Enhanced Lumbar Spine Bone Mineral Content and Bone Mineral Density in SGA Piglets Fed Arachidonic Acid and Docosahexaenoic Acid are Modulated by Birth Weight

BY

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A Thesis
Submitted to the Faculty of Graduate Studies
in Partial Fulfillment of the Requirements for the Degree of

MASTER OF SCIENCE

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THE UNIVERSITY OF MANITOBA

FACULTY OF GRADUATE STUDIES

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Enhanced Lumbar Spine Bone Mineral Content and Bone Mineral Density in SGA Piglets Fed Arachidonic Acid and Docosahexaenoic Acid are Modulated by Birth Weight

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ABSTRACT

ENHANCED LUMBAR SPINE BMC AND BMD IN SGA PIGLETS FED ARACHIDONIC ACID AND DOCOSAHEXAENOIC ACID ARE MODULATED BY BIRTH WEIGHT

The infant born small size for gestational age (SGA) has low bone mass. Dietary arachidonic acid (AA) and docosahexaenoic acid (DHA) enhance bone mass in normal birth weight (BW) piglets; however, the benefits in the SGA neonate are unknown. In this 15 d study, two levels of dietary AA + DHA (0.6 or 1.2 g/100 g of fat as AA plus DHA as 0.1 or 0.2 g/100 g of fat) versus control diet were tested for effects on growth, fatty acid status, bone mass and metabolism in SGA piglets categorized as either very low BW ($\leq$ 1.0 kg) or low BW (1.1 to 1.2 kg). Differences in outcomes for each BW category were detected by one-way ANOVA with post-hoc Bonferroni tests. Growth did not respond to diet, yet the low BW piglets fed 0.6:0.1 AA + DHA as g/100 g of fat had elevated bone mass in the spine, whereas the very low BW piglets had higher bone mass of the spine if fed the higher intake of AA + DHA. In both BW categories, the higher intake of AA + DHA lowered bone resorption relative to controls, but bone formation was unchanged. Fatty acid concentrations reflected dietary AA in all tissues, without a decline in DHA status. This study provides evidence that bone mass is enhanced by both levels of AA + DHA in the SGA piglet in a manner determined by the severity of growth restriction at birth. Interventions are needed to determine if AA + DHA can improve bone mass of the human infant born SGA.

ACKNOWLEDGEMENTS

This thesis reflects the collective contributions of many individuals who helped shape this project. I am grateful to my thesis committee members, Dr. H. Weiler, Dr. B. Watts, and Dr. M. Seshia, Dr. P. Tappia, and Dr. A. Kennedy for academic advice, encouragement, and enthusiasm. A very special thank you to my advisor, Dr. Hope Weiler, for her valuable expertise and outstanding guidance and support at every step of the way. I wish to express my appreciation to Shirley Fitzpatrick-Wong, Marilyn Latta, and Dennis Laboissiere for their superb technical assistance. I am indebted to the animal holding facility staff at the Ft. Garry campus for their knowledgeable and meticulous care of our piglets. Thank you to my fellow graduate students and summer students who helped with animal care and lab work. Heartfelt thanks to my colleague and friend Jinping Zhao for her tireless assistance and valuable insights.

Thank you to my husband Brent and daughter Brittney for their forbearance and loving support throughout my graduate program. And finally, to my parents, Stella and Maurice Kohut, who inspired me with their own achievements and contributed in countless ways to the success of this project.

This research was supported by a grant from the National Sciences and Engineering Research Council (NSERC).

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LIST OF ABBREVIATIONS

5-LO	5-Lipoxygenase
11ß-HSD	11ß-hydroxysteroid dehydrogenase
AA	Arachidonic acid
AGA	Appropriate for gestational age
ALA	Alpha-linolenic acid
ANOVA	Analysis of variance
BA	Bone area
BAP	Bone alkaline phosphatase
BFR	Bone formation rate
BMC	Bone mineral content
BMD	Bone mineral density
BMI	Basal metabolic index
BMP-2 and –4	Bone morphogenetic proteins –2 and -4
BW	Birth weight
COX	Cyclooxygenase
DHA	Docosahexaenoic acid
DHLA	Dihomo-γ-linolenic acid
DPA	Dual photon absorptiometry
DXA	Dual energy x-ray absorptiometry
EFA	Essential fatty acid
ELISA	Enzyme linked immunosorbent assay
EPA	Eicosapentaenoic acid
EPO	Evening primrose oil
FAME	Fatty acid methyl esters
FGF	Fibroblast growth factors
GC	Glucocorticoids
GH	Growth hormone
GLA	Gamma-linolenic acid
HPAA	Hypothalamic-pituitary-adrenal axis
ICTP	Cross-linked C-terminal telopeptide of type I collagen
IFN-γ	Interferon-γ
IGF-I	Insulin-like growth factor - I
IGF-II	Insulin-like growth factor - II
IGF1IR	Insulin-like growth factor – I receptor
IGF2R	Insulin-like growth factor – II receptor
IGFBP	IGF binding proteins
IL-4, -10, -17, -18	Interleukin-4, -10, -17, -18
IUGR	Intrauterine growth restriction
LA	Linoleic acid
LBM	Lean body mass
LBW	Low birth weight
LC PUFA	Long chain polyunsaturated fatty acids
LS	Lumbar spine
LTB_4	Leukotriene B_4

NTX	Cross-linked N-telopeptide of type I collagen
OB	Obese gene
PDGF	Platelet-derived growth factor
PGE_1	Prostaglandin E_1
PGE_2	Prostaglandin E_2
PGE_3	Prostaglandin E_3
$PGF_{2\alpha}$	Prostaglandin $GF_{2\alpha}$
PGH	Placental growth hormone
PGI_2	Prostaglandin I_2
PGJ_2	Prostaglandin J_2
PICP	Carboxy-terminal propeptide of type I procollagen
PLA_2	Phospholipase A_2
PPARs	Peroxisome proliferator activated receptors
PPARγ	Peroxisome proliferator activated receptor-gamma
PTH	Parathyroid hormone (Parathormone)
PUFA	Polyunsaturated fatty acids
QC	Quality control
QCT	Quantitative computed tomography
RIA	Radioimmunoassay
SD	Standard deviation
SGA	Small for gestational age
SPA	Single photon absorptiometry
TBG	Thyroid binding globulin
TGFβ-1	Transforming growth factor beta-1
TNF-α	Tumor necrosis factor- α
TNFβ	Tumor necrosis factor- β
TSH	Thyroid stimulating hormone
TXA_2	Thromboxanes
VLBW	Very low birth weight

1.0 LITERATURE REVIEW

1.1 SMALL FOR GESTATIONAL AGE BIRTH

1.1.1 Definition

Birth weight is the most widely used postnatal measurement of fetal growth. Weight at birth is "both a reflective indicator of fetal nutritional status and a prospective indicator of postnatal health and survival" (Caulfield 1991). Classification of birth weight as either appropriate (AGA) or small for gestational age (SGA) is based on evidence of significantly increased risk of neonatal morbidity and mortality in newborns with birth weight below a defined level for the corresponding gestational age (Hack and Fanaroff 1999). The 10^{th} percentile for gestational age is commonly used as a diagnostic threshold for identification of the fetus at increased risk of neonatal morbidity and mortality (Battaglia and Lubchenco 1967).

Other definitions for diagnosis of SGA birth include birth weight ≤ 2500 g (Strauss and Dietz 1998), birth weight ≤ -2 SD from the mean value for gestational age (Albertsson-Wikland et al. 1993; Lee et al. 2003a), birth weight $< 3^{rd}$ percentile (Paz et al. 1993; McIntire et al. 1999), birth length $< 3^{rd}$ percentile for gestation (Hokken-Koelega et al. 1995), or birth length ≤ -2 SD from mean reference values for height (Karlberg and Albertsson-Wikland 1995). Birth weight for gestational age is generally considered the most robust and reproducible parameter (Wales et al. 1997) and is therefore most often used. Correct classification of an infant as AGA or SGA depends upon appropriate intrauterine growth curves and accurate estimation of gestational age, preferably based on ultrasonography rather than menstrual dating (Mongelli et al.1996).

1.1.2 SGA Birth: Incidence and Risk Factors

Despite technological advances in obstetrical care, the rate of SGA births in Canada has remained relatively stable over the past two decades at approximately 9% of live Canadian births (Health Canada 2000). Predictors of SGA birth weight identified in a recent Canadian study include maternal smoking, low maternal pre-pregnancy weight ($\leq$ 45 kg), low pregnancy weight gain, advanced maternal age, alcohol and drug use, and multiple gestation (Newburn-Cook et al. 2002). Maternal factors linked to increased risk of preterm (< 37 wk. gestation) delivery were nulliparity, previous SGA birth or preterm delivery, multiple pregnancy, preexisting illness, pregnancy complications, and height < 152 cm. (Newburn-Cook et al. 2002) (see Table 1-1). Of the 33 maternal factors studied, several significantly affected gestational duration and/or birth weight; however, only a few of these were regarded as potentially modifiable (Newburn-Cook et al. 2002). Another Canadian research group has reported an upward trend in delayed childbearing among Canadian women associated with significant increases in low birth weight (<2500 g) (10.9%) and preterm (13.6%) deliveries in women over 35 years of age (Tough et al. 2002). Very young maternal age has also been identified as a risk factor for adverse pregnancy outcomes including low birth weight (<2500 g) and preterm births (Buschman et al. 2001; Statistics Canada 1998).

1.1.3 Economic and Social Impact of SGA Birth

Small for gestational age births are strongly associated with increased neonatal and infant mortality (Cnattingius et al. 2000), as well as infant and childhood morbidity

Table 1-1. Adjusted odds ratios and 95% confidence intervals (in parentheses) for risk factors associated with preterm delivery and SGA birth in a Canadian population.

Risk Factor	Preterm Delivery	Preterm SGA	Term SGA
Preexisting illness			
Diabetes mellitus	2.40 (2.08-2.72)	–	–
Hypertension	2.08 (1.75-2.41)	2.31 (1.67-2.95)	1.80 (1.49-2.11)
Chronic renal disease	2.31 (1.45-3.15)	5.02 (3.74-6.30)	
Obstetrical history			
Nulliparity	1.12 (1.03-1.21)	1.64 (1.40-1.88)	1.54 (1.46-1.62)
Past history SGA birth	1.60 (1.06-2.14)	11.62 (10.92-12.32)	8.20 (7.95-8.45)
Past history preterm delivery	3.81 (1.62-3.08)	1.41 (1.03-1.91)	–
Current pregnancy			
Multiple gestation	19.36 (19.19-19.51)	46.86 (46.54-47.18)	–
Anemia	1.56 (1.12-2.00)		
Pregnancy induced hypertension	1.68 (1.48-1.88)	5.90 (5.54-6.26)	–
Anthropometric/socio-demographic factors			
Maternal height < 152 cm	–	2.94 (2.38-3.48)	2.05 (1.87-2.23)
Pre-pregnancy weight ≤ 45 kg	–	–	3.11 (2.91-3.31)
Age ≥ 35 yr. at delivery	1.15 (1.03-1.27)	1.47 (1.19-1.75)	1.31 (1.30-1.32)
Lifestyle factors			
Smoking	1.31 (1.20-1.42)	2.16 (1.92-2.40)	2.25 (2.18-2.32)
Alcohol consumption	–	–	1.37 (1.16-1.58)
Drug use	2.50 (2.10-2.90)	2.04 (1.06-3.02)	1.42 (1.09-1.75)
Pregnancy weight gain < 0.5 kg/wk	–	–	3.11 (2.91-3.31)

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

(Goldenberg et al. 1998). SGA infants at greatest risk of neonatal complications include very small and premature infants, who often require lengthy hospitalizations and frequent readmissions to hospital for post-discharge complications (Blackburn 1995), and may suffer long-term neurocognitive deficits (Goldenberg et al. 1998).

1.1.4 Fetal Growth

Fetal growth and weight at birth reflects the effects of fetal and maternal genotypes, maternal metabolism and nutritional status during pregnancy, utero-placental function, and duration of gestation. In normal human gestation, fetal weight gain increases from about 5 g/d at 14-15 wk. gestation to 10 g/d at 20 wk., and peaks at 30-35 g/d at 32-34 wk. with subsequent slowing until term (Williams et al. 1982). A period of linear growth peak velocity begins at approximately 9-10 wk. gestation (Deter et al. 1999), peaks in the second trimester, and is followed by linear growth deceleration at approximately 17-20 wk. until term (Lee et al. 2003b).

1.1.5 Determinants of Fetal Growth

Altered fetal growth resulting in SGA birth weight may result from maternal, fetal, or placental factors, or a combination of these. Cases with no apparent cause are classified as idiopathic.

1.1.5.1 Maternal Factors

Maternal influences on birth weight include sociodemographic variables including ethnic background and socioeconomic status, lifestyle factors, chronic medical conditions, and pregnancy-related variables including gestational weight gain and certain

complications of pregnancy (Scott et al. 1981). Nutrition prior to and during pregnancy is an important determinant of fetal growth. This is reflected in positive correlations of low prepregnancy BMI and low gestational weight gain and SGA birth (Newburn-Cook et al. 2002). The association of SGA birth with maternal anthropometric measurements such as low maternal birth weight (Skjaerven et al. 1997) and short stature (reviewed by Kramer et al. 1999) may reflect genetic influences or intergenerational effects of intrauterine growth restriction.

1.1.5.2 Fetal Factors

Fetal factors associated with impaired intrauterine growth include congenital and chromosomal abnormalities, genetic defects, and certain intrauterine infections (Lee et al. 2003b).

1.1.5.3 Placental Factors

The placenta acts as an endocrine organ by production of hormones and growth factors required for maternal adaptations that facilitate tissue growth in both mother and fetus. In late gestation, placental size correlates with fetal growth rate, suggesting a regulatory role in fetal growth. The placenta is the sole means of nutrient delivery to the fetus. Through blood flow distribution and metabolic and endocrine activity, placental function determines the level of fetal nutrient supply. Adaptive mechanisms facilitate placental transfer of certain nutrients in order to preserve fetal nutritional adequacy. For example, during the period of accelerated growth and development in the third trimester of pregnancy, fetal accretion of nutrients such as calcium (Shaw 1976) and lipids

(Clandinin et al. 1980; Clandinin et al. 1989) is elevated. Placental dysfunction encompasses a broad range of conditions that affect fetal growth through reduced placental nutrient transport or by perturbations in metabolic and endocrine activities that influence fetal growth (Petraglia et al. 1996).

1.1.6 Nutrition and Fetal Growth

Adequate nutrient supply is essential to fetal growth, particularly during critical periods of development when specific tissues or organs have greater susceptibility to specific nutrient deficiencies. Nutrient delivery to the fetus is determined by maternal health and nutritional status. Maternal undernutrition and overnutrition can both impair fetal growth (Wu et al. 2004) and may program long-term metabolic alterations leading to adult chronic diseases (Hoet and Hanson 1999).

1.1.6.1 Macronutrients

All macronutrients are important to fetal growth; however, maternal intake of protein, carbohydrates, and lipids at particular times during pregnancy may influence body weight and length at birth. A high carbohydrate intake (in grams) in early pregnancy is inversely related to birth weight and placental weight, particularly if followed by low dairy protein intake in late gestation (Godfrey et al. 1996). Glucose is the primary energy substrate for the developing fetus. Placental transfer of glucose is driven by the concentration gradient between maternal and fetal circulations, and glucose uptake by fetal tissues is similarly determined by local glucose concentrations (Hay 1995). Amino acid transfer to the fetus occurs via active transport and is regulated by

placental metabolism (Aldoretta and Hay 1995). Deficient placental amino acid transport has been documented in human (Cetin et al. 1992) and porcine (Finch et al. 2004) fetal growth restriction.

Lipids contribute to fetal growth and development through a variety of mechanisms. The importance of maternal lipid transfer to fetal bone development is reflected in the positive relationship between maternal fat stores and neonatal bone mass (Godfrey et al. 2001). Maternal prepregnancy nutritional status determines the composition of lipids supplied to the fetus. Certain lipids, in particular the essential fatty acids (EFA), are supplied to the fetus entirely via maternal transfer (Uauy et al. 1999). As a result, fetal EFA status reflects the adequacy of maternal EFA adipose supply. Experimental EFA deficiency in rat pregnancy is associated with increased body weight and altered bone formation in adult offspring (Korotkova et al. 2005).

1.1.6.2 Micronutrients

Other nutrients important for fetal bone growth include calcium, phosphorus, iron, zinc, and vitamins C, D, and K. Calcium and phosphorus deposition in fetal bone depends upon adequate materno-placental transfer of calcium and phosphorus against a concentration gradient. Bone mineral acquisition is greatest during the third trimester of pregnancy, when the fetus acquires approximately 2/3 of its expected total bone mass at term (Rigo et al. 2000). In the human fetus, calcium accretion rates at 35 wk of gestation are estimated at between 150 mg/kg/d. and those of phosphorus at 75 mg/kg/d (Rigo et al. 2000). Placental transfer of calcium to the fetus is increased in response to elevated mineral demands of multiple gestation (Luke 2005), often at the expense of maternal

bone mineral reserves. Studies in the growth-restricted rat fetus have shown reduced materno-fetal calcium transfer across *in situ* perfused placentas, indicative of impaired placental mineral transport, in association with lower fetal total body calcium (Mughal et al. 1989).

Severe gestational zinc deficiency in experimental animals impairs fetal growth and shortens gestation (reviewed by Castillo-Duran and Weisstaub 2003). Isolated zinc deficiency is uncommon in the human neonate, and is usually seen in the context of global malnutrition. Although available evidence suggests that the zinc status of the SGA infant is not reduced at birth (Chunga Vega et al. 1996), the subsequent decline in zinc levels may reflect a combination of low body zinc stores and high growth demands (Domenech et al. 2001). Linear growth and motor development is improved in very low birth weight (BW < 1500 g) preterm infants supplemented with zinc (Friel et al. 1993).

Vitamin C and iron are required for hydroxylation of prolyl and lysyl residues during collagen cross-linking (Tuderman et al. 1977). Iron is also a cofactor for renal activation of vitamin D_3 (DeLuca 1976). Iron deficiency anemia in pregnancy increases with young maternal age, multiple gestation, multiparity, and vegetarian diet, and is associated with prematurity and low birth weight offspring (Scholl and Hediger 1994).

1.1.7 Timing of Fetal Growth Restriction

Both the magnitude and timing of events leading to impaired fetal growth have important consequences to neonatal outcomes and childhood growth attainment. Animals exposed to early gestational nutrient restriction are less likely to recover from their growth deficits than those who experience an adverse environment later in gestation

(Villar and Belizan 1982; McCance and Widdowson 1974). In the human neonate, first trimester growth restriction is associated with an increased risk of SGA and extremely preterm birth (Smith 2004).

Evidence from human and animal studies suggests that nutritional insults imposed at different stages of gestation cause disproportionate as well as reduced fetal growth through disruption of normal growth patterns of particular organs or tissues at "critical periods" (Milani et al 2000) in gestation (reviewed by Cameron and Demerath 2002). Disproportionate fetal growth may lead to metabolic, structural, and endocrine perturbations associated with obesity and adult chronic disease (reviewed by Cameron and Demerath 2002).

1.1.8 Genetic and Epigenetic Modulation of Fetal Growth

Heritable influences on birth weight are suggested by a positive relationship between maternal and infant birth weights (Skjaerven et al. 1997). However, maternal size may also influence infant birth weight through metabolic characteristics that determine the capacity to provide nutrients for fetal growth (Duggleby and Jackson 2001). Nutritional imbalances resulting from impaired placental delivery of nutrients to the fetus may modify expression of genetically determined metabolic processes leading to restricted fetal growth. It is often difficult to distinguish effects of the intrauterine environment from genetic influences on birth weight. For example, strong correlations between sibling birth weights (Tanner et al. 1972) and clustering of low birth weight infants within families (Wang et al. 1995) may reflect genetic or epigenetic influences.

1.1.9 Postnatal Growth of the SGA Infant

The postnatal period represents a time of rapid tissue growth. Accelerated postnatal growth occurs in the majority of SGA infants (Falkner et al. 1994), resulting in catch up in weight by approximately 6 months of age (Fitzhardinge & Inwood 1989), while recovery in length may not be apparent until 2 - 4 yr. of age (Albertsson-Wikland et al. 1993; Leger et al. 1997). Approximately 10% of children born SGA will remain ≤ -2 SD for height at 2 yr. of age (Albertsson-Wikland et al. 1993; Leger et al. 1997), while 8% demonstrate persistent short stature in adolescence (Paz et al. 1993), and adulthood (Karlberg and Albertsson-Wikland 1995; Kistner et al. 2004). In SGA-born children with delayed linear growth at 2 yr. of age, the relative risk of short stature at 18 yr. of age is 7.1 if SGA is based on birth length and 5.2 if based on birth weight (Albertsson-Wikland and Karlberg 1997). Despite evidence of capacity for rapid growth in the SGA infant, altered bone metabolism programmed in early life may not be ameliorated by catch up in height and may continue to affect skeletal function in later life.

Epidemiological and animal studies demonstrate an association between SGA birth weight and adverse adult health outcomes. The SGA neonate is predisposed to metabolic syndrome (Barker et al. 1989), low peak bone mass (Cooper et al.1995), and osteoporosis (Javaid and Cooper 2002; Yarbrough et al. 2000) in adult life. There is evidence that these relationships may be mediated in part through altered body composition. Reduced lean body mass and disproportionately high percentage body fat are reported in the SGA neonate at birth and may be linked to subsequent development of obesity and insulin resistance (Hediger et al. 1998; Lapillonne et al. 1997). Further observations from human and animal studies suggest that interactions between SGA birth weight and postnatal

growth patterns determine later health outcomes. Rapid catch up growth in the SGA infant is linked to obesity (Law 2001) and other risk factors for cardiovascular disease (Barker 2002). The same alterations in endocrine and paracrine regulation of growth stemming from adverse neonatal growth that likely lead to metabolic syndrome, obesity and cardiovascular diseases also have the capacity to affect bone. These factors may be responsible for permanent short stature and high risk of osteoporotic fracture.

1.1.10 Endocrine/Paracrine Regulation of Perinatal Growth

Endocrine control of perinatal growth involves hormones of fetal, maternal, and placental origin in addition to a wide array of peptide growth factors produced within fetal tissues. Fetal endocrine function develops early in gestation and is modified as development progresses (Fowden and Hill 2001). Intrauterine factors causing altered nutrient availability have a significant impact on fetal growth and metabolic functions. Both undernutrition and overnutrition may result in altered fetal growth. In general, nutrient deprivation suppresses production of anabolic hormones (insulin, insulin-like growth factors, and thyroxine) while increasing levels of catabolic hormones (cortisol, growth hormone), whereas nutritional excess typically produces opposing results (Fowden and Forhead 2004). The "fetal origins of disease hypothesis" proposed by Barker (1995) is based primarily on programming of the endocrine axis including IGF, GH and cortisol. The following section will thus address endocrine regulation of fetal and infant growth.

1.1.10.1 Transforming Growth Factor Beta-1

Transforming growth factor beta-1 (TGFβ) is comprised of a group of dimeric 25-kD disulfide-linked proteins that exist as three isoforms: TGFβs 1, 2, and 3. All three isoforms have specific functions in early pregnancy (Ando et al. 1998). The most common isoform, TGFβ-1, may also regulate fetal growth in later gestation (Ostlund et al. 2002). Cord blood TGFβ-1 levels are reduced in human pregnancies complicated by growth restriction, and correlate positively with birth weight and cord blood IGF-I (Ostlund et al. 2002).

1.1.10.2 Growth Hormone

Growth hormone (GH) is a 22 kD polypeptide produced by the anterior pituitary gland. Despite evidence of pituitary GH production in the human fetus (reviewed by Lee et al. 2003b), a minor role has been ascribed to GH in early intrauterine growth due to low fetal expression of GH receptors in early and mid gestation (Gluckman et al. 1981). Development of GH receptors in late gestation and early postnatal life coincides with the transition to GH-mediated growth in infancy (Simard et al. 1996). In the fetal pig, intrauterine decapitation does not affect continued fetal growth (Stryker and Dziuk 1975), suggesting minimal GH actions *in utero* in this species. Congenital GH deficiency in humans is associated with impaired growth *in utero* and in infancy (Gluckman et al. 1992), with linear growth delay evident within the first 6 months of life (Mehta et al. 2005). SGA-born children demonstrate low to normal circulating GH or have evidence of abnormal patterns of GH secretion (Albertsson-Wikland et al. 1998; Boguszewski et al. 1997).

During infancy and childhood, GH stimulates linear bone growth by promoting chondrocyte proliferation in the growth plate (Ohlsson et al. 1998). Many of the anabolic actions of GH are mediated by hepatic and locally produced IGF-I, which in turn inhibits pituitary GH gene expression in a negative feedback loop (Berelowitz et al. 1981).

Human placental GH (PGH), a placental variant of GH, is a member of a superfamily of hormones including pituitary GH and human placental lactogen (Handwerger and Freemark 2000). PGH regulates fetal growth indirectly through effects on maternal metabolism and tissue growth, thereby increasing nutrient availability to the fetus (Caufriez et al. 1990). Maternal PGH relates positively to fetal growth (Chellakooty et al. 2004).

1.1.10.3 Insulin

The fetal pancreas produces insulin early in development (Fowden and Hill 2001), and insulin levels at birth are positively related to birth weight (Fowden 1995). Insulin promotes tissue accretion of glucose, amino acids, and lipids, but has little effect on cellular differentiation (Fowden 1995). Reduced pancreatic β-cell mass and insulin content are reported in the growth-restricted fetal sheep (Limesand et al. 2005) and rat (Dahri et al. 1991). Circulating insulin levels are reduced in SGA neonates (Bajoria et al. 2002). A role for insulin in catch up growth is suggested by a positive correlation between insulin levels and postnatal growth in the SGA infant (Soto et al. 2003).

1.1.10.4 The Insulin-Like Growth Factor (IGF) System

The insulin-like growth factors (IGFs) are major determinants of perinatal growth. IGFs promote cellular proliferation and differentiation through autocrine (intracellular),

paracrine (intercellular), and classical endocrine mechanisms (Baker et al. 1993). The IGF system is comprised of two ligands (IGF-I and IGF-II), two cell surface receptors (IGF1R and IGF2R), IGF binding proteins (IGFBP 1 to 6) and IGFBP proteases. IGF-I, a 7.65 kD 70-amino acid peptide and IGF-II, a 67-amino acid peptide, share 70% amino acid sequence homology (Daughaday and Rotwein 1989) and 62% homology with proinsulin (reviewed by Dupont and Holzenberger 2003). Human and porcine IGF-I are identical in their amino acid sequences (Tavakkol et al. 1988). IGFBP modulate IGF activity by controlling their availability to cell surface receptors (Stewart and Rotwein 1996).

Detectable in the first trimester, IGFs are produced primarily in fetal liver as well as muscle, fat, and growth plate (Baker et al. 1993). In human pregnancy, the effects of IGF-II predominate during embryogenesis, while IGF-I acquires greater importance in later gestation (Baker et al. 1993). IGFBP-1 and IGFBP-2 are the main carriers of fetal circulating IGF-I, while IGFBP-3 becomes the predominant binding protein after birth (Wang et al. 1991). IGF-I levels increase throughout human gestation, and at birth, cord blood levels correlate positively with birth weight (Thieriot-Prevost et al. 1988). Among growth-restricted infants, those who experienced catch up growth during infancy had comparable IGF-I levels at 1 yr. to those of AGA infants (Thieriot-Prevost et al. 1988). During childhood, circulating IGF-I levels are predictive of linear growth velocity in the following year (Juul et al. 1994), and are highest at age 5 yr. in children with lowest birth weights and most rapid postnatal growth (Ong et al. 2002).

IGF-II is the predominant circulating form of IGF in the pig, while levels of IGF-I increase only in the last 3 weeks of gestation (Lee et al. 1991). As in the human, a

positive relationship between IGF-I and birth weight plasma is reported in the neonatal piglet (Dauncey et al. 1994; Herpin et al. 1992). IGF-I levels in the piglet increase from birth to 2 wk. of age with normal growth (Carroll et al. 2000), and exogenous IGF-I stimulates catch up growth in growth-restricted pigs (Schoknecht et al. 1997). IGF-I is highly responsive to nutritional stimuli during perinatal growth. In the fetal rat, circulating IGF-I is reduced by maternal malnutrition during gestation (Davenport et al. 1990) and recovers after postnatal nutritional repletion (Thissen et al. 1994). Nutritional restriction in the growing pig lowers both IGF-I and IGF-II (Carroll et al. 1998). Enhanced growth in response to increased energy intake in 2-month old pigs is associated with elevated hepatic and plasma IGF-I levels, suggesting endocrine regulation of growth (Weller et al. 1994).

1.1.10.5 Leptin

Leptin is the 16 kD polypeptide product of the *ob* (obese) gene. Its primary postnatal role is to regulate body weight and energy expenditure through feedback mechanisms between adipose tissue and hypothalamic satiety centers (Halaas et al. 1995). Sources of leptin during pregnancy include placenta and adipocytes of fetal and maternal origin (Kiess et al. 1998). In the human neonate, cord leptin is positively associated with birth weight (Cetin et al. 2000) and fat mass (Harigaya et al. 1997). During critical periods of growth, interactions between leptin and other hormones modulate changes in body weight, body composition, and sexual maturation (reviewed by Cameron and Demerath 2002).

Recent evidence suggests a role for leptin in the regulation of bone metabolism. Leptin stimulates bone growth in mice deficient in the *ob* gene (Steppan et al. 2000), and relates positively with bone mass and fat mass in the neonatal piglet (Weiler et al 2002). Nutritional regulation of leptin has been demonstrated in the growing pig (reviewed by Barb et al. 2001), the neonatal lamb (Ehrhardt et al. 2003), and the fetal rat (Korotkova et al. 2002). In the latter study, the ratio between the essential fatty acids linoleic acid and α-linolenic acid in the maternal rat diet determined leptin levels in the offspring (Korotkova et al. 2002). A link between leptin and IGF- I was established by Ajuwon et al. (2003) who observed dose-dependent effects of exogenous leptin on hepatic production and circulating levels of IGF-I in growing pigs.

1.1.10.6 Cortisol

Glucocorticoids (GC) may either stimulate or inhibit cellular proliferation and differentiation during fetal growth in a concentration-dependent manner (Liggins 1976; Devenport et al. 1989). In animal models, late gestational increases in cortisol are essential to fetal maturation through the induction of expression of enzymes necessary for postnatal metabolic adaptation (Fowden 1995; Liggins 1994). High GC levels, however, negatively affect fetal growth in humans (Reinisch et al. 1978), rats (Levitsky et al. 1986), and mice (Reinisch et al. 1978). In addition to direct adverse effects on cell proliferation and differentiation, excess GC suppress hepatic IGF-I production (Delany and Canalis 1995), further restricting fetal growth.

Excessive *in utero* exposure to GC has been implicated as a mechanism linking fetal growth restriction with adult chronic disease through stimulation of the

hypothalamic-pituitary-adrenal axis (HPAA) (Nyirenda and Seckl 1998). Fetal levels of GC are regulated by the placental enzyme 11ß-hydroxysteroid dehydrogenase (11ß-HSD), which acts as a barrier to the transfer of maternal cortisol to the fetus. Evidence of GC excess in fetal growth restriction is suggested by an inverse relationship between plasma cortisol and birth weight in the human (Goland et al. 1993) and the pig (Wise et al. 1991). Similar findings are reported in growth-restricted offspring of the unilaterally hysterectomized ovariectomized sow, who exhibit altered adrenocortical function including elevated plasma cortisol, higher adrenal gland weight, and increased responsiveness to adrenocorticotropic hormone stimulation (Klemcke et al. 1993).

1.1.10.7 Thyroxine

In addition to regulating metabolism, thyroid hormone exerts direct effects on tissue development (reviewed by Fowden and Forhead 2004). Levels of fetal thyroxine, thyroid stimulating hormone (TSH), and thyroid binding globulin (TBG) increase throughout normal human gestation (Thorpe-Beeston et al. 1991). In the SGA fetus, thyroxine levels are reduced and TSH levels increased (Thorpe-Beeston et al. 1992), in part attributable to decreased hepatic production of TBG (Klein et al. 1997). Fetal hypothyroidism results in growth restriction and abnormal skeletal development (Fowden 1995). Thyroxine deficiency in fetal pigs is associated with decreased hepatic and muscle IGF-I content (Latimer et al. 1993). *In vitro* triiodothyronine treatment of growth plate cartilage from the fetal pig results in enhanced chondrocyte maturation, suggesting a role in longitudinal bone development (Burch and Lebovitz 1982).

1.2 BONE

1.2.1 Fetal Bone Development

Fetal bone development occurs by two distinct mechanisms: intramembranous ossification and endochondral ossification. In both processes, a collagenous matrix is replaced by woven bone, which is then modeled by the opposing actions of bone formation and bone resorption to form mature lamellar bone.

1.2.1.1 Intramembranous Ossification

Intramembranous ossification is the process by which collagen and other proteins secreted by osteogenic cells are deposited within embryonic mesenchymal tissue to form osteoid. Subsequent mineralization of osteoid results in formation of woven bone, to be later replaced by mature lamellar bone through bone turnover. Flat bones of the skull, facial bones, and scapula are formed by this mechanism.

1.2.1.2 Endochondral Ossification

The long bones, vertebrae, and pelvis are formed by endochondral ossification in which a continuously growing cartilaginous template is progressively replaced by bone. Primordial limb buds develop in mesodermal tissue, and within these, mesenchymal cells differentiate into chondrocytes. Chondrocytic hypertrophy and proliferation produce hyaline cartilage models of the limbs covered by perichondrium at about 6 weeks gestation (Moore and Persaud 1998). Inner perichondrial cells differentiate into osteoblasts and secrete an organic matrix of type I collagen and proteoglycans (Price et al. 1994). This matrix is mineralized by deposition of calcium and phosphate

hydroxyapatite to form immature woven bone, and later replaced by mature lamellar bone.

By week 7 of gestation, a ring of ossification encircles the bone shaft, and osteoblasts have migrated into the bone cavity to form a primary ossification center (Price et al. 1994; Moore and Persaud 1998). Trabecular bone is formed within the medullary cavity and the central calcified cartilage resorbed to form the bone marrow cavity (Price et al. 1994). By 16 weeks, the basic skeletal structure and biological function are established (Miller 2003; Moore and Persaud 1998). Coincident with this is the onset of fetal movement, which through bone loading forces determines bone strength and physical geometry. The process by which bone attains its final three-dimensional shape and size for optimal bone strength and for soft tissue attachment is known as modeling.

1.2.2 Bone Modeling

Modeling is the aspect of perinatal bone development that increases bone mass and modifies bone size and shape in response to mechanical and other stimuli. In this process, resorption of existing bone by osteoclasts on the inner endosteal surface occurs concurrently with osteoblast-induced new bone formation at the outer periosteum. The overall effect during skeletal growth is to promote acquisition of bone mass and to maintain an appropriate structural form. Since bone formation and resorption occur independently, activating stimuli may initiate either process (Burr & Martin 1989). This is distinct from the cyclical process of adult bone remodeling in which the two processes are coupled in order to maintain bone mass. Changes in bone shape and size resulting

from modeling reflect the balance between osteoblast and osteoclast activity over a given period of time (Bain and Watkins 1993). Biomechanical forces are a major stimulus for modeling during growth (Carter DR. et al. 1996) and may act via PGE_2 (Yeh and Rodan 1984).

1.2.3 Regulation of Fetal Osteogenesis

Skeletal development is controlled by multiple mechanisms, including prominent actions of local growth factors such as insulin-like growth factors-1 and –II, transforming growth factor-beta (TGF-β), and fibroblast growth factors (FGFs). Maternal nutritional status before and during pregnancy determines fetal nutritional adequacy through transfer of nutrients essential for fetal bone development, while placental hormonal and metabolic activities regulate this process. The following section will discuss aspects of fetal bone mineral accretion relevant to this thesis and will give a brief overview of two key growth factors with actions in fetal bone development.

1.2.3.1 Transforming Growth Factor Beta-1 (TGFβ-1)

TGFβ-1 is expressed in embryonic and adult skeletal tissue, where its actions include modulation of cellular differentiation and phenotypic expression of osteoblasts, osteoclasts, and chondrocytes (Bonewald and Dallas 1994; Rosen et al. 1988). TGFβ-1 inhibits bone resorption *in vitro* (Pfeilschifter et al. 1988) and stimulates formation of periosteal woven bone in the neonatal rat (Noda and Camilliere 1989).

1.2.3.2 Insulin-like Growth Factors-I and -II

IGF-I and IGF-II both contribute to fetal bone development through autocrine, paracrine, and endocrine actions. IGF-I and IGF1R are expressed in osteoblasts, osteoclasts, and chondrocytes. IGF-I exerts predominantly anabolic actions, promoting bone matrix synthesis and bone formation (reviewed by Dupont and Holzenberger 2003). *In vitro* studies indicate regulatory actions of the IGFs over cellular differentiation and proliferation (Canalis 1993). The clinical disorder Laron syndrome caused by IGF-I deficiency is associated with marked osteoporosis (Laron et al. 1998) Transgenic mice deficient in IGF-I display impaired ossification and linear bone growth (Clemmons 1998), in contrast to normal skeletal size in IGF-II deficient mice (DeChiara et al. 1990). These and other studies highlight the significant role of the IGFs in bone development.

