DXA scans and milk sampling.

Unfortunately 3 sows died post-delivery after 48-72 h of lethargy, dyspnea, and lack of food and water intake. There were 3 other sows that delivered stillborn litters (**See Paper 1**). All 6 sows likely had a form of toxaemia of pregnancy (58). Risk factors include breeding at >6 mo of age, excessive fetal size, and sow obesity. Toxaemia results in stillbirth, ketosis and death may occur within 1-5 days. To the best of the researchers' ability, stress was minimized and diet intake was monitored closely to prevent toxaemia of pregnancy in this study (58).

The guinea pig proved to be an appropriate, useful model for the study of bone. The CVs for BMC of the whole body for sows and pups were <10. For all pups, 33% had a birth weight <2 SD below the mean birth weight of all pups, suggesting 1/3 of the pups in this study were born SGA. Whole body BMC (g/kg) in human infants is approximately 20 g/kg at birth (261), and similarly the control guinea pigs in this study had a mean of whole body BMC of 34 g/kg. Thus, the guinea pig appears to accrue mineral *in utero* similarly to humans. For the LP pups, whole body BMC was 29 g/kg in comparison to controls. However, the most recent report of whole body BMC at birth in human SGA neonates is 13 g/kg (8). This indicates that despite being growth restricted, the IUGR guinea pig had bone at birth that was less compromised than that in IUGR humans.

Therefore, it can not be concluded that the AA and DHA supplement would have similar effects in a human cohort. Despite this, the study provides an important contribution to the literature on bone mass and fatty acids.

5.1.2 Fatty acids, bone and IUGR

AA and DHA are highly important nutrients for neonatal neurological development and the amount required in the infant diet to complement endogenous synthesis is unclear (262). However, this study shows that a supplement of these fatty acids, in a ratio (2.5:1 AA:DHA) that provides slightly more AA than the amount typically in the milk of lactating North American women (2:1) provides some benefit to both maternal and neonatal bone.

The fatty acids in the sow diet were well incorporated into milk (**see Paper 2**). The LC PUFA enriched milk contained 80% and 40% more DHA and AA respectively. This translated into 12% and 5% greater DHA and AA respectively in sow RBC and 30% and 4% higher DHA and AA respectively in pup RBC. For all tissues, it is evident that DHA was incorporated preferentially over AA. This is a phenomenon that is reported in the literature (140). Observing the high amount of DHA in pup RBC study emphasizes the need to identify the amount of DHA that will provide benefits to growth and bone, but will not detrimentally alter AA status, which was observed in preterm infants (239). For pups, the amount of DHA in RBC was elevated by twice the concentrations observed for the sows. This may suggest that there is preferential uptake of DHA in neonatal life in the guinea pig. In rats, supplementing the diet with AA and DHA suppresses uptake of dietary long chain lipids (263), suggesting it was not diet that encouraged uptake of DHA in the pups.

The optimal amount of AA and DHA for bone has been studied, and it has been suggested that the ratio of AA:DHA is the determining factor in the effects on bone mass (14, 164). In rats, a 3:1 and 4:1 ratio of n-6:n-3 lipids increases bone calcium content but ratios of 2:1 and 1:1 have little effect on bone (163). It is

known that AA alone (18) and providing a higher AA:DHA ratio than what was given in this study elevates BMD in young piglets (151). Furthermore, a 5:1 ratio (AA:DHA) supplied in greater quantity does not provide additional benefits to bone (14), again indicating it is the ratio that is important. This study used a 2.5:1 ratio of AA:DHA to mimic the amount of LC PUFA in human milk and infant formula (129) and perhaps this provided too much DHA without AA to most favorably affect bone.

Despite small improvements in whole body BMC, femur BMD and LS BA, it is a novel, significant finding that the supplement during lactation had lasting effects until 16 wk of age. It is known that IUGR programs for outcomes in body composition (43), lipid synthesis (52), and cardiovascular health (53) in guinea pigs. In human SGA neonates, programming of cardiovascular health (264) and body composition (108) is also observed. It is agreed that the neonate is highly susceptibility of the first weeks of life to the environment and diet during this time can affect health across the lifespan (265, 266). This study not only has contributed the LP guinea pig as a novel model of *in utero* programming of bone mass, but also demonstrates that LC PUFA can correct the insult to body length and bone mass experience by this model *in utero*.

It is also important that there was benefit to both maternal and offspring bone mass. This consolidates the applicability of this animal study to the human scenario. The finding that AA and DHA improved sow bone mass during lactation is a novel contribution to the literature.

5.2 Strengths and limitations of this research

Strengths

The greatest strength of this research is the application to nutrition and health. While mechanisms responsible for the effects of IUGR and LC PUFA on bone were not investigated, the results from this study indicate that AA and DHA benefit overall whole body bone mass and growth and are not detrimental. These

LC PUFA elevate BA and BMC as measured by DXA and improve body length, which clinically, is relevant to human health.

This study was conducted long-term, and the remaining 2 time points in which these pups were sampled (wk 24 and wk 32 of life) will be included in **Paper 1** prior to publication. The duration of the trial will help conclude that findings reported were not transient and will provide foundation for future research in human infants.

This is the first study to induce IUGR in a guinea pig model using a low-protein diet. It provides data on longitudinal bone growth and mineralization in both healthy and low-birth weight guinea pigs which is not presently in the literature. While the sample size was not optimal, it is significantly higher than other recent studies in the literature that have used this model to study *in utero* programming of disease (43, 51-53). This research contributes a new animal model to the growing body of research on developmental programming of disease.

Limitations

The guinea pig is not frequently used in research trials that study bone and thus, measurements such as serum IGF-1 and PTH concentration, was not feasibly conducted. Prior to publication, the possibility of assessing these parameters will be considered. Other limitations to using the guinea pig were the variability in successful mating and deliveries. A recent publication discourages the use of guinea pigs in studies in which the diet is modified due to the poor acceptance of changes to diet in this model (57).

The sample size estimate was calculated based on whole body BMC and BMD means ± SD of 12 mo old guinea pigs (23.4 ± 2.4 g and 426 ± 14 mg/cm^2 respectively) published by Kipp and colleagues (32), and whole body and femur BMC values of guinea pig fetuses at 60 d gestation (1.45 ± 0.1 g and 18.3 ± 0.8 g) published by Rummens and colleagues (45, 267). The aim of the present study

was to detect a 5% difference in these measurements between the 4 main diet groups with a power of 0.8 and alpha of 0.05. Give the published means ± SD, it was calculated that a sample of 10-22 pups per group was necessary. Therefore, the study used 40 sows to obtain 80 pups, or 20 pups/diet group. However, with pregnancy, delivery, and litter size complications, the size of the 4 main diet groups at wk 16 ranged from 12-16 pups, which is still within the calculated required sample size of 10-22, but was not optimal.

Calculating the sample size based on the whole body BMC means ± SD of the 4 diet groups in this study (Control-Control 11.04 ± 7.8 g; Control-AA+DHA 10.7 ± 7.7 g; LP-Control 9.6 ± 7.7 g; LP-AA+DHA 9.9 ± 7.3 g), with a power of 0.8 and alpha of 0.05, a sample size of 200, or 50 pups/group (25 pups per gender) would have been optimal, given the large SD of the data. Therefore, the fact that significant results were observed in an underpowered study is fairly significant.

Lastly, this study did not explore the mechanisms by which LC PUFA are benefiting bone mass. As discussed in the literature review, there are many possible mechanisms by which AA and DHA may modulate bone mass. There are likely several factors that improved bone mass and growth in this IUGR model.

5.3 Future directions

To add to the findings in this study, future research should explore possible mechanisms for the affects of IUGR and LC PUFA on bone. This might include assessing RANK/RANKL, leptin, IGF, calcium absorption, and PTH, which appear to be promising candidates that have a role in AA and DHA metabolism and bone. To do this research, using the guinea pig or rat model would be suitable. Bone cultures from IUGR pups would also be useful to determine how the LC PUFA affects the cell proteins and gene expression. Because of the large difference in RBC DHA between the supplemented and control pups, it may be

beneficial to repeat this study with a similar design and use a higher ratio of AA:DHA.

Human studies to explore these findings further are also necessary. It would be possible to design this trial using a convenience sample of babies born IUGR who receive commercial formula fortified with AA and DHA (2:1 ratio) and measure bone mass at birth, 6 mo, 1 y, 3 y, 5 y etc. A randomized, double-blind, placebo controlled long-term trial would also ascertain whether the observed benefits of AA and DHA to bone mass in animal models is observed in human infants. Breastfeeding women could be randomized to receive an AA and DHA supplement, and milk fatty acids would have to be measured and correlated with bone mass measurements, given the variability in maternal milk. For this type of trial, the effects of AA and DHA on maternal bone mass during lactation could also be assessed.

