The Effect of Dietary Arachidonic Acid in Varying Amounts on Bone Growth and Mineralization in Formula-Fed and Sow-Fed Growing Piglets: Implications for Infant Nutrition

By

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The Effect of Dietary Arachidonic Acid in Varying Amounts on Bone Growth and Mineralization in Formula-Fed and Sow-Fed Growing Piglets: Implications for Infant Nutrition

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

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ABSTRACT

THE EFFECT OF DIETARY ARACHIDONIC ACID IN VARYING AMOUNTS ON BONE GROWTH AND MINERALIZATION IN FORMULA-FED AND SOW-FED GROWING PIGLETS: IMPLICATIONS FOR INFANT NUTRITION

Infants who are fed formula following birth do not receive arachidonic (AA) and docosahexaenoic acid (DHA), which are supplied in breast milk, and may be essential for the growing infant. The research objective was to compare the effects formula supplementation with DHA and various levels of dietary AA, with sow milk on general fatty acid status, growth, and bone metabolism and mineralization of the newborn piglet. Forty 3-day-old male piglets were randomized within litters into one of five treatment groups (n=8 per group) for fifteen days, and fed fatty acid supplemented formula + AA (0.3, 0.45, 0.60 and 0.75 % wt/wt) and DHA (0.10 % wt/wt). A sow-fed group of 8 piglets provided a comparison. Following 15 days of treatment, plasma, liver and adipose tissue were analyzed for fatty acids using gas chromatography. Biochemical markers of bone: plasma osteocalcin, urinary calcium and cross-linked N-telopeptide of type I collagen (NTx), and ex vivo release of PGE_2 in bone organ culture were measured. Bone mineral content, density and area of whole body (WB), femur and total lumber spine 1-4 were measured using dual energy x-ray absorptiometry (DEXA). Data are mean ± standard deviation (SD), and differences in outcome measurements were detected by one-way ANOVA, post hoc analyses by Student Neuman Keuls all pairwise comparison test, and correlation relationships by Pearson Product. Piglets fed formula supplemented with AA at 0.60% and 0.75% had WB bone area most similar to piglets fed sows' milk. Sow-fed piglets had elevated femur bone area and BMD, and higher BMC in all sites measured compared to formula-fed piglets. Sow-fed piglets also had elevated

levels of urinary phosphorus excretion when compared to piglets fed LC PUFA supplemented formula. Suckled piglets had significantly higher amounts of total FA/mL in plasma, and significantly lower amounts in liver, lower plasma linoleic acid (LA) but not AA, lower LA and AA, but higher eicosapentanoic acid (EPA) in liver, and lower LA and ALA in adipose, compared to formula-fed piglets. Feeding formula containing 0.45% and 0.60% AA had higher levels of ALA but not DHA compared to suckled piglets. A positive (but not significant) relationship was seen between dietary AA and WB BMC and WB bone area, as well as an inverse relationship between dietary AA and urinary NTx. Dietary enrichment of increasing amounts of AA but consistent DHA into formula, including an amount most similar to sows' milk (0.60%), did not elevate or compromise growth. Piglets fed formula supplemented with 0.60% and 0.75% AA had WB bone area most similar to the sow-fed piglets, but BMC was higher in the sow-fed piglets in all sites measured. Feeding formula containing 0.6% AA resulted in a value for bone area only 9.5% higher than the value for the group fed formula with 0.30% AA. This difference may suggest that feeding AA at a level above 0.60% provides no benefit with regard to bone content. However, the positive relationship shown between dietary AA and WB BMC demonstrates a possible positive effect of increasing amounts of AA on bone mineralization. The inverse relationship seen between dietary AA and urinary NTx suggests bone resorption decelerates as dietary AA is increased. The effect of diet on bone metabolism and mineralization is complex. The differences seen between the sow-fed and formula-fed groups suggest that sow milk may contain factors other than LC PUFA that affect bone, and further investigation is required.

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LIST OF ABBREVIATIONS

AA	arachidonic acid
ALA	alpha linolenic acid
BA	bone area
BMC	bone mineral content
BMD	bone mineral density
DEXA	dual energy x-ray absorptiometry
DGLA	dihomo-gamma-linolenic acid
DHA	docosahexaenoic acid
DPA	docosapentaenoic acid
EFA	essential fatty acid
EPA	eicosapentanoic acid
FA	fatty acid
GA	gestational age
GLA	gamma-linolenic acid
LA	linoleic acid
LBW	low birth weight
LC PUFA	long-chain polyunsaturated fatty acid
NTx	cross-linked N-telopeptide of type I collagen
PGE_2	prostaglandin E_2
PL	phospholipid
PTH	parathyroid hormone
PUFA	polyunsaturated fatty acid
RBC	red blood cells

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1.0 INTRODUCTION

Survival rates for prematurely born infants are increasing, but these infants are often found to suffer from osteopenia (rickets of prematurity), which is a condition characterized by bone mineral deficits (Hori et al, 1995). It has been postulated that bone mineralization in infancy plays a role in determining adult peak bone mass, which is a factor which influences one's risk of developing osteoporosis (Fewtrell et al, 2000A). However, Hamed et al (1993) found no relationship between prematurity (with low birth weight) and peak bone density at maturity (Hamed et al, 1993). Rubinacci et al (1993) surmised by their research that, following calculations, as long as bone mineral content (BMC) is expressed relative to body weight, early infant nutrition might have no long terms effects on BMC during childhood. Other studies have found that preterm born infants when they reached 4 to 16 years (Helin et al, 1985), and 6 to 7 years of age (Kurl et al, 1998) have lower BMC compared to age-matched controls. More recently, Fewtrell et al (2000A) found that, although genetic disposition strongly influences a preterm infant's childhood height, there is strong evidence that metabolic bone disease during the neonatal period may have a long-term stunting effect.

Osteopenia is documented as appearing by 36 to 40 weeks gestation (Ryan, 1996), and infants born prematurely tend to be shorter, weigh less, and have lower BMC than full term born infants (James et al, 1986). Adequate intakes of dietary vitamin D (Ryan, 1996) calcium and phosphorus (Hori et al, 1995) may play a role in reversing the condition, but it is also possible that dietary fats may play a role, as certain fats are precursors of eicosanoids which influence bone metabolism (Watkins et al, 1996; Watkins et al, 2000).

That dietary fats are fundamental to normal growth and development was first recognized by Burr and Burr in 1929. Dietary fats are the predominant source of energy for infants, and constitute the major storage form of energy in the body. During fetal growth, 70% of energy is devoted to brain development, and lipids make up 50-60% of the structural matter of the brain (Crawford, 2000). However, the preterm born infant has lower fat stores than the term born infant. Ziegler et al (1976), reported that the fetus at 31 weeks gestational age has 5.2% body fat, compared to 12.0% body fat at 40 weeks gestation.

Infants born before term are at a nutritional disadvantage as they do not receive the placental nourishment that a term infant receives (Crawford et al, 1998), and must often rely heavily on dietary fat intervention following birth. This may be supplied in the form of total parental nutrition or enteral nutrition, which is not modeled on preterm breast milk, but on term breast milk (Crawford, 2000), and long-chain polyunsaturated fatty acids (LC PUFA) are not added. This leads to a rapid decline in the amounts of arachidonic acid (AA) and docosahexaenoic acid (DHA) in infant plasma, to a level of one quarter or one third of the intrauterine amounts that would have been delivered by the placenta (Crawford, 2000). This inadequate supply of AA and DHA during the period of highest demand due to rapid vascular and brain growth could lead to leakage of blood into the brain as well as membrane breakdown (Crawford, 2000). Because dietary fats play a major role as determinants of infant growth and development (Koletzko et al, 1991), as well as long-term health, the supply and selection of dietary lipids during early postnatal life is very important.

The two key fatty acids currently of interest in regard to infant nutrition are the LC PUFAs, AA and DHA. These are of interest because they both are in breast milk, but have not been supplied in infant formula in North America. Studies have been undertaken to determine the influence that a supply of dietary AA and DHA have on growth, as well as their influence on the resulting LC PUFA levels found in various tissues in the human infant (Foreman-van Drongelen et al, 1995; Gibson et al, 1997; Ghebremeskel et al, 2000) and animal models for infant nutrition (Ward et al, 1998; Yeh et al, 1998). Studies have also been done in an attempt to determine appropriate amounts of AA and DHA to add into infant formula for infants who are not breast-fed (Carlson et al, 1993; Makrides et al, 1995; Bondia-Martinez et al, 1998). However, these studies have focused mainly on the effect dietary LC PUFAs have on the brain and retina, or on the effect they have on altering lipid profiles in the infant, but not on their effect on the skeleton.

At the outset of this thesis research no studies had looked at the effect that varying the amount of AA in formula, would have on bone mineral content, bone mineral density, and bone metabolism during periods of rapid growth. The main focus of this research is to determine the impact that dietary LC PUFAs have on the growing skeleton using an appropriate animal model. This will be accomplished by investigating and comparing bone development, as well as various growth parameters, in growing piglets fed varying amounts of AA into formula.

2.0 HYPOTHESIS AND OBJECTIVES

HYPOTHESIS

Formula supplemented with AA in amounts most similar to those found in sows' milk will support elevated growth, bone mass and metabolism in piglets compared to piglets fed amounts of AA not comparable to levels in sows' milk.

OBJECTIVES

1. To determine if somatic growth, fatty acid stores, bone mass and metabolism vary as a function of the varied amounts of AA in formula.
2. To determine if there are differences among the formula-fed piglets in the fatty acid supplemented formula-fed groups.
3. To determine if there are differences between the formula-fed piglets in the fatty acid supplemented groups and the sow-fed piglets, with regard to somatic growth, fatty acid storage and bone mass and metabolism.
4. To determine the ratio of dietary AA to DHA in formula that supports bone metabolism in piglets most similar to that resulting from feeding sows' milk.

3.0 LITERATURE REVIEW

3.1 IMPORTANCE OF AA AND DHA FOR NEURAL TISSUES IN INFANTS

The fatty acid status of an infant at birth determines the nutritional requirements of the individual fatty acid for the infant postnatally. In utero, AA and DHA are selected and extracted by the placenta to support fetal development (Crawford et al, 2000). Infants born prematurely are born with lower levels of AA and DHA in plasma and red blood cell phospholipids compared to full term born infants (Innis, 1991; Crawford et al, 1998). This likely occurs because the highest rate of placental transfer of both AA and DHA from mother to fetus is during the third trimester of pregnancy (Clandinin et al, 1980; Innis, 1991; Crawford, 1998), and babies born prematurely are denied this nutritional supply. Interestingly, the amount of LC PUFAs transferred from mother to infant across the placenta varies according to the gestational age of the infant. A positive correlation has also been observed in umbilical artery walls, between n-6 and n-3 LC PUFA amounts and gestational age of the infant (Foreman-van Drongelen et al, 1995), which may indicate a response of the infant for the increased demand of these fatty acids, as they are necessary for infant growth and brain development.

Anthropometric measurements, including weight, length and head circumference at birth, were also found to be significantly related to LC PUFA levels in umbilical artery walls (Foreman-van Drongelen et al, 1995) indicating LC PUFA involvement in, or association with growth. Further, there is a connection between birth weight and risk of neurodevelopmental disorders as seen in a study in Scotland (1992). Of the 896 infants born weighing <1.75 kg, 29% did not survive, 16% were disabled, 47% had cerebral

palsy, 7% were blind and 11% were deaf. Because premature infants do not receive the same supply of LC PUFAs during the last trimester, as would a term born infant, they may benefit more from dietary AA and DHA than the term infant.

AA is found throughout the body, but with a high concentration in neural and vascular tissues (Leaf et al, 1996). DHA is also found in high concentrations in the brain and retina, but is scarce in most other tissues (Clandinin, 1999). Because of the importance of AA and DHA, much research has been conducted to determine their effect on the development and function of the central nervous system (CNS), brain and retina (Innis, 1992; Crawford et al, 1997). The previous researchers have found that the brain continues to accumulate DHA for possibly as long as 40 weeks postnatally, and it has been suggested that the ability to accumulate these fatty acids in both the preterm and term infant depends on dietary DHA supply (Makrides et al, 1996; Innis, 1994). Therefore, both AA and DHA are required for the development of neural tissue and cellular differentiation.

The precursors to the fatty acids AA and DHA, which are linoleic acid (LA) and alpha linolenic acid (ALA), or else the fatty acids themselves, may be supplied via placental transfer prior to birth as mentioned earlier (Hendrickse et al, 1985), or they may be released from adipose tissue, or supplied by human milk postnatally (Clandinin et al, 1981). Because preterm infants generally have lower body fat stores compared to term infants (Ziegler et al, 1976), preterm infants are likely more dependent on a dietary supply of these fatty acids.