1.2.4 Postnatal Skeletal Growth

1.2.4.1 Longitudinal Bone Growth

At birth the long bones are completely ossified with the exception of the epiphyses. Cartilage growth plates within the epiphyses are the sites of linear bone growth. This occurs by endochondral ossification, in which chondrocyte proliferation and differentiation at the diaphyseal aspect of the growth plate leads to formation of bone matrix which is then mineralized to form bone (Price et al. 1994). During growth, the growth plate and the articular cartilage are separated by newly formed bone. Secondary ossification centers form in the cartilaginous epiphysis, resulting in conversion of central epiphyseal cartilage to bone, at which time linear growth ceases. At puberty, skeletal bone mass represents over 90% of its maximum, but does not peak until age 25-30. Peak

bone mass represents an individual's maximum bone mass and is an important determinant of risk of osteoporosis in later life.

1.2.4.2 Appositional Growth

Growth in width and thickness of the long bones occurs through modeling, in which osteoblasts produce dense cortical bone within the periosteum, and osteoclasts remove existing bone on the inner endosteal surface by resorption. The result is radial appositional growth in bone mass.

1.2.5 Bone Structure

Bone is a specialized form of connective tissue with properties of strength, rigidity, and some degree of elasticity. Bone also functions as a reservoir of calcium and other inorganic ions, and participates in calcium homeostasis. The two main morphological forms of bone are lamellar and woven bone. Lamellar bone includes dense compact or cortical bone forming the outer layer of the long bones, while trabecular (cancellous) bone is found within the central cavity of bone. Woven bone is an immature form of bone produced in early fetal bone development, in fracture repair, and in disorders associated with increased bone turnover.

1.2.5.1 Lamellar Bone

Lamellar cortical bone comprises approximately 80-85 % of total bone mass and is found primarily in the appendicular skeleton (Price et al. 1994) where it provides mechanical support and attachment to soft tissues. Compact bone is made up of multiple

columns of bones parallel to the long axis of the bone. Each column contains concentric lamellae or layers of bone within which a central neurovascular channel (Haversian canal) interconnects with periosteum and endosteum via Volkmann's canals. Deposition of new bone in lamellae reduces the diameter of the Haversian canal, trapping osteoblasts as osteocytes in lacunar spaces in the matrix. Along the outer aspect of bone, periosteal osteoblasts produce dense lamellae that become cortical bone, while at the inner endosteal surface, osteoblasts create irregular lamellae that merge with trabecular bone of the central cavity.

1.2.5.2 Trabecular (Cancellous) Bone

Cancellous or trabecular bone accounts for 15-20% of skeletal bone and is most abundant in the axial skeleton where it occupies the medullary cavities (Price et al. 1994). Trabecular bone is comprised of a network of thin spicules or trabeculae made up of irregular lamellar bone and lined by cellular elements including osteoblasts, osteoclasts, and osteo-progenitor cells. Osteocytes are imbedded within the lamellae while bone marrow occupies the inter-trabecular spaces. The extensive surface area of trabecular bone provides an active site of bone modeling highly sensitive to intrinsic and extrinsic stimuli (Price et al. 1994).

1.2.5.3 Woven Bone

Woven bone is characterized by irregularly oriented collagen fibres that subsequently undergo mineralization. In bone repair or modeling, these fibres are gradually re-arranged in parallel fashion and then mineralized to form lamellar bone.

1.2.6 Bone Composition

Bone is composed of cellular elements and an organic extracellular matrix intimately associated with the mineral phase.

1.2.6.1 Cellular Elements of Bone

The cells found in bone: osteoblasts, osteocytes, osteoclasts, and bone lining cells, participate in regulation of calcium homeostasis, bone modeling and remodeling, and maintenance of bone strength.

1.2.6.1a Osteoblasts

Osteoblasts are responsible for synthesis and secretion of osteoid, the organic component of bone matrix. In addition, osteoblastic activity promotes osteoid mineralization at periosteal and endosteal surfaces through production of growth factors and alkaline phosphatase, which hydrolyzes inhibitory organic phosphate compounds.

1.2.6.1b Osteocytes

Upon completion of matrix synthesis, osteoblasts become embedded within the matrix in lacunar spaces as osteocytes. Their function is to maintain the structural integrity of mineralized matrix by acting as mechanosensory cells through communication via gap junctions with other bone cells (Donahue et al. 1995). Osteocytes also participate in bone modeling in response to mechanical stress by mediating short-term calcium deposition or release directed by PTH and calcitonin.

1.2.6.1c Osteoclasts

Osteoclasts, multinucleated cells of macrophage-monocyte origin, are responsible for bone resorption. These are found in endosteal spaces called Howship's lacunae in which a low pH promotes enzymatic degradation of bone matrix by collagenases, proteases, cathepsins, and tartrate resistant acid phsophatase (Price et al. 1994). Differentiation and activity of osteoclasts are regulated by osteoblasts through production of local and systemic factors (Rodan 1998). Factors favoring bone resorption include calcitriol, PTH, IL-I, and TNF-α; while calcitonin, estrogens, BMP-2 and –4, PDGF, IL-4, -10, -17, -18, and calcium are antiresorptive (reviewed by Janssens et al. 2005).

1.2.6.1d Bone Lining Cells

Bone lining cells are flattened osteoblast-derived cells that form a protective layer on the bone surface. They may also differentiate into osteogenic cells under certain conditions (Miller and Jee, 1992).

1.2.6.2 Bone Matrix Proteins

Bone osteoid is primarily composed of type I collagen embedded in glycoprotein ground substance and linked to noncollagenous proteins including osteocalcin, osteonectin, osteopontin, bone sialoprotein, and fibronectin (Price et al. 1994). Bone matrix proteins are secreted and mineralized by osteoblasts and contribute to bone turnover through interactions with bone cells. Osteoid mineralization involves binding of matrix proteins to crystalline hydroxyapatite [$Ca_{10}(PO_4)_6(OH)_2$] and trace amounts of sodium, potassium, magnesium, zinc, fluoride, and carbonate (Russell et al. 1986).

Mineralized matrix imparts structure and strength to bone and functions as a mineral reserve.

1.2.6.2a Type I Collagen

Type I collagen constitutes over 90% of bone matrix proteins. Synthesis of collagen involves post-translational modifications during processing to its final form (reviewed by Muller et al. 1981). The collagen molecule (tropocollagen) is made up of three polypeptide chains (α chains) arranged in a helix. The triple helix is assembled in the rough endoplasmic reticulum of the osteoblast, where each α chain undergoes hydroxylation of proline and lysine residues to form hydroxyproline and hydroxylysine. Vitamin C is a required cofactor for this reaction. This is followed by glycosylation of certain hydroxylysine residues to form a procollagen molecule with peptide chains ending with a C terminal. Three procollagen chains are incorporated into a helical protein stabilized by cross-links at the amino (N) and carboxy (C) terminals. The N- and C-terminal extension peptides are cleaved extracellularly from the protein by specific proteases, enter the circulation and are excreted via the kidney. The remaining tropocollagen is secreted into the extracellular matrix where it is cross-linked into fibrils that further aggregate into thick collagen bundles.

1.2.6.2b Osteocalcin

Osteocalcin, a 49-amino acid 5.7 kD polypeptide specific to osteoblasts and odontoblasts, is the major non-collagenous protein in bone matrix (Price et al. 1994). While the precise physiological function of osteocalcin remains unresolved, it is generally accepted as a marker of bone turnover. Synthesis of osteocalcin by osteoblasts

is under the control of calcitriol (Puchacz et al. 1989). Vitamin K-dependent carboxylation of glutamate residues in the osteocalcin molecule facilitates binding to hydroxyapatite crystals in bone matrix (Marcus 1996; Svard et al. 1986). Carboxylated osteocalcin not incorporated into bone, in addition to uncarboxylated osteocalcin, is released into the circulation (Robins 1994).

1.2.6.2c Osteonectin

Osteonectin, a 42 kD glycoprotein specific to bone and periodontal ligament cells, may act as another local regulator of bone cell function.

1.2.6.2d Osteopontin

Osteopontin, a highly phosphorylated and glycosylated protein produced by osteoclasts, is capable of binding to type 1 collagen, osteocalcin, and osteoclasts, through which it is believed to mediate cell-matrix interactions (Denhardt & Noda 1998). Through its high affinity for hydroxyapatite crystals, osteopontin facilitates *in vitro* mineral deposition in bone matrix (Boskey et al. 1993). In addition, osteopontin modulates osteoclast differentiation in mice (Rittling et al. 1998) and *in vitro* cell signaling (Miyauchi et al. 1991).

1.2.7 Regulation of Bone Metabolism

A detailed discussion of the regulation of bone metabolism and bone formation is beyond the scope of this thesis. The following section provides a brief overview of the endocrine and local factors involved. Among a plethora of local growth factors and

cytokines active in bone, three key growth factor families: the TGF-βs, the IGF system, and the bone morphogenetic proteins (BMPs), act in concert to determine bone growth and peak bone mass (reviewed by McCarthy et al. 2000). Relevant to the topic of this thesis, eicosanoids derived from dietary LC PUFA interact with many of the above factors and thus play an integral role in bone metabolism. An abbreviated list of hormones and growth factors involved in bone metabolism is found in Table 1-2.

1.2.7.1 Calcitriol

Calcitriol (1,25-dihydroxyvitamin D_3), the biologically active form of vitamin D, is formed in the kidney by hydroxylation of 25-OH-vitamin D_3 from the liver. The overall effects of calcitriol are to provide increased substrate for bone formation. Calcitriol promotes bone mineralization through enhanced intestinal calcium absorption and reduced renal calcium clearance (Hadley 2000). When dietary calcium intake is low, calcitriol stimulates bone resorption (Holick 1996) in order to maintain calcium homeostasis. Plasma phosphate levels may also regulate production of calcitriol (Hadley 2000).

1.2.7.2 Parathyroid Hormone

Parathyroid hormone (PTH) is a peptide hormone (molecular weight 9,500) secreted by the chief cells of the parathyroid gland in response to hypocalcemia. The main actions of PTH occur in bone, kidney, and small intestine. PTH elevates circulating calcium levels through stimulation of bone resorption, renal tubular calcium reabsorption, and renal phosphate excretion (Hadley 2000). PTH induces renal 1α-OHase activity and

therefore $1,25(OH)_2$-D_3 synthesis, and participates in regulation of bone remodeling through autocrine or paracrine actions on osteoclasts mediated via osteoblasts. Small daily doses of PTH promote bone formation through enhanced synthesis of type I collagen (Gunness-Hey and Hock 1984), mediated in part by IGF-I (Canalis et al. 1989).

1.2.7.3 Calcitonin

Calcitonin is a 32-amino acid peptide secreted by thyroid clear cells in response to hypercalcemia (Hadley 2000). Calcitonin inhibits bone resorption through reduced osteoclast binding to bone, enhances renal calcium clearance (Deftos et al. 1999), and stimulates calcitriol metabolism (Hadley 2000). Regulation of intestinal calcium absorption by calcitonin has been demonstrated in the growing piglet (McKercher and Radde 1981).

1.2.7.4 Growth Hormone

Growth hormone is an important regulator of postnatal longitudinal bone growth. Deficiency of GH in childhood leads to short stature, while excess results in acromegaly (Hadley 2000). Comparisons of tibial linear growth in mice deficient in either IGF-I or GH receptors suggest dual roles for GH at the epiphyseal growth plate; an IGF-I-independent effect on chondrogenesis followed by IGF-I mediated stimulation of chondrocyte proliferation (reviewed by Isaksson et al. 1987). In addition, GH stimulates osteoblast proliferation and function including production of osteocalcin and type I collagen (reviewed by Isaksson et al. 1987).

Table 1-2. Interactions between hormones and growth factors in bone

Factor	*Effect on osteoblast (OB) and bone formation*	*Effect on osteoclast (OC) and bone resorption*	*Interactions with other growth factors and hormones*
IGF-I	+ OB proliferation + Collagen synthesis + Bone formation *in vivo*	+ Osteoclastogenesis Coupling agent between bone resorption and formation	Mediates anabolic PTH actions TGFβ-1 promotes IGF-I synthesis and release from OB PGE_2 promotes IGF-I gene expression
TGFβ-1	+ OB activity + Bone matrix formation + New bone formation *in vivo*	– Bone matrix degradation Coupling agent between bone resorption and formation	Acts synergistically with multiple growth factors in bone to modulate bone resorption and formation
BMP-2	+ OB differentiation + Ectopic bone formation *in vitro* + Fracture healing	+ OC differentiation and function	TGFβ-1 decreases BMP nRNA expression in fetal rat calvarial cell culture – GC effects on OB in vitro + TGFβ-1mRNA in human OB-like cells
IL-I	+ OB proliferation and differentiation *in vitro* – OB proliferation *in vitro* – Bone matrix synthesis	+ OC differentiation and function + Bone resorption	– TGFβ-1 secretion by OB-like cells + TGFβ-1 activity in calvaria + PGE_2 release from fetal rat calvarial cells

Table 1-2. Interactions between hormones and growth factors in bone (continued).

Factor	*Effect on osteoblast and bone formation*	*Effect on osteoclast and bone resorption*	*Interactions with other growth factors and hormones*
IL-6	+/– OB differentiation *in vitro*	+ OC differentiation and function (indirectly)	
PDGF	+ OB replication – Bone matrix formation + Fracture healing *in vivo*	+ OC replication + Bone resorption	TGFβ-1 stimulates PDGF mRNA expression in OB
bFGF	+ OB replication + Fracture healing – Type I collagen synthesis	+/– Osteoclastogenesis +/– Bone resorption	
TNF-α	+ OB proliferation + OB apoptosis – Osteocalcin production by OB – Type I collagen synthesis	+ OC formation from bone marrow macrophages	TGFβ-1 potentiates 0C formation by TNF-α TGFβ-1 suppresses OB apoptosis and bone resorption by TNF-α
IFN-γ	– Bone formation *in vivo* – Type I collagen synthesis	+/ – Osteoclastogenesis +/ – Bone resorption	TGFβ-1 antagonizes suppression of OC formation by IFN-γ
PGE_2	+ Bone formation + OB differentiation and proliferation Mediates TGFβ-1-stimulated OB proliferation	+ Bone resorption + OC differentiation and proliferation Mediates PTH-induced OC formation	+ IGF-I synthesis by OB + BMP-2 expression in mesenchymal stem cells

Table 1-2. Interactions between hormones and growth factors in bone (continued).

Hormone	*Effect on osteoblast and bone formation*	*Effect on osteoclast and bone resorption*	*Interactions with other growth factors and hormones*
$1,25(OH)_2D_3$	+ Expression of type I collagen, osteocalcin, and alkaline phosphatase in mature OB + Bone matrix mineralization +/– OB proliferation (concentration-dependent)	+ Bone resorption at high doses (indirectly) – Bone resorption *in vivo* – Bone resorption *in vitro* at physiological doses	+ intestinal calcium absorption – renal calcium excretion
PTH	+ Bone formation *in vitro* and *in vivo* (intermittent administration)	+ Bone resorption *in vivo* (indirectly)	+ Activation of vitamin D in liver and kidney
Glucocorticoids (GC)	– Type I collagen production – OB proliferation and differentiation – OB synthesis of IGF-I + OB apoptosis	+ Bone resorption *in vivo* and *in vitro*	– IGF-I actions in bone cells – IGF-I expression in growth plate – IL-I-induced bone resorption and PGE_2 production
Estrogen	+ OB proliferation and differentiation + Bone formation *in vivo*	+ OC apoptosis – OC maturation and function – Bone resorption *in vivo*	+ Synthesis of IGF-I by OB
Androgens	+ OB differentiation and proliferation + Maintenance of bone mass *in vivo*	– Bone resorption *in vitro*	

Adapted from: Janssens K, ten Dijke P, Janssens S, Van Hul W. (2005) TGF-β1 to the bone. Endoc Rev; May 18; [Epub ahead of print].

1.2.7.5 Glucocorticoids

At physiologic concentrations, glucocorticoids promote negative calcium balance through reduced renal calcium reabsorption and inhibition of vitamin D-stimulated intestinal calcium absorption (Hadley 2000). When present in excess, glucocorticoids negatively affect bone mass acquisition through suppression of osteoblastic production of IGF-I (Delany and Canalis 1995) and type I collagen (Delany et al. 1995). Longitudinal bone growth is attenuated in prepubertal mice by exogenous GC in association with reduced IGF-I expression in the epiphyseal growth plate (Smink et al. 2003).

1.2.7.6 IGF-I and IGF-II

The IGFs are the most abundant growth factors produced in bone. Skeletal IGF-I may originate from osteoblasts, circulating IGF-I, and bone marrow cells (Rosen and Donahue 1998; Hayden et al. 1995). The primary source of bone IGF-I is believed to be that produced locally (Rosen 2004), where it acts in an autocrine or paracrine manner to induce osteoblast proliferation and type I collagen synthesis (Hock et al. 1988). Targeted osteoblast-specific deletion of the IGF-I receptor gene in the mouse results in impaired bone formation and mineralization (Zhang M. et al. 2002). *In vitro* actions on osteoclast formation and activation (Mochizuki et al. 1992; Hill et al. 1995) suggest a role in bone modeling and remodeling (reviewed by Rosen 2004).

IGF-I is potently anabolic in growing bone, promoting longitudinal growth (Baker et al. 1993; Yakar et al. 2002) and bone mass accretion (Mohan et al. 2003) in animal models. Linear growth velocity during childhood correlates with plasma IGF-I levels (reviewed by Daughaday and Rotwein 1989). Regulatory control of IGF-I activity in

bone involves interactive effects of endocrine, nutritional, and local growth factors (Fowden and Forhead 2004). While GH is the primary IGF-I regulator, insulin (Baker et al. 1993), PTH (McCarthy et al. 1989), estradiol (Ernst and Rodan 1991), and PGE_2 (McCarthy et al. 1991) all act as trophic stimuli of IGF-I synthesis in bone.

Indirect actions of IGF-I on bone metabolism may be mediated through enhanced renal calcitriol synthesis as demonstrated in the rat (Wong et al 1997; Wong et al. 2000). Lack of IGF-I in genetically IGF-I-deficient mice exacerbates the effects of calcium restriction on femur BMD during pubertal growth in conjunction with reduced bone formation, increased bone resorption, and decreased serum calcitriol levels relative to controls (Kasukawa et al. 2003), adding support for the premise that IGF-I is necessary for normal calcitriol function. Evidence of IGF-I effects on calcium balance is further provided in pig studies in which exogenous GH elevates calcium absorption in addition to increasing circulating IGF-I and calcitriol (Denis et al. 1994).

1.2.7.7 Transforming Growth Factor Beta-1 (TGFβ-1)

TGFβ-1, the predominant isoform in bone, is produced by osteoblasts (Robey et al. 1987) and osteoclasts (Oursler 1994) and released into bone matrix in an inactive protein-bound form that becomes activated during bone resorption (Sporn et al. 1987). *In vitro*, TGFβ exhibits varied actions including stimulation of osteoblast activity (Reddi et al. 1987) and bone matrix formation (Noda and Camilliere 1989), and suppression of matrix degradation (Sporn et al. 1987). Subcutaneous administration of TGFβ induces new bone formation in animals (Mackie and Treshel 1990).

1.2.7.8 Bone Morphogenetic Proteins

Bone morphogenetic proteins (BMP) are a group of 8 peptides with considerable amino acid sequence homology with TGFβ. BMPs act as morphogens during fetal limb development by providing positional information that facilitates organized bone growth or skeletal patterning (reviewed by Kaplan and Shore 1996). In postnatal life, BMP contribute to fracture repair (Rasubala et al. 2003), and have been shown to induce new bone formation in the rat (Cooley et al. 2005). *In vitro*, BMP-2 reverses glucocorticoid-induced inhibition of mineralization in osteoblast cell culture (Luppen et al. 2003).

1.2.7.9 Eicosanoids

As discussed earlier, prostaglandins are key regulators of bone metabolism. PGE_2, PGE_3, and PGE_1 are more potent stimulators of resorption than the less active PGI_2 and $PGF_2\alpha$. Other eicosanoids besides prostaglandins may affect bone turnover. Leukotriene B_4 (LTB_4), the 5-LO metabolite of AA, stimulates bone resorption *in vitro* and *in vivo* (Garcia et al. 1996).

PGE_2, the most abundant prostaglandin produced in bone, exhibits biphasic concentration-dependent actions (Raisz and Fall 1990). At low concentrations it acts as a potent stimulator of bone formation in rats (Jee et al. 1990), while higher levels inhibit *in vitro* (Raisz and Fall 1990) and *in vivo* bone formation (Watkins et al. 1997), and additionally stimulate bone resorption (Akatsu et al. 1989). High intakes of AA significantly increase eicosanoid synthesis in both animal and human studies (Huang and Craig-Schmidt 1996; Whelan 1996). A high n-6 LC PUFA diet in rats is associated with greater *ex vivo* PGE_2 production in bone culture compared with an n-3 rich diet (Li et al.

1999). In addition to dietary influences, PGE_2 production is also regulated by hormonal and paracrine factors. PTH, TGFβ, IL-1 (Raisz et al. 1993), IGF (McCarthy et al. 1991), and mechanical stress (Somjen et al. 1980) are all activators of PGE_2 synthesis in bone, while estrogen (Pilbeam et al. 1989) and androgens (Pilbeam and Raisz 1990) are suppressive.

1.2.7.10 Interleukins

Interleukin-1 (IL-1) is a 17 kD peptide which exists as α and β isoforms. IL-1 inhibits bone matrix synthesis (Rosenquist et al. 1996) and promotes bone resorption *in vitro* (Dedhar 1989). IL-6 is a 23-28 kD protein produced by osteoblasts and chondrocytes which induces osteoclastogenesis in cell culture (Kukita et al. 1990).

1.2.7.11 Tumor Necrosis Factor

Tumor necrosis factor (TNF) exists as two forms, TNF-α and TNF-β, both of which inhibit type 1 collagen synthesis and promote bone resorption (Rosenquist et al. 1996). TNF-α in addition suppresses osteocalcin production by osteoblasts (Rosenquist et al. 1996).

1.2.7.12 Platelet-Derived Growth Factor

Platelet derived growth factor (PDGF) is a polypeptide stored in platelets and released in response to injury. PDGF stimulates proliferation of embryonic cells *in vitro* (Antoniades 1984) and may play a role in fetal tissue growth. In addition, PDGF is expressed at the site of fracture repair (Rasubala et al. 2003).

1.2.7.13 Interferons

Interferons are potent inhibitors of cell proliferation that exist as either α, β, and γ isoforms (Price et al. 1994). Interferon-γ (IFN-γ) is produced by activated T lymphocytes in acute inflammation (Takayanagi et al. 2000). Actions of IFN-γ in bone inhibit resorption (Takayanagi et al. 2000) and are antagonistic to those of IL-1 and TNF-α (reviewed by Udagawa 2003).

1.2.8 Bone Mineralization in the SGA Infant

At birth, the SGA infant exhibits reduced bone mass (Namgung et al 1993) relative to the neonate with AGA birth weight. As bone mass depends upon both skeletal size and mineral density, low BMC may be indicative of a deficit of either component (Molgaard et al. 1997). It is not surprising that infant bone mineral content (BMC) at birth correlates positively with birth weight, since body weight is a known predictor of BMC (Koo et al. 1996). However, there is evidence that even after adjustment for body size, preterm (Atkinson & Randall-Simpson 2000) and term (Petersen et al. 1989) SGA infants are still lower in whole body BMC those born with an AGA birth weight.

Although the basis for low bone mass in SGA-born neonates is not established, deficits in both bone size and mineralization appear to be contributory. Low cord blood osteocalcin (an index of osteoblastic activity) and low calcitriol levels are observed in the SGA compared to the AGA neonate (Namgung et al. 1993), while bone matrix collagen turnover appears unaffected (Namgung et al. 1996). The authors hypothesize that placental dysfunction constrains mineral transfer to the fetus, which together with low calcitriol levels, leads to reduced fetal bone formation.

Lower bone area and BMC in the preterm relative to the term infant suggest that intrauterine growth restriction adversely affects skeletal size; whereas poor mineral accretion due to low gestational age limits the capacity for catch up in bone mineralization (Avila-Diaz et al. 2001). The activity of specific growth factors may explain some of these differences. In a study of term neonates, Javaid et al. (2004) demonstrated a positive relationship between cord blood IGF-I and whole body BMC, which was no longer present after correction of BMC to bone size. The authors conclude that IGF-I levels at birth are more closely related to skeletal size than to mineral density.

1.2.9 Implications of Low Bone Mass in the SGA Infant

There is increasing evidence that the early manifestations of an adverse intrauterine environment continue to affect bone in adulthood. Several studies demonstrate associations of birth weight and childhood growth with later life bone mass and turnover. BMC of the hip, lumbar spine, and forearm in postmenopausal women correlate positively with birth weight (Yarbrough et al. 2000), while delayed linear growth during childhood predicts later hip fracture in both men and women (Cooper et al. 2001). Vertebral width and projected area, indicators of bone size and therefore bone growth, are significantly reduced in elderly men with osteoporotic vertebral fractures, implicating early life risk factors in the pathogenesis of osteoporosis (Vega et al. 1998). In a retrospective study of 21 yr. old females, BMC at the lumbar spine and femoral neck was positively associated with weight at 1 yr. of age and height at age 5 yr., while BMD was more strongly related to the level of physical activity in childhood and at the time of study (Cooper et al. 1995).

The authors interpret this as a predominant effect of early growth on adult skeletal size, compared to a greater influence of physical activity on bone density.

In a study of adult males 19-21 yr. of age, those born at a SGA birth weight demonstrated evidence of accelerated bone turnover including elevated serum osteocalcin, total alkaline phosphatase, and urine deoxypyridinoline, a degradation product of type I collagen (Szathmari et al. 2000). The authors speculate that programmed endocrine function involving either the GH-IGF-I or the HPAA axes contributes to enhanced bone turnover. Although the opposing processes of bone formation and bone resorption appeared to be balanced in the above study, the ramifications of the high turnover state to future bone health are of concern in light of reported associations between high bone turnover and lower bone mass in later life (Ravn et al. 1996).

Szathmari et al. (2000) further report a negative association between renal calcium excretion and birth weight in their young male subjects. A possible mechanism for this is suggested by the work of Arden et al. (2002), in which an inverse relationship between birth weight and adult intestinal calcium absorption suggests a programmed early life stimulus for calcium retention in the SGA-birth weight individual.

1.3 POLYUNSATURATED FATTY ACIDS AND PERINATAL GROWTH

Fatty acids are important for perinatal growth and development. The following sections provide an overview of fatty acid structure and metabolism with an emphasis on the essential fatty acids and the long chain polyunsaturated fatty acids derived from them.

1.3.1 Metabolism of LC PUFA

The 18-carbon essential fatty acids (EFA) linoleic acid (LA; 18:2n-6) and alpha-linolenic acid (ALA; 18:3n-3) are essential in the human diet since, unlike certain other species, man is unable to synthesize fatty acids with double bonds three (n-3) or six (n-6) carbons from the n terminus. The long chain polyunsaturated fatty acid (LC PUFA) derivatives of EFA may be synthesized by humans *de novo* from their respective n-6 or n-3 parent EFA. LC PUFA with identified biological functions are dihomo-γ-linolenic acid (DHLA; 20:3n-6), arachidonic acid (AA; 20:4n-6), eicosapentaenoic acid (EPA; 20:5n-3), and docosahexaenoic acid (DHA; 22:6n-3).

The metabolic pathway for LC PUFA synthesis involves a sequence of alternating desaturation (addition of double bonds) and elongation (addition of 2 carbons units) reactions in the hepatic endoplasmic reticulum. Desaturation of LA and ALA by Δ6-desaturase is recognized as the rate-limiting step in LC PUFA biosynthesis (Sprecher 1981). AA and EPA, the respective 20-carbon products of LA and ALA elongation and desaturation, undergo further chain elongation to yield 24:5n-3 and 24:4n-6, which in turn are desaturated by Δ6-desaturase to yield 24:6n-3 and 24:5n-6. These intermediates are translocated to the peroxisomes where partial β-oxidation produces DHA (22:6n-3) and DPA (22:5n-6) (Sprecher 1992; Sprecher et al. 1995). Figure 1-1 illustrates the metabolic pathways of the n-6 and n-3 polyunsaturated fatty acids.

1.3.2 Regulation of LC PUFA Metabolism

The Δ6- and Δ5-desaturases are regulated by nutritional (Brenner 1981; Cho et al. 1999), hormonal (Brenner 2003), and metabolic (Sprecher 2001) factors. Hepatic Δ6-desaturase is stimulated by EFA deficiency (Melin and Nilsson 1997) and suppressed by dietary LC PUFA (Cho et al. 1999). Hormonal modulation of LC PUFA synthesis occurs by activation of the desaturases by insulin, in contrast to inhibition of Δ6-desaturase activity by glucocorticoids, glucagons, and epinephrine (Brenner 2003; de Alaniz and Marra 2003). Relevant to this thesis, it is conceivable that glucocorticoid excess associated with intrauterine growth restriction may potentially suppress perinatal accretion of LC PUFA, thereby compromising the positive effects of LC PUFA on tissue growth and bone mass accretion (Blanaru et al. 2004; Weiler 2000).

The rate of production of n-6 and n-3 LC PUFA is determined by substrate availability, competition between n-6 and n-3 PUFA for the desaturase enzymes, and product inhibition of the metabolic pathway (reviewed by Innis 1991). The order of PUFA preference of the desaturase enzymes is n-3 > n-6 > n-9. Therefore, a balanced intake of both n-6 and n-3 LC PUFA is required to maintain adequate tissue status of both series of fatty acid. This has been demonstrated in infant studies in which infant formula supplemented with fish oil (a source of n-3 LC PUFA) but lacking n-6 LC PUFA suppressed growth in association with reduced circulating AA concentrations (Carlson 1996). In contrast, combined supplementation with AA and DHA supports normal growth and elevates circulating LC PUFA to approximate levels of breast-fed infants (Foreman-van Drongelen et al. 1996; Vanderhoof et al. 2000).

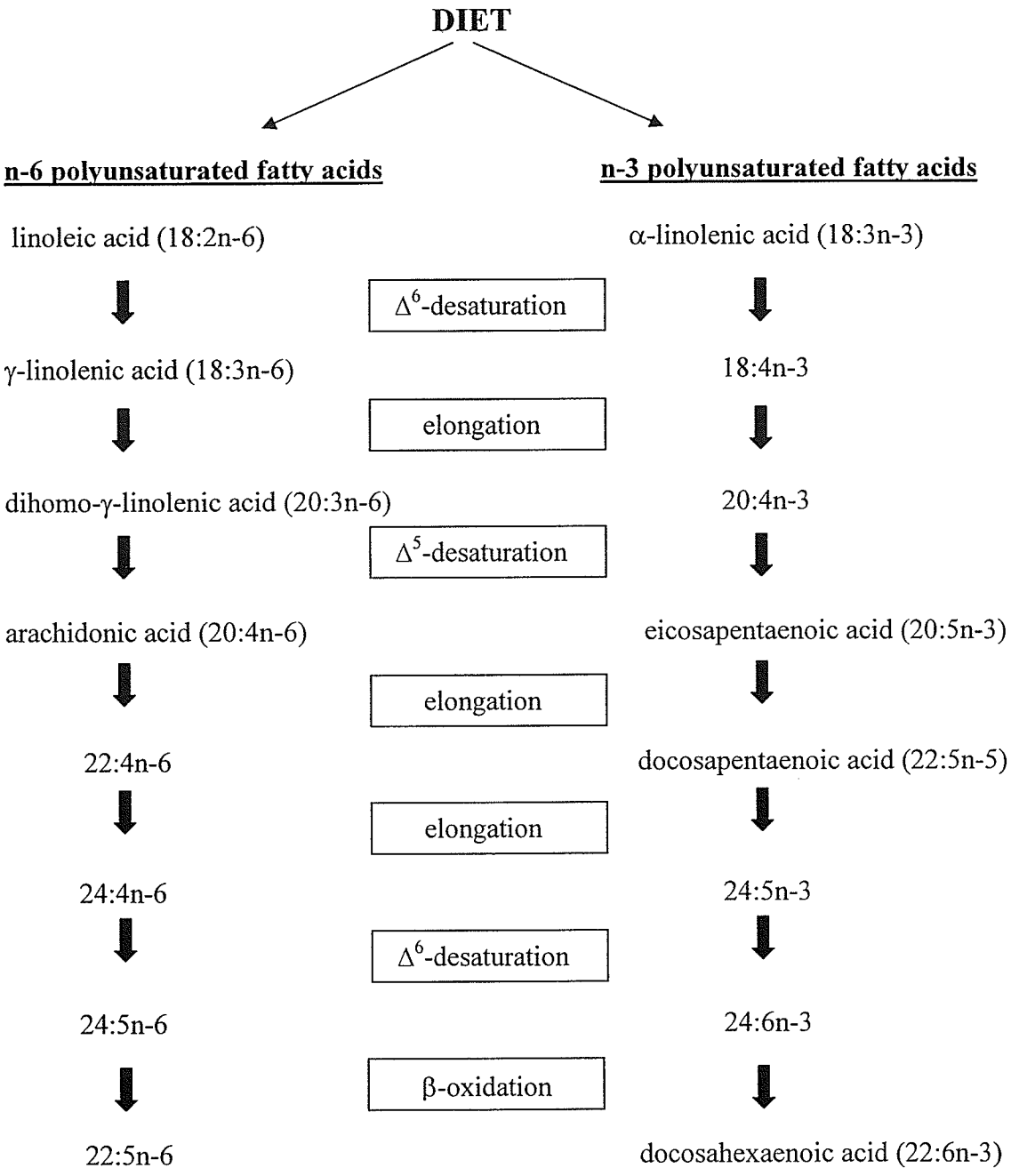

Figure 1-1. Metabolic pathways for synthesis of n-3 and n-6 polyunsaturated fatty acids.

Adapted from: Koletzko B, Edenhofer S, Lipowsky G, Reinhardt D. (1995) Effects of a low birthweight infant formula containing human milk levels of docosahexaenoic and arachidonic acids. J Ped Gastroenterol Nutr;21:201-208.

1.3.3 Perinatal Sources of LC PUFA

LC PUFA is available to the infant through adipose tissue stores, *de novo* synthesis of LC PUFA from EFA, and from dietary sources. The fetus derives much of its supply of fatty acids through materno-placental transfer, the effectiveness of which is determined by placental function and adequacy of maternal fatty acid stores. Requirements for certain fatty acids remain high in infancy and must be met through dietary sources.

1.3.3.1 Maternal LC PUFA Stores

Maternal LC PUFA status during pregnancy is highly linked to fetal growth. Significant correlations between maternal and neonatal n-6 and n-3 LC PUFA at birth in human pregnancy (Al et al. 1990; Matorras et al. 1999) highlight the importance of maternal LC PUFA supply to the fetus. The absolute and relative n-6 and n-3 LC PUFA content of the maternal diet influences fetal growth (Oken et al. 2004) and length of gestation (Olsen and Secher 2002).

1.3.3.2 Placental Transfer of LC PUFA

Preferential maternal-fetal transfer of LC PUFA in the human and the pig is suggested by the relatively greater proportions of AA and DHA in neonatal compared to maternal plasma levels (Crawford et al. 1989; Innis 1991; Rooke et al. 2001). This is explained by selective placental transport of AA and DHA to ensure high levels in fetal circulation (Campbell et al. 1998). Fetal accumulation of LC PUFA is maximal during the third trimester of pregnancy, coincident with high growth requirements for n-6 and n-

3 fatty acids estimated at 400 mg/kg/day and 50 mg/kg/day, respectively (Clandinin et al. 1981). In pregnancies complicated by growth restriction, placental transfer of fatty acids is reduced (Cetin et al. 2002; Clandinin et al. 1980), while other studies report decreased DHA levels in umbilical vessel walls of SGA neonates at birth (Foreman-van Drongelen et al. 1995).

1.3.3.3 Desaturase Capacity of the Human Neonate

Hepatic desaturase activity in the human is present by 17 wk. of gestation (Poisson et al. 1993; Rodriguez et al. 1998); however, the effectiveness of fetal desaturases and elongases in vivo has not been established (reviewed by Herrera 2002). Stable isotope techniques confirm the capability of preterm and term-born infants to convert LA and ALA to AA and DHA, respectively (Carnielli et al.1996; Demmelmair et al. 1995; Koletzko et al. 1996). However, recent evidence in human neonates indicating a minimal contribution of endogenous AA synthesis to plasma AA concentrations (Szitanyi et al. 1999) calls into question the adequacy of endogenous synthesis to meet high postnatal growth demands.

1.3.3.4 LC PUFA in Human Milk and Infant Formula

Many dietary constituents in the infant's diet vary as a function of feeding practices. Specifically, and in regard to this thesis, dietary PUFA and LCPUFA provided in human milk and certain formulas may differ in quantity and availability to the infant.