6.0 References

References

1. Gardosi J. New definition of small for gestational age based on fetal growth potential. Horm Res 2006;65 Suppl 3:15-8.
2. Saenger P, Czernichow P, Hughes I, Reiter EO. Small for gestational age: short stature and beyond. Endocr Rev 2007;28:219-51.
3. Health C. Perinatal Health Indicators for Canada: A resource manual. In: Services MoPWaG, ed. Ottawa, 2000.
4. Cooper C, Fall C, Egger P, Hobbs R, Eastell R, Barker D. Growth in infancy and bone mass in later life. Ann Rheum Dis 1997;56:17-21.
5. Helin I, Landin LA, Nilsson BE. Bone mineral content in preterm infants at age 4 to 16. Acta Paediatr Scand 1985;74:264-7.
6. Chunga Vega F, Gomez de Tejada MJ, Gonzalez Hachero J, Perez Cano R, Coronel Rodriguez C. Low bone mineral density in small for gestational age infants: correlation with cord blood zinc concentrations. Arch Dis Child Fetal Neonatal Ed 1996;75:F126-9.
7. Atkinson SA, Randall-Simpson J. Factors influencing body composition of premature infants at term-adjusted age. Ann N Y Acad Sci 2000;904:393-9.
8. Akcakus M, Kurtoglu S, Koklu E, Kula M, Koklu S. The relationship between birth weight leptin and bone mineral status in newborn infants. Neonatology 2007;91:101-6.
9. Avila-Diaz M, Flores-Huerta S, Martinez-Muniz I, Amato D. Increments in whole body bone mineral content associated with weight and length in pre-term and full-term infants during the first 6 months of life. Arch Med Res 2001;32:288-92.
10. Fewtrell MS, Prentice A, Jones SC, et al. Bone mineralization and turnover in preterm infants at 8-12 years of age: the effect of early diet. J Bone Miner Res 1999;14:810-20.
11. Jones G, Riley M, Dwyer T. Breastfeeding in early life and bone mass in prepubertal children: a longitudinal study. Osteoporos Int 2000;11:146-52.

12. Dennison EM, Syddall HE, Sayer AA, Gilbody HJ, Cooper C. Birth weight and weight at 1 year are independent determinants of bone mass in the seventh decade: the Hertfordshire cohort study. Pediatr Res 2005;57:582-6.
13. Yarbrough DE, Barrett-Connor E, Morton DJ. Birth weight as a predictor of adult bone mass in postmenopausal women: the Rancho Bernardo Study. Osteoporos Int 2000;11:626-30.
14. Mollard RC, Kovacs HR, Fitzpatrick-Wong SC, Weiler HA. Low levels of dietary arachidonic and docosahexaenoic acids improve bone mass in neonatal piglets, but higher levels provide no benefit. J Nutr 2005;135:505-12.
15. Shen CL, Yeh JK, Rasty J, Li Y, Watkins BA. Protective effect of dietary long-chain n-3 polyunsaturated fatty acids on bone loss in gonad-intact middle-aged male rats. Br J Nutr 2006;95:462-8.
16. Sun D, Krishnan A, Zaman K, Lawrence R, Bhattacharya A, Fernandes G. Dietary n-3 fatty acids decrease osteoclastogenesis and loss of bone mass in ovariectomized mice. J Bone Miner Res 2003;18:1206-16.
17. Kruger MC, Schollum LM. Is docosahexaenoic acid more effective than eicosapentaenoic acid for increasing calcium bioavailability? Prostaglandins Leukot Essent Fatty Acids 2005;73:327-34.
18. Weiler HA. Dietary supplementation of arachidonic acid is associated with higher whole body weight and bone mineral density in growing pigs. Pediatr Res 2000;47:692-7.
19. Innis SM. Essential fatty acids in growth and development. Prog Lipid Res 1991;30:39-103.
20. Bopp M, Lovelady C, Hunter C, Kinsella T. Maternal diet and exercise: effects on long-chain polyunsaturated fatty acid concentrations in breast milk. J Am Diet Assoc 2005;105:1098-103.
21. Uauy R, Mena P, Wegher B, Nieto S, Salem N, Jr. Long chain polyunsaturated fatty acid formation in neonates: effect of gestational age and intrauterine growth. Pediatr Res 2000;47:127-35.

22. Koletzko B, Agostoni C, Carlson SE, et al. Long chain polyunsaturated fatty acids (LC-PUFA) and perinatal development. Acta Paediatr 2001;90:460-4.
23. Kruger MC, Coetzer H, de Winter R, Gericke G, van Papendorp DH. Calcium, gamma-linolenic acid and eicosapentaenoic acid supplementation in senile osteoporosis. Aging (Milano) 1998;10:385-94.
24. Akesson A, Vahter M, Berglund M, Eklof T, Bremme K, Bjellerup P. Bone turnover from early pregnancy to postweaning. Acta Obstet Gynecol Scand 2004;83:1049-55.
25. Kalkwarf HJ, Specker BL. Bone mineral changes during pregnancy and lactation. Endocrine 2002;17:49-53.
26. Hsieh AT, Anthony JC, Diersen-Schade DA, et al. The influence of moderate and high dietary long chain polyunsaturated fatty acids (LCPUFA) on baboon neonate tissue fatty acids. Pediatr Res 2007;61:537-45.
27. Lian JB, Stein GS, Aubin JE. Bone formation: maturation and functional activities of osteoblast lineage cells. In: Favus MJ, ed. Primer on the Metabolic Bone Diseases and Disorders of Mineral Metabolism. Washington, D.C: American Society for Bone and Mineral Research, 2003:13-23.
28. Baron R. General principles of bone biology. In: Favus MJ, ed. Primer on Metabolic Bone Diseases and Disorders of Mineral Metabolism. Washington, DC: American Society for Bone and Mineral Research, 2003:1-7.
29. Martin TJ, Rodan GA. Coupling of bone resorption and formation during bone remodeling. In: Marcus R, Feldman D, Kelsey J, eds. Osteoporosis. USA: Academic Press, 2001:361-71.
30. Turner CH. Biomechanics of bone: determinants of skeletal fragility and bone quality. Osteoporos Int 2002;13:97-104.
31. Harvey N, Cooper C. Determinants of fracture risk in osteoporosis. Curr Rheumatol Rep 2003;5:75-81.

32. Kipp DE, Grey CE, McElvain ME, Kimmel DB, Robinson RG, Lukert BP. Long-term low ascorbic acid intake reduces bone mass in guinea pigs. J Nutr 1996;126:2044-9.
33. Turner CH, Burr DB. Basic biomechanical measurements of bone: a tutorial. Bone 1993;14:595-608.
34. Yilmaz D, Ersoy B, Bilgin E, Gumuser G, Onur E, Pinar ED. Bone mineral density in girls and boys at different pubertal stages: relation with gonadal steroids, bone formation markers, and growth parameters. J Bone Miner Metab 2005;23:476-82.
35. Yang L, Grey V. Pediatric reference intervals for bone markers. Clin Biochem 2006;39:561-8.
36. Grier SJ, Turner AS, Alvis MR. The use of dual-energy x-ray absorptiometry in animals. Invest Radiol 1996;31:50-62.
37. 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;70:719-27.
38. Njeh CF, Fuerst T, Hans D, Blake GM, Genant HK. Radiation exposure in bone mineral density assessment. Appl Radiat Isot 1999;50:215-36.
39. Fink C, Cooper HJ, Huebner JL, Guilak F, Kraus VB. Precision and accuracy of a transportable dual-energy X-ray absorptiometry unit for bone mineral measurements in guinea pigs. Calcif Tissue Int 2002;70:164-9.
40. Ward WE, Yuan YV, Cheung AM, Thompson LU. Exposure to purified lignan from flaxseed (Linum usitatissimum) alters bone development in female rats. Br J Nutr 2001;86:499-505.
41. Ward WE, Yuan YV, Cheung AM, Thompson LU. Exposure to flaxseed and its purified lignan reduces bone strength in young but not older male rats. J Toxicol Environ Health A 2001;63:53-65.
42. D'Erchia AM, Gissi C, Pesole G, Saccone C, Arnason U. The guinea-pig is not a rodent. Nature 1996;381:597-600.

43. Kind KL, Roberts CT, Sohlstrom AI, et al. Chronic maternal feed restriction impairs growth but increases adiposity of the fetal guinea pig. Am J Physiol Regul Integr Comp Physiol 2005;288:R119-26.
44. Briscoe TA, Rehn AE, Dieni S, et al. Cardiovascular and renal disease in the adolescent guinea pig after chronic placental insufficiency. Am J Obstet Gynecol 2004;191:847-55.
45. Rummens K, Van Herck E, van Bree R, Bouillon R, Van Assche FA, Verhaeghe J. Dietary calcium and phosphate restriction in guinea-pigs during pregnancy: fetal mineralization induces maternal hypocalcaemia despite increased 1 alpha,25-dihydroxycholecalciferol concentrations. Br J Nutr 2000;84:495-504.
46. Wagner JE, Manning P, J. The Biology of the Guinea Pig. New York: Academic Press Inc, 1976.
47. Poulos SP, Sisk M, Hausman DB, Azain MJ, Hausman GJ. Pre- and postnatal dietary conjugated linoleic acid alters adipose development, body weight gain and body composition in Sprague-Dawley rats. J Nutr 2001;131:2722-31.
48. Wang L, Banu J, McMahan CA, Kalu DN. Male rodent model of age-related bone loss in men. Bone 2001;29:141-8.
49. Banks R. The guinea pig: biology, care, identification, nomenclature, breeding, and genetics. USAMRIID Seminar Series, 1989.
50. Kapadia RD, Stroup GB, Badger AM, et al. Applications of micro-CT and MR microscopy to study pre-clinical models of osteoporosis and osteoarthritis. Technol Health Care 1998;6:361-72.
51. Kind KL, Clifton PM, Grant PA, et al. Effect of maternal feed restriction during pregnancy on glucose tolerance in the adult guinea pig. Am J Physiol Regul Integr Comp Physiol 2003;284:R140-52.
52. Kind KL, Clifton PM, Katsman AI, Tsiounis M, Robinson JS, Owens JA. Restricted fetal growth and the response to dietary cholesterol in the guinea pig. Am J Physiol 1999;277:R1675-82.