3.2 HUMAN MILK VS COMMERCIAL INFANT FORMULA

There are many differences between breast milk and commercial infant formula, which will be discussed further. These differences are important because infants that are not fed breast milk immediately after birth are often fed commercial formula. Science and technology have advanced in many areas, but at present, have been unable to replicate all the properties of human milk and provide them in formula.

Human breast milk is tailor-made to meet the nutrient requirements of the human infant (Goedhart and Bindels, 1994). Breast milk also includes non-nutrient components that are beneficial to the human infant such as antiviral agents, as well as infection inhibitors and immune factors that interfere with the growth of bacteria (Wright, 1988) that could attack the infant's vulnerable intestinal lining. Breast milk also contains a number of protein constituents that act as carriers for vitamins (vitamin D, folate, etc.) and hormones (thyroxine, corticosteriod binding proteins) (Picciano, 1998), as well as fat-digesting enzymes that help to ensure efficient digestion and absorption of fat by the infant (Goedhart and Bindels, 1994).

An important class of nutrients in breast milk is fat. The total fat content in breast milk may range from 31 to 53 g/L (45 to 58% total energy intake) according to the Life Sciences Research Office (LSRO, 1998). The ratio of LA to ALA often found in human breast milk is 10:1 (unpublished data presented in Koletzko et al, 1995; Carnielli et al, 1996), and the ratio of AA to DHA is commonly found to be 1:1 to 2:1 (Uauy et al, 2000). However, the composition of milk is contingent on many factors including maturity of the milk (Serra et al, 1997) and maternal diet (Agostoni et al, 1999).

Differences in fatty acid content have been reported in breast milk between colostrum, transitional milk and mature milk. As milk matured significant differences were seen in LC PUFA of the n-6 series ($p=0.002$) and n-3 series ($p=0.005$), particularly in AA ($p=0.035$) and DHA ($p=0.032$) (Serra et al, 1997). Both AA and DHA are present in small but significant amounts. Maternal diet also affects breast milk. Milk from women following a vegan diet containing high amounts of LA resulted in 2.5 times the amount of LA and ALA, half the amount of DHA and similar amounts of AA as did breast milk from women following an omnivorous diet (Sanders and Ellis, 1992). Others reported dietary effects on breast milk composition include high levels of the n-3 fatty acids, DHA and eicosapentanoic acid (EPA) of women consuming diets high in fish (Innis and Kuhnlein, 1988) and high levels of saturated fat found in the breast milk of women consuming a high carbohydrate diet (Koletzko and Abiodun, 1992).

Breast milk and commercial infant formula differ in fat content. Many current infant formulas provide fat at 35 g/L (48% total energy from fat) (LSRO, 1998). The fatty acids AA and DHA contained in human milk are currently not added to infant formula in most countries that supply formula. As in human milk, commercial formulas do contain the two essential fatty acids (EFA) LA and ALA. This addition is key as it is well known that a deficiency of these EFA results in growth retardation and skin abnormalities, which can be avoided with an intake of 0.6% energy as LA (Fomon, 1993), and an intake of approximately 0.3 to 0.5% of energy as ALA (Holman et al, 1982). The general recommendation in Canada for the addition of LA into formula is 500 mg/100 kcal or 4.5% of total energy, and 0.7% of total energy as ALA (Health Canada, 1995). Current preterm infant formulas contain LA in amounts to provide 3.0

kcal/100 kcal, and ALA in amounts to provide 0.7 kcal/100 kcal. These amounts are recommended to maximize the synthesis and membrane levels of AA and DHA. The ratio of LA/ALA are set between 4:1 and 16:1 in formulas without added C20 or C22 fatty acids (Health Canada, 1995).

As seen in Table 1, formulas do not contain preformed AA (20:2n-6) or DHA (22:6n-3) in amounts that are provided to infants via placental transfer, or those present in human breast milk. It is because of these nutritional differences between breast milk and commercial infant formula that much research in this area has taken place over the last decade. The question to be answered is whether these two LC PUFAs (AA and DHA) should be added to infant formula, and if so, how much, and from what source?

TABLE 3-1. ***Selected fatty acid composition of some currently available infant formulas (in % of total fatty acids) and breast milk.***

	Heinz-Farley's 1st Milk	*Cow & Gate Premium*	*SMA Nutrition, Wyeth Gold*	*Placental output*[1]	*Human Breast Milk Europe*[2]	*Human Breast Milk Canada*[3]
18:2 n6 (linoleic)	10.2	11.0	17.0	8.0	11.0	10.47
20:4 n6 (arachidonic)	0.035	ND[4]	ND	20.0	0.5	0.35
18:3 n3 (linolenic)	1.67	2.4	1.6	0.4	0.9	1.16
20:5 n3 (eicosapentanoic)	0.47	ND	ND	0.2	0.2	0.05
22:6 n3 (docosahexaenoic)	0.5	ND	ND	10.0	0.3	0.14

[1]Placental output = cord plasma choline phosphoglycerides, values for healthy infants with normal birth weight

[2]Human milk European: n=32, 10 days after delivery at term (values represent median and interquartile ranges)
(adapted from Crawford et al, 1998; Koletzko et al, 1995)

[3]Human milk Canada: n=198, 3 to 4 weeks postpartum (adapted from Jensen, 2001)

[4]ND = non detectable

3.3 LIPIDS

3.3a Fatty Acid Nomenclature

Lipids occur in the body mainly in the triglyceride (TG) form, which makes up approximately 95 percent of the lipid content, with the remaining 5% consisting of phospholipids and sterols. A TG consists of a glycerol backbone esterified to three fatty acids. The fatty acids on the TG may differ from each other in chain length and in the degree of saturation (Giovannini et al, 1991). A saturated fatty acid contains no double bonds, while an unsaturated fatty acid contains one or more double bonds. Fatty acids containing their first double bond located between carbon number six and seven from the methyl terminal are designated omega-6 (n-6) fatty acids, and the fatty acids containing their first double bond located between carbon three and four are designated omega-3 (n-3) fatty acids (Innis, 1991). The type of dietary fat consumed influences the type of fat in the body.

3.3b Fatty Acid Storage

Fats are the body's main energy storage molecules. In adults, fats are responsible for approximately 99% of the body's energy storage, with glycogen accounting for approximately 1%. The amount of fat stored in the term and preterm infant depends on factors such as gestational age and maternal diet as indicated earlier.

Following consumption, dietary fats are temporarily stored in the stomach, and then secreted into the small intestine where they mix with bile that has been released by the gallbladder following production by the liver. The bile surrounds the lipids allowing them to become available to the water-soluble enzymes (lipoprotein lipase) required to

split the lipid into glycerol and shorter-chain fatty acids, which then can pass through the wall of the small intestine. At this point, the smaller molecules can pass directly into the bloodstream, while the larger products of lipid digestion (longer-chain fatty acids) are reformed into TGs and incorporated into chylomicrons (Sizer et al, 2000). These are then transported through the lymph system into the bloodstream, and ultimately arrive at the adipocyte cell, which are specialized fat storing tissues that serve as the main storage house of TGs in the body (Mayes, 1996).

When fat is required for energy by the body, such as between meals, lipolysis of TGs in adipose tissue occurs, or free fatty acids may be released into plasma via action of lipoprotein lipase from TG as they are taken up into tissues (Mayes, 1996). The fatty acids are then used by the skeletal muscle and liver as a source of energy, or for structural components in tissue development. Composition of the diet is generally reflected in the type of fatty acids found in these cells. Adipocytes are located in areas of the body such as breast tissue, the insulating fat layer under the skin, in muscle and in the abdominal region. Subcutaneous adipose tissue was analyzed in this study to determine the relationship between dietary LC PUFA intake and storage.

3.3c Fatty Acid Classification and Function

Lipids form the structural components of all tissues and are indispensable for cell membrane synthesis. The second category of lipid mentioned previously is the phospholipid, which is synthesized *de novo* in the smooth endoplasmic reticulum of the cell from dietary fat (Gilder et al, 1990), mainly the unsaturated form (Mayes, 1996). Membranes of various organelles with different biological function vary in lipid

composition. The composition of the phospholipid membrane determines the cell's fluidity, ionic transport mechanisms and intracellular enzymatic reactions (Sinclair, 1984).

Fatty acids play important biological and functional roles (Mayes, 1996), in the human brain, retina and nervous system (Fliesler et al, 1983). Some are necessary for normal growth and development (Burr and Burr, 1929). The role fatty acids play is determined by their structure.

Fatty acids are classified as non-essential, (produced *de novo)*, and include omega 9 (n-9) fatty acids, or essential (not produced in the body) a group that includes n-6 and n-3 fatty acids (Xiang et al, 1999). The category of fat which the body cannot produce *de novo,* the EFA, must be provided by the diet. The essential fatty acids include the polyunsaturated fatty acid (PUFA) linoleic acid (18:2 n-6) and a second PUFA alpha-linolenic acid (18:3 n-3), both of which play a number of important roles.

Both the n-6 and n-3 fatty acids are essential for normal growth and development, as first recognized in 1929 by Burr and Burr, particularly when growth rate is high (Innis, 1991). Burr and Burr (1929) demonstrated that rats fed a fat-free diet developed dermatitis, sterility and poor growth. Studies since have shown that a diet deficient in certain PUFAs lead to a variety of deficiency states. For example, infants fed a skim milk based formula developed eczema, which was reversed with the addition of essential fatty acids back into the diet (Hansen, 1933).

The fatty acids have also been shown to play a role in vitamin D-dependent calcium absorption in the intestinal tract. Coetzer et al (1994) reported that brush border membrane vesicles isolated from adult male Sprague Dawley rats fed a diet supplemented

with fish oil, supported higher calcium transport capacity than rats fed a diet high in saturated fats. Coetzer's group observed that the lower the EFA content of the diet, the lower the calcium transport and ATPase (adenosine triphosphate enzyme) activity in the animals.

It is well known that the LC PUFAs in the n-3 series are required for optimal function and development of the human brain, retina and central nervous system (Fliesler et al, 1983; Neuringer et al, 1986), as well as other membranes throughout the body. The brain, retina and other neural tissues are particularly rich in LC PUFAs with carbon chains of 20 or longer (Giovannini et al, 1991).

The n-6 LC PUFAs such as AA, are basic constituents of cell membranes where they are metabolized by cyclo-oxygenase and lipo-oxygenase and serve as specific precursors for eicosanoid production (Giovannini et al, 1991; Watkins et al, 1996). The specific eicosanoids produced and the pathway entered, depends on the degree to which the target tissue expresses an enzyme (Ettinger, 2000). These n-6 LC PUFAs are derived either directly from the diet, or synthesized from the essential fatty acids (Watkins et al, 1996).

Eicosanoids are formed from 20-carbon LC PUFAs and make up an important group of physiological and pharmacological compounds known as prostaglandins, thromboxanes, leukotrienes and lipoxins (Mayes, 1996). These are considered powerful autocrine and paracrine regulators of numerous cells and tissues and are involved in inflammatory reactions, vasoconstriction and vasodilation, blood pressure regulation, and uterine contractions (Weber et al, 1986; Ettinger, 2000). These will be discussed in greater detail further on in this thesis.

The main fatty acids of interest in this study are the essential fatty acid LA; 18:2 n-6 and its long-chain derivative AA; 20:4 n-6 both of the n-6 series, and a second essential fatty acid ALA; 18:3 n-3, and it's long chain derivative DHA; 22:6 n-3. The n-6 fatty acids are found mainly in vegetable oils (Mayes, 1996). The 18C n-3 series of fatty acids are found in soybean oil, canola oil and green leafy vegetables, while its long chain derivative is found mainly in egg yolk, sea algae, fish and shellfish (Sizer et al, 2000).

3.3d Fatty Acid Synthesis

Infants who are not fed breast milk or formula containing LC PUFAs must synthesize AA and DHA on their own from the 18-carbon precursor. This conversion takes place in the liver. The liver performs many tasks, including: carbohydrate, protein and fat metabolism; formation and excretion of bile; conversion of ammonia to urea; and filtration. It is also involved in the storage, activation and transport of many vitamins and minerals, including all of the fat-soluble vitamins. The liver is the site of energy production by way of β-oxidation, as fatty acids from diet or adipose tissue are converted to acetyl-coenzyme A (Sizer et al, 2000).

The pathways involved in the synthesis of fatty acids in liver have been studied and documented. The endogenous synthesis of fatty acids in rat liver, from shorter chain PUFAs to their longer chain derivatives involving a series of elongation and desaturation steps as put forward originally by Sprecher et al (1975), was commonly accepted (Innis, 1992) until recently (Figure 3.1). In the n-6 FA synthesis pathway, 18:2 n-6 (the EFA linoleic acid) was thought to undergo desaturation via the enzyme delta 6 (Δ6) desaturase to 18:3 n-6, then elongate and become 20:3 n-6 followed by desaturation via Δ5

desaturase becoming 20:4 n-6 (AA, the LC PUFA of interest in this study). In the n-3 series, the chain began with the EFA 18:3 n-3 (α-linolenic acid), which would undergo desaturation via the enzyme Δ6 desaturase (shared by the n-6 series) to 18:4 n-3, elongation to 20:4 n-3, then desaturation via the enzyme Δ5 desaturase (shared with the n-6 series), to 20:5 n-3 (eicosapentanoic acid), then via elongation to 22:5 n-3 and finally to 22:6 n-3 (docosahexaenoic acid; DHA), via Δ4 desaturase.