1.3.3.4a LC PUFA Content of Human Milk

LC PUFA content of human milk is determined by maternal intake of fatty acids, duration of lactation, and certain lifestyle behaviors. High levels of n-6 and n-3 LC PUFA in colostrum decline with continued lactation, while proportions of EFA increase in mature milk (Gibson and Kneebone 1981). Levels of AA and DHA are present in term and preterm human milk in similar ratios of between 1.5:1 and 2:1 (Uauy-Dagach et al. 1998).

Maternal diet is the primary determinant of PUFA and LC PUFA in breast milk. The total n-6 to n-3 fatty acid ratio in human milk varies from 5:1 to 10:1, but may be higher with increased LA intake (Uauy-Dagach et al. 1998). Breast milk from women consuming typical Western diets contains 10-17% LA, 0.8-1.4% ALA, 0.3-0.7% AA, and 0.1-0.5% DHA (reviewed by Innis 2003). By comparison, populations with higher intakes of fish display DHA concentrations as high as 2.8% (Connor 1995).

Recent evidence shows that maternal lifestyle factors additionally impact breast milk LC PUFA composition. Maternal smoking during pregnancy, a significant risk factor for SGA birth, is also associated with decreased levels of DHA in breast milk, which may further impair neonatal growth and development (Agostoni et al. 2003).

1.3.3.4b LC PUFA in Commercial Infant Formula

The importance of dietary AA and DHA to visual and neurocognitive development has been documented in infant studies (reviewed by SanGiovanni et al. 2000). In response to this evidence, commercial preterm infant formulas have recently been enriched with AA and DHA in amounts comparable to those of human milk

(Table 1-3). While the short-term benefit of LC PUFA supplementation to visual and cognitive function is accepted in the preterm infant, conflicting reports of sustained benefit indicate the need for continued surveillance (reviewed by Simmer and Patole 2003). Notably, no short-term adverse clinical outcomes related to infant LC PUFA supplementation have been documented (Clandinin et al. 2005; Simmer and Patole 2003).

The association between dietary LC PUFA and enhanced bone mass is well established in animal research; however, with the recent advent of AA + DHA-supplemented infant formulas, surprisingly little is known about their impact on bone growth and bone mass in the human infant. In a study of term infants, Weiler et al. (in press) identified positive correlations of maternal and cord blood erythrocyte AA concentrations with infant whole body BMC, and between cord blood AA to EPA ratio and BMC of the infant femur and lumbar spine. In addition, maternal n-3 LC PUFA status related negatively to infant BMC. Preterm infants fed human milk and/or formula supplemented with AA + DHA until 12 months corrected age (AA as 0.42 g/100 g of fat + DHA as 0.26 g/100 g of fat to term, followed by AA + DHA as 0.42 and 0.16 g/100 g of fat, respectively, in discharge formula) had increased lean body mass and reduced fat mass, but no difference in body weight, BMC or BMD, compared to controls at 1 yr. of age (Groh-Wargo et al. 2005). It is clear that further study is required to clarify both short- and long-term consequences of early life LC PUFA intervention to bone health outcomes.

Table 1-3. Comparison of selected fatty acid composition of LC PUFA-supplemented commercial infant formulas and human milk (expressed as g/100g of total fat).

	Enfalac A+ (Mead Johnson Canada)	*Similac Advance (Ross Pediatrics)*	*Mature Human Milk (Canada)*[1a]	*Mature Human Milk (France)*[1b]
Linoleic acid (18:2n-6)	16.6	14.9	10.47 ± 2.62	14.67 ± 1.38
Linolenic acid (18:3n-3)	1.7	1.9	1.16 ± 0.37	0.70 ± 0.11
Arachidonic acid (20:4n-6)	0.64	0.40	0.35 ± 0.11	0.50 ± 0.06
Eicosapentaenoic acid (20:5n-3)	0	0	0.05 ± 0.05	0.02 ± 0.01
Docosahexaenoic acid (22:6n-3)	0.32	0.15	0.14 ± 0.10	0.32 ± 0.08

[1] *Adapted from:* Jensen RG. (1999) Lipids in human milk. Lipids;34:1243-1271.
[a] Values are means ± SD
[b] Values are means ± SEM

1.3.4 Determinants of Infant LC PUFA Requirements

At birth, plasma AA and DHA levels in the human neonate correlate with gestational age (Foreman-van Drongelen et al. 1995), birth weight, and head circumference (Koletzko and Braun 1991; Leaf et al. 1992a). Postnatal LC PUFA status depends upon rate of growth, adipose reserves determined *in utero*, and the metabolic capacity for LC PUFA synthesis. Available data suggest that the LC PUFA status of the SGA infant may be compromised by a combination of rapid growth (Bohler et al. 1999), low tissue stores (Clandinin et al. 1981; Clandinin et al. 1989), impaired placental transfer of LC PUFA (Cetin et al. 2002; Clandinin et al. 1980), and diminished desaturase activity (Uauy et al. 2000). In the infant born preterm as well as SGA, requirements for AA and DHA are additionally elevated by deprivation of the important third trimester placental transfer of these LC PUFA (Crawford et al.1998; Crawford 2000).

1.3.5 Assessment of Postnatal Tissue LC PUFA Status

Birth status of LC PUFA may be assessed by lipid analysis of umbilical cord blood or vessels (Hornstra et al. 1989). Postnatal AA and DHA status are commonly measured in plasma and erythrocyte lipids due to ease of access, high concentrations, and good correlation with tissue levels (Uauy-Dagach et al. 1998). Postmortem studies in the human fetus demonstrate an association between erythrocyte and cerebral cortical DHA levels (Makrides et al. 1994). Rapid tissue accretion of dietary LC PUFA in the neonatal piglet model has shown positive relationships between dietary AA and plasma AA (Blanaru et al. 2004), and between AA in plasma and liver (Rioux et al. 1997). Dietary intake of LC PUFA is similarly reflected in plasma and erythrocyte AA and DHA

concentrations in the human infant (Boehm et al. 1996; Guesnet et al. 1999), thereby facilitating monitoring of tissue response to dietary intervention

1.3.6 LC PUFA and Postnatal Growth

Normal growth requires an adequate dietary supply of the essential fatty acids (EFA), which cannot be synthesized by humans. Among the LC PUFA derived from the EFA, those of greatest importance to fetal and infant growth are AA and DHA. Quantitatively, AA is the predominant LC PUFA in tissue lipids. In contrast, DHA is localized to specific tissues such as the central nervous system and retina, where it may constitute up to 50% of phospholipids and 80% of total PUFA (Giusto et al. 2000). During late gestation and early infancy, large amounts of AA and DHA accumulate in the brain and retina (Clandinin et al. 1980; Clandinin et al. 1989), coincident with rapid growth of these organs. Additional AA and DHA are deposited in fetal adipose tissue in late gestation (Leaf et al. 1995), providing a reserve for growth and metabolic activities during the neonatal period.

Significant relationships have been demonstrated between AA and DHA status and anthropometric measurements at birth. Leaf et al. (1992a and 1992b) demonstrated significant positive correlations between plasma AA and infant birth weight, head circumference, gestational age, and placental weight. Another study of preterm infants confirmed the association between birth weight and plasma AA, and also found a significant association between birth weight and total n-6 LC PUFA and a negative correlation between birth weight and circulating ALA (Koletzko and Braun 1991). Rate of growth during the first year of life is also related positively with plasma AA

concentrations in the preterm infant (Carlson et al. 1993). In an overview of LC PUFA supplementation studies in the preterm infant, Simmer and Patole (2003) concluded that whole body length at term and at 2 months of age are enhanced by LC PUFA. The effects of LC PUFA on weight gain during infancy were less clear, however, with conflicting reports of increased weight at 2 months of age and reduced weight at 12 months compared to term infants (Simmer and Patole 2003).

Preformed dietary LC PUFA may be used by the infant as oxidative substrates for energy production; however, they are preferentially incorporated into cell membranes where they modulate membrane permeability, signaling pathways, and activities of transport proteins, receptors, and enzymes through alterations in membrane fluidity (reviewed by Sardesai 1992). The extent to which dietary LC PUFA accumulate in membranes is determined by existing LC PUFA tissue stores, desaturase activity and substrate specificity (Sprecher 1981), in addition to endocrine and genetic influences. Other LC PUFA functions include activation of transcription factors, modulation of the expression of genes involved in lipid metabolism and cell differentiation (Clarke et al. 1997; Sellmayer and Weber 2002). Further metabolism of LC PUFA leads to formation of biologically active oxygenated compounds known collectively as the eicosanoids (Innis 1991), which will be discussed in the next section.

1.3.7 Regulation of Bone Metabolism by Dietary LC PUFA

The impact of LC PUFA-enriched infant formula on bone growth and metabolism has generated recent interest due to potential implications for bone health in later life. In addition to promoting growth, LC PUFA act as modulators of bone turnover through

actions in bone and at other sites. Many of the effects of LC in bone are thought to be mediated by their eicosanoid metabolites that act as hormones in the tissues in which they are produced.

Eicosanoid synthesis is regulated by the release of LC PUFA from membrane phospholipids through an increase in phospholipase A_2 (PLA_2) activity. Cytosolic PLA_2 translocates to the cell membrane and liberates AA from the sn-2 position of membrane phospholipids. AA may be metabolized via the cyclooxygenase (COX) pathway to form prostanoids (2-series prostaglandins and prostacyclins) and thromboxanes (TXA_2); or alternatively may enter the lipoxygenase (5 LO) pathway to produce the 4-series leukotrienes and lipoxins. DGLA metabolism via COX yields the 1-series prostacyclins and thomboxanes. When EPA is the substrate for the COX pathway, prostacyclins and thromboxanes of the 3-series are formed, while the LO pathway produces the 5-series leukotrienes (reviewed by Sardesai 1992).

In the osteoblast, COX is expressed in two forms, the constitutive COX-1 enzyme and the inducible COX-2 form. COX-2 activity is regulated by several bone-resorbing factors including PTH, IL-1 (Kawaguchi et al. 1994), and TGFβ-1. The net biological effect of eicosanoids in tissues is determined by the n-6: n-3 fatty acid balance in membrane phospholipids. Figure 1-2 illustrates the metabolic pathways for eicosanoid synthesis.

Prostaglandin E_2 (PGE_2), the most abundant and biologically active eicosanoid in bone, may stimulate either bone resorption or bone formation depending on its concentration (Raisz and Fall 1990). There is evidence that PGE_2 mediates specific actions of calcitriol (Collins and Chambers 1992), mechanical forces (Somjen et al.

1980), IGF-I (McCarthy et al. 1991), and other growth factors (reviewed by Watkins et al. 2001) in bone.

PGE_2 produced in bone may modulate synthesis of IGF-I in bone and epiphyseal growth plate cartilage, with consequent effects on bone turnover. In growing chicks fed varying amounts of n-3 and n-6 PUFA (but not LC PUA), saturated, and trans fats, elevation of tibial periosteal bone formation was associated with increased cartilage IGF-I and reduced *ex vivo* PGE_2 release from bone (Watkins et al. 1997). The authors speculate that the anabolic actions of PGE_2 may have resulted from enhanced IGF-I synthesis or altered responsiveness to IGF-I. Other studies have demonstrated altered levels of circulating IGFBP in response to dietary lipids (Li et al. 1999), which may affect IGF-I availability.

LC PUFA may further enhance bone mass through effects on calcium balance. Dietary AA increases intestinal calcium absorption in young rats, possibly via PGE_2 - independent mechanisms (Song et al. 1983). Also in a rodent model, calcium absorption is enhanced by dietary fish oil (containing n-3 LC PUFA), evening primrose oil (EPO; a source of n-6 LC PUFA) (Coetzer et al. 1994), and by combined γ-linolenic acid (n-6) and EPA in a 3:1 ratio (Claassen et al. 1995). It has been suggested that membrane fatty acid content determined by dietary LC PUFA modulates calcium uptake in the enterocyte brush border (Coetzer et al. 1994).

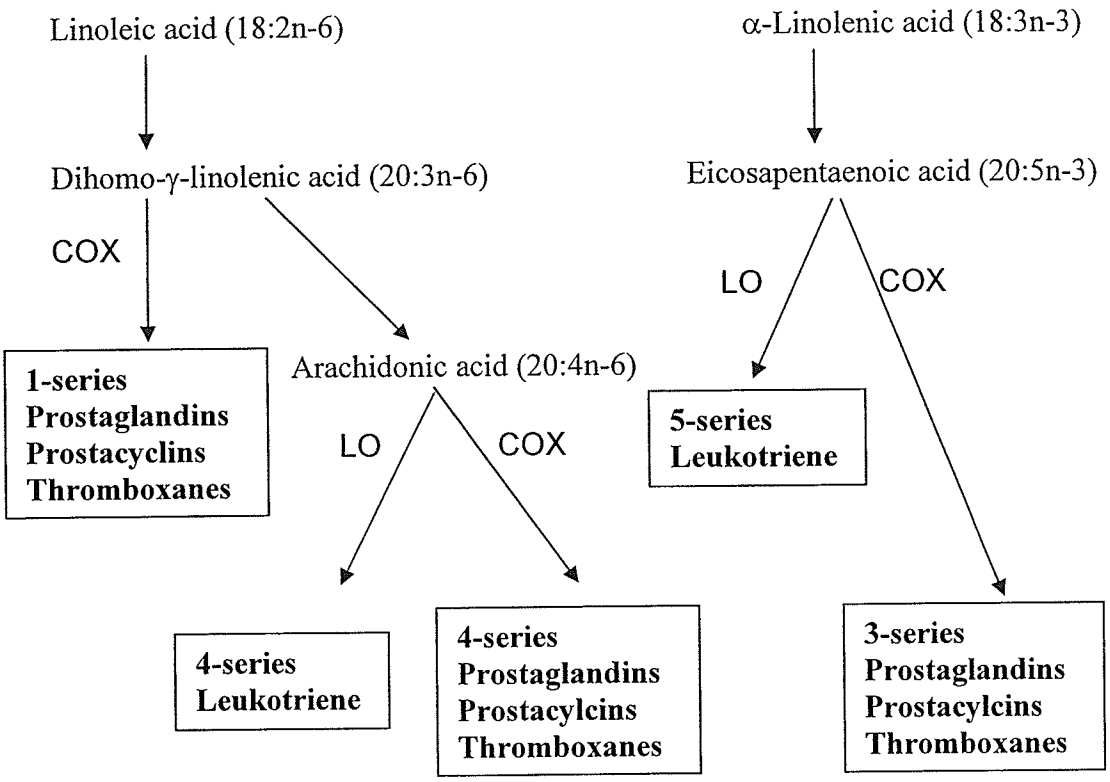

Figure 1-2. Metabolic pathways of eicosanoid synthesis

ABBREVIATIONS USED:
LO = Lipoxygenase
COX = Cyclooxygenase

Adapted from: Sardesai, V.M. (1992) Biochemical and nutritional aspects of eicosanoids. J Nutr Biochem;3,p.565.

Local production of eicosanoids from LC PUFA in the kidney may influence renal calcium excretion (Tulloch et al. 1994). EPA (n-3) and EPO (n-6) both reduce urinary calcium in experimental animals, thereby promoting positive calcium balance (Buck et al. 1991; Tulloch et al. 1994). AA, in contrast, may promote calcium excretion. In patients with calcium nephrolithiasis, elevated circulating AA levels are associated with hypercalciuria and increased intestinal calcium absorption, while dietary treatment with fish oil in these patients reduces both AA levels and calcium excretion (Baggio et al. 2000). The effects of combined n-3 and n-6 LC PUFA supplementation on renal calcium clearance are likely to be determined by the balance of eicosanoids produced due to antagonistic actions of n-6- and n-3-derived eicosanoids (Whelan 1996).

Data from a variety of animal models clearly indicate that the relative intake of dietary n-3 and n-6 LC PUFA influences bone mass and bone metabolism. High dietary ratios of GLA to EPA were found to increase femur calcium content as measured by wet ashing and to reduce type I collagen breakdown in the mature rat (Claassen et al. 1995). In growing rabbits, a high n-3 LC PUFA diet (10 g/100 g of total fat) lacking in n-6 LC PUFA negatively affected longitudinal tibial bone growth, cortical bone area, and bone structural properties in growing rabbits (Judex et al. 2000). Blanaru et al. (2004), in a study comparing several levels of AA supplementation while maintaining a constant amount of DHA (AA: DHA as 3.0:1.0; 4.5:1.0; 6.0:1.0; and 7.5:1.0 g/100 g of total fat) in the AGA neonatal piglet, report elevated whole body BMC and BMD with dietary AA + DHA as 0.6:0.1 g/100 g of total fat without adverse effects on bone turnover or growth.

For these reasons, a dietary AA to DHA ratio of 6:1 was selected for the present study. The two levels of supplementation to be tested were AA + DHA as 0.6:0.1 g/100 g

of total fat and AA + DHA as 1.2:0.2 g/100 g of total fat. These were chosen in order to evaluate the response of the more vulnerable SGA piglet to an amount with proven effectiveness in the AGA piglet compared with twice that amount.

1.3.8 Measurement of Bone Metabolism and Bone Mass

Histomorphometry and biochemical bone markers are the principal methods for assessment of bone metabolism. Histomorphometry provides qualitative and quantitative details about bone turnover at the cellular level (Eastell et al. 1988); however, the small volume of bone studied may not be representative of other bone sites or the entire skeleton (Podenphant and Engel 1987). Biochemical bone markers in plasma and urine are products of bone metabolism. Measurement of biomarkers reflects whole body bone turnover, but does not depict processes involved at the tissue level or within specific bone compartments. Bone densitometry is the preferred method for evaluation of bone mass; however, it does not provide information regarding bone turnover or bone structure and may not be sensitive to subtle changes in bone mass.

1.3.8.1 Biomarkers of Bone Formation

Markers of bone formation include osteoblast-produced enzymes and metabolites of collagen and other matrix proteins, all of which reflect osteoblast activity at specific stages of cellular differentiation (Marcus 1996).

1.3.8.1a Bone-specific Alkaline Phosphatase

Bone alkaline phosphatase (BAP), an enzyme secreted by the osteoblast, promotes bone mineralization (Anderson et al. 2004). An immunoassay specific for BAP eliminates the problem of cross-reactivity with circulating liver alkaline phosphatase (Rosalki and Foo 1984). Plasma levels of BAP increase in parallel with BMC during childhood growth (Magnusson et al. 1995); however, may be less informative during infancy (Pittard et al. 1992).

1.3.8.1b Osteocalcin

Serum osteocalcin is an index of osteoblastic activity commonly used to signify bone formation or bone turnover. Osteocalcin is elevated in high bone turnover states, and reduced when bone turnover is low (reviewed by Szulc et al. 2000). Childhood levels of osteocalcin relate positively to growth velocity (Delmas et al. 1986). Measurement of osteocalcin by radioimmunoassay (RIA) has been applied to human infants (Michaelsen et al. 1992) and the growing pig (Carter et al. 1996). Potential limitations of osteocalcin assays include cross-reactivity with osteocalcin degradation fragments, heterogeneity in carboxylation status, diurnal variation (Nielsen et al. 1990), and the requirement for normal vitamin K status (Tracy et al. 1990).

1.3.8.1c Carboxy-terminal Propeptide of Type I Collagen (PICP)

Carboxy-terminal propeptide of type I procollagen (PICP) is a polypeptide fragment removed from procollagen prior to aggregation of collagen molecules into fibres (Prockop et al.1979a). Due to its high molecular weight, PICP is not excreted by

the kidney but is instead degraded in the liver (reviewed by Szulc et al. 2000). Serum PICP levels quantitatively reflect collagen synthesis during growth (Prockop et al. 1979b) and disease processes (Simon et al. 1984), and correlate with trabecular bone formation measured histomorphometrically in adults (Parfitt et al. 1987).

1.3.8.2 Biomarkers of Bone Resorption

Markers of bone resorption represent degradation products of type I collagen derived from either cortical or trabecular bone. These include urine pyridinoline and deoxypyridinoline, which have generally been replaced by measurement of cross-linked N-telopeptide of type I collagen (NTX) and cross-linked C-terminal telopeptide of type I collagen (ICTP).

1.3.8.2a Cross-linked N-Telopeptide of Type I Collagen (NTX)

Measurement of urine NTX by ELISA is commonly used to assess bone resorption. NTX expressed in relation to urine creatinine in a spot urine sample correlates linearly with 24 hr. NTX excretion in infancy (Lapillonne et al. 2002) and has been used to detect bone resorption in pigs (Bollen et al. 1997). Urine NTX is low at birth, increases markedly during the first 10 days of life, remains stable for the next 3 months, then declines progressively until 1 yr. of age (Lapillonne et al. 2002).

1.3.8.2b Cross-linked Carboxy Terminal Telopeptide of Type I Collagen (ITCP)

ICTP is a fragment released during degradation of bone type I collagen. Circulating ICTP levels reflect bone growth or altered collagen turnover in pathological processes. In adult metabolic bone disease, serum ICTP correlates with histomorphometric measurement of bone resorption (Eriksen et al. 1993).

1.3.8.3 Measurement of Bone Mass by Dual Energy X-ray Absorptiometry

Radiological methods for assessment of bone mass include single photon absorptiometry (SPA), dual photon absorptiometry (DPA), quantitative computed tomography (QCT), and the most widely used dual energy x-ray absorptiometry (DXA). DXA measures bone mineral status in terms of bone mineral content (BMC; g hydroxyapatite) and bone mineral density (BMD; g/cm^2 hydroxyapatite). The basic principle of the DXA technique involves measurement of the attenuation of dual energy x-ray beams by tissues of differing composition. Soft tissues contain proportionately more water and organic compounds than bone and therefore restrict the number of x-rays per unit (flux) less than bone (Lukaski 1993). Preferred sites for DXA measurement in infants are the femur and the lumbar vertebrae. Radiation exposure from a DXA scan is 1-3 mrad from an AP scan of the lumbar spine or hip and 10-15 mrad from a lateral scan of the spine (Lang et al 1991), compared to 8-10 mrad from a chest x-ray (Kelly 1988).

1.3.8.3a DXA Validation in the Small Infant

The precision of DXA measurements is well established. A precision of 2.5% was demonstrated for infant whole body BMC (Venkataraman and Winters 1991), while a coefficient of variation of < 2% was determined on repeated scans of preterm infants with birth weights of 1.101- 2.440 kg followed from birth until age 3 to 4.8 yrs. (Hori et al. 1995). Assessment of DXA accuracy, the extent to which DXA measurements represent true bone mineral status, by comparison with cadaver vertebral ash weight and volume indicates significant correlations between cadaveric lumbar vertebral DXA BMC and ash weights (Ho et al. 1990). The validity of DXA scan measurements in very low birth

weight infants has been a concern due to their lower bone mass and mineralization. Ellis et al. (1994) and Koo et al. (1995a) have shown that DXA can be reliably applied to smaller preterm infants if performed at a slow scan speed and using a small pixel size. DXA has also proven useful for assessment of infant growth and body composition (Venkataraman and Ahluwalia 1992).

1.3.8.3b DXA Validation in the Neonatal Piglet

DXA validation in the piglet confirms accuracy and precision of femur BMC measurements (Brunton et al. 1997). Other groups report average coefficient of variations of <2.5% and <1.8% for BMC and BMD measurements, respectively, in piglets (Koo et al. 1995b). In addition, precision and accuracy of DXA for estimation of body composition has been established in the neonatal piglet (Fusch et al. 1999).

1.3.8.3c Advantages and Limitations of DXA

DXA is considered the reference method of bone densitometry due to its accuracy, precision (≈ 1 %), high image resolution (<1mm), rapid scan time, low radiation dose, and non-invasiveness (reviewed by Chesney 1992). In addition, DXA permits imaging of multiple sites and measurement of tissue compartments besides bone. Since DXA BMC is not affected by sample freezing, it is suitable for animal and postmortem tissue studies (Koo et al. 1995b). Sources of potential error with DXA include movement artifact, inter-operator variability, and nonmetallic orthopedic casts (Koo et al. 1995b). The main criticism of the DXA method is that it measures mineral

density by correction of BMC to projected bone area to give an areal BMD which may not reflect changes in bone thickness related to growth (Cowell et al. 1995).

1.3.9 Animal Models for the Study of Infant Nutrition

A variety of animal models have been used in the study of human nutrition. Species differences in growth rates, metabolism, nutrient requirements, and enzyme activity must be considered when extrapolating animal research observations to the human. Similarly, data obtained from adult studies may not be relevant to the neonate. While the requirement for EFA differs between species, LC PUFA absorption and metabolism are similar (Gurr 1988), and similar mechanisms of bone growth have been identified in humans and experimental animals (Loveridge and Noble 1994).

1.3.9a Comparison of Rat and Pig Models for the SGA Infant

The rat is a well-established model for adult human nutrition research; however, significant differences exist between the newborn rat and the human neonate with respect to physiologic maturity at birth, body composition, and rate of growth. The rat, in contrast to both humans and pigs, maintains early life body temperature by close maternal proximity, conserving energy for growth (Widdowson 1971a). Maturation of the HPAA axis is more advanced at birth in the humans and pig compared with rodents (Alves et al. 1997; Owen and Matthews 2003), identifying the pig as a more appropriate model for investigation of the endocrine aspects of fetal growth restriction relevant to the present thesis. For similar reasons, dietary manipulation during the neonatal period is more likely

to affect comparable stages of neuroendocrine development in human and piglet neonates.

At birth, body fat content is less than 2% in the piglet (Mellor and Cockburn 1986) and 1.1% in the rat (Gurr 1988) compared to 16.1% in the human (Gurr 1988). Most body lipids in piglets are structural constituents of tissue cell membranes, with relatively limited energy reserves particularly in the growth-restricted piglet (Mellor and Cockburn 1986). Higher postnatal growth rates in the rat and pig (Widdowson 1971a) may be advantageous in a research model by more readily demonstrating the effects of dietary manipulation on overall and specific organ growth (Fraser 1988). A comparison of rat and pig models for the human infant is summarized in Table 1-4.

1.3.9b The Neonatal Piglet Model for the SGA Infant

The piglet model has proven to be valuable for investigation of neonatal growth and fatty acid requirements (Blanaru et al. 2004; Hrboticky et al. 1991; Innis 1993; Rioux et al. 1997) due to physiologic similarities (Miller & Ullrey 1987), comparable growth patterns (Glauser et al. 1966), and tissue responsiveness to dietary LC PUFA (Blanaru et al. 2004; de la Presa-Owens et al. 1998; Huang and Craig-Schmidt 1996; Innis et al. 1996; Weiler 2000). In addition, the pig's large litter permits multiple treatments within a litter with siblings as controls. A key aspect in which the newborn piglet differs from the human neonate is its early weight bearing, which is a major influence on bone turnover through biomechanical forces (Gurr 1988). The SGA piglet may also differ from the SGA human neonate in desaturase activity at birth, as suggested by a recent report of normal Δ6- and Δ5- desaturase expression in the growth-restricted pig fetus (McNeil et al. 2005).

Table 1-4. Comparison of rat and piglet model for the study of human infant nutrition

Feature	*Rat model*	*Piglet model*
Size at birth (g)	5.5 – 6[a]	1156[b] average 1600[c]
Birth Weight variability	Less variable than piglet or human[e]	S.D. 272g[b] Range 500 – 2800g[c]
Body composition at birth	Low fat stores (1.1% body weight)[d]	Low fat stores (<2% body weight)[d]
Growth rate	Exceeds human neonate[e].	Exceeds human neonate. May double body weight in 1 week
Diet	Dam's milk or artificial formula until 21 days of age	Sow's milk or artificial formula until 3-6 wks of age
Nutrient requirements	Well defined. Modified by intestinal synthesis of nutrients and coprophagy[a].	Well defined
Digestive physiology	Differences in mineral absorption	Similar to human[f]
Bone physiology	Late epiphyseal union[a]	Similar to human
Lipid metabolism	Similar to human	Similar to human
HPA axis maturity at birth	Immature compared to human neonate[d]	Comparable maturity with human neonate[d]

[a] Nutrient Requirements of Laboratory Animals, Second Revised Edition, National Research Council, 1972, National Academy of Sciences, Washington, D.C., p. 58.

[b] Cooper, J.E. (1975): The use of the pig as an animal model to study problems associated with low birthweight. Laboratory Animals, 9, p. 331.

[c] unpublished data, Glenlea Research Station Swine Laboratory, Glenlea, Manitoba.

[d] Owen D, Matthews SG. (2003) Glucocorticoids and sex-dependent development of brain glucocorticoid and mineralocorticoid receptors. Endocrinology;144:2775-2784.

[d] Alves SE, Akbari HM, Anderson GM, Azmitia EC, McEwen BC, Strand FL. (1997) Neonatal ACTH administration elicits long-term changes in forebrain monoamine innervation. Subsequent disruptions in hypothalamic-pituitary-adrenal and gonadal function. Ann N Y Acad Sci;814:226-251.

While premature delivery does not occur in the pig, spontaneous intrauterine growth restriction affects 15-20% of offspring (Cooper 1975). Uteroplacental insufficiency is implicated in both human and porcine fetal growth restriction (Regnault et al. 2002; Warshaw 1990). Designation of newborn piglets as SGA or AGA is defined by various criteria and is highly dependent upon the birth weight distribution of the institution and the breed of pig studied. As in humans, porcine intrauterine growth restriction is associated with increased perinatal morbidity and mortality (De Roth and Downie 1976). Piglet birth weight is the major determinant of neonatal survival and body weight at weaning (Winters and Stewart 1947). Runt piglets fail to catch up to their higher birth weight littermates in weight or length (Widdowson 1971b). Of most relevance to the present thesis, the SGA piglet, like the growth-restricted human neonate, demonstrates reduced bone mass relative to its normal weight siblings (Adams 1971).

2.0 HYPOTHESES AND OBJECTIVES

2.1 HYPOTHESES

1. Dietary supplementation with AA + DHA in a 6:1 ratio supports normal growth and improves tissue LC PUFA status and bone mineralization in rapidly growing neonatal SGA piglets.

2. Dietary supplementation with AA + DHA as 1.2:0.2 g/100 g of total fat is more effective than supplementation as 0.6:0.1 g/100 g of fat in supporting postnatal growth and improving tissue LC PUFA status and bone mineralization in the neonatal SGA piglet, particularly in the very low birth weight (≤ 1.0 kg) piglet.

2.2 RESEARCH OBJECTIVES

1. To determine in the SGA piglet model, the effects of dietary LC PUFA supplementation with AA and DHA given in a 6:1 ratio as 0.6:0.1 and as 1.2:0.2 g/100 g of total fat on growth, bone mass and bone metabolism.

2. To examine differences between low birth weight (1.1-1.2 kg) and very low birth weight (≤ 1.0 kg) piglets in their response to two different levels of dietary AA + DHA with respect to growth, bone mass, and bone metabolism.

3. To investigate relationships among tissue LC PUFA status, growth parameters, biochemical markers of bone metabolism, and bone mass measurements in the SGA piglet model.

3.0 METHODS

3.1 ANIMALS AND DIETS

Thirty male Cotswold piglets with a birth weight of ≤ 1.2 kg (SGA defined as ≤ 2 SD below the mean birth weight of 1.6 ± 0.2 kg within 16 litters) were obtained at 3 d of age from Glenlea Swine Research Unit at the University of Manitoba. Piglets were identified as belonging to one of two categories defined by their birth weight: low birth weight (LBW) piglets with birth weight 1.1 - 1.2 kg and, very low birth weight (VLBW) piglets with birth weight ≤ 1.0 kg. Weight categorization was performed in order to delineate differences between LBW and VLBW piglets in their metabolic response to dietary intervention.

Newborn piglets were left with the sow for the first 48 hours of life to permit acquisition of passive immunity from colostrum. Thereafter, they were housed individually in stainless steel cages at an ambient temperature of 28 - 30 °C. Between days 3 and 5 of life the piglets were permitted a period of adaptation to the environment and artificial liquid formula. At d 5 of life, the piglets were randomly assigned to one of three dietary treatments for 15 d: 1) control (unsupplemented) formula, 2) formula supplemented with AA + DHA as 0.6:0.1 g/100 g of dietary fat, and 3) formula supplemented with AA + DHA as 1.2:0.2 g/100 g of dietary fat. Randomization was stratified by birth weight ≤ 1.0 kg or 1.1- 1.2 kg to ensure that the smallest and most vulnerable piglets were equally distributed between treatment groups. Littermates were not assigned to the same diet group. The AA was provided in the form of ARASCO (43.03% AA) and DHA as DHASCO (42.95% DHA), both obtained from Martek Biosciences Corp., Columbia, MD. The DHA is derived from a marine microalgae and

AA from a common soil bacterium. Both ARASCO and DHASCO were stored at –80 ° C to prevent oxidation. Formula was made on alternate days and kept refrigerated until used.

Piglets were fed formula at 350 ml/kg/day based on morning fasting body weight and divided equally into 3 daily feedings at 0900, 1500, and 2100 hours. All formulas met the guidelines of the National Research Council (1996) for the nutritional requirements of growing piglets and provided 1050 kcal/L, 60 g/L fat, 50 g/L protein, 2.1 g/L calcium, and 1.4 g/L phosphorus. The oil blend of the control and base diet (prior to the addition of LC PUFA) was comprised of soybean, coconut, and high oleic safflower oils with a measured total n-6 to n-3 ratio of 9.5:1. Macronutrient and micronutrient contents of the study diets are presented in Table 3-1. Diet fatty acid composition is shown in Table 3-2.

Piglets were permitted approximately 1 hour of group exercise in an enclosed pen before each feed. Animal care and all experimental procedures conformed to guidelines of the Canadian Council of Animal Care (1993) and were approved by the University of Manitoba Protocol Management and Review Committee.

3.2 ASSESSMENT OF GROWTH

Weight was measured daily at 0900 hours in the fasting state using a digital scale (Mettler-Toledo Inc, Highstown, NJ). Growth rate was calculated by the average daily weight gain ($g \cdot kg^{-1} \cdot d^{-1}$) as indicated by the following equation:

$$\left(\frac{\text{day 16 weight} - \text{day 1 weight in g}}{\text{average of day 16 weight and day 1 weight in kg}} \right) / 15 \text{ days}$$

Table 3-1. Composition of control and base diets (prior to LC PUFA supplementation)

Ingredient	Analytical Value (per litre)
Soybean oil,[1] g	23
High oleic safflower oil,[2] g	23
Coconut oil,[3] g	14
Skim milk powder,[4] g	110
Whey powder,[5] g	35
Vitamin[6] and Mineral[7] Mix	
dl-α-tocopheral acetate, mg	5
Cholecalciferol, mg	0.11
All trans-retinol acetate, mg	1
Thiamine, mg	30
Riboflavin, mg	60
Niacin, mg	400
Pantothenic acid, mg	284
Pyridoxine, mg	36
Folacin, mg	20
Vitamin B-12, mg	0.4
D-Biotin, mg	2
CaCO3, g	1
Choline Chloride, g	42.4
MnSO4, mg	40
Ferrous Sulfate, mg	167

[1]Vita Health, Canada.
[2]Bestfoods Food Service, Canada.
[3]Harlan Teklad, Madison, WI, USA.
[4]Parmalat Canada Production and Distribution.
[5]Glanbia Ingredients, Monroe, WI, USA.
[6]Harlan Teklad, Madison, WI, USA for all listed except all *trans*-retinol acetate from Sigma-Aldrich Canada.
[7]Sigma-Aldrich Canada.

Table 3-2. Fatty acid composition of experimental diets (expressed as g/100 g of total fat)

Fatty acid	Control Diet	AA:DHA as 0.6:0.1 g/100 g of fat	AA:DHA as 1.2:0.2 g/100 g of fat
C10:0	1.49 ± 0.18	1.64 ± 0.02	1.62 ± 0. 01
C12:0	10.16 ± 0.81	10.17 ± 0.10	10.77 ± 0.14
C14:0	4.13 ± 0.08	4.22 ± 0.02	4.28 ± 0.05
C15:0	0.07 ± 0.02	0.06 ± 0.00	0.07 ± 0.00
C16:0	8.69 ± 0.43	8.62 ± 0.03	8.84 ± 0.04
C16:1 n-9	0.04 ± 0.01	0.03 ± 0.00	0.01 ± 0.02
C16:1 n-7	0.12 ± 0.01	0.16 ± 0.01	0.18 ± 0.00
C17:1	0.03 ± 0.02	0.03 ± 0.02	0.00 ± 0.00
C18:0	3.53 ± 0.13	3.44 ± 0.02	3.54 ± 0.01
C18:1	36.24 ± 0.52	35.99 ± 0.39	35.31 ± 0.12
C18:2 n-6	27.61 ± 0.40	27.06 ± 0.15	26.53 ± 0.09
C18:3 n-6	0.00 ± 0.00	0.05 ± 0.00	0.05 ± 0.01
C18:3 n-3	2.94 ± 0.04	2.88 ± 0.02	2.84 ± 0.01
C20:0	0.32 ± 0.00	0.32 ± 0.00	0.32 ± 0.00
C20:1 n-9	0.21 ± 0.01	0.22 ± 0.0	0.22 ± 0.00
C20:3 n-6	0.03 ± 0.00	0.06 ± 0.00	0.11 ± 0.00
C20:4 n-6	ND	0.57 ± 0.03	1.05 ± 0.00
C20:5 n-3	ND	ND	ND
C22:0	0.31 ± 0.00	0.23 ± 0.16	0.34 ± 0.00
C22:4 n6	0.06 ± 0.01	0.04 ± 0.00	0.06 ± 0.01
C22:3 n3	ND	ND	ND
C22:6 n3	ND	0.10 ± 0.01	0.18 ± 0.01
Total n6[1] (C ≥ 18)	27.70	27.78	27. 08
Total n3[2] (C ≥ 18)	2.97	2.98	3.02
Total n6:n3[3] (C ≥ 18	9.3	9.3	9.2
AA:DHA	ND	5.70	5.83

Whole body length was measured under anesthesia on d 16 of study with a non-stretchable tape measure extended from tip of snout to base of tail.