53. Kind KL, Simonetta G, Clifton PM, Robinson JS, Owens JA. Effect of maternal feed restriction on blood pressure in the adult guinea pig. Exp Physiol 2002;87:469-77.
54. Murphy MG, Wright V, Ackman RG, Horackova M. Diets enriched in menhaden fish oil, seal oil, or shark liver oil have distinct effects on the lipid and fatty-acid composition of guinea pig heart. Mol Cell Biochem 1997;177:257-69.
55. Murphy MG, Wright V, Scott J, Timmins A, Ackman RG. Dietary menhaden, seal, and corn oils differentially affect lipid and ex vivo eicosanoid and thiobarbituric acid-reactive substances generation in the guinea pig. Lipids 1999;34:115-24.
56. Reid ME. Nutritional studies with the guinea pig. VII. Niacin. J Nutr 1961;75:279-86.
57. Thorn SL, Young GS, Kirkland JB. The guinea-pig is a poor animal model for studies of niacin deficiency and presents challenges in any study using purified diets. Br J Nutr 2007;98:78-85.
58. Noonan D. The guinea pig-Fact Sheet. Insitute of Medical and Veterinary Science, 1994.
59. Barker DJ, Bull AR, Osmond C, Simmonds SJ. Fetal and placental size and risk of hypertension in adult life. Bmj 1990;301:259-62.
60. Phillips DI, Barker DJ, Hales CN, Hirst S, Osmond C. Thinness at birth and insulin resistance in adult life. Diabetologia 1994;37:150-4.
61. Yajnik C. Interactions of perturbations in intrauterine growth and growth during childhood on the risk of adult-onset disease. Proc Nutr Soc 2000;59:257-65.
62. Martyn CN, Barker DJ, Jespersen S, Greenwald S, Osmond C, Berry C. Growth in utero, adult blood pressure, and arterial compliance. Br Heart J 1995;73:116-21.
63. Namgung R, Tsang RC, Specker BL, Sierra RI, Ho ML. Reduced serum osteocalcin and 1,25-dihydroxyvitamin D concentrations and low bone

mineral content in small for gestational age infants: evidence of decreased bone formation rates. J Pediatr 1993;122:269-75.

64. Hales CN, Barker DJ. Type 2 (non-insulin-dependent) diabetes mellitus: the thrifty phenotype hypothesis. Diabetologia 1992;35:595-601.

65. Ozaki T, Nishina H, Hanson MA, Poston L. Dietary restriction in pregnant rats causes gender-related hypertension and vascular dysfunction in offspring. J Physiol 2001;530:141-52.

66. Holness MJ, Sugden MC. Antecedent protein restriction exacerbates development of impaired insulin action after high-fat feeding. Am J Physiol 1999;276:E85-93.

67. Woodall SM, Breier BH, Johnston BM, Gluckman PD. A model of intrauterine growth retardation caused by chronic maternal undernutrition in the rat: effects on the somatotrophic axis and postnatal growth. J Endocrinol 1996;150:231-42.

68. Langley SC, Jackson AA. Increased systolic blood pressure in adult rats induced by fetal exposure to maternal low protein diets. Clin Sci (Lond) 1994;86:217-22; discussion 121.

69. Langley-Evans SC, Welham SJ, Sherman RC, Jackson AA. Weanling rats exposed to maternal low-protein diets during discrete periods of gestation exhibit differing severity of hypertension. Clin Sci (Lond) 1996;91:607-15.

70. Snoeck A, Remacle C, Reusens B, Hoet JJ. Effect of a low protein diet during pregnancy on the fetal rat endocrine pancreas. Biol Neonate 1990;57:107-18.

71. Joshi S, Rao S, Golwilkar A, Patwardhan M, Bhonde R. Fish oil supplementation of rats during pregnancy reduces adult disease risks in their offspring. J Nutr 2003;133:3170-4.

72. Das UN. Can perinatal supplementation of long-chain polyunsaturated fatty acids prevent diabetes mellitus? Eur J Clin Nutr 2003;57:218-26.

73. Korotkova M, Ohlsson C, Gabrielsson B, Hanson LA, Strandvik B. Perinatal essential fatty acid deficiency influences body weight and bone parameters in adult male rats. Biochim Biophys Acta 2005;1686 3:248-54.
74. Garofano A, Czernichow P, Breant B. In utero undernutrition impairs rat beta-cell development. Diabetologia 1997;40:1231-4.
75. Desai M, Byrne CD, Meeran K, Martenz ND, Bloom SR, Hales CN. Regulation of hepatic enzymes and insulin levels in offspring of rat dams fed a reduced-protein diet. Am J Physiol 1997;273:G899-904.
76. Desai M, Byrne CD, Zhang J, Petry CJ, Lucas A, Hales CN. Programming of hepatic insulin-sensitive enzymes in offspring of rat dams fed a protein-restricted diet. Am J Physiol 1997;272:G1083-90.
77. Park KS, Kim SK, Kim MS, et al. Fetal and early postnatal protein malnutrition cause long-term changes in rat liver and muscle mitochondria. J Nutr 2003;133:3085-90.
78. Sparre T, Reusens B, Cherif H, et al. Intrauterine programming of fetal islet gene expression in rats--effects of maternal protein restriction during gestation revealed by proteome analysis. Diabetologia 2003;46:1497-511.
79. Armitage JA, Khan IY, Taylor PD, Nathanielsz PW, Poston L. Developmental programming of the metabolic syndrome by maternal nutritional imbalance: how strong is the evidence from experimental models in mammals? J Physiol 2004;561:355-77.
80. Shepherd PR, Crowther NJ, Desai M, Hales CN, Ozanne SE. Altered adipocyte properties in the offspring of protein malnourished rats. Br J Nutr 1997;78:121-9.
81. Dahri S, Snoeck A, Reusens-Billen B, Remacle C, Hoet JJ. Islet function in offspring of mothers on low-protein diet during gestation. Diabetes 1991;40 Suppl 2:115-20.
82. Franco Mdo C, Arruda RM, Dantas AP, et al. Intrauterine undernutrition: expression and activity of the endothelial nitric oxide synthase in male and female adult offspring. Cardiovasc Res 2002;56:145-53.

83. Sherman RC, Langley-Evans SC. Antihypertensive treatment in early postnatal life modulates prenatal dietary influences upon blood pressure in the rat. Clin Sci (Lond) 2000;98:269-75.

84. Langley-Evans SC, Jackson AA. Captopril normalises systolic blood pressure in rats with hypertension induced by fetal exposure to maternal low protein diets. Comp Biochem Physiol A Physiol 1995;110:223-8.

85. Vickers MH, Ikenasio BA, Breier BH. IGF-I treatment reduces hyperphagia, obesity, and hypertension in metabolic disorders induced by fetal programming. Endocrinology 2001;142:3964-73.

86. 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:E83-7.

87. Maloney CA, Gosby AK, Phuyal JL, Denyer GS, Bryson JM, Caterson ID. Site-specific changes in the expression of fat-partitioning genes in weanling rats exposed to a low-protein diet in utero. Obes Res 2003;11:461-8.

88. Mehta G, Roach HI, Langley-Evans S, et al. Intrauterine exposure to a maternal low protein diet reduces adult bone mass and alters growth plate morphology in rats. Calcif Tissue Int 2002;71:493-8.

89. Oreffo RO, Lashbrooke B, Roach HI, Clarke NM, Cooper C. Maternal protein deficiency affects mesenchymal stem cell activity in the developing offspring. Bone 2003;33:100-7.

90. Engelbregt MJ, van Weissenbruch MM, Lips P, van Lingen A, Roos JC, Delemarre-van de Waal HA. Body composition and bone measurements in intra-uterine growth retarded and early postnatally undernourished male and female rats at the age of 6 months: comparison with puberty. Bone 2004;34:180-6.

91. Lambert J, Lamothe JM, Zernicke RF, Auer RN, Reimer RA. Dietary restriction does not adversely affect bone geometry and mechanics in rapidly growing male wistar rats. Pediatr Res 2005;57:227-31.

92. Lee PA, Chernausek SD, Hokken-Koelega AC, Czernichow P. International Small for Gestational Age Advisory Board consensus development conference statement: management of short children born small for gestational age, April 24-October 1, 2001. Pediatrics 2003;111:1253-61.

93. Newburn-Cook CV, White D, Svenson LW, Demianczuk NN, Boff N, Edwards J. Where and to what extent is prevention of low birth weight possible? West J Nurs Res 2002;24:887-904.

94. Hediger ML, Overpeck MD, Maurer KR, Kuczmarski RJ, McGlynn A, Davis WW. Growth of infants and young children born small or large for gestational age: findings from the Third National Health and Nutrition Examination Survey. Arch Pediatr Adolesc Med 1998;152:1225-31.

95. Victora CG, Barros FC, Horta BL, Martorell R. Short-term benefits of catch-up growth for small-for-gestational-age infants. Int J Epidemiol 2001;30:1325-30.

96. Javaid MK, Cooper C. Prenatal and childhood influences on osteoporosis. Best Pract Res Clin Endocrinol Metab 2002;16:349-67.

97. Hokken-Koelega AC. Timing of puberty and fetal growth. Best Pract Res Clin Endocrinol Metab 2002;16:65-71.

98. Szathmari M, Vasarhelyi B, Szabo M, Szabo A, Reusz GS, Tulassay T. Higher osteocalcin levels and cross-links excretion in young men born with low birth weight. Calcif Tissue Int 2000;67:429-33.

99. Eriksson JG, Forsen TJ, Osmond C, Barker DJ. Pathways of infant and childhood growth that lead to type 2 diabetes. Diabetes Care 2003;26:3006-10.

100. Eriksson J, Forsen T, Tuomilehto J, Osmond C, Barker D. Size at birth, childhood growth and obesity in adult life. Int J Obes Relat Metab Disord 2001;25:735-40.

101. Arden NK, Major P, Poole JR, et al. Size at birth, adult intestinal calcium absorption and 1,25(OH)(2) vitamin D. Qjm 2002;95:15-21.

102. Luo ZC, Albertsson-Wikland K, Karlberg J. Length and body mass index at birth and target height influences on patterns of postnatal growth in children born small for gestational age. Pediatrics 1998;102:E72.