More recently, an alternative pathway (Figure 3.2) of n-6 and n-3 synthesis has been elucidated in isolated rat hepatocytes (Voss et al, 1991) and human fibroblasts (Moore et al, 1995). Sauerwald et al (1997) studied whether this proposed alternative pathway is operative in the term and preterm infant, and found that human infants do utilize this pathway for endogenous synthesis of LC PUFAs.

This alternative pathway may be significant nutritionally, as it suggests there are two Δ6 desaturation steps. This would result in competition between 18:3 n-3, 18:2 n-6, 24:5 n-3 and 24:4 n-6. In the presence of this competition for the Δ6 desaturase enzyme, synthesis of the longer chain PUFAs become even more dependent on balanced optimal intake of dietary 18:2 n-6 and 18:3 n-3 fatty acids, or the possible need to supplement infant formula with the longer chain PUFAs such as 20:4 n-6 (AA) and 22:6 n-3 (DHA).

The present research analyzes the storage of fatty acids in the liver by way of lipid/fatty acid analysis following dietary intervention. Regardless of the pathways and enzymes utilized in LC PUFA synthesis, studies have shown that dietary supplementation of n-3 or n-6 LC PUFAs modify the fatty acid composition of the liver (Farquharson et al, 1995; Watkins et al, 1996). Furthermore, when n-3 FA such as EPA and DHA are increased, this modification may ultimately alter eicosanoid production in soft tissue and

bone, and therefore is likely to affect tissue metabolism (Watkins et al, 2000). Thus if DHA is provided without AA, AA status would be compromised and PGE_2 synthesis reduced. The effect of low AA in bone has yet to be reported.

Other studies conducted to determine both the preterm and term infant's ability to perform the conversion of EFAs to LC PUFAs have reported that infants fed formula without LC PUFAs developed low plasma and red blood cell AA and DHA concentrations compared to infants that were fed breast milk (Carnielli et al, 1996; Crawford et al, 1997). This suggested there was an inadequate conversion of LA to AA and ALA to DHA in infants.

These findings were challenged in a study whereby LA and ALA were labeled with carbon-13, mixed with formula, and fed to premature infants (Carnielli et al, 1996). The conversion of LA and ALA to their longer chain metabolites AA and DHA were traced into phospholipids, to confirm whether the small preterm infant is capable of synthesizing them. Using the same method, term infants were fed corn oil, which does not contain AA. It was estimated that approximately 23% of plasma AA found by day four of the study, was due to infantile LA conversion (Demmelmair et al, 1995). The question remains however, whether the amount of AA produced was enough to meet requirements in either group of subjects. Results are inconclusive at this point (Carnielli et al, 1996). However, according to Sauerwald et al (1997), regardless of how much ALA is fed, the infant is unable to provide sufficient DHA for biochemical normality due to the elongation of the n-3 series and n-6 series sharing the same enzyme system, creating competition between those two cascades.

Dietary EPA also affects the balance of n-3 and n-6 LC PUFA status in the infant.

The addition of EPA at a level of 0.3% (of total fatty acids) plus DHA at 0.1% (of total fatty acids) to preterm infants resulted in decreased AA status (Carlson, 1996). However, the author also reported that preterm infants that were fed formula containing AA at 0.43% and DHA at 0.1% experienced an increase in their AA status, as determined by assessing plasma phosphatidylcholine AA, an indicator of AA status, indicating plasma fatty acids are affected by the type of fatty acids provided in the diet.

The study of the effect of dietary AA and DHA must extend beyond current research to examine their effects on other tissues, the immune system, and bone growth and mineralization. Well-controlled, long-term trials should be conducted that combine measurements of whole body growth and development, biochemical assessment of potential sources of AA and DHA, and determination regarding the necessity of adding the LC PUFAs into infant formula, particularly for the preterm infant (Health Canada, 1995).

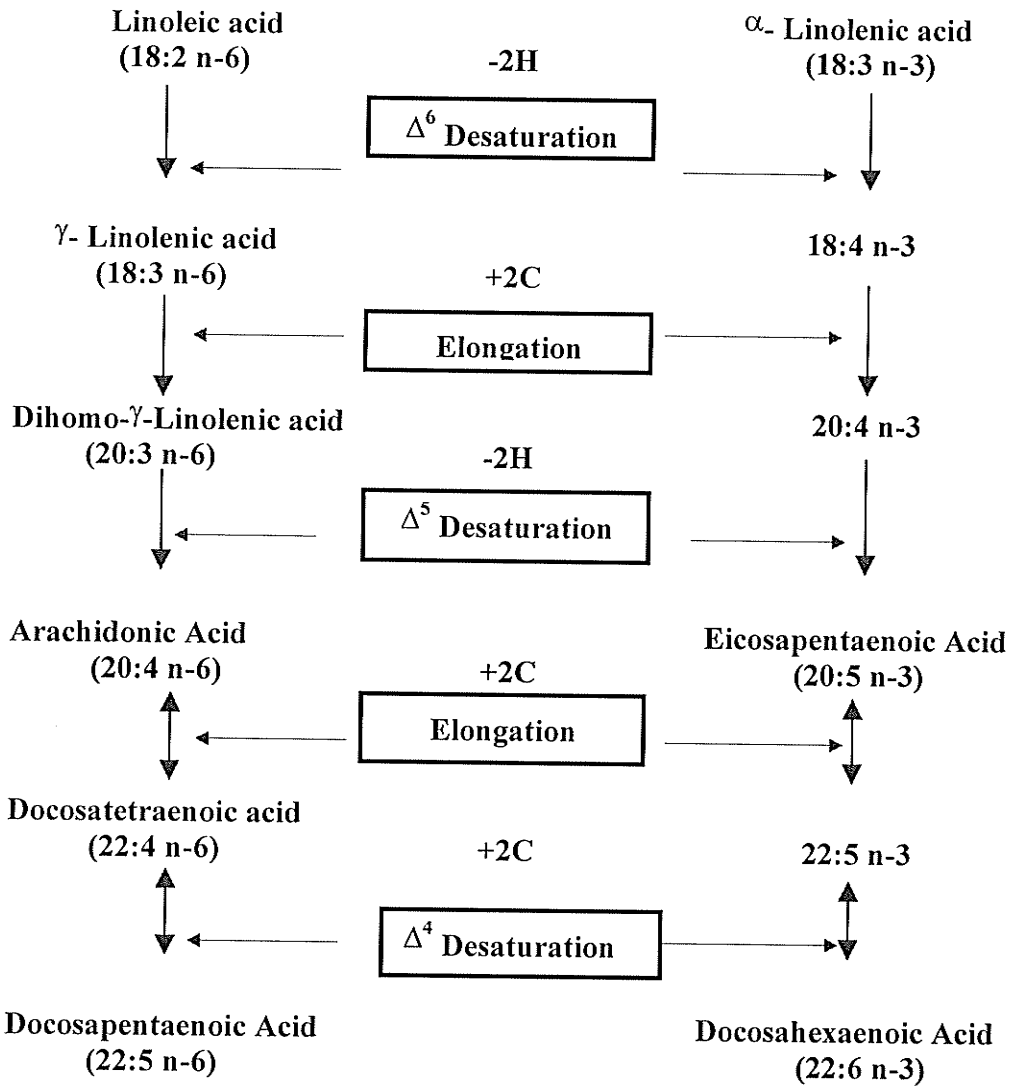
The premature infant has compromised bone growth and mineralization as well as limited fat stores, and possibly is not able to synthesize quantities of AA and DHA necessary to meet requirements for adequate growth. Therefore further studies must be conducted to determine the outcome of adding varying amounts and ratios of AA and DHA in infant formula, and their effect on whole body growth.

At the outset of this research, no studies had been published examining the effect on bone of AA and DHA in the human infant or appropriate animal model. However, a recent study has been undertaken (Weiler, 2000), which looked at the effect of a 5:1 ratio AA to DHA. Ten-day-old piglets were fed diets with or without AA and DHA for 14 days. Following the trial diet period, assessments were performed to determine fatty acid

status in plasma and liver, calcium absorption and bone mineral content and density. Supplemented piglets were heavier than the placebo-fed, and they acquired a higher whole body bone mineral content (137 g vs 112 g), and a higher bone mineral density (0.66 g/cm^2 vs 0.61 g/cm^2). Regarding fatty acid status, there were no differences observed in plasma, however in the liver, the supplemented group was higher for AA.

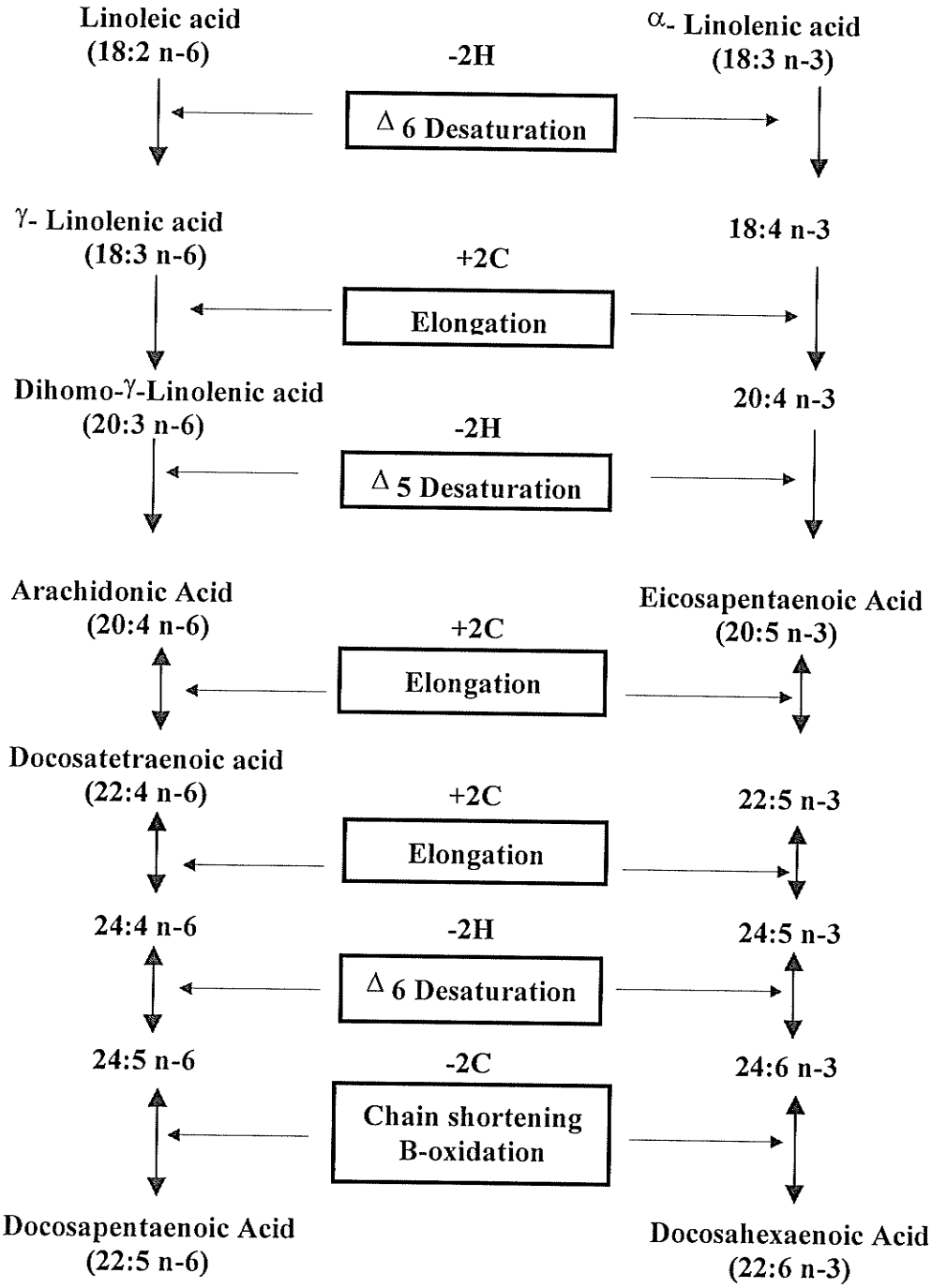
These results suggest that at a ratio of 5:1 AA:DHA, during a period of rapid growth, developing piglets increase their weight, bone mineral content and bone density compared to a standard formula-fed animal (Weiler, 2000). Further work should be undertaken to expand on this issue, which is the purpose of this research.