3.3 BASELINE BLOOD AND URINE COLLECTION

Blood samples were obtained in the non-fed state by jugular venipuncture on d 1 of study at 0900 hours. Approximately 5 ml of blood was collected in heparinized vacutainers and centrifuged at 2000 g at 4 ° C (Beckman model TJ-6R tabletop centrifuge, Palo Alto, CA, USA) to separate erythrocytes from plasma. Plasma was removed and erythrocytes then washed with an equal volume of 0.9 % NaCl solution. Erythrocytes were centrifuged again and suspended in an equal volume of distilled water. Plasma and erythrocyte samples were stored separately in glass vials at – 80 ° C under nitrogen gas until analysis. Erythrocyte lysates (containing membranous and cellular fractions) were measured for fatty acids within 28 days after collection to ensure that fatty acid degradation was not an issue. Fasting urine collection was performed using pediatric urine collection bags (U-Bag, Hollister Inc.) at 0900 hours on d 1 and d 16 of study, and samples were stored at – 20 ° C.

3.4 TISSUE COLLECTION AT THE END OF STUDY

On the morning of d 16 of study, piglets were anesthetized by intraperitoneal injection of sodium pentobarbital at 30 mg/kg (Somnotol; 65 mg/mL concentration). Once surgical anesthesia was confirmed, cardiac puncture was performed with the removal of approximately 50 ml of blood into heparinized syringes. Plasma and RBC were separated as outlined above for baseline samples. Piglets were then euthanized with

an intra-cardiac injection of Euthanol (Sodium pentobarbital; 200 mg/mL concentration). Samples of liver and adipose tissue were excised and weighed to the nearest 0.1 g. Tissue samples were frozen in liquid nitrogen and stored at – 80° C. A section of tibial diaphysis weighing approximately 1.0 g was excised, cleaned of adherent soft tissue and flushed with 0.9% NaCl solution to remove marrow cells. The bone section was placed in a vial containing 10 ml Hank's balanced salt solution (Sigma Chemical Co. Ltd) and incubated in a shaking water bath for 2 h at 39° C to allow release of PGE_2 from bone as described by Dekel et al. (1981). After incubation, the bone section and organ culture fluid (the solution in which tibial bone was incubated) were stored separately at – 20 ° C for later analysis of calcium and phosphorus content, and for PGE_2, respectively. Following tissue harvesting, the abdominal cavity was closed with suture to maintain tissue depth and the carcasses were then frozen at – 20° C in the anterior-posterior position with limbs extended.

3.5 TISSUE FATTY ACID ANALYSIS

Total lipids were extracted from plasma, erythrocytes (membranes and cytosol), liver, and adipose tissue into chloroform and methanol (2:1) after the addition of an internal standard (C17:0) according to the method of Folch et al (1957). Extracted lipids were transmethylated in methanolic hydrochloric acid-3N (Supelco Inc, Bellefonte, PA) at 80 ° C for 60 minutes. Fatty acid methyl esters were separated by gas-liquid chromatography on a Varian Star 3400 gas chromatograph (Mississauga, ON, Canada) equipped with a 30-m long capillary column made of fused silica and coated with DB225 (25% cyanopropylphenyl; J & W Scientific, Folsom, CA), an 8100 autosampler, an

integrator, and a flame ionization detector. Hydrogen was used as the carrier gas. Samples were injected at an initial temperature of 180° C followed by an increase in oven temperature at a rate of 3° C/min to reach a final temperature of 220° C. Peaks were identified by comparison with retention times of Supelco 37 component FAME mix (Supelco Inc). Fatty acid methyl esters of carbon chain length of 10 - 27 were expressed as g/100 g of total fatty acids. Samples of the three experimental diets were also analyzed for fatty acid content by the modified Folch method (1957) using C17:0 as the internal standard and results were expressed as g/100 g of total dietary fat.

3.6 BIOCHEMICAL MEASUREMENTS

Osteocalcin, an indicator of osteoblastic activity, was measured in plasma using an iodine-125 (^{125}I) radioimmunoassay kit (RIA; Diasorin, Stillwater, USA). This method uses rabbit antiserum to bovine-derived osteocalcin and has been validated for measurement of porcine osteocalcin (Pointillart et al. 1997). Bone resorption was assessed by measurement of cross-linked N-telopeptide (NTX), a breakdown product of mature type I collagen, in spot urine samples using a competitive-inhibition enzymatic immunoassay (ELISA; Osteomark, Ostex, Seattle, USA). Urine NTX was corrected to urine creatinine as measured by a colorimetric method (Sigma 555, Sigma-Aldrich Ltd., Oakville, Canada) and expressed in relation to urine creatinine as μM NTX/mM creatinine. The NTX assay has also been validated for use in piglets (Bollen et al. 1997). Urine cortisol was measured by EIA (Cedarlane). Plasma IGF-I was measured with an ELISA assay for human IGF-I, which is identical to porcine IGF-I (R&D Systems, Minneapolis, MN, USA). *Ex-vivo* PGE_2 production in bone organ culture was measured

using an ELISA method (R&D Systems, Minneapolis, MN, USA) and expressed per gram of bone sample. This method gives approximately 70% cross-reactivity with PGE_1 and 16.3% with PGE_3 and has been used in a variety of animal models including rats (Watkins et al. 2000), chicks (Watkins et al. 1996; Watkins et al. 1997), and pigs (Weiler and Fitzpatrick-Wong 2002).

Calcium (Ca) and phosphorus (P) contents of tibial cortex ($\approx$ 50 mg sample) and urine (0.25 ml sample) after digestion in 5% nitric acid were measured using inductively coupled plasma optical emission spectroscopy (ICPOES; Varian Liberty 200, Varian Canada, Mississauga, Canada). Non-fat milk powder (National Institute of Standards and Technology Standard Reference Material 1549 Non-Fat Milk Powder; Gaithersburg, MD) with known Ca and P content was used as a control. Measured Ca and P content of controls were 0.276 ± 0.002 mM/g and 0.340 ± 0.001, respectively, compared to actual contents of 0.325 mM/g and 0.342 mM/g, respectively. For each of the above assays, samples were analyzed in duplicate.

3.7 MEASUREMENT OF BONE MASS BY DUAL ENERGY X-RAY ABSORPTIOMETRY

Frozen piglet carcasses were analyzed for bone area (BA), bone mineral content (BMC), and bone mineral density (BMD) of whole body (WB), lumbar spine from L1 to L4 vertebrae (LS), and femur by dual energy x-ray absorptiometry (DXA; QDR 11.2 4500A series, Hologic Inc., Waltham, MA, USA) using infant whole body, lumbar spine, and hip subregion software. Use of the DXA method to measure bone mass has been validated in small infants (Koo et al. 1995a) and in frozen piglet carcasses (Brunton et al. 1997). Daily QC phantoms were performed with mean values of 51.652 ± 0.182 cm^2 for

BA (reference values 51.709 ± 0.171 cm^2), 52.349 ± 0.247 g for BMC (reference values 52.622 ± 0.200 g), and 1.013 ± 0.247 g/cm^2 for BMD (reference values 1.018 ± 0.002 g/cm^2) at the lumbar spine.

To obtain *ex vivo* femur DXA scans, the right femur was later dissected from each piglet carcass, cleaned of adherent soft tissue, and kept frozen at -20° C in sealed plastic bags until subsequent analysis. Femur weight (to the nearest 0.01 g) and length were measured using a digital scale and a non-stretchable plastic tape measure respectively. DXA scan measurements of BA, BMC, and BMD were performed with excised femurs submerged in a 3 cm water bath to simulate soft tissue. Use of DXA to assess BMC and BMD of isolated small bones has been validated in the piglet (Brunton et al. 1997). Values were corrected for femur weight to account for differences in size.

3.8 STATISTICAL ANALYSIS

Data was analyzed using SAS statistical software for Windows (SAS, Cary, NC, USA). Results were expressed as mean ± one standard deviation (SD) unless otherwise stated. The level of statistical significance was set at $p < 0.05$. One-way ANOVA was selected to determine differences in outcome measurements between dietary treatment groups for each of the two birth weight categories. Post-hoc analysis was performed using the Bonferroni multiple group comparison procedure. Since birth weight categories were not arrived at as a function of randomization, they were not examined for their relationship to diet in the same model, therefore, one-way ANOVA was an appropriate approach to examine diet effects for each individual birth weight category. Relationships among tissue LC PUFA status, growth parameters, biochemical bone markers, and bone

mass measurements were assessed with Pearson correlation coefficients. Backward stepwise regression analysis was conducted to identify the variables contributing to prediction of bone mass after accounting for diet effects.

An estimated sample size of n = 9 per diet group (before subclassification into LBW and VLBW categories) was based on previous studies in normal birth weight piglets (Blanaru et al. 2004) showing a difference of 9.28 ± 6.8g in whole body BMC with a power of 0.08 and alpha of 0.05. The sample size of n = 10 was established to allow for higher mortality in SGA piglets. Since no previous data were available regarding bone mass response to diet within birth weight subcategories, animals were obtained until a sample size of 10 was attained, after which within each diet group, piglets were categorized as either LBW and VLBW piglets in a balanced manner. The smaller sample size of the VLBW (n = 4) compared to the LBW category reflects their lower survival compared with higher birth weight piglets. One piglet among those fed 0.6:0.1 AA + DHA in the LBW category was excluded from the study due to chronic diarrhea and failure to thrive.

4.0 RESULTS

4.1 POSTNATAL GROWTH

Piglets in both birth weight categories gained weight during the 15-d study. Final body weight was approximately 38% greater in the LBW piglets, and end of study whole body length was 10% above that of VLBW piglets. These findings indicate incomplete catch up growth with respect to body weight and length in VLBW piglets over the 15-d study period. Within each birth weight category, there were no significant differences among diet groups with respect to birth weight, final body weight, rate of weight gain, end of study whole body length, organ weights, and formula intake. Growth measurements are presented in Table 4-1. Organ weights are found in Appendix A.

4.2 BONE MASS MEASUREMENTS

4.2.1 Whole Body Bone Mass

No significant diet effect was observed in whole body BA, BMC, and BMD among piglets in either birth weight category. These data are presented in Table 4-2.

4.2.2 Lumbar Spine Bone Mass

4.2.2a LBW Piglets

Among LBW piglets, LS BMC (Figure 4-1) and BMD (Figure 4-2), but not BA, were significantly affected by dietary AA + DHA. Supplementation with the lower level of AA + DHA elevated LS BMC by 26% compared with both controls and the higher supplementation group ($P < 0.05$ for both), while no differences were observed between

Table 4-1. Growth measurements in LBW and VLBW piglets at baseline and at end of study[1]

	Birth weight 1.1-1.2 kg			Birth weight ≤ 1.0 kg		
	DIETARY TREATMENTS[2]					
	Control n = 6	AA:DHA 0.6:0.1 n = 5	AA:DHA 1.2:0.2 n = 6	Control n = 4	AA:DHA 0.6:0.1 n = 4	AA:DHA 1.2:0.2 n = 4
Birth weight, *kg*	1.150 ± 0.055	1.120 ± 0.045	1.167 ± 0.052	0.925 ± 0.096	0.925 ± 0.096	0.925 ± 0.096
Final body weight, *kg*	4.996 ± 0.796	5.237 ± 0.287	5.649 ± 0.532	4.035 ± 0.960	4.093 ± 0.401	3.420 ± 0.397
Average weight gain, $g \cdot kg^{-1} d^{-1}$	76.65 ± 1.73	77.88 ± 3.32	76.98 ± 3.63	67.13 ± 10.01	69.30 ± 15.94	62.13 ± 5.92
Absolute weight gain, *kg*	3.617 ± 0.579	3.866 ± 0.306	4.128 ± 0.357	2.741 ± 0.899	3.019 ± 0.359	2.172 ± 0.306
Final body length, *cm*	53.87 ± 2.58	53.82 ± 2.48	55.03 ± 1.57	49.55 ± 3.16	49.15 ± 3.42	49.63 ± 1.80

[1]Values are means ± SD. Within each weight category, means in a row with different superscripts are statistically different at $P < 0.05$.
[2]AA + DHA diets expressed as g/100 g of fat

Table 4-2. Effect of supplementation with AA and DHA on whole body (WB) and lumbar spine (LS) bone mass in LBW and VLBW piglets[1]

	Birth weight 1.1-1.2 kg			Birth weight ≤ 1.0 kg		
	DIETARY TREATMENTS[2]					
	Control	AA:DHA 0.6:0.1	AA:DHA 1.2:0.2	Control	AA:DHA 0.6:0.1	AA:DHA 1.2:0.2
	n = 6	n = 5	n = 6	n = 4	n = 4	n = 4
WB BA, cm^2	342.50 ± 40.96	361.00 ± 34.10	366.70 ± 29.32	293.80 ± 50.38	302.40 ± 18.81	264.00 ± 30.97
WB BMC, *g*	73.18 ± 10.73	81.50 ± 12.51	79.68 ± 7.47	59.17 ± 12.26	60.42 ± 10.43	54.29 ± 5.85
WB BMD, g/cm^2	0.214 ± 0.013	0.225 ± 0.023	0.218 ± 0.016	0.201 ± 0.016	0.199 ± 0.021	0.206 ± 0.011
WB BMC/ final weight, *g/kg*	14.70 ± 0.81	15.54 ± 2.0	14.15 ± 1.16	14.75 ± 0.69	14.72 ± 1.52	15.92 ± 1.34
LS BA, cm^2	8.36 ± 0.90	8.25 ± 0.78	8.57 ± 1.78	6.99 ± 0.71[a]	6.94 ± 0.27[a]	8.30 ± 0.65[b]
LS BMC, *g*	1.79 ± 0.18[a]	2.26 ± 0.42[b]	1.79 ± 0.25[a]	1.75 ± 0.41[ab]	1.67 ± 0.45[a]	2.55 ± 0.31[b]
LS BMC/final weight, *g/kg*	0.37 ± 0.07	0.41 ± 0.08	0.31 ± 0.05	0.46 ± 0.14[a]*	0.41 ± 0.07 [a]*	0.75 ± 0.01 [b]*
LS BMD, g/cm^2	0.216 ± 0.030[ab]	0.276 ± 0.062[a]	0.207 ± 0.011[b]	0.255 ± 0.055	0.238 ± 0.067	0.312 ± 0.026

[1]Values are means ± SD. Within each weight category, means in a row with different superscripts are statistically different at $P < 0.05$,
* values are different at $P < 0.01$.
[2]AA + DHA diets expressed as g/100 g of fat

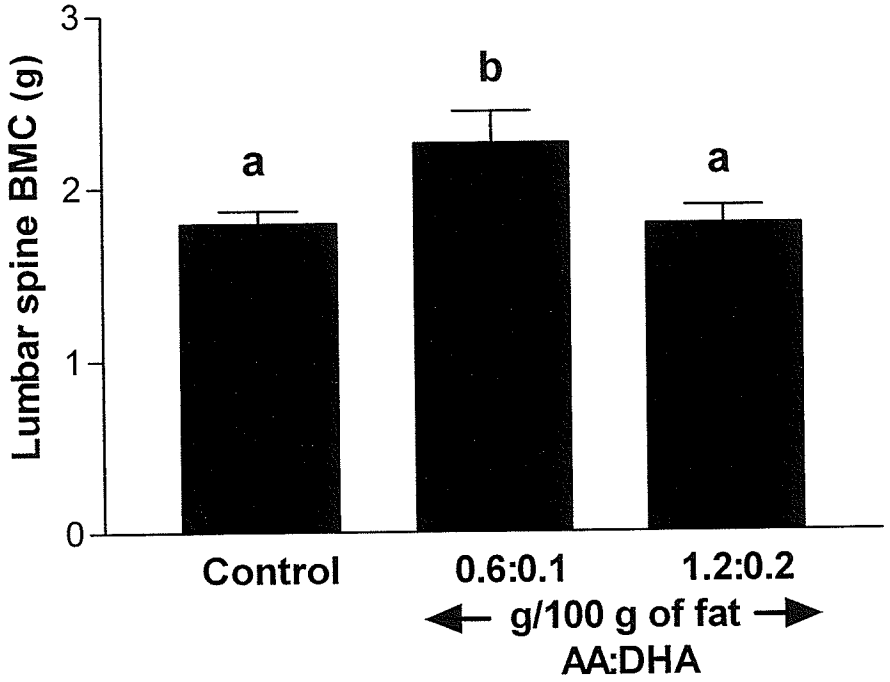

Figure 4-1. Effect of AA and DHA supplementation on lumbar spine BMC in LBW piglets. Values are means ± SD, n = 6 except 0.6:0.1 AA:DHA diet group (n = 5). Bars with different superscripts are significantly different at $P < 0.05$.

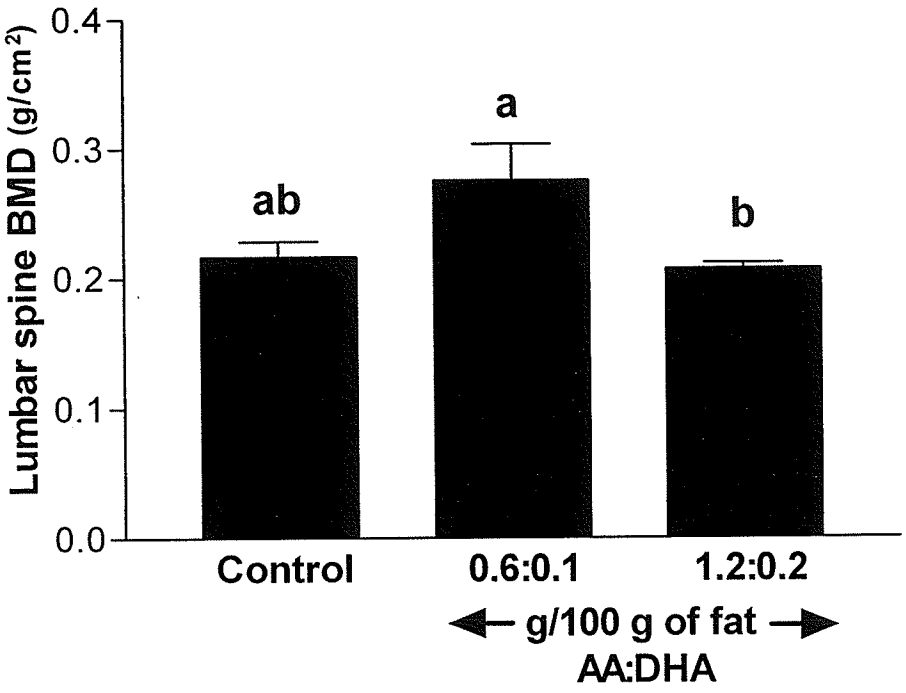

Figure 4-2. Effect of AA and DHA supplementation on lumbar spine BMD in LBW piglets. Values are means ± SD, n = 6 except 0.6:0.1 AA:DHA diet group (n = 5). Bars with different superscripts are significantly different at $P < 0.05$.

controls and the higher supplementation group. Mean values for LS BMC were: 1.79 ± 0.18g (control); 2.26 ± 0.42g (0.6:0.1 AA:DHA diet group); and 1.79 ± 0.25g (1.2:0.2 AA:DHA diet group). LS BMC corrected to final body weight did not differ between groups. LS BMD was enhanced by 28% in response to the lower compared to the higher intake of AA + DHA ($P < 0.05$), but did not differ from controls. BMD values for controls and the lower level of AA + DHA supplementation were not significantly different. LS BMD mean values were: 0.216 ± 0.029 g/cm^2 (control); 0.276 ± 0.062 g/cm^2 (0.6:0.1 AA:DHA diet group); and 0.208 ± 0.010 g/cm^2 (1.2:0.2 AA:DHA diet group).

4.2.2b VLBW Piglets

VLBW Piglets exhibited a different response to dietary AA + DHA at the LS. Among these piglets, LS BA was significantly elevated by 19% by the higher compared to the lower level of supplementation and by 20% compared to controls ($P < 0.01$ for both) (Figure 4-3). LS BMC was significantly (53%) greater at the higher compared with the lower intake of AA + DHA ($P < 0.05$), and but not compared to controls (Figure 4-4). Mean values for LS BMC were: 1.75 ± 0.41g (control); 1.67 ± 0.45 g (0.6:0.1 AA:DHA diet group); and 2.55 ± 0.31 g (1.2:0.2 AA:DHA diet group). LS BMC corrected to final body weight was significantly elevated by 67% in the higher supplementation group compared to controls and 83% above values in the lower supplementation group ($P < 0.01$ for both, Figure 4-5). No differences in LS BA, BMC, or BMC/final body weight were observed between controls and piglets fed the lower

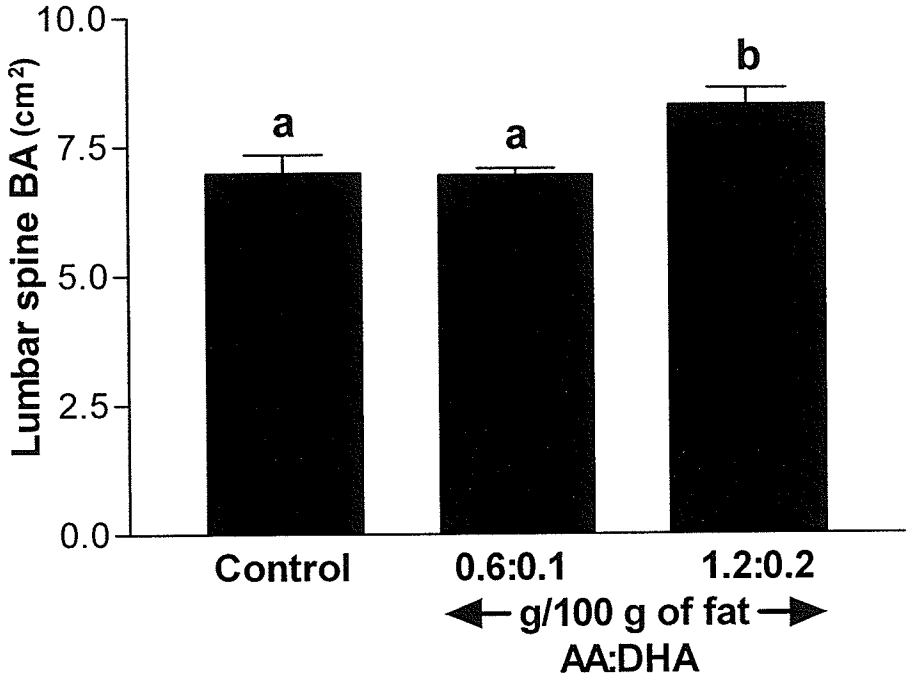

Figure 4-3. Effect of AA and DHA supplementation on lumbar spine BA in VLBW piglets. Values are means ± SD, n = 4. Bars with different superscripts are significantly different at $P < 0.05$.

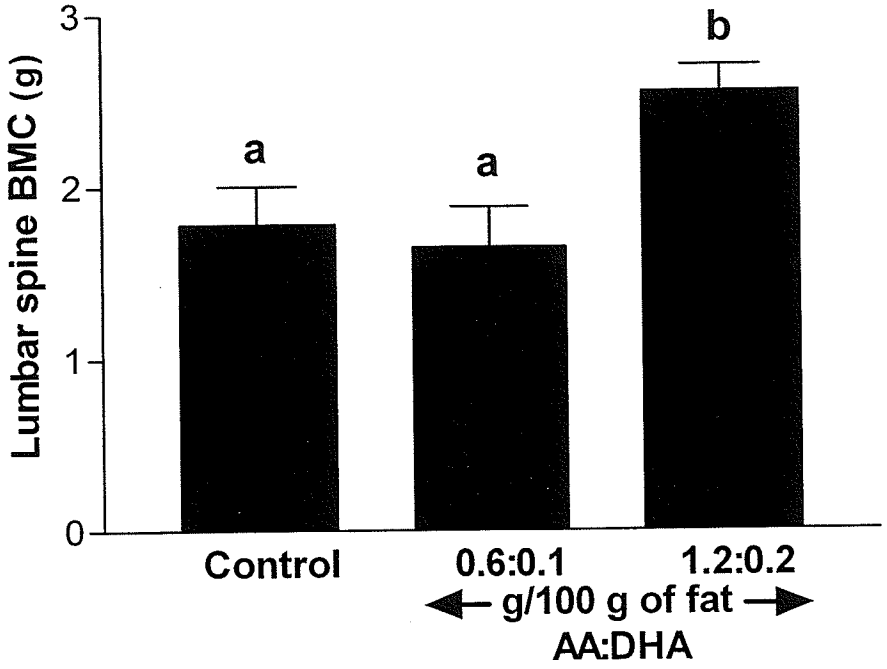

Figure 4-4. Effect of AA and DHA supplementation on lumbar spine BMC in VLBW piglets. Values are means ± SD, n = 4. Bars with different superscripts are significantly different at $P < 0.05$.

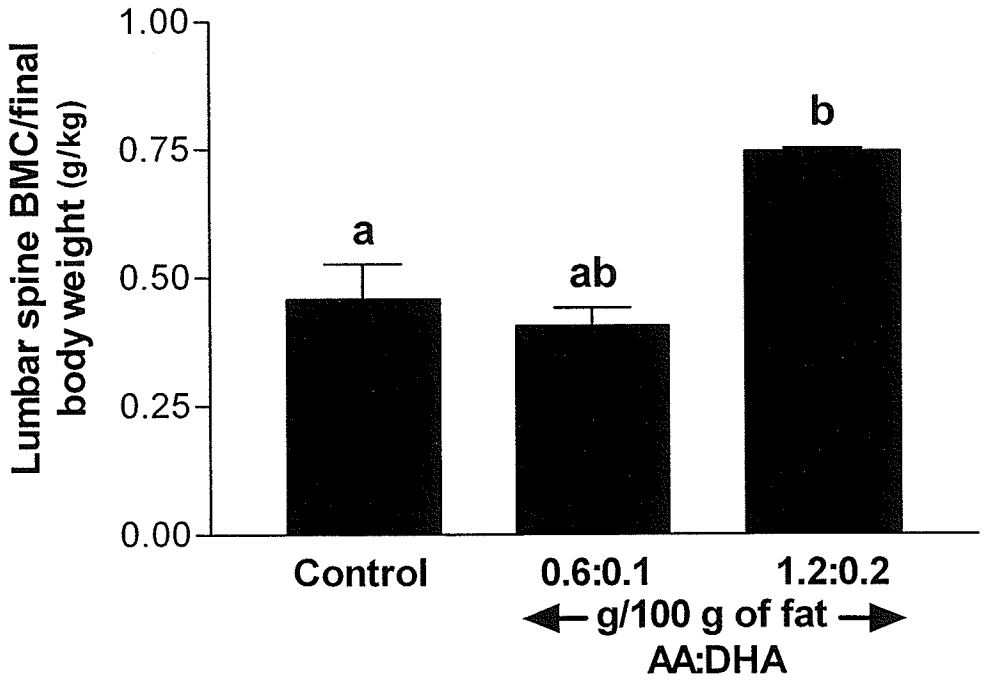

Figure 4-5. Effect of AA and DHA supplementation on lumbar spine BMC corrected to final body weight in VLBW piglets. Values are means ± SD, n = 4. Bars with different superscripts are significantly different at $P < 0.01$.

intake of AA + DHA. LS BMD was unaffected by diet, with mean values of: 0.255 ± 0.055 g/cm^2 (control); 0.238 ± 0.067 g/cm^2 (0.6:0.1 AA:DHA diet group); and 0.312 ± 0.026 g/cm^2(1.2:0.2 AA:DHA diet group). Table 4-2 summarizes LS DXA measurements for both birth weight categories.

4.2.3 *In Vivo* Femur Bone Mass

In vivo measurements of femur BA, BMC, and BMD did not differ between treatment groups for either birth weight category. Data are shown in Table 4-3.

4.2.4 *Ex Vivo* Femur Bone Mass

4.2.4a LBW Piglets

In LBW piglets, *ex vivo* femur BA was elevated in response to the higher intake of AA + DHA (by 17%), but not to the lower level of supplementation, relative to controls ($P < 0.05$) (Figure 4-6). Mean values for *ex vivo* femur BMC increased progressively as dietary intake of AA + DHA increased: 2.28 ± 0.36 g (control); 2.56 ± 0.26 g (0.6:0.1 AA:DHA diet group); 2.76 ± 0.30 g (1.2:0.2 AA:DHA diet group); however, these differences were not statistically significant ($P = 0.0551$). No effect of diet was observed on *ex vivo* femur BMC corrected for final body weight, femur weight, or on *ex vivo* femur BMD. Mean values for *ex vivo* femur BMD were: 0.230 ± 0.022 g/cm^2 (control); 0.231 ± 0.018 g/cm^2 (0.6:0.1 AA:DHA diet group); and 0.236 ± 0.013 g/cm^2 (1.2:0.2 AA:DHA diet group).

Table 4-3. Effect of supplementation with AA and DHA on *in vivo* and *ex vivo* femur bone mass in LBW and VLBW piglets[1]

	Birth weight 1.1 – 1.2 kg			Birth weight ≤ 1.0 kg		
	DIETARY TREATMENTS[2]					
	Control	AA:DHA 0.6:0.1	AA:DHA 1.2:0.2	Control	AA:DHA 0.6:0.1	AA:DHA 1.2:0.2
	n = 6	n = 5	n = 6	n = 4	n = 4	n = 4
In vivo femur BA, cm^2	7.89 ± 1.35	7.38 ± 1.01	7.76 ± 0.68	7.62 ± 1.50	6.33 ± 0.19	6.74 ± 1.43
In vivo femur BMC, *g*	1.95 ± 0.34	2.16 ± 0.36	2.25 ± 0.30	1.72 ± 0.30	1.58 ± 0.24	1.38 ± 0.10
In vivo femur BMD, g/cm^2	0.250 ± 0.043	0.294 ± 0.048	0.292 ± 0.042	0.230 ± 0.041	0.250 ± 0.036	0.215 ± 0.048
Ex vivo femur BA, cm^2	9.95 ± 1.21[a]	11.10 ± 0.62[ab]	11.69 ± 0.70[b]	7.62 ± 1.50	6.33 ± 0.19	6.74 ± 1.43
Ex vivo femur BMC, *g*	2.28 ± 0.36	2.56 ± 0.26	2.76 ± 0.30	1.72 ± 0.30	1.59 ± 0.24	1.38 ± 0.10
Ex vivo femur BMC/ body weight, *g/kg*	0.60 ± 0.04	0.49 ± 0.03	0.49 ± 0.04	0.42 ± 0.03	0.41 ± 0.04	0.44 ± 0.05
Ex vivo femur BMC/femur weight, *g/g*	0.11 ± 0.01	0.12 ± 0.01	0.12 ± 0.00	0.11 ± 0.01	0.10 ± 0.01	0.11 ± 0.02
Ex vivo femur BMD, g/cm^2	0.230 ± 0.022	0.231 ± 0.018	0.236 ± 0.013	0.230 ± 0.041	0.250 ± 0.036	0.215 ± 0.048

[1]Values are means ± SD. Within each weight category, means in a row with different superscripts are statistically different at $P < 0.05$.
[2]AA:DHA diets expressed as g/100 g of fat

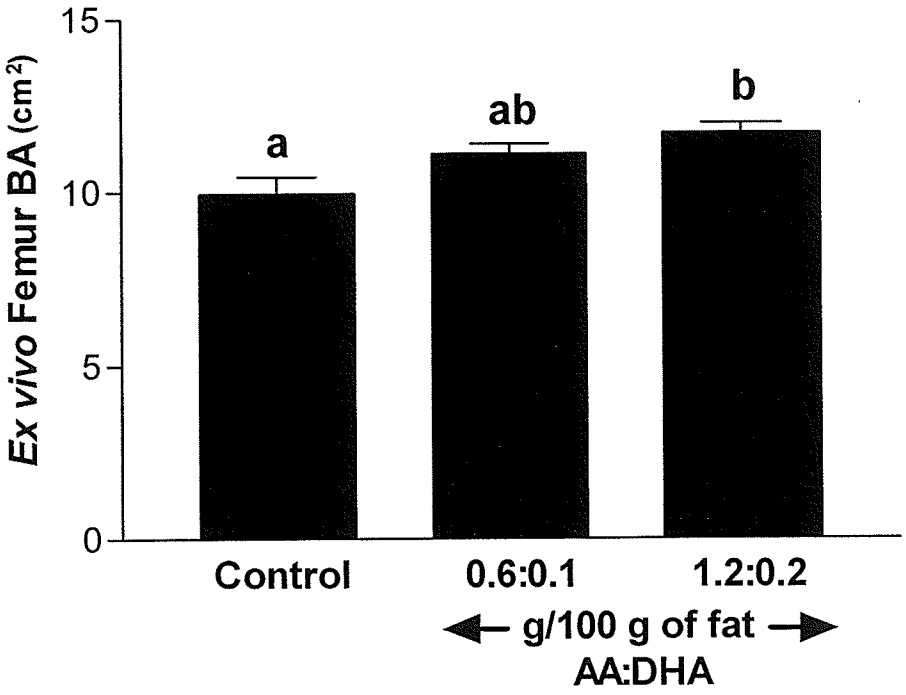

Figure 4-6. Effect of AA and DHA supplementation on *ex vivo* femur BA in LBW piglets. Values are means ± SD, n = 6 except 0.6:0.1 AA:DHA diet group (n = 5). Bars with different superscripts are significantly different at $P < 0.05$.

4.2.4b VLBW Piglets

Ex vivo femur BA, BMC, and BMD did not differ between diet groups in VLBW piglets. Results of *ex vivo* femur DXA measurements for both birth weight categories are shown in Table 4-3.

4.2.5 Body Composition

4.2.5a LBW Piglets

Body fat mass was 58% greater in the lower supplementation group compared with controls ($P < 0.05$), but did not differ between the other diet groups (Figure 4-7). Percent body fat was elevated in response to the lower intake of AA + DHA compared with the other diet groups, by 54% compared with controls and by 49% relative to the higher supplementation group ($P < 0.05$ for both) (Figure 4-8). Lean body mass (LBM), lean + BMC, and total mass were unaffected by diet.

4.2.5b VLBW Piglets

No significant effect of diet on body composition parameters was observed within this birth weight category. Table 4-4 summarizes body composition data for both birth weight categories.

4.3 TISSUE FATTY ACID COMPOSITION

4.3.1 Plasma Fatty Acids

4.3.1a LBW Piglets

Dietary AA + DHA elevated plasma AA relative to controls when supplemented as both 0.6:0.1g ($P < 0.05$) and as 1.2:0.2 ($P < 0.001$) g/100 g of fat (Figure 4-9), with no

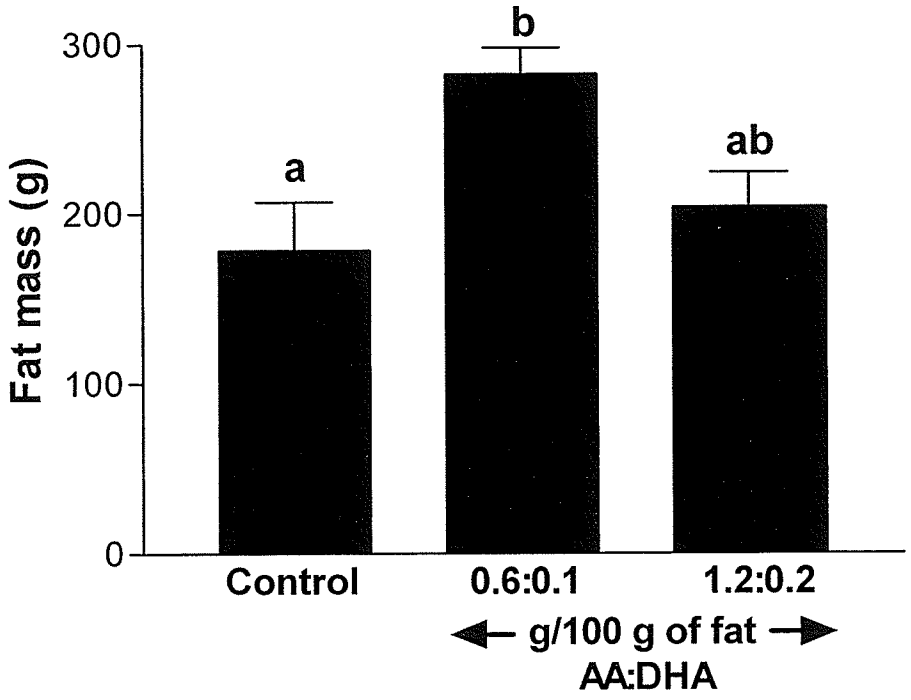

Figure 4-7. Effect of AA and DHA supplementation on total fat mass in LBW piglets. Values are means ± SD, n = 6 except 0.6:0.1 AA:DHA diet group (n = 5). Bars with different superscripts are significantly different at $P < 0.05$.