103. Luo ZC, Cheung YB, He Q, Albertsson-Wikland K, Karlberg J. Growth in early life and its relation to pubertal growth. Epidemiology 2003;14:65-73.

104. Albertsson-Wikland K, Wennergren G, Wennergren M, Vilbergsson G, Rosberg S. Longitudinal follow-up of growth in children born small for gestational age. Acta Paediatr 1993;82:438-43.

105. Hack M, Schluchter M, Cartar L, Rahman M, Cuttler L, Borawski E. Growth of very low birth weight infants to age 20 years. Pediatrics 2003;112:e30-8.

106. Koo WW, Walters JC, Hockman EM. Body composition in human infants at birth and postnatally. J Nutr 2000;130:2188-94.

107. Gale CR, Martyn CN, Kellingray S, Eastell R, Cooper C. Intrauterine programming of adult body composition. J Clin Endocrinol Metab 2001;86:267-72.

108. Hediger ML, Overpeck MD, Kuczmarski RJ, McGlynn A, Maurer KR, Davis WW. Muscularity and fatness of infants and young children born small- or large-for-gestational-age. Pediatrics 1998;102:E60.

109. Goulding A, Taylor RW, Jones IE, McAuley KA, Manning PJ, Williams SM. Overweight and obese children have low bone mass and area for their weight. Int J Obes Relat Metab Disord 2000;24:627-32.

110. Goulding A, Jones IE, Taylor RW, Williams SM, Manning PJ. Bone mineral density and body composition in boys with distal forearm fractures: a dual-energy x-ray absorptiometry study. J Pediatr 2001;139:509-15.

111. Specker BL, Johannsen N, Binkley T, Finn K. Total body bone mineral content and tibial cortical bone measures in preschool children. J Bone Miner Res 2001;16:2298-305.

112. Koo WW, Walters J, Bush AJ, Chesney RW, Carlson SE. Dual-energy X-ray absorptiometry studies of bone mineral status in newborn infants. J Bone Miner Res 1996;11:997-102.

113. Koo WW, Hockman EM. Physiologic predictors of lumbar spine bone mass in neonates. Pediatr Res 2000;48:485-9.

114. Palacios J, Rodriguez S, Rodriguez JI. Intra-uterine long bone growth in small-for-gestational-age infants. Eur J Pediatr 1992;151:304-7.

115. Pohlandt F, Mathers N. Bone mineral content of appropriate and light for gestational age preterm and term newborn infants. Acta Paediatr Scand 1989;78:835-9.

116. Minton SD, Steichen JJ, Tsang RC. Decreased bone mineral content in small-for-gestational-age infants compared with appropriate-for-gestational-age infants: normal serum 25-hydroxyvitamin D and decreasing parathyroid hormone. Pediatrics 1983;71:383-8.

117. Kohut J. Enhanced lumbar spine bone mineral content and bone mineral density in SGA piglets fed arachidonic acid and docosahexaenoic acid are modulated by birth weight. Department of Human Nutritional Sciences. Winnipeg: University of Manitoba, 2005a.

118. Kohut JR, Burr LL, Fitzpatrick-Wong SC, Weiler HA. Dietary arachidonic and docosahexaenoic acid improve bone mass and metabolism in female rat pups born small size for gestational age. submitted 2005b.

119. Fewtrell, Prentice A, Cole TJ, Lucas A. Effects of growth during infancy and childhood on bone mineralization and turnover in preterm children aged 8-12 years. Acta Paediatr 2000;89:148-53.

120. Scott KE, Usher R. Epiphyseal Development In Fetal Malnutrition Syndrome. N Engl J Med 1964;270:822-4.

121. Tsukahara H, Takeuchi M, Fujisawa K, et al. High-turnover osteopenia in preterm infants: determination of urinary pyridinium cross-links of collagen. Metabolism 1998;47:333-5.

122. Dennison EM, Syddall HE, Rodriguez S, Voropanov A, Day IN, Cooper C. Polymorphism in the growth hormone gene, weight in infancy, and adult bone mass. J Clin Endocrinol Metab 2004;89:4898-903.
123. Godfrey K, Walker-Bone K, Robinson S, et al. Neonatal bone mass: influence of parental birthweight, maternal smoking, body composition, and activity during pregnancy. J Bone Miner Res 2001;16:1694-703.
124. Cooper C, Javaid K, Westlake S, Harvey N, Dennison E. Developmental origins of osteoporotic fracture: the role of maternal vitamin D insufficiency. J Nutr 2005;135:2728S-34S.
125. Cooper C, Westlake S, Harvey N, Javaid K, Dennison E, Hanson M. Review: developmental origins of osteoporotic fracture. Osteoporos Int 2005.
126. Jaglal SB, Weller I, Mamdani M, et al. Population trends in BMD testing, treatment, and hip and wrist fracture rates: are the hip fracture projections wrong? J Bone Miner Res 2005;20:898-905.
127. Leslie WD, Anderson WA, Metge CJ, Manness LJ. Clinical risk factors for fracture in postmenopausal Canadian women: a population-based prevalence study. Bone 2007;40:991-6.
128. Groh-Wargo S, Jacobs J, Auestad N, O'Connor DL, Moore JJ, Lerner E. Body composition in preterm infants who are fed long-chain polyunsaturated fatty acids: a prospective, randomized, controlled trial. Pediatr Res 2005;57:712-8.
129. SanGiovanni JP, Berkey CS, Dwyer JT, Colditz GA. Dietary essential fatty acids, long-chain polyunsaturated fatty acids, and visual resolution acuity in healthy fullterm infants: a systematic review. Early Hum Dev 2000;57:165-88.
130. Cetin I, Giovannini N, Alvino G, et al. Intrauterine growth restriction is associated with changes in polyunsaturated fatty acid fetal-maternal relationships. Pediatr Res 2002;52:750-5.

131. Namgung R, Tsang RC. Factors affecting newborn bone mineral content: in utero effects on newborn bone mineralization. Proc Nutr Soc 2000;59:55-63.

132. Crawford MA, Doyle W, Drury P, Lennon A, Costeloe K, Leighfield M. n-6 and n-3 fatty acids during early human development. J Intern Med Suppl 1989;731:159-69.

133. Crawford MA, Costeloe K, Ghebremeskel K, Phylactos A, Skirvin L, Stacey F. Are deficits of arachidonic and docosahexaenoic acids responsible for the neural and vascular complications of preterm babies? Am J Clin Nutr 1997;66:1032S-1041S.

134. Kruger MC, Horrobin DF. Calcium metabolism, osteoporosis and essential fatty acids: a review. Prog Lipid Res 1997;36:131-51.

135. Weiler H, Fitzpatrick-Wong S, Schellenberg J, et al. Maternal and cord blood long chain polyunsaturated fatty acids are predictive of bone mass at birth in healthy, term-born infants (In press). Pediatric Research 2005;58:1-5.

136. Demmelmair H, von Schenck U, Behrendt E, Sauerwald T, Koletzko B. Estimation of arachidonic acid synthesis in full term neonates using natural variation of 13C content. J Pediatr Gastroenterol Nutr 1995;21:31-6.

137. Carnielli VP, Wattimena DJ, Luijendijk IH, Boerlage A, Degenhart HJ, Sauer PJ. The very low birth weight premature infant is capable of synthesizing arachidonic and docosahexaenoic acids from linoleic and linolenic acids. Pediatr Res 1996;40:169-74.

138. Carnielli VP, Verlato G, Pederzini F, et al. Intestinal absorption of long-chain polyunsaturated fatty acids in preterm infants fed breast milk or formula. Am J Clin Nutr 1998;67:97-103.

139. Innis SM, Gilley J, Werker J. Are human milk long-chain polyunsaturated fatty acids related to visual and neural development in breast-fed term infants? J Pediatr 2001;139:532-8.

140. Innis SM. Perinatal biochemistry and physiology of long-chain polyunsaturated fatty acids. J Pediatr 2003;143:S1-8.
141. Gray-Donald K, Jacobs-Starkey L, Johnson-Down L. Food habits of Canadians: reduction in fat intake over a generation. Can J Public Health 2000;91:381-5.
142. Smith EE, Ferguson VL, Simske SJ, Gayles EC, Pagliassotti MJ. Effects of high fat or high sucrose diets on rat femora mechanical and compositional properties. Biomed Sci Instrum 2000;36:385-90.
143. Haag M, Magada ON, Claassen N, Bohmer LH, Kruger MC. Omega-3 fatty acids modulate ATPases involved in duodenal Ca absorption. Prostaglandins Leukot Essent Fatty Acids 2003;68:423-9.
144. Watkins BA, Shen CL, Allen KG, Seifert MF. Dietary (n-3) and (n-6) polyunsaturates and acetylsalicylic acid alter ex vivo PGE2 biosynthesis, tissue IGF-I levels, and bone morphometry in chicks. J Bone Miner Res 1996;11:1321-32.
145. Weiss LA, Barrett-Connor E, von Muhlen D. Ratio of n-6 to n-3 fatty acids and bone mineral density in older adults: the Rancho Bernardo Study. Am J Clin Nutr 2005;81:934-8.
146. Terano T. Effect of omega 3 polyunsaturated fatty acid ingestion on bone metabolism and osteoporosis. World Rev Nutr Diet 2001;88:141-7.
147. Gunnes M, Lehmann EH. Physical activity and dietary constituents as predictors of forearm cortical and trabecular bone gain in healthy children and adolescents: a prospective study. Acta Paediatr 1996;85:19-25.
148. Trichopoulou A, Georgiou E, Bassiakos Y, et al. Energy intake and monounsaturated fat in relation to bone mineral density among women and men in Greece. Prev Med 1997;26:395-400.
149. Babu US, Mitchell GV, Wiesenfeld P, Jenkins MY, Gowda H. Nutritional and hematological impact of dietary flaxseed and defatted flaxseed meal in rats. Int J Food Sci Nutr 2000;51:109-17.
150. Cohen SL, Ward WE. Flaxseed oil and bone development in growing male and female mice. J Toxicol Environ Health A 2005;68:1861-70.