FIGURE 3-1. ***Classic pathway of n-3 and n-6 fatty acid metabolism utilizing Δ^6, Δ^5, and Δ^4 desaturation and elongation steps.***



(Adapted from Sauerwald et al, 1997)

FIGURE 3-2. *Currently accepted metabolic pathways for biosynthesis of n-3 and n-6 fatty acids.*



(Adapted from Koletzko et al, 1995)

3.4 EFFECT OF LC PUFA SUPPLEMENTATION ON BLOOD LEVELS OF AA AND DHA (AND REFLECTION OF VALUES OR CHANGE IN TISSUES)

The fatty acid status of infants can be determined by measuring the fatty acid composition of their blood, and their lipid profile is partially determined by their dietary fatty acid intake. Due to the uncertainty about the ability of preterm infants to manufacture appropriate amounts of the LC PUFAs AA and DHA from their precursors LA and ALA (Uauy et al, 2000), which are available preformed in breast milk, the lipid profile of infant formula is an issue.

Numerous studies have shown that, preterm infants who are fed formula without LC PUFA have lower AA and DHA contents in red blood cells and plasma (Koletzko et al, 1995; Byard et al, 1995; Foreman-van Drongelen et al, 1995; Boehm et al, 1996; Simmer, 1996; Clandinin et al, 1997; Woltil et al, 1998) as do term infants (Kohn et al, 1994; Koletzko et al, 1996; Decsi et al, 1995; Makrides et al, 1996; Bondia-Martinez, 1998; Gibson et al, 2000) compared with infants who have received human milk. Similar results have also been reported in animal studies (Yeh et al, 1998; Weiler, 2000).

Investigators have reported that infants fed formula supplemented with AA and DHA have plasma, red blood cell and/or tissue levels similar to infants who are breast-fed. For example, Clandinin and colleagues (1997) analyzed membrane PLs in preterm infants. The authors discovered that formula with the addition of 0.32 to 1.1% AA and 0.24 to 0.76% DHA were sufficient levels of these FA to achieve similar levels of PL AA and DHA in formula-fed infants as in infants fed breast milk.

Koletzko et al (1995), analyzed plasma fatty acid content (% wt/wt) of preterm infants fed either human milk (containing 0.5% AA; 0.3% DHA wt/wt), formula without

LC PUFA (0.05% AA; 0.03% DHA wt/wt) or formula with added LC PUFA (0.5% AA; 0.3% DHA wt/wt). The 3 feeds were similar in amounts of LA (11.0%, 10.8% and 13.8% wt/wt respectively) and ALA was similar in human milk (0.9% wt/wt) and supplemented formula (0.8% wt/wt), but lower in unsupplemented formula (0.4% wt/wt). At 21 days of study, the infants in the unsupplemented formula group exhibited significantly reduced levels of plasma PL AA (26% lower than values found in human milk-fed group) and DHA (36% lower than values found in human milk fed group) than values for PL AA and DHA at the start of the study. These reductions were not seen in either the human milk or formula supplemented groups, and these two groups exhibited values that were similar to each other at study end.

The Boehm (1996) group found similar results when they compared fatty acid status in serum and RBC PL in a group of very low birth weight preterm born infants (VLBW). Infants received fortified breast milk (0.36 AA, 0.22 DHA; wt % of total FA), standard formula (LC PUFA free) or supplemented formula (0.25 AA, 0.15 DHA; wt % of total FA). After two, six and 10 weeks AA levels in RBC were significantly lower in the unsupplemented infant formula fed group than in the other two groups. DHA was significantly lower after six weeks of feeding in the unsupplemented formula fed group.

In term infants, Desci et al (1995) compared infants fed unsupplemented formula to infants who were fed formula supplemented with LC PUFA in amounts similar to that found in breast milk (AA: 0.5% wt/wt, DHA: 0.3% wt/wt). The researchers found that from 1 month of age onward, AA and DHA were consistently higher in plasma of infants receiving supplemented formula than on plasma of those fed unsupplemented formula.

Kohn et al (1994) reported that the newborn term infant depends on an exogenous supply of LC PUFA during the first months of life. Term infants born between 38.5 – 41.5 weeks were fed either breast milk or one of two infant formula, one conventional, the other supplemented. The 3 feeds were similar with respect to protein, lactose and total fat content, including LA (12.28, 12.09 and 10.76 wt % of total fatty acids respectively) and ALA (0.74, 0.77 and 0.81 wt % total fatty acids respectively) acid. They differed in content of AA and DHA. Breast milk contained 0.36 wt % AA and 0.22 wt % DHA, the experimental formula contained 0.25 wt % AA and 0.18 wt % DHA, and the standard formula contained 0.02 wt % AA and <0.01% wt % DHA. Although the 3 infant groups did not differ significantly in development of weight or height during the 3-month study period, the infants fed standard formula had significantly lower values of AA in plasma PL on days 7, 30 and 90 compared with the 2 other groups. As well, content of DHA found in plasma PL was significantly more decreased in the standard formula fed group.

In 1998, Bondia-Martinez et al fed infants that were 37-42 week's gestational age human milk, a standard term formula based on cow's milk, or a supplemented formula. The standard formula contained a conventional lipid composition (including LA: 12.0 g/100 g total FA and ALA: 1.2 g/100 g total FA). The experimental formula was supplemented with egg yolk lecithin that contained AA (0.3 g/100 g total FA) and DHA (0.15 g/100 g total FA). This resulted in higher plasma n-6 PUFA in infants fed milk and experimental formula, than in those fed standard formula, at 1 and 3 months of age.

Similar results were seen by the Koletzko (1996) research group. One group of full term infants were fed breast milk, and a second group was fed formula with 13.2%

LA and 1.0% ALA (wt/wt), but no detectable AA and DHA. This resulted in significantly higher AA plasma values in the breast-fed group than the formula-fed group at day 14 (9.39 ± 1.00 vs 6.91), day 30 (9.06 ± 1.04 vs 6.16 ± 0.35) and day 60 (8.41 ± 1.19 vs 5.74 ± 0.37 % wt/wt).

The generally accepted preferred method of feeding the infant is breast-feeding. However, for those who are not breast-fed, it has been recommended that term infant formula should contain at least 0.2% of total fatty acids as DHA and 0.35% as AA. The recommendation was made by Koletzko et al (2001) in their review of available scientific information on LC PUFA during pregnancy, lactation and early life. The authors further recommended that preterm infant formula contain at least 0.35% DHA and 0.4% AA, as the infants are born with lower total body stores of DHA and AA.

As these studies have shown, both the term and preterm infant decrease their levels of AA and DHA if not fed a supply of those fatty acids.

3.5 BONE

3.5a Structure and Function

Bone is an organ composed of cortical (dense/compact) and trabecular (spongy or cancellous) bone, and cartilage, haemopoetic and connective tissues (Boskey, 1999), which together make up the skeletal system. These tissues serve 3 functions:

1 – protective: protection of internal organs and bone marrow;

2- mechanical: support to the body, movement of body parts and provision of a site for haematopoesis;

3 – metabolic: principal reservoir of calcium, phosphorus, sodium, magnesium and carbonate; principal site for mineral homeostasis (Boskey, 1999; Anderson, 2000).

Cortical bone makes up approximately 80% of the adult skeleton, where it is found mainly in the shafts (diaphyses) of large/long bones (Anderson, 2000). Cortical bone has a high mineral content (approximately 70%), and serves mainly a mechanical function.

Trabecular bone makes up approximately 20% of the adult skeleton, and is found in the knobby ends (epiphyses) of long bones, iliac crest of the pelvis, wrists, and the regions lining the marrow (Anderson, 2000). Trabecular bone is composed mainly of fine bone plates that form a lattice (lamellae), filled with haemopoetic marrow and fat containing marrow, and blood vessels (Baron, 1999). Trabecular bone serves to reduce skeletal weight without compromising strength of the skeleton, and serves as an important site of bone remodeling (Boskey, 1999).

3.5b Composition

Both cortical and trabecular bone are solidly mineralized with small canals, spaces, and bone cells. Bone contains mineral, collagen, water, noncollagenous proteins, lipids, vascular elements, and cells (in decreasing order; Posner, 1985). It gets its strength from calcium and phosphate deposited in a collagen matrix (bone matrix contains both collagen and noncollagenous proteins), which, in combination with hydroxyl ions, mature into hydroxyapatite crystals [$Ca_{10}(PO_4)_6(OH)_2$] (Anderson, 2000). These crystals represent the mineral phase characteristic of adult bone. Minerals account

for 50 to 70% of adult bone (Lian et al, 1999). In the newborn infant, the skeleton is made up of about 25 g calcium and 16 g phosphate, with about 80% being transferred to the infant during the last trimester of pregnancy (McCance in Hamed, 1993).

The second most abundant component of adult bone (mineral being the most abundant) is collagen, which is mainly type I. Type I collagen forms 90% of the organic mass (total protein) of bone and tendons, as well as forming the collagen in skin, ligaments and connective tissues (von der Mark, 1999). Collagen fibres are organized in sheets, which provide a well-organized scaffold for the deposition of mineral (Risteli et al, 1999), as well as providing bone with elasticity and flexibility (Boskey, 1999).

Water makes up 5-10% of bone tissue weight, noncollagenous proteins make up approximately 5% of weight, and lipids account for <3%. However, the composition of bone varies with age, anatomic location, diet, and health status of the individual (Lian et al, 1999).

3.5c Growth

Bone growth is a process of modeling and remodeling. Growing long bones consist of 3 major components including the diaphysis, epiphysis and epiphyseal plate. The compact cortical bone forms the diaphysis of long tubular bones and provides structure, while the epiphyses form the ends of the long bones (Seeley et al, 1992). The epiphyseal plate is the site at which growth takes place by a cycle of cartilage growth, matrix formation and cartilage calcification (Boskey, 1999). At the cessation of growth, this plate becomes ossified and forms the epiphyseal line.

3.5d Modeling and Remodeling of Bone – Bone Cells

Bone is dynamic tissue that undergoes modeling early in life, then remodeling after cessation of skeletal growth, occurring at approximately 20 years of age. Modeling of bone begins in the embryonic stage of life. During growth, there is a net increase in skeletal mass, which is referred to as bone mineral content (BMC). Bone modeling is the shaping process that maintains the morphology of the growing bone. There are 3 major types of bone cells responsible for this process, including the synthesis and resorption of bone. These bone cells are osteoblasts, osteoclasts and osteocytes. For modeling to occur, bone formation by osteoblast activity, and bone resorption via osteoclast activity occur at separate sites, and are balanced (Boskey, 1999). Osteoblasts are responsible for the formation and organization of the extracellular bone matrix and its mineralization (Menton et al, 1984). Osteoblasts are found on the surface of bone, and are derived from undifferentiated mesenchymal progenitor cells in marrow, that are capable of differentiating into chondrocytes, muscle cells and adipocytes (Rodan et al, 1981). The mature osteoblast is composed mainly of type I collagen. The linear sequence of the osteoblast progresses from osteoprogenitor to preosteoblast, to osteoblast, then to lining cells called osteocytes (Bonucci, 1990).

Osteocytes are osteoblasts that have been incorporated into the bone matrix. They are considered the most mature/terminally differentiated cells of the osteoblast lineage, and are embedded in the lacunae in the interior of the bone, throughout the mineralized matrix and cells lining the bone surface (Menton et al, 1984).

Osteoclasts are large (~ 100 μm) multinucleated, polarized cells that have the ability to degrade mineralized tissue (Lian et al, 1999). They generally appear in contact

with a calcified bone surface within a Howship's lacunae, and are responsible for resorption of the bone matrix. Bone resorption is necessary for normal development of the skeleton, its adaptability and its maintenance (Baron et al, 1993).

3.5e Regulation of Bone Formation and Resorption

The regulation of bone formation and resorption involves a combination of many factors, a few of which will be described in this thesis (Table 3-2 and 3-3). Parathyroid hormone (PTH), vitamin D and calcitonin are calcium regulating peptide hormones that help to normalize plasma calcium concentrations through intestinal absorption, renal calcium excretion, bone turnover and dietary intake of calcium (Mundy, 1993). Prostaglandins (Watkins et al, 1996) and more recently fatty acids (Weiler, 2000) also play a role in bone regulation.

3.5e-1 Parathyroid Hormone

PTH is a single-chain polypeptide consisting of 84 amino acids, and has a molecular weight of 9500. PTH helps maintain serum calcium concentrations via direct action on bone and kidney tissue, and indirectly via action on the intestinal tract (Fitzpatrick et al, 1999). Low serum calcium levels stimulate the release of PTH from the parathyroid gland, causing the reabsorption of calcium in the kidney, or via osteoclast-mediated bone resorption, which then increase calcium concentration in serum (Fitzpatrick et al, 1999).

