The effect of long chain polyunsaturated fatty acids on bone throughout the lifespan: an investigation of bone mass and its regulation by bone and mineral metabolism.

by

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A Thesis submitted to the Faculty of Graduate Studies of
The University of Manitoba
in partial fulfillment of the requirements of the degree of

Doctor of Philosophy

Department of Food and Nutritional Sciences
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THE UNIVERSITY OF MANITOBA

FACULTY OF GRADUATE STUDIES

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The Effect of Long Chain Polyunsaturated Fatty Acids on Bone Throughout the Lifespan: An Investigation of Bone Mass and its Regulation by Bone and Mineral Metabolism

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Rebecca C. Mollard

A Thesis/Practicum submitted to the Faculty of Graduate Studies of The University of Manitoba in partial fulfillment of the requirement of the degree

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ABSTRACT

The pathology of osteoporosis is low bone mass potentially leading to fractures. Dietary long chain polyunsaturated fatty acids (LCPUFA), including arachidonic acid (AA), eicosapenaenoic acid (EPA) and docosahexaenoic acid (DHA), influence bone mass, mineral content and metabolism. The amount of each of these LCPUFA required throughout the lifespan to optimize bone health is unknown. To further understand the effects of specific LCPUFA in infant bone growth, dietary AA and DHA in varying amounts at a 5:1 ratio were added to formula and fed to male piglets for 15 d. Bone mass, as measured by dual energy x-ray absorptiometry (DXA), was higher with 0.5 and 0.1 g/100 g of fat as AA and DHA than in the unsupplmented group. These effects were not explained by changes in bone (including tibia prostaglandin E_2) or mineral metabolism. Higher amounts (1.0 g AA and 0.2 g DHA/100 g of fat) reduced bone resorption, altered femur Zn and Ca content, but did not affect bone mass. To assess whether other life stages were affected by specific LCPUFA, a long-term rat study was conducted. The effects of an EPA diet (0.5 g EPA, 0.1 g AA and 0.1 g DHA/100 g of fat) or an AA diet (0.5 g AA, 0.1 g EPA and 0.1 g DHA/100 g of fat), to reflect current recommendations and/or intakes, on bone mineral content (BMC) and area (BA) using DXA and femur morphometry were compared at 3 time points (early, mid and late) from weanling to maturity (over 49 wk) in male and female rats. Compared to control, dietary EPA, regardless of when supplemented, decreased femur BA in males and decreased urinary Ca in females. At the levels studied, neither AA nor EPA affected BMC, bone resorption or femur prostaglandin E_2. Compared to control, males fed AA mid or continuously had wider femur neck and head widths, respectively. Femur Mg, Zn and P content, but not

Ca, were altered by dietary AA and EPA. The effects on Zn and P content were gender specific. Dietary AA or EPA with DHA affect mineral metabolism and bone size or mass at different stages in the life cycle. Whether higher neonatal bone mass and elevated femur neck and head width during maturity offer protection against osteoporotic fracture requires clarification.

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TABLE OF CONTENTS

Abstract ii

Acknowledgements iv

Table of Contents v

List of Tables xiii

List of Figures xvii

List of Abbreviations xviii

List of Copyright Material xx

1. GENERAL INTRODUCTION 1

1.1 Introduction 2

1.2 References 8

2. LITERATURE REVIEW 12

2.1 Polyunsaturated Fatty Acids 13

2.1.1 The essentiality of dietary n-6 and n-3 polyunsaturated fatty acids 13

2.1.2 The metabolism of n-6 and n-3 polyunsaturated fatty acids 14

2.1.3 Requirements of polyunsaturated fatty acids across the lifespan 17

Infancy (0 to 12 months) 18

Childhood (1-18 years of age) 25

Adulthood (18 years and older) 25

2.1.4 Metabolism of eicosanoids from polyunsaturated fatty acids 27

The arachidonic acid cascade in the synthesis of eicosanoids 28

Synthesis of eicosanoids from alternative polyunsaturated fatty acids 29

2.2 Bone mass and metabolism 30

2.2.1 Bone cells and bone metabolism 30

Osteoblasts 30

Osteocytes 31

Osteoclasts 32

2.2.2 Bone matrix and mineral 33

2.2.3 Bone growth: modelling 34

2.2.4 Bone remodelling 35

2.2.5 Regulation of bone metabolism 38

Insulin-like growth factors 38

Prostaglandins 40

2.2.6 Biochemical markers of bone metabolism 44

Bone formation 44

Alkaline phosphatase

Osteocalcin

Procollagen I extension peptides

Resorption 47

Hydroxyproline

Collagen crosslink molecules

2.2.7 Techniques for measurement of bone mass 48

2.3 Dietary polyunsaturated fatty acid affects on bone 51

2.3.1 Bone polyunsaturated fatty acid composition 51

2.3.2 Effects of dietary n-6 and n-3 polyunsaturated fatty acids on bone and mineral metabolism in animal models 52

Infancy 53

Weanling 58

Ovariectomized 64

2.3.3 The effects of dietary polyunsaturated fatty acids on bone in humans 66

2.3.4 Animal models 71

Piglet model 71

Rat Model 73

2.4 References 75

3. RATIONALE 101

3.1 Rationale Introduction 102

3.2 Gaps in current state of knowledge 102

3.2.1 Effects of dietary polyunsaturated fatty acids on bone mass early in life: infancy and growth 102

3.2.2 Effects of dietary polyunsaturated fatty acids on bone mass beyond infancy: programming 103

3.2.3 Healthy aging 104

3.2.4 Changing requirements across the lifespan 105

3.2.5 Amounts of n-6 and n-3 polyunsaturated fatty acids used in research 105

3.2.6 Gender differences in response to dietary n-6 and n-3 polyunsaturated fatty acids 106

3.3 Objectives and hypotheses 107

3.3.1 The effects of dietary arachidonic acid and docosahexaenoic acid on bone during infancy 107

Objectives 108

Alternate Hypotheses 108

3.3.2 The role of long chain polyunsaturated acids on bone mass beyond infancy 109

Objectives 110

Alternate Hypotheses 110

3.4 References 112

4. LOW LEVELS OF DIETARY ARACHIDONIC ACID AND DOCOSAHEXAENOIC ACID IMPROVE BONE MASS IN NEONATAL PIGLETS, WHILE HIGHER LEVELS PROVIDE NO BENEFIT 114

4.1 Abstract 117

4.2 Introduction 118

4.3 Materials and methods 122

4.3.1 Animals and Diet 122

4.3.2 Measurements 123

Growth 123

Sample collection 124

Fatty acid analysis 124

Bone mass and biochemistry 125

4.4 Statistical analysis 128

4.5 Results 129

4.6 Discussion 132

4.7 References 137

4.8 Tables 142

4.9 Figure Legend 148

5. DIETARY ARACHIDONIC ACID AND DOCOSAHEXAENOIC ACID ELEVATE FEMUR CALCIUM AND REDUCE ZINC CONTENT IN PIGLETS 151

5.1 Abstract 153

5.2 Introduction 154

5.3 Materials and methods 157

5.3.1 Animals and diet 157

5.3.2 Measurements 158

Sample collection 158

Calcium absorption 159

Bone and urine mineral analysis 159

5.4 Statistical analysis 161

5.5 Results 162

5.6 Discussion 164

5.7 References 169

5.8 Tables 173

6. CIRCULATING LONG CHAIN POLYUNSATURATED FATTY ACIDS AND BONE RESORPTION BOTH VARY AS A FUNCTION OF TIME OF DAY AND DIETARY FAT 176

6.1 Abstract 178

6.2 Introduction 179

6.3 Materials and methods 182

Animals and diet 182

Sample collection 183

Fatty acid analysis 183

Biomarkers of bone metabolism 184

6.4 Statistical analysis 185

6.5 Results 186

6.6 Discussion 189

6.7 References 194

6.8 Tables 198

6.9 Figures 200

7. DIETARY ARACHIDONIC ACID ALTERS FEMUR BONE GEOMETRY, WHILE EICOSAPENTAENOIC ACID REDUCES FEMUR BONE AREA 203

7.1 Abstract 205

7.2 Introduction 206

7.3 Methods and Materials 210

Study design 210

Weight and length 212

Bone mass 213

Femur measurements 213

Bone and urine mineral content 213

Biochemistry 214

Fatty acid analysis 215

7.4 Statistical analysis 217

7.5 Results 218

Serial measurements 218

Feed intake, body weight and weight gain

Bone area and bone mineral content

Urinary calcium

Bone resorption

End point measurements 219

Body and tail length

Liver polyunsaturated fatty acid proportions

Adipose polyunsaturated fatty acid proportions

Femur measurements

Femur mineral composition

Prostaglandin E_2

7.6 Discussion 223

7.7 References 228

7.8 Tables 235

7.9 Figure Legend 243

8.0 DISCUSSION 247

8.1 Contribution to present state of knowledge 248

8.2 Summary of major contributions 253

Study during infancy 253

Study from weanling to maturity 253

8.3 Conclusions 254

8.4 Implications and suggested future research 254

8.5 References 258

Appendix A. Supplemental tables 264

LIST OF TABLES

Table 2.1.1 Recommendations for n-6 and n-3 polyunsaturated acids and/or long chain polyunsaturated acids. 19

Table 2.3.1 The effects of dietary polyunsaturated fatty acids on bone in infant animal models. 54

Table 2.3.2 The effects of dietary polyunsaturated fatty acids on bone in growing animal models. 59

Table 2.3.3 The effects of dietary polyunsaturated fatty acids on bone in ovariectomized rodents. 65

Table 2.3.4 Epidemiological studies investigating the effects of n-6 and n-3 polyunsaturated fatty acids on bone in humans. 68

Table 2.3.5 The effects of alterations in dietary polyunsaturated fatty acids on bone in humans. 69

Table 4.8.1 Dietary PUFA (g/100 g of fat) measured in formula fed to piglets for 15 d. 142

Table 4.8.2 Composition control of diet fed to piglets for 15 d. 143

Table 4.8.3 Whole body, lumbar spine and femur BA and BMC of piglets fed AA:DHA supplemented formula for 15 d versus control. 144

Table 4.8.4 Erythrocyte PUFA (g/100 g of fat) of piglets fed AA:DHA supplemented formula for 15 d versus control. 145

Table 4.8.5 Liver PUFA (g/100 g) of piglets fed AA:DHA supplemented formula for 15 d versus control. 146

Table 4.8.6 Adipose PUFA (g/100 g) of piglets fed AA:DHA supplemented formula ... 147

Table 5.8.1 Femur wet weight, length and ash weight of piglets fed formula with AA:DHA for 15 d versus control. 173

Table 5.8.2. Femur mineral content of piglets fed formula with AA:DHA for 15 d versus control. 174

Table 5.8.3 Urinary mineral excretion of piglets fed formula supplemented with AA:DHA for 15 d versus control. 175

Table 6.8.1 The effect of dietary AA:DHA and time on plasma PUFA proportions (g/100 g) in piglets fed formula for 15 d versus control. 198

Table 6.8.2 Relationships among urinary N-telopeptide:creatinine with time of d and selected plasma n-6 and n-3 polyunsaturated fatty acids in piglets fed formula with AA and DHA. 199

Table 7.8.1 Study Design: Three phases of 15 weeks each. 235

Table 7.8.2 Polyunsaturated fatty acid (g/100 g) measured in diet fed to rats for 45 weeks. 236

Table 7.8.3 Body weight and weight gain in rats measured following early, mid and late phases of 15 weeks each. 237

Table 7.8.4 Whole body, lumbar spine and femur bone area and bone mineral content in rats measured following early, mid and late phases of 15 weeks each. 238

Table 7.8.5 The effects of dietary long chain polyunsaturated fatty acids and gender on liver polyunsaturated fatty acid proportions (g/100 g of fat) following 45 weeks of study. 239

Table 7.8.6. The effects of dietary long chain polyunsaturated fatty acids and gender on adipose polyunsaturated fatty acid proportions (g/100 g of fat) following 45 weeks of study......240

Table 7.8.7 The effects of dietary long chain polyunsaturated fatty acids and gender on femur measurements following 45 weeks of study......241

Table 7.8.8. The effects of long chain polyunsaturated fatty acids and gender on femur mineral composition following 45 weeks......242

Table A.1 The effect of AA and DHA supplementation on bone mineral density of the whole body, lumbar spine and femur in piglets fed formula for 15 d.....265

Table A.2 The effect of AA and DHA supplementation on urinary NTx, plasma OC and ex vivo femur PGE_2 in piglets fed formula for 15 d.....266

Table A.3 The effect of AA and DHA supplementation and time on urinary NTx and plasma OC in piglets fed formula for 15 d.....267

Table A.5 The interaction between dietary AA and DHA and time on plasma EPA and DHA proportions in piglets fed formula for 15 d.....268

Table A.6 The effect of long chain polyunsaturated fatty acids on whole body, lumbar spine and femur bone mineral density in rats over 45 weeks.....269

Table A.7 The effect of long chain polyunsaturated fatty acids on femur bone area and urinary Ca in males and female rats.....270

Table A.8 The effects of dietary long chain polyunsaturated fatty acids on femur head and neck width when incorporated at different stages (early, mid or late) or continuously across the lifespan compared to control in male and female rats.....271

Table A.9 The effects of dietary long chain polyunsaturated fatty acids on femur zinc and phosphorus (μmol/g of bone) when incorporated at different stages (early, mid or late) or continuously across the lifespan compared to control in male and female rats.....272

LIST OF FIGURES

Figure 2.1.1 Metabolic transformation of essential polyunsaturated fatty acids to form long chain polyunsaturated fatty acids. 15

Figure 4.9.1 The effect of AA and DHA supplementation on whole body BMD (a), lumbar spine BMD (b) and femur BMD (c) in piglets fed formula for 15 d. 149

Figure 4.9.2 The effect of AA and DHA supplementation on urinary NTx concentration (a) and plasma osteocalcin concentration (b) in piglets fed formula for 15 d. 150

Figure 6.9.1 The effect of dietary AA:DHA and time on urinary NTx concentration (a) and plasma OC concentration (b) in piglets fed formula for 15 d. 201

Figure 6.9.2 The interaction effect of dietary AA:DHA and time on EPA (a) and DHA (b) proportions in piglets fed formula for 15 d. 202

Figure 7.9.1 The main effect of dietary LCPUFA on BA (a) and urinary Ca (b) in male and female rats over 45 weeks compared to control. 244

Figure 7.9.2 The effect of dietary LCPUFA on femur neck width (a) and head width (b) when fed early, mid or late (15 wks) or continuously (45 wks) compared to control. 245

Figure 7.9.3 The effect of dietary LCPUFA on femur Zn content (a) and P content (b) when fed early, mid or late (15 wks) or continuously (45 wks) compared to control 246

LIST OF ABBREVIATIONS

AA	arachidonic acid
AI	adequate intakes
ALA	α-linolenic acid
BA	bone area
BMC	bone mineral content
BMD	bone mineral density
BSAP	bone specific alkaline phosphatase
Ca	calcium
CTx	C-telopeptide of type-1 collagen cross-links
DGLA	dihomo-gamma linolenic acid
DHA	docosahexaenoic acid
DRI	dietary reference intakes
DXA	dual-energy X-ray absorptiometry
Dpd	deoxypyridinolines
EPA	eicosapentaenoic acid
FA	fatty acids
GLA	gamma-linolenic acid
GH	growth hormone
IGF	insulin-like growth factor
IGFBP	insulin-like growth factor-binding proteins
IGF-1	insulin-like growth factor-1
IGF-2	insulin-like growth factor-2

IP	intraperitoneal
HP	hydroxyproline
LA	linoleic acid
LCPUFA	long-chain polyunsaturated fatty acids
Mg	magnesium
NTx	N-telopeptides of type 1 collagen cross-links
OC	osteocalcin
P	phosphorus
PG	prostaglandins
PGE_2	prostaglandin E_2
PICP	carboxyterminal propeptide of type I collagen
PINP	aminoterminal propeptide of type I collagen
PTH	parathyroid hormone
PUFA	polyunsaturated fatty acids
Pyd	pyridinolines
USDA	United States Department of Agriculture
WHO	World Health Organization
Zn	zinc

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LOW LEVELS OF DIETARY ARACHIDONIC ACID AND DOCOSAHEXAENOIC ACID IMPROVE BONE MASS IN NEONATAL PIGLETS, WHILE HIGHER LEVELS PROVIDE NO BENEFIT.....95

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CHAPTER 1.

GENERAL INTRODUCTION

1.1 Introduction

Osteoporosis is a "skeletal disorder characterized by compromised bone strength predisposing a person to an increased risk of fracture. Bone strength reflects the integration of 2 main features: bone density and bone quality" (National Institute of Health, 2001). Low bone density and microarchitectural deterioration of bone tissue (reduced quality) results in the occurrence of fractures from low-energy traumas that are associated with mortality and both short and long-term morbidity (Akesson, 2003). Clinically, osteoporosis is recognized by the occurrence of fractures, which typically arise at the hip, spine and distal forearm (Cooper et al, 2003). This is because bone loss has no symptoms and goes undetected until bones fracture (Dempster et al, 1993).

Over the past 15 years, evidence suggests that dietary fat may influence bone growth, mineral mass and strength. Alterations in dietary fat could potentially reduce the risk of osteoporosis, a common health problem with increasing prevalence in both sexes. In 2000, prevalence of osteoporosis in Canadian women aged $\geq$ 50 years was 12.1% at the lumbar spine and 7.9% at the femoral neck; in Canadian men $\geq$ 50 years, it was 2.9% at the lumbar spine and 4.8% at the femoral neck (Tenenhouse et al, 2000). By 2041, one quarter of the population will be over 65. As a result, the incidence of osteoporosis is expected to rise steeply over the next few decades (Papadimitropoulos et al, 1997).

The health implications of osteoporosis are attributed to fractures and the associated mortality and morbidity (Brown and Josse, 2002). The greatest fracture-attributable mortality and morbidity arise from hip fracture. Within one year following hip fracture, the mortality rate is 21% for men and 36% for women (Cooper et al, 2003). Reducing the incidence of osteoporosis and fractures has not only health benefits but also

economic benefits. For example, 50% of women who suffer from a hip fracture do not return to their previous functional state and become dependent on others for daily functioning and 40% of those require long-term care (Chrischilles et al, 1991). In 1993, total health care costs attributable to osteoporosis and related fractures were estimated to be $1.3 billion (Goeree et al, 1996).

From a health care prospective, an effective strategy to reduce the incidence of osteoporotic fracture may be to maximize bone mass early in life. Major determinants of bone mass during adulthood include bone growth and mineralization achieved by the end of infancy (1 year), as well as bone growth and mineral accretion during the pubertal growth spurt. After puberty (closure of the growth plates), there is a consolidation of bone (until peak bone mass is achieved), followed by a period of maintenance (~25-39 years of age) and then bone loss with aging. Prevention of bone loss due to aging can also slow the development of osteoporosis. There is also the possibility that dietary treatments could restore bone integrity, preventing or reversing osteoporosis. Thus, it is important to research the effects of dietary treatments on bone during these important periods of the lifespan in order to optimize bone health.

The polyunsaturated fatty acids (PUFA) currently recognized as essential in the diet are linoleic acid (LA, 18:2n-6) of the n-6 series and alpha-linolenic acid (ALA, 18:3n-3) of the n-3 series (Innis et al, 1999). Once consumed, these PUFA can be converted to more unsaturated PUFA of the same series, gamma-linolenic acid (GLA, 18:3n-6) from LA and more highly unsaturated and longer chain PUFA including arachidonic acid (AA, 20:4n-6) from LA and eicosapentaenoic acid (EPA, 20:5n-3) and

docosahexaenoic acid (DHA, 22:6n-3) from ALA (Carlson 1997). PUFA containing 20 or more carbons are considered long chain PUFA (LCPUFA).

It is known that LA and ALA have a role in bone health. A deficiency of both LA and ALA leads to bone fractures in neonatal rats (Stepankova et al, 1996). In rats a deficiency of ALA reduces midshaft cross-sectional dimension of the tibia compared to a diet with adequate ALA (Reinwald et al, 2004). Following repletion of ALA, bone modelling was accelerated and the tibial midshaft cross-sectional dimension improved (Reinwald et al, 2004). Whether bones fully recovered from ALA deficiency was not stated (Reinwald et al, 2004). Conversely, the dietary intake required to optimize bone health throughout the lifespan is not known (Weiler and Fitzpatrick-Wong, 2002).

Alteration in the dietary ratio of n-6:n-3 PUFA and supplementation of specific PUFA and LCPUFA in the diet can potentially increase peak bone mass and slow bone loss later in life (Watkins et al, 2001). The effect of different dietary n-6 and n-3 PUFA ratios and specific PUFA and LCPUFA on bone has been investigated in bone of neonatal piglets, weanling rats and rabbits and ovariectomized rats and mice. These studies have helped to reveal some of the roles that n-6 and n-3 PUFA and LCPUFA play in bone health across the lifespan.

In neonatal piglets, supplementation of small amounts of dietary AA plus DHA in formula were shown to elevate bone mass (Weiler, 2000; Weiler and Fitzpatrick-Wong; Blanaru et al, 2004). In weanling rats, supplementation of GLA and EPA were shown to increase bone Ca content, increase Ca balance and reduce bone resorption (Claassen et al, 1995a; Claassen et al, 1995b). Such improvements in bone mineral mass during growth could potentially increase peak bone mass and thereby reduce the risk of osteoporosis and

fracture later in life. In weanling rats, the addition of fish oil (high in EPA and DHA) to reduce the total n-6:n-3 ratio resulted in elevated bone formation (Watkins et al, 2000). In another study; however, the addition of fish oil (similar to the amount given by Watkins et al, 2000) had no affect on femur mineral density or Ca in weanling male rats (Kelly et al, 2003).

Diets high in EPA and DHA also have been shown to have negative effects on bone quality during periods of growth. In weanling female rats, but not in males, a high fish oil diet had a negative effect on vertebrae strength and growth (Sirois et al, 2003). High-fat diets with fish oil in weanling rabbits resulted in reduced bone growth and mechanical properties (Judex et al, 2000). Whether these detrimental effects in rabbits were due to the high fat diet (31% versus 8%) or to inclusion of fish oil requires further investigation. Thus, establishing the optimal amount of the specific n-6 and n-3 PUFA and LCPUFA is important to maximizing bone mass.

In rodents, bone loss from estrogen deficiency (following ovariectomization mimicking a postmenopausal state) may be reduced by the inclusion of n-6 and n-3 PUFA and/or LCPUFA in the diet. In ovariectomized rats, a diet supplemented with EPA prevented the loss of bone weight and strength (Sakaguchi et al, 1994). In ovariectomized rats, n-3 PUFA (EPA and DHA) increased bone mineral content (BMC) and bone length of the tibia compared to sham-operated rats fed a high n-6 PUFA diet (LA) (Watkins et al, 2003). In ovariectomized mice, n-3 LCPUFA (EPA and DHA) maintained bone mineral density (BMD) of the lumbar spine and distal femur (Sun et al, 2003). A GLA plus EPA supplement resulted in increased femur Ca and reduced bone resorption in ovariectomized rats compared to sham-operated rats (Schlemmer et al, 1999). These

studies support the thesis that dietary n-6 and n-3 PUFA and/or LCPUFA slow the loss of bone due to estrogen deficiency, thus reducing the risk of osteoporosis.

There is very little research on n-6 and n-3 PUFA, including LCPUFA, in humans as it relates to bone. In men and women, an increasing n-6:n-3 ratio appears to be associated with lower BMD of the hip (Weiss et al, 2005). In that study, the mean n-6:n-3 ratios met current recommendations with the average ratio of n-6:n-3 of 8.4 ± 2.5 (SD) for men and 7.9 ± 2.2 (SD) for women. While the full range for the data was not provided, statistically, data points should fall within 3 SD of the mean. Some ratios of n-6:n-3 in the study by Weiss et al (2005) may have been as low as 0.8:1 and as high as 18.4:1. In preterm infants, the addition of AA and DHA at levels found in human milk to infant formula did not influence bone mass (Groh-Wargo et al, 2005). In Japan, a dietary EPA supplement elevated transmission index (similar to BMD) of calcaneous bone in women (Terano, 2001). Postmenopausal women with low bone mass, taking primrose oil (high in GLA) and fish oil (high in EPA and DHA) had increased bone mass of the lumbar spine and femoral neck (Kruger et al, 1998). In contrast, Bassey et al (2000) failed to show an effect of Efacal ® (4 g primrose oil and 440 mg fish oil per d) on whole body BMD in pre- and postmenopausal women. Further research is needed to determine whether specific dietary n-6 and n-3 PUFA and LCPUFA can reduce the risk of osteoporosis and fracture in humans.

Overall, studies investigating the effects of dietary n-6 and n-3 PUFA and LCPUFA on bone suggest a delicate balance between specific dietary n-6 and n-3 PUFA or LCPUFA. Dietary n-6 and n-3 PUFA and LCPUFA appear to have the potential to reduce the risk and slow progression of osteoporosis by optimizing bone mass during

growth and slowing and possibly preventing the loss of bone mass later in life. The PUFA and LCPUFA with the highest potential to affect bone mass seem to be GLA, AA, EPA and DHA. However, research is needed to determine the optimal amount of the specific n-6 and n-3 PUFA and LCPUFA to maximize bone mass at various stages in the lifespan.

The overall hypothesis of this thesis is that specific n-6 and n-3 LCPUFA, specifically AA, EPA and DHA will imporve bone mass and the effects of AA, EPA and DHA will be explained by changes in bone and mineral metabolism. This thesis will advance the scientific understanding of the process of bone growth, mineralization and maintenance as influenced by LCPUFA (AA, EPA and DHA) and the mechanism(s) of their effect at different stages in across the lifespan. The following literature review (Chapter 2) will provide an overview of n-6 and n-3 PUFA physiology/chemistry, bone physiology and a review of the research regarding dietary n-6 and n-3 PUFA and LCPUFA and their effects on bone and mineral metabolism.

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CHAPTER 2.

LITERATURE REVIEW

2.1 Polyunsaturated Fatty Acids

2.1.1 The essentiality of dietary n-6 and n-3 polyunsaturated fatty acids

The n-6 and n-3 PUFA are important components of membrane phospholipids (Innis, 1993), which are fundamental cell membrane components (Martinez and Mougan, 1998). Tissue n-6 and n-3 PUFA compositions differ considerably among the different membranes and cells, leading to a microenvironment that facilitates optimum function of the particular membrane and associated proteins (Innis, 1993). The PUFA currently recognized as dietary essential fatty acids are LA of the n-6 series and ALA of the n-3 series (Innis et al, 1999). Their biological importance derives in part from their role as constituents of structural lipids, which influences the activities of membrane-linked functional molecules (receptors, enzymes, transporters) (Reviewed by Fernstrom, 2000). In addition to their structural roles, LA and ALA serve as precursors for production of AA and EPA, respectively, which in turn are precursors for the production of biologically active eicosanoids (Uauy and Hoffman, 2000). LA and its metabolites are important for growth, dermal integrity, wound healing, liver and kidney function and protection against infection, whereas ALA and its metabolites are important in sensory and other neural-based behaviours (Carlson, 1997).

Once consumed, LA and ALA can also be converted to more unsaturated PUFA, including GLA from LA, and longer-chain, more highly unsaturated PUFA of the same series termed LCPUFA, including AA from LA and EPA and DHA from ALA (Carlson, 1997). LCPUFA contain a minimum of 20 carbon atoms and 3 methylene-interrupted cis-double bonds (Woltil et al, 1999). Accretion of AA and DHA in membrane lipids occurs during growth and development (Innis, 1991). AA is quantitatively the most important

LCPUFA and is widespread through out the body (Leaf et al, 1992). DHA has a highly specific distribution, being the predominant membrane FA of synaptosomes, retinal photoreceptors, mitochondria, spermatozoa, but scarce in other tissues (Reviewed by Leaf et al, 1992).

2.1.2 The metabolism of n-6 and n-3 polyunsaturated fatty acids

It has long been accepted that LA and ALA are converted to their respective LCPUFA, including AA, EPA and DHA (Gibson and Makrides, 1998). However, the details of the synthetic pathway have been subject to many investigations (Gibson and Makrides 1998). Initially, it was proposed that both animals and humans convert the 18-carbon PUFA to LCPUFA through a simple series of desaturation (adding a double bond each time) and elongation (adding 2 carbons each time) steps in the endoplasmic reticulum (Gibson and Makrides 1998). While this holds true for LCPUFA with 20 carbons or less, such as AA and eicosapentaenoic acid (EPA, 20:5n-3), the final steps in the synthesis of the 22 carbon LCPUFA with 5 or 6 double bonds such as DHA involves a complex process that occurs in the peroxisomes (Sprecher et al, 1995). The metabolic pathway for the conversion of LA and ALA to their respective LCPUFA is presented in **Figure 1.1**.

What regulates the transfer of FA to the peroxisomes for partial β-oxidation, rather than the mitochondria for complete oxidation, and whether they move as free FA or acyl-CoA is not known (Innis et al, 1999). Since chain elongation, desaturation and partial β-oxidation occur only at the carboxyl end of the molecule, the PUFA series is not subject to change as it is metabolized and products remain a part of the same family (Salem and Pawlosky, 1994). The essentiality of n-6 and n-3 PUFA for humans is

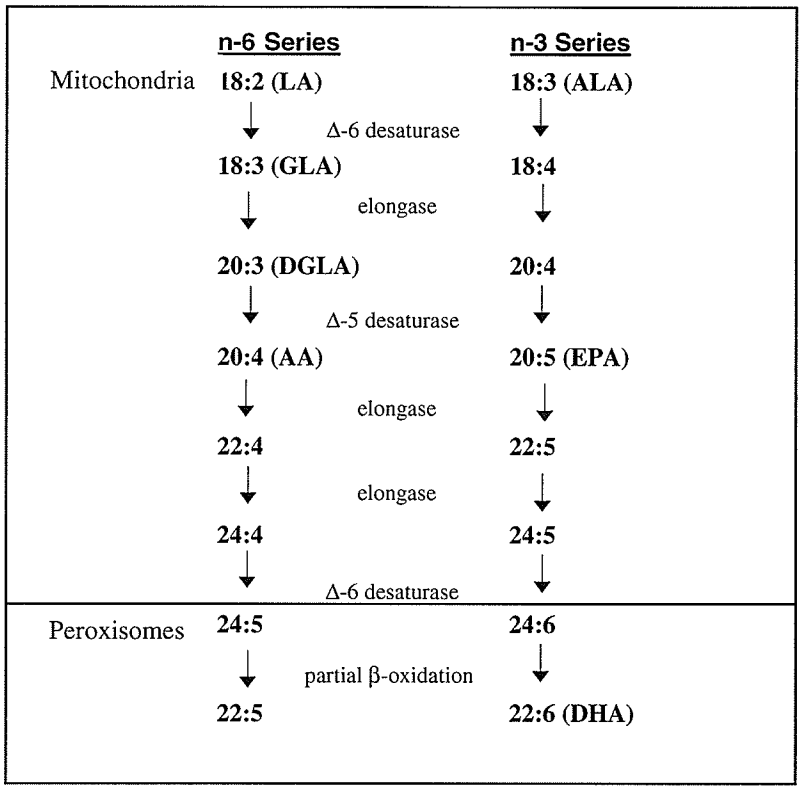

Figure 2.1.1 Metabolic transformation of essential polyunsaturated fatty acids to form long chain polyunsaturated fatty acids (Adapted from Uauy and Castillo, 2003). Abbreviations: AA=arachidonic acid, DGLA=dihommo-gamma-linolenic acid, DHA=docosahexaenoic acid, EPA=eicosapentaenoic acid

explained by the inability of animal tissues to introduce double bonds in positions before carbon 9, counting from the methyl-terminus (Uauy and Hoffman, 2000). The conversion of LA to AA and ALA to DHA depends on the dietary contents of LA and ALA and the ratio of one to the other (Innis, 1992a). The n-6 and n-3 series compete for synthesis and incorporation into membranes (Carlson, 1997). It is known that n-3, n-6 and n-9 PUFA are processed by the same enzyme system with the substrate preference of n-3>n-6>n-9 (Classeen et al, 1995b). Because the n-6 and n-3 PUFA compete for enzymes, the dietary ratio of n-6:n-3 plays an important role in their metabolism (Arbuckle et al, 1994). Since they have different functional roles, competition between them can have functional consequences (Carlson, 1997). The concentrations of AA and DHA found in tissue phospholipids are the net result of the rates of endogenous synthesis from LA and ALA and the amount of preformed AA and DHA in the diet (Innis, 2000).

Desaturation and elongation is one of the several pathways by which dietary LA and ALA can be metabolized (Innis 1992a). Dietary LA and ALA can undergo β-oxidation in the mitochondria to produce energy or can be directly incorporated into tissue phospholipids (Innis 1992a). What controls the partitioning of dietary LA and ALA among potential pathways of metabolism is not known (Innis, 1992a). It is expected that if energy requirements are not met, dietary LA and ALA would be used to meet energy needs (Innis 1992a). Tests with 14C and tritium-labelled PUFA and LCPUFA showed that the proportions of brain AA recovered from AA itself was 10 times higher than recovered from its precursor LA (Sinclair, 1975). Similar results were described for the comparison of ALA and DHA. This illustrates the preferential uptake of AA and DHA compared with the biosynthetic route (Crawford, 2000). Oxidation of these and other

carbon chain 20-22 FA involves initial chain shortening and retroconversion in the peroxisomes, before mitochondrial β-oxidation (Reviewed by Innis 1992a). Only 15% of AA and DHA are oxidized in a 24-h period compared to 50-60% of LA and ALA under normal metabolic conditions (Sinclair, 1975). Differences in metabolism and the specificities of the acyltransferases involved in phospholipid synthesis and remodelling may explain why dietary AA and DHA are better sources of tissue AA and DHA than LA and ALA (Innis 1992a). As a result, the appropriate ranges of intake for AA and DHA would be much lower and narrower than those for LA and ALA (e.g., 6-20% of dietary FA as LA compared with 0.5-1% as AA) (Innis, 2000).

2.1.3 Requirements of polyunsaturated fatty acids across the lifespan

LCPUFA are not considered dietary essential fatty acids. Nonetheless, a relevant question is do humans need to receive LCPUFA from the diet for optimal health or can dietary LA and ALA be converted to their respective LCPUFA in sufficient amounts consistent with optimal health, thereby fulfilling requirements for n-6 and n-3 PUFA? The following sections of PUFA requirements across the lifespan are organized into stages of life that are relevant to this thesis regarding bone acquisition and maintenance: infancy, childhood and adulthood. The ages used to define infancy, childhood and adulthood are based on the age ranges used for recommendations in the Dietary Reference Intakes (DRI). Major sources of LA in the diet are from oils, including safflower oil, sunflower oil, soybean oil and canola oil. Good sources of dietary ALA include soybean oil, canola oil, linseed oil and flax seed oil. Animal products (meat, eggs and milk) are dietary sources LA, ALA, AA, EPA and DHA. Whereas, fish and marine foods are excellent sources of EPA and DHA and contain small amounts of AA.

Infancy (0 to 12 months)

Recommendations for dietary PUFA and LCPUFA are summarized in **Table 2.1.1.** At birth, PUFA and LCPUFA status is a reflection of the maternal diet during pregnancy. Currently, there is no recommendation for LCPUFA during pregnancy. The DRI set for pregnancy are 13 g/g of LA and 1.3 g/d of ALA; these are adequate intake (AI) recommendations (DRI, 2002). The International Society for the Study of Fatty Acids and Lipids (ISSFAL) recommends that pregnant women consume 0.3 mg of DHA per d (Simopoulos, 1999). Innis and Elias (2003) found that pregnant Canadian women consume low amounts of DHA, 16.7 % consume <67 mg, 60% consume <150 mg and 16 % consume > 300 mg per day. A strong positive maternal-fetal correlation for all n-6 and n-3 PUFA and LCPUFA has been reported (Al et al, 1995; Al et al, 1996). Supplementation of DHA during pregnancy can lead to higher plasma DHA in mothers and newborn infants when supplemented as 4 g of fish oil (van Houwelingen et al, 1995; Dunsten et al, 2004). Other studies have shown that smaller amounts of DHA supplementation (200 mg/d and 400 mg/d) during pregnancy leads to higher maternal plasma DHA without altering the infants plasma DHA status (Montgomery et al, 2003; Sanjurjo et al, 2004). Whether increasing DHA above recommendations during pregnancy is beneficial for the fetus is unknown. In population studies, higher intakes of EPA and DHA were associated with longer gestational periods and larger birth weights (Olsen, 1989). In 1992, Olsen et al found that supplementation of 10.8 g of n-3 LCPUFA from fish oil resulted in increased gestational period by 4 days. During the last trimester of gestation, large amounts of n-6 and n-3 LCPUFA are required to support rapidly growing tissues (Koletzko et al, 1995). In humans, the demand is greatest in the

Table 2.1.1 Dietary Reference Intake (DRI, 2002) and International Society for the Study of Fatty acids and Lipids Adequate Intake Recommendations (Simopoulos, 1999) for Infants, Children and Adults.

	Gender and/or Age	LA	ALA	AA	EPA	DHA	Total n-6	Total n-3
Infants								
	Boys and Girls							
DRI (g/d)	0-6 mo	NR	NR	NR	NR	NR	4.4	0.5
	7-12 mo	NR	NR	NR	NR	NR	4.6	0.5
ISSFAL* (% of fat)	Infancy	10.00	1.50	0.50	<0.10	0.35	NR	NR
Children								
DRI (g/d)	Boys and Girls							
	1-3 y	7	0.7	NR	NR	NR	NR	NR
	4-8 y	10	0.9	NR	NR	NR	NR	NR
	Boys							
	9-13 y	12	1.2	NR	NR	NR	NR	NR
	14-18 y	16	1.6	NR	NR	NR	NR	NR
	Girls							
	9-13 y	10	1.0	NR	NR	NR	NR	NR
	14-18 y	11	1.1	NR	NR	NR	NR	NR
Adults								
DRI (g/d)	Men							
	19-50	17	1.6	NR	NR	NR	NR	NR
	≥51	14	1.6	NR	NR	NR	NR	NR
	Women							
	19-50	12.0	1.1	NR	NR	NR	NR	NR
	≥51	11.0	1.1	NR	NR	NR	NR	NR
ISSFAL g/d	Males and Females	4.44 6.67¤	2.2	NR	0.22♦	0.22♦	NR	NR
ISSFAL % energy	Males and Females	2.0 3.0¤	1.0	NR	0.1	0.3	NR	NR

*Recommendations for infant formula

¤ Upper Limit

♦ Minimum

Abbreviations: ALA= α-linolenic acid, AA=arachidonic acid, DRI=daily recommended intake, DHA=docosahexaenoic acid, EPA=eicosapentaenoic acid, ISSFAL=International Society for the Study of Fatty Acids and Lipids, LA=linoleic acid, PUFA=polyunsaturated fatty acid, NR=no recommendation at this time

third trimester of gestation, since the velocity of growth is exponentially high and remains rapid until 6 mo of life (Dobbing and Sands, 1979). Between 6 and 18 mo of life, the velocity of growth slows (Dobbing and Sands, 1979) and the demand for LCPUFA declines. Initially, the placenta controls the passage and quantity of AA and DHA available to the fetus (Clandinin, 1999). Then, by its own metabolism, the fetus starts to synthesize AA and DHA (Reviewed by Clandinin, 1999). The age at which this progression begins and meets requirements is not known (Clandinin, 1999). However, preterm infants as young as 28 wk postconceptual age can make AA and DHA from their essential PUFA precursors (Carnielli et al, 1996).

After birth, breast milk lipids provide a relatively stable supply of n-6 and n-3 PUFA and LCPUFA sufficient to meet the need for LCPUFA deposition in growing tissues for infants (Reviewed by Koletzko et al, 1995). Breast milk usually contains 8-16% LA, 0.5-1% ALA and a variety of n-6 and n-3 LCPUFA (Innis, 1992b). In Canada, the amount of AA varies from 0.4 to 0.8 g/100 g of fat, EPA varies from 0.12 to 0.2 g/100 g of fat and DHA varies from 0.2 to 0.5 g/100 g of fat (Reviewed by Hibbeln, 2002). In the U.S., the amount of AA varies from 0.29 to 0.69 g/100 g of fat, EPA varies from 0.04 to 0.1 g/100 g of fat and DHA varies from 0.06 to 0.27 g/100 g of fat (Reviewed by Hibbeln, 2002). It is known that the variation of n-6 and n-3 PUFA concentrations in breast milk is largely explained by variations in the fat composition of the mother's diet (Reviewed by Innis, 2000). Diets high in marine foods result in a much higher DHA and EPA content in breastmilk (Koletzko et al, 1992), whereas, breastmilk AA content is not as influenced by dietary AA content (Koletzko et al, 1992; Sanders and Reddy 1992; Villalpando et al, 1998). Supplementation of fish oil or DHA results in

higher DHA concentrations in breast milk (Harris et al 1984; Makrides et al, 1996, Helland et al, 1998; Fidler et al, 2000), mother's plasma (Makrides et al, 1996) and in the infant's plasma (Makrides et al, 1995). It is interesting to note that the addition of AA alone in the maternal diet did not alter the AA content and decreased the content of EPA and DHA in breast milk, however, the supplementation of AA, DHA and EPA increased all three LCPUFA in breast milk (Smit et al, 2000). Recommendations during lactation for LA are 13 g/d and 1.3 g/d for ALA (DRI, 2002). There are no DRI for LCPUFA during lactation; however, ISSFAL recommends 0.3 g/d of DHA (Simopoulos, 1999).

Previous to 2002, all infant formulas in North America contained only LA and ALA. This was thought to provide adequate fat nutrition since formula-fed infants grew as well as breast-fed infants (Gibson and Makrides, 1998), however, breastfeeding has been associated with a higher cognitive development than formula feeding (Anderson et al, 1999). These observations have created interest in the possible role of breast milk nutrients in optimizing neurodevelopment (Innis et al, 1999). The cortices of infants fed breast milk have higher concentrations of DHA than those fed infant formula (Makrides et al, 1994; Farquharson et al, 1992). This observation has been attributed to the fact that breast milk is a rich source of DHA, whereas most infant formulas do not contain DHA and resulted in the speculation that adding DHA to infant formulas will result in improved neural function (Makrides and Gibson 2000). This hypothesis has been central to several randomized clinical trials of formula feeding in attempts to mimic the beneficial effects of breastfeeding on early childhood development (Makrides and Gibson 2000). In preterm infants, improved visual development is reported for those who are fed the formula supplemented with DHA (Uauy et al, 1994; Carlson et al, 1993; Carlson et al,

1996); however, studies have found reduced growth most likey due to a decrease in AA status (Carlson et al, 1996, Ryan et al 1999). A subsequent study with preterm infants who were fed formula supplemented with AA plus DHA, improved visual and cognitive development was found with no effects on growth (O'Connor et al, 2001).

Similar studies with fullterm infants and supplementation of formula with DHA or AA and DHA on tests of visual and cognitive development have yielded conflicting results. Visual and cognitive development were not improved with supplementation in some studies (Auestad et al, 1997; Scott et al, 1998; Lucas et al, 1999; Makrides et al, 2000; Auestad et al, 2001), whereas other studies reported benefits of supplementing formulas with DHA or both DHA and AA (Agostoni et al, 1995; Makrides et al, 1995; Birch et al, 1998; Willatts et al, 1998; Birch et al, 2000; Birch et al, 2002). No studies showed adverse effects of either DHA or AA and DHA on growth. Conflicting results are also also seen in studies that included comparisons between formula-fed and breastfed infants (Agonstoni et al, 1995; Auestad et al, 1997; Birch et al, 1998; Jorgensen et al, 1998; Scott et al, 1998; Lucas et al, 1999; Makrides et al, 2000). For example, studies have shown that infants fed formula containing adequate ALA (>2.0 g/100 g of fat) had similar neurodevelopment compared to those fed breastmilk (Auestad et al 1995, Scott et al, 1998, Auestad et al, 2001).