151. Weiler HA, Fitzpatrick-Wong SC. Modulation of essential (n-6):(n-3) fatty acid ratios alters fatty acid status but not bone mass in piglets. J Nutr 2002;132:2667-72.

152. Dodin S, Lemay A, Jacques H, Legare F, Forest JC, Masse B. The effects of flaxseed dietary supplement on lipid profile, bone mineral density, and symptoms in menopausal women: a randomized, double-blind, wheat germ placebo-controlled clinical trial. J Clin Endocrinol Metab 2005;90:1390-7.

153. Lucas EA, Wild RD, Hammond LJ, et al. Flaxseed improves lipid profile without altering biomarkers of bone metabolism in postmenopausal women. J Clin Endocrinol Metab 2002;87:1527-32.

154. Griel AE, Kris-Etherton PM, Hilpert KF, Zhao G, West SG, Corwin RL. An increase in dietary n-3 fatty acids decreases a marker of bone resorption in humans. Nutr J 2007;6:2.

155. Watkins BA, Li Y, Allen KG, Hoffmann WE, Seifert MF. Dietary ratio of (n-6)/(n-3) polyunsaturated fatty acids alters the fatty acid composition of bone compartments and biomarkers of bone formation in rats. J Nutr 2000;130:2274-84.

156. Watkins BA, Shen CL, McMurtry JP, et al. Dietary lipids modulate bone prostaglandin E2 production, insulin-like growth factor-I concentration and formation rate in chicks. J Nutr 1997;127:1084-91.

157. Coetzer H, Claassen N, van Papendorp DH, Kruger MC. Calcium transport by isolated brush border and basolateral membrane vesicles: role of essential fatty acid supplementation. Prostaglandins Leukot Essent Fatty Acids 1994;50:257-66.

158. Lucia VD, Fitzpatrick-Wong SC, Weiler HA. Dietary arachidonic acid suppresses bone turnover in contrast to low dosage exogenous prostaglandin E(2) that elevates bone formation in the piglet. Prostaglandins Leukot Essent Fatty Acids 2003;68:407-13.

159. Sirois I, Cheung AM, Ward WE. Biomechanical bone strength and bone mass in young male and female rats fed a fish oil diet. Prostaglandins Leukot Essent Fatty Acids 2003;68:415-21.

160. Poulsen RC, Kruger MC. Detrimental effect of eicosapentaenoic acid supplementation on bone following ovariectomy in rats. Prostaglandins Leukot Essent Fatty Acids 2006;75:419-27.

161. Lucey AJ, Paschos GK, Cashman KD, Martinez JA, Thorsdottir I, Kiely M. Influence of moderate energy restriction and seafood consumption on bone turnover in overweight young adults. Am J Clin Nutr 2008;87:1045-52.

162. Claassen N, Coetzer H, Steinmann CM, Kruger MC. The effect of different n-6/n-3 essential fatty acid ratios on calcium balance and bone in rats. Prostaglandins Leukot Essent Fatty Acids 1995;53:13-9.

163. Claassen N, Potgieter HC, Seppa M, et al. Supplemented gamma-linolenic acid and eicosapentaenoic acid influence bone status in young male rats: effects on free urinary collagen crosslinks, total urinary hydroxyproline, and bone calcium content. Bone 1995;16:385S-392S.

164. Blanaru JL, Kohut JR, Fitzpatrick-Wong SC, Weiler HA. Dose response of bone mass to dietary arachidonic acid in piglets fed cow milk-based formula. Am J Clin Nutr 2004;79:139-47.

165. Green KH, Wong SC, Weiler HA. The effect of dietary n-3 long-chain polyunsaturated fatty acids on femur mineral density and biomarkers of bone metabolism in healthy, diabetic and dietary-restricted growing rats. Prostaglandins Leukot Essent Fatty Acids 2004;71:121-30.

166. Liu D, Veit HP, Wilson JH, Denbow DM. Long-term supplementation of various dietary lipids alters bone mineral content, mechanical properties and histological characteristics of Japanese quail. Poult Sci 2003;82:831-9.

167. Liu D, Denbow DM. Maternal dietary lipids modify composition of bone lipids and ex vivo prostaglandin E2 production in early postnatal Japanese quail. Poult Sci 2001;80:1344-52.

168. Mimouni F, Campaigne B, Neylan M, Tsang RC. Bone mineralization in the first year of life in infants fed human milk, cow-milk formula, or soy-based formula. J Pediatr 1993;122:348-54.
169. Schanler RJ, Burns PA, Abrams SA, Garza C. Bone mineralization outcomes in human milk-fed preterm infants. Pediatr Res 1992;31:583-6.
170. Bishop NJ, Dahlenburg SL, Fewtrell MS, Morley R, Lucas A. Early diet of preterm infants and bone mineralization at age five years. Acta Paediatr 1996;85:230-6.
171. Chan GM. Growth and bone mineral status of discharged very low birth weight infants fed different formulas or human milk. J Pediatr 1993;123:439-43.
172. Specker BL, Beck A, Kalkwarf H, Ho M. Randomized trial of varying mineral intake on total body bone mineral accretion during the first year of life. Pediatrics 1997;99:E12.
173. Weiler HA, Fitzpatrick-Wong S. Dietary long-chain polyunsaturated fatty acids minimize dexamethasone-induced reductions in arachidonic acid status but not bone mineral content in piglets. Pediatr Res 2002;51:282-9.
174. Lillycrop KA, Phillips ES, Jackson AA, Hanson MA, Burdge GC. Dietary protein restriction of pregnant rats induces and folic acid supplementation prevents epigenetic modification of hepatic gene expression in the offspring. J Nutr 2005;135:1382-6.
175. Berger A, Mutch DM, German JB, Roberts MA. Dietary effects of arachidonate-rich fungal oil and fish oil on murine hepatic and hippocampal gene expression. Lipids Health Dis 2002;1:2.
176. Maurin AC, Chavassieux PM, Meunier PJ. Expression of PPARgamma and beta/delta in human primary osteoblastic cells: influence of polyunsaturated fatty acids. Calcif Tissue Int 2005;76:385-92.
177. Maurin AC, Chavassieux PM, Vericel E, Meunier PJ. Role of polyunsaturated fatty acids in the inhibitory effect of human adipocytes on osteoblastic proliferation. Bone 2002;31:260-6.

178. Kliewer SA, Sundseth SS, Jones SA, et al. Fatty acids and eicosanoids regulate gene expression through direct interactions with peroxisome proliferator-activated receptors alpha and gamma. Proc Natl Acad Sci U S A 1997;94:4318-23.
179. Khan E, Abu-Amer Y. Activation of peroxisome proliferator-activated receptor-gamma inhibits differentiation of preosteoblasts. J Lab Clin Med 2003;142:29-34.
180. Akune T, Ohba S, Kamekura S, et al. PPARgamma insufficiency enhances osteogenesis through osteoblast formation from bone marrow progenitors. J Clin Invest 2004;113:846-55.
181. Lecka-Czernik B, Gubrij I, Moerman EJ, et al. Inhibition of Osf2/Cbfa1 expression and terminal osteoblast differentiation by PPARgamma2. J Cell Biochem 1999;74:357-71.
182. Cock TA, Back J, Elefteriou F, et al. Enhanced bone formation in lipodystrophic PPARgamma(hyp/hyp) mice relocates haematopoiesis to the spleen. EMBO Rep 2004;5:1007-12.
183. Siddhivarn C, Banes A, Champagne C, Riche EL, Weerapradist W, Offenbacher S. Prostaglandin D pathway and peroxisome proliferator-activated receptor gamma-1 expression are induced by mechanical loading in an osteoblastic cell line. J Periodontal Res 2006;41:92-100.
184. Lecka-Czernik B, Moerman EJ, Grant DF, Lehmann JM, Manolagas SC, Jilka RL. Divergent effects of selective peroxisome proliferator-activated receptor-gamma 2 ligands on adipocyte versus osteoblast differentiation. Endocrinology 2002;143:2376-84.
185. Gat-Yablonski G, Phillip M. Leptin and regulation of linear growth. Curr Opin Clin Nutr Metab Care 2008;11:303-308.
186. Hess R, Pino AM, Rios S, Fernandez M, Rodriguez JP. High affinity leptin receptors are present in human mesenchymal stem cells (MSCs) derived from control and osteoporotic donors. J Cell Biochem 2005;94:50-7.