Vitamin D (1,25 dihydroxyvitamin D) plays a role in the regulation of PTH gene expression. PTH increases the renal synthesis of $1,25(OH)_2D_3$, thereby increasing serum

calcium and decreasing serum phosphate concentrations via increased calcium and phosphate intestinal absorption (Juppner et al, 1999; Holick, 1999). PTH is thought to stimulate the osteoclast to activate bone resorption having a catabolic effect, and induce catabolic activation in the osteoblast, thus influencing the osteoclast. PTH has also been found to enhance collagenase synthesis, inhibit type I collagen synthesis, and reduce alkaline phosphatase activity in the osteoblast-like cell line, indicating PTH is selective (Midura et al, 1994).

3.5e-2 Vitamin D and Calcitonin

Vitamin D can be provided by the diet, or vitamin D_3 is produced in the skin from 7-dehydrocholesterol (a final intermediate in cholesterol biosynthesis). This conversion occurs via a nonenzymatic, photochemically mediated action, using energy from ultraviolet (UV) light between 290 and 315 nm (Tenenhouse, 1990).

The liver is the principal physiological site for the 25-hydroxylation of Vitamin D, which is hydroxylated at the C-25 position, via liver 25-hydroxylase enzyme (Tenenhouse, 1990). This vitamin D metabolite can then be transported in the blood, bound to vitamin D-binding protein (DBP), to the kidney where it can undergo further hydroxylation to form $1,25(OH)_2D_3$, the active vitamin D hormone (Tenehouse, 1990). This active metabolite of Vitamin D, ($1,25(OH)_2D_3$) then plays a major role, which is related to bone metabolism and mineral homeostasis. This form of vitamin D functions by increasing intestinal and phosphate absorption, as well as calcium renal absorption (Raisz, 1993). It can either inhibit or induce osteoblast differentiation, depending on the

stage at which it is given or produced, as well as promote osteoclast differentiation (Carmeliet et al, 1999).

The requirement of vitamin D for normal bone and cartilage mineralization was first suggested by the observation that epiphyseal cartilage and trabecular bone did not mineralize adequately in animals with rickets, who were also fed a diet either poor in phosphate or a high Ca/P ratio (Harrison et al, 1958).

Calcitonin is a hormone produced by cells in the thyroid gland. The hormone lowers the levels of calcium and phosphate in the blood, and is believed to inhibit the resorption of bone (Mundy, 1993).

3.5e-3 Prostaglandins

In many cells, prostaglandins along with thromboxanes and leukotrienes are synthesized from arachidonic acid, following their release from phospholipids, by the action of phospholipase A_2. Prostaglandins are localized tissue hormones that are synthesized then released by osteoblastic bone cells. The production of prostaglandins are regulated by growth factors, cytokines, systemic hormones and mechanical force (Kawaguchi et al, 1995).

Prostaglandin E_2 (PGE_2), a cyclo-oxygenase product of AA, was found to be a simulating substance of bone resorption first by Klein and Raisz (1970). PGE_2 is involved in paracrine (affecting adjacent cells) and autocrine (affecting cells in which they are synthesized) regulation of bone formation (Kimmel et al, 1994), and appears to have both stimulatory and inhibitory effects on bone formation and resorption.

PGE_2 is hypothesized to be a potent stimulator of bone formation in vivo (Jorgensen et al, 1988), as well as a powerful anabolic agent capable of preventing disuse-induced cortical bone loss, while stimulating bone formation associated remodeling. This anabolic effect has been demonstrated by Jee et al (1992) and Akamine et al (1997) when PGE_2 was injected into 13-month-old female Sprague-Dawley rats, at doses of 1 to 6 mg PGE_2/kg/day.

PGE_2 apparently affects bone by increasing collagen formation, thereby increasing bone formation. However, PGE_2 also inhibits bone resorption at low levels (Kimmel, 1994; Quinn et al, 1996). At low concentrations, PGE_2 affects DNA synthesis and stimulates alkaline phosphatase activity of osteoblasts, possibly through cyclic AMP production (Hakeda et al, 1986), thereby increasing osteoblastic cyclic AMP production (Partridge et al, 1986). The exact mechanism of action of PGE_2 is still unknown.

In vitro, PGE_1 and PGE_2 have been shown to stimulate bone nodule formation in calvarial rat cells (Flanagan et al, 1992). At high concentrations, PGE_2 appears to have an opposite effect, inhibiting collagen formation.

TABLE 3-2. ***Some mediators of bone modeling.***

OSTEOCLASTS	
Stimulatory	***Inhibitory***
Vitamin D	Bisphosphonates
Thyroxin	Calcitonin
Prostaglandins	Estrogen
Osteoclast-activating factors	Anabolic agents
Interleukin 1	Fluorides
Transforming growth factors	
OSTEOBLASTS	
Vitamin D	Glucocorticoids
Estrogen	
Fluorides	
Prostaglandins	
Insulin-like growth factor	

Adapted from BoneKey-Osteovision

TABLE 3-3. ***Some factors influencing bone strength.***

Quantative	*Chemical*
Bone density	Matrix components
Compact/spongy bone	Mineral components
Degree of mineralization	
Structural	*Miscellaneous*
Architecture of compact bone	Rate of bone turnover
Trabecular architecture	Vascular supply
Lamellar arrangement	Bone marrow status
Collagen bundling	
Cross-linking	

Adapted from BoneKey-Osteovision

3.5e-4 Fatty Acids

Fatty acids also play a role in bone metabolism (Weiler, 2000) as modulators of bone remodeling (Watkins et al, 2001) through calcium absorption (Claassen et al, 1995; Kruger et al, 1997) and are reflected in the fatty acid composition of bone (Watkins et al, 1996). Alam et al (1993) discovered that the type of dietary fats fed to rats strongly influenced the fatty acid composition of bone lipids. Three groups of 4-week-old Sprague-Dawley rats were fed nutritionally adequate, semi purified diets containing either 10% corn oil (control diet; high in n-6 FA), 9% butter + 1% corn oil (high in saturated FA), or 9% ethyl esther concentrate (EEC) of n-3 FA + 1% corn oil (high in n-3 FA) for 10 weeks. Fatty acid composition of maxillae and mandible PL were measured resulting in a higher level of LA in the 10% corn oil group (control, high in 18:2 n-6) than the other two groups. Groups fed 9% EEC + 1% corn oil (high in n-3 FA) contained significantly higher amounts of n-3 FA (20:5, 22:5 and 22:6) than the control group, and the concentration of total PL AA was significantly lower in the maxillae and mandible of the group fed fish oil (EEC) + corn oil than the other groups.

Kruger et al (1997) found a correlation between EFAs and parameters of bone formation and degradation in a study designed to investigate the effect of various dietary ratios of n-6:n-3 on calcium homeostasis. Female Sprague Dawley ovariectomised rats were supplemented from 11 weeks of age, for 6 weeks with different ratios (9:1; 3:1, 1:3; 1:9) of (gamma-linolenic acid; 18:3 n-6) GLA:EPA (Eicosapentaenoic acid; 20:5 n-3) + DHA and were compared to a control. Following dietary treatment, bone parameters and RBC fatty acid profiles were measured. GLA increased significantly in all groups, as well as DGLA (GLA is elongated rapidly to DGLA) at $p \leq 0.05$. AA was significantly

reduced in groups fed ratios of 1:3 and 1:9 (n-6:n-3) compared to control. DGLA was closely correlated with bone calcium and negatively with deoxypyridinoline, indicating an anabolic effect on bone, or less bone resorption, as suggested by the researchers.

The Watkins group (1996) reported that chicks fed soybean oil (high in 18:2 n-6) compared to those fed menhaden oil (high in 20:5 n-3 and 22:6 n-3) had increased the concentration of 18:2 n-6, 20:4 n-6, 22:4 n-6 and 22:5 n-6 (the last three being the long chain derivatives of 18:2 n-6) in liver and bone. Alternatively, chicks fed menhaden oil had increased 20:5 n-3 and 22:6 n-3 concentrations in liver and bone, again indicating an effect of dietary fatty acids. Watkins et al (2000) investigated the effects of varying dietary ratios of n-6:n-3 PUFAs on bone compartments and biomarkers of bone formation in male Sprague-Dawley rats. Feeding rats a diet with a low ratio of n-6:n-3 (2.6 or 1.2) fatty acids resulted in significantly lower ex vivo PGE_2 production in liver and bone organ cultures (right femur and tibia) and reduced AA concentration, compared to rats fed diets at a higher ratio (23.8 or 9.8). The authors reported that as the dietary ratio of n-6:n-3 fatty acids declined, bone modeling condition improved in the rats.

Children born prematurely may have similar growth compared to children born at term, but their bone mineralization can remain low until 12 years of age, compared to term-born children (Fewtrell et al, 2000B). This suggests that the premature infant does not catch up to the term infant with regard to bone mass. To assess the dietary needs of the infant with respect to bone metabolism and potential associations with LC PUFA, we must be able to measure BMC and BMD.

3.6 BONE MARKERS FOR ASSESSMENT OF GROWTH AND METABOLISM

The activity of bone may be measured with the use of biochemical markers, which are currently available to determine the state of both bone formation and resorption. Biochemical markers of bone can be measured in serum and urine, and each reflects a different stage of osteoblast differentiation. During bone formation, the matrix must be produced before mineralization can occur, therefore early markers of bone formation are released and available for measurement (Naylor et al, 1999). These markers specific to formation include serum bone-specific alkaline phosphatase, carboxy-terminal propeptide of type I collagen, amino-terminal propeptide of type I collagen and osteocalcin. Those specific to resorption include urinary hydroxyproline, pyridinolines, C-telopeptide of collagen cross-links, and N-telopeptide of type I collagen.

3.6a Osteocalcin and Bone Modeling

Osteocalcin (OC) is a major noncollagenous, vitamin K dependent extracellular protein with a molecular weight of 5700 (Lian et al, 1987). This protein is synthesized and secreted exclusively by osteoblasts, and in small amounts by the ondoblasts (Bronkers et al, 1985), in the late stage of maturation. OC contains 3 residues of γ-carboxyglutamic acid, which is a calcium-binding amino acid that allows it to bind with hydroxyapatite (Lian et al, 1987). OC is specific to bone and dentin (Bronckers et al, 1985). The majority of this protein accumulates in bone, but very small amounts (nanomoles) circulate in the blood (Lian et al, 1987).

Serum osteocalcin assay has been found to be a sensitive marker of bone formation, which parallels the growth velocity curve during childhood and adolescence (Michaelsen et al, 1992), as OC is released into circulation at a proportional rate to new bone formation (Seki et al, 1994). Evidence suggests that OC production occurs late in osteogenesis, and that its biological functions are also related to bone resorption (Lian et al, 1984, Khosla et al, 1999).

The OC radioimmunoassay (RIA) was used by the Michaelsen (1992) research group, to show significant differences and correlation of serum OC levels in infants (gestational age 37-42 weeks). Infants fed human milk ($n = 48$) were compared to infants fed formula ($n = 14$). At 2 months of age, the breast-fed infants had significantly elevated serum OC values (275 ± 87 ng/ml) than the formula-fed infants (80 ± 44 ng/ml), at $p<0.001$. Serum OC levels were also found to be positively correlated to breast milk intake compared to formula intake (ml/kg body weight; $r = 0.59$, $p \leq 0.001$) in this study.

Nielsen et al (1990) investigated the diurnal pattern and 24-hour integrated serum concentration of OC (with circadian rhythm characterized by high nocturnal values and minimum noon-time values), and found there was no influence of age or sex on the diurnal variation.

3.6b N-telopeptide and Bone Remodeling

Type I collagen is synthesized in both soft and hard tissues. In soft tissue it is mainly produced by fibroblasts, which also produce large amounts of type III collagen. Bone collagen is produced by osteoblasts, which normally do not produce type III collagen (Risteli et al, 1999), therefore making type I collagen the best source for

quantification of the metabolic turnover of the skeleton (Risteli et al, 1997). Also, these metabolites released from bone can be directly released into circulation, making them available for measurement.

The main molecular sites involved in collagen cross-linking are the short, nonhelical peptides located at both ends of the collagen molecule, the amino (N) and carboxy (C) terminal telopeptides. Normally, these cross-linked telopeptides are linked via pyridinium or pyrrole compounds to the helical portion of neighboring collagen molecules (Eyre et al, 1984). Then during collagen breakdown, these telopeptides are released into circulation while still attached to the helical portions of nearby molecules by pyridinium cross-links. The majority of these cross-links are cleared by the kidneys (Knott et al, 1997) where they can be measured.