Several approaches have been used to study infant PUFA requirements. These include extrapolation from the PUFA composition of breast milk, analyses of fetal and infant autopsy tissue and comparison of PUFA in plasma and erythrocytes of infants fed various milk or formula diets (Innis 1992a) and clinical studies of visual and cognitive development (those previously mentioned). Amounts of n-6 and n-3 PUFA vary

considerably in breast milk, depending on the type and quantity of fat in the mother's diet (Innis 1992b). The variability in breast milk PUFA (Innis 1992b) and the influence of dietary fat composition on circulating PUFA (Innis, 1992a) limits the validity of establishing infant requirements from these measures (Innis, 1993). Although formula-fed infants without AA and DHA have lower concentrations of AA and DHA in plasma and erythrocytes than those fed breast milk, the extent to which they provide information about LCPUFA amounts in tissues is unclear (Lucas et al, 1999). It is important to note that studies have shown that infants fed human milk or formulas supplemented with DHA or AA and DHA consistently have similar and higher plasma and red blood cell levels of DHA or AA and DHA than infants fed unsupplemented formulas (Auestad et al, 2001). Data from a small number of studies on formula-fed infants who died in infancy indicate that although the levels of AA in the brain were similar to those of breast-fed infants, levels of DHA were lower (Farquharson et al, 1992; Makrides et al, 1994). However, the significance of this finding is unknown because the range of cortical DHA content associated with normal function has not been defined (Lucas et al, 1999).

In piglet research, supplementation with AA and DHA results in alterations in the PUFA composition of tissues. Weiler (2000) found that supplementation of AA and DHA as 0.5:0.1 g/100 g of fat resulted in lower LA and higher AA in liver and bone. Blanaru et al (2004) fed piglets varying ratios of AA:DHA, 0.3:0.1, 0.45:0.1, 0.6:0.1 and 0.75:0.1 g/100 g of fat. These researchers found that AA increased in plasma, liver and adipose, but not in brain with increased supplementation of AA, where supplementation of AA as 0.45 g/100 g of fat led to a decrease in DHA in brain. Plasma PUFA and LCPUFA proportions reflected the PUFA and LCPUFA proportions in liver and adipose, but not in

brain. In 2002, Petryk and Weiler found the same relationship between liver and plasma AA and DHA proportions. In chicks, n-6 and n-3 PUFA composition of bone was reflective of alterations in the total n-6:n-3 PUFA ratio, as well as the supplementation of EPA plus DHA (Watkins et al, 1996). Together, these data suggest that supplementation of dietary AA and DHA during infancy alters PUFA composition of multiple tissues.

In 1999, a workshop on the essentiality of and recommendations for n-6 and n-3 PUFA and LCPUFA recommended that the adequate intakes (AI) of AA and DHA for infant formula were 0.50 and 0.35g/100 g of FA, respectively (Simopoulos, 1999). These amounts were empirically-derived and set at quantities that would support growth and neural development similar to breastfeeding. In North America, following approval from the FDA in 2001 and Health Canada in 2002, infant formula companies started supplementing infant formulas with AA and DHA. The addition of AA and DHA to formula is not mandatory, thus some formulas on the market contain only LA and ALA. Formulas containing AA and DHA are marketed as those that support optimal neural and retinal development compared to formulas containing only LA and ALA. The amount of AA and DHA currently added to formula is similar to the AI recommended by ISSFAL in 1999. For example in Canada, Enfalac A^+ has AA:DHA equal to 0.64:0.32 g/100 of fat and Nestle Good Start with DHA and ARA (i.e. AA) has AA:DHA equal to 0.58:0.29 g/100 g of fat. In Canada and Australia, the total n-6:n-3 PUFA ratio in formula has been recommended to be within the range of 4:1 to 10:1 (Jumpsen et al, 1997). The current DRI have AI recommendations for total n-6 PUFA and total n-3 PUFA, but not specific n-6 or n-3 PUFA or LCPUFA.

Several sources of LCPUFA are currently available for supplementation of diets (Innis, 2000). These include fish and other marine oils, egg total lipids or PL and oils derived from micro-algae and fungi (single-cell organisms) (Innis et al, 1999). Currently, Martek Biosciences Corporation supplies the source of AA and DHA currently used in infant formula available in Canada, Enfamil A^+ and Nestle Good Start with DHA and ARA (i.e. AA). The oil that provides DHA is made by microalgae (Crypthecodinium cohnii) and oil that provides the AA is made by fungi (Mortierella alpina).

Childhood (1-18 years of age)

Recommendations for dietary PUFA and LCPUFA are summarized in Table 2.1.1 DRI recommendations for children focus on LA and ALA (DRI, 2002). They are calculated as AI and are based on current mean intakes. After the age of 2, the composition of dietary fat should be aimed at reducing the risk of chronic disease (Uauy and Castillo, 2003), yet still supporting continued development towards optimal health. Uauy and Catillo (2003) recommended a total n-6 PUFA of 4-13 % of energy, total n-3 PUFA of 1-2% and a n-6:n-3 ratio of 5:1 to 10:1.

Adulthood (18 years and older)

Recommendations for dietary PUFA and LCPUFA are summarized in Table 2.1.1 Most adults are able to meet requirements for LCPUFA by consuming adequate amounts of the 18 carbon precursors (Innis, 1991). The current DRI AI recommendations for n-6 and n-3 PUFA do not include LCPUFA (DRI, 2002). However, current recommendations set by ISSFAL for adults include the LCPUFA (Simopoulos, 1999). Uauy (1999) suggested that to prevent cardiovascular and other non-communicable chronic diseases, fish should be eaten 2-3 times a week (approximately 35 g/d). Whether

these recommendations apply to bone health is unclear. Current intakes for adults are reported to be lower than recommended in the U.S and other countries. Simopoulos (1999) stated that intakes of EPA plus DHA were from 0.1- 0.2 g/d and total n-3 PUFA intakes of 1.1 to 1.6 in the U.S. Weiss et al (2005) found that in California, adults aged 45-90 were consuming 0.2 g/d of EPA plus DHA and a total n-3 PUFA of 1.3 g/d. Cadler and Grimble (2002) estimated that the intake of EPA plus DHA in Western populations was 0.25 g/d for adults. In French men the mean intake of EPA was 0.150 g/d and DHA was 0.273 g/d (Astorg et al, 2004). In French women, the mean intake of EPA was 0.118 g/d and DHA was 0.226 g/d (Astorg et al 2004). In Australian adults the mean intake of EPA was 0.056 g/d and DHA was 0.106 g/d (Meyer et al, 2003). Thus, intakes of EPA and DHA are much lower than current recommendations in not only in the U.S., but also in various industrialize countries all over the World. It is important to note that dietary intakes of EPA and DHA are much higher in Countries that consume more fish or marine life. For example, Japanese adults consume much higher amounts of EPA and DHA. Those living in a fishing village consume an average of 0.634 g/d of EPA and 1.141 g/d of DHA, whereas, those living in a farming village consume 0.383 g/d of EPA and 0.711 g/d of DHA (Yamada et al, 2000).

A balanced ratio of n-6:n-3 is required for optimal health and prevention of chronic disease (Simopoulos, 2002; Kris-Etherton et al, 2000). In the past, it is estimated that hunter-gatherer populations consumed close to a 1:1 ratio of n-6:n-3 PUFA. In our society, there has been an increase in n-6 PUFA consumption (increased intake of corn, sunflower and sesame oils) due to modern agricultural practices and changes in food processing (Kris-Etherton et al, 2000; Simopoulos, 1999). There has also been a

reduction in cold-water fish consumption, changes in animal production and increased processing of grains leading to a reduced consumption of n-3 PUFA (Kris-Etherton et al, 2000; Simopoulos, 1999). These changes in dietary n-6 and n-3 have resulted in consumption of a high ratio of n-6:n-3 PUFA. For example, the average ratio of total n-6:n-3 PUFA in the American diet has been estimated as approximately 9.8:1 (Kris-Etherton et al, 2000), but has been reported as high as 15:1 and 16.7:1 (Simopoulos, 2002). The World Health Organization (WHO) recommends a n-6:n-3 ratio of 5:1 to 10:1, while others suggest the optimal ratio of n-6 to n-3 PUFA varies from 1:1 to 4:1 depending on the disease (Simopoulos, 2002). Nutritionists suggest that an increased intake of foods containing n-3 PUFA and a decreased consumption of foods containing high amounts of n-6 is required to reduce the risk of chronic disease (Simopoulos, 2002; Kris-Etherton et al, 2000). This does not mean that n-6 PUFA are harmful, but that a balance of n-6 to n-3 PUFA is required for optimal health and that the current intake of n-6 PUFA is too high and n-3 PUFA is too low.

2.1.4 Biosynthesis of eicosanoids from polyunsaturated fatty acids

AA and EPA are related to chronic disease through their metabolism to eicosanoids. The term eicosanoids describes the oxygenated derivatives of LCPUFA containing 20 carbon atoms (Sellmayer and Koletzko, 1999). Eicosanoids include prostanoids, leukotrienes, lipoxins and epoxegenases. The prostanoids include the prostaglandins (PG), prostacyclins and thromboxanes (Mayes, 2000a). Physiologically, eicosanoids are considered to act as local hormones through G-protein linked receptors to elicit their biochemical effects (Mayes, 2000b). AA is the predominant precursor because it is very abundant in phospholipid (Sellmayer and Koletzko, 1999). Other LCPUFA

serve as precursors for enzymatically oxygenated FA with 20 carbons, including dihomo-γ-linolenic acid (DGLA) and EPA (Sellmayer and Koletzko, 1999). PUFA with 18 or 22 carbons, such as LA and DHA, are also oxygenated by some, but not all the enzymes in the cascade (Sellmayer and Koletzko, 1999). For years, alteration in the PUFA and LCPUFA precursors to eicosanoids has been studied for benefits to health and prevention of chronic diseases such as cardiovascular disease (Ruxton et al, 2004) and more recently for benefits to bone. Overall it is believed that diets consistent with a high n-6 PUFA or resulting in high AA status are associated with chronic diseases including low bone mass (Weiss et al, 2005). Thus a discussion of the AA cascade in the synthesis of eicosanoids is warranted.

The arachidonic acid cascade in the synthesis of eicosanoids

AA can be oxygenated by three different enzyme systems: cyclooxygenases, lipoxygenases and cytochrome P450 monooxygenases (Sellmayer and Koletzko, 1999). The expression of these are specific for the type of cell (Sellmayer and Koletzko, 1999). After release from its storage in membrane phospholipid by phospholipase A_2, or after its transcellular exchange, metabolism of AA by the cyclooxygenases leads to the formation of thromboxane A_2 and PG (Sellmayer and Koletzko, 1999). The PG include, PGE_2 and $PGF_{2\alpha}$ (Sellmayer and Koletzko, 1999). Metabolism by lipoxygenases results in the formation of leukotrienes and subsequent metabolism results in the formation of lipoxins (Sellmayer and Koletzko, 1999). Metabolism of AA by the cytochrome P450 monooxygenases gives rise to epoxides, the epoxyeicosatrienoic acids and ω-hydroxylated derivatives of the precursor PUFA or other eicosanoids (Sellmayer and Koletzko, 1999).

Synthesis of eicosanoids from alternative polyunsaturated fatty acids

AA is not the only LCPUFA metabolized to eicosanoids. DGLA and EPA are also precursors to eicosanoids (Sellmayer and Koletzko, 1999). DGLA is formed from LA and GLA is principally converted to the 1-series PG (PGE_1 and $PGF_{1\alpha}$) and the 3-series leukotrienes (Sellmayer and Koletzko, 1999). *In vivo* formation of these eicosanoids however is barely detectable (Sellmayer and Koletzko, 1999). EPA is readily metabolized to 3-series PG (PGE_3 and $PGF_{3\alpha}$) and 5-series leukotrienes (Sellmayer and Koletzko, 1999). In addition, DGLA and EPA can be oxygenated in vitro to various monohydroxylated or epoxide derivatives by lipoxygenase and cytochrome P450 monooxygenase activities, respectively (Sellmayer and Koletzko, 1999). However, the physiological effects are unknown (Sellmayer and Koletzko, 1999).

2.2 Bone mass and metabolism

Bone is a specialized connective tissue that together with cartilage, forms the skeletal system (Baron, 1999). It consists of living cells embedded within or lining surfaces of a mineralized organic matrix (Watkins, 1998). Bone provides mechanical support for the body and locomotive movement through the attachment of muscles (Watkins, 1998). In addition, it protects vital organs and acts as a reservoir for minerals (Watkins, 1998).

Bone is a multifunctional connective tissue that consists of a structural framework of mineralized matrix containing osteoblasts, osteocytes and osteoclasts (as well as other cells) (Watkins, 1998). These cells produce a variety of biological regulators that control local bone metabolism (Watkins, 1998). Systemic calciotropic hormones, such as parathyroid hormone (PTH) and 1,25$(OH)_2$vitamin D_3 (calcitriol), and autocrine and paracrine factors, such as eicosanoids (PGE_2) and growth factors (insulin like growth factor), control the activities of bone modelling to alter length, diameter and shape of bones in children (Watkins, 1998). Bone growth and maintenance thereafter includes the activities of bone formation, mineralization and resorption (Watkins, 1998). This thesis focuses on bone growth, metabolism and mineral mass. The following sections are devoted to bone mineralization, modelling and remodelling and the cells responsible for those processes.

2.2.1 Bone cells and bone metabolism

Osteoblasts

Osteoblasts are mononucleated bone-forming cells that originate locally from mesenchymal stem cells (Baron, 1999). These precursors multiply and differentiate into

preosteoblasts and then mature osteoblasts (Baron, 1999). The osteoblast is the cell responsible for the production of matrix constituents, collagen and ground substance (Baron, 1999). Osteoblasts line the layer of bone matrix they produce before it is calcified, known as osteoid tissue (Baron, 1999). Osteoid tissue represents the transitional state between matrix formation and mineralization, which is approximately 10 d (Baron, 1999). Cytoplasmic processes, located on the secreting side of the osteoblast, extend deep into the osteoid matrix and are in contact with the osteocyte processes in their canaliculi (Baron, 1999). The plasma membrane of the osteoblast is rich in alkaline phosphatase and has been shown to have receptors for PTH (Baron, 1999). Osteoblasts have receptors for estrogens and calcitriol in their nuclei, as well as receptors for cytokines (Baron, 1999). Toward the end of the matrix-secreting period, the osteoblast becomes either a bone lining cell or osteocyte (Baron, 1999).

Osteocytes

Calcified bone matrix is metabolically active. Osteocytes are found embedded deep within bone in small osteocytic lacunae (Baron, 1999). Osteocytes were originally osteoblasts that became trapped in matrix they produced, which in turn became calcified (Baron, 1999). Osteocytes have several long cell processes (filopodial) that are rich in microfilaments and in physical contact with either filopodial from other osteocytes or with processes from the cells lining the bone surface (osteoblasts or bone lining cells) (Baron, 1999). These processes are organized during the formation of matrix and before its calcification and form a network of thin canaliculi or tunnels that permeate the entire bone surface (Baron, 1999). Osteocytes can synthesize new bone matrix at the surface of osteocytic lacunae, which can subsequently become calcified (Baron, 1999). Osteocytes

are phagocytized and digested with other components of bone during osteoclastic resorption and may play a role in local activation of bone turnover or remodelling (Baron, 1999).

Osteoclasts

The osteoclast is a multinucleated bone-lining cell responsible for bone resorption (Baron, 1999). The osteoclast is usually found in contact with a calcified bone surface and within lacuna (the result of its own resorptive activity) (Baron, 1999). It is possible to find up to five osteoclasts at a resorptive site, although, there is normally only one or two (Baron, 1999). The osteoclast contains transport vesicles loaded with lysosomal enzymes (Baron, 1999). The most prominent feature of the osteoclast is at the point of contact with the bone matrix characterized by deep foldings of the plasma membrane referred to as a ruffled border, surrounded by a ring of contractile proteins (sealing zone) that serve to attach the cell to the bone surface and seal off the subosteoclastic bone-resorbing compartment (Baron, 1999). The plasma membrane at the ruffled border contains proteins also found on the lyzosome membrane and related organelles and a specific type of electrogenic proton adenosine triphosphatase involved in acidification (Baron, 1999). The basolateral membrane of the osteoclast is specifically enriched in sodium-potassium pumps, $HCO3^-/Cl^-$ exchangers and Na^+/H^+ exchangers (Baron 1999).

During bone resorption, lysosomal enzymes are actively synthesised by the osteoclast (Baron, 1999). The enzymes are secreted through the ruffled border into the extra-cellular bone-resorbing compartment (Baron 1999). They reach adequate concentrations because this area is sealed off (Baron 1999). The cell also secretes several metalloproteinases such as collagenase and gelatinase (Baron, 1999). The osteoclast

acidifies the extracellular compartment by secreting protons across the ruffled border membrane (Baron, 1999). The extracellular bone-resorbing compartment is characterized by a low pH, lysosomal enzymes and substrate (Baron, 1999). The low pH dissolves the hydroxyapatite crystals, exposing the matrix (Baron, 1999). The lysosomal enzymes (collagenase and cathepsins), now in an optimal pH, degrade matrix components (Baron, 1999). The residues are either internalized or transported across the cell and released in the basolateral domain or released into circulation during periods of relapse of the sealing zone (Baron, 1999). Whether the osteoclast has receptors for PTH, estrogen or calcitriol is controversial (Baron, 1999). Evidence suggests that the osteoclast undergoes apoptosis after a cycle of resorption (Baron, 1999).

2.2.2 Bone matrix and mineral

Bone is formed by mineralization of collagen fibres (type I, 90% of total protein) and noncollagenous proteins (Baron, 1999). Crystals of hydroxyapatite $[3Ca_3(PO4)_2]\cdot(OH)_2$ are found on and within collagen fibres and in the ground substance (Baron, 1999). The ground substance is mainly composed of glycoproteins and proteoglycans (Baron, 1999). These have high ion bonding capacity and play an important role in the calcification process and the fixation of hydroxyapatite crystals to collagen fibres (Baron, 1999). Collagen fibres alternate in adult bone from layer to layer (preferential organization), giving the bone a lamellar structure that creates the highest density of collagen per unit of tissue (Baron, 1999). The lamellae can be parallel to each other if deposited along a flat surface as in trabecular bone and periosteum or cylindrical if deposited on a surface surrounding a channel centred on a blood vessel (haversian system) (Baron, 1999). However, when bone is formed very rapidly, (e.g. during

development) there is no preferential organization of collagen fibres (Baron, 1999). Here, the collagen fibres are not packed as tightly and are in randomly-oriented bundles, referred to as woven bone (Baron, 1999). At birth the mineral mass of the skeleton is approximately 70-95 g and this increases to 2400 g in young women and 3300 g in young men (Trotter and Hixon, 1974). Bone mineral accretion continues until peak bone mass (highest amount of bone mass attained in life) is reached late in the second or early in the third decade of life. Gains of bone mineral are rapid during adolescence and up to 25% of peak bone mass is acquired during the 2-year period across peak height velocity (Bailey et al, 1999). At peak height velocity, males and females have reached approximately 90% of their adult height, but only 57% of their adult BMC (Bailey et al, 1999). By the age of 18, a minimum of 90% of peak bone mass is acquired (Bailey et al, 1999).

2.2.3 Bone growth: modelling

Bone growth by modelling is influenced by complex interactions between genetic potential, environmental influences and nutrition (Watkins, 1998). Bones increase in length and diameter by a process called modelling (Watkins, 1998). This process of generalized and continuous growth and reshaping of bone is governed by the activities of the osteoblasts and osteoclasts until the adult bone structure is attained (Watkins, 1998). Bone modelling provides order to the increase in bone mass that accompanies body growth (Watkins, 1998). During modelling, the diameter of bones increases by apposition of bone matrix by osteoblasts located within the periosteum (outer surface of the bone) (Watkins, 1998). Long bones grow not only in diameter, but also in length. Linear growth of the long bones, primarily responsible for linear growth of the skeleton, is dependent upon proliferation and sequential mineralization of cartilage cells in the epiphyseal

growth plate (Gertner, 1999). The location of the growth plate, between the epiphyseal and metaphyseal regions of bone, allows the bones to increase in length (Watkins, 1998). As the columns of chondrocytes grow outwards from the growth plate, they mature and eventually undergo apoptosis, while their surrounding matrix mineralizes (Gertner, 1999). A zone of the growth plate is always in the process of maturation (Gertner, 1999). Eventually the balance between proliferation and maturation shifts in the direction of maturation, with a reduction in the width of the zone of proliferating chondrocytes until the epiphysis fuses and the growth plate disappears (Gertner, 1999).

2.2.4 Bone remodelling

The skeletal morphology of the adult represents a compromise between structural and metabolic responsibilities - providing support and locomotion, while participating in the regulation of mineral homeostasis (Watkins, 1998). This compromise is accomplished through the genetic potential for growth and the interactions between nutrition, metabolism and endocrine factors (Watkins, 1998). Hormones and certain nutrients regulate the autocrine and paracrine cellular relationships responsible for the maintenance of bone mass and structure (Watkins, 1998). In the adult skeleton, the coordination of bone-resorbing and bone-forming activities is termed the bone remodelling cycle (Watkins, 1998). In a normal adult skeleton (following growth and development), bone formation only occurs where bone resorption has taken place (Baron, 1999). This sequence of events - activation, resorption and formation, occurs at the remodelling site (Baron, 1999). The complete remodelling cycle at each site takes from 3 to 6 mo (Baron, 1999). Bone remodelling involves the removal and internal restructuring of existing bone

and is responsible for the maintenance of mass and structure in the adult skeleton (Reviewed by Watkins et al, 2001).

Bone remodelling is in balance until the 4th decade of life, when resorption becomes greater than formation, resulting in a net loss of bone mass (Percival, 1999). In males, this loss of bone is equivalent to approximately 1% per year (Newton-John and Morgan, 1970). This loss also occurs in women, but is accelerated at menopause for 10-15 y when bone loss is approximately 3-5% per year (Newton-John and Morgan, 1970).

There are two types of bone, cortical and trabecular, which differ in their structure and degree of mineralization (Baron, 1999). A thick, dense layer of calcified tissue, the cortical bone forms the external part of the bone (Baron 1999). In the diaphysis or midshaft, the cortex bone encloses the medullary cavity where the haematopoietic bone marrow is located (Baron 1999). The cortex becomes progressively thinner toward the metaphysis and the epiphysis; and the internal space is filled with a network of thin, calcified trabeculae known as trabecular bone (Baron 1999). The spaces enclosed by these thin trabeculae are filled with haematopoietic bone marrow (Baron 1999). Cortical and trabecular bone are made up of the same cells and matrix components, but have structural and functional differences (Baron 1999). The primary structural difference is that 80-90% of the volume of compact bone is calcified, whereas 15-25% of the trabecular bone is calcified (Baron 1999). The remainder of the trabecular bone is made up of bone marrow, blood vessels and connective tissue (Baron 1999). As a result, 70-85% of the interface with soft tissues is at the endosteal bone surface, which leads to the functional difference - the cortical bone fulfills mainly the mechanical and protective role and the trabecular bone a metabolic function (Baron 1999).

Postmenopausal bone loss (primary postmenopausal osteoporosis) occurs primarily in trabecular bone, whereas age-associated bone loss (senile osteoporosis) occurs in cortical and trabecular bone (Abraham, 1991). Bone loss occurs following menopause as a result of estrogen deficiency. Loss of estrogen results in increased rates bone turnover and increased bone resorption (Eastell, 2003). The exact effects of estrogen on bone are unknown, but it has been suggested that estrogen increases the production of IGF-1 and reduces the production of inflammatory cytokines (Eastell, 2003). The estrogen deficiency has been shown to increase the lifespan of osteoclasts and reduce the lifespan of osteoblasts and it is believed this is due to an increase in inflammatory cytokines (interleukin-1, interleukin-6, tumor necrosis factor) (Mangolagas, 2000). In response to aging there is a number of osteoblasts resulting in a decrease in bone formation (Monolagas, 2000). During a lifetime, women will lose one-half of their trabecular bone and one-third of their cortical bone; men will lose about two-thirds of these amounts (Mazess, 1982). A greater amount of trabecular bone is lost because it has a greater surface area compared to cortical bone (Reid, 2003). Quantitatively, aging is the most important factor, accounting for 62% of the total loss, with menopause accounting for 38% (Norden and Heaney, 1990). These losses are extensive and therapies to slow this are required for the prevention of osteoporosis. Current therapies for used for the treatment of both types of osteoporosis in women, include antiresorbing agents (bisphosphonates, estrogen replacement therapy and calcitonin) and anabolic agents (intermittent PTH therapy). In men, with the exception of estrogen replacement therapy, similar treatments are used. Nutritional interventions include dietary Ca, vitamin D and Mg. Based on research in animals and humans there is potential alterations in dietary

total n-6 and n-3 PUFA (including LCPUFA) and individual fatty acids (GLA, AA, EPA and DHA) to influence bone mass and metabolism and potentially reduce the risk of osteoporosis.

2.2.5 Regulation of bone metabolism

Bone formation and resorption are regulated by systematic hormones (including calcitiol and PTH) and locally produced growth factors (insulin-like growth factors) within the skeleton (Reviewed by Watkins et al, 1996). Other local modifiers of bone metabolism are eicosanoids, which include PG (Watkins et al, 1996). The following is a discussion focusing on insulin-like growth factors (IGF) and PG - the production of these regulators of bone cell metabolism are influenced by dietary n-6 and n-3 PUFA.

Insulin-like Growth Factors

Two insulin-like growth factors have been identified, IGF-1 and IGF-2 (Lian et al, 1999). These peptides are present in systemic circulation and are synthesized by multiple tissues, including bone, where they act as local regulators of cell metabolism (Lian et al, 1999). A major site for the synthesis of IGF-1 is in the liver, which secretes IGF-1 into the blood stream (Straus, 1994). IGF-1 is produced at a low level by many tissues in the fetus and its production is not regulated by growth hormone (Straus, 1994). Postnatally, IGF-1 production increases and comes under the control of growth hormone (Straus, 1994). There is a high expression of IGF-2 in the fetus, suggesting a role of IGF-2 as a fetal growth factor (Straus, 1994). IGF-1 and IGF-2 bind to high affinity receptors, IGF-1 or type 1 and IGF-2 or type 2 receptors (Straus, 1994). The liver releases systemic IGF-1 and its synthesis is growth hormone dependent, however, synthesis in peripheral tissues is regulated by many different hormones (Delany et al, 1994; Jones and Clemmons, 1995).

Hormones and growth factors regulate the synthesis of skeletal IGF-1, whereas IGF-2 is regulated only by growth factors (Delany et al, 1994; Gangji et al, 1998). PTH and other stimulators of cAMP in bone cells, as well as growth hormone, are stimulators of IGF-1 synthesis (Delany et al, 1994).

IGF-1 and IGF-2 have similar biological activities, but IGF-1 is more potent in bone (Lian et al, 1999). In vitro, IGF enhance bone collagen and matrix synthesis and stimulate the replication of osteoblast lineage cells (Hock et al, 1988). The effects of IGF on the matrix synthesis are in part dependent on an increased number of cells (Lian et al, 1999). IGF directly adjust the differentiated function of the osteoblasts (Lian et al, 1999). IGF increase type 1 collagen transcription and decrease the transcription of collagenase 3 or matrix metalloproteinase (MMP)-13, a collagen-degrading enzyme (Canalis et al, 1995). This leads to the inhibition of collagen breakdown (Lian et al, 1999). This dual effect is central to maintaining bone matrix and mass (Lian et al, 1999). IGF-1 is produced by osteoblasts and stored in the bone matrix (Stall et al, 1998). This is important for the coupling of bone resorption and formation during remodelling (Watkins et al, 2001). During resorption, IGF-1 is released from bone stimulating formation in an autocrine or paracrine fashion (Linkhart et al, 1996).

In circulation, IGF-1 is bound to IGF-binding proteins (IGFBP) (Jones and Clemmons et al, 1995). The most abundant IGFBP is IGFBP-3, which is GH dependent (Lian et al, 1999). Bone cells secrete 6 known IGFBP (from 1 to 6) (Lian et al, 1999). However, their precise role is not known (Lian et al, 1999). They could be involved in extending the half-life of IGF, in neutralizing or enhancing their activity or in the transport to target cells (Lian et al, 1999). Some IGFBP have inhibitor effects (i.e.

IGFBP-4) and some have stimulatory effects (i.e. IGFBP-5) (Reviewed by Lian et al, 1999). The regulation of IGFBP synthesis is complex. IGFBP-3, 4, 5 are controlled by cAMP, while others are controlled by IGF-1 or IGF-2 (Lian et al, 1999).

Serum IGF-1 and IGFBP-3 positively correlate with BMD of the lumbar spine (Kim et al, 1999) and decreased serum IGF-I are strongly associated with increased risk of osteoporotic fractures in postmenopausal women (Garnero et al, 2000). A lower IGF-1 concentration is associated with lower bone mass in hip fracture patients compared to age-matched controls (Calo et al, 2000). IGF-1 declines with age (Nicolas et al, 1994) and this decline may account for the progressive loss of bone that occurs with age (Calo et al, 2000). This decline is due to impaired synthesis of IGF-1 and essential bonding proteins, which may be the result of declining concentrations of growth hormone (Boonen et al, 1996).

Prostaglandins

Although several localized compounds act on bone, the PG seem to be the principle mediators of bone cell function and their biosynthesis and release from bone cells can be induced by many other hormones and factors (Watkins et al, 2001). As mentioned, the PG are derivatives of 20 carbon carboxylic acids formed by cyclooxygenase pathway (Watkins et al, 1996) synthesized from DGLA, AA and EPA. Adbel-Hakim et al (1994) investigated whether the PGE_2 production is altered in essential PUFA deficiency (ie. LA deficiency). Three groups of rats were fed one of three diets - an essential FA deficient diet, a marginally essential FA deficient diet or a control diet. AA levels in total phospholipids and the *ex vivo* production of PGE_2 were measured in the submandibular salivary glands. The AA levels were significantly different among

the groups fed the different diets. The control group had the highest level of AA and the essential FA deficient diet group had the lowest level of AA and much higher levels of n-9 PUFA. A reduction in the n-6 PUFA and an increase in n-9 PUFA in total phospholipid indicated an essential FA deficiency. The FA patterns of the marginally essential FA deficient diet group were intermediate between those of the control and the essential fatty acid deficiency group. *Ex vivo* production of PGE_2 was significantly lower in the essential FA deficient diet group, but not in the marginally essential FA deficient diet group. The changes in PUFA composition, AA levels and *ex vivo* PGE_2 production were reversed after 5 wk of feeding the control diet to the essential FA deficient diet group and to the marginally essential FA deficient diet group. Thus, dietary PUFA intake affects PG synthesis.

PGE_2 is the primary PG affecting bone metabolism (Watkins et al, 2001). Studies have shown that PGE_2 is the most abundant eicosanoid in bone and that PG of the E series are the most potent bone resorption stimulators (Reviewed by Kawaguchi et al, 1995). Prostacyclin is the next most abundant PG in bone, but does not appear to be an important mediator of bone resorption (Reviewed by Kawaguchi et al, 1995). Small amounts of $PGF_{2\alpha}$ are produced in bone cells and exogenous $PGF_{2\alpha}$ can stimulate bone resorption (Reviewed by Kawaguchi et al, 1995). However, its effect is controlled by an increase in PGE_2 production (Reviewed by Kawaguchi et al, 1995).

PG are potent multifunctional regulators of bone formation and resorption (Raisz, 1995). PG are local mediators, produced in skeletal tissues (Quinn et al, 1997). They are produced in abundance in bone cells and the cells adjacent to the bone (in the marrow and periosteal tissues) (Raisz, 1995). There is evidence that both osteocytes and osteoblasts

can produce PG (Raisz, 1995) and that they are potent stimulators of bone formation (Raisz and Fall, 1990; Igarashi et al, 1994; Igarashi et al, 1997).

The concentration of PGE_2 produced locally in bone is critical; at moderate levels it is stimulatory for bone formation and inhibitory at high levels (Raisz and Fall, 1990). PGE_2 increases *in vitro* bone formation, but has an inhibitory effect on formation after an extended period of time (Reviewed by Schlemmer et al, 1999). Diets that moderate *ex vivo* PGE_2 production in bone organ culture increased markers of bone formation in human and marine osteoblast cell culture (Igarashi et al, 1994; Igarashi et al, 1997). In MC3T3-E1 osteoblast-like cell lines, EPA reduced PGE_2 production and increased biochemical markers of bone formation compared to those treated with AA (Lorenzo et al, 1987). Following fracture, endogenous PGE_2 production is increased (Dekel et al, 1981) suggesting a role for PGE_2 in bone formation and repair. When PG are administered to animals or humans, the major effect seems to be a stimulation of bone formation (Reviewed by Kawaguchi et al, 1995). PGE_1 and PGE_2 injections cause periosteal bone formation in infants (Poznanski et al, 1985). PGE_2 was reported to increase cortical bone mass and intracortical bone remodelling in both intake in normal and ovariectomized rats (Jee et al, 1990) and increased proximal tibial metaphysical bone area in ovariectomized rats (Mori et al, 1990). PGE_2 at 3 mg/kg body weight per d increased tibial diaphysis cortical bone formation rate by enhancing modelling in aged male rats (Yao et al, 1999). PGE_2 strongly inhibits bone resorption in monocyte UMR106 cocultures (Quinn et al, 1994). The strength and duration of this inhibitory effect suggested that it was not caused by an inhibitory effect on the activity of mature osteoclasts, but due to an effect on osteoclast differentiation (Quinn et al, 1994). PGE_2

inhibited osteoclast differentiation in a concentration dependent manner, inhibition was evident at concentrations as low as 10^{-8}M and at 10^{-6}M was also mediated by PGE_1 and $PGF_{2\alpha}$ (Quinn et al, 1997).

PG influence the synthesis and action of IGF, which are major bone derived growth factors (Baylink et al, 1993). In organ culture, this stimulation appears to be due to an increase in the replication and differentiation of osteoblast precursors (Reviewed by Raisz, 1995). This effect is most likely mediated by cAMP and may be associated with an increased production of IGF-1 (Raisz et al, 1993). Thus, some of the effects of PGE_2 on bone formation and resorption may be controlled locally by stimulating the biosynthesis of IGF-1. Once secreted and deposited in bone matrix, the IGF are released during resorption and act to stimulate new bone formation and matrix production (Watkins et al, 2001). IGF-1 is important in the coupling of bone formation with resorption during remodelling (Watkins et al, 2001). The relationship between PG and IGF is also important in optimizing the attainment of mass during stages of skeletal growth and development and maintenance of mass during aging (Watkins et al, 2001).

There are two distinct PG G/H synthase or cyclooxygenase enzymes, COX-1 and COX-2 (Raisz, 1995). Transcription of COX-2 mRNA is increased by almost all the factors that stimulate PG synthesis in bones (Raisz ,1995). It has been found that PG can amplify their own production in bone by inducing COX-1 and COX-2 (Pilbeam et al, 1995). PTH is the most potent stimulator of PG production, while calcitriol has a smaller stimulatory effect (Reviewed by Kawaguchi et al, 1995).

The effect of PGE_2 on bone metabolism is based on the amount of PGE_2 found in bone. Dietary n-6 and n-3 PUFA can influence the bone's capacity to synthesize PGE_2.

For example, a diet higher in n-6 PUFA (LA) would have higher PGE_2 production versus a diet lower in n-6 PUFA (Watkins et al, 1996; Watkins et al, 2000). Therefore, by altering the amount of n-6 and n-3 PUFA and by including n-6 or n-3 LCPUFA (AA or EPA) it is possible to influence the amount of PGE_2 in bone and reach an amount that would favor bone formation versus resorption. Thus, research is needed to determine the optimal amount of AA and EPA to maximize bone mass during growth and prevent bone loss that occurs naturally later in life.

2.2.6 Biochemical markers of bone metabolism

One way to assess bone cell metabolism is to measure biochemical markers of bone metabolism in the blood and urine. Blood sampling and urine collection allow for easy and frequent measurements over time without undue risk or discomfort to the subject (Calvo et al, 1996). Markers of bone formation and resorption are of value in identifying individuals suffering from high bone turnover rates and may be useful predictors of fracture risk and bone loss (Brown and Josse, 2002). Studies are needed to confirm the usefulness of bone biochemical markers in individual patients (Brown and Josse, 2002). These markers are also helpful in designing potential therapies in the prevention and management of bone diseases. The following is a discussion of the bone biochemical markers that are relevant to the thesis and the existing literature pertaining to effects of dietary PUFA and LCPUFA on bone.

Bone formation

Alkaline Phosphatase

There are four isoenzymes of alkaline phosphatase: placental, germ cell, intestinal and liver/bone/kidney (Price and Thompson, 1995). The liver/bone/kidney isoenzyme

represents the predominant form found in serum and exhibits a range of isoforms (Price and Thompson, 1995). There are predominant isoforms associated with each of the tissue sources, liver, kidney and bone (Price and Thompson, 1995). It is generally believed that only one isoform is derived from bone (Price and Thompson, 1995). Elevation of serum phosphatase is most often due to changes in the liver and bone. In the past, this has created a diagnostic problem because it was difficult to differentiate between the isoforms (Price and Thompson, 1995). However, due to current techniques, this is now possible (Price and Thompson, 1995). Bone specific alkaline phosphatase (BSAP) is associated with the cell membrane of the osteoblast, but its function is unknown (Price and Thompson, 1995). It has been shown to be increased by a variety of factors including PTH, calcitriol and growth factors (Reviewed by Price and Thompson, 1995). BSAP is a marker of differentiated osteoblasts and is present in hypertrophic chondrocytes of the epiphyseal growth plate (Crofton et al, 1999). The largest increase in BSAP is found in conditions associated with increased mineralization (Price and Thompson, 1995). An increase has also been seen in conditions associated with demineralization (reflecting a response of osteoblasts to loss of mineral) (Price and Thompson, 1995). As an indicator of bone metabolism, it is less sensitive than osteocalcin (OC) (Azria, 1989).

Osteocalcin

OC is a small non-collagenous protein found only in bone tissue and dentine (Price and Thompson, 1995). OC is secreted by osteoblasts, is widely used as a marker for osteoblastic activity and bone formation (Khosla and Kleerekoper, 1999). It is incorporated into the extracellular matrix of bone bound to hydroxyapatite crystals (Power and Fottrell, 1991). A small proportion is released into circulation (Price and

Thompson, 1995). The precise function is not known (Price and Thompson, 1995). Several possible functions have been suggested including involvement in the mineralization process, acting as a messenger for calcitriol in bone resorption and an inhibitor of growth factor activity (Power and Fottrell, 1991). The expression of OC is enhanced by calcitriol, but PTH does not influence it (Price and Thompson, 1995). A decrease of serum OC may be an indicator of lowered bone formation (Koo, 1996). Both serum and urinary OC have been used as markers for bone formation (Taylor et al, 1990). High OC may also suggest increased bone turnover; this is because OC can be incorporated into the matrix and released during bone resorption (Khosla and Kleerekoper, 1999). *In vivo* studies suggest that OC is associated with matrix mineralization rather than matrix synthesis (Calvo et al, 1996). However, because matrix mineralization and synthesis are linked in most clinical situations, it is appropriate to interpret OC data in terms of changes in formation (Calvo et al, 1996). OC levels follow a circadian rhythm characterized by a decline during the morning to a noontime low followed by a gradual rise that peaks after midnight (Calvo et al, 1996). As a result, it is important that serum samples be collected at the same time each day (Taylor et al, 1990).

Procollagen I Extension Peptides

The major synthetic product of osteoblasts is type I collagen. Thus, indices of type I collagen synthesis would appear to be ideal bone formation markers (Khosla and Kleerekoper, 1999). Several assays have been developed for measuring either the carboxy- or amino-extension peptides of the procollagen molecule (Khosla and Kleerekoper, 1999). These extension peptides, carboxyterminal propeptide of type I collagen (PICP) and aminoterminal propeptide of type I collagen (PINP), guide assembly

of the collagen triple helix and are cleaved from the newly-formed molecule in relation with collagen biosynthesis (Khosla and Kleerekoper, 1999). However, because type I collagen is not specific to bone, these peptides are produced by other tissues that synthesize collagen (Khosla and Kleerekoper, 1999)

Resorption

Hydroxyproline

Hydroxyproline (HP), an amino acid, is mainly found in collagen (Price and Thompson, 1995). Most of the HP found in body fluids is from collagen breakdown and its concentration in urine is used as a marker for bone resorption (Reviewed by Price and Thompson, 1995). Most of the collagen is broken down into free amino acids, which are filtered by the kidney and reabsorbed for oxidation by the liver (Price and Thompson, 1995). The breakdown of collagen is not the only source of urinary HP (Price & Thompson, 1995). Urinary levels of HP reflect the turnover of extra-skeletal as well as skeletal proteins (Reviewed by Azria, 1989). HP does not have a good correlation with other markers of bone resorption, such as bone histomorphometry (Delmas, 1992). HP assays are tedious and time consuming (Calvo et al, 1996). HP excretion as a marker of bone resorptive activity is less than ideal (Calvo et al, 1996).

Collagen Crosslink Molecules

The extracellular matrix is stabilized by the formation of covalent crosslinks between adjacent collagen chains, referred to as collagen crosslink molecules (Price and Thompson, 1995). Pyridinolines (Pyd) and deoxypyridinolines (Dpd), two major crosslinking molecules, are unique to collagen and elastin and play an important role in determining the physical and chemical characteristics of collagen (Price and Thompson,

1995). Urine contains both free collagen crosslink molecules and fragments (Price and Thompson, 1995). Crosslink molecules are only found in mature collagen and do not include collagen that has been synthesized but not incorporated into collagen fibrils (Price and Thompson, 1995). Secretion of collagen crosslink is positively correlated with other measures of bone resorption (Price and Thompson, 1995). There is, however, variability in specificity among assays (Price and Thompson, 1995). It has been argued that the measurement of the crosslinks is not specific for type I collagen, but the ability to measure the telopeptide, a peptide crosslink fragment, would offer greater specificity (Price and Thompson, 1995). Measurement of urinary N-telopeptide of collagen cross-links (NTx) (Hanson et al, 1992) and serum NTx (Clemens et al, 1997) have been reported as sensitive and specific markers of bone resorption. Also, urinary C-telopeptide of collagen cross-links (CTx) (Bonde et al, 1994; Qvist et al, 2002) and serum CTx (Christgau et al, 2000; Qvist et al, 2002) have been shown to be specific and sensitive markers of bone resorption. The crosslinked peptides are derived specifically from bone collagen degradation and are not metabolized (Hanson et al, 1992). Crosslinked peptides are useful in showing acute changes in bone resorption (Qvist et al, 2002).

2.2.7 Techniques for measurement of bone mass

Single-photon absorptiometry is a precise method for measuring bone mineral content (BMC) at peripheral individual bone sites (Greer et al, 1983). It uses a collimated monoenergetic photon beam emitted from a low energy source that is moved at a constant speed across bone and soft tissue (Barden and Mazess, 1988). Single-photon absorptiometry has a very low radiation dose that is limited to the site of scanning (Barden and Mazess, 1988). The technique requires a constant soft tissue thickness at the

measuring site and is normally used on the forearm or heel (Price and Thompson, 1995). Dual photon absorptiometry involves the use of two photon energies (to overcome some of the variations seen in single photon absorptiometry) on different sites of the body with different tissue and fat content (Price and Thompson, 1995). This technique can be performed on any part of the body, however, the most common sites are the lumbar spine and various areas of the hip. This is a precise and accurate method for measuring bone of the axial skeleton, but has a longer scanning time then single-photon absorptiometry (Reviewed by Barden and Mazess, 1988). It is also difficult to measure small bones because of its larger beam size (Barden and Mazess, 1988).