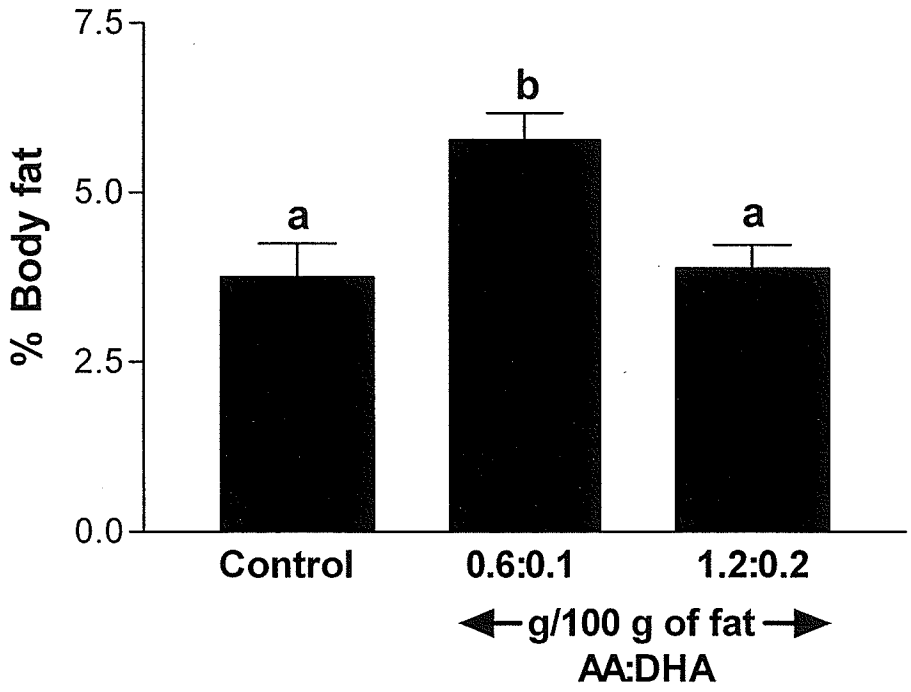

Figure 4-8. Effect of AA and DHA supplementation on percent body fat in LBW piglets. Values are means ± SD, n = 6 except 0.6:0.1 AA:DHA diet group (n = 5). Bars with different superscripts are significantly different at $P < 0.05$.

Table 4-4. Effect of supplementation with AA and DHA on body composition in LBW and VLBW piglets[1]

	Birth weight 1.1 – 1.2 kg			Birth weight ≤ 1.0 kg		
	DIETARY TREATMENTS[2]					
	Control	AA:DHA 0.6:0.1	AA:DHA 1.2:0.2	Control	AA:DHA 0.6:0.1	AA:DHA 1.2:0.2
	n = 6	n = 5	n = 6	n = 4	n = 4	n = 4
Fat mass, *g*	178.3 ± 70.1[a]	282.2 ± 35.0[b]	203.8 ± 49.9[ab]	192.3 ± 108.8	146.7 ± 63.6	122.6 ± 65.4
Lean and BMC, *g*	4498 ± 803.2	4394 ± 645.9	5030 ± 423.9	3526 ± 827	3693 ± 269	3146 ± 451
Lean mass, *g*	4604 ± 837	4823 ± 343.7	5154 ± 435.0	3659 ± 885	3754 ± 283	3215 ± 499
Total mass, *g*	4677 ± 846.6	4905 ± 349.6	5234 ± 440.7	3718 ± 897	3814 ± 290	3269 ± 502
% Body fat	3.8 ± 1.2[a]	5.8 ± 0.9[b]	3.9 ± 0.9[a]	5.0 ± 2.2	3.8 ± 1.4	3.7 ± 1.8

[1]Values are means ± SD. Within each weight category, means in a row with different superscripts are statistically different at $P < 0.05$.
[2]AA:DHA diets expressed as g/100 g of fat

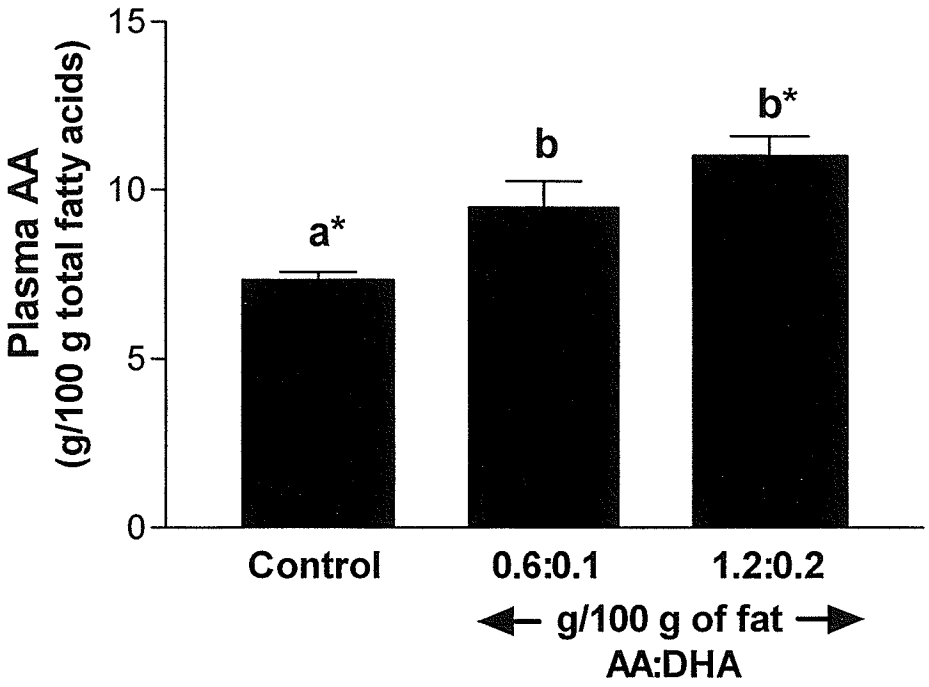

Figure 4-9. Effect of AA and DHA supplementation on plasma AA (expressed as g/100 g of total fatty acids) in LBW piglets. Values are means ± SD, n = 6 except 0.6:0.1 AA:DHA diet group (n = 5). Bars with different superscripts are significantly different at $P < 0.05$, *indicates significant difference at $P < 0.001$.

differences between supplemented groups. Plasma DHA showed an upward trend with increasing intake of AA + DHA; however, this was nonsignificant, possibly due to high inter-subject variability. Mean plasma DHA values (expressed as g/100 g of total fatty acids) were: 2.04 ± 0.40 (control); 2.26 ± 0.63 (0.6:0.1 AA:DHA diet group); and 2.46 ± 0.38 (1.2:0.2 AA:DHA diet group). Piglets fed the higher amount of AA + DHA had an elevated plasma AA:EPA ratio compared to controls ($P < 0.01$), while no differences were observed between the other diet groups. LA in plasma was increased in the control group compared to the higher supplementation group ($P < 0.01$), but did not differ between other diet groups. Plasma ALA, EPA, total n-6 and total n-3 fatty acids, and the AA:DHA ratio were not altered by diet.

4.3.1b VLBW Piglets

In VLBW piglets, significant differences between diet groups were observed only with respect to AA and DHA proportions in plasma. The higher level of supplementation increased plasma AA above controls ($P < 0.01$), with no differences noted between other diet groups (Figure 4-10). Mean plasma AA values (expressed as g/100 g of total fatty acids) were: 7.18 ± 1.38 (control); 9.21 ± 0.34 (0.6:0.1 AA:DHA diet group); and 10.35 ± 1.35 (1.2:0.2 AA:DHA diet group). Plasma DHA was significantly elevated by AA + DHA at both levels of intake compared to controls (both $P < 0.05$), while no difference was detected between the two supplemented groups (Figure 4-11). Mean plasma DHA values (expressed as g/100 g of total fatty acids) were: 1.49 ± 0.20 (control); 2.11 ± 0.16 (0.6:0.1 AA:DHA diet group); and 2.01 ± 0.35 (1.2:0.2 AA:DHA diet group). Plasma

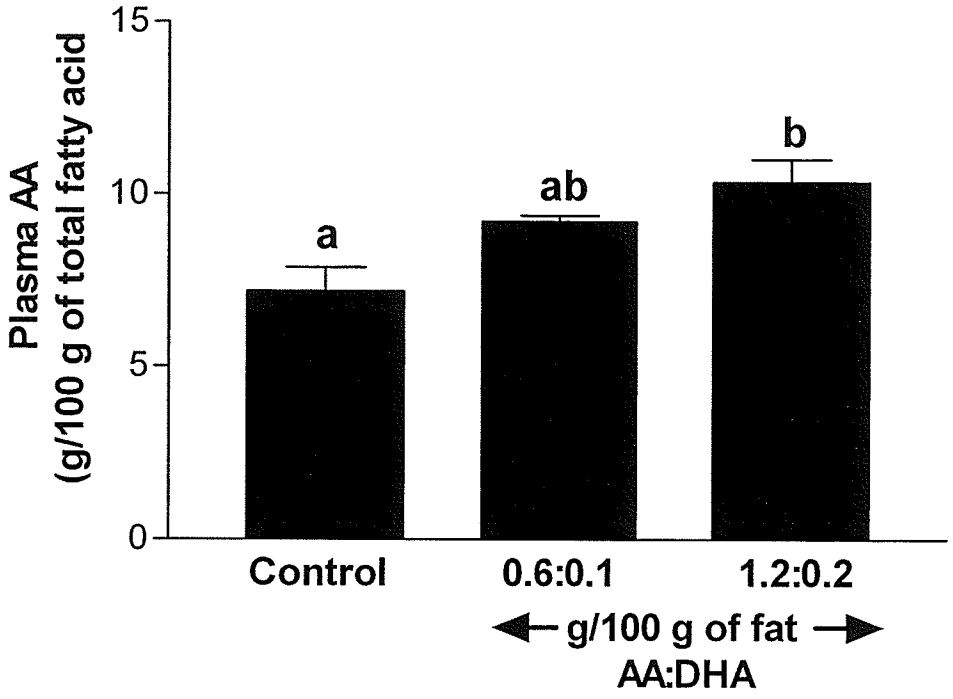

Figure 4-10. Effect of AA and DHA supplementation on plasma AA (expressed as g/100 g of total fatty acids) in VLBW piglets. Values are means ± SD, n = 4. Bars with different superscripts are significantly different at $P < 0.01$.

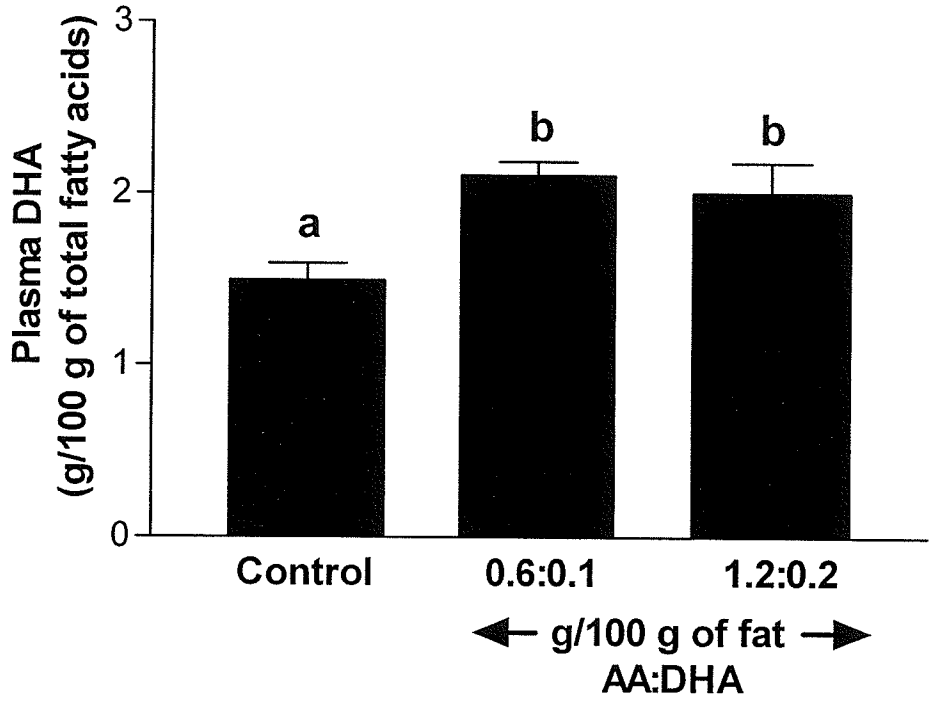

Figure 4-11. Effect of AA and DHA supplementation on plasma DHA (expressed as g/100 g of total fatty acids) in VLBW piglets. Values are means ± SD, n = 4. Bars with different superscripts are significantly different at $P < 0.05$.

LA, ALA, EPA, total n-6 and n-3 fatty acids, and AA:DHA ratio were unaffected by diet. Plasma fatty acid concentrations for both birth weight categories are shown in Table 4-5.

4.3.2 Erythrocyte Fatty Acids

4.3.2a LBW Piglets

Erythrocyte AA content was elevated relative to controls by AA + DHA at both levels (both $P < 0.01$), with no difference detected between supplemented groups (Figure 4-12). No effect of diet was observed for erythrocyte DHA, EPA, LA, ALA, total n-6 and total n-3 fatty acids, n-6:n-3 fatty acid ratio, or AA:DHA ratio, while the ratio of AA to EPA was significantly elevated by both levels of AA + DHA ($P < 0.05$ and $P < 0.001$, respectively).

4.3.2b VLBW Piglets

Among VLBW piglets, erythrocyte AA content was significantly elevated in response to both levels of AA + DHA (both $P < 0.01$, Figure 4-12). Mean values (expressed as g/100 g of total fatty acids) were: 3.52 ± 0.65 (control); 3.50 ± 0.48 (0.6:0.1 AA:DHA diet group); and 5.24 ± 0.07 (1.2:0.2 AA:DHA diet group). Erythrocyte DHA content was increased with the higher AA + DHA intake compared to controls ($P < 0.001$) and the lower level of AA + DHA diet group ($P < 0.01$), with no difference between the lower AA + DHA intake and controls. Mean values (expressed as g/100 g of total fatty acids) were: 1.24 ± 0.24 (control); 1.36 ± 0.10 (0.6:0.1 AA:DHA diet group); and 2.03 ± 0.17 (1.2:0.2 AA:DHA diet group) (Figure 4-13). Piglets fed the higher amount of AA + DHA had LA concentrations significantly greater than those of the other

Table 4-5. Selected fatty acid composition of plasma lipids in LBW and VLBW piglets at the end of study (expressed as g/100 g of total fatty acids)[1]

	Birth weight 1.1 – 1.2 kg			Birth weight ≤ 1.0 kg		
	DIETARY TREATMENTS[2]					
	Control	AA:DHA 0.6%:0.1%	AA:DHA 1.2%:0.2%	Control	AA:DHA 0.6%:0.1%	AA:DHA 1.2%:0.2%
	n = 6	n = 5	n = 6	n = 4	n = 4	n = 4
18:2 n-6	33.55 ± 1.26^{a}*	30.69 ± 2.81^{ab}	29.10 ± 1.11^{b}*	32.01 ± 1.96	30.64 ± 1.93	29.60 ± 1.53
18:3 n-3	1.12 ± 0.10	1.11 ± 0.25	1.11 ± 0.15	1.13 ± 0.35	1.23 ± 0.16	1.20 ± 0.20
20:4 n-6	7.34 ± 0.57^{a}†	9.48 ± 1.74^{b}	11.02 ± 1.43^{b}†	7.18 ± 1.38^{a}*	9.21 ± 0.34^{a}	10.35 ± 1.35^{b}*
20:5 n-3	0.21 ± 0.04	0.19 ± 0.06	0.18 ± 0.04	0.19 ± 0.08	0.20 ± 0.05	0.24 ± 0.06
22:6 n-3	2.04 ± 0.40	2.26 ± 0.63	2.46 ± 0.38	1.49 ± 0.20^{a}	2.11 ± 0.16^{b}	2.01 ± 0.35^{b}
Total n-6 (C ≥ 18)	42.37 ± 1.14	41.67 ± 1.64	42.30 ± 3.00	41.42 ± 2.44	41.34 ± 1.97	41.59 ± 0.78
Total n-3 (C ≥ 18)	4.16 ± 0.32	4.18 ± 0.72	4.33 ± 0.38	3.46 ± 0.45	4.18 ± 0.47	4.02 ± 0.40
n-6:n-3 (C ≥ 18)	10.25 ± 0.91	10.24 ± 2.10	9.66 ± 0.78	12.17 ± 2.07	10.10 ± 0.54	10.43 ± 1.17
AA:DHA	3.68 ± 0.60	4.32 ± 0.67	4.52 ± 0.64	4.78 ± 0.34	4.38 ± 0.27	5.21 ± 0.65
AA:EPA	35.50 ± 6.83^{a}*	53.72 ± 10.31^{ab}	64.80 ± 15.94^{b}*	44.55 ± 19.58	47.49 ± 11.66	42.55 ± 2.91

[1]Values are means ± SD. Within each weight category, means in a row with different superscripts are statistically different at $P < 0.05$.

* values are different at $P < 0.01$, †values are different at $P < 0.001$

[2]AA:DHA diets expressed as g/100 g of fat

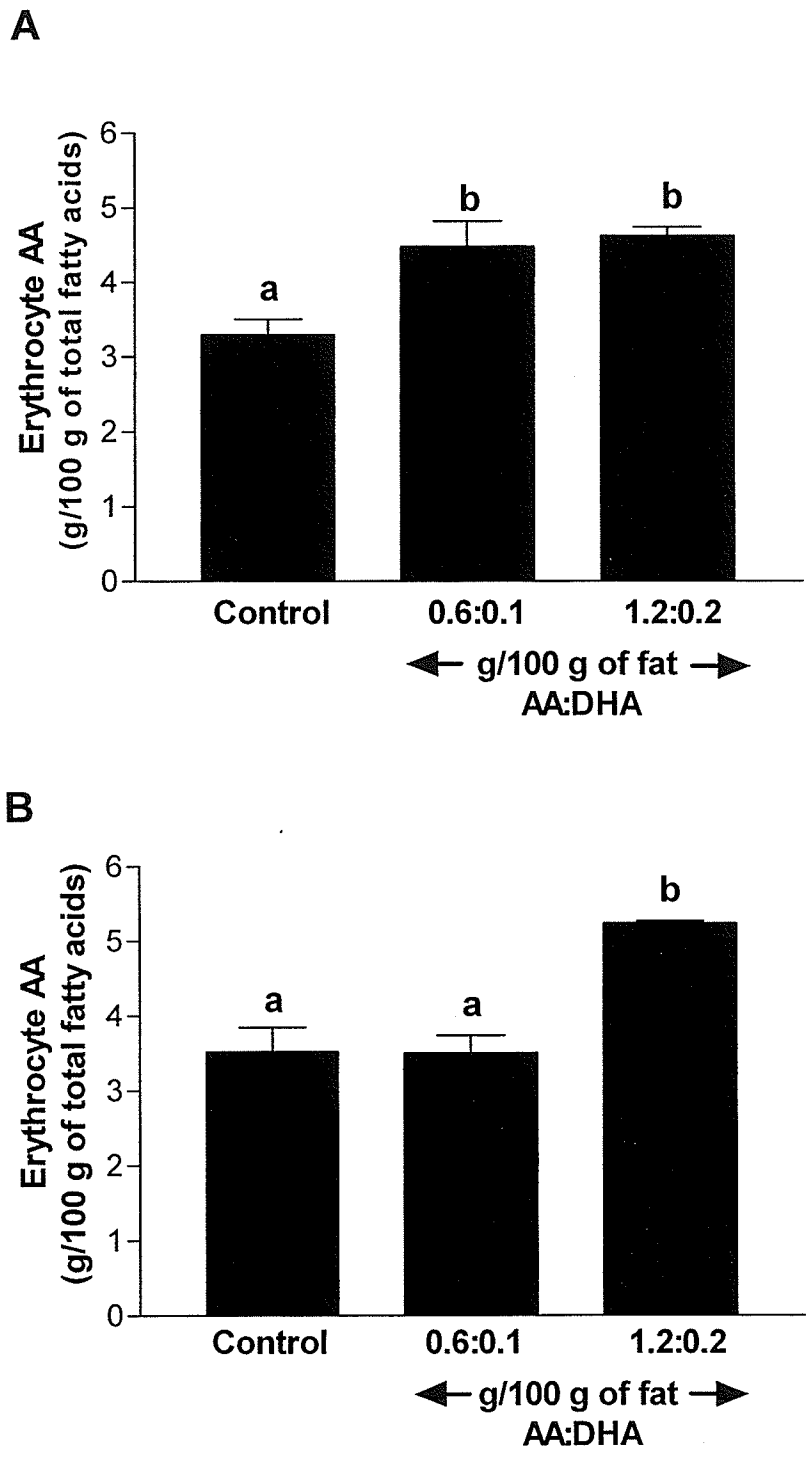

Figure 4-12. Effect of AA and DHA supplementation on erythrocyte AA (expressed as g/100 g of total fatty acids) in LBW (**A**) and VLBW (**B**) piglets. Values are means ± SD, n = 6 for LBW piglets, except 0.6:0.1 AA:DHA diet group (n = 5), n = 4 for VLBW piglets. Bars with different superscripts are significantly different at $P < 0.01$.

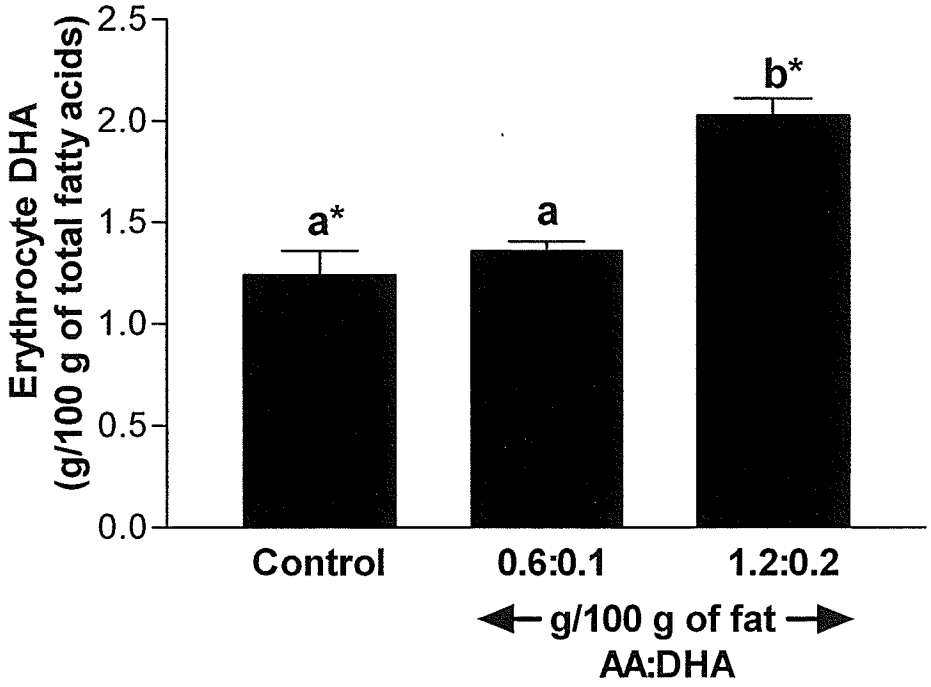

Figure 4-13. Effect of AA and DHA supplementation on erythrocyte DHA (expressed as g/100 g of total fatty acids) in VLBW piglets. Values are means ± SD, n = 4. Bars with different superscripts are significantly different at $P < 0.01$, * indicates significant difference at $P < 0.001$.

two diet groups ($P < 0.05$ for both), while their erythrocyte ALA levels were elevated only above those of the lower AA + DHA diet group ($P < 0.05$). Total n-6 and total n-3 fatty acids were elevated by 1.2:0.2 AA + DHA compared to controls ($P < 0.05$ and $P < 0.01$, respectively) and compared to the lower supplementation group ($P < 0.01$ for both). In contrast to the LBW piglets whose n-6:n-3 fatty acid ratio was unaffected by diet, the n-6:n-3 fatty acid ratio in the VLBW piglets was significantly reduced by the higher intake of AA + DHA compared to both other dietary treatments ($P < 0.05$ for both). Erythrocyte EPA, AA:DHA and AA:EPA ratios did not differ between diet groups. Erythrocyte fatty acid composition for both birth weight categories is presented in Table 4-6.

4.3.3 Percent Change in Erythrocyte Fatty Acids

Change in erythrocyte fatty acid proportions from baseline to end of study, expressed as percent change from baseline, was calculated for LA, ALA, AA, DHA, and EPA. Regardless of dietary treatment and birth weight category designation, erythrocyte content of AA, DHA, and EPA declined over the 15-d period, and both levels of dietary AA + DHA attenuated the decline in erythrocyte AA and DHA proportions.

4.3.3a LBW Piglets

In LBW piglets, % change in erythrocyte AA showed a reciprocal relationship with dietary AA + DHA intake, with mean values of: -45.7 ± 7.2 % (control); -21.2 ± 13.0 % (0.6:0.1 AA:DHA diet group); and -17.9 ± 6.8 % (1.2:0.2 AA:DHA diet group). Both low and high intakes of AA + DHA significantly attenuated the % decrease in erythrocyte AA compared to controls ($P < 0.01$ and $P < 0.001$, respectively); however, no

Table 4-6. Selected fatty acid composition of erythrocyte lipids in LBW and VLBW piglets at the end of study (expressed as g/100 g of total fatty acids)[1]

	Birth weight 1.1 – 1.2 kg			Birth weight ≤ 1.0 kg		
	DIETARY TREATMENTS[2]					
	Control	AA:DHA 0.6:0.1	AA:DHA 1.2:0.2	Control	AA:DHA 0.6:0.1	AA:DHA 1.2:0.2
	n = 6	n = 5	n = 6	n = 4	n = 4	n = 4
18:2 n-6	19.77 ± 2.01	18.13 ± 2.30	16.87 ± 2.23	17.56 ± 1.20^{a}	16.76 ± 0.72^{a}	18.25 ± 0.49^{b}
18:3 n-3	0.58 ± 0.11	0.53 ± 0.16	0.50 ± 0.13	0.45 ± 0.09^{ab}	0.44 ± 0.02^{a}	0.56 ± 0.03^{b}
20:4 n-6	3.29 ± 0.52^{a}*	4.48 ± 0.76^{b}*	4.62 ± 0.30^{b}*	3.52 ± 0.65 ^{a}*	3.50 ± 0.48 ^{b}*	5.24 ± 0.07 ^{b}*
20:5 n-3	0.08 ± 0.02	0.07 ± 0.01	0.06 ± 0.01	0.05 ± 0.01	0.05 ± 0.01	0.07 ± 0.01
22:6 n-3	1.42 ± 0.12	1.51 ± 0.14	1.73 ± 0.38	1.24 ± 0.24^{a}†	1.36 ± 0.10^{a}*	2.03 ± 0.17^{b}*†
Total n-6 (C ≥ 18)	24.33 ± 2.08	23.95 ± 2.63	22.81 ± 2.57	22.19 ± 1.90^{a}	21.37 ± 0.47^{a}*	24.81± 0.48^{b}*
Total n-3 (C ≥ 18)	2.79 ± 0.21	2.74 ± 0.26	2.93 ± 0.63	2.49 ± 0.38^{a}*	2.38 ± 0.11^{a}*	3.31 ± 0.29^{b}*
n-6:n-3 (C ≥ 18)	8.75 ± 0.66	8.78 ± 0.99	7.99 ± 1.29	8.97 ± 0.70^{a}	9.00 ± 0.35^{a}	7.54 ± 0.68^{b}
AA:DHA	2.33 ± 0.32	3.02 ± 0.75	2.75 ± 0.46	2.85 ± 0.16	2.60 ± 0.46	2.60 ± 0.17
AA:EPA	46.43 ± 13.44^{a}*	69.77 ± 11.08^{b}	82.73 ± 16.04^{b}*	70.82 ± 22.72	72.25 ± 26.13	77.91± 13.59

[1]Values are means ± SD. Within each weight category, means in a row with different superscripts are statistically different at $P < 0.05$.

* values are different at $P < 0.01$, †values are different at $P < 0.001$

[2]AA:DHA diets expressed as g/100 g of fat

difference between supplemented groups was noted (Figure 4-14). Erythrocyte retention of DHA was significantly improved by the higher supplementation level compared to controls ($P < 0.05$), but not to the lower AA + DHA diet group (Figure 4-15). Mean values for % change in erythrocyte DHA were: -30.9 ± 11.7 % (control); -20.1 ± 15.9 % (0.6:0.1 AA:DHA diet group); and -3.2 ± 20.0 % (1.2:0.2 AA:DHA diet group). Percent increase in erythrocyte LA was reduced significantly by both supplemented diets compared to controls ($P < 0.05$), but not compared to each other. Values for % change in LA were: 87.8 ± 16.4 % (control); 53.8 ± 11.7 % (0.6:0.1 AA:DHA diet group); and 60.4 ± 21.9 % (1.2:0.2 AA:DHA diet group). No diet effect on % change in ALA or EPA was observed.

4.3.3b VLBW Piglets

Percent changes in erythrocyte AA, EPA, LA, and ALA were unaffected by diet in VLBW piglets. A significant attenuation of erythrocyte DHA decline was observed with both the low and high levels of supplementation relative to controls ($P < 0.05$ and $P < 0.01$, respectively), while no difference between the supplemented diets was detected (Figure 4-15). Mean values for % change in erythrocyte DHA were: -22.7 ± 5.8 % (control); -14.6 ± 11.3 % (0.6:0.1 AA:DHA diet group), and 5.2 ± 11.5 % (1.2:0.2 AA:DHA diet group). The results of percent change in erythrocyte fatty acids for both birth weight categories are presented in Table 4-7.

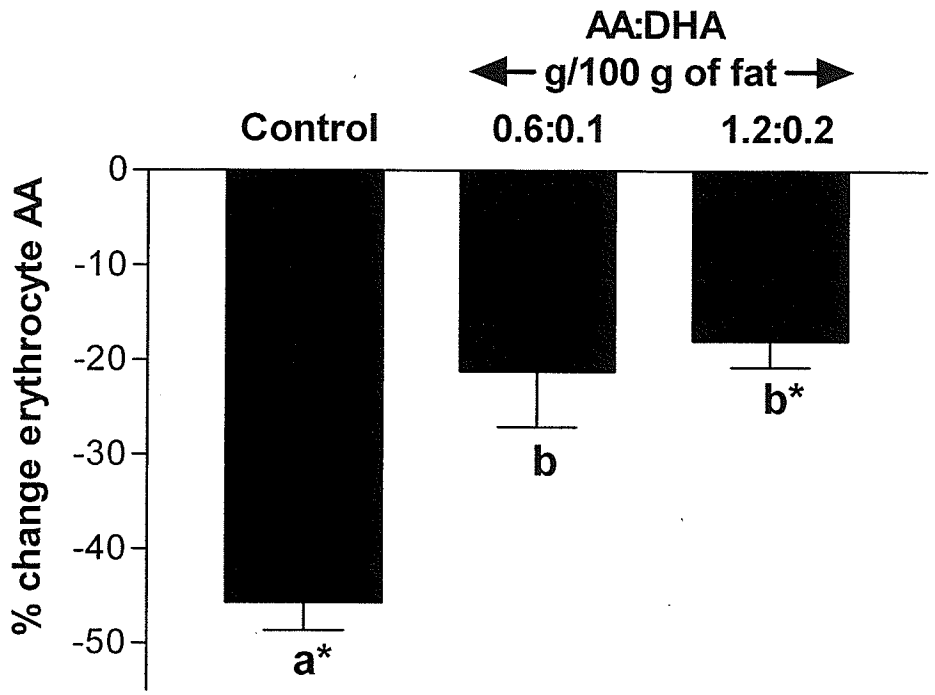

Figure 4-14. Effect of AA and DHA supplementation on percent change in erythrocyte AA in LBW piglets. Values are means ± SD, n = 6 except 0.6:0.1 AA:DHA diet group (n = 5). Bars with different superscripts are significantly different at $P < 0.01$, *indicates significant difference at $P < 0.001$.

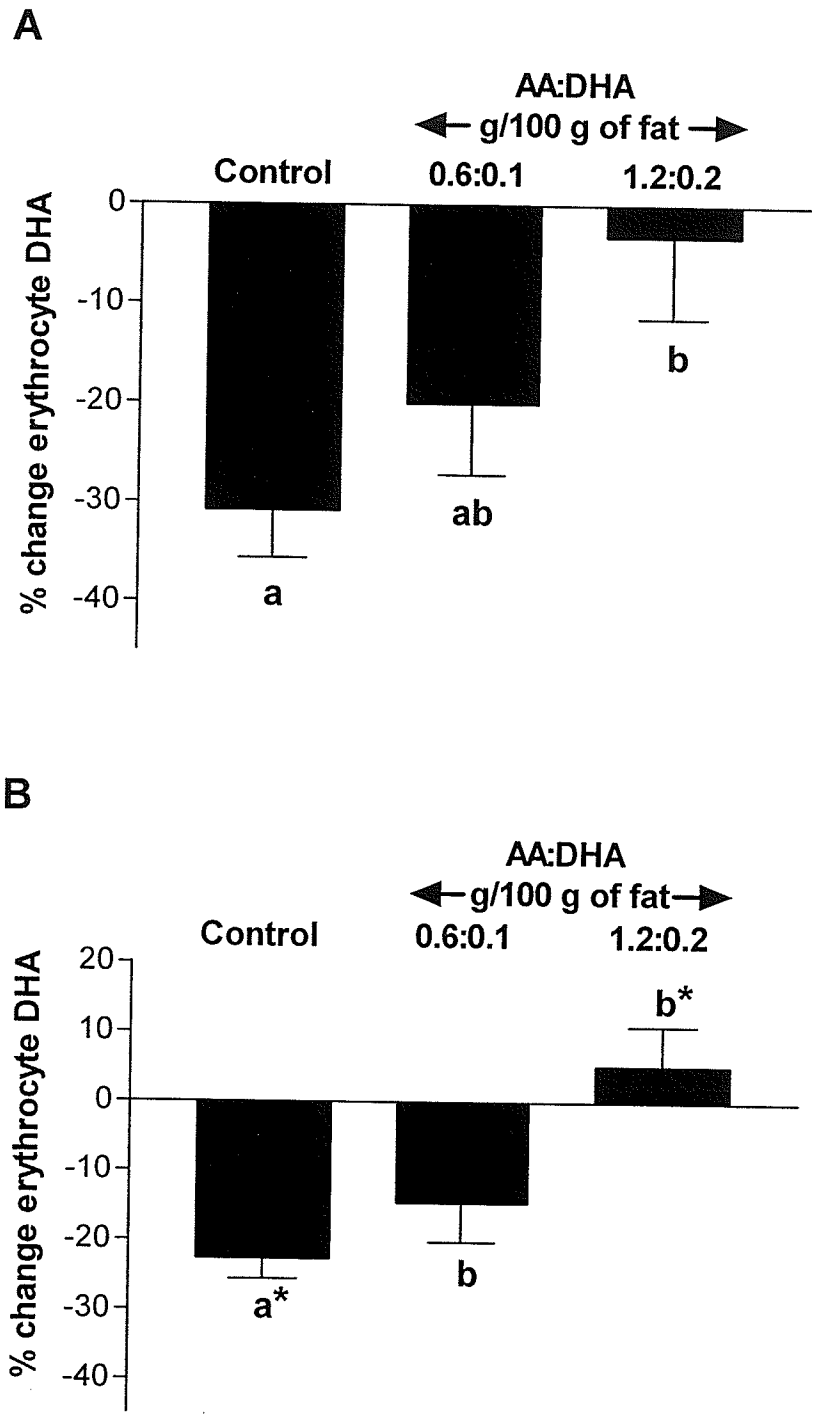

Figure 4-15. Effect of AA and DHA supplementation on percent change in erythrocyte DHA in LBW (**A**) and VLBW (**B**) piglets. Values are means ± SD, n = 6 for LBW piglets except 0.6:0.1 AA:DHA diet group (n = 5), n =4 for VLBW piglets. Bars with different superscripts are significantly different at $P < 0.05$, *indicates significance at $P < 0.01$.