Urinary N-telopeptide of type I collagen (NTx) is currently used as a marker of bone resorption. This type I collagen protein makes up approximately 90% of the organic matrix in bone. During periods of bone resorption, when the 2 major cross-links, pyridinoline and deoxypyridinoline are released into circulation, the collagen undergoes partial breakdown and the remnants are released into the urine and can then be measured. The most common ELISA assay used to measure NTx is based on an antibody binding to the cross-linked peptides that arise from type I collagen degradation (Gfatteral et al, 1997). This method was used to measure bone resorption in term and preterm infants.

This NTx assay has been shown to be a useful tool, as it is sensitive enough to detect changes in bone resorption in pigs (Bollen et al, 1997). NTx is also a stable assay and has been shown to be more sensitive and less variable than pyridinolines (Weiler, 1996). Thus NTx has been reported to be a more dynamic marker of bone resorption

than other markers, showing greater response to change in therapy than pyridinium cross-links (Blumsohn et al, 1995).

3.7 MEASUREMENT OF MINERAL ACCRETION IN GROWING BONE

There are various methods that can be used to provide measurements of bone density in children. Quantitative computed tomography is an expensive method involving levels of radiation that preclude the use on children (100 mRem). Single photon absorptiometry, is a method that has been used most extensively to study preterm infants, but has a limitation of examining only bones in the peripheral skeleton, which are mainly cortical bones in children that have low turnover rates (Shaw et al, 1995).

Another method, dual photon absorptiometry, uses two separate energy levels giving it the capability of measuring trabecular bone, but with the limitation of using radioisotopes which decay with time, therefore reducing the precision of the measurement. Finally, dual-energy x-ray absorptiometry (DEXA), which is the more cost effective method, delivering a low radiation dose (3-5 mRem), with an accuracy and precision level of 1-2%, is currently the most widely used and favored method (Shaw et al, 1995). Newer DEXA models make use of a fan beam to enable faster scan times.

DEXA was developed for the purpose of measuring bone mineral at multiple sites (Shore et al, 1999). It works by producing a pencil beam of x-rays that are transmitted from a source providing alternating pulses of 70 kVp and 140 kVp through a rotating calibration disk of bone and soft tissue equivalent materials, which are measured longitudinally by a detector located above the subject. By correcting the bone mineral content for the projected area of bone, a measurement of bone mineral density (g/cm^2) is

produced (Shaw et al, 1995). By analyzing photon absorption at two different sites, the amount of bone mineral, soft tissue and fat can be calculated without requiring the need for constant body thickness. DEXA has the ability of estimating total lean body mass and fat mass (Shaw et al, 1995), therefore providing whole body and regional measurements of bone, lean and fat mass (Glastre et al, 1990).

Work has been done to study the applicability of DEXA in newborns by measuring precision and accuracy (Braillon et al, 1992). Using a Hologic QDR 1000 instrument, very small quantities of mineral from 0.5g to 4g of hydroxyapatite were tested in vitro. The mean precision for six femurs for BMC and BMD was 1.2 and 0.8%, respectively. The mean precision measured in lumbar spine (L1 to L5) in 10 newborn infants, with two or three repetitions was 2.4 and 1.5% for BMC and BMD, respectively. Brunton et al (1997) also evaluated the efficacy of DXA (Hologic QDR-1000/W) for estimating BMC and BMD is small subjects, and found that DEXA is a useful tool for estimating total body composition of rapidly growing infants provided it is used with caution in respect to anticipated increments over time.

The drawbacks of DEXA bone densitometry in children is that the method measures the attenuation of an x-ray beam across a projected cross-section of bone, which depends on the actual density of skeletal mineral, and on the dimensions of he bone along the axis of the beam. These dimensions along with their relation to one another change as children grow. Therefore, corrections based on the child's height and weight must be applied to the machine's output (Friedl et al, 1993).

The advantages of DEXA for measurement in children are that the scan occurs over a short period of time (3-6 minutes), which is a reasonable amount of time for a

child to lie motionless. The low radiation exposure from the Hologic QDR-4500W model (1.5 R/2.6 μSv per whole body scan) would allow 385 scans to be performed on an individual per year, and be within the legal limit of radiation exposure. The coefficients of variances of this machine are 1.2% and 0.2% for whole body fat (g) and lean body mass (g) respectively (Weiler, 2000). Studies analyzing the accuracy of DEXA with analysis in pigs of lean and fat mass, have found an excellent correlation ($r = 0.96$) in lean tissue, and $r = 0.83$ in fat tissue (Svendsen et al, 1993), suggesting DEXA is a reliable tool. Venkataraman et al (1992) also found that DEXA provides reproducible measurements of body composition including total BMC, fat and lean body mass in newborn infants.

3.8 THE PIGLET AS A MODEL FOR HUMAN NUTRITION

Animal studies are critical in research, as they allow us to advance in the study of areas such as metabolism, while allowing the researcher to manipulate the diet of the animal. Various fatty acids can be added to formula, for example, and the effects of the various intakes can be analyzed by assessing membrane composition, plasma, tissue, bone structure and others. Advantages of animal over human studies include the ability to strictly control dietary intake by including only those nutrients required for study. Timing as well as duration of the specific dietary regime can be controlled. We can control or eliminate various confounding factors by controlling the animal's environment. Finally, we are able to extract tissue for observation at study end (Innis, 2000). When relating animal study outcomes to human infants, it is necessary to choose an appropriate animal species.

The piglet model has proven to be valuable for human investigation for a number of reasons. Some advantages include their rapid growth rate, doubling or tripling their size in approximately 2 weeks (Weiler et al, 1995), large litters, similar digestive system to the human infant including fat digestion and absorption due to their similar gut enzymes and pH. The large size of the animal allows for easy handling and provides ample tissue for investigation. The piglet is also responsive to manipulations in dietary fatty acids (Innis, 1993).

For the above reasons, the piglet model was used for study purposes in this thesis.

4.0 MATERIALS AND METHODS

4.1 EXPERIMENTAL DESIGN

The experimental design used was a 15-day researcher blind randomized block design. Forty-five 3-day-old male piglets were obtained from Glenlea Research Station, University of Manitoba. All piglets were randomized within litters into one of five groups, by drawing a pre-coded number out of an envelope. Nine of the piglets remained with the sow and were sow-fed for the remainder of the study. Thirty-six piglets were removed from the sow and transported to the University of Manitoba Animal Care Facility. Piglets in treatment groups were randomized to receive standard formula with the addition of a varied ratio of AA to DHA for 15 days. The formula provided optimal nutrition for healthy growing piglets as set out by the National Research Council, 1998. Group 1 received AA:DHA at a 3.0:1.0 ratio (AA:DHA 0.3:0.1% wt/wt of total dietary fat); Group 2 received AA:DHA at a 4.5:1.0 ratio (AA:DHA 0.45:0.1% wt/wt); Group 3 received AA:DHA at a 6.0:1.0 ratio (AA:DHA 0.6:0.1% wt/wt); Group 4 received AA:DHA at a 7.5:1.0 ratio (AA:DHA 0.75:0.1% wt/wt: oils added to standard formula to achieve above ratios are presented in Table 4-1; actual measured fatty acid composition of supplemented formula are provided in Table 4-2). Ratios of AA to DHA referred to in dietary treatment groups in this thesis are the target ratios (actual measured ratios are provided in Table 4-2). Group 5 was sow-fed. Standard formula contained a total n-6 to n-3 fatty acid ratio of 8.4:1.0. The formula used in the study was provided in kind, from Ross-Abbott Laboratories, Columbus Ohio (Product Number P96-AV2). The composition of the base formula is presented in Table 4-3. The AA was provided in the form of RBD-ARASCO: 40.6% AA, and DHA as RBD-DHASCO: 40.0% DHA.

Martek's Oils are blended triglyceride vegetable oils, highly enriched in DHA and AA. The DHA is derived from a marine microalgae (DHASCO®), and the AA is derived from a common soil organism (ARASCO®). Both were provided, in kind, by Martek Biosciences Corp., Columbia, ML. The AA and DHA concentration measured in sows' milk from the sows in this research, approximates AA = 0.8% and DHA = 0.1% fat, wt/wt. The oil blend that was used to make the standard formula, contained soybean, coconut and high oleic safflower oils, and will be referred to as Ross oil. Ross oil was added to AA and DHA oil mixtures to make up the balance of oil required to create 500 ml of oil in total, and in the correct ratios and amounts.

Each treatment oil was blended and frozen (-20°C) in individual 100 ml containers until used. Formulas were blended with appropriate thawed oils at each feed. Once oils were thawed, they were kept refrigerated at 4°C.

Formula-fed piglets were housed individually in stainless steel cages under heat lamps to maintain an ambient temperature of 28-30°C.

On the first day of arrival, the piglets were taught to lap, and were fed standard formula at half strength. By the end of day two, formula was increased to three quarters then full strength. Beginning on treatment day #1 (or day 5 of life), the piglets in each of the four formula treatment groups, and the group on the farm were weighed by digital scale (Mettler-Toledo Inc., Highstown, NJ) with an animal weighing program (average of 3 weights) before each morning feeding, which represents a sow-fed state (sow-fed piglets were weighed at 9 am, which does not represent a non-fed state). Based on their fasting weight, the formula-fed piglets received formula at 350 ml/kg/day that was divided into three equal amounts, plus oil at 0.55 ml/kg/feed, and fed to piglets at 0900,

1500 and 2100 hours. Piglets were allowed approximately 1 hour of exercise in a pen outside of their cages before each feed.

Piglets in Group 5 (sow-fed) were maintained according to standard housing at the research station. Conditions included: 16 hours of light with 8 hours of darkness; piglets were penned with the sow allowing some room for physical activity, but not an overabundance; piglets were allowed to cross-foster to sows in this study, but only to a maximum of 10 piglets per sow.

TABLE 4-1. ***Amounts of ARASCO and DHASCO and Ross Oils added to each of the 4 diet treatments to make the varying ratios of AA:DHA. Total of 500 grams of oil.***

	Diet 1 (3.0:1.0)	**Diet 2 (4.5:1.0)**	**Diet 3 (6.0:1.0)**	**Diet 4 (7.5:1.0)**
AA ARASCO (grams)	51.1	76.7	102.2	127.8
DHA DASCO (grams)	17.3	17.3	17.3	17.3
Ross Oil (grams)	431.6	406.0	380.1	354.9

Sample calculation:
Diet 2: 0.45% AA; 0.1% DHA = 4.5:1.0 ratio
AARASCO = 0.22998 g ÷ 1.5 x 500 = 76.7 g
DASCO = 0.0519 g ÷ 1.5 x 500 = 17.3 g
∴ Ross Oil = $\frac{1.218\ g \div 1.5 \times 500}{1.5\ \text{total oil}} = \frac{406.0\ g}{500\ g}$

*Ross oil was the oil used to make the standard formula.

TABLE 4-2. ***Average fatty acid composition of piglet formula, diet treatments and sows' milk.***[1]

Fatty Acid (% wt/wt of total lipid)	Standard Formula[4]	Diet 1[5] (AA:DHA 3.0:1.0)	Diet 2 (AA:DHA 4.5:1.0)	Diet 3 (AA:DHA 6.0:1.0)	Diet 4 (AA:DHA 7.5:1.0)	Sows' Milk Average
Nonessential Fatty Acids						
8:0	1.94	1.98 ± 0.27	2.16 ± 0.05	1.97 ± 0.05	2.01 ± 0.10	0.04 ± 0.01
10:0	1.41	1.44 ± 0.19	1.58 ± 0.03	1.43 ± 0.02	1.45 ± 0.05	0.21 ± 0.06
12:0	0.47	10.65 ± 1.51	11.68 ± 0.27	10.41 ± 0.21	10.59 ± 0.26	0.29 ± 0.08
14:0	4.15	4.22 ± 0.55	4.66 ± 0.08	4.11 ± 0.10	4.15 ± 0.06	3.32 ± 0.58
16:0	6.21	6.36 ± 0.04	7.10 ± 0.12	6.33 ± 0.16	6.36 ± 0.08	26.01 ± 2.01
18:0	3.11	3.23 ± 0.32	3.57 ± 0.04	3.23 ± 0.06	3.25 ± 0.03	4.31 ± 0.87
20:0	0.26	0.27 ± 0.03	0.30 ± 0.00	0.27 ± 0.01	0.27 ± 0.00	0.11 ± 0.02
22:0	0.23	0.24 ± 0.03	0.27 ± 0.01	0.25 ± 0.00	0.25 ± 0.00	0.06 ± 0.01
24:0	0.11	0.13 ± 0.01	0.15 ± 0.01	0.14 ± 0.01	0.14 ± 0.00	0.08 ± 0.02
16:1 n-9	0.09	0.08 ± 0.01	0.09 ± 0.00	0.08 ± 0.00	0.08 ± 0.00	0.61 ± 0.18
16:1 n-7	ND[2]	ND	ND	ND	ND	8.31 ± 1.49
18:1 n-9	29.75	30.97 ± 3.24	30.30 ± 0.74	30.48 ± 0.84	30.34 ± 0.45	31.62 ± 2.09
18:1 n-7	0.47	0.38 ± 0.05	0.44 ± 0.05	0.40 ± 0.01	0.44 ± 0.01	2.32 ± 0.52
20:1 n-9	0.15	0.16 ± 0.02	0.17 ± 0.00	0.16 ± 0.00	0.16 ± 0.00	0.39 ± 0.05
22:1 n	ND	ND	ND	ND	ND	0.08 ± 0.01
24:1 n-9	0.07	0.07 ± 0.01	0.08 ± 0.00	0.07 ± 0.00	0.07 ± 0.00	0.10 ± 0.02