In dual-energy X-ray absorptiometry (DXA), dual-energy x-ray sources are used rather than the radionuclide sources used for single-photon absorptiometry and dual-photon absorptiometry (Barden and Mazess, 1988). X-ray sources have much greater radiation flux, which are used to achieve improved precision and image resolution and reduced scanning times (Reviewed by Barden and Mazess, 1988). The dose is as low as or lower than conventional single-photon absorptiometry and dual-photon absorptiometry because the beam is highly collimated and energies are optimized for contrast (Barden and Mazess, 1988). DXA has now replaced the dual photon method. It has a similar technique, with an X-ray source replacing the photon source (Price and Thompson, 1995). DXA was developed to measure bone mineral at multiple sites (Shore and Poznanski, 1999). By analyzing at two different energies, the amount of bone mineral, soft tissue and fat can be calculated, without the need for constant body part thickness (Shore and Poznanski, 1999). This allows for determination of whole-body composition, as well as BMC of multiple skeletal sites (Shore and Poznanski, 1999). In validation

studies of DXA performed on piglets and in neonates, it has been found that BMC and bone area measurements were accurate (Brunton et al, 1993; Koo et al, 1995; Picaud et al, 1996). Due to the advantages of DXA, it is recommended for research across the lifespan. DXA is currently the best available measurement of BMD (Ross et al, 1990) and our best gauge of bone strength (Stein and Ashok, 2000). BMD is the most readily quantifiable predictor of fracture risk for those who have not yet suffered from a fracture (Brown and Josse, 2002). Osteoporosis is defined as a BMD value more than 2.5 SD below the mean value of a young adult 25 years of age (Kanis et al, 1997).

2.3 Dietary polyunsaturated fatty acids affects on bone

2.3.1 Bone polyunsaturated fatty acid composition

Dietary n-6 and n-3 PUFA are reflected in the PUFA composition in bone. Alam et al (1993) fed male weanling rats one of three diets: a n-6 PUFA diet (high in LA), a saturated fat diet or a n-3 PUFA diet (high in ALA). AA concentrations were significantly lower in the mandibles and maxillae of rats fed the saturated FA and n-3 PUFA diets compared with those fed the n-6 PUFA diet. The proportions of AA as a percentage of total FA were lowest in the n-3 PUFA group.

Alterations in dietary EPA, AA and DHA also change the composition of LCPUFA in bone. Kokkinos et al (1993) compared a diet high in n-6 PUFA (LA) to a diet high in n-3 LCPUFA (EPA and DHA). AA was significantly lower in alveolar bone of rats fed the diet high in n-3 LCPUFA when compared to the rats fed the n-6 PUFA rich diet. Chicks given a diet high in n-3 LCPUFA (EPA and DHA) had lower concentrations of AA and higher concentrations of EPA and DHA in bone than those given a diet high in n-6 PUFA (LA) (Watkins et al, 1996). In rats, reducing the dietary PUFA ratios of n-6:n-3 from 23.8 to 1.2 by incorporating n-3 LCPUFA (EPA and DHA) into the diet increased EPA and decreased AA in bone femur compartments (Watkins et al, 2000). Even very small amounts of dietary LCPUFA can change the LCPUFA composition of bone. Incorporation of 0.5 g/100 g of fat as AA in the diet increased the amount of AA in bone (Weiler, 2000). This research clearly indicates that dietary PUFA and LCPUFA are factors in determining bone tissue content of LCPUFA. However, the amount of the specific n-6 and n-3 LCPUFA in the diet or in bone required for optimization of bone mass and mineralization are not known.

2.3.2 Effects of dietary n-6 and n-3 polyunsaturated fatty acids on bone and mineral metabolism in animal models

The effects of alterations in dietary PUFA and LCPUFA on bone have been studied at different stages of the lifespan using various animal models. There are two main mechanisms that have been suggested for the increases in BMC and mass after alteration of dietary PUFA and LCPUFA, which are changes in Ca metabolism (Ca absorption or excretion) and/or altered bone metabolism (bone resorption or formation). The exact effects of PUFA and LCPUFA on Ca and bone metabolism are unknown. There are different possible mechanisms for their effect on Ca absorption, including modulation of membrane lipid composition, conversion to PG, inhibiting PG synthesis or activation of protein kinases A and C via protein phosphorylation (Reviewed by Haag and Kruger, 2001). Recently, the effect of n-6 and n-3 LCPUFA on calcium uptake in the intestine by direct or indirect (via protein kinase A or protein kinase C) action on ATP-dependent pumps in the basolateral membrane of intestinal cells has received attention (Haag and Kruger, 2001; Haag et al, 2003). It has been suggested that AA influences Ca excretion through a PGE_2 and calcitriol pathway (Baggio et al, 2000; Baggio, 2002; Baggio et al, 2002). This pathway has been proposed based on research in rats where feeding of fish oil reduced plasma PGE_2, plasma AA content, serum calcitriol, Ca absorption and Ca excretion (Baggio et al, 2000). Also, there was a strong positive correlation between plasma AA content and both intestinal Ca absorption and urinary Ca excretion (Baggio et al, 2000). There was also a strong positive correlation between plasma PGE_2 concentration and serum calcitriol concentrations (Baggio et al, 2000) and PGE_2 has been shown to alter the activity of 1,α-hydroxylase (the rate limiting step in

calcitriol synthesis) (Kurokawa, 1987). For bone metabolism, it is has been hypothesized that dietary n-6 and n-3 PUFA impact bone formation and resorption activities by modulating PGE_2 biosynthesis (Watkins et al, 2001). As the types of dietary PUFA and LCPUFA can modulate the levels of AA in phospholipids, they can change PGE_2 synthesis. AA is the precursor of PGE_2, thus a significant decrease in AA leads to a decrease in PGE_2 levels in bone (Kokkinos et al, 1993, Watkins et al, 1996).

Following is a discussion of the effects of dietary n-6 and n-3 PUFA on bone across the lifespan in animal models and the suggested mechanisms of action. For the purpose of this thesis, the discussion will be focused on changes in bone growth, mass and quality through alterations in Ca metabolism and bone metabolism in animals. Specific cellular effects of PUFA and LCPUFA on bone and other tissues will not be discussed; they are beyond the scope of this thesis. This research can be separated into 3 stages of life: infancy, childhood and adolescence (weanling/growing) and postmenopause/estrogen deficiency (ovariectomization) and is summarized in **Tables 2.3.1-2.3.3**. Very few studies investigating the effects of dietary PUFA and LCPUFA on bone mass in animal models during the attainment of peak bone mass and into maturity have been conducted.

Infancy

Research investigating the effects of dietary n-6 and n-3 PUFA on bone and mineral metabolism is summarized in Table 2.3.1. The majority of animal research on the effects of dietary PUFA and LCPUFA during infancy has been done in piglets. In piglets, dietary AA (0.5 g/100 g of fat) and DHA (0.1 g/100 g of fat) with a total n-6:n-3 ratio of 5:1 significantly increased BMD of the whole body, lumbar spine and femur compared to

Table 2.3.1. The effects of dietary polyunsaturated fatty acids on bone in infant animal models.

Reference	Model and Sample Size	Age	Fatty Acid Supplementation (expressed as g/100 g of diet, g/100 g of fat and/or n-6/n-3 ratio)	Length of Study	Results
Watkins et al. 1996	Male chicks n=120	1 d	G1 – 90 g/kg as SO G2 – 39.2 g/kg as MO (56 g/100 g of fat) plus 30.8 g/kg as SFO (44 g/100 g of fat SO -total n-6n-3 ratio of 7.19 MO plus SFO -total n-6:n-3 ratio of 1.98 ratio	19 d	• trabecular bone surfaces and bone formation rates were significantly ↑ in chicks fed MO + SFO • ↑bone formation rate in chicks given MO + SFO was associated with a 3.5 fold ↓ in tibial *ex vivo* PGE_2 production • EPA concentration 10.8 times ↑ and AA 50% ↓ in the tibia in the G fed MO • no differences in body weight, weight gain, feed intake, feed conversion, bone length, bone ash or BMC • serum alkaline phosphatase activity was ↑ in chicks given MO plus SFO diet
Weiler 2000	Male piglets n=12	10 d	G1 - Standard formula LA and ALA G2 - standard formula + AA (0.5 g/100 g of fat) and DHA (0.1 g/100 g of fat) Both diets had a total n-6:n-3 ratio of 5:1	14 d	• AA:DHA ↑ BMD of the whole body, lumbar spine and femur. • no differences in whole body length, Ca absorption or biomarkers • AA:DHA ↑ AA and ↓ LA in liver and bone but no difference in DHA level • liver AA was positively related to growth, AA in bone, BMC, BMD and urinary PGE_2 but negatively related to LA in bone
Blanaru et al. 2004	Male piglets n=40	5 d	G1-4 - AA (0.30, 0.45 0.60 or 0.75 g/100 g of fat) and DHA (0.1 g/100 g of fat) All diets had a total n-6:n-3 ratio of 9:1	15 d	• AA ↑ with ↑ supplementation of AA in the plasma, liver and adipose but not in the brain, where supplementation of AA as 0.45 g/100 g of fat led to ↓ DHA • plasma PUFA and LCPUFA proportions reflected the PUFA and LCPUFA proportions in liver and adipose, but not brain • 0.6 and 0.75 g/100 g of fat as AA lead to ↑ whole body BMC compared to those receiving 0.3 g/100 g of fat. • no effect on femur BA, femur BMC or whole body BA • no effect on plasma IGF-1, calcitriol, PTH or OC • no effect on *ex vivo* release of bone PGE_2 or urinary NTx • liver AA was positively related with plasma IFG-1 and calcitriol and urinary NTx. /continued

Table 2.3.1 continued. The effects of dietary polyunsaturated fatty acids on bone in infant animal models.

Reference	Model and Sample Size	Age	Fatty Acid Supplementation (expressed as g/100 g of diet, g/100 g of fat and/or n-6/n-3 ratio)	Length of Study	Results
Lucia et al. 2004	Male piglets n=28	5 d	G1 - Control diet G2 - AA(0.8 g/100 g of fat) G3 - Standard diet + PGE2 injection (0.1mg/kg/d) G4 - AA (0.8 g/100 g of fat) + PGE_2 injection (0.1mg/kg/d) All diets had a total n-6:n-3 ratio of 9:1	15 d	• plasma OC levels ↑ due to PGE_2 injection (0.1mg/kg/d), not seen in the G supplemented with dietary AA • AA and PGE_2 interaction ↓ femur BMC and BA • no effect of diet alone on femur length, weight, BA, BMC or BMD • AA ↓ *ex vivo* PGE_2 release from bone

Abbreviations: AA: arachidonic acid, BMD: bone mineral density, BMC: bone mineral content, Ca: Calcium, DHA: docosahexanoic acid, FO: fish oil, G: group, IGF-1: insulin-like growth factor 1, LCPUFA: long chain polyunsaturated fatty acids, OC: osteocalcin, MO: menhaden oil, PGE_2: prostaglandin E_2, PTH: parathyroid hormone, SO: soy oil, SFO: safflower oil

those receiving only LA and ALA with the same total n-6:n-3 ratio (Weiler, 2000). Piglets fed 0.60 and 0.75 g/100 g of fat as AA had higher BMC compared to those fed 0.30 and 0.45 g/100 g of fat as AA. All the diets had 0.1 g/100 g of fat as DHA and a total n-6:n-3 ratio of 9:1 (Blanaru et al, 2004). In contrast, piglets fed 0.8 g/100 g of fat as AA and 0.1 g/100 g of fat as DHA with a total n-6:n-3 ratio of 9:1 had no affect on BMC or BMD (Lucia et al, 2003). Therefore, based on research conducted in male neonatal piglets, dietary AA ranging from 0.5 to 0.75 g/100 with 0.1 g/100 g of fat as DHA (AA:DHA ratios of 5:1 to 7.5:1) result in improved bone mass in piglets (Weiler, 2000; Blanaru et al, 2004), while higher amounts of AA offer no benefit (Lucia et al, 2003). However, supplementation of higher amounts of AA may increase bone mass if more DHA was incorporated in the diet to maintain the AA:DHA ratio between 5:1 to 7.5:1. Changes in bone mass in piglets are not explained by changes in bone metabolism, PGE_2 production or Ca metabolism. Weiler (2000) supplemented AA:DHA as 0.5:0.1 g/100 g of fat and Blanaru et al (2004) supplemented AA:DHA as 0.75:0.1 and 0.60:0.1 and saw improved bone mass without changes in bone resorption (indicated by urinary NTx) or bone turnover (indicated by plasma OC). There were also no changes in *ex vivo* release of PGE_2 from the tibia (Blanaru et al, 2004) or concentrations excreted in the urine (Weiler, 2000). Supplementation of AA and DHA as 0.8:0.1 g/100 g of fat reduced urinary NTx and decreased bone PGE_2 concentrations, without resulting in changes in bone mass (Lucia et al, 2003). Supplementation of 0.5:0.1 g of fat as AA:DHA did not alter Ca absorption, tibial diaphysis Ca content, plasma Ca or urinary Ca (Weiler, 2000). Unaltered Ca absorption and urinary Ca in response to LCPUFA supplementation could have been the result of (1) already optimal Ca transport and high mineral retention during

periods of rapid growth in the piglets, (2) supplementation of total LCPUFA as 0.6% of fat by weight may not have been enough to alter intestinal Ca transport or retention (3) the method used (in situ ligated loop) to measure Ca absorption may not have represented whole intestinal absorption.

In piglets, dietary AA and DHA does not affect whole body, lumbar spine or femur bone area (BA) (measured by DXA) compared to a diet with the same total n-6:n-3 ratio, but only containing LA and ALA (Weiler, 2000, Blanaru et al, 2004). In contrast, reducing the total n-6 to n-3 PUFA ratio (from 7.6 to 1.4) through incorporation of n-3 LCPUFA (EPA and DHA) into the diet increased total BA and cortical BA (measured by histomorphometric analysis), but did not affect length of the tibia in chicks at 21 d of age (Watkins et al, 1996). However, this effect was not sustained following 42 d of study. Bone formation rate and serum alkaline phosphatase activity were significantly higher in chicks fed the high n-3 LCPUFA diet, which may explain the changes in BA. Increased bone formation rates in chicks given EPA and DHA were associated with a 3.5 fold decrease in *ex vivo* PGE_2 production in the tibia (Watkins et al, 1996). However, ASA treatment inhibited PGE_2 production in the soybean group, but did not alter the bone formation rate (Watkins et al, 1996). This suggests that under these circumstances bone formation is not regulated by PGE_2. IGF-1 concentrations in liver, cortical and cartilage following 21 d were increased, but after 42 d concentrations were no longer higher (actually lower in the group fed n-3 LCPUFA versus those fed n-6 PUFA). It is important to note that the research conducted in chicks is not representative of current recommendation for EPA (<1 g/100 g of fat), DHA (0.3 g/100 of fat) or for the total n-6:n-3 ratio (5:1 to 10:1) for infant formula. The amounts of AA and DHA used in piglet

research are similar to that found in sow's milk (Blanaru et al, 2004) and are close to current recommendations for infant formula (0.5 g/100 g of fat as AA and 0.3 g of fat as DHA). The total n-6:n-3 PUFA ratio (5:1 to 9:1) is also within the recommended range.

Weanling

The effects of dietary PUFA on bone and mineral metabolism introduced after weanling are summarized in **Table 2.3.2**. In rats, alterations in dietary GLA plus EPA to achieve total n-6:n-3 ratios of the 3:1 (7.4 g/100 g of fat as GLA and 2.5 g/100 g of fats as EPA plus DHA) and 1:1 (GLA 6.6g/100 of fat and EPA plus DHA 5.0 g/100 g of fat) resulted in higher Ca content in bone (24.7% and 9.0%, respectively) compared to rats fed a diet containing only LA and ALA (total n-6:n-3 ratio of 3:1) (Claassen et al 1995 a). Changes in bone Ca content were not seen when dietary GLA and EPA were altered to achieve a total n-6:n-3 ratio of 1:3 (GLA 5.2 g/100 g of fat and EPA plus DHA 12.4 g/100 g of fat). There was no effect on femur length, indicating no affect on growth. Bone resorption (indicated by Pyd, Dpd and HP excretion) was significantly decreased in the GLA:EPA dietary groups indicating reduced bone resorption (Claassen et al, 1995b). The GLA and EPA 3:1 group had increased Ca absorption and increased Ca balance but urinary Ca was not affected. The GLA and EPA 1:1 and 1:3 groups had reduced Ca absorption and Ca excretion, which may reflect a lower Ca load compared to the 3:1 group (Claassen et al 1995a). Thus, the inclusion of GLA and EPA in the diet likely elevated Ca content as a result of either enhanced mineralization and/or reduced bone resorption. Similarly, supplementation with GLA and EPA plus DHA enhanced Ca

Table 2.3.2 The effects of dietary polyunsaturated fatty acids on bone in growing animal models.

Reference	Model and Sample Size	Age	Fatty Acid Supplementation (expressed as g/100 g of diet, g/100 g of fat and/or n-6/n-3 ratio)	Length of Study	*Results*
Watkins et al. 2000	Male rats n=60	3 wk	G1 - 90 g/100 g as SFO + 10 g/100 g of fat as MO G2 - 80 g/100 g as SFO + 20 g/100 g of fat as MO G3 - 50 g/100 g as SFO + 50 g/100 g of fat as MO G4 - 30 g/100 g as SFO + 70 g/100 g of fat as MO total n-6:n-3 ratios of 23.8, 9.8, 2.6 or 1..2	42 d	• as the dietary ratio of n-6/n-3 ↓ and the EPA level ↑, the concentration of EPA ↑ while the AA ↓ in bone compartments • the ↑ n-3 PUFA found in bone was associated with ↓ *ex vivo* PGE_2 production in bone and ↑ serum level of BSAP • a significant positive correlation between bone PGE_2 production and the ratio of AA/EPA • a significant negative correlation between bone formation rate and either the ratio of AA/EPA or PGE_2 in bone
Kelly et al 2003	Male rat n=40	28 d	G1 - 100 g/100 g SO G2 - 56 g/100 g of diet as MO plus 44 g/100 g of diet as SFO	8 wk	• SO G had ↑ *ex vivo* release of PGE_2 and ↑ urinary pyridinium cross links • no differences in serum OC or IGF-1 • no differences in femur measurements - length, dry weight, ash weight, density, bone mineral mass, Ca, P or Mg • no differences mineral absorption - Ca, Mg or P
Sirois et al 2003	Male and female rats n=36-48	3 wk	G1 - 70 g/kg SO G2 - 60 g/kg (86 g/100 g of fat) MO plus 10 g/kg (14 g/100 g of fat) SO	5 wk	• no effects on femur measurements – length, weight, width, BMC, BMD, yield load, resilience, peak load or toughness • no effects on vertebrae height, width, weight • vertebrae peak load ↓ in MO females but no effect on males • no effect on urinary Ca or serum IGF-1 • ↓ feed intake in G fed MO
Liu et al 2003	Male Japanese Quail n=120	4 wk	G1 - 50 g/kg SO G2 - 50 g/kg FO (27.3 g/100 g of fat as n-3 LCPUFA) SO total n-6:n-3 ratio = 12.55:1 FO total n-6:n-3 ratio =0.66:1	7 mo	• SO ↓ tibia shear force and shear stress, but ↑ shear fracture energy • SO ↓ tibia proximal cortical density, diaphysis cortical density, diaphysis thickness and distal end cortical thickness • SO ↓ tibia ash weight, BMC, Ca, P • No differences in body weight, feed intake, tibia length, tibia diameter, tibia weigh, tibia proximal end cortical thickness or tibia distal end cortical density /continued

Table 2.3.2 continued. The effects of dietary polyunsaturated fatty acids on bone in growing animal models.

Reference	Model and Sample Size	Age	Fatty Acid Supplementation (expressed as g/100 g of diet, g/100 g of fat and/or n-6/n-3 ratio)	Length of Study	Results
Green et al 2004	Male rats n=80	4 wk	G1 - diabetic receiving 70 g/kg of SO G2 - healthy receiving 70 g/kg of SO G3 - diabetic receiving 40 g/kg (57 g/100g of fat) of MO and 30 g/kg (43 g/100 g of fat) of CO G4 - diabetic receiving 40 g/kg (57 g/100g of fat) of MO and 30 g/kg (43 g/100 g of fat) of CO SO total n-6:n-3 ratio = 7.1:1 MO-CO- total n-6:n-3 ratio = 1.4:1	5 wk	• Femur BMD was higher in the MO-CO G versus the SO G in healthy rats but no difference in the diabetic rats • ↓ OC in the diabetic G receiving MO-CO compared to SO but no difference in the healthy rats • ↓ *Ex vivo* release of PGE_2 with MO-CO feeding compared to SO in both diabetic and healthy rats • no changes in urinary NTx, plasma IGF-1, plasma calcitriol or urinary ca
Reinwald et al 2004	Male and female rats n=44	49 d	G1 - n-3 adequate G2 - n-3 deficient G3 - n-3 repleted	8 wk	• n-3 deficiency resulted in ↓ n-3 PUFA and ↑ n-6 PUFA in bone and muscle • n-3 deficiency diminished structural integrity (measured by energy to peak load) of the tibia • n-3 repletion restored the n-6:n-3 PUFA ratio in bone and muscle • length of femur not affected by n-3 deficiency • length of the tibia ↓ with n-3 deficiency • n-3 repletion ↑ bone modeling (cross-sectional geometry) and ↑ second moment in tibia compared to n-3 adequate G following 4 wk of treatment (whether the n-3 repleted g was different from control at end of study was not stated)
Liu et al 2004	Male Japanese Quail n=80	4 wk	G1 - 50 g/kg SO G2 - 50 g/kg FO (27.3 g/100 g of fat as n-3 LCPUFA) SO total n-6:n-3 ratio = 12.55:1 FO total n-6:n-3 ratio =0.66:1	7 mo	• No differences in body weight, feed intake, tibia length, tibia diameter, tibia weigh, • SO ↓ femur ash, tibia pyridinoline and tibia deoxypyridinoline • SO ↑ PGE_2 in bone marrow supernantant and ex vivo production in bone organ culture • PGE_2 was negatively correlated with ash, pyridinoline and total cross-links, but positively correlated with collagen content

Abbreviations: AA: arachidonic acid, BMD: bone mineral density, Ca: Calcium, co: corn oil, DHA: docosahexaenoic acid, EPA: eicosapentaenoic acid, FO: fish oil, G: group, IGF-1: insulin like growth factor –1, m: month(s), Mg: magnesium, MO: menhaden oil, P: phosphorus, PGE_2: prostaglandin E_2, PUFA: polyunsaturated fatty acids, OC: osteocalcin, SFO: safflower oil, SNO: sunflower oil

transport across the intestinal wall in more mature rats (Coetzer et al, 1994). *In vivo* studies of the effect of dietary supplementation on Ca transport showed that changes in the dietary content of PUFA influenced the brush border membrane lipid composition as well as Ca transport in the intestine (Coetzer et al, 1994; Baggio et al, 1996; Baggio, 2000; Baggio et al, 2002). In male rats, a high n-3 LCPUFA diet (fish oil as 10g/100 g of diet) reduced bone resorption and osteoclast activity compared to those fed a high n-6 PUFA diet (corn oil as 10g/100 g of diet) (Iwami-Morimoto et al, 1999). Similarly in male rats, dietary n-3 LCPUFA (56 g/100 g of fat as menhaden oil) reduced bone resorption (indicated by decreases in urinary Pyd and Dpd values) and also reduced *ex vivo* bone PGE_2 concentrations compared to a soybean diet. However, diet had no effect on bone turnover (indicated by serum OC), Ca absorption or femur measurements (length, ash weight, density or Ca) (Kelly et al, 2003). There was a trend towards higher dry weight (P=0.075) and mineral mass (P=0.058) for those receiving n-3 LCPUFA. In healthy male rats, a similar amount of n-3 LCPUFA diet (menhaden-corn oil diet, 57 g/100 g of fat) elevated femur BMD but had no affect in diabetic rats compared to a n-6 PUFA group (soybean oil) (Green et al 2004). Changes in BMD were not explained by changes in bone resorption (indicated by urinary NTx). In the diabetic rats, plasma OC values were reduced, but OC values were not changed in the healthy group. Femur Ca and urinary Ca were unaffected, however, *ex vivo* PGE_2 production was reduced with dietary n-3 LCPUFA in diabetic and healthy rats (Green et al, 2004). A reduction in the n-6:n-3 ratio from 23.8 to 1.2 reduced *ex vivo* PGE_2 production and increased bone formation (increases in BSAP and bone formation rates) in male rats (Watkins et al, 2000).

Although the research is inconclusive, dietary n-3 LCPUFA or total n-6:n-3 ratios of $\leq$3:1 are shown to improve bone mass, reduce bone resorption and increase bone formation during growth. To alter femur Ca, there may be a need for inclusion of an n-6 PUFA or LCPUFA beyond the rate-limiting step of the Δ-6 desaturase enzyme. Consistently, reductions in total n-6 PUFA through the inclusion of n-3 LCPUFA (EPA and DHA) in the diet reduce *ex vivo* PGE_2 production in rats (Watkins et al, 2000; Kelly et al, 2003; Green et al, 2004). In male rats, there was a positive correlation between bone PGE_2 production and the ratio of AA:EPA, but negative correlations between bone formation rate and either the ratio of AA:EPA or PGE_2 in bone (Watkins et al, 2000). It is important to note that reductions in *ex vivo* PGE_2 is not always related to improved bone mass or mineral content (Kelly et al, 2003).

There are few studies investigating the effects of dietary n-6 and n-3 PUFA on bone during growth that are followed into maturity (after attainment of peak bone mass). In Japanese quail, dietary soybean oil (50 g/kg, total n-6:n-3 ratio of 12.6:1) resulted in reduced BMC in the tibia and collagen cross links compared to those fed fish oil (50 g/kg, 27.33 g of n-3 LCPUFA, total n-6:n-3 ratio of 0.7:1). Those fed the soybean oil also had higher PGE_2 and PGE_2 was negatively correlated with tibia ash weight and collagen cross links (Liu et al, 2004). In a similar study with Japanese quail and the same dietary treatments, those animals fed the fish oil diet had the highest percentage of tibial ash (Liu et al, 2003). The fish oil diet also increased tibial strength and improved cortical thickness and density compared to the soybean oil group (Liu et al, 2003). There was no affect of diet on tibia weight, length or diameter (Liu et al, 2003; Liu et al, 2004). Thus,

long-term dietary soybean oil during growth had significant adverse effect on mature bone metabolism, impaired mechanical properties and histological characteristics.

The changes in bone in response to altered PGE_2 may occur through IGF-1. Li et al (1999) reported that *ex vivo* PGE_2 production was higher in rats given a diet high in n-6 PUFA (n-6:n-3 ratio of 7.3:1) and serum IGF-1 was decreased compared to a group fed a diet higher in n-3 PUFA (n-6:n-3 of 1.8). Those fed the higher n-3 diet had increased ash weight per mm of humeri versus those fed higher the n-6 PUFA diet. Although, in other studies high dietary n-3 LCPUFA have reduced *ex vivo* PGE_2 production without changes in serum IGF-1 (Kelly et al, 2003; Green et al, 2004).

There is a balance of total n-6:n-3 PUFA required for bone health indicated by the negative effects of low ratios of total n-6:n-3 PUFA (0.05:1 to 0.6 :1) on bone quality. In female growing rats, a fish oil diet (12.63 g/100 g of fat as EPA plus DHA) with a total n-6:n-3 ratio of 0.6:1 had a significant negative effect on vertebrae strength and growth (measured by length) versus a soybean oil diet with a ratio total n-6:n-3 PUFA ratio of 6.6:1. It is important to note that these changes were not accompanied by other negative effects on bone or Ca excretion but were accompanied with reduced feed intake. There was no effect on male rats (Sirois et al, 2003). Sirois et al (2003) speculated that their results conflict with other studies because of the amount of ALA in their control diet. Their control diet had ALA as 8.65 g/100 g of fat, while, Watkins et al (2000) had a control diet of 2.85 g/100 g of fat as ALA due to the different types of oils used to formulate the diets. They speculated that higher ALA may dilute the beneficial effects of fish oil. It is also important to point out that due to the differences in ALA content, the ratio of total n-6:n-3 was very different between Watkins et al (2000) and Sirois et al

(2003), 1.2: to 2.6:1 versus 0.6:1 for the menhaden oil groups, important in the effect dietary PUFA on bone. The researchers also indicated that alterations in bone markers may not translate into functional differences in bone i.e. strength. It is also important to note that there was a detrimental effect in female rats, but not in male rats. Other researchers have only investigated the effects of LCPUFA on bone in male animals. In growing male rabbits, a high fat fish oil (30.15 g/100 g of fat as n-3 LCPUFA) with a total n-6:n-3 ratio of 0.05:1 resulted in reduced tibial bone quantity (smaller middiaphyseal areal properties and shorter tibiae) and compromised structural properties. There was a pair-fed group to control for feed intake. These effects could have been due to the higher fat content (31% versus 8%) of the fish oil diet. High fat diets (rich in saturated fat) reduce trabecular BMC and reduced bone strength in roosters (Wohl et al, 1998). Further research is needed to determine the dietary ratio of n-6:n-3, as well as the amount of the specific PUFA and LCPUFA, that are required during bone growth and mineralization to optimize bone mass and strength.

Ovariectomized

Studies in ovariectomized rodent models show that dietary PUFA and/or LCPUFA could play a role in prevention of bone loss due to estrogen deficiency (summarized in **Table 2.3.3**). Ovariectomized rats fed a low Ca diet supplemented with EPA (0.32 g/100 g of fat) prevented the decrease in femur weigh and strength seen in a low Ca group, but had no effect when dietary Ca was normal (Sakaguchi et al, 1994). Ovariectomized rats fed GLA plus EPA (1 g/kg) as a diester plus estrogen and 1% linoleic acid plus estrogen increased the amount of femur Ca/mm (12.6% and 17.5% respectively) and reduced bone resorption (indicated by reduced Dypd concentrations)

Table 2.3.3 The effects of dietary polyunsaturated fatty acids on bone in ovariectomized rodents.

Reference	Model and Sample Size	Age	Fatty Acid Supplementation (% wt/wt of total dietary fat or n-6/n-3 ratio)	Length of Study	Results
Schlemmer et al, 1999	Female ovx rats n=50	11 wk	G1 - LA and sham operated G2 - LA and ovx G3 - LA and estrogen G4 - LA plus 1g/kg of diester, estrogen and ovx G5 - LA plus 1 g/kg, placebo and ovx	3 wk	• estrogen and diester alone increased Ca per femur to sham levels • estrogen plus the diester increased the effectiveness of estrogen on bone Ca and urinary excretion of bone resorption markers
Sun et al, 2003	Female mice n=20	8 wk	G1 - 5 g/100 g of diet as CO and sham-operated G2 - 5 g/100 g of diet as FO plus 0.5 g/100 g of diet as CO	2 m of feeding diets, operated on, followed by 4 m of feeding diets	• ovx rats fed CO diet, BMD of the distal left femur ↓ by 20% and 22.6 % in the lumbar spine • ovx rats fed FO diet, BMD of the femur only ↓ by 10% and no change in the lumbar spine • in both Gs of ovx rats, BMD of the distal femur was ↓ compared to sham-operated animals, only the lumbar spine was different for those fed CO • no effect of diet on BMD in the sham-operated animals
Watkins et al, 2003	Female Rats n=24	8 wk	G1 - 110.4 g/kg as SFO and sham- operated G2 - 100.4 g/kg of SFO and ovx G3 - 32.7 g/kg of MO plus 77.7 g/kg as SFO and ovx SFO total n-6:n-3 ratio of 479:1 MO plus SFO total n-6:n-3 ratio of 5:1	12 wk	• MO feeding ↓ pyridinoline • MO ↑ tibia length and BMC compared to sham-operated animals (fed SFO), however, ovx animals fed SFO were intermediate.

Abbreviations: BMC: bone mineral content, BMD: bone mineral density, Ca: calcium, co: corn oil, FO: fish oil, G: group, LA: linoleic acid, m: month(s), MO: menhaden oil, ovx: ovariectomized, PUFA: polyunsaturated fatty acids, SFO: safflower oil, SNO: sunflower oil

compared to the ovariectomy plus placebo group; the effect of estrogen was enhanced by the diester. Only the groups with estrogen showed significant increases in bone Ca and significant decreases in bone turnover, although the diester alone did increase bone Ca levels towards baseline (Schlemmer et al, 1999). In ovariectomized rats, n-3 PUFA (menhaden oil plus safflower, total n-6:n-3 ratio of 5:1) increased BMC and bone length of the tibia compared to sham-operated rats fed a high n-6 PUFA diet (safflower oil) devoid of n-3 PUFA (total n-6:n-3 ratio of 479:1) (Watkins et al, 2003). It is interesting to note that ovariectomized rats fed the high n-6 diets had intermediate values for both tibia BMC and length. The n-6 PUFA diet used had an extremely high n-6:n-3 ratio and could be considered a diet deficient in n-3 PUFA. The addition of fish oil has also been investigated in ovariectomized mice where diets made with fish oil (5 g/100 of diet as fish oil plus 0.5 g/100 g of diet as corn oil) prevented BMD loss while a corn oil diet (5 g/100 g of diet as corn oil) offered no benefit (Sun et al, 2003). There was no effect of fish oil on BMD in the sham-operated mice. This data indicates that modification in dietary PUFA and LCPUFA could potentially slow or prevent bone loss resulting from estrogen deficiency reducing the risk or slowing of the progression of osteoporosis.

2.3.3 The effects of dietary polyunsaturated fatty acids on bone in humans

Very little research has been done to investigate the effects of dietary PUFA and LCPUFA on bone in humans. Total dietary PUFA intake has been related to measurements of bone mass in humans. In young girls (11-17 years of age), total PUFA intake was positively associated with the change in BMD of ultradistal forearm (representing trabecular bone), but not in distal forearm over 1 year (Gunnes and Lehmann, 1996). In boys, associations were not seen between PUFA intake and the

change in ultradistal or distal forearm BMD (Gunnes and Lehmann, 1996). In women (aged 45-55 at baseline), total PUFA intake was associated with femoral BMD loss over a 5 - 7 year period (MacDonald et al, 2004). It is important to note that the researchers looked at total PUFA intake and not the specific classes (n-6 versus n-3), which could explain why the results of these studies conflict.

Only two epidemiological studies have investigated the relationship between dietary n-6 and n-3 PUFA and LCPUFA separately on bone (summarized in **Table 2.3.1**). Only one large epidemiological study has focused on the association between ratios of PUFA and BMD in both men and women (Weiss et al, 2005). The researchers found that the ratio of LA:ALA was negatively associated with BMD of the hip in men and women (with and without hormone replacement therapy) following adjustment for age and other variables (body mass index, Ca intake, exercise, smoking, alcohol intake, use of thiazides and use of thyroid hormones). They also found that an increasing n-6:n-3 ratio was significantly associated with lower BMD in the hip of all women and the spine in women not receiving hormone replacement therapy. In men, hip BMD was negatively associated with total n-6:n-3 PUFA when adjusted for age but not for other variables. Terano (2001) compared the BMD of men and women (38 to 80 years of age) from a fishing village in Japan to urban matched controls and found that the women living in the fishing village (who consumed higher amounts of n-3 LCPUFA) had higher radial BMD. The researchers saw no differences between the two groups of men.

Very few studies have investigated the effects of supplemented dietary n-6 and n-3 PUFA and LCPUFA on bone (summarized in **Table 2.3.2**). In preterm infants, formula containing 0.42:0.26 g/100 g of fat as AA:DHA until hospital discharge and 0.42:0.15

2.3.4 Epidemiological studies investigating the effects of n-6 and n-3 polyunsaturated fatty acids on bone in humans.

Reference	Model and Sample Size	Age	Study Design	Length of Study	Results
Terano, 2001	Men and women n=132	38-80 yr	132 residents from a fishing village age-matched to 332 controls living in an urban centre	NA	• fishing village residents had higher BMD of the proximal and distal radium • serum EPA and DHA proportions were higher in fishing village residents reflecting a daily intake of fish
Weiss et al, 2005	Men and women n=1532	40 to 90 yr	642 men, 564 women not on HRT, 326 women on HRT	4 yr	• when adjusted for age, total n-6:n-3 PUFA was negatively associated with BMD of the hip in men, once adjusted for BMI and lifestyle factors this relationship was lost • when adjusted for age, BMI and lifestyle factors total n-6:n-3 PUFA ratio was negatively associated with BMD of the spine in women not receiving HRT and hip for both Gs of women • ratio of LA:ALA was positively associated with BMD of the hip in men and all women after adjusting for age, BMI and lifestyle factors • mean total n-6:n-3 ratio for men was 8.4 and for women was 7.9 • the mean ratio for LA:ALA for men was 10:0 and for women it was 9.4

Abbreviations: ALA: a-linolenic acid, BMD: bone mineral density, DHA: docosahexaenoic acid, EPA: eicosapentaenoic acid, HRT: hormone replacement therapy, LA: linoleic acid, NA: not applicable

Table 2.3.5 The effects of alterations in dietary polyunsaturated fatty acids on bone in humans.

Reference	Model and Sample Size	Age	Fatty Acid Supplementation (% wt/wt of total dietary fat or n-6/n-3 ratio)	Length of Study	Results
Kruger et al, 1998	Women n=65	Mean age of 79.5 y	G1 - GLA plus EPA (10:1 ratio) and Ca (600 mg) G2 - coconut capsules and Ca (600 mg)	2 phases, 18 m each	After the first 18 months: • OC and Dpd ↓ in both Gs and BSAP ↑ • BMD of the lumbar spine BMD ↓ 3.2% in the placebo and remained the same in the GLA plus EPA G • BMD of the femur ↓ by 2.1% in the placebo G and ↑ by 1.3% in the GLA plus EPA G After the second 18 months all patients received GLA plus EPA: • those who continued on GLA + EPA treatment had a 3.1% ↑ in BMD in the lumbar spine, while those that switched to treatment had a 2.3% ↑ in BMD • those who continued the treatment had no change in BMD in the femur but those that switched had a ↑ of 4.7%
Terano, 2001	Hyper-lipid-emic women n=33	Mean age of 58 y	G1 - 1.8 g of EPA plus HMGCoA reductase inhibitors G2 - HMGCoA reductase inhibitors	1 yr	• EPA ↑ BMD (measured by ultrasound)
Bassey et al, 2000	Pre- and post-m women n=85	25-40 y for pre-m, 50-65 y for post- m	G1 - Efcal (4 g EPO, 440 mg of FO, 1 g of Ca) G2 - 1.0 g of Ca	1 yr	• Efcal did not alter BMD, urinary NTx, urinary HP, serum OC or serum BSAP
Groh-Wargo et al, 2005	Preterm infants n=60	<33 wk gesta-tional age (750 – 1800 g)	G1 - control G2 - AA plus DHA from fungal/FO G3 - AA plus DHA from FO/egg 0.42:0.26 AA:DHA until discharge 0.42:0.16 AA:DHA from discharge to end of study Breast feeding was up to the mother	Until 1 yr gestational-corrected age	• no differences in whole body BMC or BMD

Abbreviations: BMC: bone mineral content, BMD: bone mineral density, BSAP: bone specific alkaline phosphatase, Ca: Calcium, Dpd: deoxypyridinolines, DHA: docosahexaenoic acid, EPA: eicosapentaenoic acid, FO: fish oil, GLA: gamma-linolenic acid, G: group, HP: hydroxyproline, NTx: N-telopeptides of type 1 collagen cross-links

g/100 g of fat as AA:DHA up to 1 year of gestation-corrected age did not alter whole body BMC or BMD (Groh-Wargo et al, 2005). Also in preterm infants, supplementation of AA and DHA had no effect on mineral balance of Ca, P, Mg and Zn (Martinez et al, 2002). Postmenopausal women with low bone mass randomized to a 6 g of oil mixture containing primrose oil (high in GLA) and fish oil (high in EPA and DHA) had higher bone mass compared to those receiving a placebo (Kruger et al, 1998). Following 18 months, subjects receiving the placebo lost 3.2 % of bone mass of the lumbar spine and 2.1% of the femoral neck, while those receiving the oil mixture maintained bone mass of the lumbar spine and had a 1.3% increase in the femoral neck. There was also a significant reduction in turnover (decreased plasma OC and urinary Dpd). For all subjects receiving treatment for 18 m, lumbar spine increased - 3.1% in those on the treatment from the beginning and 2.3% in those switching from placebo to active treatment. In the femur, subjects receiving the treatment from the beginning of study saw no changes, while, those who switched from the placebo to active treatment, femoral BMD increased by 4.1 %. The CV for DPX measurement of the spine BMD is 0.9% and femoral neck is 1.6% (based on mean values of 44 independent researchers). Hyperlipidemic females randomized to receive 1.8 g of EPA with HMGCoA reductase inhibitors had higher BMD and elasticity (measured by the transmission index of ultrasound) than those given only HMGCoA reductase inhibitors (Terano, 2001). In contrast, Bassey et al (2000) failed to show an effect of Efacal ® (4 g primrose oil, 1 g Ca and 440 mg fish oil per d) achieving a total n-6:n-3 ratio of approximately 10:1 versus placebo on whole body BMD or markers of bone turnover in pre- and postmenopausal women. From this research, it is reasonable to conclude that both the dietary ratio of n-6 to n-3 PUFA and the particular n-

6 and n-3 PUFA influence bone metabolism, and that future research on both PUFA is required to fully understand the effect of dietary n-6 and n-3 PUFA on bone. More randomized supplementation trials are needed to determine the amount of n-6 and n-3 PUFA and LCPUFA required to optimize bone mass during growth and slow bone loss later in life.

In studies conducted on humans, the effects of dietary PUFA and LCPUFA on bone do not investigate changes in Ca metabolism. In patients with kidney stones and hypercalciuria, diets rich in fish oil reduce the urinary excretion of Ca (Buck et al, 1991; Baggio et al, 1996; Baggio et al, 2000). It would be useful to determine how these changes in Ca metabolism affect bone mass and future studies need to include measurements in Ca metabolism to help explain changes seen in bone.

2.3.4 Animal models

There are various animal models that can be used for determining the effects of dietary treatments on bone during growth and maturity. The closest model to humans is the nonhuman primate, however, they are very expensive and their use is restricted. Following is a discussion of two animal models used in this thesis, piglets and rats, highlighting why they were chosen for this research. It is important to note that it remains to be determined if there are major species differences among piglets, rats and humans in n-6 and n-3 PUFA metabolism and which animal model best represents what takes place in humans (Innis et al, 1999).

Piglet model

The early-weaned piglet is an excellent model for the human neonate. Advantages in using the piglet model in infant nutrition studies include: highly adaptive to metabolic

cages, can be weaned at birth and reared artificially, large litter size, readily available and inexpensive (Moughan and Rowan, 1989). Also, piglets can be fed by hand with ease, normally suckle only 20-25 d and during this relatively short period of time increase body weight by ~ 500% (Innis, 1993). The piglet provides ample tissue and tissue growth to allow for analyses of the effects of diet on the FA composition of tissues (Innis, 1993). Much is known about the nutrition and growth in the pig (Moughan and Rowan, 1989) and nutritional requirements are very similar between piglets and humans in infancy and growth (Miller and Ullrey, 1987). The piglet has similar development of the intestine, fat digestion and absorption, similar pathways of lipid and lipoprotein metabolism, similar essential fatty acid (LA and ALA) requirements and natural milk fatty acid composition (Reviewed by Innis, 1993). Research has shown that LA and ALA are elongated and desaturated by the same enzyme system as in humans and that Δ-6 desaturase is the rate limiting step (Innis et al, 1999). It has, however, been shown that the piglet has a higher rate of LA and ALA conversion to their respective LCPUFA (Reviewed by Innis et al, 1999). This is most likely due to the higher growth rate of piglets as compared to human infants. As indicated by the fact that at 25 days old the piglet increases its body weight by approximately 500% (Innis, 1993) and by 3 weeks old the piglet is representative of a 3 month old infant (Moughan and Rowan, 1989).