Table 4-7. Percent change in selected erythrocyte fatty acids in LBW and VLBW piglets at the end of study[1]

	Birth weight 1.1- 1.2 kg			Birth weight ≤ 1.0 kg		
	DIETARY TREATMENTS[2]					
	Control	AA:DHA 0.6%:0.1%	AA:DHA 1.2%:0.2%	Control	AA:DHA 0.6%:0.1%	AA:DHA 1.2%:0.2%
	n = 6	n = 5	n = 6	n = 4	n = 4	n = 4
18:2 n-6	87.8 ± 16.4^{a}	53.8 ± 11.7^{b}	60.4 ± 21.9^{b}	61.3 ± 15.6	68.2 ± 13.5	44.6 ± 13.5
18:3 n-3	100.7 ± 51.0	89.0 ± 27.5	117.4 ± 67.2	70.4 ± 33.2	65.7 ± 24.6	68.8 ± 23.7
20:4 n-6	−45.7 ± 7.2^{a}*†	−21.2 ± 13.0^{b}*	−17.9 ± 6.8^{b}†	−43.0 ± 11.1	−39.1 ± 7.9	−29.2 ± 17.0
20:5 n-3	−39.1 ± 19.6	−37.0 ± 23.2	−56.0 ± 7.9	−38.3 ± 14.5	−49.5 ± 25.6	−64.3 ± 25.4
22:6 n-3	−30.9 ± 11.7^{a}	−20.1 ± 15.9^{ab}	−3.2 ± 20.0^{b}	−22.7 ± 5.8^{a}*	−14.6 ± 11.3^{b}	5.2 ± 11.5^{b}*

[1]Values are means ± SD. Within each weight category, means in a row with different superscripts are statistically different at $P < 0.05$.

* values are different at $P < 0.01$, †values are different at $P < 0.001$

[2]AA:DHA diets expressed as g/100 g of fat

4.3.4 Adipose Tissue Fatty Acids

Adipose AA concentrations rose in response to both levels of AA + DHA supplementation in both birth weight categories, with significant differences between all three diet groups ($P < 0.001$ for all comparisons, Figure 4-16). Proportions of LA, ALA, EPA, and total n-6 fatty acids did not differ with dietary treatment in either birth weight category. Table 4-8 shows adipose tissue fatty acid composition for both birth weight categories.

4.3.4a LBW Piglets

DHA content of adipose tissue in the LBW piglets reflected dietary AA + DHA intake, with control levels significantly lower than both supplemented groups (both $P < 0.001$) and intermediate tissue DHA concentrations in the lower supplementation group ($P < 0.01$ for comparison between supplementation groups) (Figure 4-17). Total n-6 and n-3 fatty acids, and the n-6 to n-3 fatty acid ratio did not differ with dietary treatment. The ratio of AA to DHA rose in parallel with increasing dietary intake of AA + DHA. Significant differences in adipose AA: DHA ratios were found in comparisons between the higher supplementation group and both controls ($P < 0.001$) and the lower AA + DHA diet group ($P < 0.01$), but not between the lower supplementation group and controls. Adipose AA:EPA ratios increased in response to both levels of AA + DHA supplementation compared to controls (both $P < 0.001$), but did not differ between supplemented groups.

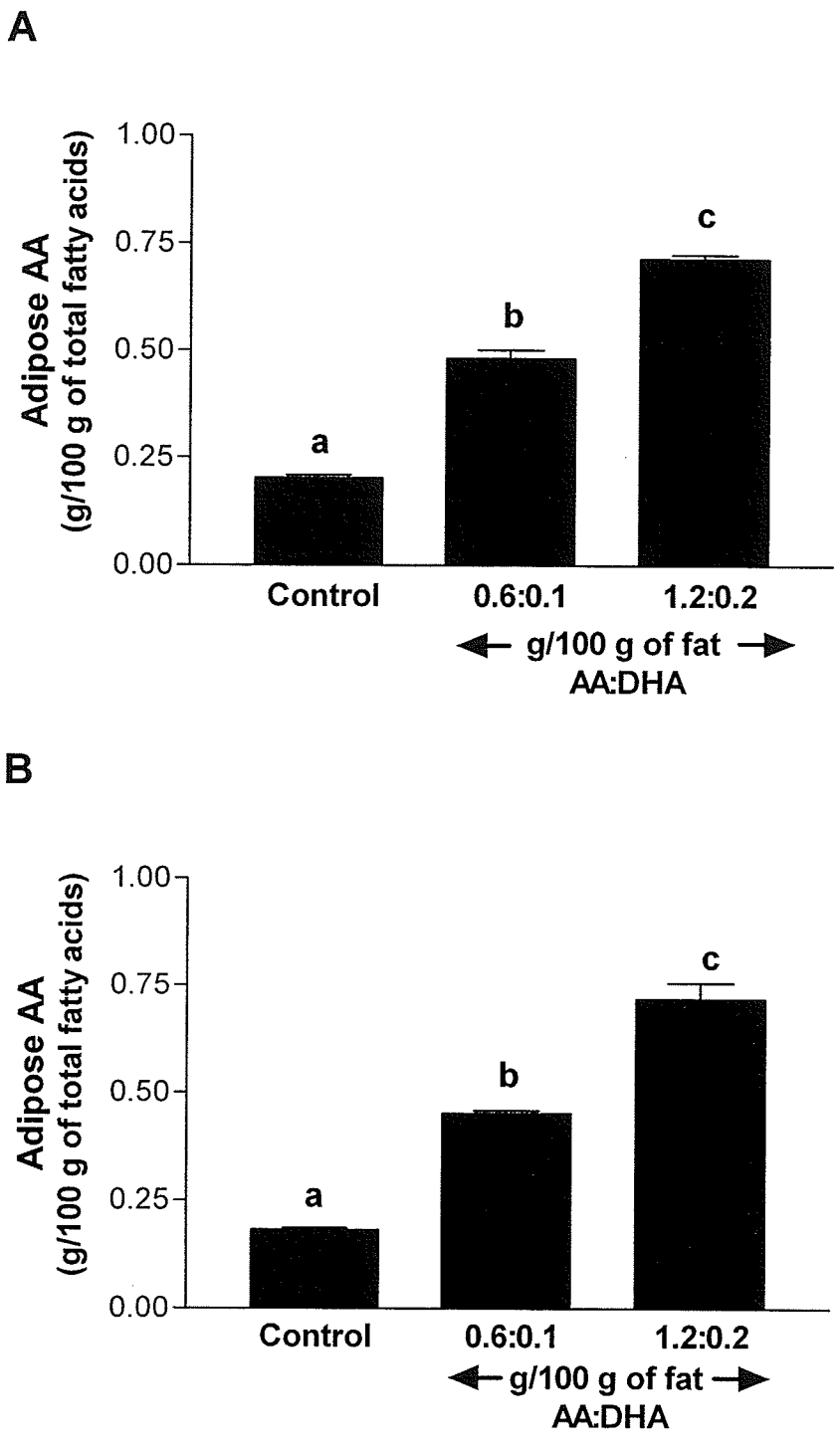

Figure 4-16. Effect of AA and DHA supplementation on adipose AA in LBW (**A**) and VLBW (**B**) piglets. Values are means ± SD, n = 6 for LBW piglets except 0.6:0.1 AA:DHA diet group (n = 5), n =4 for VLBW piglets. Bars with different superscripts are significantly different at $P < 0.001$.

Table 4-8. Selected fatty acid composition of adipose lipids in LBW and VLBW piglets at the end of study (expressed as g/100 g of total fatty acids)[1]

	Birth weight 1.1- 1.2 kg			Birth weight ≤ 1.0 kg		
	DIETARY TREATMENTS[2]					
	Control	AA:DHA 0.6%:0.1%	AA:DHA 1.2%:0.2%	Control	AA:DHA 0.6%:0.1%	AA:DHA 1.2%:0.2%
	n = 6	n = 5	n = 6	n = 4	n = 4	n = 4
18:2 n-6	26.78 ± 1.23	25.21 ± 1.69	25.77 ± 1.12	26.60 ± 1.56	26.83 ± 0.68	26.69 ± 1.18
18:3 n-3	2.74 ± 0.15	2.38 ± 0.40	2.59 ± 0.12	2.29 ± 0.44	2.70 ± 0.12	2.21 ± 0.19
20:4 n-6	0.20 ± 0.02^{a}†	0.48 ± 0.04^{b}†	0.72 ± 0.02^{c}†	0.18 ± 0.01^{a}†	0.45 ± 0.01^{b}†	0.72 ± 0.07^{c}†
20:5 n-3	0.06 ± 0.01	0.05 ± 0.01	0.06 ± 0.01	0.04 ± 0.01	0.06 ± 0.02	0.04 ± 0.01
22:6 n-3	0.06 ± 0.01^{a}†	0.12 ± 0.02^{b*}†	0.16 ± 0.02^{c*}†	0.08 ± 0.03^{a*}	0.11 ± 0.01^{a}	0.16 ± 0.02^{b*}
Total n-6 (C ≥ 18)	27.83 ± 1.26	26.63 ± 1.62	27.48 ± 1.09	27.65 ± 1.52	28.20 ± 0.65	28.42 ± 1.23
Total n-3 (C ≥ 18)	3.13 ± 0.15	2.79 ± 0.42	3.07 ± 0.13	2.52 ± 0.53^{ab}	3.15 ± 0.13^{a}	2.48 ± 0.20^{b}
n-6:n-3 (C ≥ 18)	8.90 ± 0.28	9.65 ± 1.04	8.97 ± 0.25	11.21 ± 1.49^{a}	8.97 ± 0.25^{a}	11.47 ± 0.52^{b}
AA:DHA	3.42 ± 0.40^{a}†	3.97 ± 0.23^{a}	4.59 ± 0.45^{b}†	2.79 ± 1.21^{a}	4.28 ± 0.21^{b}	4.45 ± 0.13^{b}
AA:EPA	3.57 ± 0.49^{a}†	10.14 ± 3.32^{b}*†	12.37 ± 1.53^{c}*†	4.26 ± 1.20^{a}†	8.73 ± 4.84^{a}	16.39 ± 1.45^{b}†

[1]Values are means ± SD. Within each weight category, means in a row with different superscripts are statistically different at $P < 0.05$.

* values are different at $P < 0.01$, †values are different at $P < 0.001$

[2]AA:DHA diets expressed as g/100 g of fat

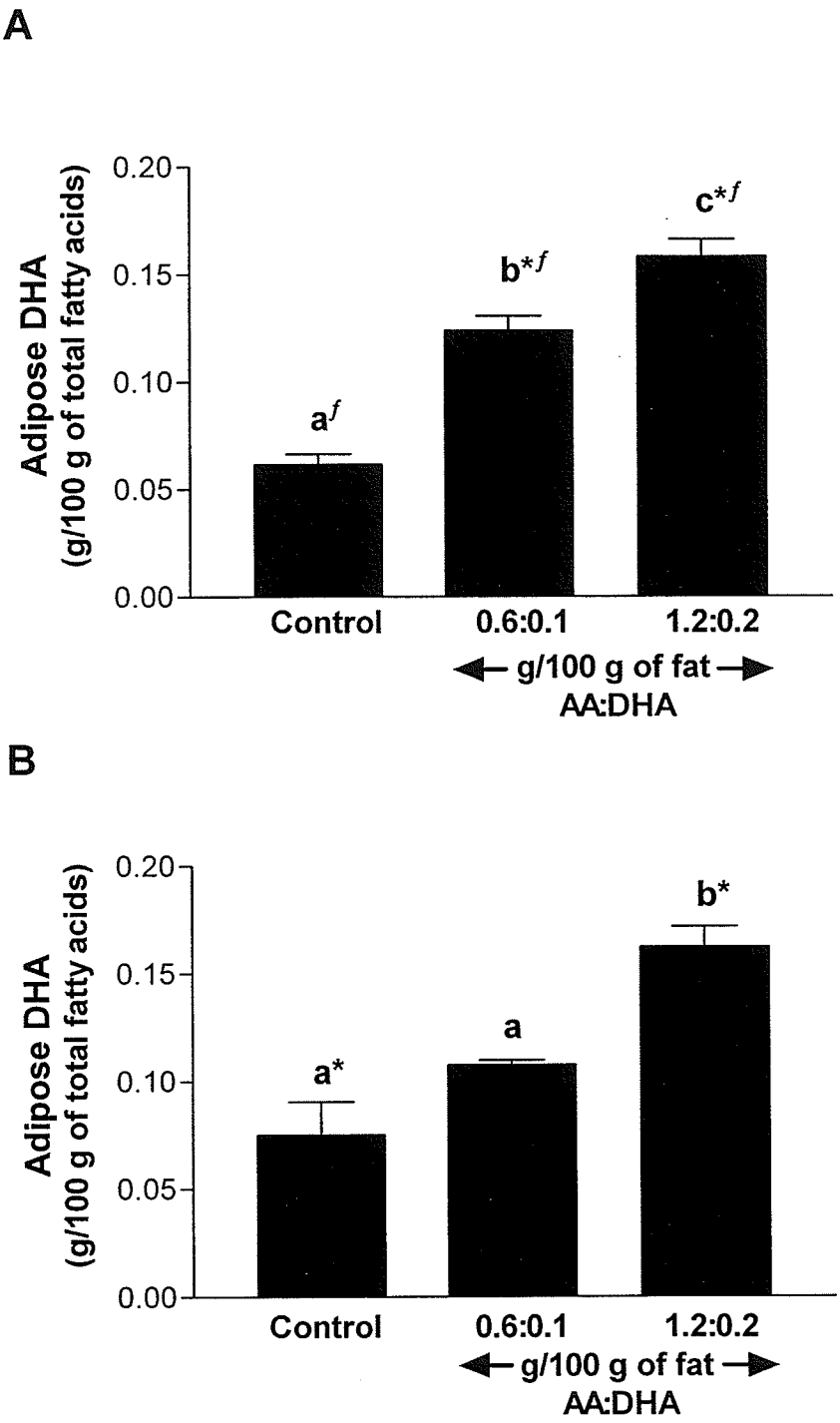

Figure 4-17 Effect of AA and DHA supplementation on adipose DHA (expressed as g/100 g of total fatty acids) in LBW (**A**) and VLBW (**B**) piglets. Values are means ± SD, n = 6 for LBW piglets except 0.6:0.1 AA:DHA diet group (n = 5), n = 4 for VLBW piglets. Bars with different superscripts are significantly different at $P < 0.05$, *indicates significance at $P < 0.01$, ^{f} indicates significance at $P < 0.001$.

4.3.4b VLBW Piglets

In VLBW piglets fed 1.2:0.2 g/100 g AA + DHA, adipose DHA rose significantly above controls ($P < 0.001$) and the lower supplementation group ($P < 0.05$) (Figure 4-17). Total n-3 fatty acids increased in response to the lower dietary intake of AA + DHA compared with the higher supplementation group ($P < 0.05$), but not relative to controls. While total n-6 fatty acids were unaffected by diet, the n-6:n-3 fatty acid ratio was significantly lower in piglets fed the lower AA + DHA intake compared to both other diet groups ($P < 0.05$ for both). The ratio of AA to DHA in adipose tissue reflected the level of dietary AA + DHA intake, with values highest in the higher supplementation group compared to controls ($P < 0.05$), and intermediate values in the lower supplementation group compared to controls ($P < 0.05$). The AA to DHA ratio did not differ significantly between the two supplemented groups. AA to EPA ratios also rose with increasing dietary AA + DHA intake; with significant differences were found between the higher supplementation group and controls ($P < 0.001$) and between the two supplemented groups ($P < 0.05$).

4.3.5 Liver Fatty Acids

4.3.5a LBW Piglets

Liver AA was elevated by both low and high levels of AA + DHA compared to controls ($P < 0.05$ and $P < 0.001$, respectively, Figure 4-18). DHA concentrations rose progressively as intake of AA + DHA increased; however, group means differed significantly only between the higher supplementation and control groups ($P < 0.05$, Figure 4-19). Similar EPA concentrations in both supplemented groups were lower

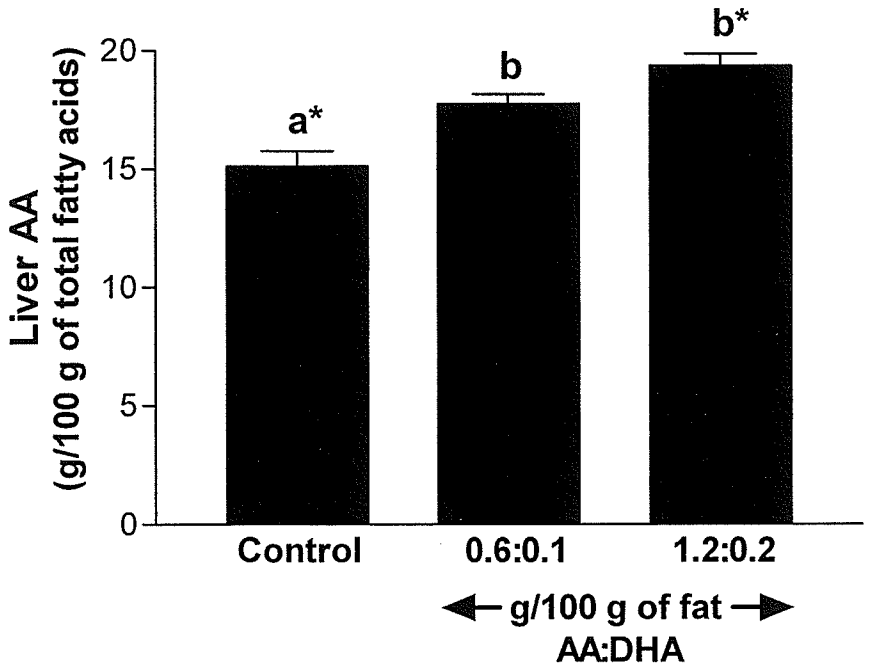

Figure 4-18. Effect of AA and DHA supplementation on liver AA (expressed as g/100 g of total fatty acids) in LBW piglets. Values are means ± SD, n = 6 except 0.6:0.1 AA:DHA diet group (n = 5). Bars with different superscripts are significantly different at $P < 0.05$, * indicates significant difference at $P < 0.001$.

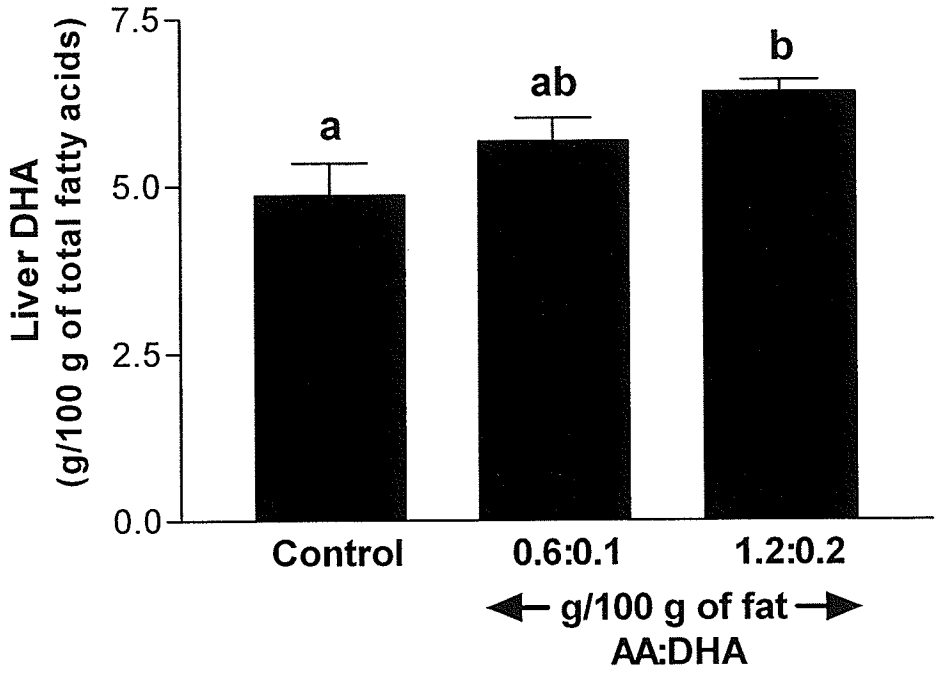

Figure 4-19. Effect of AA and DHA supplementation on liver DHA (expressed as g/100 g of total fatty acids) in LBW piglets. Values are means ± SD, n = 6 except 0.6:0.1 AA:DHA diet group (n = 5). Bars with different superscripts are significantly different at $P < 0.05$.

compared to controls, but these differences did not attain statistical significance after application of the Bonferroni test ($P = 0.038$). Liver LA content was significantly lowered in piglets fed the low and high AA + DHA intakes relative to controls ($P < 0.05$ and $P < 0.001$, respectively), but supplemented groups did not differ from each other. ALA concentrations showed a nonsignificant downward trend as level of AA + DHA intake increased. Total n-3 fatty acids tended to increase with increasing dietary AA + DHA ($P = 0.0585$), while total n-6 fatty acids and the n-6 to n-3 ratio were unaffected by diet. The AA to EPA ratio was elevated in response to both low and high supplementation compared to controls ($P < 0.05$ and $P < 0.01$, respectively), but did not differ from each other. Liver fatty acid data for both birth weight categories are summarized in Table 4-9.

4.3.5b VLBW Piglets

Both the low and high AA + DHA intakes increased liver AA compared to controls ($P < 0.05$ and $P < 0.01$, respectively) in VLBW piglets (Figure 4-20). Liver DHA content was elevated by both the low and high supplementation diets compared with controls ($P < 0.05$ and $P < 0.01$, respectively), with no difference between supplemented groups (Figure 4-21). EPA concentrations declined as AA + DHA intakes increased; however differences between group means were not significant. Liver AA to DHA ratio showed a nonsignificant downward trend as the level of AA + DHA intake increased ($P = 0.07$), while AA to EPA ratios were significantly greater in piglets fed the higher intake of AA + DHA compared with controls ($P < 0.05$), but not relative to the lower AA + DHA diet group. Liver LA was elevated in response to both the low and high

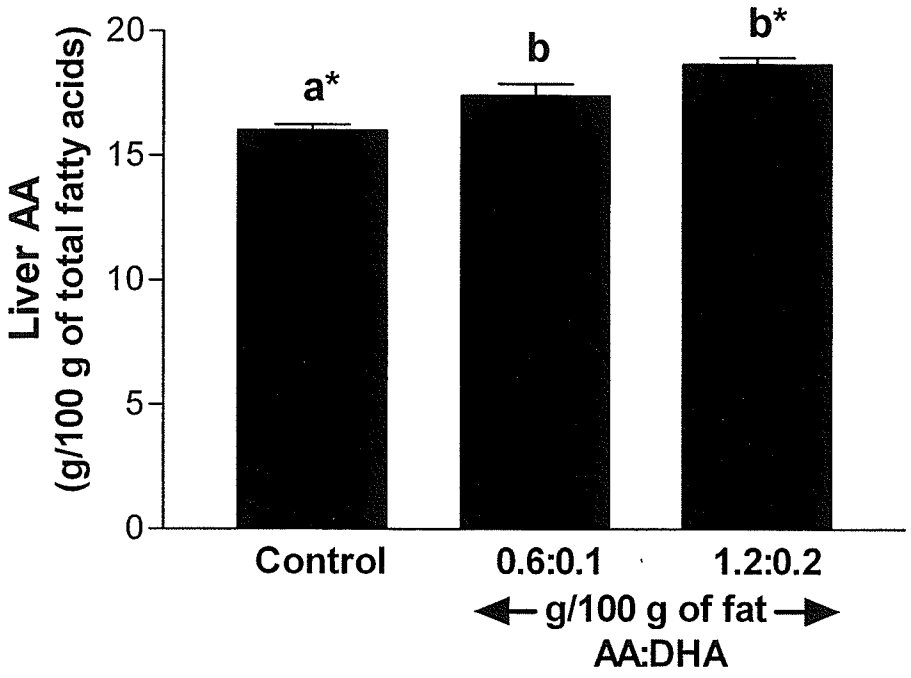

Figure 4-20. Effect of AA and DHA supplementation on liver AA (expressed as g/100 g of total fatty acids) in VLBW piglets. Values are means ± SD, n = 4. Bars with different superscripts are significantly different at $P < 0.05$, * indicates significant difference at $P < 0.01$.

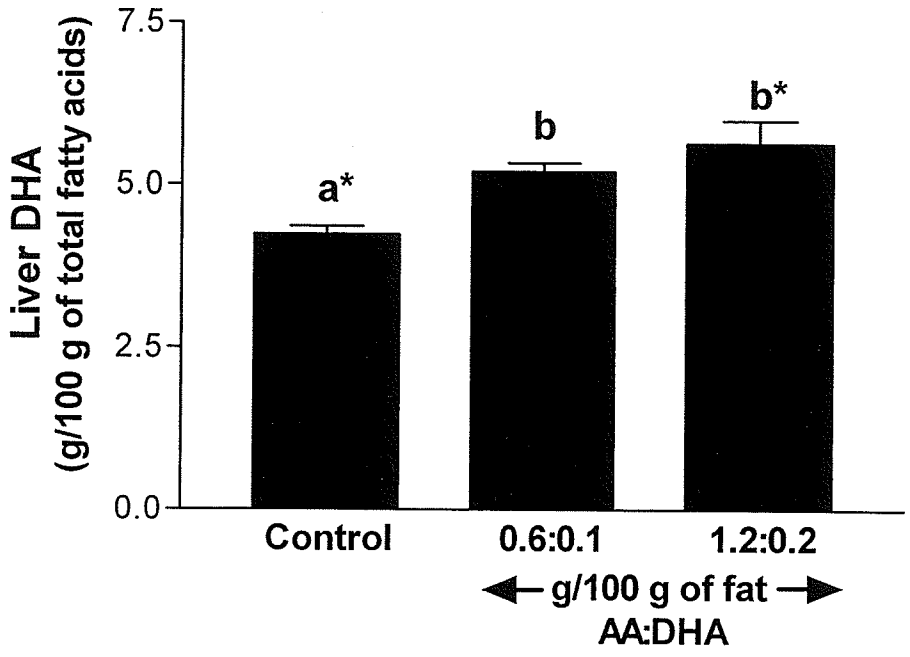

Figure 4-21. Effect of AA and DHA supplementation on liver DHA (expressed as g/100 g of total fatty acids) in VLBW piglets. Values are means ± SD, n = 4. Bars with different superscripts are significantly different at $P < 0.05$, * indicates significant difference at $P < 0.01$.

Table 4-9. Selected fatty acid composition of liver lipids in LBW and VLBW piglets at the end of study (expressed as g/100 g of total fatty acids)[1]

	Birth weight 1.1 – 1.2 kg			Birth weight ≤ 1.0 kg		
	DIETARY TREATMENTS[2]					
	Control	AA:DHA 0.6:0.1	AA:DHA 1.2:0.2	Control	AA:DHA 0.6:0.1	AA:DHA 1.2:0.2
	n = 6	n = 5	n = 6	n = 4	n = 4	n = 4
18:2 n-6	20.36 ± 1.99^{a}†	17.37 ± 1.15^{b}	15.91 ± 1.00^{b}†	19.26 ± 1.10^{a}*	17.53 ± 0.60^{b}	16.53 ± 0.67^{b}*
18:3 n-3	0.52 ± 0.21	0.45 ± 0.16	0.38 ± 0.08	0.49 ± 0.13	0.49 ± 0.08	0.34 ± 0.09
20:4 n-6	15.13 ± 1.56^{a}†	17.75 ± 0.90^{b}	19.38 ± 1.21^{b}†	16.03 ± 0.47^{a}*	17.43 ± 0.93^{b}	18.69 ± 0.57^{b}*
20:5 n-3	0.21 ± 0.04	0.16 ± 0.03	0.16 ± 0.03	0.26 ± 0.08	0.19 ± 0.03	0.18 ± 0.03
22:6 n-3	4.86 ± 1.19^{a}	5.67 ± 0.78^{ab}	6.40 ± 0.45^{b}	4.24 ± 0.25^{a}*	5.20 ± 0.26^{b}	5.64 ± 0.70^{b}*
Total n-6 (C ≥ 18)	37.53 ± 1.06	37.09 ± 0.32	37.33 ± 0.49	37.17 ± 0.79	37.02 ± 0.57	37.16 ± 0.55
Total n-3 (C ≥ 18)	7.31 ± 0.82	7.93 ± 0.85	8.46 ± 0.51	6.65 ± 0.56	7.52 ± 0.37	7.58 ± 0.96
n-6:n-3 (C ≥ 18)	5.18 ± 0.73	5.06 ± 0.81	4.23 ± 0.47	5.66 ± 0.43	4.97 ± 0.58	4.23 ± 0.47
AA:DHA	3.26 ± 0.70	3.22 ± 0.30	3.04 ± 0.29	3.79 ± 0.13	3.35 ± 0.12	3.34 ± 0.44
AA:EPA	74.84 ± 20.00^{a}*	110.60 ± 17.34^{b}	125.30 ± 20.96^{b}*	65.64 ± 21.82^{a}	94.13 ± 9.44^{a}	111.00 ± 25.45^{b}

[1]Values are means ± SD. Within each weight category, means in a row with different superscripts are statistically different at $P < 0.05$,

* values are different at $P < 0.01$, †values are different at $P < 0.001$.

[2]AA:DHA diets expressed as g/100 g of fat

supplementation levels ($P < 0.05$ and $P < 0.01$, respectively). No differences between diet groups were observed for ALA, total n-6 fatty acids, total n-3 fatty acids, and the n-6 to n-3 fatty acid ratio.

4.4 BIOCHEMICAL MEASUREMENTS

4.4.1 LBW Piglets

No differences in plasma osteocalcin (range 6.58 - 12.71 nM/L), plasma IGF-I (range 5.24 - 17.16 nM/L), bone PGE_2 (range 2.45 - 8.41nM/L), or urine cortisol (range 0.03-0.48 mM/mM creatinine) were observed among diet groups at the end of study. Urine NTX, an index of bone resorption, showed a trend downwards with increasing levels of AA + DHA supplementation, with mean values (as μM/mM creatinine): 14.56 ± 3.93 (control); 8.89 ± 5.58 (0.6:0.1 AA:DHA diet group); 7.52 ± 1.56 (1.2:0.2 AA:DHA diet group). Piglets supplemented with the higher level of AA + DHA demonstrated a significantly lower urine NTX compared to controls ($P < 0.01$), but no differences were observed between the other diet groups (Figure 4-22). Urine Ca and P, Ca/P ratio, bone Ca and P content, and bone Ca/P ratio did not differ between diet groups.

4.4.2 VLBW Piglets

There was no significant difference in plasma osteocalcin (range 5.90-11.65 nM/L), plasma IGF-I (range 3.75-17.04 nmol/L), or bone PGE_2 (range 1.31-6.54 nM/L) among diet groups. Urine NTX values decreased numerically but not significantly ($P = 0.0494$) with increasing intake of AA + DHA; however, group differences failed to reach statistical significance after Bonferroni testing. Mean values for NTX (expressed asμM

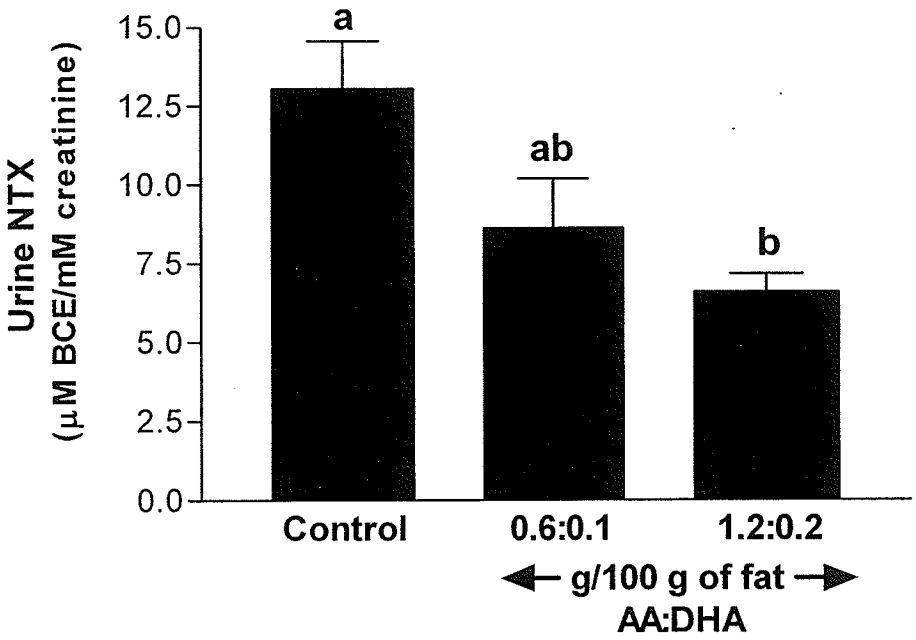

Figure 4-22. Effect of AA and DHA supplementation on urine N-telopeptide (NTX) in LBW piglets. Values are means ± SD, n = 6 except 0.6:0.1 AA:DHA diet group (n = 5). Bars with different superscripts are significantly different at $P < 0.05$.

BCE/mM creatinine) were: 7.29 ± 3.93 (control); 7.06 ± 2.33 (0.6:0.1 AA:DHA diet group); and 3.44 ± 1.13 (1.2:0.2 AA:DHA diet group). Bone Ca content was higher in the 0.6:0.1 AA + DHA diet group compared to the other dietary treatments (both $P < 0.001$, Figure 4-23), and bone Ca/P ratio was elevated by 0.6:0.1 AA + DHA supplementation compared to both controls ($P < 0.05$) and to the higher supplementation group ($P < 0.01$) (Figure 4-24). Urine cortisol (range 0.05-0.42 mM/mM creatinine) was significantly elevated by the lower level of supplementation compared to controls ($P < 0.05$), but otherwise did not differ between diet groups (Figure 4-25). Biochemical data for both birth weight categories are found in Tables 4-10 and 4-11.

4.5 PEARSON PRODUCT CORRELATION ANALYSIS

Correlation analysis was conducted to delineate relationships between outcome measurements of growth and bone mass, bone biomarkers, and LC PUFA status.

4.5.1 Whole Body Bone Mass

In correlation analysis of all piglets ($n = 29$), whole body BMC and BMD correlated negatively with plasma osteocalcin ($r = -0.69$, $P < 0.0001$; $r = -0.38$, $P = 0.04$, respectively). In addition, whole body BMD for all piglets ($n = 29$) correlated positively with IGF-I ($r = 0.39$, $P = 0.04$) and negatively with cortisol ($r = -0.41$, $P = 0.03$).

Significant positive correlations of whole body BA and BMC, but not BMD, with final body weight were observed in LBW piglets ($r = 0.87$, $P < 0.0001$; $r = 0.69$, $P = 0.002$ respectively) and VLBW piglets ($r = 0.92$, $P < 0.0001$; $r = 0.87$, $P = 0.0002$,

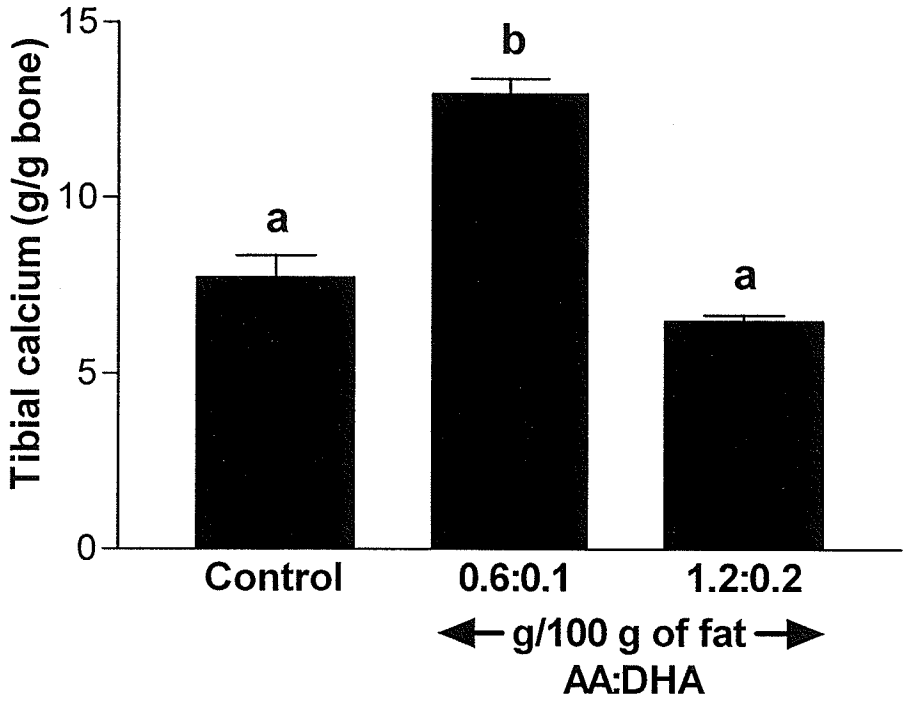

Figure 4-23. Effect of AA and DHA supplementation on tibial calcium in VLBW piglets. Values are means ± SD, n = 4. Bars with different superscripts are significantly different at $P < 0.001$.