187. Goldstone AP, Howard JK, Lord GM, et al. Leptin prevents the fall in plasma osteocalcin during starvation in male mice. Biochem Biophys Res Commun 2002;295:475-81.
188. Elefteriou F, Takeda S, Ebihara K, et al. Serum leptin level is a regulator of bone mass. Proc Natl Acad Sci U S A 2004;101:3258-63.
189. Zambrano E, Bautista CJ, Deas M, et al. A low maternal protein diet during pregnancy and lactation has sex- and window of exposure-specific effects on offspring growth and food intake, glucose metabolism and serum leptin in the rat. J Physiol 2006;571:221-30.
190. Perez-Matute P, Marti A, Martinez JA, Moreno-Aliaga MJ. Effects of arachidonic acid on leptin secretion and expression in primary cultured rat adipocytes. J Physiol Biochem 2003;59:201-8.
191. Soliman M, Kimura K, Ahmed M, et al. Inverse regulation of leptin mRNA expression by short- and long-chain fatty acids in the cultured bovine adipocytes. Domest Anim Endocrinol 2006.
192. Chen X, Li D, Yin J, et al. Regulation of dietary energy level and oil source on leptin and its long form receptor mRNA expression of the adipose tissues in growing pigs. Domest Anim Endocrinol 2006;31:269-83.
193. Perez-Matute P, Marti A, Martinez JA, et al. Eicosapentaenoic fatty acid increases leptin secretion from primary cultured rat adipocytes: role of glucose metabolism. Am J Physiol Regul Integr Comp Physiol 2005;288:R1682-8.
194. Okada T, Sato NF, Kuromori Y, et al. Characteristics of obese children with low content of arachidonic acid in plasma lipids. Pediatr Int 2007;49:437-42.
195. Xu H, Watkins BA, Adkisson HD. Dietary lipids modify the fatty acid composition of cartilage, isolated chondrocytes and matrix vesicles. Lipids 1994;29:619-25.
196. Norrdin RW, Jee WS, High WB. The role of prostaglandins in bone in vivo. Prostaglandins Leukot Essent Fatty Acids 1990;41:139-49.

197. Sone K, Tashiro M, Fujinaga T, Tomomasa T, Tokuyama K, Kuroume T. Long-term low-dose prostaglandin E1 administration. J Pediatr 1980;97:866-7.

198. Miller SC, Marks SC, Jr. Effects of prostaglandins on the skeleton. Clin Plast Surg 1994;21:393-400.

199. Raisz LG, Fall PM. Biphasic effects of prostaglandin E2 on bone formation in cultured fetal rat calvariae: interaction with cortisol. Endocrinology 1990;126:1654-9.

200. McCarthy TL, Centrella M, Raisz LG, Canalis E. Prostaglandin E2 stimulates insulin-like growth factor I synthesis in osteoblast-enriched cultures from fetal rat bone. Endocrinology 1991;128:2895-900.

201. Collin P, Guenther HL, Fleisch H. Constitutive expression of osteoclast-stimulating activity by normal clonal osteoblast-like cells: effects of parathyroid hormone and 1,25-dihydroxyvitamin D3. Endocrinology 1992;131:1181-7.

202. Hakeda Y, Nakatani Y, Kurihara N, Ikeda E, Maeda N, Kumegawa M. Prostaglandin E2 stimulates collagen and non-collagen protein synthesis and prolyl hydroxylase activity in osteoblastic clone MC3T3-E1 cells. Biochem Biophys Res Commun 1985;126:340-5.

203. Hakeda Y, Nakatani Y, Hiramatsu M, et al. Inductive effects of prostaglandins on alkaline phosphatase in osteoblastic cells, clone MC3T3-E1. J Biochem (Tokyo) 1985;97:97-104.

204. Sato K, Fujii Y, Kasono K, Saji M, Tsushima T, Shizume K. Stimulation of prostaglandin E2 and bone resorption by recombinant human interleukin 1 alpha in fetal mouse bones. Biochem Biophys Res Commun 1986;138:618-24.

205. Hoffmann O, Klaushofer K, Gleispach H, et al. Gamma interferon inhibits basal and interleukin 1-induced prostaglandin production and bone resorption in neonatal mouse calvaria. Biochem Biophys Res Commun 1987;143:38-43.

206. Rosenquist JB, Ohlin A, Lerner UH. Cytokine-induced inhibition of bone matrix proteins is not mediated by prostaglandins. Inflamm Res 1996;45:457-63.

207. Raisz LG, Simmons HA. Effects of parathyroid hormone and cortisol on prostaglandin production by neonatal rat calvaria in vitro. Endocr Res 1985;11:59-74.

208. Klein-Nulend J, Pilbeam CC, Harrison JR, Fall PM, Raisz LG. Mechanism of regulation of prostaglandin production by parathyroid hormone, interleukin-1, and cortisol in cultured mouse parietal bones. Endocrinology 1991;128:2503-10.

209. Tang LY, Cullen DM, Yee JA, Jee WS, Kimmel DB. Prostaglandin E2 increases the skeletal response to mechanical loading. J Bone Miner Res 1997;12:276-82.

210. Weinreb M, Suponitzky I, Keila S. Systemic administration of an anabolic dose of PGE2 in young rats increases the osteogenic capacity of bone marrow. Bone 1997;20:521-6.

211. Weinreb M, Rutledge SJ, Rodan GA. Systemic administration of an anabolic dose of prostaglandin E2 induces early-response genes in rat bones. Bone 1997;20:347-53.

212. Abrahamsen B, Rohold A, Henriksen JE, Beck-Nielsen H. Correlations between insulin sensitivity and bone mineral density in non-diabetic men. Diabet Med 2000;17:124-9.

213. Li M, Jee WS, Ke HZ, et al. Prostaglandin E2 restores cancellous bone to immobilized limb and adds bone to overloaded limb in right hindlimb immobilization rats. Bone 1993;14:283-8.

214. Ito H, Ke HZ, Jee WS, Sakou T. Anabolic responses of an adult cancellous bone site to prostaglandin E2 in the rat. Bone Miner 1993;21:219-36.

215. Jee WS, Ke HZ, Li XJ. Long-term anabolic effects of prostaglandin-E2 on tibial diaphyseal bone in male rats. Bone Miner 1991;15:33-55.

216. Yao W, Jee WS, Zhou H, et al. Anabolic effect of prostaglandin E2 on cortical bone of aged male rats comes mainly from modeling-dependent bone gain. Bone 1999;25:697-702.

217. Sibonga JD, Zhang M, Ritman EL, Turner RT. Restoration of bone mass in the severely osteopenic senescent rat. J Gerontol A Biol Sci Med Sci 2000;55:B71-8; discussion B79-84.

218. Keila S, Kelner A, Weinreb M. Systemic prostaglandin E2 increases cancellous bone formation and mass in aging rats and stimulates their bone marrow osteogenic capacity in vivo and in vitro. J Endocrinol 2001;168:131-9.

219. Ke HZ, Jee WS, Zeng QQ, Li M, Lin BY. Prostaglandin E2 increased rat cortical bone mass when administered immediately following ovariectomy. Bone Miner 1993;21:189-201.

220. Jee WS, Mori S, Li XJ, Chan S. Prostaglandin E2 enhances cortical bone mass and activates intracortical bone remodeling in intact and ovariectomized female rats. Bone 1990;11:253-66.

221. Kokkinos PP, Shaye R, Alam BS, Alam SQ. Dietary lipids, prostaglandin E2 levels, and tooth movement in alveolar bone of rats. Calcif Tissue Int 1993;53:333-7.

222. Venkatraman JT, Chu WC. Effects of dietary omega-3 and omega-6 lipids and vitamin E on serum cytokines, lipid mediators and anti-DNA antibodies in a mouse model for rheumatoid arthritis. J Am Coll Nutr 1999;18:602-13.

223. Evans DB, Bunning RA, Russell RG. The effects of recombinant human interleukin-1 beta on cellular proliferation and the production of prostaglandin E2, plasminogen activator, osteocalcin and alkaline phosphatase by osteoblast-like cells derived from human bone. Biochem Biophys Res Commun 1990;166:208-16.

224. Evans DB, Thavarajah M, Kanis JA. Involvement of prostaglandin E2 in the inhibition of osteocalcin synthesis by human osteoblast-like cells in

response to cytokines and systemic hormones. Biochem Biophys Res Commun 1990;167:194-202.

225. Jones DH, Kong YY, Penninger JM. Role of RANKL and RANK in bone loss and arthritis. Ann Rheum Dis 2002;61 Suppl 2:ii32-9.

226. Roodman GD. Cell biology of the osteoclast. Exp Hematol 1999;27:1229-41.

227. Han SY, Lee NK, Kim KH, et al. Transcriptional induction of cyclooxygenase-2 in osteoclast precursors is involved in RANKL-induced osteoclastogenesis. Blood 2005;106:1240-5.

228. Gasparoni A, Ciardelli L, Avanzini A, et al. Age-related changes in intracellular TH1/TH2 cytokine production, immunoproliferative T lymphocyte response and natural killer cell activity in newborns, children and adults. Biol Neonate 2003;84:297-303.

229. Berrington JE, Barge D, Fenton AC, Cant AJ, Spickett GP. Lymphocyte subsets in term and significantly preterm UK infants in the first year of life analysed by single platform flow cytometry. Clin Exp Immunol 2005;140:289-92.

230. Conover CA, Lee PD, Riggs BL, Powell DR. Insulin-like growth factor-binding protein-1 expression in cultured human bone cells: regulation by insulin and glucocorticoid. Endocrinology 1996;137:3295-301.

231. Rosen CJ, Donahue LR. Insulin-like growth factors and bone: the osteoporosis connection revisited. Proc Soc Exp Biol Med 1998;219:1-7.

232. Yakar S, Rosen CJ, Beamer WG, et al. Circulating levels of IGF-1 directly regulate bone growth and density. J Clin Invest 2002;110:771-81.

233. Ueland T. GH/IGF-I and bone resorption in vivo and in vitro. Eur J Endocrinol 2005;152:327-32.

234. Terzolo M, Piovesan A, Osella G, et al. Serum levels of bone Gla protein (osteocalcin, BGP) and carboxyterminal propeptide of type I procollagen (PICP) in acromegaly: effects of long-term octreotide treatment. Calcif Tissue Int 1993;52:188-91.