Table continued on next page

n-6 Polyunsaturated Fatty Acids						
18:2n-6	16.77	17.27 ± 1.78	19.13 ± 0.28	17.06 ± 0.39	17.04 ± 0.19	12.51 ± 1.06
18:3n-6	0.10	0.11 ± 0.01	0.12 ± 0.00	0.10 ± 0.00	0.10 ± 0.00	0.14 ± 0.08
20:2n-6	ND	ND	ND	ND	ND	0.35 ± 0.07
20:3n-6	ND	0.04*[3]	0.05 ± 0.00	0.05 ± 0.00	0.07 ± 0.00	0.12 ± 0.02
20:4n-6 **AA**	ND	0.25 ± 0.03	0.47 ± 0.01	0.52 ± 0.01	0.65 ± 0.02	0.54 ± 0.15
22:4n-6	ND	ND	ND	ND	ND	0.09 ± 0.03
Total n-6	16.87	17.67	19.77	17.73	17.86	13.75
n-3 Polyunsaturated Fatty Acids						
18:3n-3	1.95	1.99 ± 0.16	2.16 ± 0.02	1.97 ± 0.03	1.97 ± 0.02	1.66 ± 0.57
22:5n-3	0.05	0.06 ± 0.01	0.05 ± 0.01	0.06 ± 0.02	0.05 ± 0.00	0.26 ± 0.10
22:6n-3 **DHA**	ND	0.08 ± 0.01	0.10 ± 0.01	0.09 ± 0.01	0.08 ± 0.01	0.07 ± 0.03
Total n-3	2.00	2.13	2.31	2.12	2.10	1.99
Total n-6/n-3	8.44	8.30	8.56	8.36	8.50	6.91
Actual Ratio AA:DHA	ND	3.1	4.7	5.8	8.1	7.7

[1]Values are mean ± SD where > one sample was analyzed.

[2]ND = not detectable

[3]*Detected in only 1 sample

[4]Standard formula = 1 analysis

[5]Diet 1 = 5 replications (pooled); Diet 2 = 5 replications (pooled); Diet 3 = 6 replications (pooled); Diet 4 = 6 replications (pooled); Sows' Milk Samples n = 6 (pooled)

TABLE 4-3. ***Content of micro and macro nutrient components of piglet base formula.***

Nutrient	Analytical Value (per liter)
Protein, g	60.5
Fat, g	57.2
Carbohydrate, g	58.5 – 65.5
Energy, Kcal	961 – 1050
Ash, g	13.20
Linoleic Acid, g	13.10 – 22.20
Calcium, mg	2395
Phosphorus, mg	1791
Magnesium, mg	195.8
Sodium, mg	830
Potassium, mg	2603
Chloride, mg	1322
Iron, mg	20.0 – 30.0
Zinc, mg	20.00 – 30.00
Copper, mg	1.0 – 2.0
Manganese, μg	600 – 1000
Iodine, mg	0.090 – 0.750
Selenium, μg	21.000 – 47.000
Vitamin A, IU	886
Vitamin D, IU	100 – 250
Vitamin K, μg	40.0 – 80.0

Vitamin C, mg	118
Thiamine (B1), mg	1.83
Riboflavin (B2), mg	0.900 – 3.000
Pyridoxine (B6) mg	0.670
Vitamin B12, μg	6.00 – 10.00
Pantothenic Acid, mg	4.0 – 10.0
Folic Acid, μg	237
Niacin, mg	6.00 – 20.00
Biotin, μg	10.0 – 90.0
Choline, mg	270 – 330
m-Inositol, mg	47.0 – 66.0
Taurine, mg	66 – 104

[1]

[1] Values according to the manufacturer measured per litre.

4.2 EXPERIMENTAL PROTOCOL

The experimental procedures were approved by the "Fort Garry Campus Protocol Management and Review Committee" University of Manitoba, and were in agreement with the Guide for the Care and Use of Experimental Animals (Canadian Council on Animal Care, 1993).

4.3 ASSESSMENT OF GROWTH AND BODY COMPOSITION

Formula-fed piglets were weighed prior to each morning feed (and suckled pigs weighed at 9 am). This determined the amount of formula plus oils given per day. Growth rate was determined by calculating an average daily weight gain ($g \cdot kg^{-1} \cdot d^{-1}$). (Formula = [change of weight in grams/(weight on day 1 + day 15/2)] ÷ 15 = $g \cdot kg^{-1} \cdot d^{-1}$). Length was measured from tip of snout to base of tail using a plastic tape after day 15, piglets were in an anesthetized state.

Whole body composition was measured by dual energy X-ray absorptiometry (DEXA, QDR4500W; Hologic Inc., Waltham, MA). Frozen piglet carcasses were transported to the dual energy x-ray absorptiometer. Scans were performed with the piglet in the anterior-posterior position with limbs extended. Scans were performed to determine bone mineral content (BMC), density (BMD) and bone area, using software version V8.16a:5. Lumbar 1-4 were assessed using the low density spine program, and the right femur using the subregion array hip program (V8.20a:5). Femurs were scanned in vivo. Then a femur scan in water was conducted using a plastic container filled to a level that covered the bone sample. Weight (grams) and length (to nearest 0.1 cm) of femurs were measured after excision of soft tissue.

4.4 COLLECTION OF BLOOD AND URINE

Blood was collected using internal jugular blind stab technique on mornings of day 1 of treatment, from animals at 9 am. Animals were placed in a V-shaped holder and were non-anesthetized. Five mL (<10% blood volume assuming 60-65 ml blood/kg) blood samples were collected in heparanized syringes from each piglet. Samples were centrifuged (Beckman model TJ-6R, tabletop centrifuge, Palo-Alto, CA) as quickly as possible, to separate red blood cells (RBC) from plasma at 2000 x g at 4°C. RBC were then washed with an equal volume of saline solution (0.9% NaCl) and centrifugation process was repeated. Saline was removed and RBC stored in fresh saline solution of equal volume. Plasma and RBC were placed in glass vials and flushed with liquid nitrogen, then stored at -80°C until analyzed for fatty acid composition. Urine was collected from formula-fed piglets in the non-fed state on day 1 using plastic urine collection bags (U-Bag, Hollister Inc. company, state) taped to the underbelly, then stored at -20°C, and later analyzed for calcium and phosphorus content, creatinine and N-telopeptide of type 1 collagen.

4.5 COLLECTION OF BLOOD, URINE AND TISSUE SAMPLES AT TERMINATION

Sow-fed piglets were transferred from the Glenlea Research Station to the University of Manitoba on the evening of the 15th treatment day. All piglets were fed only water ad libitum after 1800 hours. In the morning of the 16th day, piglets were anaesthetized one by one, by intra-peritoneal injection of sodium pentobarbital (Somnotol; 65mg/ml concentration), into lower abdomen (varying piglet order from

highest to lowest, then reversed with each litter). Once deep anesthesia was achieved, cardiac puncture was performed drawing approximately 50 ml of blood into heparinized syringes. Following removal of blood, animals were terminated with euthanol, sodium pentobarb (200 mg/ml), using the inserted syringe in left ventricle. The animal's length was measured using a plastic measuring tape from tip of snout to the base of the tail (to nearest 0.1 cm). Heart, liver, kidneys, spleen, adipose tissue and tibia (~ 1.0 g) samples were excised, rinsed with 0.9% NaCl and weighed to the nearest 0.1 g. Urine was collected in a syringe via bladder puncture and stored at -20°C. Tissues were flash frozen in liquid nitrogen and stored at -80°C. Tibia samples (~ 1.0 g) were removed and cleaned of soft tissue and marrow, followed by incubation 10 ml Hanks Balanced Salt Solution (1x; 90 ml distilled water + 10 ml Hanks Solution) and placed into a shaking water bath at 37-39°C for 2 hours. The bone segment was removed from Hanks solution and stored at -20°C for Ca/P analysis. Hanks solution was stored at -20°C for PGE_2 analysis.

4.6 FATTY ACID EXTRACTION AND METHYLATION

4.6a Plasma Lipid Extraction

Plasma samples were extracted using a modified Folch method (Folch et al, 1957). One hundred µl of C17 (1 mg/ml heptadecanoic acid in chloroform) were added to glass screw top tubes as a standard followed by 0.5 ml samples of plasma, then by 10 ml of 2:1 chloroform: methanol (containing 0.01% butylated hydroxytoluene:BHT – added as an antioxidant to limit lipid peroxidation) and then vortexed for 30 seconds. Two ml of methanol were added and tube vortexed for 15 additional seconds, then centrifuged for 10 to 15 minutes at 2000 x g (2000 rpm) in the Beckman CS-6 centrifuge

(Beckman Coulter Canada Inc., Mississauga, Ontario). The solvent layers were then transferred to clean glass tubes with the addition of 4 ml of chloroform and 3 ml of 0.73% sodium chloride solution. The samples were then capped, vortexed for 30 seconds, and centrifuged (as above) for 5 to 10 minutes. The top layers were removed and discarded. The tubes were then washed with 2 to 3 ml of TUP (total upper phase; chloroform:methanol:water 3:48:47), the top layers removed and discarded, then this step repeated. The chloroform layers were then flushed with nitrogen and frozen at -20°C until methylated. Duplicate samples were extracted.

4.6b Liver Lipid Extraction

Liver samples were extracted using a modified Folch method (Folch et al, 1957). Tissue samples (1.0 g) were weighed and transferred to 50 ml glass screw top tubes, followed by 10 ml of 2:1 chloroform: methanol (containing 0.01% BHT), flooded with nitrogen gas and stored in a freezer at -20°C for 24 hours. After this time period, samples were removed from freezer and homogenized using a Polytron (Brinkmann Instruments, Rexdale, Ontario). The samples were then transferred into clean 20 ml screw top tubes and centrifuged for 15 minutes at 1500 x g (1500 rpm) in the Beckman GS-6 centrifuge. The liquid was carefully transferred into a clean screw top tubes, leaving the pellets behind. An amount equal to the amount of sample of 0.73% sodium chloride solution was added, the tubes capped and vortexed for 30 seconds, then centrifuged (1500 rpm as above) for 10 to 15 minutes at approximately 1500 rpm (as above). The upper phases and inter phase layers were removed and discarded. Twice, the sides of the tubes were rinsed with 1-2 ml of TUP (chloroform:methanol:water 3:48:47), vortexed and

centrifuged (1500 rpm as above). The bottom chloroform layers were then transferred to clean 15 ml glass screw top tubes and evaporated under nitrogen at 30°C to dryness. Tube walls were washed down twice with 1 ml of chloroform, lipid then transferred to a 4 ml screw top vials with teflon lids and were ready for methylation. Samples were analyzed in duplicate.

4.6c Plasma and Liver Lipid Methylation

Frozen chloroform layers were transferred into clean screw top containers and evaporated to dryness under nitrogen in a 30°C water bath. One ml of toluene and 1.2 ml of methanolic HCL were added to dried tubes, capped and vortexed for 30 seconds. The tubes were placed in a rack and put into a preheated 80°C oven for 1 hour. The rack was removed then cooled for 15 minutes. One ml of deionized water was added to tubes, vortexed for 15 seconds, and centrifuged (2000 x g) for 5 minutes. The top layers were transferred to clean glass tubes to which 1 ml of petroleum ether was added. Tubes were then vortexed for 15 seconds, centrifuged (2000 rpm as above) for 5 minutes, then top layers transferred to previously transferred top layers. Deionized water was added at 2 ml, tubes vortexed 15 seconds, centrifuged (2000 rpm as above) for 5 minutes, then top layers transferred into conical gas-liquid chromatography (GC) vials and dried under nitrogen. Once dry, 100 µl of hexane was immediately added to the tubes, then capped and ready for separation and measurement using gas-liquid chromatography (GC).