Fan beam-DXA has been validated for use to measure body composition of piglets. Koo et al (1995) found that measurements predicted the scale weight of the piglets and chemical body composition with a high degree of accuracy and precision. The precise accuracy of the fan beam-DXA technique was supported by the extremely high adjusted r^2 in the relation between DXA measurements with the respective scale weights

and chemical component weights from carcass analysis. The precision was supported by extremely high reliability coefficients and low residuals. DXA bone mass measurement as BMC and BMD was validated against both carcass ash and Ca content. Also, the fan beam-DXA can be used to predict soft tissue content, specifically lean and fat mass. Thus, the piglet is well suited for investigation of the early life benefits of dietary treatments, including PUFA, on bone mass during rapid growth.

Rat Model

Animal models are commonly used in aging research because they allow researchers to obtain data that is difficult or impossible to obtain from humans (Sprott, 1997). Some studies that can, in principle, be done in humans are much easier to do in animals because of their shorter life spans (Sprott, 1997). For example, measurements of changes over entire life spans, which would take almost a century to complete in humans based on current life expectancy, can be accomplished in rodents in 2 to 3 years (Sprott, 1997). Rats provide indispensable tools for the study of mammalian aging and the development of methods for ameliorating aging-related diseases in humans (Sprott, 1997). Rats are widely used as an animal model for bone research (Kalu, 1991; Frost and Jee, 1992; Vanderscheuren et al, 1992; Jee and Yao, 2001; Ke et al, 2001). The Food and Drug Administration guidelines have indicated a need for rat experimentation in the evaluation of any agent used in the treatment or prevention of osteoporosis (Thompson et al, 1995). Rats are good models for skeletal research because the same mechanisms control gains in bone mass and losses in young and aged rats as in humans (Frost and Jee, 1992). Multiple studies investigate the effects of dietary n-6 and n-3 PUFA and LCPUFA on bone in the rat model (Claassen et al, 1995a; Claassen et al, 1995b;Watkins et al,

2000; Kelly et al 2003; Watkins et al, 2003; Green et al, 2004). It has been shown that LA and ALA are converted to their respective LCPUFA by elongation and desaturation and that, as in humans, the rate limiting step is the Δ-6 desaturase enzyme in rats (Bernet and Sprecher, 1975). The rat model has been used to determine n-6 and n-3 PUFA (LA and ALA) conversion to their respective LCPUFA in multiple tissues (brain, liver, kidney and heart) using multiple techniques including stable and radio isotopes (Innis et al, 1999). Thus, overall the rat is a good model for the combined investigation of bone and PUFA metabolism.

Significant correlations have been demonstrated in rats between percent ash from chemical analysis and percent BMC by pencil beam DXA (Casez et al, 1994; Lu et al, 1994; Jebb et al, 1996). Makan et al (1997) demonstrated that fan beam based DXA technology can be used to provide reproducible measurements of BMC, lean body mass and fat mass in rats and that results similar to older pencil beam systems can be obtained. Lean body mass and fat mass results are accurate throughout the weight range of animals studied. The researchers found that in smaller animals (<270 g) BMC was underestimated. With BMC there is a size-dependent error, which needs to be considered, especially when longitudinal measurements of bone mass in growing animals are contemplated. Because DXA does not require killing the rat, longitudinal assessment of body composition is possible (Rose et al, 1998), which can be very valuable in determining changes in body composition in response to treatment over the lifespan.

2.4 References

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CHAPTER 3.

RATIONALE

3.1 Rationale introduction

In 2002, the Clinical Practice Guidelines for the Diagnosis and Management of Osteoporosis in Canada stated there was no good-quality evidence to support or refute the benefits of essential fatty acids on bone mass or fracture risk (Brown and Josse, 2002). GLA, AA, EPA, and DHA and/or modifications of the total n-6:n-3 ratios have been shown to affect various bone processes (modelling, remodelling and mineralization) and bone characteristics (including size, mass, mineral content and strength). These effects have been seen across the life span in animal models representing infancy (piglets, chicks), growth (rats, rabbits) and postmenopause (rats, mice). Although limited, human research supports a role for n-6 and n-3 PUFA and LCPUFA in bone mass during adulthood. This research has improved our understanding of the roles that n-6 and n-3 PUFA play in bone health. However, on the basis of the studies, the amount and type of PUFA required to maximize the attainment of peak bone mass and maintain bone mass thereafter is unknown.

3.2 Gaps in the current state of knowledge

3.2.1 Effects of dietary polyunsaturated fatty acids on bone mass early in life: infancy and growth

The effects of n-6 and n-3 PUFA and LCPUFA on bone mass and mineralization are not fully elucidated. Differences in types and amounts of n-6 and n-3 PUFA and LCPUFA, as well as the use of different animal models, measurements techniques and life stages make studies of the effects of PUFA and LCPUFA on bone difficult to compare. Changes seen in bone in response to dietary n-6 and n-3 PUFA and LCPUFA modifications are not fully understood or explained through changes in Ca metabolism,

bone metabolism or PGE_2 concentrations. Differences among studies may be due to the type of PUFA and LCPUFA supplemented, amount supplemented or the different total n-6:n-3 ratios used. Research is needed to clarify the amount of n-6 and n-3 PUFA, including LCPUFA, required to optimize bone mass and understand the mechanism(s) of their effects.

3.2.2 Effects of dietary polyunsaturated fatty acids on bone mass beyond infancy: programming

Alterations in dietary n-6 and n-3 FA can improve bone mass and increase bone Ca content during infancy and growth. These improvements could potentially result in the attainment of higher peak bone mass. At the age of 70, half the variance in bone mass is accounted for by peak bone mass (Hui et al, 1990) and the amount of bone accrued early in life has an important effect on fracture risk in later life (Harvey and Cooper, 2004). It is unknown whether improvements gained during infancy and periods of growth would continue after supplementation has stopped due to sustained alterations in metabolism. Alterations in Ca metabolism and bone cell metabolism are potential mechanisms responsible for changes seen in bone in response to n-6 and n-3 FA. Over the past 15 years, research has indicated that experiences in early life can have a long-term impact on health (Kuh and Davie-Smith, 1993). The focus has been on the relationship between early growth and chronic disease in adulthood. This area of research is inspired by the work of Barker (Moore and Davies, 2001). Barker proposed a "fetal origins theory of adult disease", where alterations in fetal nutrition and endocrine status result in developmental adaptations that permanently change structure, physiology and metabolism predisposing individuals to disease in adult life (cardiovascular, metabolic

and endocrine) (Barker, 1995). This is due to programming or the process whereby a stimulus or insult at a sensitive or critical period of development has long-term effects (Lucas, 1991). Early-life programming research has also investigated the relationships between factors in early infancy and risk of disease later in life, e.g. programming effects of childhood growth on future risk of chronic disease (Eriksson et al, 1999; Forsen et al, 1999). No research has been conducted investigating the modification of dietary PUFA or LCPUFA during infancy or periods of growth and subsequent risk of osteoporosis. Programming effects on Ca metabolism or bone metabolism could result in sustained improvements in bone mass once the dietary treatment has ceased compared to those who did not receive dietary treatment. Previous research has shown that early life programming pertains to bone. Weight in infancy (Cooper et al, 1997; Duppe et al, 1997; Fall et al, 1998) is related to bone mass in adulthood; and rate of growth throughout childhood is linked to risk of hip fracture (Cooper et al, 2001). Research is needed to understand environmental determinants (including nutrition) on growth, peak bone mass, bone loss and risk of osteoporosis. Following animals after the supplementation of dietary n-6 and n-3 FA would identify whether early introduction results in alterations in the risk of osteoporosis due to programming effects on bone or Ca metabolism.

3.2.3 Healthy aging

Despite the immense potential of PUFA or LCPUFA to program and/or increase bone mass attained early in life, the potential for PUFA and LCPUFA to improve bone at other ages is also very high. Very little research has been conducted investigating the effects of dietary PUFA and LCPUFA during adulthood following the attainment of peak bone mass before menopause. This is the period in the lifespan when bone mass is

maintained through remodelling (resorption = formation) and eventually starts to become compromised with age as resorption exceeds the capacity for formation. Potentially increasing bone mass or slowing/preventing the onset of bone loss at this period of time could reduce the risk of osteoporosis.

3.2.4 Changing requirements across the lifespan

It is important to determine when specific LCPUFA should be supplemented during the lifespan and how much. As well as, it is necessary to determine whether the requirements or effects of AA, EPA and DHA change over the lifespan. Current DRI recommendations for n-6 and n-3 PUFA are based on the median intake in that age range and energy expenditure and thus suggest a lower recommendation for n-6 PUFA for those over 50 years of age. Whether n-6 and n-3 PUFA requirements decrease with age and LCPUFA increase is unknown. In addition, there are no DRI recommendations for LCPUFA, but research suggests that n-6 and n-3 LCPUFA could potentially reduce the risk of chronic diseases. Research is needed to determine the optimal amount of n-6 and n-3 LCPUFA required at different stages across the life span to optimize bone health and reduce the risk of osteoporosis.

3.2.5 Amounts of n-6 and n-3 polyunsaturated fatty acids used in research

Only studies involving neonatal piglets have investigated the effects of current LCPUFA recommendations and intakes on bone. These amounts ranged from 0.3 to 0.8 g/100 g of fat as AA with 0.1 g/100 g of fat as DHA and had total AA:DHA ratios ranging from 3:1 to 8:1 (Weiler, 2000; Lucia et al, 2003; Blanaru et al, 2004). The amounts of AA and DHA vary in breast milk. In the U.S., the average amount of AA is 0.54 g/100 g of fat and the average amount of DHA is 0.17 g of fat (Hibbeln, 2002). In

Canada, it is reported as slightly different, AA as 0.57 g/100 g of fat and DHA as 0.35 g/100 of fat (Hibbeln, 2002). While the amounts currently added to infant formula in North America are 0.40 to 0.64 g/100 g of fat as AA and 0.15 to 0.32 g/100 g of fat as DHA (ratios of AA:DHA of 2:1 to 2.7:1). The amounts of n-3 LCPUFA used in previous research in chick, rat and mice research were much higher than current intakes and recommendations at any stage in the lifespan. As an example, a research study conducted in chicks used diets containing either no EPA or DHA or 8.66 g/100 g of fat as EPA and 5.31 g/100 g of fat as DHA (Watkins et al, 1996). To obtain amounts used in previous research, specific oils such as fish oil would need to be supplemented into the diet.

To date, no study has investigated the range of current recommendations of EPA on bone mass following infancy. There are no recommendations for AA past infancy; whether AA is required for bone growth and mineralization during childhood or bone mass maintenance has not been investigated. There is a need for research investigating the effects of LCPUFA consumption reflective of current intakes and recommendations on bone mass and quality.

3.2.6 Gender differences in response to dietary n-6 and n-3 polyunsaturated fatty acids

The effects of dietary PUFA and LCPUFA in studies over the lifespan are not conducted equally in males and females; studies conducted during specific periods of life focus on only one gender. During periods of growth, investigations are mainly conducted in males. Studies investigating the prevention of bone loss are conducted in ovariectomized female animals representing a postmenopausal state or in postmenopausal women. Research has not been conducted on the prevention of bone loss in males by

modification of n-6 and n-3 PUFA and LCPUFA. Research should be conducted in both genders at every life stage to determine whether they respond differently to treatment.

3.3 Objectives and hypotheses

3.3.1 The effects of dietary arachidonic acid and docosahexaenoic acid on bone during infancy

The research conducted in piglets investigated the effects of increasing amounts of AA:DHA (0.5:0.1, 1.0:0.2 and 2.0:0.4 g/100 g of fat) maintained at a ratio of 5:1 on bone mass, bone metabolism, Ca absorption, bone composition and mineral excretion. These results will help determine the optimal amount of AA and DHA during the neonatal period by incorporating higher amounts, while maintaining a constant ratio. In previous piglet research, a range of AA amounts (0.3 to 0.8 g/100 g of fat) and a range of AA:DHA ratios (3:1 to 8:1) have been studied in infant piglets. The amount of AA was chosen based on the amount of AA found in sow and human milk. The amount of DHA was also chosen based on amounts found in both breast milk and sows milk, but also to test the effects of different AA:DHA ratios on bone. The optimal ratio of AA:DHA based on research in piglets is between 5:1 and 7.5:1 for improving bone mass. This research will determine whether higher amounts of AA and DHA are beneficial while maintaining a constant AA:DHA ratio of 5:1. This research also increases the understanding of how AA and DHA affect bone metabolism and whether higher amounts will alter Ca absorption and/or retention. One piglet study was designed to address the following objective and hypotheses.

Objectives:

To determine:

1. the amount of dietary AA plus DHA (0.5:0.1, 1.0:0.2, 2.0:0.4 g/100 g of fat) that influences bone formation, resorption and bone mass (addressed in Chapter 4)
2. the effect(s) of physiological amounts of dietary AA plus DHA on bone PGE_2 and plasma IGF-I concentrations (addressed in Chapter 4)
3. the amount of dietary AA plus DHA (0.5:0.1,1.0:0.2,2.0:0.4 g/100 g of fat) required to elevate Ca absorption and mineral retention (addressed in Chapter 5)
4. the amount of AA plus DHA required to increase the bone mineral content of specific minerals (addressed in Chapter 5)
5. the circadian rhythm of bone metabolism in response to dietary AA and DHA (addressed in Chapter 6)
6. the relationship between plasma PUFA and LCPUFA proportions and biomarkers of bone metabolism (addressed in Chapter 6)

Alternate Hypotheses

- there will be a dose response on bone mass and bone cell metabolism to dietary AA and DHA supplementation over the ranges studied
- dietary AA and DHA will alter bone mass and metabolism via PGE_2 and IGF-1

- AA and DHA supplementation will increase intestinal Ca absorption and mineral retention when fed at the higher amounts of 1.0:0.2 and 2.0:0.4 g/100 g of fat
- AA and DHA supplementation will lead to changes in bone composition of specific bone minerals consistent with increases in BMD
- alterations in the circadian rhythm of bone metabolism will better describe the responses to LCPUFA and the mechanisms behind the responses than single-end points
- circulating plasma PUFA and LCPUFA proportions will be related biochemical markers of bone metabolism, which will better explain the effects of dietary LCPUFA on bone mass

3.3.2 The role of long chain polyunsaturated acids on bone mass beyond infancy

The research conducted in rats investigated the effects of LCPUFA throughout the lifespan - specifically AA, DHA and EPA - to determine how these individual LCPUFA affect bone metabolism and bone mass within the same animal model and in the same amounts over a long period of time in both males and females. This study began at weaning to compare to previous research in growing (weanling) animals and to determine whether changes sustained during early life remain once supplementation stops (i.e. programming). The results will also help identify the requirements of specific LCPUFA across the lifespan and whether they change with age. Following the animals until 49 wk of age allows the determination of whether specific LCPUFA slow the loss of bone that accompanies age. Female rats begin to loss bone mass at approximately 9 mo of age (Ji et al, 1991). This research will also identify whether requirements and actions of LCPUFA

differ between males and females at different stages of life. Animals were fed AA, EPA and DHA during one of three 15-wk phases (early, mid and late) or continuously throughout life. When they were not receiving a supplemented diet, they were fed control. One rat study was designed to address the following objectives and hypotheses (addressed in Chapter 7).

Objectives:

To determine:

1. the type of LCPUFA that elevate bone mineral, growth and size at different life stages
2. what stage of life LCPUFA have the greatest impact on bone by studying early, mid and late introduction
3. the effects of dietary LCPUFA in amounts similar to current intakes and recommendations for prevention of osteoporosis
4. whether benefits obtained during early and mid inclusion of LCPUFA are sustained once dietary LCPUFA treatment has ceased
5. whether males and females respond differently to dietary LCPUFA with respect to bone mass, bone growth and mineral metabolism
6. whether LCPUFA requirements change among life stages

Alternate Hypotheses

- supplementation of AA versus EPA at different time points throughout the lifespan will result in different effects on bone mass and mineralization
- dietary AA will be beneficial during growth by increasing bone mass

- dietary EPA will be beneficial following the attainment of peak bone mass by reducing bone resorption and urinary Ca excretion
- males and females would respond differently to LCPUFA supplementation

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CHAPTER 4.

LOW LEVELS OF DIETARY ARACHIDONIC ACID AND DOCOSAHEXAENOIC ACID IMPROVE BONE MASS IN NEONATAL PIGLETS, WHILE HIGHER LEVELS PROVIDE NO BENEFIT

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Running Title: Long-Chain Polyunsaturated Fatty Acids and Bone Mass

Low Levels of Dietary Arachidonic Acid and Docosahexaenoic Acid Improve Bone Mass in Neonatal Piglets, But Higher Levels Provide No Benefit[*^]

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Key Words: Bone mass, long-chain polyunsaturated fatty acids, piglets, prostaglandin E_2, bone modeling

4.1 Abstract

In piglets, feeding arachidonic acid (AA) and docosahexaenoic acid (DHA) in a 5:1 ratio leads to elevated bone mass, but the optimal total quantity requires clarification. We studied bone mass and modeling of piglets that were randomized to receive 1 of 4 formulas for 15 days: control or with AA:DHA (0.5:0.1 g, 1.0:0.2 g or 2.0:0.4 g AA:DHA/100 g of fat). Measurements included: bone area (BA), mineral content (BMC) and density (BMD) of whole body, lumbar spine and excised femurs; biomarkers of bone modeling were plasma osteocalcin and urinary N-telopeptide (NTx); tibial *ex vivo* release of prostaglandin E_2 (PGE_2); plasma insulin-like growth factor-1 (IGF-1); and tissue fatty acids. Main effects were identified using ANOVA and post hoc Bonferroni t-tests. In supplemented piglets, relationships among liver fatty acid proportions and bone mass were detected using Pearson correlations. Whole body (P=0.028) and lumbar spine (P=0.043) BMD were higher in the group supplemented with 0.5:0.1 g AA:DHA/100 g of fat. Tissue AA and DHA increased in proportion to diet. Liver EPA positively correlated ($r= 0.38$, $P\leq 0.05$) with whole body and femur BMC and BMD and lumbar spine BMC. Liver AA:EPA ratio negatively correlated ($r=- 0.039$, $P\leq 0.05$) with whole body, femur and lumbar spine BMC plus whole body and femur BMD. Dietary 1.0:0.2 g AA:DHA/100 g reduced NTx relative to 2.0:0.4 g AA:DHA/100 g of fat (P=0.039). No other dietary effects were observed in the biochemistry. Supplemental AA:DHA (0.5:0.1 g/100 g of fat) elevate bone mass, but higher amounts are not beneficial.

4.2 Introduction

At present the majority of infant formula companies throughout the world add arachidonic acid (AA) and docosahexaenoic acid (DHA) to at least one of their product lines even though they are not considered essential fatty acids. The basis for this addition is: 1) the potential inadequate synthesis of AA and DHA relative to requirements of the neonate (Carlson, 1997; Bowen et al, 1999; Innis, 2000) and 2) potential benefits to retinal and cognitive development (San Giovanni et al, 2000a; San Giovanni et al, 2000b; Simmer, 2000a; Simmer, 2000b). In previous years, our research group has investigated the effects of these long-chain polyunsaturated fatty acids (LCPUFA) on bone mass and observed higher values with dietary supplementation of AA and DHA in a n-6:n-3 ratio of 5:1 to 7.5:1 (Weiler, 2000; Blanaru et al, 2004;Wieler and Fitzpatrick-Wong, 2004).

A number of other research groups also report that LCPUFA influence bone mass in various animal models (Watkins et al, 1997; Judex et al, 2000; Watkins et al, 2000). Part of the mechanism may involve modification of bone tissue LCPUFA concentrations, as demonstrated in chicks and rats (Watkins et al, 1997; Watkins et al, 1996), and the resulting changes in eicosanoids (Watkins et al, 1996). Although various eicosanoids act on bone, the prostaglandins (PG) seem to be the principle mediators of bone cell function (Watkins et al, 2001). Prostaglandin E_2 (PGE_2) is the principal PG affecting bone metabolism (Watkins et al, 2001). PGE_2 exhibits biphasic effects on bone – stimulating formation at low concentrations but inhibiting formation at high concentrations (Raisz and Fall, 1990). The anabolic effect of PGE_2 may occur through stimulation of insulin-growth factor-1 (IGF-1) production in bone or by increased responsiveness to IGF-1 in bone (Baylink et al, 1993; Raisz et al, 1993). Another PG in bone, PGE_3, is synthesised

from eicosapentaenoic acid (EPA, 20:5n-3). The amounts of the specific precursors and PG required for optimal bone growth and mineralization is currently unknown. Very few studies have investigated the potential for AA and DHA to influence bone cell function and modeling during periods of rapid growth and no reports exist for human infants.

Supplementation of AA plus DHA (0.5:0.1 g/100 g of fat as AA:DHA) with a total n-6:n-3 ratio of 5:1 was associated with higher total body and regional bone mineral density (BMD) in neonatal piglets (Weiler, 2000). Subsequently, supplementation of AA and DHA in the same proportions, but with a total ratio of 9:1, in neonatal piglets had significantly elevated bone mineral content (BMC) in femur compared to control formula (Weiler and Fitzpatrick-Wong, 2004). Feeding 0.6 and 0.75 g/100 g of fat as AA with a constant amount of DHA (0.1 g/100 g of fat) elevated BMC in piglets compared to those fed lower amounts of AA (0.3 and 0.45 g/100 g of fat) (Blanaru et al, 2004).

Regarding the mechanism(s) responsible for increases in bone mass due to supplementation of AA and DHA (0.5:0.1 g/100 g of fat), only one of our studies has been shown to elevate bone PGE_2 concentrations relative to control, but this only applied to piglets treated with glucocorticoids (Weiler and Fitzpatrick-Wong, 2004). Likewise, in piglets fed AA and DHA, plasma IGF-1 was not altered (Blanaru et al, 2004). In chicks, IGF-1 is elevated after consuming 14 d of a fish-oil-based diet, but bone formation rate was unaffected relative to a soybean-oil-based diet (Watkins et al, 1997). In rats fed a fish-oil-based diet, bone formation rate is negatively associated with PGE_2 (Watkins et al, 2000). In addition to changes in PGE_2 or IGF-1 with dietary LCPUFA in rats, bone resorption is reduced in piglets by dietary LCPUFA (AA at 0.8:0.1 g/100 g of fat) (Lucia

et al, 2003). Thus the mechanisms responsible for the LCPUFA-induced elevations in bone mass require further clarification.

Ratios of AA:DHA (0.5-0.75:0.1) and total n-6:n-3 ≤ 9:1 support normal growth and elevate bone mass during periods of rapid growth. These ratios are similar to those used by Watkins et al (2000, 1996) and Claassen et al (1995a). For example, in growing rats supplementation of n-6/n-3 fatty acids as gamma-linolenic acid (GLA, 18:3n-6) and EPA plus DHA, in ratios of 3:1 also reduced bone resorption and elevated bone calcium content (Claassen et al, 1995a). However, the optimal amount of specific LCPUFA, including AA and DHA is still unknown since the amounts used in our piglet studies have always been less that 1 g/100 g of fat and are very low in contrast to studies in chicks and rats (Watkins et al, 2000; Watkins et al, 1996; Claassen et al, 1995). Watkins et al (1996, 1997, 2000) supplemented fish oil at 35-50 g/ kg of diet in chicks and rats. Claassen et al (1995a) altered the n-6:n-3 ratio by supplementing rats with varying amounts of GLA and EPA plus DHA. The amount of GLA ranged from 5.2 to 7.4 g/100 g, EPA ranged from 1.8 to 9.2 g/100 g and DHA ranged from 0.7 to 3.2 g/100 g. The ratios of AA:DHA that best support bone in neonatal piglets in studies conducted in our laboratory ranged from 5:1 to 8:1, but whether this applies when higher amounts are supplemented in the same ratio is unclear. A ratio of AA:DHA of 5:1 (Weiler, 2000; Weiler and Fitzpatrick-Wong, 2002) and a total n-6:n-3 ratio of 9:1 (Weiler and Fitzpatrick-Wong, 2002; Blanaru et al, 2004) were chosen for this study since two previous studies also used this ratio and demonstrated higher bone mass over 15 d in piglets. The objectives of this study were to determine the amount of dietary AA plus

DHA that best influences bone mass and biomarkers of formation and resorption plus bone PGE_2 and plasma IGF-1 concentrations.

This study was conducted in the piglet in order to build upon previous work from our laboratory. In addition, the study is designed to test infant formula and the piglet normally suckles over as little as 20-25 d (Miller and Ullrey, 1987). Piglets respond to lipid nutrition in a similar manner as human infants (Innis, 1993), have similar nutrient requirements to human term born infants (Miller and Ullrey, 1987) and have been used to validate the software used to measure infant bone mass (Brunton et al, 1997).

4.3 Materials and methods

4.3.1 Animals and diet

Male piglets (n=36), born at The Glenlea Swine Research Unit, University of Manitoba were transported to the housing facility at the main campus of the University. The mean birth weight (± SD) of piglets born at this institution is 1.6 ± 0.2 kg. Animal care and procedures were reviewed and approved by the University of Manitoba Committee on Animal Use and were within the guidelines of the Canadian Council of Animal Care (1993). Piglets were selected with a birth weight of ≥ 1.4 kg, from 10 litters consisting of 10-12 piglets each with at least 4 males. Piglets arrived on day 3 of life and were taught to lap liquid formula. Piglets were fed by a combination of gavage feeding, plus lapping of formula to ensure enough formula was consumed to continue growth for 2 d of adaptation. The strength of control formula was progressively increased from half strength on d 3 to full strength by the end of d 4 of life, followed by experimental or control formula on d 5. Piglets were housed individually in stainless steel cages and room temperature was maintained at 29-30 °C. Based on 09:00 h weight, the piglets were offered 350 mL/kg of liquid formula per d. This amount was divided into 3 equal portions provided at 09:00, 15:00 and 21:00 for 15 d (from 5 to 21 d of life) as per Weiler and Fitzpatrick-Wong (2002). Food intake was monitored at each feeding, but formula was readily consumed without waste.

Piglets were randomized to receive one of four dietary treatments (n=9 per group). Treatments were standard formula (control) containing no AA:DHA or the same formula but made with oil containing with 0.5:0.1 g AA:DHA, 1:0.2 g AA:DHA or 2:0.4 g AA:DHA/100 g of fat (**Table 4.8.1**). Supplementation was held at a constant n-6:n-3

ratio of 5:1 (Table 4.8.1). The AA was provided in the form of RBD-ARASCO® (40.6 g/100 g of fatty acids as AA) and DHA in the form of RDB-DHASCO® (40.0 g/100 g of fatty acids as DHA). AA was derived from a common soil fungi and DHA was derived from a marine microalgae (Martek Biosciences Corp). These sources were chosen because they were previously used in our laboratory and because they are used in the manufacturing of many North American infant formula products. Formulas were isocaloric with equal amounts of fat. The formula was based on nutritional requirements for healthy growing piglets between 3 and 10 kg as set by The National Research Council (1996) and currently proven to support growth in our laboratory (Weiler and Fitzpatrick-Wong, 2002) **(Table 4.8.2)**. Formula contained 1050 kcal/L, 60 g/L fat, 50 g/L protein, 2.1 g/L calcium and 1.4 g/L phosphorous. Piglets were allowed approximately 1 h of exercise before each feed.

4.3.2 Measurements

Growth

Weight (kg) was measured daily at 09:00 h by digital scale (Mettler-Toleto Inc., Highstown, NJ) in the non-fed state from day 1 to day 16 of study with an animal weighing program (mean of 3 weights). Weight gain ($g \cdot kg^{-1} \cdot d^{-1}$) was calculated at the end of study.

Weight gain:

$$\left[\frac{\text{weight in g on d 16} - \text{weight in g on d 1}}{\text{(weight in kg d 1 + d 16)/2}}\right] \text{ /15 d}$$

Length (cm) was determined by measuring from tip of snout to base of tail (does not include the tail) to the nearest cm using a non-stretchable measuring tape while the piglets were anaesthetized on d 16 of study.

Sample collection

Urine was collected over 2 d on d 14 and 15 using metabolic cages and measured as 2-24 h pooled samples. To ensure no sample breakdown, urine was collected between 09:00 to 1500 h, 1500 to 21:00 and 21:00 to 09:00 h the next day. Samples were stored at –20 °C. Blood (5 mL) was taken from all piglets using the internal jugular blind stab technique on day 16 between 09:00 h and 10:00 h to remove circadian rhythm as a variable in the biomarkers of bone. Anticoagulated blood (heparin) was separated into plasma and erythrocyte fractions. Plasma and erythrocytes were obtained by centrifugation at 2000 g for 10 min at 4 °C and stored at –80 °C until analysis of other outcome measurements including erythrocyte fatty acids.

Following blood collection piglets were anaesthetized by I.P. injection of sodium pentobarbital (30 mg/kg, 65 mg/mL concentration) or isofluorane gas then terminated using sodium pentobarbital overdose (180 mg/kg). The liver was excised and weight to the nearest 0.1 g. A section of white adipose was removed from between the shoulders. The liver and adipose tissue were flash frozen in liquid nitrogen prior to storage at –80 °C until analyzed for fatty acids.

Fatty acid analysis

Liver and adipose fatty acids were measured to reflect body stores of AA and DHA in response to diet. Erythrocyte fatty acids were measured to enable future comparison to human neonates where only plasma or erythrocyte fatty acids are feasible

in vivo. Total lipids from tissues were extracted according to an adapted method of Folch et al as previously described (1957). Erythrocytes were extracted in chloroform containing 0.01% butylated hydroxytoluene (BHT) and adipose and liver were extracted in chloroform:methanol 2:1 containing 0.01% BHT. An internal standard, heptadecaenoic acid (C17:0) was added to each erythrocyte and liver sample. Liver and adipose were homogenized. Crude lipid extracts were transmethylated in 1.2 mL of methanolic HCl (3N, Supleco Inc., Bellefonte, PA) at 80 °C for one h. Fatty acid methyl esters were separated by gas-liquid chromatograph (Varian Star 3400, Varian, Mississauga, Ont, Can), equipped with a 30 m capillary column (J&W Scientific, Folsom, U.S.), a flame ionization detector and using hydrogen as the carrier gas. The column is made of fused silica coated with DB225 (25% cyanopylphenyl) and run at 180-220 °C with a between sample temperature of 240 °C to clean the column. The detector oven is set at 300 °C and produces a sensitivity of 1-5 μg/L. Fatty acid methyl esters (C12-24) were identified by comparison with retention times of Supelco 37 component FAME mix (Supelco Inc.) and expressed as percent of total fatty acids (g/100 g of fatty acids).

Bone mass and biochemistry

Small sections of the tibia cortex were excised from the mid-diaphysis (~ 1.0 g) cleaned of marrow and rinsed with 0.9% NaCl prior to bone organ culture as described by Dekel et al (1981). Tibia sections were incubated in 1x Hank's Balanced Salt solution (Sigma, St. Louis, MO, U.S.A.) for 2 h at 39 °C in a shaking water bath, followed by the removal of bone and rapid freezing of solution. This method was chosen as it has been used in chicks (Watkins et al, 1996), rats (Watkins et al, 2000) and piglets (Blanaru et al, 2004) to assess PG metabolism after dietary treatment. Samples were stored at –20 °C

until duplicate analysis of PGE_2 by enzyme linked immunosorbent assay (R&D Systems, Minneapolis, MS, U.S.A.) and corrected to the weight of the tibia segment studied. To minimize interference of the Hank's Balanced Salt solution with the alkaline phosphatase enzyme, standards were reconstituted using this solution as opposed to the buffer provided with the kit. In addition, the kits cross-reacts with PGE_1 (70%) and PGE_3 (16.3%). For PGE_2 the CV% was < 15%.

Following removal of tissues at termination the abdominal cavity was closed with suture to maintain tissue depth. The piglet carcasses were then transported to the dual energy x-ray absorptiometer (DXA) (QDR 11.2, 4500A series, Hologic Inc.) and assessed using infant whole body, lumbar spine and hip subregion software. All scans were performed with the piglet in the anterior-posterior position with limbs extended. Whole body and lumbar spine bone area (BA), BMC and BMD were measured. Whole body and lumbar spine BMC were corrected to length and weight to account for potential size differences. Right femurs were then excised and freed of soft tissue for determination of weight, length, BMC, BA and BMD. For the DXA scan, femurs were placed in a small plastic water bath (tested for interference with the scan accuracy) with 3 cm depth and aligned in an anterior-posterior position. DXA has been shown to be a simple, accurate and precise technique for measuring BMC and BMD in isolated small animal bones (Brunton et al, 1997; Kastle et al, 2002). Only water should be used for determination of BMC and BMD by DXA (Kastle et al, 2002). These DXA measurements are precise with CV % less than 4 % in piglets of a similar size (Weiler, 2000; Weiler and Fitzpatrick-Wong, 2002; Blanaru et al, 2004).

Plasma osteocalcin was measured in duplicate using an I^{125} radioimmunoassay (DiaSorin, Stillwater, M.N., U.S.A.). This assay is based on rabbit antiserum to bovine osteocalcin that has been proven to be a valid approach for measuring porcine osteocalcin (Pointillart et al, 1997). Plasma IGF-1 was measured in duplicate using an enzyme-linked immunosorbent assay (Quantikine, R&D Systems, Minneapolis, U.S.A.). NTx in urine was measured in duplicate by a competitive inhibition enzyme-linked immunosorbent assay (Osteomark, Ostex, Seattle, U.S.A.). Although a human assay, it has been validated for the use in samples from growing piglets (Bollen et al, 1997). Urinary NTx sample values were corrected to creatinine as determined by the Jaffe method (procedure no. 555; Sigma-Aldrich Ltd, Oakville, Canada) to account for urinary dilution. For NTx the CV % was <20% and for osteocalcin and IGF-1 the CV % was < 15%. Creatinine in urine was measured colorimetrically (Sigma, St. Louis, U.S.A.). The CV % for triplicate analysis of creatinine in all samples was <10%.

4.4 Statistical analysis

The estimated sample size required to observe a difference in whole body BMC of 25 ± 13 g (Weiler, 2000) with a power of 0.80 and an α value of 0.05 was calculated to be eight per group. Main effects were detected using two-way ANOVA (diet and litter, general linear model) using SigmaStat statistical software (Jandel Scientific, San Rafael, CA, U.S.A.). Litter was included in the statistical analysis as a main effect since previous research demonstrated that litter has a strong effect on bone parameters (Blanaru et al, 2004). Post-hoc analysis using Bonferroni t-tests was conducted when main effects were detected by the two-way ANOVA. Relationships between variables were detected by Pearson Correlation analysis using GraphPad Prism Version 3.02 software (GraphPad Software, San Diago, CA, U.S.A.). Correlations between PUFA in liver, selected to reflect whole body PUFA status, and bone outcomes were conducted with the control group excluded since it was not a component of the dose-response relationship. A P-value of less than 0.05 was accepted as significant. Data is expressed as mean ± standard error of the mean (SEM).

4.5 Results

At baseline there were no significant differences among the groups in weight (range 2.00 to 2.14 kg). By the end of the feeding trial, weight was not different among the groups, 5.48 to 5.63 kg. Accordingly, there were also no differences among groups in rate of weight gain or end of study length. Rate of weight gain ranged from 58.7 to 62.5 ($g \cdot kg^{-1} \cdot d^{-1}$) and length from 53.7 to 54.2 cm.

There was a significant effect of LCPUFA supplementation on BMD in whole body, (P=0.028) **(Figure 4.9.1a)** and lumbar spine (P=0.043) **(Figure 4.9.1b)**. Post hoc analysis indicated that the group supplemented with 0.5:0.1 g/100 g of fat as AA:DHA had significantly higher whole body (6 %) and lumbar spine (9 %) BMD than control (Figure 1a-b). There was no effect of dietary treatment on BA or BMC of the whole body or lumbar spine **(Table 4.8.3)**. There was a significant treatment effect of LCPUFA supplementation on whole body BMC corrected to final length, however, post hoc analysis did not show significant differences among the groups (Table 2). There was no effect of LCPUFA supplementation on femur BA, BMC (Table 2) or BMD **(Figure 4.9.1c)**. There was also no effect on femur weight or length (data not shown).

To examine the influence of LCPUFA supplementation on bone metabolism, urinary NTx:creatinine (a marker of osteoclast activity) and plasma osteocalcin (a marker of osteoblast activity) were measured. There was a significant effect of LCPUFA supplementation on urinary NTx:creatinine (P=0.039) **(Figure 4.9.2a).** Posthoc analysis showed that the group receiving 1.0:0.2 g/100 g of fat as AA:DHA had significantly lower urinary NTx:creatinine than those receiving 2.0:0.4 g/100 g of fat. Those receiving control formula or 0.5:0.1 g/ 100 g fat had intermediate concentrations of urinary

NTx:creatinine. There were no significant differences in plasma osteocalcin (P=0.077) between the groups **(Figure 4.9.2b).**

Ex vivo release of PGE_2 from bone was measured to determine if the LCPUFA supplementation alters the concentration of PGE_2 in bone. No significant differences were found among the groups (range 13.62 to 15.88 pmol/g bone), although the measurement was highly variable. No significant differences in plasma IGF-1 concentrations were found among the groups, with values ranging from 14.01 to 15.54 nmol/L.

The proportion of erythrocyte **(Table 4.8.4)**, liver **(Table 4.8.5)** and adipose **(Table 4.8.6)** LCPUFA varied according to the amount of AA and DHA in the diet. The control group had the lowest proportion of AA and DHA in all tissues and as the dietary AA and DHA increased the concentration of AA and DHA significantly increased. In erythrocytes the proportion of LA, ALA, EPA, AA:DHA, AA:EPA+DHA and total n-6:n-3 were unchanged; total n-6, total n-3 and AA:EPA increased with supplementation. In liver the proportion of LA and ALA plus total n-6:n-3 were decreased; total n-6, AA:DHA and AA:EPA + DHA did not change; total n-3 and AA:EPA increased with supplementation. Liver EPA proportions were not significantly lower in the group receiving 0.5:0.1 g/100 g of fat as AA:DHA, but were significantly lower in the groups supplemented with larger amounts. Amounts of fatty acids (mg/g) in liver were altered in the same way as proportional values (data not shown). In the adipose, the proportion of total n-3 significantly increased with supplementation of AA:DHA at 1.0:0.2 g and 2.0:0.4 g/100 g of fat. EPA was not detected in adipose tissue. A dietary treatment effect was observed for total n-6 fatty acid proportions in adipose tissue, however, post hoc

analysis did not identify differences among the groups. The rest of the fatty acid proportions were not altered in adipose tissue with supplementation

Correlations were conducted to determine the relationship between liver LA, ALA, EPA, AA and DHA proportions and bone mass measurements (whole body, femur and lumbar spine BMC and BMD) in the piglets supplemented (n=27) with AA and DHA. LA was significantly correlated with whole body BMD (r=0.43, P=0.0236). LA was not correlated with any regional measurements of BMD. ALA was significantly correlated with the lumbar spine BMD (r=0.49, P=0.009). ALA was not correlated with whole body or femur BMD. LA and ALA were not correlated with whole body or regional measurements of BMC. EPA proportions were still significantly correlated with whole body BMC (r=0.58, P=0.0014), whole body BMD (r=0.38, P=0.0494), femur BMC (r=0.61, P=0.0008) and femur BMD (r=0.51, P=0.0062) and lumbar spine BMC (r=0.45, P=0.0186). The AA:EPA ratio was significantly correlated with whole body BMC (r=-0.49, P=0.0098), whole body BMD (r=-0.42, P=0.0311), femur BMC (r=-0.48, P=0.0122), femur BMD (r=-0.39, P=0.0466) and lumbar spine BMC (r=-0.39, P=0.0440).

4.6 Discussion

Since AA and DHA are added to some infant formulas, there is a need to examine the response of growing tissues, including bone to these LCPUFA. This study represents the third in a series (Weiler, 2000; Weiler and Fitzpatrick-Wong, 2002) that used a ratio of 5:1 AA:DHA and confirms that 0.5:0.1 g/100 g fat as AA:DHA enhances bone mass in neonatal piglets. In a similar study, supplementation of AA at 0.60 g and 0.75 g/100 g of fat with 0.1 g DHA/100 g of fat resulted in elevated whole body BMC in piglets (Blanaru et al, 2004). These studies used small amounts of AA and DHA (< 1 g/100 g fat) and the present study suggests that higher proportions of supplementation are not beneficial to neonatal bone. Similarly, dietary n-6:n-3 fatty acid ratios between 1:1 and 3:1 enhance bone mass in weaned rats (Claassen et al, 1995a; Watkins et al, 2000), although the amounts of dietary (n-6) (GLA) and (n-3) (EPA and DHA) fatty acids were much greater (>7 g total/100g of dietary fat). While these studies are quite different in terms of the age of the animals (neonate vs. weanling), the animal models (piglets vs. rats), the type of LCPUFA supplemented, the actual amount of PUFA per kg body weight was similar. For example, the rats in Claassen et al (1995b) ate ~20-22 g food/d and had a final weight of ~350 g. Given the proportion of LCPUFA in the diets, the rats consumed ~ 0.3g GLA/kg, 0.07 g EPA/kg and ~0.02 g DHA/kg in the 3:1 n-6:n-3 group. In our piglet study, the amounts of LCPUFA were 0.11 g AA/kg and 0.02 g DHA/kg throughout the study.

In regard to bone metabolism, the only biomarker that changed significantly was NTx; 1.0:0.2 g/ 100 g of fat as AA:DHA had lower urinary NTx:creatinine values than 2.0:0.4 g/100 g of fat as AA:DHA, while the other two groups had intermediate

concentrations. Alterations in urinary NTx:creatinine did not affect bone mass over the short duration of the study. Similarly, piglets fed 0.8:0.1 g/100 g of fat as AA:DHA had lower urinary NTx:creatinine concentrations without alterations in bone mass (Lucia et al, 2003). It is possible that had the diets been continued for longer and to adulthood that bone mass would reflect resorption. The highest level might be limiting over the longer-term due to elevation of bone resorption while the intermediate diet might enhance bone mass due to reduced resorption. In healthy (Claassen et al, 2005a) and ovariectomized rats (Schlemmer et al, 1999) dietary GLA and EPA reduce resorption and enhance bone mass. Dietary EPA alone improves bone strength in ovariectomized rats (Sakaguchi et al, 1994) and fish oil decreases osteoclastogenesis and loss of bone mass in ovariectomized mice (Sun et al, 2003). Even in humans, GLA and EPA reduce bone turnover and enhance bone mass in 80-year old women (Kruger et al, 1998). Thus based on our research, the optimal amount of LCPUFA supplementation during growth might lie between 0.5 and 1.0 g/100 g fat. Following growth and the attainment of peak bone mass, continued moderated bone resorption and turnover could improve bone mass, reduce risk of fracture and osteoporosis.

The mechanisms by which LCPUFA enhance bone mass cannot be explained by the biochemistry used in this study. Only the lowest level of supplement was associated with enhanced BMD yet no alterations were observed in urinary NTx, plasma osteocalcin and IGF-1 or bone PGE_2. Similarly, no differences in these measurements were observed in piglets fed dietary AA (0.3 g – 0.75 g/100 g of fat) with DHA held constant (0.1 g/100 g of fat) and an overall n-6/n-3 ratio of approximately 9:1 (Blanaru et al, 2004). While NTx and osteocalcin are accepted markers of bone metabolism, it is currently uncertain

whether changes in circulating concentrations of IGF-1 reflect bone tissue concentration and the amount in bone maybe more important for bone formation (Rodan and Rodan, 1995). It is not surprising that no change in PGE_2 was observed in our piglets since the n-6:n-3 ratio was held constant. Researchers have shown that dietary PUFA, specifically the n-6:n-3 ratio, is a major factor in determining bone tissue content of AA and EPA and this, in turn, affects the capacity to synthesize PGE_2 (Watkins et al, 1996; Watkins et al, 2000). The overall n-6:n-3 ratio of our formula was ~ 9:1 for all our diets and was not significantly reduced by the addition of AA and DHA to the formula. However, a limitation of the current research is that the concentration of *ex vivo* PGE_2 measured may not reflect the true levels of PGE_2 in bone and the ELISA kit used cross-reacts with PGE_1 (70%) and PGE_3 (16.3%).