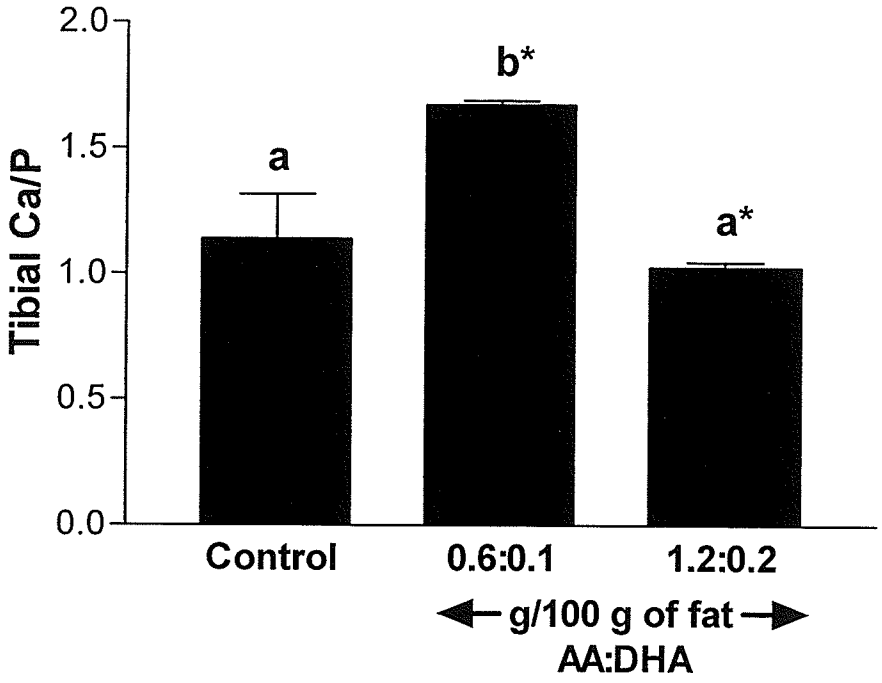

Figure 4-24. Effect of AA and DHA supplementation on tibial Ca/P ratio in VLBW piglets. Values are means ± SD, n = 4. Bars with different superscripts are significantly different at $P < 0.05$, *indicates significant difference at $P < 0.01$.

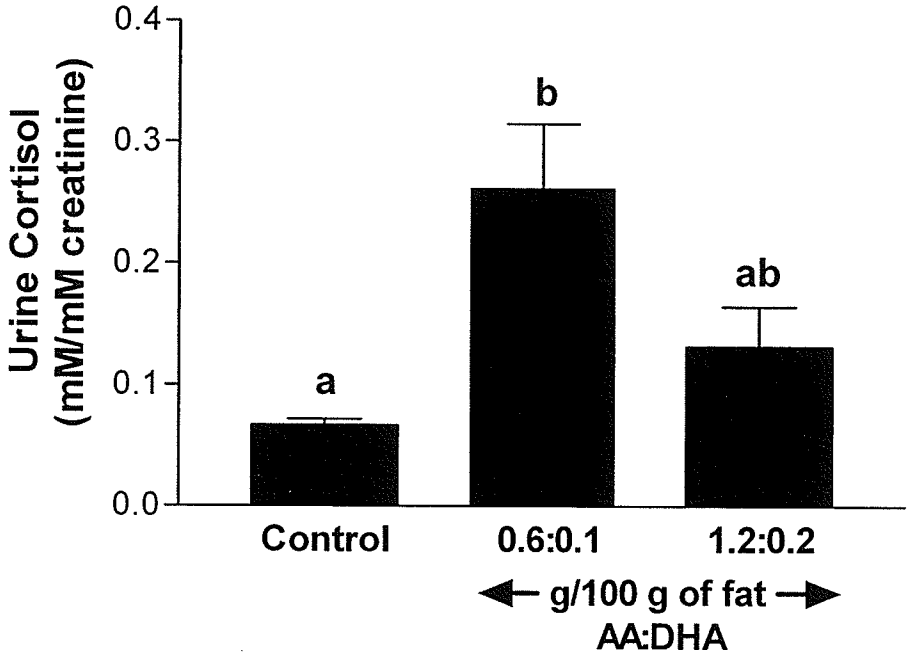

Figure 4-25. Effect of AA and DHA supplementation on urine cortisol in VLBW piglets. Values are means ± SD, n = 4. Bars with different superscripts are significantly different at $P < 0.05$.

Table 4-10. Plasma and urine biochemistry in LBW and VLBW piglets at the end of study[1]

	Birth weight 1.1 – 1.2 kg			Birth weight ≤ 1.0 kg		
	DIETARY TREATMENTS[2]					
	Control n = 6	AA:DHA 0.6:0.1 n = 5	AA:DHA 1.2:0.2 n = 6	Control n = 4	AA:DHA 0.6:0.1 n = 4	AA:DHA 1.2:0.2 n = 4
Plasma osteocalcin, *nMl/L*	8.78 ± 1.82	10.50 ± 1.80	8.86 ± 1.65	8.82 ± 2.40	8.25 ± 0.88	10.22 ± 0.64
Plasma IGF-I, *nM/L*	11.40 ± 3.87	11.71 ± 4.42	11.42 ± 4.00	10.05 ± 5.11	11.52 ± 3.24	7.97 ± 4.62
Urine NTX, *μM BCE/mM creatinine*	14.56 ± 3.93[a]	8.89 ± 5.58 [ab]	. 7.52 ± 1.56[b]	7.29 ± 2.52	7.06 ± 2.33	3.44 ± 1.13
Urine Ca *nM/M creatinine*	0.2 ± 0.1	0.2 ± 0.1	0.2 ± 0.2	0.4 ± 0.2	0.7 ± 0.3	0.8 ± 0.9
Urine P, *nM/M creatinine*	4.0 ± 5.4	4.2 ± 5.5	8.2 ± 4.9	2.5 ± 4.8	7.3 ± 5.1	0.7 ± 1.0
Urine Ca/P	0.1 ± 0.1	0.9 ± 1.0	ND	2.1 ± 1.8	0.5 ± 0.8	2.8 ± 4.0
Urine cortisol, *mM/mM creatinine*	0.18 ± 0.15	0.12 ± 0.07	0.17 ± 0.11	0.07 ± 0.01[a]	0.26 ± 0.11[b]	0.13 ± 0.07[ab]

[1]Values are means ± SD. Within each weight category, means in a row with different superscripts are statistically different at $P < 0.05$.
[2]AA:DHA diets expressed as g/100 g of fat

Table 4-11. Bone biochemistry in LBW and VLBW piglets at the end of study[1]

	Birth weight 1.1 – 1.2 kg			Birth weight ≤ 1.0 kg		
	DIETARY TREATMENTS[2]					
	Control n = 6	AA:DHA 0.6:0.1 n = 5	AA:DHA 1.2:0.2 n = 6	Control n = 4	AA:DHA 0.6:0.1 n = 4	AA:DHA 1.2:0.2 n = 4
PGE_2 in bone culture fluid, *ng/g of bone*	5.27 ± 1.84	4.50 ± 2.02	4.59 ± 2.16	4.22 ± 1.89	3.44 ± 0.82	3.24 ± 1.37
Tibial Ca, *g/g bone*	0.13 ± 0.01	0.10 ± 0.04	0.13 ± 0.18	$0.08 \pm 0.01^{a\dagger}$	$0.13 \pm 0.01^{b\dagger}$	$0.07 \pm 0.00^{a\dagger}$
Tibial P, *g/g bone*	0.08 ± 0.01	0.07 ± 0.01	0.08 ± 0.01	0.07 ± 0.01	0.08 ± 0.01	0.06 ± 0.01
Tibial Ca/P	1.7 ± 0.0	1.4 ± 0.4	1.7 ± 0.0	1.1 ± 0.4^{a}	$1.7 \pm 0.0^{b*}$	$1.0 \pm 0.0^{a*}$

[1]Values are means ± SD. Within each weight category, means in a row with different superscripts are statistically different at $P < 0.05$, * values are different at $P < 0.01$, †values are different at $P < 0.001$.
[2]AA:DHA diets expressed as g/100 g of fat

respectively). Among LBW piglets, whole body BA was negatively correlated with EPA in plasma and liver ($r = -0.67$, $P = 0.003$; $r = -0.56$, $P = 0.02$, respectively), and bone PGE_2 ($r = -0.75$, $P = 0.0005$). Similarly, whole body BMC was inversely related to plasma and liver EPA ($r = -0.79$, $P = 0.0002$; $r = -0.61$, $P = 0.009$, respectively), and bone PGE_2 ($r = -0.67$, $P = 0.003$). Whole body BMC and BMD correlated negatively with cortisol ($r = -0.57$, $P = 0.02$; $r = -0.52$, $P = 0.03$, respectively) in these piglets.

In VLBW piglets, whole body BA related positively to urine NTX ($r = 0.64$, $P = 0.02$) and bone Ca/P ($r = 0.61$, $P = 0.03$), and inversely with erythrocyte AA ($r = -0.71$, $P = 0.009$). Significant negative relationships were observed between whole body BMC and % change in erythrocyte AA ($r = -0.64$, $P = 0.03$) and final body weight as discussed earlier. Whole body BMD in this birth weight category was positively related to erythrocyte EPA ($r = 0.66$, $P = 0.02$).

4.5.2 Lumbar Spine Bone Mass

In correlation analysis of all 29 piglets, LS BMC was inversely related to urine NTX ($r = -0.41$, $P = 0.03$, Figure 4-26). The relationship between NTX and LS BMC was stronger for VLBW ($r = -0.65$, $P = 0.02$) than LBW piglets ($r = -0.50$, $P = 0.04$) (Figures 4-27 and 4-28, respectively).

Among LBW piglets, LS BMC was positively associated with urine Ca/P ($r = 0.73$, $P = 0.0009$) and negatively related to bone Ca/P ($r = -0.72$, $P = 0.001$) and EPA in plasma and liver ($r = -0.50$, $P = 0.04$; $r = -0.48$, $P = 0.046$, respectively). LS BMD correlated positively with urine Ca/P ($r = 0.85$, $P < 0.0001$) and negatively with bone Ca/P ($r = -0.87$, $P < 0.0001$) and adipose EPA ($r = -0.65$, $P = 0.004$).

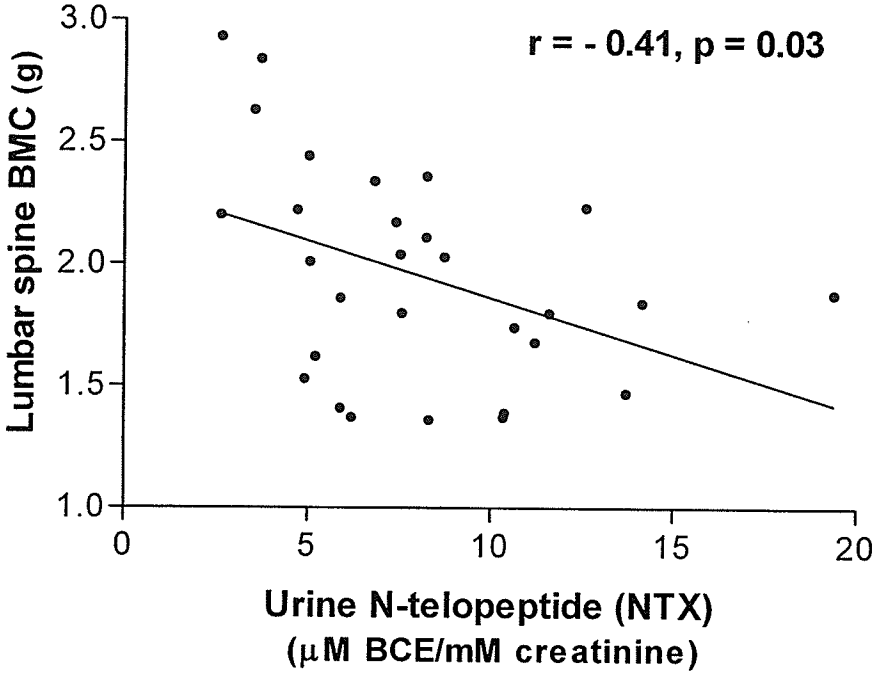

Figure 4-26. Relationship between urine N-telopeptide (NTX) and lumbar spine BMC in all piglets (n = 29).

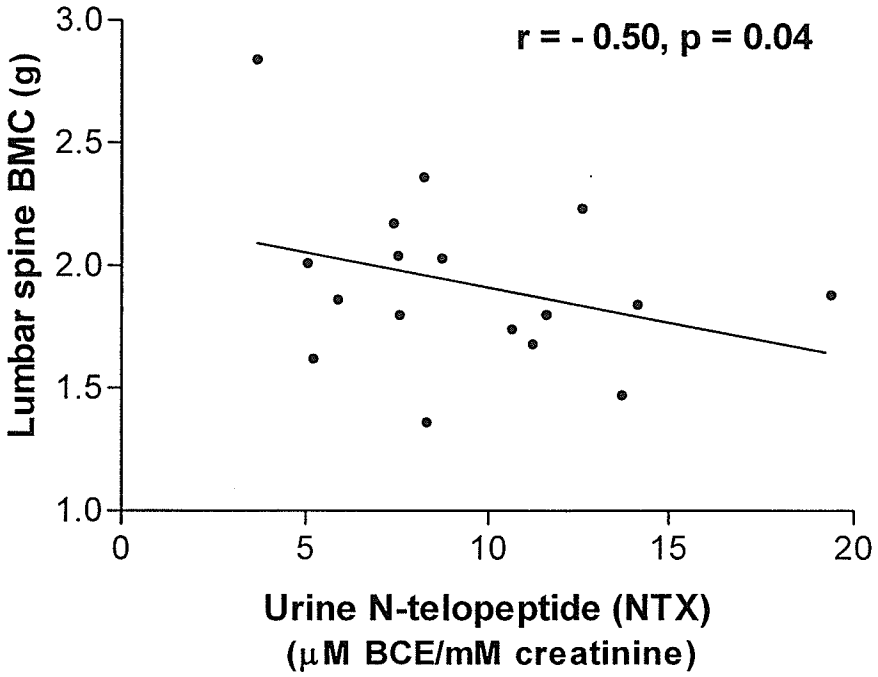

Figure 4-27. Relationship between urine N-telopeptide (NTX) and lumbar spine BMC in LBW piglets (n = 17).

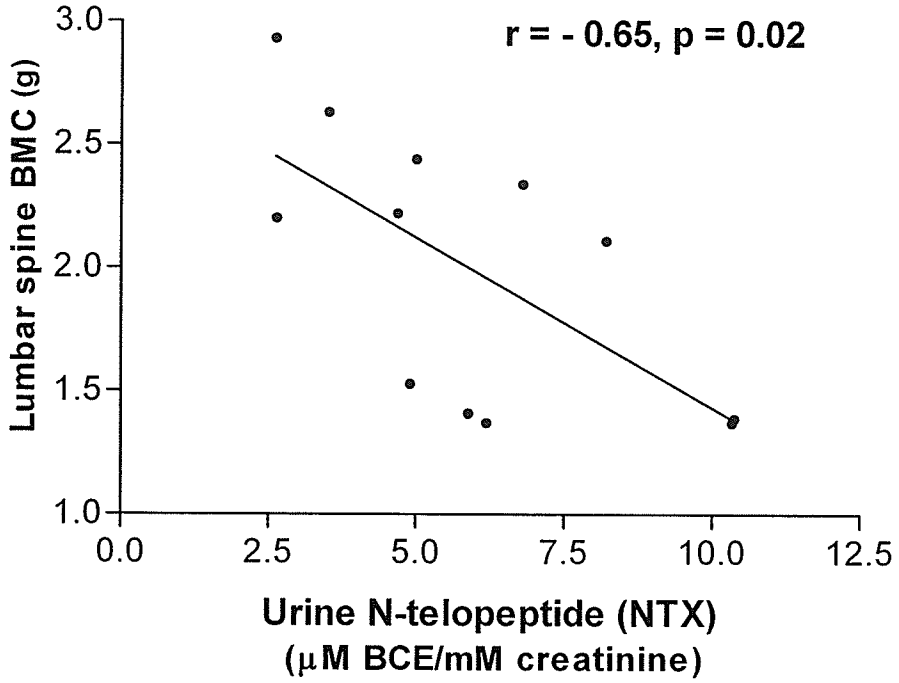

Figure 4-28. Relationship between urine N-telopeptide (NTX) and lumbar spine BMC in VLBW piglets (n = 12).

LS BMC in VLBW piglets correlated positively with erythrocyte DHA ($r = 0.60$, $P = 0.02$), liver AA ($r = 0.64$, $P = 0.02$), and inversely with bone Ca/P ($r = -0.58$, $P = 0.048$). A positive relationship between LS BMC and osteocalcin was near significance ($r = 0.57$, $P = 0.054$). As in LBW piglets, VLBW piglets showed an inverse relationship between LS BMD and bone Ca/P ($r = -0.58$, $P = 0.0488$). A negative relationship between LS BMD and NTX did not reach statistical significance ($r = -0.57$, $P = 0.051$).

4.5.3 *Ex Vivo* Femur Bone Mass

Correlation analysis of all piglets ($n = 29$) demonstrated negative correlations between *ex vivo* femur BA and BMC and plasma osteocalcin ($r = -0.82$, $P < 0.0001$; $r = -0.74$, $P < 0.0001$, respectively). *Ex vivo* femur BA and BMC were strongly associated with final body weight in LBW piglets ($r = 0.87$, $P < 0.0001$; $r = 0.86$, $P < 0.0001$, respectively) and VLBW piglets ($r = 0.92$, $P < 0.0001$; $r = 0.90$, $P < 0.0001$, respectively).

Among LBW piglets, both *ex vivo* femur BMC and BMD were inversely related to bone PGE2 ($r = -0.63$, $P = 0.007$; $r = -0.65$, $P = 0.005$, respectively), while BMD additionally showed negative correlations with plasma osteocalcin ($r = -0.52$; $P = 0.03$) and liver EPA ($r = -0.49$, $P = 0.045$). *Ex vivo* femur BMC correlated positively with adipose and liver AA ($r = 0.57$, $P = 0.02$; $r = 0.51$, $P = 0.04$, respectively), and negatively with NTX ($r = -0.61$, $P = 0.009$) and plasma and liver EPA ($r = -0.76$, $P = 0.0004$; $r = -0.79$, $P = 0.0002$, respectively). In VLBW piglets, an inverse relationship was observed between *ex vivo* femur BMC and erythrocyte AA ($r = -0.68$, $P = 0.02$), and

between *ex vivo* femur BMD and % change in erythrocyte AA levels (r = – 0.63, P = 0.03).

4.5.4 Body Composition, Growth Parameters and Biochemistry

Analysis of all piglets (n = 29) showed a positive correlation between birth weight and end of study fat mass (r = 0.42, P = 0.03, Figure 4-29). No associations were observed between birth weight and other body composition parameters. Final body weight and body composition measurements were not significantly related; however, fat mass related positively to absolute weight gain (D15 weight in kg – D0 weight in kg) in all piglets (n = 29) (r = 0.61, P = 0.0005, Figure 4-30). IGF-I was not significantly associated with either birth weight or final body weight; however, analysis of all piglets (n = 29) demonstrated a significant positive correlation between IGF-I and absolute weight gain (D15 weight in kg – D0 weight in kg) (r = 0.39, P = 0.04). We also observed positive correlations of plasma IGF-I with percent body fat (r = 0.58, P = 0.0495) and absolute fat mass (r = 0.61, P = 0.036, Figure 4-31) in VLBW piglets, and negative correlations with urine cortisol in the LBW category (r = – 0.63, P = 0.007, Figure 4-32). A significant negative correlation between urine cortisol and *ex vivo* femur length in LBW piglets (r = – 0.50, P = 0.04) was not apparent in the VLBW category.

4.5.5 Fatty Acid Correlations

Correlation analysis of all piglets (n = 29) revealed negative correlations between NTX and adipose tissue DHA (r = – 0.65, P = 0.0001) and between NTX and erythrocyte AA (r = – 0.50, P = 0.006, Figure 4-33). The NTX/erythrocyte AA relationship was

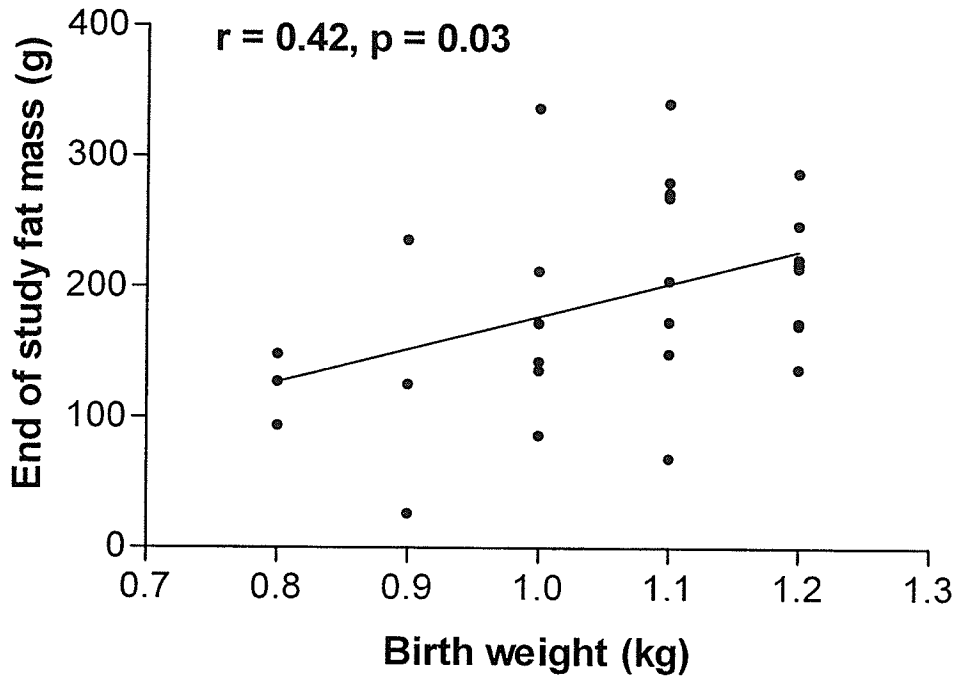

Figure 4-29. Relationship between birth weight and end of study fat mass in all piglets (n = 29).

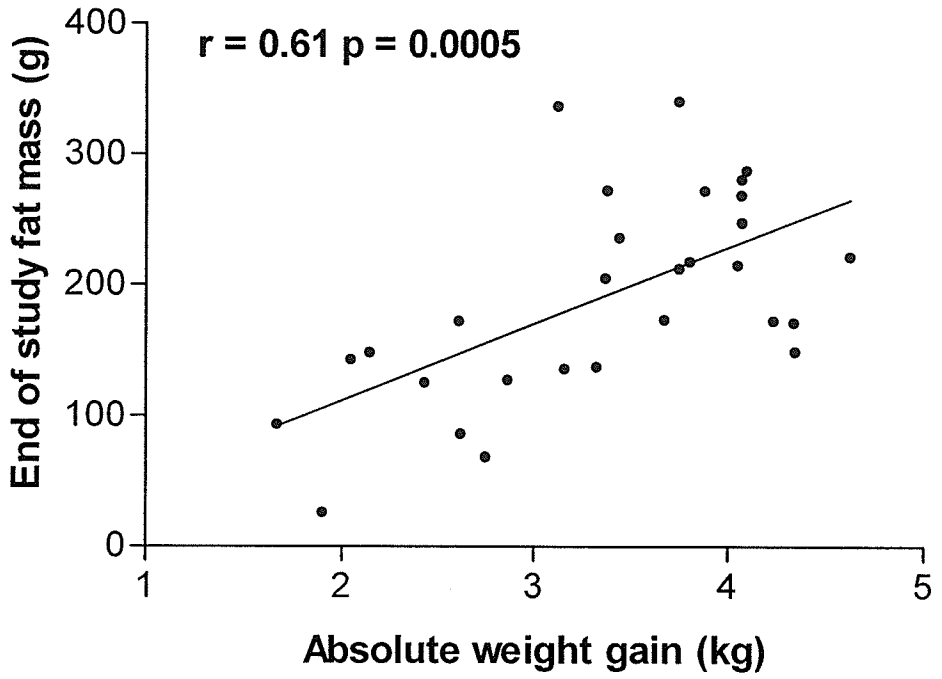

Figure 4-30. Relationship between absolute weight gain (D15 – D0 weight) and end of study fat mass in all piglets (n = 29).

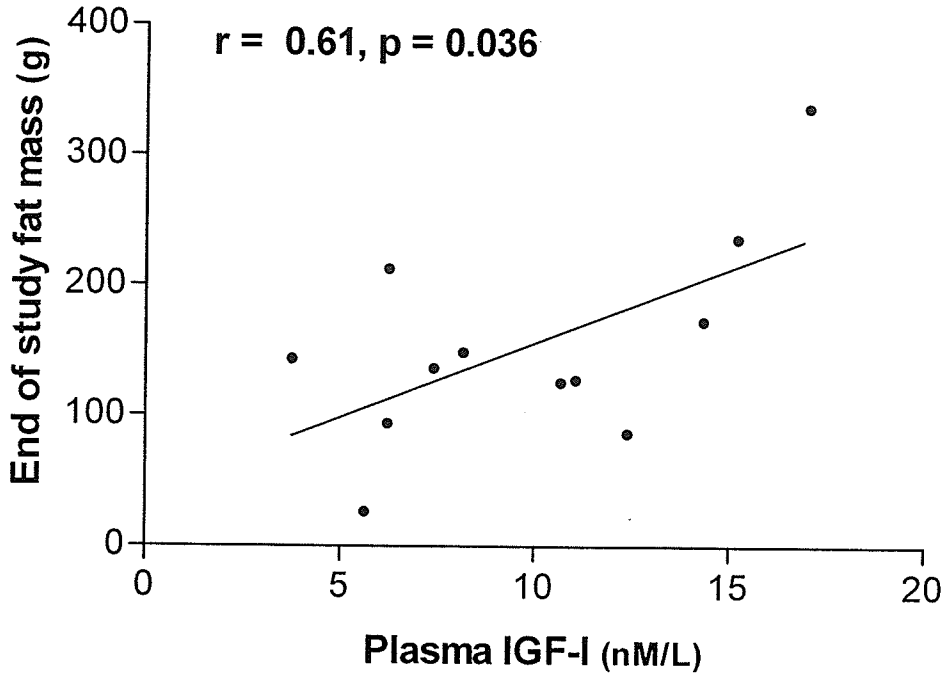

Figure 4-31. Relationship between plasma IGF-I and end of study fat mass in VLBW piglets (n = 12).

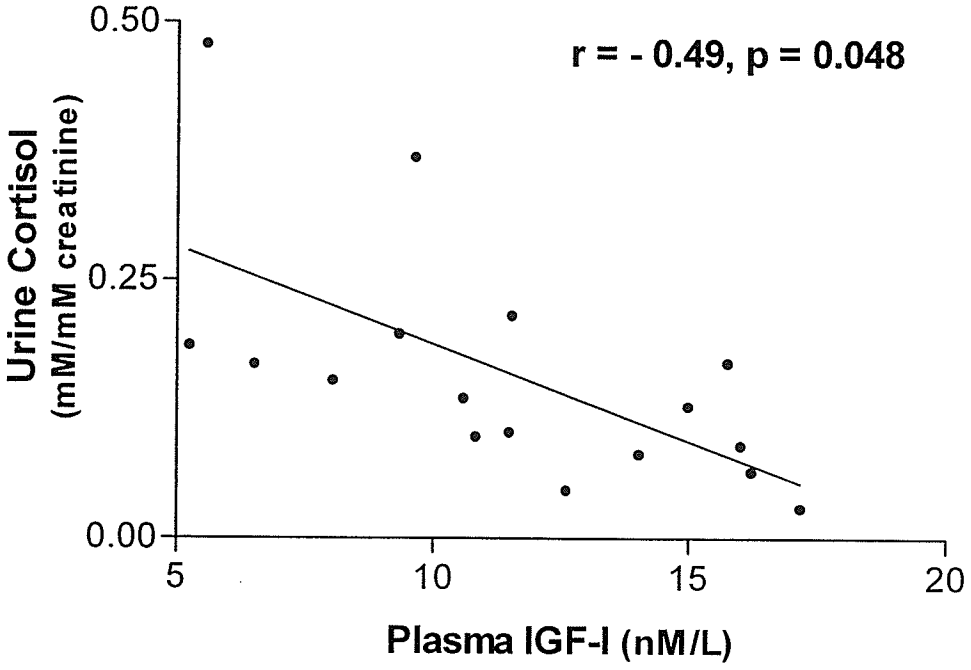

Figure 4-32. Relationship between plasma IGF-I and urine cortisol (expressed in relation to urine creatinine) in LBW piglets (n = 17).

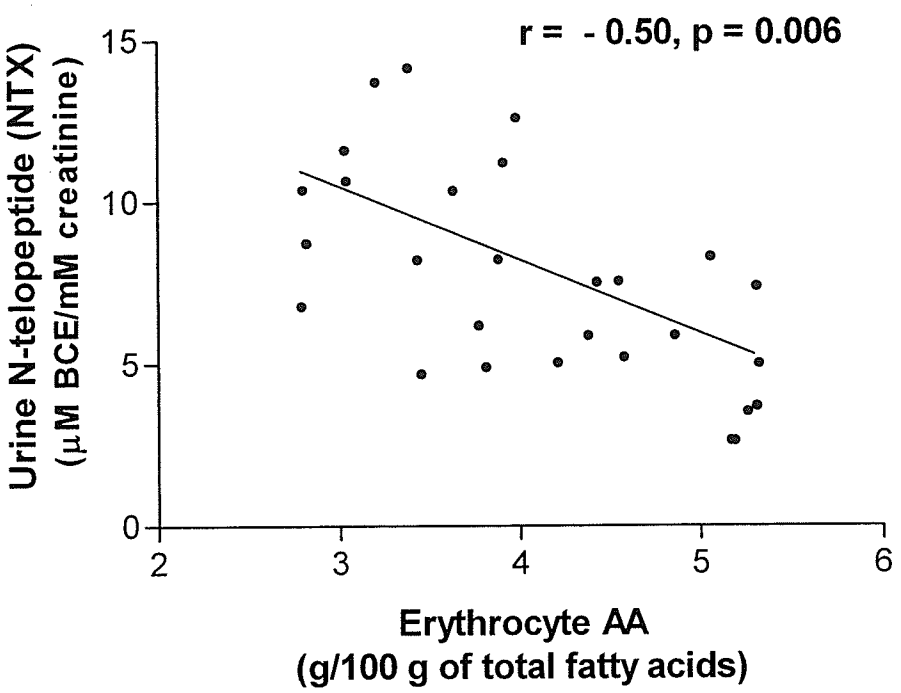

Figure 4-33. Relationship between erythrocyte AA (expressed as g/100 g of total fatty acids) and urine N-telopeptide (NTX) in all piglets (n = 29).

stronger for VLBW ($r = -0.75$, $P = 0.005$) than LBW piglets ($r = -0.64$, $P = 0.006$). In contrast, the NTX/adipose DHA relationship was stronger in LBW ($r = -0.70$, $P = 0.001$) compared to VLBW ($r = -0.64$, $P = 0.03$) piglets.

In LBW piglets, negative correlations between NTX and tissue fatty acid content were also observed for liver AA ($r = -0.50$, $P = 0.01$) and a near significant relationship with plasma AA ($r = -0.46$, $P = 0.053$). Liver EPA, in contrast, exhibited a positive association with urine NTX ($r = 0.62$, $P = 0.006$). Within the VLBW category, NTX was inversely related to AA concentrations in plasma ($r = -0.59$, $P = 0.045$), erythrocytes ($r = -0.75$, $P = 0.005$), and liver ($r = -0.78$, $P = 0.003$). Significant negative correlations were found between plasma IGF-I and total n-6 fatty acids in both adipose tissue and liver ($r = -0.63$, $P = 0.03$; $r = -0.60$, $P = 0.04$, respectively) in the VLBW piglets.

4.6 REGRESSION ANALYSIS

4.6.1 Whole Body Bone Mass

4.6.1a LBW Piglets

In backward stepwise regression analysis, plasma DHA, EPA, AA:EPA ratio, AA:DHA ratio, and osteocalcin, bone PGE_2, and urine cortisol were significant ($r^2 = 0.97$) predictors of WB BMC in LBW piglets in the regression equation: $y = 122.39 + 14.9$*(plasma DHA as g/100 g of total fatty acids) – 288.8*(plasma EPA as g/100 g of total fatty acids) – 0.7*(plasma AA:EPA ratio) + 9.7*(plasma AA:DHA ratio) – 1.7*(plasma osteocalcin in nM/L) – 1.2*(bone PGE_2 in nM/L) – 41.9*(urine cortisol as mM/mM creatinine). WB BMD was best predicted ($r^2 = 0.79$) by the regression equation: $y = 0.41 - 0.02$*(final body weight in kg) – 0.24*(plasma EPA as g/100 g of total fatty

acids) – 0.005*(plasma osteocalcin in nM/L) – 0.1*(urine cortisol as mM/mM creatinine).

4.6.1b VLBW Piglets

Final body weight was the only factor retained in the regression analysis for WB BMC ($r^2 = 0.90$, P = 0.0002) in the equation y = 10.79 + 12.26*(final body weight in kg). Plasma IGF-I was a small but significant positive predictor of WB BMD in addition to three negative factors: plasma EPA, AA:EPA ratio, and urine NTX ($r^2 = 0.88$) in the regression equation: y = 0.33 – 0.3*(plasma EPA as g/100 g of total fatty acids) – 0.002*(plasma AA:EPA ratio) + 0.004*(plasma IGF-I in nM/L) – 0.000004*(urine NTX asμM BCE/mM creatinine).

4.6.2 Lumbar Spine Bone Mass

4.6.2a LBW Piglets

Plasma EPA was the only significant factor ($r^2 = 0.51$) contributing to LS BMC among LBW piglets in the regression equation: y = 2.67 – 3.89*(plasma EPA as g/100 g of total fatty acids). No variables in the regression model predicted LS BMD ($r^2 = 0.85$).

4.6.2b VLBW Piglets

LS BMC was best predicted by the regression equation: y = – 0.33 + 0.55*(final body weight in kg) + 1.09*(plasma DHA as g/100 g of total fatty acids) – 4.39*(urine cortisol as mM/mM creatinine) – 0.20*(urine NTX as μM BCE/mM creatinine)

($r^2 = 0.97$). Urine cortisol was the strongest predictor of LS BMD (total $r^2 = 0.95$), while urine NTX and plasma AA and DHA were additional contributors in the regression equation: y = 0.42 – 0.63*(urine cortisol as mM/mM creatinine) – 0.03*(urine NTX as μM BCE/mM creatinine) – 0.06*(plasma AA as g/100 g of total fatty acids) + 0.35*(plasma DHA as g/100 g of total fatty acids).

4.6.3 *Ex Vivo* Femur Bone Mass

4.6.3a LBW Piglets

In backward stepwise regression analysis, final body weight, plasma AA:EPA, and IGF-I were identified as significant factors ($r^2 = 0.96$) contributing to *ex vivo* femur BMC in the regression equation y = 0.45 + 0.36*(final body weight in kg) + 0.009*(plasma AA:EPA ratio) – 0.03*(plasma IGF-I in nM/L). *Ex vivo* femur BMD was best explained by plasma AA:EPA and osteocalcin ($r^2 = 0.84$) in the regression equation: y = 0.26 + 0.0004*(plasma AA:EPA ratio) – 0.03*(plasma osteocalcin in nM/L).

4.6.3b VLBW Piglets

Ex vivo femur BMC was best predicted by final body weight ($r^2 = 0.99$) in the regression equation: y = – 0.03 + 0.43*(final body weight in kg). None of the variables entered into the regression model were predictive of *ex vivo* femur BMD ($r^2 = 0.93$).

4.6.4 Body Composition

4.6.4a LBW Piglets

In regression analysis, urine cortisol was identified as the only significant predictor of fat mass ($r^2 = 0.75$) in the regression equation: y = 263.9 – 287.6*(urine cortisol as mM/mM creatinine). Lean mass + BMC was best explained by final body weight, plasma AA, EPA, and AA:EPA ($r^2 = 0.97$) in the regression equation: y = 2458.9 + 806.6*(final body weight in kg) + 165.8*(plasma AA as g/100 g of total fatty acids) – 9834.1*(plasma EPA as g/100 g of total fatty acids) – 32.6*(plasma AA:EPA ratio). LBM was best predicted by final body weight, plasma AA, EPA AA:EPA, and bone PGE_2 ($r^2 = 0.98$) in the regression equation: y = 3505.6 + 702.6*(final body weight in kg) + 210.8*(plasma AA as g/100 g of total fatty acids) – 11274*(plasma EPA as g/100 g of total fatty acids) – 37.5*(plasma AA:EPA ratio) – 51.1*(bone PGE_2 in nM/L). The variables contributing to total mass ($r^2 = 0.98$) are given in the regression equation: y = 3638.8 + 213.5*(plasma AA as g/100 g of total fatty acids) – 11573*(plasma EPA as g/100 g of total fatty acids) – 38.1*(plasma AA:EPA ratio) – 52.3*(bone PGE_2 in nM/L). None of the variables entered into the regression model predicted percent body fat in LBW piglets.