235. Schmid C, Schlapfer I, Waldvogel M, Zapf J, Froesch ER. Prostaglandin E2 stimulates synthesis of insulin-like growth factor binding protein-3 in rat bone cells in vitro. J Bone Miner Res 1992;7:1157-63.
236. Holick MF. Vitamin D and bone health. J Nutr 1996;126:1159S-64S.
237. Chen JY, Ling UP, Chiang WL, Liu CB, Chanlai SP. Total body bone mineral content in small-for-gestational -age, appropriate-for-gestational -age, large-for-gestational -age term infants and appropriate-for-gestational -age preterm infants. Zhonghua Yi Xue Za Zhi (Taipei) 1995;56:109-14.
238. Mollard RC, Kohut J, Zhao J, Weiler HA. Proximal intestinal absorption of calcium is elevated in proportion to growth rate but not bone mass is small for gestational age piglets. J Nutr Biochem 2004;15:149-54.
239. Ryan AS, Montalto MB, Groh-Wargo S, et al. Effect of DHA-containing formula on growth of preterm infants to 59 weeks postmenstrual age. Am J Hum Biol 1999;11:457-467.
240. Llanos A, Li Y, Mena P, Salem N, Jr., Uauy R. Infants with intrauterine growth restriction have impaired formation of docosahexaenoic acid in early neonatal life: a stable isotope study. Pediatr Res 2005;58:735-40.
241. Koletzko B, Cetin I, Brenna JT. Dietary fat intakes for pregnant and lactating women. Br J Nutr 2007;98:873-7.
242. Care CCoA. Guide to the care and use of experimental animals, 1984.
243. Reeves PG. Components of the AIN-93 diets as improvements in the AIN-76A diet. J Nutr 1997;127:838S-841S.
244. Huebner JL, Hanes MA, Beekman B, TeKoppele JM, Kraus VB. A comparative analysis of bone and cartilage metabolism in two strains of guinea-pig with varying degrees of naturally occurring osteoarthritis. Osteoarthritis Cartilage 2002;10:758-67.
245. van der Sluis IM, de Muinck Keizer-Schrama SM. Osteoporosis in childhood: bone density of children in health and disease. J Pediatr Endocrinol Metab 2001;14:817-32.

246. Lu PW, Briody JN, Ogle GD, et al. Bone mineral density of total body, spine, and femoral neck in children and young adults: a cross-sectional and longitudinal study. J Bone Miner Res 1994;9:1451-8.
247. Starfield B, Shapiro S, McCormick M, Bross D. Mortality and morbidity in infants with intrauterine growth retardation. J Pediatr 1982;101:978-83.
248. Ong KK. Catch-up growth in small for gestational age babies: good or bad? Curr Opin Endocrinol Diabetes Obes 2007;14:30-4.
249. Namgung R, Tsang RC. Bone in the pregnant mother and newborn at birth. Clin Chim Acta 2003;333:1-11.
250. Pearson D, Kaur M, San P, Lawson N, Baker P, Hosking D. Recovery of pregnancy mediated bone loss during lactation. Bone 2004;34:570-8.
251. Henderson PH, 3rd, Sowers M, Kutzko KE, Jannausch ML. Bone mineral density in grand multiparous women with extended lactation. Am J Obstet Gynecol 2000;182:1371-7.
252. Sowers MF, Scholl T, Harris L, Jannausch M. Bone loss in adolescent and adult pregnant women. Obstet Gynecol 2000;96:189-93.
253. Miller SC, Anderson BL, Bowman BM. Weaning initiates a rapid and powerful anabolic phase in the rat maternal skeleton. Biol Reprod 2005;73:156-62.
254. Silva HG, Tortora RP, Farias ML. Increased bone turnover during the third trimester of pregnancy and decreased bone mineral density after parturition in adolescents as compared to age-matched control patients. Gynecol Endocrinol 2005;21:174-9.
255. Health C. Nutrition for healthy term infant. Ottawa, Ontario: Minister of Health, 1998.
256. Helland IB, Smith L, Saarem K, Saugstad OD, Drevon CA. Maternal supplementation with very-long-chain n-3 fatty acids during pregnancy and lactation augments children's IQ at 4 years of age. Pediatrics 2003;111:e39-44.

257. Chan GM, Roberts CC, Folland D, Jackson R. Growth and bone mineralization of normal breast-fed infants and the effects of lactation on maternal bone mineral status. Am J Clin Nutr 1982;36:438-43.
258. Ulrich U, Miller PB, Eyre DR, Chesnut CH, 3rd, Schlebusch H, Soules MR. Bone remodeling and bone mineral density during pregnancy. Arch Gynecol Obstet 2003;268:309-16.
259. Helland IB, Saugstad OD, Saarem K, Van Houwelingen AC, Nylander G, Drevon CA. Supplementation of n-3 fatty acids during pregnancy and lactation reduces maternal plasma lipid levels and provides DHA to the infants. J Matern Fetal Neonatal Med 2006;19:397-406.
260. Sciences NAo. Nutritional requirements of the guinea pig. Nutritional requirements of laboratory animals 4th edition, 1995.
261. Javaid MK, Godfrey KM, Taylor P, et al. Umbilical cord leptin predicts neonatal bone mass. Calcif Tissue Int 2005;76:341-7.
262. Carnielli VP, Simonato M, Verlato G, et al. Synthesis of long-chain polyunsaturated fatty acids in preterm newborns fed formula with long-chain polyunsaturated fatty acids. Am J Clin Nutr 2007;86:1323-30.
263. Perin N, Jarocka-Cyrta E, Keelan M, Clandinin T, Thomson A. Dietary lipid composition modifies intestinal morphology and nutrient transport in young rats. J Pediatr Gastroenterol Nutr 1999;28:46-53.
264. Cottrell EC, Ozanne SE. Early life programming of obesity and metabolic disease. Physiol Behav 2008;94:17-28.
265. Lucas A. Long-term programming effects of early nutrition -- implications for the preterm infant. J Perinatol 2005;25 Suppl 2:S2-6.
266. Maurage C. Children's nutrition and health in adulthood. Appetite 2008.
267. Rummens K, van Bree R, Van Herck E, et al. Vitamin D deficiency in guinea pigs: exacerbation of bone phenotype during pregnancy and disturbed fetal mineralization, with recovery by 1,25(OH)2D3 infusion or dietary calcium-phosphate supplementation. Calcif Tissue Int 2002;71:364-75.

Appendix A

Animal Ethics Certificate

Appendix B

Diet specifications

Purified Guinea Pig Diet, Apple Flavored — 5TYJ

DESCRIPTION

Purified guinea pig diet.

Storage conditions are particularly critical to TestDiet® products, due to the absence of antioxidants or preservative agents. To provide maximum protection against possible changes during storage, store in a dry, cool location. Storage under refrigeration (2° C) is recommended. Maximum shelf life is six months. (If long term studies are involved, storing the diet at -20° C or colder may prolong shelf life.) Be certain to keep in air tight containers.

*Product Forms Available**	*Catalog #*
3/16" Pellet	1811248

*Other Forms Available By Request

INGREDIENTS (%)

Sucrose	28.9009
Corn Starch	21.8000
Soy Protein Isolate	16.7171
Powdered Cellulose	14.0000
Soybean Oil	6.0000
Casein - Vitamin Free	5.0000
Dicalcium Phosphate	2.8434
Potassium Citrate, Tribasic Monohydrate	1.1323
Calcium Carbonate	1.1263
Potassium Carbonate	0.6799
Salt	0.5808
Artificial Flavors	0.5000
Choline Chloride	0.2643
Ascorbic Acid	0.2335
Magnesium Oxide	0.1468
Vitamin and Mineral Premix	0.1468
Vitamin K Premix	0.0628
L-Methionine	0.0539
Ethoxyquin (a preservative)	0.0012

NUTRITIONAL PROFILE [1]

Protein, %	***19.3***	***Minerals***	
Arginine, %	1.43	Calcium, %	1.08
Histidine, %	0.56	Phosphorus, %	0.65
Isoleucine, %	1.06	Phosphorus (available), %	0.52
Leucine, %	1.79	Potassium, %	0.80
Lysine, %	1.42	Magnesium, %	0.10
Methionine, %	0.40	Sodium, %	0.35
Cystine, %	0.24	Chlorine, %	0.40
Phenylalanine, %	1.14	Fluorine, ppm	15.9
Tyrosine, %	0.92	Iron, ppm	75
Threonine, %	0.81	Zinc, ppm	70
Tryptophan, %	0.31	Manganese, ppm	76
Valine, %	1.04	Copper, ppm	13.0
Alanine, %	0.94	Cobalt, ppm	3.1
Aspartic Acid, %	2.31	Iodine, ppm	0.59
Glutamic Acid, %	4.62	Chromium, ppm	1.6
Glycine, %	0.80	Molybdenum, ppm	0.58
Proline, %	1.51	Selenium, ppm	0.30
Serine, %	1.15		
Taurine, %	0.00	***Vitamins***	
Fat, %	***6.1***	Vitamin A, IU/g	33.0
Cholesterol, ppm	0	Vitamin D-3 (added), IU/g	1.2
Linoleic Acid, %	3.07	Vitamin E, IU/kg	50.0
Linolenic Acid, %	0.47	Vitamin K (as menadione), ppm	2.40
Arachidonic Acid, %	0.00	Thiamin Hydrochloride, ppm	9.3
Omega-3 Fatty Acids, %	0.47	Riboflavin, ppm	6.2
Total Saturated Fatty A	0.88	Niacin, ppm	66
Total Monounsaturated Fatty Acids, %	1.26	Pantothenic Acid, ppm	19
Polyunsaturated Fatty Acids, %	3.53	Folic Acid, ppm	3.0
Fiber (max), %	***14.0***	Pyridoxine, ppm	4.1
Carbohydrates, %	***50.8***	Biotin, ppm	0.3
Energy (kcal/g) [2]	***3.33***	Vitamin B-12, mcg/kg	10
		Choline Chloride, ppm	1,850
		Ascorbic Acid, ppm	2,300.0

From:	kcal	%
Protein	0.772	23.0
Fat (ether extract)	0.548	16.4
Carbohydrates	2.031	60.6

1. Based on the latest ingredient analysis information. Since nutrient composition of natural ingredients varies, analysis will differ accordingly. Nutrients expressed as percent of ration on an As Fed basis except where otherwise indicated.
2: Energy (kcal/gm) - Sum of decimal fractions of protein, fat and carbohydrate x 4,9,4 kcal/gm respectively.