In all tissues measured, AA and DHA increased significantly in proportion to the diet indicating that the diets were capable of enriching multiple tissues. Supplementation of AA:DHA at 0.5:0.1 g/100 g of fat elevated the proportions of AA (11%) and DHA (25%) yet it did not alter the proportion of EPA or the AA:EPA ratio in the liver. Supplementation of higher amounts of AA and DHA may not be beneficial to bone due to resulting reductions in liver EPA and elevations in AA:EPA. The ratio of AA:EPA in liver was negatively correlated with whole body, femur and lumbar spine BMC plus whole body and femur BMD. These data suggest that reduced EPA in tissues relative to AA represents a less than optimal condition for bone mineralization. A positive affect on bone mass may have resulted if EPA was supplemented in low amounts with AA and DHA to prevent the decline in the tissues. Whether EPA has a specific role to play in bone modeling during growth is unclear. Similar to our results, Watkins et al (2000)

found that the ratio of AA:EPA in bone was negatively correlated with bone formation rate in rats. From studies in n-3 deficient rats it is clear that n-3 PUFA have a role to play in normal bone modeling (Reinwald et al, 2004). Together, these data support the idea that there is a delicate balance in n-6 and n-3 PUFA that is required for a benefit to be observed in bone. There was no effect of fatty acid supplementation on growth measured by final body weight, final body length, rate of weight gain, femur weight and femur length. Previously, in piglets of identical age, growth was also unaffected by AA and DHA (Lucia et al, 2003; Blanaru et al, 2004), but one study demonstrated enhanced growth (Weiler and Fitzpatrick-Wong, 2002). In a safety study in piglets of equal age, supplemental AA (0.62 to 0.96 mg/g formula vs. ours at 0.30 to 1.20 mg/g) and DHA (0.37 to 0.55 mg/g formula vs ours at 0.06 to 0.24 mg/g) did not alter growth (Merritt et al, 2003). Thus, while the mechanism(s) for enhanced growth in some studies, but not others using very similar formulas is unclear, the discrepancy might be related to fatty acid status at study inception due to maternal diets. Future studies should include assessment of fatty acid status of the sows or the gestation diets.

In summary, LCPUFA supplemented at 0.5:0.1 g/100 g of fat as AA:DHA elevated bone mass and greater amounts had no additional benefit. In fact the highest level might be limiting over the longer-term due to elevation of bone resorption. Also, the mechanism by which AA and DHA enhance bone mass does not seem to be altered bone turnover or PGE_2 release from bone. Increases in bone mass early in life may be retained throughout life resulting in attainment of higher peak bone mass and reduced risk of fracture and osteoporosis. Future research is needed to determine: 1) how dietary LCPUFA affect bone; 2) whether the elevations in bone mass continue with longer-term

supplementation; and 3) determine if supplementation of these LCPUFA at other points in the life cycle improve bone mass and/or slow the loss of bone mass that occurs naturally later in life.

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Table 4.8.1 Dietary PUFA (g/100 g of fat) measured in formula fed to piglets for 15 d[1].

	Control Diet (g/100 g fatty acids)	AA:DHA Diets (g/100 g of fatty acids) 0.5:0.1	1.0:0.2	2.0:0.4
C12:0	10.78	10.64	10.50	10.24
C14:0	4.34	4.33	4.32	4.30
C16:0	8.04	8.12	8.19	8.34
C18:0	3.11	3.20	3.27	3.43
C18:1 n-9	38.33	37.97	37.63	36.96
C18:1 n-7	1.79	1.77	1.75	1.71
C18:2 n-6, LA	27.12	26.82	26.53	25.97
C18:3 n-3, ALA	3.11	3.07	3.03	2.95
C20:0	0.31	0.32	0.33	0.34
C20:1	0.15	0.15	0.15	0.15
C20:4 n-6, AA	0	0.49	0.96	1.88
C20:5 n-3, EPA	0	0	0	0
C22:0	0.25	0.26	0.28	0.31
C22:5 n-3	0	0	0	0
C22:6 n-3, DHA	0	0.10	0.19	0.38
20:4 n-6:22:5 n-3 AA:DHA	0	5.05	5.05	5.05

[1]Data expressed as g/100 g of fatty acids. Fatty acids with zero for specific fatty acids indicate not detected.
Abbreviations: LA=linoleic acid, ALA=alpha-linolenic acid, AA=arachidonic acid, EPA=eicosapentaenoic acid, DHA=docosahexaenoic acid.

Table 4.8.2 Composition Control of diet fed to piglets for 15 d.

Diet Composition of Control Formula	Unit/L
Oil Blend:	
Soybean,[1] g	23
High oleic safflower,[2] g	23
Coconut,[3] g	14
Dry Mix:	
Skim milk powder,[4] g	110
Whey powder,[5]g	35
Vitamin[6] and Mineral[7] Mix:	
dl-α-tocophyeryl acetate, mg	5
Cholecalciferol, mg	0.11
All trans-retinol acetate, mg	1
Thiamine, mg	30
Riboflavin, mg	60
Niacin, mg	440
Pantothenic acid, mg	284
Pyridoxine, mg	36
Folacin, mg	20
Vitamin B-12, mg	0.4
D-Biotin, mg	2
$CaCO_3$, g	1
Choline chloride, g	42.4
$MnSO_4$, mg	40
Ferrous sulphate, mg	167

[1]Vita Health, Winnipeg, Manitoba
[2]Bestfoods Food Service, Division of Bestfoods, Toronto, Canada
[3]Harlan Teklad, Madison, WI
[4]Parmalat Canada Production and Distribution, Winnipeg Canada
[5]Lactose reduced whey powder (as Avaonlac 134). Glanbia Ingredients, Monroe, WI
[6]Harlan Teklad, Madison, WI, for all listed except all trans-retinol acetate from Sigma-Aldrich
[7]Sigma-Aldrich Canada, Oakville, Canada

Table 4.8.3 Whole body, lumbar spine and femur BA and BMC of piglets fed AA:DHA supplemented formula for 15 d versus control[1].

	Control Diet	AA:DHA Diets (g/100 g of fat)		
		0.5:0.1	1.0:0.2	2.0:0.4
Whole Body BA (cm^2)	454.80 ± 16.06	465.85 ± 25.99	456.47 ± 18.83	455.32 ± 19.06
Whole Body BMC (g)	120.82 ± 6.42	131.72 ± 8.99	124.64 ± 7.16	120.67 ± 5.57
Whole Body BMC/ Final Length $(g/cm)^2$	2.43 ± 0.09	2.42 ± 0.11	2.30 ± 0.09	2.24 ± 0.06
Lumbar Spine BA (cm^2)	9.44 ± 0.56	10.56 ± 0.71	10.34 ± 0.63	10.20 ± 0.40
Lumbar Spine BMC (g)	2.35 ± 0.17	2.87 ± 0.24	2.58 ± 0.11	2.49 ± 0.11
Femur BA (cm^2)	12.76 ± 0.86	12.23 ± 0.54	12.18 ± 0.56	11.90 ± 0.56
Femur BMC (g)	2.99 ± 0.22	3.22 ± 0.24	2.98 ± 0.21	2.94 ± 0.14

[1] Data expressed as mean ± SEM, n=35.
[2] Identifies a main effect of diet, $P<0.05$.
Abbreviations: AA=arachidonic acids, DHA= docosahexaenoic acid, BA=bone area, BMC=bone mineral content.

Table 4.8.4 Erythrocyte PUFA (g/100 g of fat) of piglets fed AA:DHA supplemented formula for 15 d versus control[1].

	Control Diet	AA:DHA Diets (g/100 g of fat)		
		0.5:0.1	1.0:0.2	2.0:0.4
LA	17.87 ± 0.84	17.37 ± 0.77	16.45 ± 0.77	15.89 ± 0.77
ALA	0.04 ± 0.01	0.05 ± 0.01	0.05 ± 0.01	0.07 ± 0.01
AA	3.64^{a} ± 0.19	4.59^{b} ± 0.18	5.51^{c} ± 0.18	6.15^{c} ± 0.18
EPA	0.08 ± 0.01	0.08 ± 0.01	0.09 ± 0.01	0.08 ± 0.01
DHA	1.17^{a} ± 0.09	1.54^{ab} ± 0.09	1.64^{b} ± 0.09	2.03^{c} ± 0.09
Total n-6	22.48 ± 0.94	23.08 ± 0.87	23.17 ± 0.87	23.34 ± 0.87
Total n-3	1.85^{a} ± 0.12	2.21^{a} ± 0.11	2.29^{ab} ± 0.11	2.68^{b} ± 0.11
AA:DHA	3.12 ± 0.23	3.08 ± 0.21	3.46 ± 0.21	3.07 ± 0.21
AA:EPA	46.74^{a} ± 5.14	60.17^{ab} ± 4.76	67.77^{b} ± 4.76	78.18^{b} ± 4.76
AA:EPA+DHA	2.91 ± 0.21	2.92 ± 0.19	3.28 ± 0.19	2.94 ± 0.19
Total n-6:n-3	12.23^{c} ± 0.45	10.57^{bc} ± 0.41	10.22^{ab} ± 0.41	8.80^{a} ± 0.41

[1]Data expressed as mean ± SEM, n=35. Different subscripts identify differences among dietary treatment groups where a<b<c.
Abbreviations: PUFA=polyunsaturated fatty acids, LA=linoleic acid, ALA=alpha-linolenic acid, AA=arachidonic acid, EPA=eicosapentaenoic acid, DHA=docosahexaenoic acid.

Table 4.8.5 Liver PUFA (g/100 g) of piglets fed AA:DHA supplemented formula for 15 d versus control[1].

	Control Diet	AA:DHA Diets (g/100 g of fat)		
		0.5:0.1	1.0:0.2	2.0:0.4
LA	$18.83^{c} \pm 1.50$	$17.07^{b} \pm 1.36$	$16.63^{b} \pm 0.89$	$14.58^{a} \pm 0.65$
ALA	$0.50^{b} \pm 0.05$	$0.37^{a} \pm 0.04$	$0.41^{a} \pm 0.04$	$0.36^{a} \pm 0.03$
AA	$15.91^{a} \pm 0.52$	$17.73^{b} \pm 0.49$	$18.46^{b} \pm 0.23$	$20.01^{c} \pm 0.27$
EPA	$0.22^{a} \pm 0.02$	$0.22^{a} \pm 0.02$	$0.18^{ab} \pm 0.02$	$0.15^{b} \pm 0.02$
DHA	$4.30^{a} \pm 0.98$	$5.39^{b} \pm 0.68$	$5.75^{b} \pm 0.53$	$6.51^{c} \pm 0.42$
Total n-6	36.01 ± 0.37	36.12 ± 0.27	36.41 ± 0.34	35.95 ± 0.18
Total n-3	$6.05^{a} \pm 0.35$	$6.86^{b} \pm 0.21$	$7.22^{bc} \pm 0.18$	$7.70^{c} \pm 0.15$
AA:DHA	3.89 ± 0.38	3.32 ± 0.11	3.23 ± 0.09	3.08 ± 0.06
AA:EPA	$75.86^{a} \pm 8.53$	$86.06^{ab} \pm 7.89$	$107.90^{b} \pm 7.89$	$147.40^{c} \pm 7.89$
AA:EPA+DHA	3.66 ± 0.19	3.19 ± 0.17	3.13 ± 0.17	3.02 ± 0.17
Total n-6:n-3	$6.10^{b} \pm 0.38$	$5.31^{a} \pm 0.17$	$5.06^{a} \pm 0.13$	$4.68^{a} \pm 0.09$

[1]Data expressed as mean ± SEM. Different subscripts identify differences among dietary treatment groups where a<b<c.

Abbreviations: PUFA=polyunsaturated fatty acids, LA=linoleic acid, ALA=alpha-linolenic acid, AA=arachidonic acid, EPA=eicosapentaenoic acid, DHA=docosahexaenoic acid.

Table 4.8.6 Adipose PUFA (g/100 g) of piglets fed AA:DHA supplemented formula for15 d versus control[1].

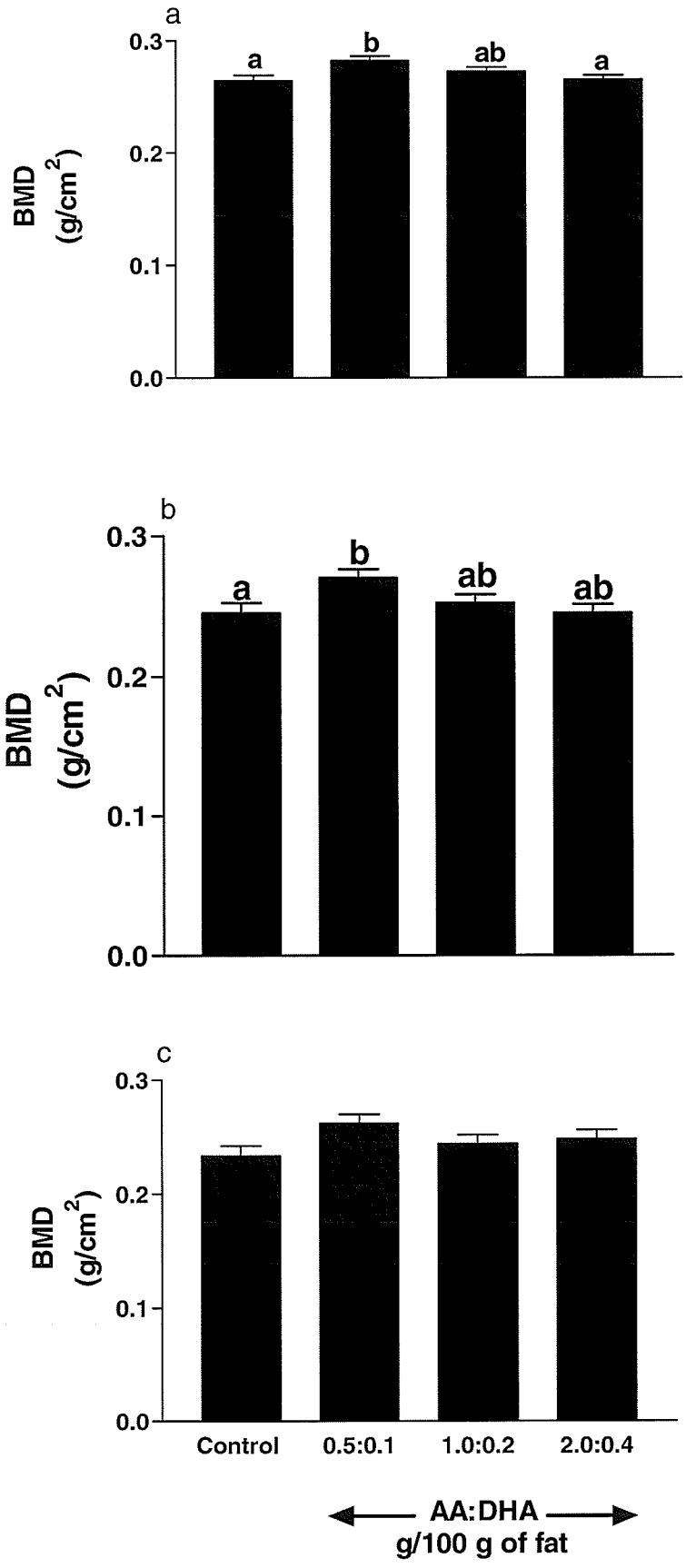
	Control Diet	AA:DHA Diets (g/100 g of fat)		
		0.5:0.1	1.0:0.2	2.0:0.4
LA	24.72 ± 0.46	24.49 ± 0.40	24.89 ± 0.41	24.58 ± 0.38
ALA	2.17 ± 0.06	2.13 ± 0.05	2.18 ± 0.05	2.17 ± 0.07
AA	0.28^{a} ± 0.09	0.46^{b} ± 0.01	0.62^{c} ± 0.03	1.01^{d} ± 0.03
EPA	ND	ND	ND	ND
DHA	0.07^{a} ± 0.02	0.11^{b} ± 0.01	0.15^{c} ± 0.01	0.24^{d} ± 0.01
Total n-6*	25.26 ± 0.45	25.29 ± 0.40	25.94 ± 0.44	26.18 ± 0.40
Total n-3	2.45^{a} ± 0.06	2.46^{a} ± 0.05	2.53^{b} ± 0.06	2.62^{c} ± 0.06
AA:DHA	3.99 ± 0.30	4.18 ± 0.12	4.27 ± 0.07	4.25 ± 0.12
Total n-6:n-3	10.33 ± 0.12	10.29 ± 0.12	10.24 ± 0.08	10.01 ± 0.09

[1]Data expressed as mean ± SEM. Different subscripts identify differences among dietary treatment groups where a<b<c.
*Identifies a main affect of diet.
Abbreviations: PUFA=polyunsaturated fatty acids, LA=linoleic acid, ALA=alpha-linolenic acid, AA=arachidonic acid, EPA=eicosapentaenoic acid, DHA=docosahexaenoic acid.

4.9 Figure Legend

Figure 4.9.1. The effect of AA and DHA supplementation on whole body BMD (a), lumbar spine BMD (b) and femur BMD (c) in piglets fed formula for 15 d. Data are mean ± SEM, n=35. Bars with different subscripts identify significant differences where a<b<c, $P<0.05$. Abbreviations: AA=arachidonic acid, BMD=bone mineral density, DHA=docosahexaenoic acid.

Figure 4.9.2. The effect of AA and DHA supplementation on urinary NTx concentration (a) and plasma osteocalcin concentration (b) in piglets fed formula for 15 d. Data are mean ± SEM, n=35. Bars with different subscripts identify significant differences where a<b<c, $P<0.05$. Abbreviations: AA=arachidonic acid, DHA=docosahexaenoic acid, NTx= N-telopeptides of type 1 collagen cross links.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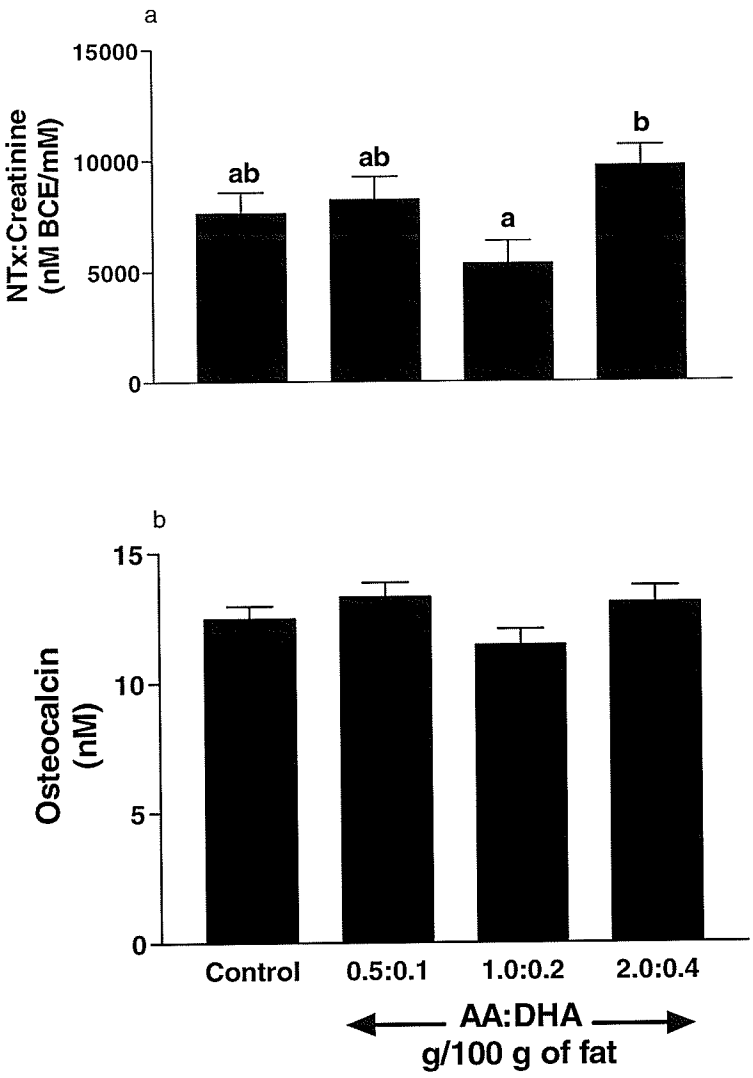
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1.0:0.2
2.0:0.4
AA:DHA
g/100 g of fat

CHAPTER 5.

DIETARY ARACHIDONIC ACID AND DOCOSAHEXAENOIC ACID ELEVATE FEMUR CALCIUM AND REDUCE ZINC CONTENT IN PIGLETS[1]

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Running title: PUFA and Minerals

Dietary Arachidonic Acid and Docosahexaenoic Acid Elevate Femur Calcium and Reduce Zinc Content in Piglets

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5.1 Abstract

Dietary polyunsaturated fatty acids (PUFA) of the n-6 series and long chain PUFA (LCPUFA) of the n-3 series elevate femur Ca content in association with enhanced Ca balance. Mother's milk is also associated with enhanced calcium balance and contains LCPUFA, specifically arachidonic acid (AA) and docosahexaenoic acid (DHA). However, the effect of AA and DHA on calcium metabolism and other bone minerals during infancy has not been fully investigated. Thus, piglets were randomized to receive 1 of 4 formulas for 15 d: control or control with AA:DHA, 0.5:0.1 g, 1.0:0.2 g or 2.0:0.4 g/100 g of fat. Measurements included calcium absorption using an *in situ* ligated loop technique and femur mineral composition and urinary mineral excretion of Ca, P, Zn, and Mg. Main effects were identified using 2-way ANOVA (litter, diet) and post hoc analysis was conducted using Duncan's Multiple Range Test. Significant effects of the diet were observed in femur Ca and Zn content, but not Ca absorption, urinary mineral excretion (Ca, P, Mg or Zn), total femur ash weight, femur length, femur P or femur Mg content. The piglets receiving AA:DHA as1.0:0.2 g/100 g of fat had 1.9 % higher mg Ca/g of ash but 8.6% lower μg Zn/g of ash than the control group. In conclusion, femur ash weight was not affected by dietary AA and DHA, however, the Ca content was elevated and Zn content was reduced by AA and DHA (1.0:0.2 g/100 g of fat as AA:DHA). These data suggest that the dietary ratio of AA to DHA of 5:1 does not affect mineral accretion but that amounts of AA plus DHA, similar to the upper limit of human milk, might be detrimental to bone mineralization over time due to low storage of Zn in bone.

5.2 Introduction

Mother's milk is accepted as optimal with respect to Ca absorption in the infant (Institute of Medicine, 1997). Whether this is attributable to specific constituents of mother's milk is unclear. Dietary polyunsaturated fatty acids (PUFA) and long chain polyunsaturated fatty acids (LCPUFA) affect Ca absorption and Ca balance in animals (Claassen et al, 1995). Arachidonic acid (AA) and docosahexaenoic acid (DHA) are present in breast milk (Hoffman et al, 2003), but are not considered essential fatty acids. Until recently, formula available in North America contained only the essential n-6 and n-3 PUFA, linoleic acid (LA) and alpha-linolenic acid (ALA). In 2002, the FDA and Health Canada approved the supplementation of infant formula with AA and DHA, the LCPUFA products of LA and ALA respectively. Since then, most companies have marketed at least one type of formula containing AA and DHA. Whether AA and DHA are responsible for enhanced Ca absorption in mother's milk is difficult to address. Inclusion of AA and DHA in formula might offer clarification. To date, the effect of formula with and without AA and DHA on bone mineralization and Ca balance remains unclear.

In preterm infants, inclusion of 0.5 g/100 g of fat as AA and 0.3 g/100 g of fat as DHA in formula had no effect on mineral balance (Ca, P, Mg, Zn) (Martinez et al, 2002). Also in preterm infants, dietary AA (0.46 g/100 g of fat) and DHA (0.26 g100 g until hospital discharge followed by 0.16 g/100 g of fat) in formula observed no benefits to bone mass through to 1 yr corrected age (Groh-Wargo et al, 2005). This is in contrast to animal-based research whereby feeding formula with added AA and DHA (0.5:0.1 g/100 g fat) to piglets increases bone mass by 8 to 22% compared to formula with only LA and

ALA (Weiler, 2000; Weiler and Fitzpatrick-Wong, 2002; Mollard et al, 2005). However, higher amounts (1.0:0.2 and 2.0/0.4 g/100 g of fat as AA:DHA) offered no additional benefit despite keeping the ratio of AA:DHA constant at 5:1 (Mollard et al, 2005).

A proposed mechanism for elevations in bone mass in response to n-6 and n-3 PUFA and LCPUFA supplementation is an alteration in mineral metabolism (Claassen et al, 1995). Bone contains many minerals, including Ca, P, Mg and Zn, each of which is essential to infant growth and bone mineralization. Calcium provides structural integrity in the skeleton; 99% of Ca is in the skeleton in the form of hydroxyapatite (Broadus, 1999). Phosphorus is also important in maintaining the structural integrity of the skeleton, 85% is found in the skeleton as hydroxyapatite (Broadus, 1999). Approximately 67% of the body's Mg is located in bone on the hydroxyapatite crystal surface (Broadus, 1999). Magnesium plays an important role in Ca metabolism, since it is a cofactor of enzymes required for synthesis of calcitriol (active form of vitamin D) (Connor et al, 1972) and parathyroid hormone (Connor et al, 1972; Rude et al, 1976; Rude et al, 1978; Rude et al, 1985). Thus, Mg is involved in bone mineral homeostasis and also influences hydroxyapatite crystal formation (Wallach, 1990). Mg affects bone cell function, during Mg deficiency the number of osteoblasts is decreased and the number of osteoclasts is increased (Rude et al, 1999; Rude et al, 2003). Approximately 29% of Zn is found in bone (Jackson et al, 1989). Zn is essential in bone metabolism as a cofactor of enzymes (Saltman and Strause, 1993), including alkaline phosphatase, an important enzyme in bone mineralization (Yamaguchi and Yamguchi, 1986). Elevations in bone Ca, P, Mg or Zn content could enhance bone mineralization and may explain the higher bone mass seen with supplementation of LCPUFA.

In growing rats, dietary gamma-linolenic acid (GLA) plus eicosapentaenoic acid and DHA increased Ca absorption, Ca balance and bone Ca content (Claassen et al, 1995). Inclusion of 0.5:0.1 g/100 g of fat as AA:DHA had no affect on Ca absorption in piglets following 15 d (Weiler, 2000) in addition to no changes in Ca or P in tibial diaphysis, plasma or urine (Weiler, 2000; Weiler and Fitzpatrick-Wong, 2002). It is important to note that bone Ca and P were measured in tibia diaphysis sections, which may not reflect total tibia content. The effects of AA and DHA supplementation on bone Mg and Zn content have not been previously investigated.

In previous research involving piglets, a ratio of AA:DHA ranging from 5:1 to 7.5:1 elevates bone mass (Weiler, 2000; Weiler and Fitzpatrick-Wong, 2002, Blanaru et al, 2004). A ratio of AA:DHA of 5:1 was chosen for this study since two previous studies also used this ratio and demonstrated higher bone mass over 15 d in piglets (Weiler, 2000; Weiler and Fitzpatrick-Wong, 2002). Ca and P metabolism did not change when AA and DHA were supplemented at 0.5:0.1 g/100 g (Weiler, 2000; Weiler and Fitzpatrick-Wong, 2002). It is possible that a higher amount of AA and DHA, as used by Claassen et al (7.4 g/100 g of fat as GLA, 2.5 g/100 g of fat as EPA and 0.7 g/100 g as DHA) (1995), might enhance mineral balance during growth. Thus, the objectives of this study were to determine the effects of formula containing varying amounts of AA:DHA (0.5:0.1, 1.0:0.2, 2.0:0.4 g/100 g of fat) at a 5:1 ratio versus control formula on: (1) Ca absorption; (2) femur mineral composition (Ca, P, Mg, Zn); and (3) mineral excretion (Ca, P, Mg, Zn). These objectives were a part of and accomplished within a large piglet study that was also designed to study the effects of dietary AA and DHA on bone mass (Mollard et al, 2005).

5.3 Materials and methods

5.3.1 Animals and diet

Male piglets (n=40) born at The Glenlea Swine Research Unit, University of Manitoba were transported to the housing facility at the main campus of the University. The average birth weight (± SD) of piglets born at this institution is 1.6 ± 0.2 kg. Piglets were selected with a birth weight of ≥ 1.4 kg, from 10 litters consisting of 10-12 piglets each with at least 4 males. Piglets arrived on d 3 of life and were taught to lap liquid formula. Piglets were fed by a combination of gavage feeding, plus lapping of formula to ensure enough formula was consumed to continue growth for 2 d of adaptation. The strength of control formula was progressively increased from half strength on d 3 to full strength by the end of d 4 of life, followed by experimental or control formula on d 5. Piglets were housed individually in stainless steel cages and room temperature was maintained at 29-30 °C. Based on 0900 h weight, the piglets were offered 350 mL/kg of liquid formula per d. This amount was chosen based on energy requirements (The National Research Council, 1996) and has been shown to support growth in piglets (Weiler and Fitzpatrick-Wong, 2002; Lucia et al, 2003; Blanaru et al, 2004). This amount was divided into 3 equal portions provided at 0900, 1500 and 2100 for 15 d (from 5 to 21 d of life) as per Weiler and Fitzpatrick-Wong (2002). Animal care and procedures were examined by the University of Manitoba Committee on Animal Use and were within the guidelines of the Canadian Council of Animal Care (1993).

Piglets were randomized upon arrival and within litters to receive one of four dietary treatments. Treatments were control formula or control supplemented with 0.5:0.1 g AA:DHA, 1:0.2 g AA:DHA or 2:0.4 g AA:DHA per 100 g of fat. Supplementation

was held at a constant n-6:n-3 ratio of 5:1. The AA was provided in the form of RBD-ARASCO (40.6 g/100 g of fatty acids as AA) and DHA in the form of RDB-DHASCO (40.0 g/100 g of fatty acids as DHA). AA was derived from a common soil fungi and DHA was derived from a marine microalgae (Martek Biosciences Corp). These sources were chosen because they were previously used in our laboratory and because they are used in the manufacturing of many infant formula products. Formulas were isocaloric with equal amounts of fat. The formula was based on nutritional requirements for healthy growing piglets between 3 and 10 kg as set by The National Research Council (1996) and currently proven to support growth in our laboratory (Weiler and Fitzpatrick-Wong, 2002). The dietary composition of the control formula and dietary composition of PUFA in each treatment formula has been previously published (Mollard et al, 2005). Formula contained 1050 kcal/L, 60 g/L fat, 50 g/L protein, 2.1 g/L Ca, 1.4 g/L P and 6.3 mg/L Zn. Piglets were allowed approximately 1 h of exercise before each feed.

5.3.2 Measurements

Sample collection

Urine was collected over a 24 h period using metabolic cages starting on d 14 and ending on d 15 of study. Urine was collected from 21:00 to 09:00, 09:00 h to 15:00 h and 15:00 to 21:00 and then pooled for analysis. On d 16, the piglets were anaesthetized by I.P. injection of sodium pentobarbital (30 mg/kg, 65 mg/mL concentration) or isofluorane gas, Ca absorption was measured and then animals were terminated using sodium pentobarbital overdose (180 mg/kg) and femurs were excised.

Calcium absorption

Calcium absorption was measured using an intestinal ligated loop technique as described previously by Weiler et al (1995). The intestinal ligated loop technique is designed to assess Ca absorption by testing the disappearance of a radiolabeled Ca isotope across the layers of the intestinal tract. This technique was conducted on the morning of the 16^{th} d of study. Food was removed 12 h prior to the procedure. Piglets were anaesthetized and a 5-10 cm section of duodenum distal to the ligament of Treitz was ligated at both ends using suture and the duodenal section was then filled with radioisotope buffer solution (pH of 7.4, mOsmol of 315) containing: ^{45}Ca (18 MBq/L; Amersham Ltd), Poly R-478 (100 mg/L; Sigma-Aldrich Ltd), mannitol (20 mmol/L; Fisher Scientific) and $CaCl_2$ (2 mmol/L; Fisher Scientific). Samples were taken every 5 min for a 30-min period. Samples were analyzed for ^{45}Ca by scintillation counting (Model LS 6000TA; Beckman Instruments Inc.). Poly R-478 was measured by UV spectrometry as described by (Stahl et al, 1991). Ca absorption was calculated according to the method of Ghishan et al (1980).

Bone and urine mineral analysis

Once removed, femurs were cleaned and measurements were taken. Measurements included wet weight (g) and length (mm). Length was measured using callipers to the nearest 0.01 mm. Whole femurs were defatted in acetone (Optima, Fisher Scientific, Allentown, PA, U.S.A.) over 5 d and then ashed in a muffle furnace at 600°C for 6 h. The femurs were then ground and weighed to determine ash weight. Femur ash (200 mg) was digested in 1.5 mL concentrated nitric acid and 0.25 mL of pooled 24-h urine samples were digested in 0.5 mL concentrated nitric acid for measurement of

minerals. Both urine and femur digests were left at room temperature for 48 h and then diluted to obtain a final concentration of 5% (vol/vol) nitric acid in deionized water. Femur and urinary Ca, P, Mg and Zn determined by inductively coupled plasma optical emission spectroscopy (Varian Liberty 200, Varian Canada, Mississauga, ON, CAN). The coefficient of variance for each mineral was <10%.

5.4 Statistical Analysis

Main effects were detected using two-way ANOVA (diet and litter) using SAS statistical software (SAS software release 8.2; SAS Institute, Cary, NC). Litter was included in the statistical analysis as a main effect since previous research demonstrated that litter has a strong effect on bone parameters (Blanaru et al, 2004). Post-hoc analysis was conducted using Duncan's Multiple Range Test when main effects were detected by the two-way ANOVA. A P-value of less than 0.05 was accepted as significant. Data is expressed as mean ± standard error of the mean (SEM).

5.5 Results

At the start of the study, there were no differences among the groups in weight (range 2.00 to 2.14 kg). By the end of the feeding trial, weight remained consistent among the groups and ranged on average from 5.48 to 5.63 kg among the dietary groups. Accordingly, there were also no differences among groups in length, ranging from 53.7 to 54.2 cm, at the end of the study. There were no differences among groups in feed intake since the piglets consumed all the formula they were given at each feed (350 ml/kg/d).

There were no dietary effects on Ca absorption corrected to intestinal weight (control 10.5 ± 1.1; 0.5:0.1 g AA:DHA 9.0 ± 0.5; 1.0:0.2 g AA:DHA 9.4 ± 1.1; 2.0:0.4 g AA:DHA 9.2 ± 0.7 mmol/30 min/g intestine). The average Ca absorption for all animals was 84 %. No dietary effects were observed on femur wet weight, length or ash weight (**Table 5.8.1**). There was a significant main effect of diet on femur Ca when expressed as mg/g of femur ash (P=0.0498) (**Table 5.8.2**). The 1.0:0.2 g AA:DHA group had significantly higher femur Ca than control and the 2.0:0.4 g AA:DHA group, while the 0.5:0.1 g AA:DHA had intermediate values. There were no diet effects on femur Ca when expressed per whole femur (g) (Table 5.8.2) or per length (control 25.3 ± 1.7; 0.5:0.1 g AA:DHA 25.8 ± 1.1; 1.0:0.2 g AA:DHA 25.1 ± 1.3; 2.0:0.4 g AA:DHA 24.1 ± 1.0). There was a significant main effect of diet on femur Zn when expressed as µg/g of femur ash (P=0.0227) (Table 5.8.2). The 1.0:0.2 g AA:DHA group had significantly lower values compared to control, while the 0.5:0.1 g AA:DHA and 2.0:0.4 g AA:DHA groups had intermediate values. There was no effect of diet on femur Zn when expressed per whole femur (µg/g) (Table 5.8.2) or per femur length (control 9.5 ± 0.8; 0.5:0.1 g AA:DHA 9.3 ± 0.5; 1.0:0.2 g AA:DHA 8.5 ± 0.5; 2.0:0.4 g AA:DHA 8.5 ± 0.4 µg/mm).

There were no main effects of diet on femur P expressed per g, whole femur (Table 5.8.2) or per femur length (control 15.8 ±1.1; 0.5:0.1 g AA:DHA 16.0 ± 0.7; 1.0:0.2 g AA:DHA 15.6 ± 0.8; 2.0:0.4 g AA:DHA 15.1 ± 0.6 mg/mm). There were no main effects of diet on femur Mg expressed per g, whole femur (Table 5.8.2) or per femur length (control 273 ± 18; 0.5:0.1 g AA:DHA 273 ± 18; 1.0:0.2 g AA:DHA 262 ± 12; 2.0:0.4 g AA:DHA 262 ± 13 mg/mm).

Diet did not affect urinary Ca, P, Mg or Zn when expressed as over a 24 h period (mmol) or when corrected to the weight of the animal (mmol/kg) (**Table 5.8.3**).

5.6 Discussion

This is the first study to report upon the effects of dietary AA and DHA on the composition of bone mineral including Ca, P, Mg and Zn during infancy. Dietary AA:DHA did not alter femur growth or mineral content when added in amounts (0.5:0.1 g/100 g of fat) similar to that observed in human milk of Canadian's (Hibbeln et al, 2002). Higher amounts of AA:DHA at 1.0:0.2 g/100 g of fat, but not 2.0:0.4 g/100 g of fat elevated Ca and reduced Zn, but had no effect on Mg or P in the femur. Previously in piglets, Ca and P were measured in a tibial diaphysis sections and were unchanged with supplementation of 0.5:0.1 g/100 g of fat as AA:DHA (Weiler, 2000; Weiler and Fitzpatrick-Wong, 2002). The current study supports those findings, with similar results in the whole femur with supplementation of AA:DHA as 0.5:0.1 g/100 g of fat. The alterations in femur Ca and Zn might be related to previously reduced bone resorption observed at 1.0:0.2 g/100 g of fat as AA:DHA (Mollard et al, 2005). Nonetheless, supplementation of AA:DHA did not significantly increase bone mineral content (BMC) or BMD of the femur in these piglets (Mollard et al, 2005). This is also supported by the fact that total ash weight and Ca per total ash were not affected.

Other investigators have studied the effects of dietary PUFA and LCPUFA on femur mineral content and growth in rats. In growing rats, GLA and EPA supplemented to achieve a total n-6:n-3 ratio of 3:1 (GLA 7.4 g/100 g of fat and EPA/DHA 2.5 g/100 g of fat) and 1:1 (GLA 6.6g/100 of fat and EPA plus DHA 5.0 g/100 g of fat) resulted in higher amounts of Ca in bone compared to control, but a ratio of 1:3 (GLA 5.2 g/100 g of fat and EPA plus DHA 12.4 g/100 g of fat) had no effect (Claassen et al, 1995). GLA and EPA did not alter femoral ash weight or growth indicated by length (Claassen et al,

1995). In another study, soybean oil (70 g/kg diet) versus menhaden oil (high in EPA and DHA) plus safflower oil (39.2 plus 30.8 g/kg diet, respectively) did not result in any changes in femur measurements including weight, length, ash weight, Ca, Mg or P in growing rats (Kelly et al, 2003). Although the amounts of particular PUFA and LCPUFA and total n-6:n-3 PUFA ratios were not stated, it can be assumed that the diet containing soybean had no LCPUFA and had a much higher total n-6:n-3 PUFA ratio than the menhaden-safflower diet. In healthy and diabetic growing rats, the same amount of soybean oil (70 g/kg) compared to a similar diet of menhaden oil (40 g/kg) made with corn oil (30 g/kg) found no effect of diet on femur measurements including Ca, P, weight or length. The total n-6:n-3 ratio of the soybean diet was 7.1:1 and the total n-6:n-3 ratio of the menhaden diet was 1.4:1 (Green et al, 2004). All of these studies have not investigated the effects of dietary n-6 and n-3 PUFA and LCPUFA on bone Zn content.

Although results are conflicting, research does support a role for different PUFA and LCPUFA in the femur Ca content depending upon the amount of the n-6 and n-3 PUFA or LCPUFA used. The rats used by Claassen et al (1995) ate 20-22 g food/d and had a final weight of ~350 g. Given the proportion of LCPUFA in the diets, the rats consumed ~ 0.3g GLA/kg, 0.07 g EPA/kg and ~0.02 g DHA/kg of body weight in the 3:1 (n-6)/(n-3) group. In our piglet study, the amounts of LCPUFA for the group receiving 1.0:0.2 g/100 g of fat as AA:DHA were 0.22 g AA/kg and 0.04 g DHA/kg of body weight. In the studies conducted by Kelly et al (2003) and Green et al (2004), the amount of EPA plus DHA based on the composition of menhaden oil is approximately 11.2 g/100 g of fat, calculated the same way as done for Claassen et al (1995) the amount consumed was ~0.49 g EPA plus DHA/kg of body weight. Overall, it appears that dietary n-6 (GLA

or AA) and n-3 (EPA and/or DHA) PUFA that bypass the rate-limiting step of Δ-6 desaturase are needed in a ratio from 3:1 to 5:1 to observe elevations in bone Ca.

AA:DHA supplemented at 1.0:0.2 g/100 g of fat reduced femur Zn. In a normal adult, approximately 29% of body Zn is found in bone (Jackson et al, 1989). During Zn deficiency the amount of Zn in the bone decreases because bone is an endogenous source of zinc when the dietary supply is low (Jackson et al, 1982). Dietary Zn intake was not different among the groups of piglets, which suggests that Zn metabolism is altered in response to LCPUFA. Retention of Zn was not affected by diet as indicated by urinary Zn values. Possible explanations for lower femur Zn are that intestinal absorption is reduced or bone uptake is reduced because it is being accumulated or used in other tissues.

An important objective of this study was to determine whether dietary AA plus DHA alters Ca absorption. Supplementation of AA:DHA at 0.5:0.1 g/100 g of fat in formula containing a total n-6:n-3 ratio of 5:1 does not alter Ca absorption in piglets (Weiler, 2000). The current study supports that observation and indicates that higher amounts (1.0:0.2 and 2.0:0.4 g/100 g of fat) are also ineffective. In growing rats, dietary GLA and EPA plus DHA with a total n-6:n-3 ratio of 3:1 and 1:1 increased Ca absorption by 45% and 21.4% respectively, compared to control rats fed LA and ALA (total n-6:n-3 ratio of 3:1) (Claassen et al, 1995). The lack of increase in Ca absorption in piglets may be the result of: already optimum Ca transport (84% absorption) during periods of rapid growth in piglets compared to the older growing rats (developmental stage of the animal model); the different animal models (rat vs. piglet); and the method used (in situ ligated loop) may not have represented whole intestinal absorption. It is most likely that the differences between these two studies were due to the developmental age differences in

the animals studied rather than the specific amounts of PUFA, since in human preterm infants dietary AA and DHA did not affect mineral balance (Martinez et al, 2002). In piglets, Ca absorption was measured at 84%, whereas in rats, although the percentage of Ca absorbed was not stated, Ca absorption was increased by up to 45% (Claassen et al 1995). This indicates that Ca absorption in the older rats fed the control diet was much lower compared to piglets fed the control diet.

The final objective of this study was to determine whether dietary AA and DHA alter urinary mineral excretion - no effects of the LCPUFA diets were observed. In growing rats, dietary GLA, EPA and DHA supplemented to achieve a total n-6:n-3 ratio of 1:3 (GLA 5.2 g/100 g of fat and EPA plus DHA 12.4 g/100 g of fat) reduced urinary Ca excretion without changes in femur Ca content (Claassen et al, 1995). Dietary GLA and EPA with a total n-6:n-3 ratio of 3:1 increased urinary Ca but also increased Ca balance and femur Ca content (Claassen et al, 1995). In contrast, higher AA status resulting from a high consumption of n-6 PUFA leads to increased Ca excretion (Baggio et al, 2002). In piglets, supplementation of 0.5:0.1 g/100 g of fat as AA:DHA did not alter urinary Ca or P (Weiler, 2000; Weiler and Fitzpatrick-Wong, 2002), possibly since the n-6 PUFA was not modified. The current study supports previous findings in piglets and shows that 2 to 4-fold higher amounts of AA and DHA, while maintaining the total n-6 PUFA constant, have no effect on Ca excretion. However, diets made with menhaden oil versus those made with soybean oil had no effect on urinary Ca in healthy (Kelly et al, 2003, Green et al, 2004) or diabetic rats (Green et al, 2004). A menhaden oil diet has a much lower total n-6:n-3 ratio compared to a soybean oil diet. Whether it is the total n-

6:n-3 ratio or the amount of specific PUFA or LCPUFA that influence Ca excretion requires further investigation.