4.6.4b VLBW Piglets

Fat mass was best predicted by plasma IGF-I ($r^2 = 0.85$) in the regression equation: y = 1.77 + 0.24*(plasma IGF-I in nM/L). Factors contributing to total body mass ($r^2 = 0.93$) included plasma AA, EPA, osteocalcin, IGF-I, and urine cortisol in the regression equation: y = 1886.8 – 450.4*(plasma AA as g/100 g of total fatty acids) +

10416*(plasma EPA as g/100 g of total fatty acids) + 34.5*(plasma osteocalcin in nM/L) + 85.4*(plasma IGF-I in nM/L) + 3836.2*(urine cortisol as mM/mM creatinine). Lean mass + BMC was best explained by the same variables as total mass ($r^2 = 0.91$) in the regression equation: y = 1932.9 – 419.6*(plasma AA as g/100 g of total fatty acids) + 9589.5*(plasma EPA as g/100 g of total fatty acids) + 215.8*(plasma osteocalcin nM/L) + 70.8*(plasma IGF-I in nM/L) + 3963.1*(urine cortisol as mM/mM creatinine). LBM was predicted by the regression equation ($r^2 = 0.93$): y = 1838.2 – 445.8*(plasma AA) + 10329*(plasma EPA as g/100 g of total fatty acids) + 232.6*(plasma osteocalcin in nmol/L) + 84.2*(plasma IGF-I in nmol/L) + 3812.7*(urine cortisol as mM/mM creatinine). Percent body fat was best explained ($r^2 = 0.95$) by plasma AA, IGF-I, and bone PGE_2 in the regression equation: y = 298.1 – 20.2*(plasma AA as g/100 g of total fatty acids) + 15.9*(plasma IGF-I in nM/L) – 33.3*(bone PGE_2 in nM/L).

5.0 DISCUSSION

5.1 BONE MINERALIZATION

The primary objective of the present study was to determine the response in the growth-restricted piglet to dietary supplementation with AA + DHA at two different levels over 15 days in early neonatal life. The bone mineral findings in this study demonstrate enhanced lumbar spine bone mass in response to both levels of LC PUFA, and increased femur bone area in response to the higher level of supplementation, with specific effects dependent upon birth weight classification. Bone resorption as reflected by urine NTX was suppressed by both levels of dietary AA + DHA in both birth weight categories; however, these differences reached statistical significance only in the LBW category. Negative correlations between urine NTX and AA concentrations in liver and erythrocytes in the LBW piglets, and between NTX and plasma, erythrocyte, adipose tissue, and liver AA content in the VLBW piglets suggests that improvement of tissue AA status by dietary AA + DHA is linked to suppression of bone resorption. The mechanistic basis for these associations is not identified in the present study.

Since vertebral bone is predominantly trabecular (Seeman et al. 1982), the present data suggest that trabecular bone sites were more affected than cortical by dietary AA and DHA. Enhancement of bone mass in the femur, a predominantly cortical bone site, was limited to the LBW piglets fed the greater amount of AA + DHA. Our observation of differential effects of dietary LC PUFA on the appendicular and axial skeleton may be explained by the higher metabolic turnover of trabecular bone and its greater surface area relative to bone volume (Price et al. 1994). Data from human and animal studies indicate a heightened sensitivity of trabecular bone sites to dietary manipulation. A calcium-

deficient diet fed to young rats was associated with decreased BMD in tibial trabecular, but not cortical bone, in as little as 24 hr. (Seto et al. 1999), and in growing piglets leads to reduced trabecular bone volume and mineral apposition rate (Eklou-Kalonji et al. 1999).

Differential effects of AA + DHA at trabecular and cortical bone sites in the neonatal period may be influenced by regional differences in bone mass at birth. The effects of prenatal protein-energy malnutrition in the rat are expressed differently at various bone sites. Nakamoto et al. (1983) found lower calcium content and increased calcium metabolism in the rat mandible compared with the long bones, accompanied by evidence of altered HPAA activity. Gudehithlu and Ramakrishnan (1990a) observed more pronounced effects of intrauterine protein restriction (5% vs. 20% protein) in the trabecular-rich calvarium compared to the femur in the rat, whereas the identical dietary treatment during suckling had a greater impact upon the femur (Gudehithlu and Ramakrishnan 1990b). It is therefore conceivable that if trabecular bone was relatively more affected by an adverse intrauterine environment than cortical bone, its responsiveness to subsequent nutritional repletion may be heightened.

An important finding of the present study is that bone mass outcomes of LC PUFA supplementation were modulated by birth weight classification. Piglets with birth weight of 1.1 – 1.2 kg responded to the lower level of AA + DHA supplementation with an increase in lumbar spine BMD; whereas in piglets ≤ 1.0 kg at birth, an effect of dietary AA + DHA at the lumbar spine was observed only at the higher level of supplementation. VLBW piglets fed AA + DHA as 1.2:0.2 g/100 g of fat had elevated LS BA and BMC, but unlike LBW piglets, showed no diet effect on LS BMD. These observations suggest

that bone mineral gain occurred with lower amounts of dietary LC PUFA in the LBW piglets; whereas the VLBW animals required higher levels of AA + DHA to sustain an increase in bone size and quantity of bone matrix, but not mineral density.

In skeletal development, bone matrix synthesis precedes mineral deposition and requires an adequate supply of amino acids. Deficient placental amino acid transport in human (Cetin et al. 1992) and porcine (Finch et al. 2004) fetal growth restriction may compromise production of bone matrix proteins. Significantly lower lean body mass in VLBW (n = 12) compared with LBW (n = 17) piglets (3659 ± 885 vs. 4604 ± 837 g, $P < 0.001$, 2-tailed t-test) in the present study is reflective of reduced overall protein accretion *in utero*. Rats exposed to protein-energy malnutrition in fetal life show biochemical alterations of bone matrix proteoglycans that may interfere with normal bone mineral deposition (Miwa et al. 1989; Miwa et al. 1990). Consistent with the above findings, fetal growth restriction induced by a low protein diet in the rat results in reduced whole body bone area and BMC, but not BMD, in the offspring, indicating predominant effects on bone quantity (Mehta et al. 2002).

The differences in bone response to dietary LC PUFA between LBW and VLBW piglets may parallel the temporal pattern of postnatal bone mineralization observed in the SGA infant. Avila-Diaz et al. (2001) reported lower whole body BA and BMC corrected for age and weight in preterm infants at 6 months of age compared with term infants, despite higher daily mineralization rates. They concluded that delayed growth in bone size as reflected by BA was a limiting factor in catch up of whole skeletal mineralization in preterm infants. A longitudinal study of bone growth and mineralization in VLBW AGA preterm infants similarly showed that growth in radial bone width exceeded the rate

of accretion in BMC in the first 4 months of life, implying that catch up in bone matrix formation precedes mineral deposition (Pittard et al. 1990). Kanbe et al. (2002) determined that LS BMD at term equivalent age in VLBW preterm infants lags behind that of normal term infants, and is strongly influenced by birth weight and length, implying that intrauterine determinants of fetal ponderal and linear growth continue to affect bone formation in infancy. These investigators further propose that postnatal factors are more important than size at birth to bone growth after the age of 2 yr.

Although the present study did not measure LC PUFA in bone, significant relationships were identified between tissue AA + DHA status and bone mineral status. Whole body and *ex vivo* femur BMD correlated negatively with % change in erythrocyte AA levels ($r = -0.64$, $P = 0.03$; $r = -0.63$, $P = 0.03$, respectively) in the VLBW piglets, suggesting a link between enhanced postnatal AA status and bone mineral deposition. Negative relationships of whole body BA and *ex vivo* femur BMC with erythrocyte AA ($r = -0.71$, $P = 0.009$; $r = -0.68$, $P = 0.02$, respectively) in the VLBW piglet may be explained by the level of eicosanoid production in bone in response to increasing levels of dietary AA. In rats fed various n-6 to n-3 ratios (23.8, 9.8, 2.6, or 1.2), bone formation rate (BFR) and alkaline phosphatase were highest with the lowest n-6:n-3 diet (Watkins et al. 2000). BFR related negatively with bone PGE_2 and the AA to EPA ratio in bone, suggesting a negative effect of high AA intake on bone formation that may be mediated by PGE_2. Inverse relationships between PGE_2 and whole body BA and BMC ($r = -0.75$, $P = 0.0005$; $r = -0.67$, $P = 0.003$, respectively) and *ex vivo* femur BMC and BMD ($r = -0.63$, $P = 0.007$; $r = -0.65$, $P = 0.005$, respectively) in LBW piglets, suggests

that even within the physiological range of PGE_2 values, higher concentrations favor bone resorption over bone formation.

The lack of a diet effect on bone PGE_2 may be indicative of the small increments in PGE_2 concentrations in response to the low levels of LC PUFA which did not significantly alter total n-6 to n-3 LC PUA ratios in either of the supplemented diets compared to control diet. Alternatively, the changes in regional bone mass may have been mediated by eicosanoids other than PGE_2 or may indicate the net effect of opposing eicosanoid actions. PGE_3, derived from EPA, is potently pro-resorptive (Raisz et al. 1989), while PGJ_2, another AA-derived eicosanoid, inhibits preosteoblast differentiation in vitro (Khan and Abu-Amer 2003). Another explanation may be the low specificity of the ELISA assay for the 2 series prostaglandins, as indicated by 70% cross-reactivity of the PGE_2 antibody against PGE_1 and 16.3% against PGE_3.

Differences in bone outcomes in LBW and VLBW piglets may also be explained by mineral status at birth. Bone mineralization may have been constrained by lack of sufficient calcium and phosphorus in the severely growth-restricted piglet. Evidence of impaired placental mineral transfer in experimental intrauterine growth restriction (Mughal et al. 1989) suggests that the SGA neonate starts life with a deficit of minerals, particularly calcium, required for optimal postnatal bone growth. If this theory is correct, then one might expect enhanced calcium retention as a compensatory mechanism. In fact, elevated intestinal calcium absorption is reported in the human SGA neonate (Picaud et al. 1994) and likely reflects increased requirements of catch up bone mineralization. Transient neonatal hypocalcemia with secondary hyperparathyroidism may occur in the

human newborn in the first 24 h of life (Tsang et al. 1975) and acts as a stimulus for enhanced calcium absorption and production of calcitriol (Steichen et al. 1980).

Intestinal calcium absorption measured in a small subset of control SGA piglets (n = 15) from the present study was elevated compared to that of unsupplemented AGA piglets of the same age (n = 15) (Mollard et al. 2004), suggesting an increased demand for bone growth or for maintenance of calcium homeostasis. The observation of an inverse relationship between calcium absorption and birth weight ($r = -0.61$, $P = 0.002$) in the above study further supports the inference of increased absorption in response to elevated needs. It is also possible that enhanced calcium absorption in the growth-restricted neonate is mediated by a stimulus for calcium retention programmed *in utero* as reported in adults who were born SGA (Arden et al. 2002; Szathmari et al. 2000).

The increased bone calcium content observed in VLBW piglets fed AA + DHA as 0.6: 0.1 g/100 g of fat is not explained by the biochemical findings. It is possible that improved calcium retention contributed to higher bone calcium. Dietary AA, EPO, fish oil, and GLA + EPA in a 3:1 ratio all stimulate intestinal calcium absorption in rats (Song et al. 1983; Coetzer et al. 1994; Claassen et al. 1995); however, the underlying mechanisms and the dose-response relationship between dietary LC PUFA and calcium absorption are not established. Renal excretion of calcium and phosphorus was not affected by diet in the present study, suggesting that the net eicosanoid effect in the renal tubules was unchanged. The negative relationship between LS BMD and bone calcium in both LBW and VLBW piglets is unexpected; however, since the bone sample was derived from tibial cortical bone, it may reflect localized changes in mineral deposition

that differ from events at trabecular bone sites. In fact, the negative relationship may reflect competition between trabecular and cortical bone sites for available calcium.

Bone turnover was assessed by plasma osteocalcin (an index of osteoblastic activity) and urine NTX (a marker of osteoclast activity). Similar osteocalcin values among treatment groups within each birth weight category indicate that whole body osteoblast activity was unaffected by AA + DHA. Consistent with this observation, whole body BMC and BMD and linear growth in terms of whole body length and femur length were similar among diet groups. These findings do not, however, exclude growth in other bone dimensions such as vertebral thickness or long bone cross-sectional diameter through appositional bone growth. In accord with our results, supplementation of AGA piglets with AA + DHA as 0.6:0.1 g/100 g of fat from d 3 to d 20 of life similarly did not alter plasma osteocalcin levels (Blanaru et al. 2004). Comparison of osteocalcin values of AGA piglets with those of our LBW and VLBW piglets at the same age indicates lower levels in SGA (LBW: 10.50 ± 1.8 nM/L; VLBW: 8.25 ± 0.88 nmol/L) relative to AGA (16.2 ± 5.0 nM/L) piglets (Blanaru et al. 2004), signifying reduced bone turnover in growth-restricted piglets. Low osteocalcin in SGA compared to AGA piglets is consistent with suppression of osteoblast proliferation and function due to glucocorticoid excess programmed *in utero*.

Osteocalcin related inversely with whole body BMC and BMD in all piglets in the current study (n = 29). Negative associations of circulating osteocalcin with bone strength and femur ash weight have similarly been reported in other studies of growing pigs (Carter S. et al. 1996). Generally regarded as an indicator of bone turnover, osteocalcin may also act to limit bone matrix synthesis at a specific time in the process of bone

formation (Ducy et al. 1996). On the basis of the above data, the relationship between whole body BMC and BMD and osteocalcin may be explained by reduced osteocalcin production in piglets having more mature mineralized bone compared to those in the earlier stages in the bone formation process.

Bone resorption as assessed by urine NTX was suppressed by dietary AA + DHA in both birth weight categories, but was significant only in LBW piglets. Inverse relationships between NTX and both LS BMD and erythrocyte AA suggest that elevated bone mass at the lumbar spine is linked to enhanced tissue AA status and to suppression of bone resorption. While the basis for attenuated bone resorption is not explained by our biochemical data, it may reflect improved calcium balance through increased intestinal calcium absorption (Mollard et al. 2004), thereby reducing the stimulus for calcium mobilization from bone.

Comparison of urine NTX values in AGA piglets (Blanaru et al. 2004) with those of the SGA piglets in the present study in response to dietary AA + DHA as 0.6:0.1 g/100 g of total fat over 15 days (AGA: 10.7 ± 4.0; LBW: 8.89 ± 5.58; VLBW: 7.06 ± 2.33; all expressed as μM/mM) indicates that this level of AA + DHA attenuated bone resorption of growth-restricted piglets into the "normal" range of the AGA piglet. In addition, the higher level of AA + DHA intake further suppressed NTX levels to at or below those of the sow-fed AGA piglet in the study of Blanaru et al. (2004): sow-fed AGA: 10.2 ± 4.0; LBW: 7.52 ± 1.56; VLBW: 3.44 ± 1.13; all expressed as μM/mM. The implications of this observation are that fetal growth restriction in piglets is associated with elevated bone turnover with respect to bone resorption, and that the higher level of AA + DHA supplementation more effectively suppressed resorption in SGA piglets.

Potential pitfalls in interpretation of the NTX assay result from diurnal variations in NTX production that may confound spot urine measurements. This is unlikely in the present study since all urine specimens were obtained in the early a.m. Another limitation of this method is the wide variability in NTX values observed in this and other animal studies using crosslink excretion as a measure of bone resorption (Blanaru et al. 2004).

Circulating IGF-I was unaffected by either level of AA + DHA, suggesting that hepatic IGF-I production was not altered, since the liver is its principal source. This is consistent with the similar weight gain and linear growth within each birth weight category, given the importance of IGF-I for postnatal growth in the piglet (Carroll et al. 2000). Analysis of all 29 piglets revealed a positive relationship between plasma IGF-I and absolute weight gain, suggesting IGF-I involvement in ponderal growth irrespective of birth weight or dietary treatment. Equivalent IGF-I levels among diet groups at 20d of life is an important observation, in light of recent evidence that elevated IGF-I in prepubertal children may be programmed by early life influences (de Waal et al. 1994; Fall et al. 2000). The implications of our findings are that dietary AA + DHA at the two levels studied did not contribute to programming of the IGF system over the 15d study period.

Another SGA piglet study found low cord blood IGF-I levels at birth; however, by 2 weeks of age, plasma and hepatic IGF-I were equivalent in SGA and control piglets, despite greater relative growth in the SGA piglets (Ritacco et al. 1997). This parallels postnatal changes in human IGF-I levels which are low at birth in the SGA neonate and subsequently increase after the first week of life to reach levels comparable with those of AGA infants (Domenech et al. 2001). While IGF-I was not measured at birth in the

current study, based on the above findings, one might expect it to be lower in the VLBW compared to LBW piglets. If so, then similar plasma IGF-I levels between the LBW (11.40 ± 3.87 nmol/L) and VLBW (10.05 ± 5.11 nmol/L) control groups at the end of study suggest that IGF-I increased more rapidly in the more growth-restricted piglets, likely in support of catch up growth.

An important finding in the present study was the association of IGF-I with fat mass in VLBW piglets. The positive relationship between IGF-I and fat mass implies that even within the normal range, higher levels of IGF-I favored accretion of adipose tissue over lean mass. This together with the finding of a positive correlation between IGF-I and absolute weight gain suggests that in piglets with more rapid postnatal growth and higher IGF-I levels, ponderal gain occurred predominantly in adipose tissue. Evidence from in vitro, animal, and human studies points towards a role for IGF-I in accretion of both fat and muscle tissue. IGF-I is expressed in human and porcine adipocytes (reviewed by Louveau and Gondret 2004) and stimulates in vitro adipocyte differentiation (Jia and Heersche 2000). Obesity and increased body weight in mini-piglets fed a hypercaloric diet is accompanied by elevated plasma IGF-I levels and IGF-I gene expression in fat and muscle at sexual maturity, suggesting that energy excess stimulated IGF-I synthesis and consequent tissue anabolism (Sebert et al. 2005). This is substantiated by studies in the growth-restricted piglet, in which exogenous IGF-I in the neonatal period elevates circulating IGF-I and stimulates accretion of both protein and lipids (Schoknecht et al 1997).

A regulatory role for IGF-I in tissue compartment growth is corroborated by positive relationships between plasma IGF-I and energy intake, ponderal index, and

skinfold measurements in the SGA infant (Domenech et al. 2001). Other investigations have previously identified significant correlates of infant cord blood IGF-I with fat and muscle mass at birth (Javaid et al. 2004). After correction to total body mass as measured by DXA, resulting values for proportionate fat mass remained positively correlated with serum IGF-I, while those of proportionate muscle mass were negatively related to IGF-I (Javaid et al. 2004).

The stimulus for adipogenesis in the severely growth-restricted piglets may originate *in utero*, with consequent programming of the HPAA; however, without a reference group of AGA piglets for comparison, biochemical evidence of HPAA dysregulation cannot be established. Nonetheless, the inverse relationship of urine cortisol with plasma IGF-I and with *ex vivo* femur length in LBW piglets is compatible with cortisol-mediated effects on linear bone growth. Additional evidence for programmed alteration of endocrine function arises from a piglet study demonstrating increased adrenal gland size and increased stimulated HPAA activity at 3 months of age in LBW (defined as < 1.47 kg) piglets with poor postnatal growth compared to higher birth weight piglets (defined as > 1.53 kg) (Poore and Fowden 2003). The authors conclude that low birth weight followed by postnatal growth failure contribute to altered HPAA responsiveness. The relationships among IGF-I, cortisol, and femur length documented in the current study are strikingly similar to those reported by Cianfarani et al. (1998) who found negative correlations of plasma cortisol with IGF-I in the AGA infant and with linear growth in the SGA infant. The above data together with *in vitro* evidence that cortisol inhibits IGF-I transcription in human osteoblasts (Swolin et al. 1996) point towards altered HPAA activity as a likely mechanism for our observations.

A positive association between whole body BMD and IGF-I ($r = 0.39$, $P = 0.04$) for all 29 piglets suggests anabolic effects of plasma IGF-I on mineral deposition. While circulating IGF-I is known to contribute to bone growth and mineral density (Yakar et al. 2002), systemic concentrations may not reflect changes in local tissue levels of IGF-I in bone, which may be more important to bone metabolism than hepatic IGF-I (Rodan and Rodan 1995). In addition, regulatory mechanisms likely differ between sites of IGF-I production (Rosen 2000), since growth hormone is the predominant endocrine influence on hepatic IGF-I synthesis while local growth factors and cytokines are more active in regulating bone metabolism. It is therefore conceivable that the observed elevation in bone mass at the lumbar spine was mediated by local IGF-I acting in an autocrine or paracrine fashion. This would also explain the fact that the gain in bone mass at the lumbar spine is not reflected in whole body bone mineralization.

Another factor to consider in the interpretation of serum IGF-I levels is the potential modulation of IGF-I bioactivity by the IGFBP. Serum IGFBP-1 increases with birth weight in the human and rat (Verhaeghe et al. 1993; Unterman et al. 1993) and thus may be modified by conditions that promote fetal growth restriction. Significant associations of cord blood cortisol with IGF-I (negative) and IGFBP-1 (positive) in the AGA infant suggests modulation of the IGF system by glucocorticoids *in utero* (Cianfarani et al. 1998).

5.2 BODY COMPOSITION

Analysis of body composition suggests a negative effect of AA and DHA on fat deposition without altering body weight or lean mass. As the present study was designed for the short-term assessment of LC PUFA effects, tissue compartment measurements may not reflect long-term consequences. A review of animal studies of early life LC PUFA supplementation suggests no effect on body weight or tissue composition. Short-term supplementation of the AGA piglet using comparable levels of AA + DHA report no effect on body weight (Blanaru et al. 2004; Lucia et al. 2003; Mollard et al. 2005), with the exception of one report of increased weight gain in slightly older piglets fed AA + DHA as 0.5:0.1 g/100 g of dietary fat for 14 days (Weiler 2000). Body weight was similarly unaffected by dietary AA + DHA in artificially reared rat pups in the first 3 weeks of life (Ward et al. 1998), or by various levels of n-6 and n-3 LC PUFA in growing chicks (Watkins et al. 1997). Results of several prospective infant studies are equivocal regarding effects of LC PUFA on weight gain (Clandinin et al. 2005; Groh-Wargo et al. 2005); however there are indications that body composition is favorably affected. Groh-Wargo et al. (2005) report an increase in lean body mass and reduction in fat mass at 1 year of age in infants supplemented with AA + DHA as 0.42:0.26 g/100 g of fat to term age followed by post-discharge supplementation of AA + DHA as 0.42:0.16 g/100 g of fat (Groh-Wargo et al. 2005).

The effects of AA + DHA on body composition in the present study may involve prostaglandin derivatives of AA that either promote or suppress adipogenesis via opposing effects on nuclear receptors known as peroxisome proliferator activated receptors (PPARs) (Reginato et al. 1998). Derivatives of PGJ_2, promote adipogenesis

through activation of the adipogenic nuclear receptor PPARγ (Forman et al. 1995), while $PGF_{2\alpha}$ inhibits adipocyte differentiation *in vitro* (Serrero et al. 1992). The role of metabolic or endocrine alterations programmed *in utero* must also be considered. Elevated urine cortisol in VLBW piglets fed the lower amount of AA + DHA result from programmed metabolic alterations inappropriate to postnatal nutritional adequacy. It has been proposed that poor fetal growth followed by rapid catch up growth in infancy predisposes to obesity in later life (reviewed by Cameron and Demerath 2002). In addition to the HPAA axis, activities of leptin and the IGF system may be altered by the intrauterine environment. Programming of postnatal leptin expression by maternal nutrition during pregnancy has been demonstrated in piglets, with an inverse relationship between birth weight and adipose tissue leptin at 59 d of age (Ekert et al. 2000). This observation may reflect leptin resistance or alternatively may result from rapid catch up growth with disproportionate deposition of fat mass.

5.3 TISSUE FATTY ACID COMPOSITION

The prominent tissue accretion of both AA and DHA argue against glucocorticoid suppression of desaturase activity, and is corroborated by a recent report of normal activity of Δ-6 and Δ-5 desaturases in piglet intrauterine growth restriction (McNeil et al. 2005).

5.3.1 Plasma Fatty Acids

In both birth weight categories, supplementation with AA + DHA was reflected in plasma fatty acid content. In LBW piglets, enrichment of plasma AA, but not DHA, was

observed at both levels of supplementation. These findings suggest that AA was more readily incorporated into plasma lipids than DHA, possibly because AA does not require further desaturation and elongation for tissue lipid incorporation. Evidence that many tissues accumulate AA in a dose-dependent manner even when provided at low dietary intakes has been demonstrated in the rat (Danone et al. 1975) and hamster (Whelan et al. 1993). Alternatively, our findings may indicate preferential uptake of DHA by developing tissues with high DHA requirements such as the brain and retina (Clandinin et al. 1980; Clandinin et al. 1989; Giusto et al. 2000; Uauy et al. 1992). This is supported by investigations of Crawford et al. (1976) in which AA and DHA concentrations rose progressively from maternal liver to placenta to fetal liver to fetal brain in what has been termed "biomagnification" of tissue LC PUFA.

In VLBW piglets, plasma AA increased in response to the higher intake of AA + DHA compared to the other diet groups, while both supplementation levels enhanced DHA relative to controls. This may reflect increased requirements for preformed DHA in the more severely growth-restricted piglets with smaller fat stores (Widdowson 1971b) and lower endogenous synthesis as observed in the SGA infant (Uauy et al. 2000).

5.3.2 Erythrocyte Fatty Acids

In both birth weight categories, erythrocyte AA was significantly elevated by the higher level of supplementation, while in VLBW piglets, the higher AA + DHA intake additionally increased erythrocyte DHA. Similar to the plasma fatty acid profile, the relatively greater incorporation of AA vs. DHA into erythrocyte membrane lipids may be explained by fatty acid partitioning to meet tissue requirements for specific LC PUFA.

Notably, the higher level of dietary AA + DHA in LBW piglets resulted in a 22% increase in erythrocyte DHA concentrations compared to those of controls; whereas the same intake in VLBW piglets produced a 64% increase in DHA. The dramatic increase in DHA levels in VLBW piglets may be compensatory to DHA deficiency at birth. In human infants, DHA concentrations in the cord vessel wall relate positively to prenatal growth (Foreman-van Drongelen et al. 1995), while in piglets, improved neonatal survival is accompanied by increased tissue DHA and reduced 22:5n-6 in offspring of sows fed 10g salmon oil/kg during gestation, suggesting that prenatal n-3 LC PUFA alleviates a postnatal deficit in DHA in offspring (Cordoba et al. 2000). In the present study, plasma DHA was identified as a significant positive predictor of LS BMC and BMD in VLBW piglets, which may signify a greater postnatal requirement for n-3 LC PUFA for bone mass accretion in severe intrauterine growth restriction.

Enrichment of erythrocyte AA by the higher AA + DHA intake was accompanied by a 17% reduction in erythrocyte LA in LBW piglets, in contrast to a 4% increase in LA in VLBW piglets. Reciprocal relationships between LA and AA in plasma (Sinclair and Mann 1996) and lung (Huang and Craig-Schmidt 1996) phospholipids have been interpreted as a competitive interaction (Whelan et al. 1993). The above findings together with the striking increases in erythrocyte AA and DHA levels in VLBW compared to LBW piglets (AA: 49% vs. 40%; DHA: 64% vs. 22%) suggest that reduced tissue AA, DHA, and LA at birth in the more severe growth-restricted piglets was the primary stimulus for rapid accretion of these nutrients.

5.3.3 Percent Change in Erythrocyte Fatty Acids

The commonly observed postnatal decline in tissue AA and DHA concentrations in infants and experimental animals has been explained as a dilutional effect of tissue expansion (Farquharson et al. 1995). Attenuation of the % decline in erythrocyte AA levels over 15 days occurred in response to both AA + DHA diets and similar effects on DHA concentrations in response to the higher level of supplementation in LBW piglets. In the VLBW category, the % change in erythrocyte DHA was attenuated by both supplementation levels, suggesting compensatory DHA accretion in response to low DHA status at birth. Percent gain in erythrocyte LA content was attenuated by both levels of supplementation among LBW piglets ($P < 0.05$), likely due to displacement of membrane lipid LA by AA.

5.3.4 Adipose Tissue and Liver Fatty Acids

In both LBW and VLBW piglets, adipose tissue and liver accumulation of AA and DHA and ratios of AA to DHA and to EPA reflected dietary intake. These results differ from patterns of liver and adipose tissue AA and DHA accretion in the AGA piglet. Blanaru et al. (2004) found no effect of dietary AA + DHA on adipose tissue AA or DHA content and on liver DHA concentrations. The implication of these comparisons is that greater amounts of AA and DHA are required by the SGA neonate to compensate for low adipose tissue reserves at birth. Elevation of liver DHA by LC PUFA in the SGA but not the AGA piglet may reflect greater uptake of circulating DHA in response to lower DHA status at birth. A recent report of normal desaturase activity in the spontaneously growth-

restricted piglet (McNeil et al. 2005) suggests that different desaturase activity between SGA and AGA piglets is not a contributing factor.

5.4 POSTULATED MECHANISMS FOR ACTIONS OF ARACHIDONIC ACID AND DOCOSAHEXAENOIC ACID

PGE_2 as measured in the present study was not identified as a mechanism for improvement of bone mass by supplementation with AA + DHA. Recent evidence that DHA and EPA inhibit *in vitro* osteoclast generation and activation in response to receptor activator of NFκB ligand (RANKL) (Sun et al. 2003), also known as osteoclast differentiation factor (ODF), suggests another mechanism by which AA + DHA may attenuate bone resorption. Nitric oxide (NO), a free radical produced from arginine by the enzyme nitric oxide synthase (NOS), inhibits *in vitro* bone resorption when present in high concentrations (reviewed by van't Hof and Ralston 2001). Fetal and maternal synthesis of NO is necessary for normal fetal and placental growth (reviewed by Wu et al. 2004) and is impaired in SGA infants at birth (Hata et al. 1998). *In vitro* induction of inducible NOS gene expression in human osteoblast-like cells by AA, but not EPA, suggests another method by which dietary AA may enhance bone mass (Priante et al. 2005). The recently identified transcription factor core binding factor 1 (Cbfa1) regulates osteoblast differentiation (Kobayashi et al. 2000) and function (Ducy et al. 1999), and may represent another potential site for LC PUFA actions in bone (Zhang X. et al. 2002).

5.5 CONCLUSION

The objectives of this study were to examine the bone response of SGA piglets based on designation of birth weight as either LBW or VLBW to dietary AA + DHA as either 0.6:0.1 or as 1.2:0.2 g/100 g of total fat. Our results demonstrate that both levels of AA + DHA supplementation improve bone mass at the lumbar spine in a manner determined by birth weight classification. The lower intake of AA + DHA improved LS BMC and BMD in LBW piglets, while a higher intake was required to elevate LS BA and BMC but not BMD in VLBW piglets, implying that severely growth-restricted animals may benefit from more LC PUFA. Enhanced LS bone mass was linked to reduced bone resorption and to improved tissue AA status as indicated by lower NTX in supplemented piglets, and by the negative associations of NTX with LS BMC and tissue AA content.

Data from this study support the fetal origins of adult disease hypothesis by demonstrating that birth weight predicts fat mass at day 20 of life in the growth-restricted piglet. Although ponderal and linear growth were unaffected by dietary LC PUFA, fat mass was unexpectedly elevated in LBW piglets and urine cortisol increased in VLBW piglets fed AA + DHA as 0.6:0.1 g/100 g of fat. These results together with the positive association of fat mass with urine cortisol may signify interactive effects of postnatal nutrition and *in utero* programming of endocrine activity. Significant associations of IGF-I with whole body BMD and fat mass suggest a postnatal role for IGF-I in bone mineralization and adipose tissue accretion. The present data are unable to explain the relationship between IGF-I and fat deposition; however, programmed alterations of several endocrine axes including HPAA, leptin, and IGF-I may be implicated.

In summary, supplementation with AA and DHA in the SGA neonatal piglet enhances bone mass at the lumbar spine through attenuation of elevated bone resorption and possibly through indirect effects on body fat content. These findings have important implications for both short- and long-term consequences to the human SGA neonate. Further investigation in the SGA piglet model is necessary to delineate the underlying mechanisms and to clarify the long-term impact of dietary AA + DHA intervention on bone and other health outcomes.

5.6 STRENGTHS AND LIMITATIONS OF THIS STUDY

5.6.1 Strengths

The primary strength of the present study is in its subclassification of SGA piglets into two birth weight categories based on severity of growth restriction at birth in recognition of the fact that SGA neonates do not behave as a homogenous group. This study expands on information derived from previous studies in the AGA piglet and permits comparisons between SGA and AGA neonates that may be of relevance to the human infant. A further outcome of our study is to confirm the suitability of the SGA neonatal piglet as a model for investigation of the impact of nutritional interventions on growth and development of the whole animal as well as isolated tissues and organs.

5.6.2 Limitations

Several limitations of the present study deserve mention. These include the small sample size, particularly for the most severely growth-restricted piglets, and the short duration of the study, which limits the extrapolation of findings to long-term outcomes.

Statistical analysis of the data could be improved by use of newer statistical approaches such as the mixed model which accounts for random and fixed effects and therefore permits analysis of the entire sample as one group.

Measurement of endocrine and growth factors at baseline would have enhanced the interpretation of end of study results by delineating temporal patterns in expression and relationships to skeletal and growth outcomes. Given the importance of endocrine influences to intrauterine and postnatal growth, assessment of leptin, insulin, calcitriol, and PTH should be considered. In addition, circulating levels of growth factors active in bone may not accurately reflect their concentrations and activity in bone; therefore, measurement of bone IGF-I, osteocalcin, and fatty acid composition would be of great value. Interpretation of changes in bone and circulating IGF-I in relation to size at birth and subsequent growth would benefit from assessment of IGFBP-1. And finally, newer biomarkers of bone turnover such as plasma ICTP and PICP do not require correction to urine creatinine, thereby minimizing potential variance in measurements.

5.7 FUTURE RESEARCH DIRECTIONS

Several observations in the present study warrant further investigation in the SGA piglet model. First, a larger study of longer duration should be undertaken to evaluate long-term sequelae of dietary AA and DHA intervention at the levels studied. A greater focus on measurements of nutritional and LC PUFA status of the sow is needed to better explain the birth status of offspring. Important parameters in the sows include anthropometric measurements of pregestational body weight, whole body length, and birth weight if available, as well as an analysis of plasma, erythrocyte, and milk fatty acid

composition. Outcomes in SGA offspring should include overall growth and body composition, bone growth and histology at cortical and trabecular sites, bone turnover histomorphometric analysis, and testing of bone mechanical properties. The poorly understood renal contribution to LC PUFA effects might be clarified by measurement of local LC PUFA and eicosanoid levels. Adipose tissue response to dietary LC PUFA, growth rate, and systemic hormones may be explained by measurement of IGF-I, eicosanoids, and PPARγ expression in subcutaneous or visceral fat. These measurements together with bone densitometry, bone biomarkers, and endocrine parameters would provide a more complete picture of the events occurring in bone and other tissues in relation to LC PUFA status over time frames representative of the neonatal period, prepubertal growth, and sexual maturation.

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7.0 APPENDIX A

7.1 Table A-1. Piglet organ weights at the end of study[1]

	Birth weight 1.1- 1.2 kg			Birth weight ≤ 1.0 kg		
	DIETARY TREATMENTS[2]					
	Control	AA:DHA 0.6:0.1	AA:DHA 1.2:0.2	Control	AA:DHA 0.6:0.1	AA:DHA 1.2:0.2
	n = 6	n = 5	n = 6	n = 4	n = 4	n = 4
Brain, *g*	34.6 ± 6.9	31.6 ± 4.1	33.5 ± 3.1	28.5 ± 8.4	28.3 ± 7.1	29.8 ± 5.8
Liver, *g*	148.1 ± 24.0	172.5 ± 40.7	145.2 ± 38.0	127.2 ± 21.0	121.2 ± 14.9	126.2 ± 21.3
Right kidney, *g*	22.6 ± 6.4	18.3 ± 2.7	18.7 ± 3.2	14.2 ± 3.2	16.8 ± 4.4	12.0 ± 1.3
Left kidney, *g*	23.5 ± 7.1	18.9 ± 3.7	17.2 ± 3.1	14.5 ± 3.4	14.9 ± 4.3	12.0 ± 0.6
Spleen, *g*	14.8 ± 4.0	13.8 ± 5.9	14.0 ± 4.0	9.4 ± 2.6	8.1 ± 1.9	11.2 ± 4.1
Left femur, *g*	20.5 ± 3.5	21.3 ± 1.4	22.9 ± 2.4	15.9 ± 4.1	16.1 ± 1.4	13.6 ± 2.0

[1]Values are means ± SD. Within each weight category, means in a row with different superscripts are statistically different at $P < 0.05$.
[2]AA:DHA diets expressed as g/100 g of fat