FEEDING DIRECTIONS

Feed ad libitum. Plenty of fresh, clean water should be available at all times.

CAUTION:
Perishable, upon receipt store in a cool dry place, refrigeration recommended.

For laboratory animal experimental use only, NOT for human consumption.

3/24/2006

ISO 9001:2000 CERTIFIED

TestDiet
www.testdiet.com

Low Protein Purified Guinea Pig Diet, Apple Flavor — 5TYN

DESCRIPTION

Modification of TestDiet® 5TYJ Semi-Purified Diet with low crude protein and apple flavoring.

Storage conditions are particularly critical to TestDiet® products, due to the absence of antioxidants or preservative agents. To provide maximum protection against possible changes during storage, store in a dry, cool location. Storage under refrigeration (2° C) is recommended. Maximum shelf life is six months. (If long term studies are involved, storing the diet at -20° C or colder may prolong shelf life.) Be certain to keep in air tight containers.

*Product Forms Available**	*Catalog #*
3/16" Pellet	1811266

*Other Forms Available By Request

INGREDIENTS (%)

Corn Starch	31.3237
Sucrose	29.0000
Powdered Cellulose	14.0000
Soy Protein Isolate	8.0400
Soybean Oil	6.0000
Dicalcium Phosphate	3.0699
Casein - Vitamin Free	2.6500
Potassium Citrate, Tribasic Monohydrate	2.1898
Calcium Carbonate	0.9060
Salt	0.6128
Artificial Flavors	0.5000
Magnesium Oxide	0.4053
L-Methionine	0.3000
Vitamin/Mineral Premix	0.2736
Choline Chloride	0.2643
Ascorbic Acid	0.2335
Sodium Phosphate	0.1171
Vitamin K Premix	0.0628
L-Cystine	0.0500
Ethoxyquin (a preservative)	0.0012

NUTRITIONAL PROFILE [1]

Protein, %	***9.9***
Arginine, %	0.70
Histidine, %	0.28
Isoleucine, %	0.52
Leucine, %	0.88
Lysine, %	0.70
Methionine, %	0.47
Cystine, %	0.16
Phenylalanine, %	0.56
Tyrosine, %	0.46
Threonine, %	0.40
Tryptophan, %	0.15
Valine, %	0.51
Alanine, %	0.46
Aspartic Acid, %	1.13
Glutamic Acid, %	2.27
Glycine, %	0.39
Proline, %	0.75
Serine, %	0.56
Taurine, %	0.00
Fat, %	***6.1***
Cholesterol, ppm	0
Linoleic Acid, %	3.07
Linolenic Acid, %	0.47
Arachidonic Acid, %	0.00
Omega-3 Fatty Acids, %	0.47
Total Saturated Fatty A	0.88
Total Monounsaturated Fatty Acids, %	1.26
Polyunsaturated Fatty Acids, %	3.53
Fiber (max), %	***14.0***
Carbohydrates, %	***60.4***
Energy (kcal/g) [2]	***3.34***

From:	kcal	%
Protein	0.396	11.8
Fat (ether extract)	0.548	16.3
Carbohydrates	2.415	71.9

Minerals	
Calcium, %	1.08
Phosphorus, %	0.66
Phosphorus (available), %	0.60
Potassium, %	0.80
Magnesium, %	0.25
Sodium, %	0.35
Chlorine, %	0.41
Fluorine, ppm	15.8
Iron, ppm	75
Zinc, ppm	70
Manganese, ppm	76
Copper, ppm	13.1
Cobalt, ppm	3.3
Iodine, ppm	0.59
Chromium, ppm	1.6
Molybdenum, ppm	0.47
Selenium, ppm	0.24
Vitamins	
Vitamin A, IU/g	33.0
Vitamin D-3 (added), IU/g	1.2
Vitamin E, IU/kg	49.9
Vitamin K (as menadione), ppm	2.40
Thiamin Hydrochloride, ppm	9.3
Riboflavin, ppm	6.1
Niacin, ppm	66
Pantothenic Acid, ppm	19
Folic Acid, ppm	3.0
Pyridoxine, ppm	4.1
Biotin, ppm	0.3
Vitamin B-12, mcg/kg	10
Choline Chloride, ppm	1,850
Ascorbic Acid, ppm	2,300.0

1. Based on the latest ingredient analysis information. Since nutrient composition of natural ingredients varies, analysis will differ accordingly. Nutrients expressed as percent of ration on an As Fed basis except where otherwise indicated.
2. Energy (kcal/gm) - Sum of decimal fractions of protein, fat and carbohydrate x 4,9,4 kcal/gm respectively.

FEEDING DIRECTIONS

Feed ad libitum. Plenty of fresh, clean water should be available at all times.

CAUTION:
Perishable, upon receipt store in a cool dry place, refrigeration recommended.

For laboratory animal experimental use only, NOT for human consumption.

4/10/2006

TestDiet
www.testdiet.com

Purified Guinea Pig Diet, Apple Flavor, w/DHASCO and ARASC 5A3Z

DESCRIPTION

Purified guinea pig diet with apple flavoring and DHASCO and ARASCO.

Storage conditions are particularly critical to TestDiet® products, due to the absence of antioxidants or preservative agents. To provide maximum protection against possible changes during storage, store in a dry, cool location. Storage under refrigeration (2° C) is recommended. Maximum shelf life is six months. (If long term studies are involved, storing the diet at -20° C or colder may prolong shelf life.) Be certain to keep in air tight containers.

*Product Forms Available**	*Catalog #*
3/16" Pellet	1811497
3/16" Pellet	1811497

*Other Forms Available By Request

INGREDIENTS (%)

Sucrose	28.2685
Corn Starch	21.8000
Soy Protein Isolate	16.7171
Powdered Cellulose	14.0000
Casein - Vitamin Free	5.0000
Soybean Oil	4.8511
Dicalcium Phosphate	2.6990
Potassium Citrate, Tribasic Monohydrate	2.1667
Calcium Carbonate	1.1029
ARASCO	0.8205
Salt	0.5934
Artificial Flavors	0.5000
Magnesium Oxide	0.4048
DHASCO	0.3283
Choline Chloride	0.2643
Ascorbic Acid	0.2335
Vitamin and Mineral Premix	0.1320
Vitamin K Premix	0.0628
L-Methionine	0.0539
Ethoxyquin (a preservative)	0.0012

NUTRITIONAL PROFILE [1]

Protein, %	***19.3***
Arginine, %	1.43
Histidine, %	0.56
Isoleucine, %	1.06
Leucine, %	1.79
Lysine, %	1.42
Methionine, %	0.40
Cystine, %	0.24
Phenylalanine, %	1.14
Tyrosine, %	0.92
Threonine, %	0.81
Tryptophan, %	0.31
Valine, %	1.04
Alanine, %	0.94
Aspartic Acid, %	2.31
Glutamic Acid, %	4.62
Glycine, %	0.80
Proline, %	1.51
Serine, %	1.15
Taurine, %	0.00
Fat, %	***6.1***
Cholesterol, ppm	0
Linoleic Acid, %	2.54
Linolenic Acid, %	0.40
Arachidonic Acid, %	0.00
Omega-3 Fatty Acids, %	0.38
Total Saturated Fatty A	0.71
Total Monounsaturated Fatty Acids, %	1.02
Polyunsaturated Fatty Acids, %	2.86
Fiber (max), %	***14.0***
Carbohydrates, %	***50.1***
Energy (kcal/g) [2]	***3.31***

From:	kcal	%
Protein	0.772	23.2
Fat (ether extract)	0.548	16.5
Carbohydrates	2.005	60.3

Minerals	
Calcium, %	1.08
Phosphorus, %	0.66
Phosphorus (available), %	0.53
Potassium, %	0.80
Magnesium, %	0.25
Sodium, %	0.35
Chlorine, %	0.40
Fluorine, ppm	15.9
Iron, ppm	75
Zinc, ppm	70
Manganese, ppm	76
Copper, ppm	13.0
Cobalt, ppm	3.1
Iodine, ppm	0.59
Chromium, ppm	1.6
Molybdenum, ppm	0.57
Selenium, ppm	0.24
Vitamins	
Vitamin A, IU/g	33.0
Vitamin D-3 (added), IU/g	1.2
Vitamin E, IU/kg	53.9
Vitamin K (as menadione), ppm	2.40
Thiamin Hydrochloride, ppm	9.3
Riboflavin, ppm	6.2
Niacin, ppm	66
Pantothenic Acid, ppm	19
Folic Acid, ppm	3.0
Pyridoxine, ppm	4.1
Biotin, ppm	0.3
Vitamin B-12, mcg/kg	10
Choline Chloride, ppm	1,850
Ascorbic Acid, ppm	2,300.0

1. Based on the latest ingredient analysis information. Since nutrient composition of natural ingredients varies, analysis will differ accordingly. Nutrients expressed as percent of ration on an As Fed basis except where otherwise indicated.
2. Energy (kcal/gm) - Sum of decimal fractions of protein, fat and carbohydrate x 4,9,4 kcal/gm respectively.

FEEDING DIRECTIONS

Feed ad libitum. Plenty of fresh, clean water should be available at all times.

CAUTION:
Perishable, upon receipt store in a cool dry place, refrigeration recommended.

For laboratory animal experimental use only, NOT for human consumption.

4/11/2006

ISO 9001:2000 CERTIFIED

TestDiet
www.testdiet.com

Appendix C

Author waiver