In conclusion, higher whole body or lumbar spine bone mass found in the piglets supplemented with 0.5:0.1g/100 g of fat as AA:DHA was not explained by alterations in Ca absorption, mineral composition or mineral excretion. In preterm infants, dietary AA and DHA in formula had no effect on mineral balance (Ca, P, Mg, Zn) (Martinez et al, 2002) or whole body bone mass (Groh-Wargo et al, 2005). The piglets used in the study were appropriate for gestational age (born at an appropriate weight), which is a model for term infants. Whether dietary AA and DHA alter mineral balance or bone mass of human infants born at term requires examination. The specific minerals in the whole body and lumbar spine were not measured, therefore, alterations in mineral composition cannot be ruled out as the difference responsible for improvements in BMD. Dietary AA:DHA as 1.0:0.2 g/100 g of fat increased femur Ca (per g of femoral ash) and reduced bone resorption (Mollard et al, 2005). A longer-term study may have shown improved bone mass through reduced bone resorption and higher bone Ca. In addition, mineral balance was not measured in this study and could potentially explain why dietary AA:DHA at 1.0:0.2 g/100 g of fat resulted in altered bone Ca and Zn content. Research is needed to determine the mechanism for increased femoral Ca and reduced femoral Zn content. Whether the decrease in femur Zn seen here is detrimental to bone is unknown and requires further investigation.

5.7 References

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Table 5.8.1 Femur wet weight, length and ash weight of piglets fed formula with AA:DHA for 15 d versus control[1].

	Control Diet	AA:DHA Diets (g/100 g of fat)			
		0.5:0.1	**1.0:0.2**	**2.0:0.4**	**P-Value**
Wet Weight (g)	23.00 ±5.93	23.67 ±6.31	22.58 ±6.80	22.66 ±4.92	0.8254
Length (mm)	77.9 ±0.8	79.0 ±0.8	78.1 ±0.8	77.8 ±0.8	0.7337
Ash Weight (g)	3.45 ±0.26	3.54 ±0.26	3.37 ±0.25	3.28 ±0.19	0.7512

[1] Data expressed as mean ± SEM, n=32

Abbreviations: AA=arachidonic acid, DHA= docosahexaenoic acid, LCPUFA=long chain polyunsaturated fatty acids

Table 5.8.2 Femur mineral content of piglets fed formula with AA:DHA for 15 d versus control[1].

	Control Diet	AA:DHA Diets (g/100 g of fat)			
		0.5:0.1	**1.0:0.2**	**2.0:0.4**	**P-Value**
Ca					
mg/g of ash	573.59[a] ±2.25	580.56[ab] ±3.05	584.21[b] ±2.59	573.75[a] ±1.24	0.0498
mg/femur	1974.07 ±146.14	2051.85 ±142.23	1968.76 ±139.41	1881.62 ±111.05	0.6745
Zn					
µg/g of ash	215.46[b] ±2.93	210.36[ab] ±6.38	196.91[a] ±6.89	202.30[ab] ±5.14	0.0227
µg/femur	745.92 ±65.19	742.10 ±53.99	666.12 ±56.47	664.04 ±43.35	0.1891
P					
mg/g of ash	357.87 ±1.33	359.67 ±2.27	364.37 ±3.60	358.61 ±1.02	0.2236
mg/femur	1231.67 ±91.30	1305.72 ±88.14	1221.29 ±93.18	1174.33 ±76.18	0.7673
Mg					
mg/g of ash	6.21 ±0.12	6.12 ±0.10	6.12 ±0.15	6.22 ±0.12	0.8860
mg/femur	21.35 ±1.57	21.78 ±1.82	20.55 ±1.35	20.46 ±1.40	0.7539

[1]Data expressed as mean ± SEM, n=32. Different subscripts identify differences among dietary treatment groups where a<b<c.
Abbreviations: AA=arachidonic acid, DHA= docosahexaenoic acid, Ca=calcium, LCPUFA=long chain polyunsaturated fatty acids, Mg=magnesium, P=phosphorus, Zn=zinc.

Table 5.8.3 Urinary mineral excretion of piglets fed formula supplemented with AA:DHA for 15 d versus control[1].

	Control Diet	AA:DHA Diets (g/100 g of fat)			
		0.5:0.1	1.0:0.2	2.0:0.4	P-Value
Ca					
mmol/24 h	925.3 ±139.0	870.2 ±249.4	1593.1 ±557.7	789.9 ±202.9	0.5603
mmol/24 h/kg	166.6 ±27.2	162.1 ±50.2	270.3 ±89.9	144.4 ±37.7	0.2187
P					
mmol/24 h	625.8 ±40.1	483.5 ±157.1	569.6 ±330.7	564.2 ±181.6	0.9416
mmol/24 h/ kg	115.3 ±46.8	90.0 ±30.3	82.6 ±37.3	97.0 ±27.4	0.9311
Mg					
mmol/24 h	1322.7 ±211.0	1244.1 ±190.2	1206.6 ±275.6	1258.5 ±230.3	0.9998
mmol/24 h/ kg	242.4 ±39.2	219.6 ±29.1	205.8 ±43.7	225.2 ±41.4	0.9924
Zn					
µmol/24 h	16.1 ±6.3	18.4 ±6.2	11.3 ±1.6	225.2 ±41.4	0.5974
µmol/24 h/kg	3.1 ±1.4	3.3 ±1.1	2.0 ±0.2	2.1 ±0.5	0.4937

[1]Data expressed as mean ± SEM, n=32.
Abbreviations: AA=arachidonic acid, Ca=calcium, DHA=docosahexaenoic acid, LCPUFA=long chain polyunsaturated fatty acids, Mg=magnesium, P=phosphorus, Zn=zinc

CHAPTER 6.

CIRCULATING LONG CHAIN POLYUNSATURATED FATTY ACIDS AND BONE RESORPTION BOTH VARY AS A FUNCTION OF TIME OF DAY AND DIETARY FAT.[1]

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Running title: LCPUFA and circadian rhythm of bone metabolism

Circulating long chain polyunsaturated fatty acids and bone resorption both vary as a function of time of day and dietary fat*^

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6.1 Abstract

Dietary arachidonic acid (AA) and docosahexaenoic acid (DHA) reduce biomarkers of bone metabolism, urinary N-telopeptide (NTx) and osteocalcin (OC). The objective of this study was to examine the effect of AA and DHA on the circadian rhythm of NTx and OC. Piglets were randomized to receive 1 of 4 formulas for 15 d: control or control with AA:DHA as 0.5:0.1 g, 1.0:0.2 g or 2.0:0.4 g/100 g of fat. Measurements included: plasma OC and polyunsaturated fatty acids (PUFA) (sampled at 0900 h, 1500 h and 2100 h on d 15) and urinary NTx:creatinine (samples collected from 2100 h on d 14 to 0900 h, 0900 h to 1500 h and 1500 h to 2100 h on d 15). Main effects (litter, diet, time) were identified by a mixed model repeated measures ANOVA. In those fed AA and DHA, relationships among plasma PUFA proportions and bone resorption were detected using regression. Diet and time did not affect OC. There was an effect of diet ($P=0.0467$) and time ($P<0.0001$) on urinary NTx:creatinine, whereby the those receiving 0.5:0.1 g/100 g of fat as AA:DHA had the lowest values and values were lowest at 2100 h. Likewise, diet ($P=0.0001$) and time ($P<0.0001$) affected plasma AA and DHA; diet elevated values and time reduced values. There was a diet by time interaction on EPA and DHA proportions, suggesting dietary AA and DHA altered their circadian rhythm. Plasma LCPUFA were not associated with urinary NTx:creatinine. Therefore, dietary AA and DHA influence plasma EPA and DHA proportions, but do not alter the circadian rhythm of bone resorption.

6.2 Introduction

Arachidonic acid (AA) and docosahexaenoic acid (DHA) are not considered essential fatty acids, but may be beneficial for a variety of health outcomes when included in the diet. Over the past decade, our research group has investigated the effects of these long chain polyunsaturated fatty acids (LCPUFA) supplemented in formula on bone mass in piglets and observed higher values of bone mineral content or density with dietary supplementation of AA:DHA in amounts ranging from 0.5:0.1 to 0.75:0.1 g/100 g (Weiler, 2000; Weiler and Fitzpatrick-Wong, 2002a; Blanaru et al, 2004; Mollard et al, 2005). It has been proposed that these changes in bone mass result from alterations in bone metabolism, which has been examined by measurements of biomarkers of bone metabolism.

There are several biomarkers currently available and validated for use of measuring bone metabolism, including osteocalcin (OC) and N-telopeptide of type 1 collagen crosslinks (NTx) (Khosla and Kleerekoper, 1999). OC is a noncollagenous protein secreted by osteoblasts and is accepted as a marker of bone formation (Khosla and Kleerekoper, 1999). OC is also released into circulation when bone is broken down, so the amount found in the plasma can represent both bone formation and resorption (Khosla and Kleerekoper, 1999). Thus, OC is an indicator of bone remodelling during growth or turnover during maintenance of bone mass (Khosla and Kleerekoper, 1999). NTx refers to the amino-terminal telopeptide that is found in type I collagen and is released during bone resorption (Khosla and Kleerekoper, 1999). Measurement of urinary NTx is reported as sensitive and specific markers of bone resorption (Hanson et al, 1992).

A circadian rhythm is a pattern based on a 24 h cycle. An example of a type of circadian rhythm is a diurnal rhythm, which peaks only once over the 24 h period. OC levels follow a circadian rhythm characterized by a decline during the morning (0300 h) to a noontime low followed by a gradual rise that peaks after midnight in humans (Calvo et al, 1996) and in piglets (Guo et al, 2000). NTx has been shown to be highest in the early morning (peak excretion between 0300-0700 h) with a decline over the d to a nadir in the afternoon (between 1500-1900 h) (Blumsohn et al, 1994). Other researchers have only found a circadian rhythm for NTx when corrected to creatinine (Bollen et al, 1995; Ju et al, 1997; Wolthers et al, 2001). Consequently, time of sampling is a major consideration when measuring these biochemical markers.

In many studies conducted within our research group, the elevations in bone mass in response to dietary AA and DHA (0.5:0.1 to 0.75:0.1) are not clearly explained by biomarkers of bone metabolism (Weiler, 2000; Blanaru et al, 2004; Mollard et al, 2005). In addition, 0.8:0.1(Lucia et al, 2003) and 1.0:0.2 g/100 g of fat (Mollard et al, 2005) as AA:DHA suppresses bone resorption (Lucia et al, 2003; Mollard et al, 2005). In each of these piglet studies, the biomarkers of bone metabolism were measured at one time of day - typically between 0800 and 1000 h and in the non-fed state. In one research study using piglets, DHA status was inversely related to bone resorption (Weiler and Fitzpatrick-Wong, 2002b) and in another study liver AA was positively associated with bone resorption (Blanaru et al, 2004).

To our knowledge, there is no research examining how plasma PUFA and biomarkers of bone metabolism change over the day in response to feeding (versus the non-fed state) and whether the circadian rhythms of OC and NTx are altered by dietary

LCPUFA. The circadian rhythm (also referred to as circadian variation) of bone resorption over the day is associated with food intake and is diminished during fasting (Bjarnason et al, 2002). The response of biomarkers of bone resorption to fat, glucose and protein were similar (Bjarnason et al, 2002). In the fed state, urinary NTx and plasma OC are lower then during the non-fed state (Clowes et al, 2002).

In order to establish if consuming dietary LCPUFA alters bone metabolism throughout the day, samples collected at multiple time points across the day and from both fasted and fed states should be examined. Hence, the objectives of this study were to determine: (1) the effects of varying amounts of dietary AA and DHA at a constant ratio (5:1) on the circadian rhythms of biomarkers of bone metabolism; (2) whether time of sampling affects the identification of differences between dietary groups; and (3) the associations between plasma PUFA proportions and biomarkers of bone metabolism. These objectives were a part of and accomplished within a large piglet study that was also designed to study the effects of dietary AA and DHA on bone mass (Mollard et al, 2005).

6.3 Materials and methods

Animals and diet

Male piglets (n=32), born at The Glenlea Swine Research Unit, University of Manitoba were transported to the housing facility at the main campus of the University. Piglets were selected with a birth weight of ≥ 1.4 kg, from 8 litters consisting of 10-12 piglets each with at least 4 males. Piglets were adapted over 2 d to housing and diet as per Mollard et al (2005). Throughout the study and based on 0900 h weight, the piglets were offered 350 mL/kg of liquid formula per d. This amount was divided into 3 equal portions provided at 0900, 1500 and 2100 for 15 d (from 5 to 21 d of life) as per Weiler and Fitzpatrick-Wong (2002a). Piglets consumed formula within 2 h following feeding. Animal care and procedures were examined by the University of Manitoba Committee on Animal Use and were within the guidelines of the Canadian Council on Animal Care (1993).

Piglets were randomized to receive one of four dietary treatments as per Mollard et al (2005). Treatments were control formula or control supplemented with 0.5:0.1 g AA:DHA, 1:0.2 g AA:DHA or 2:0.4 g AA:DHA per 100 g of fat. Supplementation was held constant at an AA:DHA ratio of 5:1 and a total n-6:n-3 ratio of 9:1. The AA was provided in the form of RBD-ARASCO (40.6 g/100 g of fatty acids as AA) and DHA in the form of RDB-DHASCO (40.0 g/100 g of fatty acids as DHA). AA was derived from a common soil fungi and DHA was derived from a marine microalgae (Martek Biosciences Corp). These sources were chosen because they were previously used in our laboratory and because they are used in the manufacturing of many infant formula products. Formulas were isocaloric with equal amounts of fat. The formula was based on

nutritional requirements for healthy growing piglets between 3 and 10 kg as set by The National Research Council (1996) and currently proven to support growth (Weiler and Fitzpatrick-Wong, 2002a). The dietary composition of the control formula and dietary composition of PUFA in each treatment formula has been previously published (Mollard et al, 2005). Formula contained 1050 kcal/L, 60 g/L fat, 50 g/L protein, 2.1 g/L calcium and 1.4 g/L phosphorous. Piglets were allowed approximately 1 h of exercise before each feed.

Sample collection

Urine was collected at intervals between feedings over a 24 h period using metabolic cages starting on d 14 and ending on d 15 of study. Urine was collected from 2100 to 0900, 0900 h to 1500 h and 1500 to 2100 h. Samples were stored at -20 °C until analysis of NTx and creatinine. On d 15 before piglets were fed (0900, 1500 and 2100 h), blood (5 mL) was sampled using the internal jugular blind stab technique. Anticoagulated blood (heparin) was separated into plasma and erythrocyte fractions. Plasma and erythrocytes were obtained by centrifugation at 2000 g for 10 minutes at 4 °C, and plasma was stored at -80 °C until analysis of OC and polyunsaturated fatty acids. The next morning, piglets were anaesthetized by I.P. injection of sodium pentobarbital (30 mg/kg, 65 mg/mL concentration) or isofluorane gas and terminated using sodium pentobarbital overdose (180 mg/kg).

Fatty acid analysis

Total lipids from were extracted according to an adapted method of Folch et al (1957) as previously described (Blanaru et al, 2004). Plasma was extracted in chloroform:methanol 2:1 containing 0.01% BHT. An internal standard, heptadecanoic acid (C17:0) was added to each sample. Crude lipid extracts were transmethylated in 1.2

mL of methanolic HCl (3N, Supleco Inc., Bellefonte, PA) at 80 °C for one hour. Fatty acid methyl esters were separated by gas-liquid chromatograph (Varian Star 3400, Varian, Mississauga, ON, Can), equipped with a 30 m capillary column (J&W Scientific, Folsom, U.S.), a flame ionization detector and using hydrogen as the carrier gas. The column is made of fused silica coated with DB225 (25% cyanopylphenyl) and run at 180-220 °C with a between sample temperature of 240 °C to clean the column. The detector oven is set at 300 °C and produces a sensitivity of 1-5 ng/mL. Fatty acid methyl esters (C12-24) were identified by comparison with retention times of Supelco 37 component FAME mix (Supelco Inc.) and expressed as proportion of total fat (g/100 g of fat).

Biomarkers of bone metabolism

Plasma OC was measured in duplicate using an I^{125} radioimmunoassay (DiaSorin, Stillwater, M.N., U.S.A.). This assay is based on rabbit antiserum to bovine OC that has been proven to be a valid approach (porcine OC standard) for measuring porcine OC (Pointillart et al, 1997). NTx in urine was measured in duplicate by a competitive inhibition enzyme-linked immunosorbent assay (Osteomark, Ostex, Seattle, U.S.A.). Although a human assay, it has been validated for the use in samples from growing piglets by cross-calibrating to a human standard (Bollen et al, 1997). Urinary NTx sample values were corrected to creatinine as determined by the Jaffe method (procedure no. 555; Sigma-Aldrich Ltd, Oakville, Canada) to account for urinary dilution. For NTx the CV % was <20% and for OC it was < 15%. Creatinine in urine was measured colorimetrically (Sigma, St. Louis, U.S.A.). The CV % for triplicate analysis of creatinine in all samples was <10%.

6.4 Statistical analysis

Main effects were detected using mixed model repeated measures ANOVA using with diet and time as fixed effects; the random effect was litter. Residual plots were done to determine outliers. Differences among times and groups were identified with estimate statements (t-tests with Bonferroni correction). Relationships between plasma PUFA and bone resorption were detected by linear regression analysis while also accounting for the effect of time of d. Relationships between PUFA in plasma, selected to reflect whole body PUFA status, and bone resorption were conducted with the control group excluded since it was not a component of the dose-response relationship. A P-value of less than 0.05 was accepted as significant. Data is expressed as mean ± standard error of the mean (SEM). All data was analyzed using SAS statistical software (SAS software release 8.2; SAS Institute, Cary, NC).

6.5 Results

To examine the influence of dietary LCPUFA on the circadian rhythm of biomarkers of bone modeling, urinary NTx:creatinine (a marker of osteoclast activity) and plasma OC (a marker of osteoblast activity) were measured. There was a significant effect of diet on NTx (P=0.0467) (**Figure 6.9.1a**). The 1.0:0.2 g AA:DHA group had lower NTx:creatinine compared to all other groups. There was also a significant effect of time on NTx values (P<0.0001), with lower NTx values at 2100 h compared to 0900 and 1500 h (Figure 6.9.1a). There was no time by diet interaction on NTx:creatinine (P=0.4093). There were no significant main effects of diet (P=0.4445), time (P=0.0808) or time by diet interaction (P=0.1605) on OC values (**Figure 6.9.1b**).

The effect of diet on plasma PUFA and LCPUFA proportions are presented in **Table 6.8.1**. There was a significant main effect of diet on the proportion of AA in plasma. The control group had the lowest proportion of AA in the plasma and as the dietary AA increased the proportion of AA significantly increased. There was a significant effect of diet on LA, with proportions decreasing as AA and DHA increased in the diet. There was no dietary effect on the proportion of ALA. There was a significant main effect of diet on the proportion of total n-6 PUFA; however, posthoc analysis did not identify differences among groups. There was also a significant main effect of diet on total n-3 PUFA - proportions decreased with supplementation of AA and DHA.

The effect of time on plasma PUFA and LCPUFA proportions are presented in Table 6.8.1. There was a significant main effect of time on the proportion on AA in the plasma, it was significantly higher at 0900 (12 h postprandial) compared to 1500 (6 h postprandial) and 2100 h (6 h postprandial). There was a significant effect of time on

plasma PUFA proportions; LA and ALA significantly increased with time, with proportions significantly lower at 0900 h compared to 1500 and 2100 h. Total n-6 PUFA and total n-3 PUFA significantly decreased with time. Total n-6 was significantly higher at 0900 h compared to 1500 and 2100 h, while total n-3 was significantly higher at 0900 compared to 1500 and 2100 h, but also significantly higher at 1500 h compared to 2100 h.

There was a main effect of diet on plasma DHA, but not EPA proportions. However, both EPA and DHA decreased over time. A diet by time interaction was found for EPA (P=0.0243) (**Figure 6.9.2a**) and DHA (P=0.0187) (**Figure 6.9.22b**). Posthoc analysis identified that plasma EPA and DHA responded differently to dietary AA and DHA than control formula between 0900 h and 1500 h, but were not different between 1500 and 2100 h. The 1.0:0.2 g AA:DHA diet had a different effect on DHA proportions compared to the 0.5:0.1 g AA:DHA diet from 0900 to 2100 h. These interactions indicate that the response of plasma EPA and DHA proportions to dietary AA and DHA is different in a fasted versus fed state. It also indicates that dietary AA and DHA alter the change in EPA and DHA over the day versus control.

Regression analysis was conducted to determine whether changes in urinary NTx:creatinine related to changes in specific plasma n-6 and n-3 PUFA and LCPUFA when adjusted for time (**Table 6.8.2**). An example of the regression equation (relationship between NTx:creatinine and LA after accounting for time) is y=17302 (intercept coefficient) – 177.88 (time coefficient) –771.14 (LA coefficient). Only plasma LA was negatively associated with urinary NTx:creatinine. ALA, AA, EPA, DHA, total n-6 PUFA and total n-3 PUFA were not related to urinary NTx. This was also true for the

total n-6:n-3 ratio; however, it was very close to significant (P=0.0579) and may be a reflection of the LA and urinary NTx:creatinine relationship.

6.6 Discussion

Previously reported in these piglets, NTx in overnight (12 h) samples was significantly lower in the group fed 1.0:0.2 g/100 g of fat as AA:DHA compared to the group fed 2.0:0.4 g/100 g of fat as AA:DHA, but neither had values different from control or 0.5:0.1 g/100 g as AA:DHA (P=0.039) (Mollard et al, 2005). A similar observation was made herein when urine was sampled over the d, except 1.0:0.2 g/100 g of fat as AA:DHA resulted in significantly lower values compared to all groups. Thus a 24 h sample seems superior to a 12 h sampling with respect to identifying differences in NTx due to these dietary LCPUFA. Other dietary PUFA such as gamma-linolenic acid (GLA) plus EPA and DHA in a 3:1 ratio (Claassen et al, 1995) or fish oil (EPA and DHA) (Kelley et al, 2003) have been shown to reduce bone resorption as indicated by urinary pyridinolines measured over a 4 to 5 d period. Overall, it seems that a sample collected over a 24 h period is better than an overnight (12 h) sample collection to accurately assess bone resorption.

In contrast, OC values were not affected by dietary AA and DHA over the range of intakes studied in plasma sampled at 0900 h (Mollard et al, 2005) and this finding is the same when plasma is sampled at different time points across the day. Previously, plasma OC values were lower in response to AA:DHA supplementation at 0.5:0.1 g/100 g of fat compared to a similar control formula (Weiler and Fitzpatrick-Wong, 2002a). Other studies have not found differences in plasma OC values in response to dietary AA and DHA at 0.5 and 0.1 g/100 g of fat, respectively (Weiler, 2000). However, changes in bone formation have been seen in growing rats fed EPA and DHA at high amounts using different biomarkers. Watkins et al (2000) found that a diet high in n-3 LCPUFA (EPA

and DHA) increased serum bone-specific alkaline phosphatase compared to those fed a diet high in n-6 PUFA (LA) but only observed a downward trend when OC was used to assess bone formation. Thus, it seems likely that OC is not sensitive enough to detect changes in bone formation due to PUFA.

In piglets, dietary AA:DHA at 0.8:0.1 (Lucia et al, 2003) and 1.0:0.2 g/100 g of fat (Mollard et al, 2005) reduce bone resorption without resulting in changes in bone mass. While other studies have shown that 0.5:0.1 g/100 g of fat as AA:DHA increase bone mass without altering bone metabolism in piglets (Weiler, 2000; Blanaru et al, 2004; Mollard et al, 2005). The conflicting results cannot be explained by the dietary amounts and/or ratios of AA:DHA. Bone metabolism may follow a circadian rhythm reflected in the circadian rhythms of biomarkers of bone metabolism and changes in this rhythm could explain changes seen in bone mass. Research suggests that the majority of variation seen in biochemical markers of bone resorption over the day and night are not purely circadian but rather induced by food intake (Bjarnason et al, 2002). The circadian variation in bone resorption is diminished during fasting and food intake reduces bone resorption (Bjarnason et al, 2002; Clowes et al, 2002) and formation (Clowes et al, 2002) acutely. This is the first study to investigate the effects of dietary LCPUFA supplementation on the circadian variation of biomarkers of bone metabolism. Piglets fed 0.5:0.1 g/100 g of fat as AA:DHA had higher whole body and lumbar spine BMD, but urinary NTx collected overnight (12 h postprandial) and 0900 h plasma OC (12 h postprandial) did not explain bone mass changes (Mollard et al, 2005). Here we show that the circadian rhythm of these two specific biomarkers of bone metabolism are unaltered

by supplementation of AA:DHA at 0.5:0.1, 1.0:0.2 and 2.0:0.4 g/100 g of fat as indicated by the lack of a diet by time interaction.

In piglets, the circadian rhythm of NTx adjusted to creatinine (highest in the morning with a significant drop over the d) is similar to that in humans (Bollen et al, 1995; Ju et al, 1997; Wolthers et al, 2001). Plasma OC did not significantly drop over the day. This could be due to the fact that blood was sampled over a 12 h period rather than 24 h. If blood had been sampled at 0300 h, this may have indicated that the OC follows a similar rhythm as reported in humans. Previously in piglets, researchers found that plasma OC was significantly lower at 0900 h and 1700 h compared to 0100 h (Guo et al, 2000).

Tissue proportions of PUFA are influenced by and reflect the amount of PUFA in the diet, including plasma (Blanaru et al, 2004). Changes in plasma PUFA and LCPUFA concentrations over the d in response to feeding have not been reported. Plasma LCPUFA proportions decreased over the d. The essential PUFA responded differently than the LCPUFA; LA and ALA proportions increased and the LCPUFA decreased over the d suggesting that plasma PUFA and LCPUFA have their own circadian rhythm in response to feeding. Both total n-6 and n-3 PUFA in the plasma decrease over the day. It has been shown that fatty acids with a higher number of double bonds are transfered from the intestine to plasma and from plasma to liver and skeletal muscle at a higher rate then those that are more saturated (Bessesen et al 2000). There was a significant interaction between diet and time for plasma EPA and DHA, which suggest that without dietary AA and DHA, plasma EPA and DHA may not decrease over the d. The same interaction was not seen for plasma AA proportions. In response to supplementation or feeding, tissues

may be removing LCPUFA from the plasma for metabolic processes (eicosanoid production, phospholipid membranes) or storing them in tissues such as adipose.

Plasma LA was negatively associated with urinary NTx, even after accounting for time. This is surprising, since a reduction of dietary LA by inclusion of GLA with EPA and DHA or EPA and DHA alone decreases bone resorption (Claassen et al, 1995; Kelly et al, 2003). Plasma AA proportions were not related to urinary NTx after adjusting for time. A positive relationship was found in piglets between liver AA and urinary NTx when AA:DHA was supplemented as 0.30, 0.45, 0.60, or 0.75:0.1 g/100 g of fat (Blanaru et al, 2004). In rats, a significant negative correlation was found between bone formation rate and the ratio of AA:EPA in bone (Watkins et al, 2000). In the present study, there was no relationship between plasma EPA, AA:EPA or DHA proportions and urinary NTx. In piglets fed a PUFA diet with no DHA or AA, higher plasma DHA concentrations were associated with reduced bone resorption (Weiler and Fitzpatrick-Wong, 2002b). Thus short-term changes in circulating AA and DHA due to consumption do not relate bone metabolism, but changes due to more chronic consumption are associated bone metabolism. Plasma should not be used when determining the relationships among body PUFA, including LCPUFA proportions, and bone metabolism. This is due to the fact that plasma proportions are influenced by both time and diet, whereas in tissues such as liver, proportions would be relatively constant over the day.

In conclusion, dietary AA:DHA at 1.0:0.2 g/100 g of fat resulted in lower bone resorption compared to all other groups, however, there was no effect of dietary LCPUFA on the circadian rhythms of biomarkers of bone resorption. Whether higher amounts of dietary LCPUFA, different types of LCPUFA or changes in the total n-6:n-3

ratio lead to changes in the circadian rhythm of biomarkers of bone metabolism requires further investigation. Here, plasma was sampled before feeding and urine was collected for a period of 6 h following feeding. It would be interesting to determine how bone biomarkers and plasma LCPUFA respond to dietary LCPUFA sooner after feeding (within 1 h) and multiple time points (every h) to determine if there is a more immediate response and whether effects were missed because of measuring only 6 h post-prandial. There is the potential for LCPUFA to result in acute changes in bone turnover. Plasma OC was not affected by time, measurements earlier in the morning may have identified its reported circadian rhythm and an effect of dietary LCPUFA on that rhythm. Plasma LCPUFA proportions seem to follow a circadian rhythm in response to feeding. Whether this rhythm is controlled by the amount of LCPUFA in the diet warrants additional study. Future research is needed to determine whether changes in plasma LCPUFA and biomarkers of bone metabolism over the d are in response to time or diet. Bone loss as a result of aging and estrogen deficiency (menopause) occurs due to an increase in resorption over formation (Williams and Wallach, 1997). If diet does influence the circadian rhythm of biomarkers of bone formation and resorption, there is potential for reducing bone loss later in life. Whether changes in bone resorption in response to dietary AA and DHA result in changes in bone mass over a longer period of supplementation and whether changes in plasma PUFA proportions alter bone metabolism requires further clarification.

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Table 6.8.1 The effect of dietary AA:DHA and time on plasma PUFA proportions (g/100 g) in piglets fed formula for 15 d

	Diet				Time (h)			P-value		
	Control	AA:DHA (g/100 g of fat)								
		0.5:0.1	1.0:0.2	2.0:0.4	0900	1500	2100	Diet	Time	Time x Diet
LA	33.37^{d} ±0.37	31.73^{c} ±0.38	30.73^{b} ±30.73	28.10^{a} ±0.37	30.15^{x} ±0.40	30.98^{y} ±0.30	31.42^{y} ±0.26	<0.0001	0.0011	0.2652
ALA	1.47 ±0.05	1.44 ±0.05	1.38 ±0.05	1.40 ±0.05	1.16^{x} ±0.03	1.53^{y} ±0.04	1.51^{y} ±0.04	0.3671	<0.0001	0.7513
AA	6.05^{a} ±0.47	7.65^{b} ±0.49	9.12^{c} ±0.49	10.99^{d} ±0.48	9.80^{y} ±0.42	7.86^{x} ±0.42	7.70^{x} ±0.43	<0.0001	<0.0001	0.2049
EPA	0.15 ±0.02	0.17 0.02	0.14 ±0.02	0.15 ±0.02	0.20^{y} ±0.01	0.14^{x} ±0.01	0.12^{x} ±0.01	<0.0001	<0.0001	0.0243
DHA	1.29^{a} ±0.14	1.61^{b} ±0.14	1.73^{b} 0.14	1.89^{b} ±0.14	2.03^{y} ±0.13	1.46^{x} ±0.13	1.40^{x} ±0.13	0.1520	<0.0001	0.0187
Total n-6 PUFA	39.58 ±0.40	39.77 ±0.42	40.49 ±0.42	40.43 ±0.41	41.28^{y} ±0.56	40.08^{x} ±0.54	39.45^{x} ±0.30	0.0492	<0.0001	0.8182
Total n-3 PUFA	3.37^{a} ±0.13	3.67^{b} ±0.13	3.65^{b} ±0.13	3.81^{c} ±0.13	3.91^{y} ±0.13	3.55^{x} ±0.13	3.40^{x} ±0.13	<0.0001	<0.0001	0.2718

versus control[1].

[1]Data expressed as means ± SEM, n=32. Differences among groups are identified by different subscripts, for dietary differences a<b<c<d, for time differences x<y<z. Abbreviations: ALA=α-linolenic acid, AA=arachidonic acid, DHA=docosahexaenoic acid, EPA=eicosapentaenoic acid, LA=linoleic acid, LCPUFA=long chain polyunsaturated fatty acids, PUFA=polyunsaturated fatty acids

Table 6.8.2 Relationships among urinary N-telopeptide:creatinine with time of d (h) and selected plasma n-6 and n-3 polyunsaturated fatty acids (g/100 g of fat) in piglets fed formula with AA and DHA[1,2].

Predictor	R^2	Coefficient	P-value
Time alone	0.1273		0.0033
Intercept		10027	<0.0001
Time		-253.53	0.0033
Time and LA	0.1857		0.0015
Intercept		20814	0.0002
Time		-232.41	0.0058
LA		-368.87	0.0375
Time and ALA	0.1374		0.0095
Intercept		11498	<0.0001
Time		-203.18	0.0503
ALA		-1586.39	0.3946
Time and AA	0.1479		0.0065
Intercept		7067.05	0.0120
Time		-204.42	0.0294
AA		240.57	0.2219
Time and EPA	0.1346		0.0105
Intercept		8429.02	0.0016
Time		-208.41	0.0490
EPA		6056.44	0.4692
Time and DHA	0.1704		0.0028
Intercept		5493.77	0.0557
Time		-151.88	0.1301
DHA		1723.58	0.0753
Time and Total n-6 PUFA	0.1393		0.0089
Intercept		17140	0.0298
Time		-282.55	0.0022
Total n-6 PUFA		-165.83	0.3527
Time and Total n-3 PUFA	0.1441		0.0074
Intercept		5284.10	0.2411
Time		-199.89	0.0411
Total n-3		1053.93	0.2710
Time and Total n-6:n-3 PUFA	0.1761		0.0022
Intercept		17302	<0.0001
Time		-177.88	0.0530
Total n-6:n-3 PUFA		-771.14	0.0579

[1]n=24

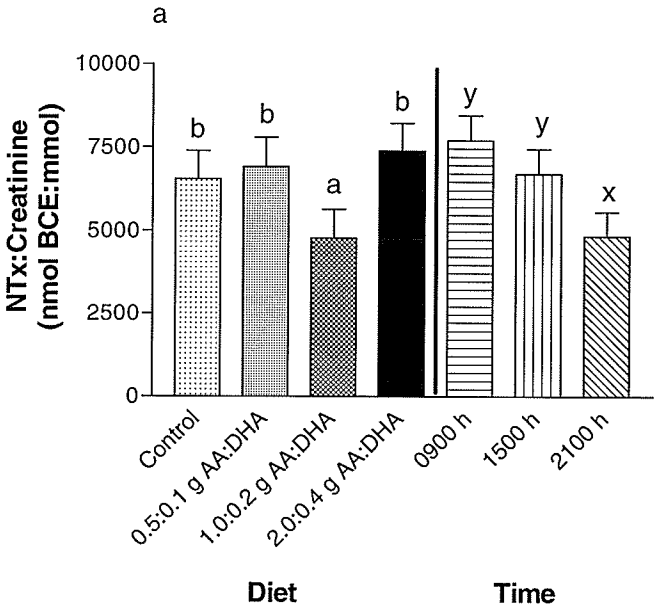
[2]Equation y= intercept coefficient – time coefficient – PUFA coefficient

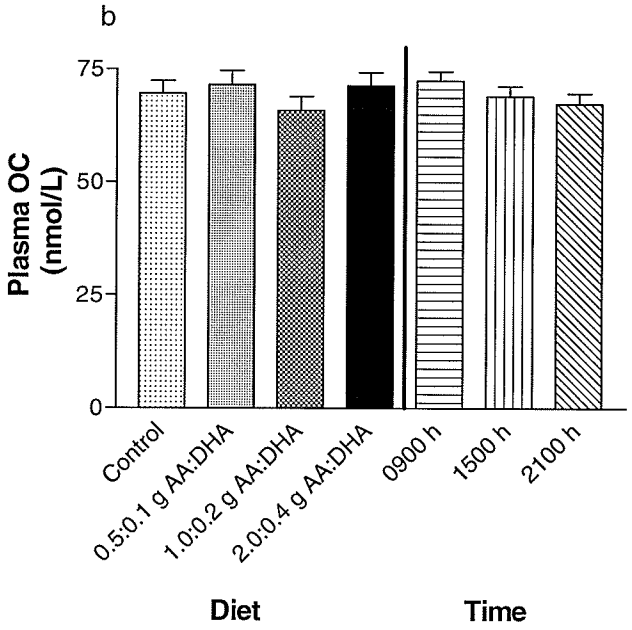
Abbreviations: ALA=α-linolenic acid, AA=arachidonic acid, DHA=docosahexaenoic acid, EPA=eicosapentaenoic acid, LA=linoleic acid, LCPUFA=long chain polyunsaturated fatty acids, PUFA=polyunsaturated fatty acid

6.9 Figure Legend

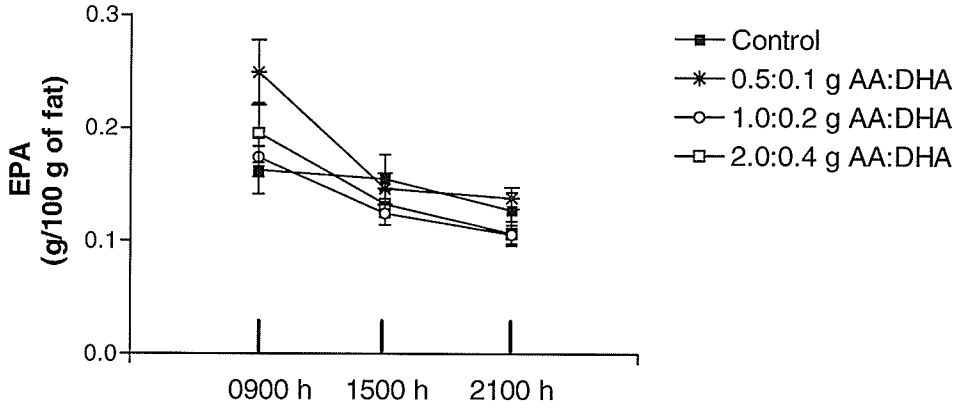
Figure 6.9.1. The effect of dietary AA:DHA and time on urinary NTx concentration (a) and plasma OC concentration (b) in piglets fed formula for 15 d. Data are mean ± SEM, n=32. Bars with different subscripts indicate significant differences where a<b for diet and x<y, $P<0.05$. Abbreviations: AA=arachidonic acid, BCE=bone collagen equivalents, DHA=docosahexaenoic acid, NTx= cross-linked N-telopeptides of type 1 collagen, OC=osteocalcin

Figure 6.9.2. The interaction effect of dietary AA:DHA and time on EPA (a) and DHA (b) proportions in piglets fed formula for 15 d. Plasma EPA and DHA decreased from 0900 to 1500 h in the 0.5:0.1 g AA:DHA, 1.0:0.2 g AA:DHA and 2.0:0.4 g AA:DHA groups, but did not change in the control group. The 1.0:0.2 g AA:DHA diet had a different effect on DHA proportions compared to the 0.5:0.1 g AA:DHA diet from 0900 to 2100 h. Data are mean ± SEM, n=32. Abbreviations: AA=arachidonic acid, DHA=docosahexaenoic acid, EPA=eicosapentaenoic acid

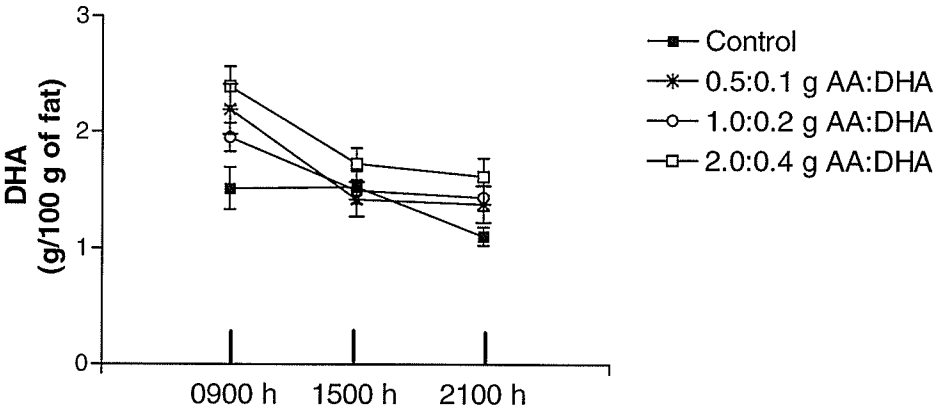

a
NTx:Creatinine (nmol BCE:mmol)
10000
7500
5000
2500
0
b
b
a
b
y
y
x
Control
0.5:0.1 g AA:DHA
1.0:0.2 g AA:DHA
2.0:0.4 g AA:DHA
0900 h
1500 h
2100 h
Diet
Time


b
Plasma OC (nmol/L)
75
50
25
0
Control
0.5:0.1 g AA:DHA
1.0:0.2 g AA:DHA
2.0:0.4 g AA:DHA
0900 h
1500 h
2100 h
Diet
Time

a


EPA
(g/100 g of fat)
0.3
0.2
0.1
0.0
0900 h
1500 h
2100 h
Control
0.5:0.1 g AA:DHA
1.0:0.2 g AA:DHA
2.0:0.4 g AA:DHA

b


DHA
(g/100 g of fat)
3
2
1
0
0900 h
1500 h
2100 h
Control
0.5:0.1 g AA:DHA
1.0:0.2 g AA:DHA
2.0:0.4 g AA:DHA

CHAPTER 7.

DIETARY ARACHIDONIC ACID ALTERS FEMUR BONE GEOMETRY, WHILE EICOSAPENTAENOIC ACID REDUCES FEMUR BONE AREA.[1]

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[1]Prepared in manuscript format.

Running title: LCPUFA and Femur Mineral Content and Morphology

Dietary Arachidonic Acid Alters Femur Bone Geometry, While Eicosapentaenoic Acid Reduces Femur Bone Area

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Key words: femur morphology, urinary Ca, long chain polyunsaturated fatty acids, bone mass

7.1 Abstract

Dietary long chain polyunsaturated fatty acids (LCPUFA) elevate bone mass; but requirements of individual LCPUFA, arachidonic acid (AA) and eicosapentaenoic acid (EPA) for optimizing bone mass throughout the lifes is unknown. The objective of this study was to determine how AA and EPA, in proportions similar to human intake, would affect bone when introduced at different points or maintained throughout life. Weanling male and female rats were randomized to control or one of two LCPUFA diets: AA diet (0.5 g AA, 0.1 g EPA and 0.1 g DHA /100 g of fat) or EPA diet (0.5 g EPA, 0.1 g AA and 0.1 g DHA /100 g of fat). LCPUFA diets were fed in 4 patterns: early (4-18.9 wk of age), mid (19-34.9 wk of age) or late (35-49 wk of age) introduction or continuously (4-49 wk of age). When rats were not fed LCPUFA diets they received control and one group was fed control for the total study period. Serial measurements included: weight, bone area (BA), bone mineral content (BMC), plasma c-telopeptide of type I collagen and urinary Ca. End of study measurements included: body and tail length, femur morphometry and mineral composition, liver and adipose LCPUFA proportions and *ex vivo* femur prostaglandin E_2. Whole body and lumbar spine BMC and BA plus femur BMC were not affected by diet. There was a diet by sex effect on femur BA, and neck and head width. Compared to control, males fed EPA had lower values for BA and males fed AA either mid or continuously had wider femur neck and head widths, respectively. Dietary EPA reduced urinary Ca compared to the AA diet. Femur Mg, Zn and P were altered by dietary LCPUFA. Dietary EPA was reflected in changes in adipose and in liver; however, dietary AA was only reflected in adipose. Whether changes in femur size, morphology and mineral composition influence bone strength requires further study.

7.2 Introduction

Osteoporosis affects approximately 1.4 million Canadians (Goerree, 1996) with a prevalence of 16% in women and 6.5 % in men over 50 years of age (Tenenhouse, 2000). There are two types of osteoporosis postmenopausal and senile. Postmenopausal osteoporosis occurs in women due to an increase in bone resorption in response to estrogen deficiency and occurs mostly in trabecular bone (Willams and Wallach, 1997). Senile osteoporosis, affecting both men and women, is the result of reduced cortical and trabecular bone formation due to aging (Willams and Wallach, 1997). Thus, bone loss in postmenopausal and senile osteoporosis is due to a relative or absolute increase in resorption over formation (Willams and Wallach, 1997).

Two major factors influence the risk of osteoporosis; the level of bone mass achieved at skeletal maturity (peak bone mass) and the rate of bone loss in later years (Percival, 1999). Maximization of bone mass during growth and reducing the rate of bone loss due to menopause and aging could prevent or slow the progression of osteoporosis. In primary health care, diet and lifestyle are ideal targets for use in designing interventions. Dietary Ca and vitamin D are the mainstay of dietary advice for the primary prevention of osteoporosis, but other nutrients such as polyunsaturated fatty acids (PUFA), require further study before use in primary health care (Brown and Josse, 2002).

Dietary PUFA, including long chain PUFA (LCPUFA), affect bone during growth, as observed in neonatal male piglets; dietary AA and DHA (5 to 7.5:1 ratio) added to formula elevated bone mass (Weiler, 2000; Weiler and Fitzpatrick-Wong, 2002; Blanaru et al, 2003; Mollard et al, 2005a) and in some cases, reduced bone resorption

(Lucia et al, 2003; Mollard et al, 2005a). In more developed, yet growing male rats, dietary gamma-linolenic acid (GLA, n-6 PUFA) plus EPA and DHA (total n-6:n-3 PUFA ratios of 1:1 and 3:1) (Claassen et al, 1995) and EPA plus DHA alone (Kelly et al, 2003) reduced bone resorption; only one study has observed elevated bone Ca (Claassen et al, 1995). In Japanese quail, long-term dietary fish oil increases tibial BMC, Ca and P content, strength, cortical thickness and density compared to dietary soybean oil (Liu et al, 2003; Liu et al, 2004). These types of diets also protect against bone loss due to estrogen deficiency, as observed in ovariecomized rats fed a low Ca diet with EPA (Sakaguchi et al, 1994), GLA plus EPA (Schlemmer et al, 1999) or fish oil (Watkins et al, 2003). However, in growing female rats, a high n-3 LCPUFA diet had negative effects on vertebrae strength and growth, but had no effect on males (Sirois et al, 2003). Thus, modification of dietary n-6 and n-3 PUFA can elevate bone mass during growth and protect against bone loss due to estrogen deficiency in animals, suggesting that modification of dietary PUFA might reduce the risk of postmenopausal or senile osteoporosis in humans. In fact, in women ~ 80 yr of age, dietary GLA and EPA elevated bone mass over 18 mo (Kruger et al, 1998).

Despite studies indicating a protective role of dietary PUFA, including LCPUFA, in the primary prevention of osteoporosis, there are multiple gaps in the present state of knowledge regarding the effects of n-6 and n-3 PUFA on bone. Research has not investigated whether elevations in bone mass are sustained (i.e. programmed) once consumption of dietary PUFA stops. Programming is the process whereby a stimulus or insult at a sensitive or critical period of development has long-term effects (Lucas, 1991). Early-life programming research has also investigated the relationships between factors

in early infancy and risk of disease later in life (Eriksson et al, 1999; Forsen et al, 1999). No research has been conducted investigating the modification of dietary PUFA or LCPUFA during infancy or periods of growth and subsequent risk of osteoporosis. Programming effects on Ca metabolism or bone metabolism could result in sustained improvements in bone mass once the dietary treatment has ceased compared to those who did not receive dietary treatment. In addition, a variety of n-6 and n-3 PUFA seem to affect bone, AA and DHA in piglets or combinations of GLA, EPA and DHA in rats. To date, most of the PUFA work on bone during growth is in males and research investigating bone loss has focused on ovariectomized females. Establishing if gender effects exist is important since Sirois et al (2003) demonstrated that males and females respond differently to dietary PUFA during growth. No other studies exist where males and females were tested in the same design. There is also a limited amount of research that traverses the lifespan; there are two studies conducted in male Japanese quail that follow them from growth (1 mo) into maturity (8 mo) (Lui et al, 2003; Lui et al, 2004).

In humans, it is unknown when specific LCPUFA should be incorporated into the diet and if needs for LCPUFA with respect to bone change across the lifespan. No study has investigated the effects of LCPUFA consistent with the range of current intakes and/or recommendations on bone mass except for those conducted in neonatal piglets. In rats, it is not clear if dietary PUFA can maximize peak bone mass, since longitudinal studies between birth and the ages at which peak bone mass is achieved have not been conducted. The longest PUFA intervention study regarding bone mass was conducted using male weanling rats 4 to 12 wk old (Kelly et al, 2003). In male rats, peak bone mass occurs by 12 mo and trabecular bone loss occurs after 12 mo (Ke et al, 2001). In

ovariectomized female rats, the longest intervention study was from 8 to 20 wk old (Watkins et al 2003). In female rats, peak bone mass is achieved by 7.5 mo (Jiang et al, 1997) and trabecular bone loss occurs after 9 mo (Ji et al, 1991). Although long-term studies have been conducted in quail, the amounts of n-3 LCPUFA were much higher then recommended (n-3 LCPUFA as 27.33 g/100 g of fat). Thus, the current study was designed to investigate whether dietary LCPUFA reflecting current recommendations and/or intakes affect bone mass, composition and bone size throughout different life stages. The specific objectives were to determine: (1) the type of LCPUFA that elevate bone mineral, growth and size at different life stages; (2) what stage of life LCPUFA have the greatest impact on bone by studying early, mid and late introduction; (3) the effects of dietary LCPUFA in amounts similar to current intakes and recommendations for prevention of osteoporosis; (4) whether benefits obtained during early and mid inclusion of LCPUFA are sustained (5) whether males and females respond differently to dietary LCPUFA; and (6) whether the affects of LCPUFA on bone change among the different life stages.

7.3 Methods and materials

Study design

The Sprague Dawley rat was selected for this long-term study since it is the most common strain and species used in PUFA and bone research (Claassen et al, 1995, Watkins et al, 2000; Kelly et al, 2003; Sirois et al, 2003; Watkins et al, 2003; Green et al, 2004). In addition, the rat is appropriate for serial (Rose et al, 1998) and reproducible (Makhan et al, 11997) measurements of BMC using dual energy x-ray absorptiometry (DXA) (Makhan et al, 1997).

Male and female Sprague Dawley rats (n=108) were obtained at 21 d of life and immediately randomized to treatments, but fed an AIN-93G diet (control diet) for one week of adaptation. Throughout the adaptation and study periods, the rats were maintained in a controlled environment with a 14-h light, 10-h dark cycle. Rats were provided with deionized water ad libitum, available in glass bottles with rubber stoppers and stainless steel sipper tubes. Feed intake and body weights were recorded weekly. Animal care procedures were based on the guidelines outlined in the Canadian Council on Animal Care (1993). The University of Manitoba, Health Science Centre Protocol Management and Review Committee approved the study protocol.

After adaptation, the rats were randomized to one of nine groups (1 through 9) receiving control or one of two dietary treatment diets during three different phases (early, mid or late) over a 45 wk period (**Table 7.8.1**). Diets were nutritionally adequate and based on the AIN-93G formulation (Reeves 1997). Dietary treatments were either control or one of two LCPUFA diets 1) AIN-93G with 0.5 AA, 0.1 EPA and 0.1 DHA g/100 g of fat 2) AIN-93G with 0.5 EPA, 0.1 AA and 0.1 DHA g/100 g of fat. The fatty

acid compostion of each diet is presented in **Table 7.8.2**. Each feeding phase lasted 15 weeks. The AA was provided in the form of RBD-ARASCO® (40.6 g/100 g of fatty acids as AA). EPA and DHA were provided in triglyceride form (42.3 g/100 g of fatty acids as EPA and 40.0 g/100 g of fat as DHA). AA was derived from a common soil fungus (Martek Biosciences), while EPA and DHA were derived from fish oil (wild Sardine, Anchovy, and Mackerel) (Ocean Nutrition Canada).

The amounts of LCPUFA used were chosen based on either current intakes and/or current recommendations. The amounts of dietary PUFA and LCPUFA used in previous rat research were much higher then current intakes and recommendations. For example, Claassen et al (1995) used amounts of GLA ranging from 5.2 to 7.4 g/100 g, EPA ranging from 1.8 to 9.2 g/100 g and DHA ranging from 0.7 to 3.2 g/100 g in growing rats. At this time, there are no recommendations for LCPUFA in childhood. In 1999, the adequate intake value for LCPUFA recommendations for adults were based on a 2000 kcal/day diet and were as follows, DHA plus EPA as 0.65 g/day, DHA should be at least 0.22 g/d and EPA should be at least 0.22 g/day (Simpoulos, 1999). Based on 60 g of fat per day, recommendations for EPA and DHA of 0.22 g/day are a minimum of 0.37 g/100 g of fat and EPA plus DHA of 0.65 g/day is 1.08 g/100 g of fat. Current intakes for adults are reported to be lower than recommended. Intakes of EPA plus DHA range from 0.1-0.25 g/day (Simopoulos, 1999; Calder and Grimble, 2002; Weiss et al, 2005). Current intakes based on 60 g of fat/d of EPA plus DHA range from 0.17 to 0.33 g/100 g of fat. There are no recommendations for AA past infancy, but mean intakes in western diets are reported as 0.06 to 0.30 g/d of fat (Cadler and Grimble, 2002; Weiss et al, 2005). In French men the intake of AA was 0.204 g/d and in women was 0.152g/d (Astorg et al,

2004). Lower amounts were found in the diets of Austrailian adults; AA intake was 0.052 g/d (Meyer et al, 2003). The amounts of AA and DHA used were chosen to build upon previous research done in neonatal piglets, where dietary AA as 0.5 g/100 g of fat and DHA as 0.1 g/100 g of fat increased bone mass (Weiler et al, 2000; Weiler and Fitzpatrick-Wong, 2002; Mollard et al, 2005a). The total n-6:n-3 ratio of the control diet (7.9:1) is similar to the recommended ratio for children and adults.

Following each 15 wk phase, early, mid and late, blood was sampled, urine was collected and bone mass was measured. Blood was collected from the saphenous vein (150-500 μL, based on body weight) between 0800 and 0900 h for measurement of bone resorption. Urine was collected from 0800 to 1400 h for measurement of urinary Ca and creatinine. Rats were anaesthetized (using isoflorane gas) for measurement of bone area (BA) and BMC using DXA. At end of study, the rats were anesthetized using isoflorane gas (^{Pr}AErrane, Baxter Corp. Mississauga, ON, Canada). While anaesthetized, blood was collected via cardiac puncture and the inferior vena cava was cut after abdominal incision. Tissues were collected, weighed, immediately frozen in liquid nitrogen and stored at -80°C.

Weight and length

Body weight was measured weekly at 0900 h to the nearest g by a digital scale (Mettler-Toleto Inc., Highstown, NJ) using an animal weighing program. The mean of three weights was used to reduce variability. From body weight the rate of weight gain (g $\cdot$ kg^{-1} $\cdot$ week^{-1}) was calculated for each 15 wk phase.

Weight gain:

$$\left[\frac{\text{weight in g wk 1} - \text{weight in g wk 15}}{(\text{weight in kg wk 1} + \text{wk 15})/2}\right] / 15 \text{ wk}$$

At end of study, body length was measured from nose tip to anus and tail length was measured from anus to tail tip. Both measurements were done while the animal was under anaesthetic with the animal placed on its front and measured to the nearest 0.1 cm with a non-stretchable tape measure.

Bone mass

Bone mass of whole body, lumbar spine 1-4 and left femur was assessed by measuring BA and BMC using DXA (4500A, Hologic Inc., Bedford, MA, U.S.A.; small animal software option) while anesthetized at early, mid and late time points. All scans were performed with the rat in the posterior position with limbs extended.

Femur measurements

Following termination, right femurs were excised, cleaned of soft tissue and weighed to the nearest mg. Femur length and morphometry measurements (thickness/width of diaphysis, neck, head, proximal epiphysis and knee) were obtained using a calliper to the nearest 0.01 mm as described by Reichling et al (2000). Following measurements, femurs were dried in an oven at 85°C for 48 h and weighted to obtain a dry weight.

Bone and urine mineral content

Following drying, femur Ca, Mg, P and Zn content were determined. Femurs were wet ashed using 1.5 mL of concentrated nitric acid (trace metal grade, Fischer Scientific, Allentown, PA, U.S.A) at room temperature for 48 h and then heated on a

heating block set at 85°C to ensure that all mineral was digested. Urinary Ca was measured in 6 h urine collections (0800-1400 h) by wet ashing 250 μL of urine in 0.5 mL of concentrated nitric acid for 48 h. The bone and urine digest were then diluted to obtain a final concentration of 5% (vol/vol) nitric acid in deionized water and then measured using inductively coupled plasma optical emission spectroscopy (Varian Liberty 200, Varian Canada, Mississauga, ON, CAN). Mineral content was expressed per g of dry weight, per mm of length and per femur. Urinary Ca values were corrected to creatinine as determined by the Jaffe method (procedure no. 555; Sigma-Aldrich Ltd, Oakville, Canada) to account for urinary dilution. Creatinine in urine was measured colorimetrically (Sigma, St. Louis, U.S.A.). The CV % for triplicate analysis of creatinine in all samples was <10%.

Biochemistry

Osteoclast activity (bone resorption) was determined by measuring bone-related serum degradation products of C-terminal telopeptides of type I collagen using an ELISA (RatLaps, Osteometer BioTech A/S, Herlev, Denmark). The calculated % agreement (average difference from the mean/mean expressed as a percent) was >80% for C-terminal telopeptides of type I collagen. Bone organ culture was performed on left femur diaphysis (~1 g), obtained immediately after termination, as described by Dekel et al (1981). Femur sections were incubated in 0.5 mL Hanks' Balanced Salt Solution (Sigma, St. Louis, MO, U.S.A.) for 2 h at 37 °C in a shaking water bath, followed by the removal of bone and rapid freezing of both the bone and solution. Samples were stored at -20 °C until duplicate analysis of PGE_2 by ELISA (R&D Systems, Minneapolis, MS, U.S.A.). Values were corrected to the weight (g) of the femur segment studied. To minimize

interference of the Hanks' Balanced Salt Solution with the alkaline phosphatase enzyme, standards were reconstituted using this solution as opposed to the buffer provided with the kit. In addition, the antibody cross-reacts with PGE_1 (70 %) and PGE_3 (16.3 %). For PGE_2 the calculated % agreement was >85 %.

Fatty acid analysis

Dietary oils were transmethylated in 1.2 mL of methanolic HCl (3N, Supleco Inc., Bellefonte, PA) at 80 °C for one h. Fatty acid methyl esters were separated by gas-liquid chromatograph (Varian Star 3400, Varian, Mississauga, Ont, Can), equipped with a 30 m capillary column (J&W Scientific, Folsom, U.S.), a flame ionization detector and using hydrogen as the carrier gas. The samples were run at 180-220 °C and the detector oven was set at 300 °C. Fatty acid methyl esters (C12-24) were identified by comparison with retention times of Supelco 37 component FAME mix (Supelco Inc.) and expressed as percent of total fatty acids (g/100 g of fatty acids).

Liver and adipose fatty acids were measured at end of study to reflect body stores of LCPUFA in response to diet and to determine whether early and mid supplementation alters stores once supplementation has ceased. Total lipids from tissues were extracted according to an adapted method of Folch et al (1957) as previously described (Mollard et al, 2002a). Adipose and liver were extracted in chloroform:methanol 2:1 containing 0.01% BHT. An internal standard, heptadecaenoic acid (C17:0) was added to each liver sample. Liver and adipose samples were homogenized and crude lipid extracts were transmethylated in 1.2 mL of methanolic HCl (3N, Supleco Inc., Bellefonte, PA) at 80 °C for 1 h. Fatty acid methyl esters were separated by gas-liquid chromatograph (Varian Star CP-3800, Varian, Mississauga, Ont, Can), equipped with a Chrompack CP-Select

CB column (Varian, Mississauga, Ont, Can) for FAME 100 m x 0.25 mm diameter with a 0.25 μm film thickness. The machine is equipped with a flame ionization detector and uses hydrogen as the carrier gas. The detector oven is set at 300 °C. Fatty acid methyl esters (C12-24) were identified by comparison with retention times of NuChekPrep standard 461 (Elysian, MN, USA). Fatty acids were expressed as a percent of total fatty acids (g/100 g of fatty acids).

7.4 Statistical analysis

The samples size was a total n=108, over 9 groups and n=12 per group. The sample estimate was based on previous studies and the ability to detect a 10% difference in bone mass (prevention of bone loss) over the long term considering a power of 0.80 and alpha of 0.05. For measurements made following each phase; main (diet, time and gender) and interaction effects were determined by mixed model repeated measures ANOVA. Time was included as a main effect in the model to identify time by diet interactions. Main effects of time are not included in this manuscript because they do not relate to the objectives of the study. Residual plots were used to determine outliers and these were then deleted. Post hoc analysis was conducted by estimate statements using t-tests. The correlation structure was examined to determine an appropriate repeated measure structure. Males and females were analyzed together, unless their measurement values over time had different correlation structures, which was the case for weight gain, femur BMC and C-terminal telopeptides of type I collagen. Main effects and interactions on whole body, lumbar spine and femur BMD were not tested; the mixed model is not appropriate because BMD is a ratio of two normally distributed variables resulting in a Cauchy distribution (has no mean or variance). For measurements made at end of study, main and interaction effects were identified by 2-way ANOVA (group and gender). Post hoc analysis was conducted using Duncan's Multiple Range Test. Data is presented as means ± standard error of the means (SEM) and a P-value <0.05 was considered significant.

7.5 Results

Serial measurements

Feed intake, body weight and weight gain

Measurements of body weight and weight gain are presented in **Table 7.8.3.** Feed intake was not altered by dietary treatment, but males consumed more diet then females (females 21 ± 1 g/d, males 29 ± 1 g/d). For body weight, males and females were analyzed together. There was no effect of diet on body weight, but there was an effect of gender. There was no diet by time or diet by gender interactions. For weight gain, males and females were analyzed separately. There was no effect of diet on weight gain for males or females. There was no diet by time interaction on rate of weight gain for either males or females.

Bone area and bone mineral content

Measurements of BA and BMC at each phase in males and females are presented in **Table 7.8.4**. For whole body and lumbar spine BA and BMC, males and females were analyzed together. There was no effect of diet, diet by time or gender by diet for whole body or lumbar spine BA or BMC. There was an effect of gender ($P<0.0001$) on whole body BA, whole body BMC, lumbar spine BA and lumbar spine BMC. Males and females were analyzed together for femur BA. There was a main effect of diet ($P=0.0208$), gender and a diet by gender interaction ($P=0.0214$), but no time by diet interaction. Males had higher femur BA compared to females. Males receiving EPA have lower BA than those receiving control (**Figure 7.9.1a**) and those receiving AA had intermediate values. There was no effect of diet on female BA. Males and females were

analyzed separately for femur BMC. There were no diet effects or time by diet effects on femur BMC in males or females.

Urinary calcium

For analysis of urinary Ca excretion, males and females were analyzed together. There was a main effect of gender; males had lower urinary Ca compared to females. There was no effect of diet on urinary Ca, however, there was a diet by gender interaction (P=0.0030) **(Figure 7.9.1b).** Females fed the EPA diet had lower urinary Ca values compared to those fed the AA diet. Those fed the control had intermediate values compared to those fed either AA or EPA.

Bone resorption

Males and females were analyzed separately for C-terminal telopeptides of type I collagen. There was no diet or diet by time effects on C-terminal telopeptides of type I collagen in males or in females (data not shown).

<u>End point measurements</u>

For the following end point measurements (body and tail length, liver PUFA proportions, adipose PUFA proportions, femur morphology and femur mineral composition), males and females were statistically analyzed together.

Body and tail length

There were no effects of diet or diet by gender on body length or tail length. There was a main effect of gender on body length (males 29.1 ± 0.2 cm, females 24.4 ± 0.1 cm) and tail length (males 24.7 ± 0.2 cm, females 22.2 ± 0.2 cm).

Liver polyunsaturated fatty acid proportions

The effects of diet and gender on liver PUFA proportions are presented in **Table 7.8.5**. There was no effect of diet on LA, ALA, AA and total n-6 proportions. Those fed EPA late had higher DHA proportions compared to control and females had higher DHA proportions then males. There was a main effect of diet on of EPA proportions. Those fed the EPA diet late or continuously had higher values then control. Those fed the AA diet continuously or the EPA late or continuously had higher proportions compared to control. There was a main effect of gender on LA, AA, DHA, total n-6 and total n-3 but not on ALA or EPA. Males had higher LA and total n-6 compared to females, whereas females had higher AA, DHA and total n-3 then males. There were no diet-by-gender interactions for any of the liver PUFA measured.

Adipose polyunsaturated fatty acid proportions

The effect of diet and gender on adipose PUFA proportions are presented in **Table 7.8.6**. There was no effect of diet on LA, ALA, DHA, total n-6 and total n-3 proportions. There was a significant effect of diet on AA proportions. The rats receiving AA continuously had higher proportions then control and those receiving AA early. There was a significant main effect of diet on EPA. Those receiving EPA continuously had higher EPA proportions then those receiving control. There was an effect of gender on LA, AA, EPA and DHA proportions. Males had higher LA and lower AA, EPA and DHA proportions compared to females. Whereas, females had lower total n-6 PUFA and total n-3 proportions compared to males. There was no effect of gender on ALA proportions. There were no diet-by-gender interactions for any of the PUFA measured

Femur measurements

The effects of diet and gender on femur measurements are presented in **Table 7.8.7**. There was an effect of gender on all femur measurements. There was no effect of diet on femur head width, but there was a diet by gender (P=0.0239) (**Figure 7.9.2a**). Males receiving AA through the three phases had higher femur head size than those fed control, EPA during any phase and AA during the early phase. There was no effect of diet on neck width, but there was an interaction between diet and sex (P=0.0244) (**Figure 7.9.2b**). Males fed AA during the middle phase had significantly larger neck width then those fed EPA during the early or late phases, but were not different from control. There was no effect of diet on head or neck width in the females. There was no diet or diet by gender effects on femur length, wet weight, dry weight, diaphysis width, proximal epiphysis width or knee width.

Femur mineral composition

Main effects of diet and gender on femur Ca, Mg, P and Zn are presented in **Table 7.8.8**. Minerals are expressed per g of femur, per femur and per mm of femur. There was no effect of diet on Ca content, but there was a significant gender effect. Males had higher Ca/femur and Ca/mm; however, females had higher Ca/g. There was a significant effect of diet and a diet-by-gender interaction on Zn/g (P=0.0094). Zn/g of bone was affected by diet in females but not in males (**Figure 7.9.3a**). Females fed AA during the early phase had lower Zn/g of femur compared to control. There was no effect of gender on femur Zn/g. There was no effect of diet, but a significant effect of gender on Zn/femur and Zn/mm where females have higher values then males. There were no diet-by-gender interactions on Zn/femur or Zn/mm. There was no main effect of diet on femur P/g,

P/femur or P/mm. There was no gender effect on P/g, but there was a diet-by-gender interaction (P=0.0144) (**Figure 7.9.3b**). Males fed AA during the middle phase had significantly lower P/g, but there was no effect in females. There was a gender effect on P/femur and P/mm., but no diet-by-gender interactions. Females had higher P/bone and P/mm than males. There were no diet or gender effects on Mg/g, but there were on Mg/bone and Mg/mm. Mg/femur was higher in those fed AA early, EPA early, EPA late and EPA continuous compared to control. Mg/mm was higher in those fed AA early, EPA late and EPA continuously compared to control. Mg/femur and Mg/mm was higher in females compared to males. There were no diet-by-gender interactions for Mg/g, Mg/femur or Mg/mm.

Prostaglandin E_2

There were no diet, gender or diet-by-gender interaction for femur *ex vivo* PGE_2 concentrations (Table 7.8.7).

7.6 Discussion

The primary objective of this study was to establish if bone growth and mineralization could be elevated (programmed) by dietary LCPUFA given early in life and sustained thereafter to counteract bone loss with age. While gross measures of whole body and long bone growth were not altered by the LCPUFA diets, in agreement with other shorter-term studies (Watkins et al, 1996; Kelly et al, 2003; Blanaru et al; 2004; Green et al, 2004; Mollard et al, 2005a; Mollard et al, 2005b), the most novel observations herein were altered neck and head width in response to dietary LCPUFA in males. Rats fed the AA diet during the time at which peak bone mass of the femur was attained (mid phase) had greater neck width compared to male rats fed the EPA diet during early growth (early phase) or with aging (late phase). Femur head width was elevated with dietary AA throughout the study period compared to control, those fed EPA during any period and AA during early growth (early phase). In contrast, rats fed EPA also had lower femur BA overall. In chicks, dietary lipid combinations affected tibia morphometry at 21 d of age, but this was not sustained to 42 d of age (Watkins et al, 1997). The current study suggests that the femoral neck geometry can be programmed by dietary AA with lesser amounts of EPA and DHA after infancy but before peak bone mass is achieved. This observation is promising in the prevention of osteoporosis, at least in men, since in patients with proximal hip fractures, femur neck BMD is reduced as a result of eroding cortices (Jordan, 2000). Determining whether a wider femoral neck or head proves advantageous to bone strength is a logical next step, since bone morphometry is a well-known determinant of bone strength (Frost, 2002).

Both dietary LCPUFA interventions altered femur P, Mg or Zn and thus have the potential to impact the mechanical properties of bone (Peng et al, 1997). The AA diet given during achievement of peak bone mass in males programmed for reduced P, suggesting higher proportions of hydroxyapatite, a predominant feature of cortical bone. This correlates with the higher neck width. Programming of bone mineral composition also seemed evident in both genders fed either LCPUFA diet early in life since Mg content was elevated. Such elevations would be protective against the age-related depletion of Mg stores in rats (Peng et al, 1997) and humans (Durlach et al, 1998) and potential reductions in bone strength. Further, the predominantly EPA diet demonstrated ability to enhance Mg stores in bone at any life stage. In females, withdrawing dietary AA after early growth led to subsequent reductions in AA and Zn stores by late maturity. Dietary AA enhances Zn transport in intestine (Song and Mooradian, 1998) and thus dietary AA is required throughout life in females to achieve higher Zn status, as observed herein. Previously in male piglets, dietary AA plus DHA caused reductions in femur Zn (Mollard and Weiler, Chapter 5) but this was not evident in male rats, regardless of age suggesting that testosterone preserves Zn stores. This is a reasonable explanation since withdrawal of testosterone in rats leads to reduced Zn status (Linet, 1972). It is also possible that males and females metabolize or store Zn in different tissues in response to LCPUFA. It is possible that males use more Zn due to a higher lean mass and LCPUFA could influence the amount of lean mass, thus there is less Zn stored in bone.

Elevated Ca excretion is included in the factors predisposing individuals to osteoporosis (Seeman, 2002; Kelepouris et al, 1995; Perry et al, 1982). Dietary n-3 LCPUFA at relatively low amounts compared to n-6 LCPUFA at the same amount,

reduced Ca excretion in females, but not in males. This is supported by the fact that estrogen and LCPUFA are additive in increasing bone Ca content (Schlemmer et al, 1999). Other studies suggest that higher proportions of n-3 LCPUFA are required for an effect in males. For example, dietary GLA, EPA and DHA supplemented to achieve a total n-6:n-3 ratio of 1:3 reduced urinary Ca excretion in young male rats (Claassen et al, 1995). In contrast, a 3:1 ratio increased urinary Ca and a ratio of 1:1 had no effect on urinary Ca. Menhaden oil (total n-6:n-3 ratio of 1.4:1) versus soybean oil (total n-6:n-3 ratio of 7.1:1) had no effect on urinary Ca in healthy or diabetic male rats (Green et al, 2004). Thus, dietary n-3 LCPUFA in higher proportions would reduce urinary excretion of Ca, as seen in male and female patients with kidney stones or hypercalciuria (Baggio et al, 1996; Baggio 2002). In contrast to the EPA diet, the AA diet elevated urinary Ca in females. The higher excretion of Ca concurs with Baggio et al (2002), whereby AA status is associated with urinary Ca excretion. This may not be observed during infancy since in male piglets, elevation of AA status did not lead to changes in Ca excretion (Weiler, 2000; Weiler and Fitzpatrick-Wong, 2002; Mollard et al, 2005a). It is also possible that to see differences in urinary Ca excretion, LCPUFA of opposing series (n-6 versus n-3) are required in the diets being compared, since neither the AA or EPA diet were different from control. Future studies are needed to determine the specific n-6 and n-3 PUFA and LCPUFA and the amount required to alter Ca excretion.

Regardless of gender, the AA diet given early seems to program for lower adipose AA stores later in life, and if given continuously supports AA storage. Reduced storage might be beneficial to Ca excretion. Dietary intervention to reduce Ca excretion through dietary EPA might be more feasible since the EPA diet given either continuously or late

in life enhanced EPA status and reduced Ca excretion. Males had higher liver and adipose LA than females, while females had higher LCPUFA proportions. In adult humans, females have a higher conversion rate of ALA to EPA and DHA in plasma than men (Burge and Wooton, 2002; Burge et al, 2002). This may also be true for the n-6 series. This suggests that females have a higher Δ-6 desaturase activity compared to males and requirements for LCPUFA may be higher for males than for females. Thus, it makes sense that the requirements of LCPUFA for males might be higher to observe an effect in mineral metabolism.

Despite changes in bone geometry, bone mineral composition and mineral excretion, plus LCPUFA status; the dietary LCPUFA interventions used in this study did alter bone mass or metabolism. This was unexpected since dietary AA and DHA has repeatedly elevated bone mass of piglets (Weiler, 2000; Weiler and Fitzpatrick-Wong, 2002; Blanaru et al, 2004; Mollard et al, 2005a) and reduced bone resorption (Lucia et al, 2003; Mollard et al, 2005a). Likewise, dietary n-3 LCPUFA elevate bone mass in rats in association with reduced PGE_2, (Watkins et al 2003; Liu et al, 2003; Green et al 2004; Liu et al, 2004). In this study, we saw no differences in femur *ex vivo* PGE_2 suggesting the mechanism(s) responsible for changes in the femur BA and morphology are independent of PGE_2 metabolism. In the present study, only femur BA was reduced by inclusion of dietary higher amounts EPA at any life stage studied. The lower BA of the femur, but not femur weight or length, suggests that other aspects of bone geometry than those studied were also modified and requires clarification through use of CT scanning. In the present study there were no programming effects of LCPUFA intervention beyond infancy on bone mass, only geometry. Whether dietary LCPUFA in infancy programs

final bone mass was not addressed in this study; since it was not conducted during infancy.

This study was designed to assess the effects of dietary LCPUFA on accretion of bone mass before, during and following the attainment of peak bone mass, in addition to maintenance during healthy aging. The repeated measures design and multi-site approach to studying bone across the major life stages of growth maturity and aging is a major strength of this study. The major limitation of this study was the inability to statistically evaluate changes in BMD. In males but not females, only BMD of lumbar spine declined by the end of study. Thus, the benefits of LCPUFA to BMD remain unclear. Future studies need to extend further into aging and use techniques such as CT that might permit statistical evaluation of bone mass. In conclusion, inclusion of dietary LCPUFA during growth did not program higher bone mass or altered bone resorption. Dietary EPA and AA in amounts similar to current intakes did not alter bone mass; however, it did alter femur morphology and mineral composition. Dietary EPA reduced urinary Ca compared to dietary AA in females, but did not alter femur Ca content; however, studies in older animals may show a protective effect on Ca loss from bone. Whether AA and EPA requirements change between life stages requires further study. As indicated by this study, investigations of the effects of dietary treatments on bone should be designed to include males and females, since there are many possible gender-specific effects.

7.7 References

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Table 7.8.1 Study Design: Three phases of 15 weeks each.

Group	Early	Mid	Late
Control	Control	Control	Control
AA Early	AA diet	Control	Control
AA Mid	Control	AA Diet	Control
AA Late	Control	Control	AA Diet
AA Continuous	AA Diet	AA Diet	AA Diet
EPA Early	EPA diet	Control	Control
EPA Mid	Control	EPA Diet	Control
EPA Late	Control	Control	EPA Diet
EPA Continuous	EPA Diet	EPA Diet	EPA Diet

Abbreviations: AA=arachidonic acid, EPA=eicosapentaenoic acid

Table 7.8.2 Polyunsaturated fatty acid (g/100 g) measured in diet fed to rats for 45 weeks[1].

	Control	AA Diet	EPA Diet
16:0	10.80	10.96	10.84
18:0	22.71	22.93	22.84
18:1n-9	1.41	1.44	1.49
18:2n-6, LA	50.99	51.08	51.02
18:3n-6	0.23	0.27	0.24
18:3n-3, ALA	6.35	6.35	6.36
20:0	0.41	0.42	0.42
20:1n-9	0.20	0.22	0.24
20:2n-6	0.07	0.08	0.08
20:3n-6	0.00	0.02	0.01
20:4n-6, AA	0.00	0.51	0.13
20:5n-3, EPA	0.00	0.13	0.53
22:0	0.39	0.41	0.40
22:5n-3	0.00	0.02	0.02
22:6n-3, DHA	0.00	0.12	0.13
24:0	0.14	0.16	0.14
AA:EPA+DHA	0.00	2:1	0.2:1
Total n-6:n-3	7.9:1	8.1:1	7.3:1

[1]Data expressed as g/100 g of fatty acids. Fatty acids with zero for specific fatty acids indicate not detected. Abbreviations: ALA=α-linolenic acid, AA=arachidonic acid, DHA=docosahexaenoic acid, EPA=eicosapentaenoic acid, LA=linoleic acid

Table 7.8.3 Body weight and weight gain in rats measured following early, mid and late phases of 15 weeks each[1].

	Females	Males
Body Weight (g)		
Early	359 ± 6^a	642 ± 10^a
Mid	429 ± 7^b	788 ± 14^b
Late	506 ± 10^c	877 ± 16^c
Weight Gain ($g \cdot kg^{-1} \cdot week^{-1}$)		
Early	63 ± 1^b	85 ± 1^c
Mid	9 ± 1^a	9 ± 1^b
Late	9 ± 1^a	6 ± 1^a

[1]Data expressed as means ± SEM, n=106. Different subscripts indicate differences among phases within each gender; a<b<c.

Table 7.8.4 Whole body, lumbar spine and femur bone area and bone mineral content in rats measured following early, mid and late phases of 15 weeks each.

	Females	Males
Whole Body BA (cm^2)		
Early	69.40 ± 0.74^a	95.00 ± 0.91^a
Mid	78.50 ± 0.80^b	109.72 ± 1.02^b
Late	82.09 ± 0.99^c	114.68 ± 1.19^c
Whole Body BMC (g)		
Early	11.25 ± 0.15^a	17.07 ± 0.24^a
Mid	13.48 ± 0.17^b	21.42 ± 0.28^b
Late	14.55 ± 0.22^c	23.13 ± 0.31^c
Lumbar Spine BA (cm^2)		
Early	1.81 ± 0.02^a	2.44 ± 0.02^a
Mid	2.00 ± 0.17^b	2.81 ± 0.03^b
Late	2.10 ± 0.02^b	2.87 ± 0.03^c
Lumbar Spine BMC (g)		
Early	0.41 ± 0.01^a	0.58 ± 0.01^a
Mid	0.48 ± 0.01^b	0.72 ± 0.01^b
Late	0.50 ± 0.01^c	0.72 ± 0.02^b
Femur BA (cm^2)		
Early	1.37 ± 0.01^a	1.82 ± 0.02^a
Mid	1.46 ± 0.02^b	1.93 ± 0.03^b
Late	1.50 ± 0.02^c	2.01 ± 0.03^c
Femur BMC (g)		
Early	0.46 ± 0.01^a	0.68 ± 0.01a
Mid	0.53 ± 0.01^b	0.81 ± 0.01b
Late	0.56 ± 0.01^c	0.84 ± 0.01c

Data expressed as means ± SEM, n=106. Different subscripts indicate differences among phases within each gender a>b>c.
Abbreviations: BA=bone area, BMC=bone mineral content

Table 7.8.5 The effects of dietary long chain polyunsaturated fatty acids and gender on liver polyunsaturated fatty acid proportions (g/100 g of fat) following 45 weeks of study[1].

	Diet										Gender		
	Control	AA				EPA				P-Value	Females	Males	P-Value
		Early	Mid	Late	Continuous	Early	Mid	Late	Continuous				
LA	14.20	14.75	14.19	14.93	16.70	16.89	17.82	16.62	18.60	0.0874	13.558	18.596	<0.0001
	0.88	1.49	1.09	1.65	1.81	1.85	1.31	1.56	1.36		0.487	0.702	
ALA	0.40	0.52	0.41	0.58	0.41	0.66	0.42	0.54	0.44	0.8445	0.454	0.519	0.4613
	0.12	0.13	0.09	0.15	0.12	0.17	0.12	0.15	0.13		0.038	0.078	
AA	6.94	7.95	7.25	6.94	9.00	7.95	8.30	9.02	9.29	0.3532	9.466	6.697	<0.0001
	0.88	1.18	0.72	0.67	1.00	1.18	0.77	1.53	1.09		0.524	0.311	
EPA	0.16^{a}	0.17^{ab}	0.16^{a}	0.20^{abc}	0.26^{abc}	0.25^{abc}	0.20^{abc}	0.30^{c}	0.27^{bc}	0.0128	0.201	0.233	0.1481
	0.02	0.03	0.02	0.05	0.04	0.03	0.02	0.04	0.03		0.013	0.019	
DHA	2.41^{ab}	2.55^{ab}	2.31^{a}	2.43^{ab}	2.97^{abc}	2.87^{abc}	2.98^{abc}	3.79^{c}	3.54^{bc}	0.0353	3.854	1.915	<0.0001
	0.45	0.53	0.28	0.31	0.44	0.51	0.40	0.79	0.54		0.250	0.093	
Total n-6 PUFA	22.11	23.74	22.42	22.84	26.86	26.05	27.26	26.83	29.02	0.0576	24.003	26.489	0.0412
	1.51	2.25	1.67	2.12	2.01	1.99	1.32	1.60	1.68		0.840	0.904	
Total n-3 PUFA	3.34^{a}	3.68^{ab}	3.31^{a}	3.65^{ab}	4.92^{bc}	4.35^{abc}	4.12^{abc}	5.23^{c}	4.92^{bc}	0.0480	4.979	3.211	<0.0001
	0.55	0.61	0.32	0.48	0.64	0.57	0.42	0.84	0.64		0.293	0.170	

[1]Data expressed as means ± SEM. Different subscripts identify differences among dietary groups a>b>c. Abbreviations ALA=α-linolenic acid, AA=arachidonic acid, DHA= docosahexaenoic acid, EPA=eicosapentaenoic acid, LA=linoleic acid, PUFA=polyunsaturated fatty acid

Table 7.8.6 The effects of dietary long chain polyunsaturated fatty acids and gender on adipose polyunsaturated fatty acid proportions (g/100 g of fat) following 45 weeks of study[1].

	Diet									Gender		
	Control	AA				EPA				Group P-Value	Females	Males	Gender P-Value
		Early	Mid	Late	Continuous	Early	Mid	Late	Continuous				
LA	24.22 ± 0.76	25.46 0.93	24.27 ± 0.88	25.78 ± 0.88	26.99 ± 1.04	25.76 ± 1.31	25.50 ± 1.41	24.87 ± 0.88	26.99 ± 1.04	0.6400	23.97 ± 0.40	27.00 ± 0.50	<0.0001
ALA	0.10 ± 0.01	0.11 ±0.01	0.11 ± 0.01	0.11 ± 0.01	0.12 ± 0.01	0.11 ± 0.01	0.09 ± 0.02	0.09 ± 0.01	0.10 ± 0.01	0.7812	0.10 ± 0.01	0.10 ± 0.01	0.6754
AA	0.51^{ab} ± 0.02	0.48^{a} ± 0.05	0.53^{abc} ± 0.03	0.59^{bc} ± 0.03	0.62^{c} ± 0.03	0.52^{ab} ± 0.03	0.54^{abc} ± 0.03	0.49^{a} ± 0.03	0.50^{ab} ± 0.02	0.0268	0.56 ± 0.01	0.51 ± 0.02	0.0118
EPA*	0.039^{c} ± 0.005	0.030^{a} ± 0.003	0.033^{ab} ± 0.002	0.039^{ab} ± 0.002	0.040^{abc} ± 0.003	0.033^{ab} ± 0.002	0.034^{ab} ± 0.002	0.043^{cd} ± 0.002	0.047^{d} ± 0.002	<0.0001	0.041 ± 0.001	0.034 ± 0.002	<0.0001
DHA	0.12 ± 0.01	0.09 ± 0.02	0.11 ± 0.01	0.13 ± 0.01	0.13 ± 0.01	0.11 ± 0.01	0.12 ± 0.01	0.14 ± 0.01	0.13 ± 0.01	0.0598	0.14 ± 0.01	0.10 ± 0.01	<0.0001
Total n-6 PUFA	25.10 ± 0.76	26.36 ± 0.92	25.19 ± 0.87	26.79 ± 0.87	27.29 ± 1.16	26.66 ± 1.30	26.41 ± 1.42	25.72 ± 0.91	27.86 ± 1.05	0.6203	24.93 ± 0.40	27.88 ± 0.50	<0.0001
Total n-3 PUFA	1.58 ±0.06	1.60 ± 0.05	1.59 ± 0.04	1.74 ± 0.05	1.75 ± 0.06	1.67 ± 0.08	1.72 ± 0.08	1.63 ± 0.06	1.81 ± 0.08	0.0757	1.63 ± 0.03	1.73 ± 0.04	0.0352

[1]Data expressed as means ± SEM. * Data expressed to 3 decimal points otherwise the error would appear to be zero. Different subscripts identify differences among dietary groups a>b>c. Abbreviations ALA=α-linolenic acid, AA=arachidonic acid, DHA= docosahexaenoic acid, EPA=eicosapentaenoic acid, LA=linoleic acid, PUFA=polyunsaturated fatty acids

Table 7.8.7 The effects of dietary long chain polyunsaturated fatty acids and gender on femur measurements following 45 weeks of study[1].

	Diet									Gender		
	Control	AA				EPA				Group	Females	Males	Gender
		Early	Mid	Late	Continuous	Early	Mid	Late	Continuous	P-Value			P- Value
Length (mm)	39.93 ± 0.78	41.32 ± 1.11	41.10 ± 0.95	40.76 ± 0.85	39.96 ± 1.07	40.82 ± 0.91	40.48 ± 0.85	40.90 ± 0.80	40.50 ± 0.97	0.2655	37.86 ± 0.12	43.52 ± 0.16	<0.0001
Wet Weight (g)	1.24 ± 0.09	1.29 ± 0.10	1.20 ± 0.10	1.31 ± 0.11	1.29 ± 0.12	1.26 ± 0.10	1.26 ± 0.09	1.30 ± 0.09	1.24 ± 0.09	0.5597	0.97 ± 0.01	1.56 ± 0.02	<0.0001
Dry Weight (g)	0.92 ± 0.07	0.97 ± 0.08	0.93 ± 0.08	0.97 ± 0.07	0.96 ± 0.09	0.94 ± 0.07	0.94 ± 0.06	0.97 ± 0.07	0.91 ± 0.07	0.8572	0.72 ± 0.01	1.16 ± 0.01	<0.0001
Diaphysis Width (mm)	3.42 ± 0.17	3.44 ± 0.12	3.37 ± 0.16	3.47 ± 0.16	3.38 ± 0.11	3.38 ± 0.11	3.41 ± 0.10	3.54 ± 0.15	3.38 ± 0.11	0.7954	3.03 ± 0.03	3.82 ± 0.04	<0.0001
Knee Width (mm)	7.63 ± 0.22	7.45 ± 0.21	7.63 ± 0.19	7.68 ± 0.24	7.58 ± 0.25	7.53 ± 0.19	7.60 ± 0.18	7.63 ± 0.21	7.50 ± 0.18	0.6332	7.00 ± 0.05	8.15 ± 0.05	<0.0001
Proximal Epiphysis Width (mm)	8.89 ± 0.28	8.88 ± 0.28	8.97 ± 0.25	9.05 ± 0.24	9.02 ± 0.34	9.00 ± 0.27	9.18 ± 0.22	8.99 ± 0.22	9.01 ± 0.25	0.7551	8.22 ± 0.04	9.75 ± 0.06	<0.0001
Head Width (mm)	4.44 ± 0.10	4.48 ± 0.12	4.50 ± 0.13	4.57 ± 0.11	4.56 ± 0.18	4.48 ± 0.12	4.54 ± 0.11	4.47 ± 0.09	4.42 ± 0.10	0.4054	4.16 ± 0.02	4.82 ± 0.03	<0.0001
Neck Width (mm)	2.21 ± 0.07	2.26 ± 0.08	2.28 ± 0.10	2.23 ± 0.08	2.23 ± 0.10	2.19 ± 0.07	2.28 ± 0.05	2.21 ± 0.04	2.21 ± 0.07	0.7876	2.03 ± 0.02	2.43 ± 0.02	<0.0001
PGE_2 (ng/g)	7.30 ± 0.73	8.14 ± 1.01	7.28 ± 1.08	7.65 ± 0.75	6.86 ± 0.70	6.97 ± 0.90	7.57 ± 0.74	6.29 ± 0.57	8.45 ± 1.10	0.8747	7.08 ± 0.38	7.55 ± 0.41	0.4172

[1]Data expressed as means ± SEM. Different subscripts identify differences among dietary groups a>b>c. Abbreviations: AA=arachidonic acid, DHA= docosahexaenoic acid, EPA=eicosapentaenoic acid

Table 7.8.8 The effects of long chain polyunsaturated fatty acids and gender on femur mineral composition following 45 weeks of study[1].

	Diet										Gender		
	Control	AA Early	Mid	Late	Continous	EPA Early	Mid	Late	Continuos	Group P-value	Females	Males	Gender P-value
Ca													
mmol/femur	5.61 ± 0.38	5.45 ± 0.42	5.37 ± 0.43	5.68 ± 0.38	5.63 ± 0.42	5.59 ± 0.45	5.20 ± 0.39	5.59 ± 0.32	5.37 ± 0.39	0.9681	4.65 ± 0.105	6.33 ± 0.17	<0.0001
mmol/g	6.18 ± 0.30	5.76 ± 0.31	5.849 ± 0.259	6.041 ± 0.309	6.03 ± 0.29	5.98 ± 0.22	5.58 ± 0.25	5.91 ± 0.35	6.13 ± 0.44	0.9089	6.45 ± 0.13	5.44 ± 0.12	<0.0001
***mmol/mm**	0.140 ± 0.008	0.131 ± 0.008	0.129 ± 0.008	0.138 ± 0.007	0.139 ± 0.009	0.136 ± 0.009	0.127 ± 0.008	0.137 ± 0.007	0.132 ± 0.010	0.8996	0.122 ± 0.003	0.147 ± 0.004	<0.0001
P													
mmol/ femur	2.42 ± 0.14	2.95 ± 0.21	2.88 ± 0.19	2.57 ± 0.18	2.47 ± 0.16	2.88 ± 0.18	2.88 ± 0.21	2.74 ± 0.19	3.05 ± 0.21	0.1681	2.91 ± 0.09	2.63 ± 0.09	0.0202
mmol/g	3.09 ± 0.22	2.89 ± 0.16	2.66 ± 0.20	2.78 ± 0.14	3.33 ± 0.22	3.13 ± 0.11	2.97 ± 0.17	3.06 ± 0.07	3.15 ± 0.14	0.0894	3.08 ± 0.08	2.92 ± 0.07	0.1470
***mmol/ mm**	0.061 ± 0.004	0.073 ± 0.007	0.071 ± 0.006	0.064 ± 0.005	0.062 ± 0.006	0.071 ± 0.004	0.072 ± 0.006	0.068 ± 0.005	0.075 ± 0.006	0.3434	0.076 ± 0.003	0.061 ± 0.002	<0.0001
Mg													
µmol/femur	93.55^{a} ± 9.51	135.96^{b} ± 14.24	119.87^{ab} ± 13.46	114.87^{ab} ± 11.75	89.56^{a} ± 6.75	131.88^{b} ± 10.77	120.47^{ab} ± 13.57	131.24^{b} ± 9.35	140.57^{b} ± 15.00	0.0260	128.42 ± 5.99	112.42 ± 5.69	0.0362
µmol/g	119.37 ± 12.03	134.99 ± 13.28	111.70 ± 13.91	125.31 ± 11.72	121.54 ± 9.91	141.53 ± 7.22	124.70 ± 12.26	146.70 ± 5.23	139.35 ± 8.01	0.1796	133.90 ± 4.67	125.18 ± 5.51	0.1664
µmol/ mm	2.35^{ab} ± 0.23	3.37^{c} ± 0.42	2.99^{abc} ± 0.38	2.86^{abc} ± 0.32	2.23^{a} ± 0.18	3.22^{bc} ± 0.25	3.01^{abc} ± 0.36	3.25^{c} ± 0.26	3.45^{c} ± 0.39	0.0420	3.36 ± 0.16	2.61 ± 0.14	0.0002
Zn													
µmol/femur	3.01 ± 0.17	3.27 ± 0.15	3.40 ± 0.01	3.04 ± 0.17	3.18 ± 0.22	3.01 ± 0.15	3.34 ± 0.16	3.00 ± 0.15	3.21 ± 0.15	0.5232	3.23 ± 0.08	3.12 ± 0.07	0.2383
µmol/g	3.85 ± 0.27	3.24 ± 0.15	3.15 ± 0.16	3.30 ± 0.14	4.28 ± 0.30	3.45 ± 0.21	3.51 ± 0.25	3.44 ± 0.21	3.44 ± 0.28	0.0107	3.50 ± 0.13	3.50 ± 0.09	0.6580
***µmol/mm**	0.076 ± 0.005	0.080 ±0.005	0.083 ± 0.003	0.075 ± 0.005	0.080 ± 0.008	0.076 ± 0.004	0.083 ± 0.004	0.074 ± 0.004	0.078 ± 0.004	0.7273	0.085 ± 0.002	0.073 ± 0.002	<0.0001

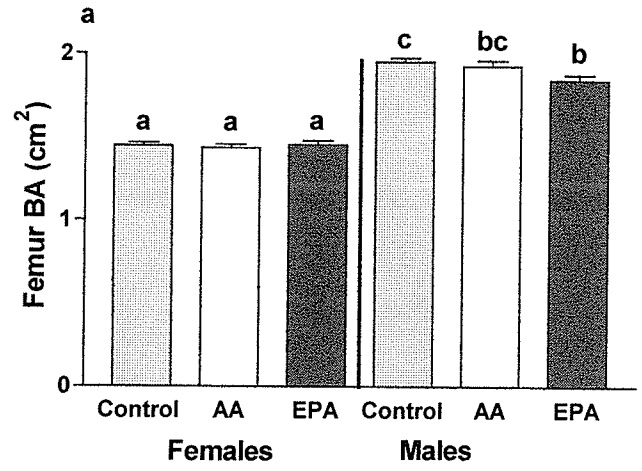
[1]Data expressed as means ± SEM. * Data expressed to 3 decimal points when the error would appear to be zero. Different subscripts identify differences among dietary groups a>b>c. Abbreviations: AA=arachidonic acid, DHA= docosahexaenoic acid, EPA=eicosapentaenoic acid

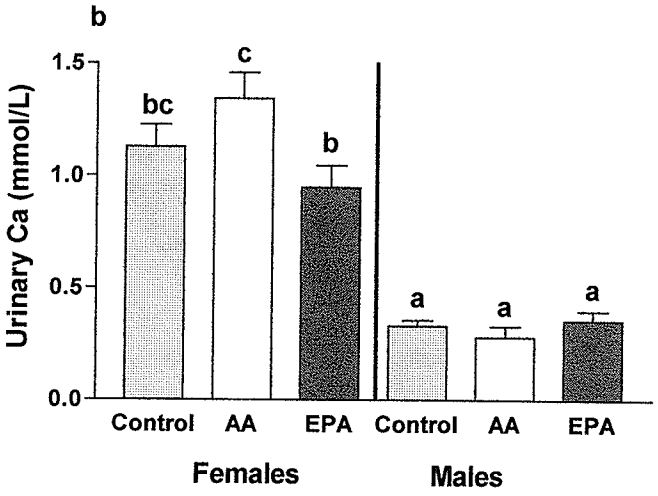
7.9 Figure Legends

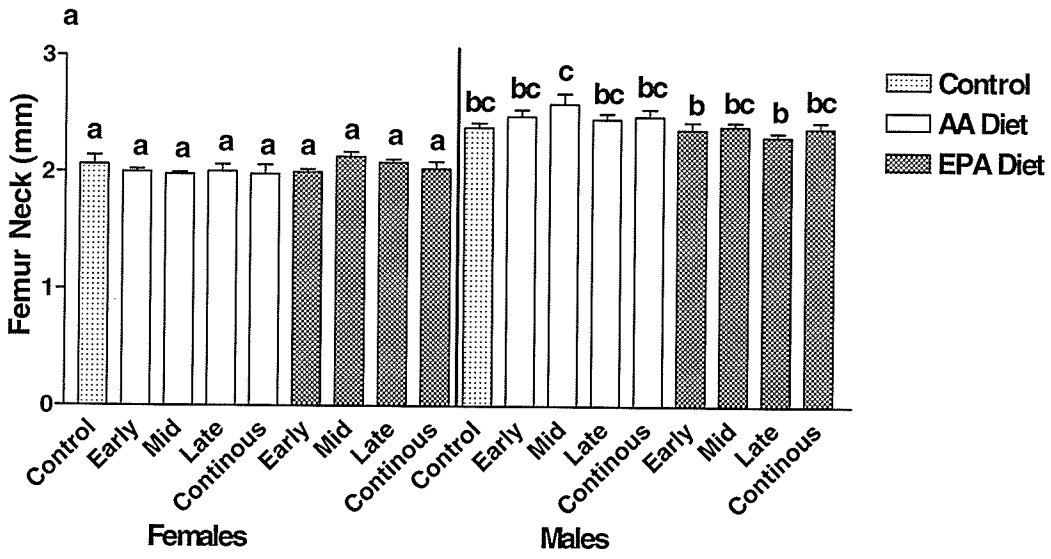
Figure 7.9.1 The main effect of dietary LCPUFA on BA (a) and urinary Ca (b) in male and female rats over 45 weeks compared to control. Means ± SEM, n=106. Different subscripts identify differences among groups across the genders, a<b<c. Abbreviations: AA=arachidonic acid, BA=bone are, EPA=eicosapentaenoic acid, LCPUFA=long chain polyunsaturated fatty acids.

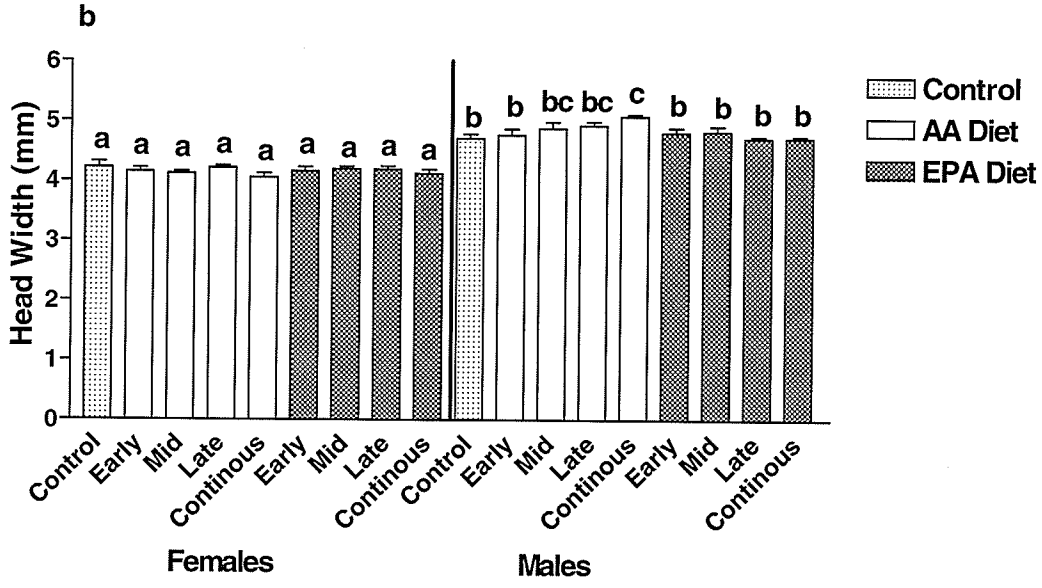
Figure 7.9.2 The effect of dietary LCPUFA on femur neck width (a) and head width (b) when fed early, mid or late (15 wks) or continuously (45 wks) compared to control. Means ± SEM, n=106. Different subscripts identify differences among groups across the genders, a<b<c<d. Abbreviations: AA=arachidonic acid, EPA=eicosapentaenoic acid, LCPUFA=long chain polyunsaturated fatty acids.

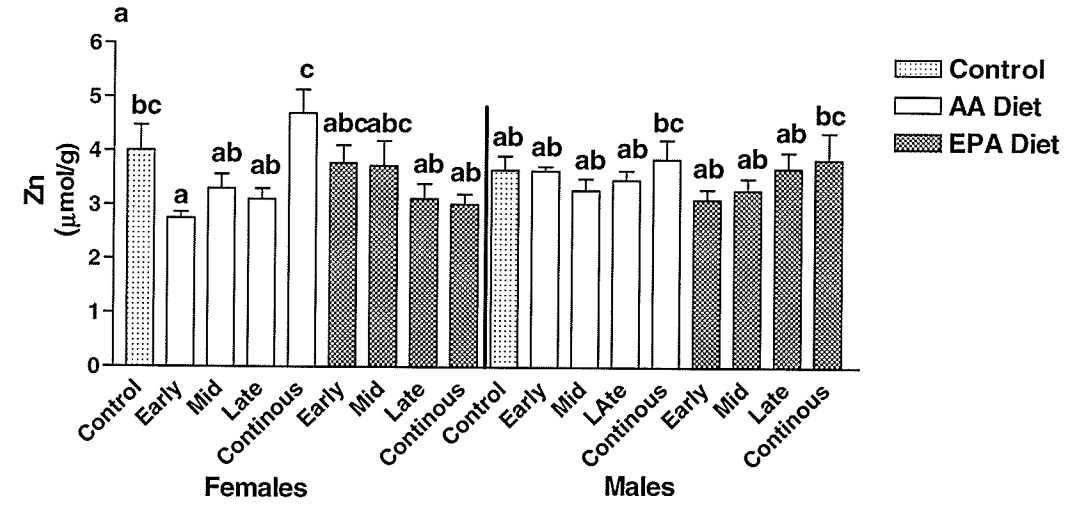
Figure 7.9.3 The effect of dietary LCPUFA on femur Zn content (a) and P content (b) when fed early, mid or late (15 wks) or continuously (45 wks) compared to control. Means ± SEM, n=106. Different subscripts identify differences among groups across the genders, a<b<c<d. Abbreviations: AA=arachidonic acid, EPA=eicosapentaenoic acid, LCPUFA=long chain polyunsaturated fatty acids, P=phosphorus, Zn=zinc.

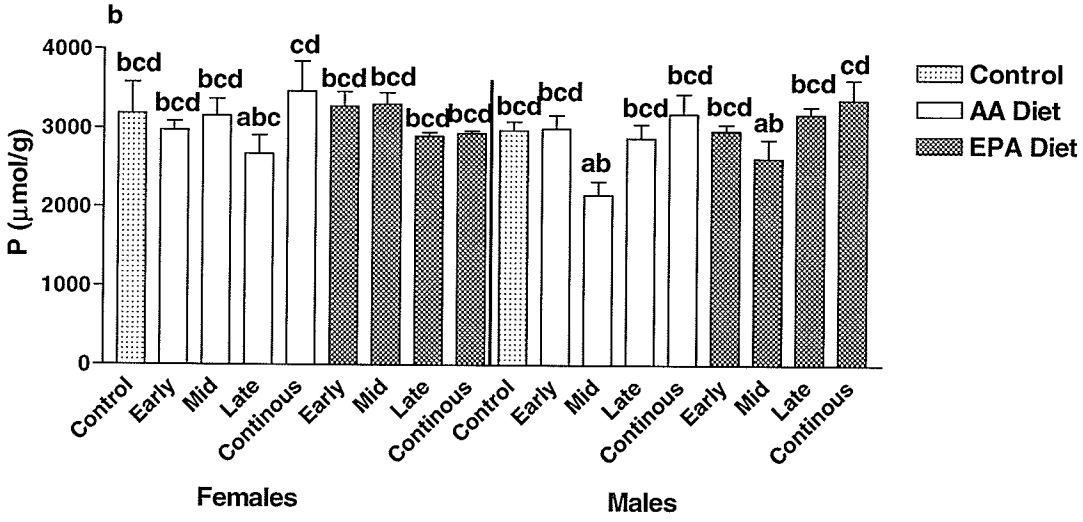

a
2
1
0
Femur BA (cm^2)
a
a
a
c
bc
b
Control
AA
EPA
Control
AA
EPA
Females
Males


b
1.5
1.0
0.5
0.0
Urinary Ca (mmol/L)
bc
c
b
a
a
a
Control
AA
EPA
Control
AA
EPA
Females
Males


a
Femur Neck (mm)
3
2
1
0
a a a a a a a a a
bc bc c bc bc b bc b bc
Control
AA Diet
EPA Diet
Control Early Mid Late Continous Early Mid Late Continous
Females
Control Early Mid Late Continous Early Mid Late Continous
Males


b
Head Width (mm)
6
5
4
3
2
1
0
a a a a a a a a a
b b bc bc c b b b b
Control
AA Diet
EPA Diet
Control Early Mid Late Continous Early Mid Late Continous
Females
Control Early Mid Late Continous Early Mid Late Continous
Males


a
Zn (µmol/g)
Control
AA Diet
EPA Diet
Females
Males
Control
Early
Mid
Late
Continous


b
P (µmol/g)
Control
AA Diet
EPA Diet
Females
Males
Control
Early
Mid
Late
Continous

CHAPTER 8.

DISCUSSION

8.1 Contribution to present state of knowledge

The studies encompassed within this thesis were designed to address a number of objectives and hypotheses in propelling knowledge of the effects of specific LCPUFA on bone at various stages of life. The first set of studies focused on identifying the optimal amount of AA:DHA required to elevate bone mass in infancy. Amounts ranging from 0.5:0.1 to 0.75:0.1 g/100 g of fat were previously shown to increase bone mass (Weiler, 2000; Weiler and Fitzpatrick-Wong, 2002; Blanaru et al, 2004), however, whether higher amounts in the same AA:DHA ratio (5:1 to 7.5:1) lead to greater improvements was unknown. This thesis research confirmed that 0.5:0.1 g/100 g of fat as AA:DHA increases whole body and lumbar spine BMD without affecting bone growth or mineral metabolism. In addition, it found that two and four-fold increments of AA and DHA, similar to intakes of n-6 and n-3 PUFA as used in older animals (Claassen et al, 1995), are not beneficial to bone during infancy. Higher amounts might be worthy of caution in infancy since Zn status, assessed using femur Zn, was reduced by ~10%. It is important to note that at the higher intakes of dietary AA and DHA, EPA declined in the liver. Liver EPA was positively associated and AA:EPA was negatively associated with bone mass measurements. We speculate that higher amounts of AA and DHA would potentially improve bone mass if small amounts of EPA were also given to prevent the decline of EPA in the tissues.

Studies postulate that the mechanism behind changes in bone mass in response to dietary n-6 and n-3 PUFA and LCPUFA are linked to PGE_2 and IGF-1 metabolism (Watkins et al, 1996; Watkins et al, 2000), bone metabolism (Claassen et al, 1995) and Ca metabolism. However, the amount of AA and DHA that was shown to elevate BMD

in piglets was not associated with any alterations in these features of metabolism in infancy. Higher amounts of dietary AA:DHA (1.0:0.2 g/100 g of fat) did reduce bone resorption and increase femur Ca content; in spite of this, bone mass was not altered.

The second major focus of this thesis was to examine if dietary LCPUFA also affected bone following infancy. While BMD was not able to be assessed, long bone growth seemed to be affected as indicated by reductions in femur BA with dietary 0.5 g/100 g of fat as EPA with 0.1 g/100 g of fat as DHA and AA in males. As well, dietary LCPUFA affected femur morphology measurements, including neck width and head width in males. The same effects on femur BA and morphology measurements were not seen for females. There is a possibility that bone growth and mineralization could be elevated (programmed) by dietary LCPUFA given early in life and sustained thereafter to counteract bone loss with age in males. This is indicated by the fact that femoral neck geometry can be programmed by dietary AA, with lesser amounts of EPA and DHA after infancy but before peak bone mass is achieved. Both dietary LCPUFA interventions altered femur P, Mg and Zn with no affect on Ca content. The predominately AA diet given during achievement of peak bone mass in males was programmed for reduced P. Programming of bone mineral composition also seemed evident in both genders fed either LCPUFA diet early in life, since Mg content was elevated. Further, the predominantly EPA diet demonstrated ability to enhance Mg stores in bone at any life stage. In females, withdrawing dietary AA after early growth led to subsequent reductions in AA and Zn stores by late maturity.

As in the studies of piglets, changes in femur morphology and BA were not explained by alterations in bone resorption or PGE_2. Dietary n-3 EPA and DHA reduce

bone resorption in growing rats (Claassen et al, 1995; Kelly et al, 2003). The amounts of n-3 EPA and DHA used in this study (0.6 g/100 g of fat as EPA plus DHA) are much lower than those used in previous research (2.5 to 12.4 g/100 g of fat as EPA plus DHA) (Claassen et al, 1995). Thus, higher amounts of EPA may be required to see a reduction in bone resorption. The amount of EPA in the predominantly EPA diet are similar to that recommended for prevention of chronic disease (Simopoulos, 1999). Current recommendations of EPA could be too low to prevent bone loss that occurs naturally with aging. Here, the rats were followed into maturity; whether bone resorption is altered in the aging rat cannot be elucidated. Dietary DHA was included in both studies at 0.1 g/100 g of fat. It is also possible that higher amounts of DHA would result in beneficial effects on bone mass. Studies showing reduced resorption had a lower ratio of EPA to DHA (~2:1) (Claassen et al, 1995). *Ex vivo* femur PGE_2 was measured at the end of the study. It is possible that PGE_2 concentrations were altered in response to LCPUFA at other points i.e. following the early and mid phases. It would have been advantageous to collect teeth samples following each phase to determine whether PGE_2 changed over time in response to dietary LCPUFA. This approach is currently under validation in the same laboratory as the research within this thesis. The predominately EPA diet reduced Ca excretion in females compared to similar amounts of n-6 LCPUFA (predominantly AA), but had no affect in the males. These changes in urinary Ca excretion did not alter BMC or femur Ca content, however, during bone loss reduced urinary Ca may be beneficial. This data indicates that AA has positive effects during growth, whereas, higher EPA might limit growth but could be beneficial in the prevention of bone loss that occurs naturally with aging.

Research in the field of LCPUFA and bone suggests that the mechanisms behind the observations within this thesis might be related to bone cell proliferation (Manolagas, 1998; Maurin et al, 2002), bone cell differentiation and apoptosis (Manolagas, 1998), cytokine production (Endres et al, 1989; Meydani et al, 1991; Kettler et al, 2001; Priante et al, 2002) and leukotriene metabolism (Gallwitz et al, 1993; Garcia et al, 1996a; Garcia et al, 1996b; Traianedes et al, 1998).

The following are examples of the relationships of LCPUFA to cell proliferation and cytokine production. PUFA and LCPUFA can act as signalling molecules involved in the regulation of gene expression (Duplus et al, 2000; Jump, 2002; Pegorier et al, 2004), which has been emphasized by the discovery of peroxisome proliferators-activated receptors (PPAR). PPAR are nuclear receptors that are activated by the binding of fatty acids and their metabolites (Kliewer et al, 1997). The ligand-receptor complex acts as a transcription factor on target genes (Maurin et al, 2005). Activation of PPAR, specifically PPARγ induce adipogenesis and inhibit osteoblastogenesis (Ogawa et al, 1999; Diascro et al, 1998; Lecka-Czernik et al, 2002). AA and DHA increase PPARγ mRNA expression (Maurin et al, 2005). Thus, AA and DHA could decrease the number of osteoblasts and increase the number of adipocytes found in bone. Adipocytes inhibit osteoblast cell proliferation and it has been proposed that this effect is mediated by LCPUFA (Maurin et al 2000). The presence of adipocytes induces an inhibition of osteoblast cell proliferation (Maurin et al, 2000). This effect was mediated by a factor released by adipocytes that is present in large amounts in the osteoblast supernatant after co-culture with adipocytes, mainly AA and DHA (Maurin 2000). AA and DHA have been reported to dose-

dependently inhibit the proliferation of primary osteoblast cells through cell cycle arrest (Maurin et al, 2002).

Interleukin-1 (IL-1), interleukin-6 (IL-6) and tumor necrosis factor α (TNFα) are inflammatory cytokines (Grimble, 1998) that increase osteoclast formation, activity and lifespan (Manolagas and Jilka, 1995). Dietary EPA (2.7 g/d) and DHA (1.85 g/d) reduces IL-1 and TNFα in the blood of young males (Endres et al, 1989). Dietary EPA and DHA had a similar effect in in the blood of young and older women; decreased IL-1, IL-6 and TNFα (Meydani et al, 1991). AA increases the IL-1 and TNFα mRNA expression in what tissue, whereas, EPA prevents the effect of AA on cytokine gene expression when added simultaneously (Priante et al, 2002). The effect of AA on cytokine mRNA expression was mediated by a protein kinase C-dependent mechanism (Priante et al, 2002). Similar results were seen in another study where AA induced the expression of IL-6 mRNA through activation of protein kinase C in osteoblasts and EPA had no effect (Bordin et al, 2003). Osteoclast resorption is dependent upon the interaction between receptor activator of nuclear factor-$_k$b ligand (RANKL) on cells of the osteoclast lineage with RANK on osteoblast cells (Yasuda et al, 1998; Nakagawa et al 1998). Osteoclast formation is inhibited by osteopotegrin (OPG) a decoy receptor for RANKL (Yasuda et al, 1998). PGE_2 has been shown to stimulate RANKL and inhibit OPG production (Brandstrom et al, 2001; Kanematsu et al, 2000; O'Brien et al, 2001). Fish oil has been shown to decrease the mRNA expression of RANKL and increase the mRNA expression of OPG in mice (Bhattacharya et al, 2005). This is only a short discussion of how LCPUFA (AA, EPA and DHA) influence bone metabolism at a cellular level. Whether the concentrations of LCPUFA used in this *in vitro* work are physiologically relevant to the

skeleton is unclear. Future studies whereby dietary LCPUFA and bone mass are investigated should incorporate these areas of study along with effects on mineral and bone metabolism to determine the specific mechanism of LCPUFA action on bone.

8.2 Summary of major contributions

Study during infancy

- the optimal amount of AA:DHA is 0.5:0.1 g/100 g fat with respect to growth and bone mass in infancy and does not affect mineral or bone metabolism
- amounts of dietary AA and DHA higher than 0.5:0.1 g/100 g of fat at that the same ratio (5:1) are not effective since they reduce bone resorption (ie modelling may be suppressed) and alter the composition of bone mineral without increasing bone mass
- confirmation that dietary AA and DHA elevate bone mass and reduce bone resorption in infancy but not in association with PGE_2 and IGF-1 in infancy
- response of bone resorption to AA and DHA is best assessed using a 24-h sample
- assessment of LCPUFA status using plasma samples should be conducted in a time-controlled manner since AA, EPA and DHA decline over the day and EPA and DHA decline in response to dietary AA

Study from weanling to maturity

- dietary AA, EPA and DHA did not alter BMC in growing and mature rats when given in amounts similar to current intakes and/or recommendations (amounts easily attained in the diet through food)
- amounts of EPA similar to current recommendations reduced femur BA in males, but a similar amount of AA had no affect

- dietary AA during growth appeared to program increased femur neck width in males, potentially increasing proximal femur strength and reducing the risk of fracture
- dietary AA, EPA and DHA altered femur mineral composition when fed at different time points across the life span
- dietary EPA reduced urinary Ca excretion in relation to dietary AA without altering femur Ca content
- males and females respond differently to dietary LCPUFA

8.3 Conclusions

Among the dietary intakes studied at a ratio of 5:1, the amount of AA:DHA to optimize bone mass during infancy was 0.5:0.1 g/100 g of fat and higher amounts offer no additional benefit. Dietary EPA reduced femur appositional growth following weanling, since BA was reduced but length was unaffected. Both dietary EPA and AA influenced mineral composition of bone without changing total BMC. The effects of diets with AA and DHA in infancy and AA, EPA and DHA in older growing animals on bone seen in this thesis were not explained by changes in mineral, PGE_2 or bone metabolism. Males and females responded differently to dietary AA, EPA and DHA, and future studies should continue to investigate responses to LCPUFA on bone in both genders.

8.4 Implications and suggested future research

The implications of this research cannot be directly translated to human nutrition, but rather used to continue to design future studies to elucidate the effects of LCPUFA on bone and direct future studies in humans. The ratio of AA to DHA found to support higher bone mass in piglets is higher than that added to infant formula (0.4-0.6 and 0.15-

0.30, respectively) and current adequate intake recommendations (Simpopoulos, 1999). At present, infant formula companies market formula with added AA and DHA as formula that supports neural and retinal development. Our data indicates that dietary AA and DHA may also be linked to higher bone mass. The piglet research was planned and implemented before infant formula companies began adding AA and DHA to their products. The amount of DHA added to formula is higher than those used in the piglet research, 0.15 to 0.30 versus 0.1 g/100 g of fat as DHA. Future research is needed to investigate how the amounts currently added to infant formula affect bone mass in piglets and the benefits of AA and DHA at 0.5:0.1 g/100 g fat in human infants. To date, there is only one study investigating the effects of dietary AA and DHA on bone mass in human preterm infants. The effects on term infants have not been investigated. The requirements for LCPUFA could be different among preterm and term infants, since LCPUFA stores at birth are limited by preterm birth. It is also important to note that all the research conducted in piglets on the effects of AA and DHA on bone mass has been conducted in males. Future research is needed to determine whether AA and DHA supplementation affects the bone mass of female piglets and whether their response to LCPUFA is the same.

Based on the research conducted in piglets, there is a need for investigation into whether the elevations in bone mass continue with longer-term supplementation and if such benefits are sustained once supplementation ceases. Whether dietary LCPUFA in infancy program final bone mass was not addressed in the rat research and requires further study. Longer-term studies are needed to determine whether improvements in

bone mass during infancy are sustained throughout life increasing the potential for peak bone mass.

Dietary AA and EPA similar to current intakes do influence femur morphology and mineral composition. Intakes of EPA at amounts similar to those recommended for prevention of chronic disease affect femur BA, but not bone resorption or BMC. To observe benefits in bone mass and bone resorption in the prevention of osteoporosis, there may be a need for higher n-3 LCPUFA requirements. There are no current recommendations for dietary AA; however, this data indicates that if total n-6:n-3 ratio is within the recommended range of 5:1 to 10:1 (Simopoulos, 1999), dietary AA may be beneficial to femur morphology. Whether greater femur head and neck widths increase femur bone strength requires testing. Recently in guinea pigs, measurement of femur neck BMD was validated using DXA. Whether DXA can be used to measure neck BMD in the rat requires investigation. The lower BA of the femur, but not femur weight or length, suggests that other aspects of bone geometry than those studied were also modified and requires clarification through use of CT scanning. Future studies need to extend further into aging, using techniques such as CT, that could permit statistical evaluation of bone mass. Currently, whether the use of multivariate analysis of covariance will allow for statistical analysis of whole body, lumbar spine and femur BMD is under investigation.

It is currently unknown whether changes in mineral composition seen with dietary LCPUFA, without alterations in BMC, is beneficial to bone strength and overall body health; and whether reductions of urinary Ca excretion are beneficial to bone or are reflective of a reduced Ca load (i.e. reduced Ca absorption). Mineral balance studies

could clarify femur mineral composition and if Ca excretion is altered in response to dietary LCPUFA; and may indicate whether these changes are due to mineral metabolism i.e. absorption or tissue uptake (including bone). In addition, further research is needed to determine how dietary LCPUFA influence bone mass, BA, femur mineral composition and femur morphology. The mechanisms studied in this thesis did not explain changes seen in bone in response to dietary LCPUFA.

This thesis has added to the current knowledge of the effects of dietary LCPUFA on bone and mineral metabolism. The research findings contribute to the current state of knowledge of the effects of LCPUFA throughout life. A greater understanding of how LCPUFA affect bone will assist experts in determining LCPUFA recommendations based over all health, including bone outcomes. As well, this study has identified new areas of study for the effects of LCPUFA on bone mineral composition and femur morphology.

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APPENDIX A.

SUPPLEMENTAL TABLES

Table A.1 The effect of AA and DHA supplementation on bone mineral density of the whole body, lumbar spine and femur in piglets fed formula for 15 d[1] (Chapter 4, Figure 4.8.1).

	Control Diet	AA:DHA Diets (g/100 g of fat)		
		0.5:0.1	**1.0:0.2**	**2.0:0.4**
Whole Body BMD (g/cm^2)	0.264^a ±0.005	0.282^b ±0.004	0.272^{ab} ±0.004	0.265^a ±0.004
Lumbar Spine BMD (g/cm^2)	0.245^a ±0.007	0.270^b ±0.006	0.252^{ab} ±0.006	0.245^{ab} ±0.006
Femur BMD (g/cm^2)	0.233 ±0.009	0.262 ±0.008	0.244 ±0.008	0.248 ±0.008

[1]Data are mean ± SEM, n=35. Different subscripts identify differences among groups, a<b<c.

Abbreviations: AA=arachidonic acid, BMD=bone mineral density, DHA=docosahexaenoic acid.

Table A.2 The effect of AA and DHA supplementation on urinary NTx, plasma OC and ex vivo femur PGE_2 in piglets fed formula for 15 d[1] (Chapter 4, Figure 4.8.2).

	Control Diet	AA:DHA Diets (g/100 g of fat)		
		0.5:0.1	**1.0:0.2**	**2.0:0.4**
Urinary Ntx:creatinine (nmol BCE:mmol)	7601^{ab} ±941	8201^{ab} ±1027	5315^{a} ±1027	9714^{b} ±940.7
Plasma OC (nmol/L)	12.47 ±0.50	13.31 ±0.54	11.44 0.61	13.08 0.64
Femur PGE_2 (ng/g of bone)	5.50 ±0.90	4.59 ±0.66	5.18 ±0.67	4.85 ±0.47

[1]Data are mean ± SEM, n=35. Different subscripts identify differences among groups, a<b<c.

Abbreviations: AA=arachidonic acid, DHA=docosahexaenoic acid, NTx= N-telopeptides of type 1 collagen cross-links, OC=osteocalcin, PGE_2=prostaglandin E_2

Table A.3 The effect of AA and DHA supplementation and time on urinary NTx and plasma OC in piglets fed formula for 15 d[1] (Chapter 6, Figure 6.8.1).

	Diet				Time (h)		
	Control	AA:DHA (g/100 g of fat)					
		0.5:0.1	1.0:0.2	2.0:0.4	0900	1500	2100
Urinary NTx:Creatinine (nmol BCE:mmol)	7007^{b} ±727	6650^{b} ±642	4471^{a} ±597	7386^{b} ±826	7647^{y} ±600	6928^{y} ±692	4723^{x} ±518
Plasma OC (nmol)	11.98 ±0.32	12.301 ±0.37	11.37 ±0.34	12.31 ±0.37	72.48 ±0.31	11.89 ±0.30	11.61 ±0.28

[1]Data are mean ± SEM, n=32. Different subscripts identify differences among diets (a<b<c) and time (x<y).

Abbreviations: AA=arachidonic acid, DHA=docosahexaenoic acid, NTx= N-telopeptides of type 1 collagen cross-links, OC=osteocalcin

Table A.5 The interaction between dietary AA and DHA and time on plasma EPA and DHA proportions in piglets fed formula for 15 d[1] (Chapter 6, Figure 6.8.2).

	Control	AA:DHA (g/100 g of fat)		
		0.5:0.1	1.0:0.2	2.0:0.4
EPA				
0900 h	0.16 ± 0.02	0.25 ± 0.03	0.17 ± 0.02	0.20 ± 0.03
1500 h	0.16 ± 0.02	0.15 ± 0.01	0.12 ± 0.01	0.13 ± 0.01
2100 h	0.13 ± 0.02	0.14 ± 0.01	0.11 ± 0.01	0.11 ± 0.01
DHA				
0900 h	1.51 ± 0.18	2.20 ± 0.22	1.95 ±0.12	2.39 ± 0.17
1500 h	1.53 ± 0.14	1.42 ± 0.15	1.50 ± 0.08	1.73 ± 0.14
2100 h	1.11 ± 0.08	1.38 ± 0.16	1.44 ± 0.11	1.62 ± 0.16

[1]Data are mean ± SEM, n=35.
AA=arachidonic acid, DHA=docosahexaenoic acid, EPA=eicosapentaenoic acid

Table A.6 The effect of long chain polyunsaturated fatty acids on whole body, lumbar spine and femur bone mineral density in rats over 45 weeks[1].

	Control Diet	AA Diet	EPA Diet
Whole Body BMD (g/cm^2)	0.182 ± 0.001	0.182 ± 0.001	0.182 ± 0.001
Lumbar Spine BMD (g/cm^2)	0.241 ± 0.002	0.241 ± 0.002	0.238 ± 0.002
Femur BMD (g/cm^2)	0.380 ± 0.003	0.380 ± 0.003	0.378 ± 0.004

[1]Data presented as means ± SEM, n=106.
Control Diet: no AA, EPA or DHA, AA Diet: 0.5 g AA, 0.1 g EPA, 0.1 g DHA /100 g of fat, EPA diet: 0.5 g EPA, 0.1 g AA, 0.1 g DHA /100 g of fat,
Abbreviations: AA=arachidonic acid, BMD=bone mineral density, DHA=docosahexaenoic acid, EPA=eicosapentaenoic acid

Table A.7 The effect of long chain polyunsaturated fatty acids on femur bone area and urinary Ca in males and female rats[1] (Chapter 7, Figure 7.8.1).

	Females			Males		
	Control Diet	**AA Diet**	**EPA Diet**	**Control Diet**	**AA Diet**	**EPA Diet**
Femur BA (cm^2)	1.45 ±0.02[a]	1.43 ± 0.02[a]	1.45 ± 0.02[a]	1.95 ± 0.02[b]	1.93 ± 0.03[b]	1.84 ± 0.03[a]
Urinary Ca (mmol/L)	1.13 ± 0.10	1.34 ± 0.11	0.95 ± 0.10	0.33 ± 0.03	0.28 ± 0.05	0.36 ± 0.04

[1]Data presented as means ± SEM, n=106. Different subscripts identify differences among the groups

Control Diet: no AA, EPA or DHA, AA Diet: 0.5 g AA, 0.1 g EPA, 0.1 g DHA /100 g of fat, EPA diet: 0.5 g EPA, 0.1 g AA, 0.1 g DHA /100 g of fat,

Abbreviations: AA=arachidonic acid, BA=bone area, Ca=calcium, DHA=docosahexaenoic acid, EPA=eicosapentaenoic acid

Table A.8 The effects of dietary long chain polyunsaturated fatty acids on femur head and neck width when incorporated at different stages (early, mid or late) or continuously across the lifespan compared to control in male and female rats[1] (Chapter 7, Figure 7.8.2).

	Control Diet	AA Diet				EPA Diet			
		Early	Mid	Late	Continuous	Early	Mid	Late	Continuous
Femur Neck Width (mm)									
Females	2.06 ± 0.08	2.00 ± 0.03	1.99 ± 0.02	2.00 ± 0.06	1.98 ± 0.08	2.00 ± 0.03	2.13 ± 0.05	2.08 ± 0.03	2.03 ± 0.06
Males	2.38^{ab} ± 0.04	2.48^{ab} ± 0.06	2.59^{b} ± 0.09	2.46^{ab} ± 0.05	2.48^{ab} ± 0.06	2.37^{a} ± 0.07	2.40^{ab} 0.04	2.31^{a} ± 0.03	2.38^{ab} ± 0.06
Femur Head Width (mm)									
Females	4.22 ± 0.10	4.15 ± 0.07	4.12 ± 0.03	4.21 ± 0.04	4.05 ± 0.07	4.16 ± 0.07	4.19 ± 0.04	4.19 ± 0.06	4.12 ± 0.07
Males	4.71^{a} ± 0.07	4.76^{a} ± 0.11	4.88^{ab} ± 0.11	4.92^{ab} ± 0.07	5.08^{b} ± 0.04	4.81^{a} ± 0.08	4.83^{a} ± 0.09	4.71^{a} ± 0.03	4.72^{a} ± 0.03

[1]Data presented as means ± SEM, n=106. Different subscripts identify differences within the gender, a<b<c.
Control: no AA, EPA or DHA, AA diet: 0.5 g AA, 0.1 g EPA, 0.1 g DHA/ 100 g of fat, EPA diet 0.5 g EPA, 0.1 g AA, 0.1 g DHA/ 100 g of fat
Abbreviations: AA=arachidonic acid, DHA=docosahexaenoic acid, EPA=eicosapentaenoic acid

Table A.9 The effects of dietary long chain polyunsaturated fatty acids on femur zinc and phosphorus (μmol/g of bone) when incorporated at different stages (early, mid or late) or continuously across the lifespan compared to control in male and female rats[1] (Chapter 7, Figure 7.8.3).

	Control Diet	AA Diet				EPA Diet			
		Early	Mid	Late	Continuous	Early	Mid	Late	Continuous
Zn (μmol/g of bone)									
Females	4.01^{bc} ± 0.47	2.76^{a} ± 0.11	3.30^{ab} ± 0.27	3.11^{ab} ± 0.20	4.70^{c} ± 0.44	3.78^{abc} ± 0.33	3.73^{abc} ± 0.46	3.12^{ab} ± 0.27	3.02^{ab} ± 0.19
Males	3.66 ± 0.26	3.65 ± 0.07	3.29 ± 0.22	3.48 ± 0.17	3.90 ± 0.35	3.12 ± 0.19	3.29 ± 0.22	3.70 ± 0.29	3.85 ± 0.50
P (μmol/g of bone)									
Females	3185.83 ± 396.95	2979.46 ±112.92	3159.80 ± 21183	2677.99 ± 234.08	3468.86 ± 379.87	3277.06 ± 188.17	3307.66 ± 146.36	2904.22 ± 43.55	2941.55 ± 26.87
Males	2979.46^{cd} ± 112.92	3000.09^{bcd} ± 171.07	2155.64^{ab} ± 171.21	2880.95^{bcd} ± 177.42	3184.10^{bcd} ± 258.19	2972.86^{bcd} ± 83.26	2624.39^{ab} ± 236.43	3182.51^{bcd} ± 95.36	3367.33^{cd} ± 454.22

[1]Data presented as means ± SEM, n=106. Different subscripts identify differences within the gender, a<b<c<d.
Control: no AA, EPA or DHA, AA diet: 0.5 g AA, 0.1 g EPA, 0.1 g DHA/ 100 g of fat, EPA diet 0.5 g EPA, 0.1 g AA, 0.1 g DHA/ 100 g of fat
Abbreviations: AA=arachidonic acid, EPA=eicosapentaenoic acid,P=phosphorus, Zn=zinc