4.6d Adipose Lipid Extraction

Adipose samples were extracted using a modified Folch method (Folch et al, 1957). Tissue samples (1.0 g) were weighed and transferred to 50 ml glass screw top tubes, followed by 10 ml of 2:1 chloroform: methanol (containing 0.01% BHT) and homogenized with the Polytron unit (Brinkmann Instruments Canada Ltd.; Rexdale, Ontario). Fats were then filtered through Whatman #4 filter paper (7 cm) into clean 20 ml screw top tubes. To the tubes, 2.3 ml of 0.73% sodium chloride solution were added, the tubes capped and vortexed for 30 seconds, then centrifuged for 10 to 15 minutes at 1500 x g (approximately 1500 rpm) in the Beckman CS-6 centrifuge. The top layers were removed and discarded. The sides of the tubes were rinsed with 1-2 ml of TUP (chloroform:methanol:water 3:48:47), the top layers removed and discarded, then this step repeated. The bottom chloroform layers were then transferred to clean 15 ml glass screw top tubes and evaporated under nitrogen at 30°C to dryness. Tube walls were washed down twice with 1 ml of chloroform, lipid then transferred to 4 ml screw top vials with teflon lid and were ready for methylation. Duplicate samples were extracted.

4.6e Adipose Lipid Methylation

Methylation began with 100 µl of extracted lipid transferred into 8 ml screw top glass tubes and evaporated to dryness under nitrogen in a 30°C water bath. One ml of toluene and 1.2 ml of methanolic HCL were added to the tubes, capped and vortexed for 30 seconds. The tubes were placed in a rack and put into an 80°C preheated oven for 1 hour. The rack was removed and tubes allowed to cool for 15 minutes. Then 1 ml of deionized water was added to the tubes, capped and vortexed for 15 seconds, then

centrifuged (1500 rpm as above) for 5 minutes. The top layers were transferred to clean 8 ml glass tubes, 1 ml of petroleum ether added to the bottom layers, capped and vortexed for 15 seconds, then centrifuged (1500 rpm as above) for 5 minutes. The top layers were then transferred to the previously removed top layers, to which 2 ml of deionized water was added, the tubes capped and vortexed for 15 seconds, then centrifuged (1500 rpm as above) for 5 minutes. Part of the petroleum ether layers were transferred into a conical GC vials, capped, then stored in a freezer (-20°C) or separated and measured using GC.

4.6f Oil Methylation

Oil samples (0.11 g) were measured and placed into 5 ml vials to which 1.0 ml hexane (concentration of 100 mg/ml) was added, then vortexed 5 to 10 seconds. Then 0.1 mL of diluted oil samples (10 mg) were pipetted into 8 ml screw top tubes, solvents evaporated under nitrogen in a warm water bath (35°C). One ml of toluene was added to the tubes and vortexed 5 to 10 seconds, 1.0 ml of methanolic HCl was added, tubes were capped with teflon lined lids and vortexed 5 to 10 seconds. Tubes were then placed in an 80°C oven for 1 hour, then removed from oven and allowed to cool to room temperature. Distilled water (1.0 ml) then hexane (1.0 ml) were added to the tubes, capped and vortexed for 20 seconds. Tubes were centrifuged in the CS-6 centrifuge at 2000 x g, as above, for approximately 4 minutes. The hexane (upper layers) were transferred to clean 8 ml glass tubes to which 1.0 ml of distilled water was added, tubes capped and vortexed for 20 seconds. Tubes were then centrifuged (2000 x g as above) for approximately 4 minutes, followed by the transferal of part of the hexane layers to GC vials, ready for separation and measurement in GC (2 μl injection).

4.6g Formula Lipid and Fatty Acid Extraction and Methylation

Each of the four dietary treatments were mixed in a blender, with appropriate amounts of formula and oil, in triplicate, as were fed at one feed to piglets. Portions (0.1 g) of the mixture were placed in 2-50 ml screw top tubes (in duplicate), and to each 10 ml of 2:1 chloroform:methanol and BHT were added, tubes were then capped and vortexed for 15 seconds. Three ml methanol was added, capped and vortexed 15 seconds, then centrifuged 10 to 15 minutes (1500 x g) in the GS-6 centrifuge. Solvent layers were transferred to clean 50 ml screw top tubes, to which 6 ml chloroform and 5 ml 0.73% NaCl were added, capped and vortexed 30 seconds. Tubes were then centrifuged 5 to 10 minutes (1500 x g). Top layers were then removed and discarded. Sides of tubes were rinsed with TUP (1 to 2 ml), then the top layer was removed and discarded (twice). Bottom layers were transferred to 15 ml screw top tubes and evaporated to dryness under nitrogen in 30°C water bath. Five ml chloroform was added, of which a 500 µl aliquot was removed and added to 8 ml screw top tubes. 0.1 ml C17 (10 mg/ml chloroform or heptane) was added to the tubes containing the samples. Samples were evaporated and methylated (as per section 4.6c), to dryness under nitrogen in a 30°C water bath. One ml of toluene and 1.2 ml methanolic HCl were added to tubes, capped and vortexed 30 seconds. Tubes were racked and placed into an 80°C preheated oven for one hour, then removed and allowed to cool for 10 to 15 minutes. Deionized water (1.0 ml) was added to tubes, followed by 1.0 ml hexane, capped and vortexed 20 seconds, then centrifuged (Beckman GS-6) for 4 minutes at 1500 x g). Top layers were transferred to clean 8 ml tubes, to which 1.0 ml distilled water was added, then capped, vortexed 20 seconds, and

centrifuged 4 minutes (as above). Top layers were then transferred to GC vials ready for separation and measurement on GC.

4.6h Sow Milk Lipid and Fatty Acid Extraction and Methylation

The procedure was followed as for formula (as per section 4.6g), using 10 mg/ml of C17 (100 µl). Duplicate samples were measured.

4.7 LIPID AND FATTY ACID ANALYSIS VIA GAS-LIQUID CHROMATOGRAPHY

GC analysis varied slightly depending on the sample being analyzed. Fatty acids (FA) of interest were 18:2 n-6, 18:3 n-3, 20:4 n-6, 20:5 n-3 and 22:6 n-3. Assessment of total lipids in plasma, adipose tissue and liver were performed using gas-liquid chromatography (Gas Chromatograph, Varian Star 3400 Varian Canada Inc., Mississauga Ontario) equipped with a 30 m x 0.25 mm capillary column (ID Model #DB-225, J & W Scientific high resolution gas chromatography column, Folsom, CA) with a film thickness of 0.25 microns. The carrier gas was helium. The sample volume injected was 1 µl (splitless injection). Temperature of the column was programmed from 180° C and held for 5 minutes, to 220°C at a rate of 3° per minute and held for 13 minutes. Quantitation of fatty acids for plasma and liver samples was based on peak areas comparison of peak area for a C:17 internal standard. The assay for adipose samples were to determine percentage of fatty acids only, therefore heptadecanoic acid was not added as an internal standard.

Data was expressed as % wt/wt of fatty acids for measurement of fatty acids greater than 8 carbons.

4.8 BIOCHEMICAL ASSESSMENT OF BONE METABOLISM

4.8a Urinary N-telopeptide of Type I Collagen

Collagen matrix breakdown was analyzed in urine samples, by measuring N-telopeptide of type I collagen (NTx) through a competitive enzyme-linked immunosorbent assay (ELISA). Briefly, 25 µl of urine sample, control or standard was added to each well of a microtiter plate precoated with human NTx, followed by 200 µl of diluted conjugate solution (enzyme horseradish peroxidase covalently linked to labeled monoclonal antibody that was directed against the cross-linked NTx in the urine samples). The plate was incubated at 25°C for 90 minutes. Microtiter plate was then washed 5 times with 350 µL of wash solution. After blotting dry, 200 µl of chromogen/buffered substrate (1:101 dilution) was added to wells. The plate was covered and incubated 15 minutes to allow color development (blue color appears in wells with bound Ab-horseradish peroxidase conjugate). Sulfuric acid (100 µl) was then added to each well to stop the reaction (wells with blue turned yellow). Following competition for binding sites, the optical density was measured at a wavelength of 450 nm, using Spectramax 340 Microplate Spectrophotometer (Molecular Devices Corporation, Sunnyvale, California). The procedure followed was as outlined by the manufacturer (Osteomark, INCSTAR Corporation, Stillwater, MN). Duplicate measurements were performed, and the results were expressed as nanomoles per litre of urine, then corrected for creatinine as micromolar per millimolar creatinine. The higher

the absorbance reading (optical density), the less NTx in the sample. This assay was used to illustrate the impact of AA on bone matrix formation. NTx can be found in urine during periods of type I collagen breakdown. High levels reflect periods of somatic growth or increased metabolic rate of bone turnover.

Level I Control: 281-377 nM BCE (Mean 329 nM BCE)
Measured: 329.255 nM BCE
Accuracy: 100%

Level 2 Control: 1159-1397 nM BCE (Mean 1278 nM BCE)
Measured: 1156.822 nM BCE
Accuracy: 91%

4.8b Osteocalcin Radioimmunoassay (RIA)

Plasma osteocalcin (OC) was measured to determine osteoblast activity. Osteocalcin is the most abundant noncollagenous protein found in bone and dentin, and is synthesized predominantly by mature osteoblasts and incorporated into bone matrix. Because OC is secreted by osteoblasts, it is commonly accepted as a marker for osteoblastic activity and as a marker of bone formation (Khosla et al: in Bone Primer 4th Ed). During periods of bone formation and turnover, OC is released into urine and nanomolar concentrations circulate in the blood (Lian et al, 1987). OC has therefore been found to be a direct reflection of bone turnover. During periods of new bone formation and high bone turnover, circulating OC levels are elevated (Lian et al, 1987).

This assay was conducted using plasma samples following the procedure in the instruction manual Osteocalcin I^{125} RIA Kit, INCSTAR Corp, Stillwater, MN. (Catalogue No./REF./KAT.-NR.: 15065 or 15130). This kit has been tested for use in swine, however it was done using purified porcine OC (Pointillart et al, 1997). This is an

immunochemical binder-ligand reaction assay that measures the quantity of indicator molecules (osteocalcin) labeled with a radioisotope (I^{125}) by counting radioactive decay in a scintillation counter (Packard Cobra, model #D5002 gamma counter). An OC standard was made containing 25 ng/ml of bovine OC, buffer, sodium azide (preservative) and distilled water. This was used to make a serial dilution ranging from 25 to 0.78 ng/ml, from which a standard curve was produced. Competition between the OC in the plasma sample (50 µl, 1:10 dilution) and I^{125} labeled bovine OC occurred for a binding site on the rabbit anti-bovine antibody. Phase separation was carried out by the addition of a precipitating complex, goat anti-rabbit serum, with carrier rabbit serum and polyethylene glycol. Following centrifugation (20 minutes, Beckman model TJ-6R, 1500 x g), samples were decanted of supernatant, and tubes containing precipitate were placed in Packard Cobra gamma counter (as above), and counts per million read. Samples were interpolated and fit on the standard curve. The lower the number of counts the higher the amount of OC in the sample. Samples were measured in duplicate.

Lot #: 100360A
Osteocalcin Control: 3.6-5.8 ng/ml
Measured: 4.185 ng/ml (as measured in our lab)
Accuracy: 89%

4.8c Urinary Calcium/Phosphorus Analysis

Urine samples were diluted in concentrated nitric acid prior to addition of distilled water and measurement. Urine samples (0.25 ml) were placed in labeled plastic vials, to which 0.5 ml nitric acid was added. Containers were sealed and placed under a fume hood for 24 to 48 hours. Distilled water (9.5 ml) was then added to each container (to arise as 0.5%), and sealed. To determine accuracy, milk samples were prepared as the

above samples, void of urine but instead with 0.25 mg of non-fat milk powder. Three control samples were done for reference. Control samples were stored under fume hood along with urine samples. Calcium in all samples were then measured by Inductively Coupled Plasma Optical Emission Spectroscopy (ICPOES: Varian Liberty 200, Varian Canada, Mississauga, Canada). Samples were measured in duplicate with random samples analyzed in triplicate to permit estimation of coefficient of variation. The average coefficient of variance (CV %) for urine (on day 16) was 20% and 11% for calcium and phosphorus, respectively.

4.8d Plasma Calcium/Phosphorus Analysis

Plasma samples were diluted in concentrated nitric acid prior to addition of water and measurement. Plasma samples (0.25 ml) were placed in labeled plastic vials. Procedure was then followed as for urine above. Samples were measured in duplicate with random samples measured in triplicate to permit estimation of coefficient of variation. The average CV % for samples measured in triplicate (on day 16) was 19% and 35% for calcium and phosphorus, respectively.

4.8e Tibia Calcium/Phosphorus Analysis

Tibia bone samples were diluted in concentrated nitric acid (0.5%) prior to addition of distilled water and measurement. Cortical bone samples (50 to 200 mg), free of periosteum and marrow were crushed with mortar and pestle, then placed into labeled glass containers to which 1.0 ml of nitric acid was added. Samples were placed under a fume hood and left for 2 days to digest the bone samples. Distilled water (19 ml) was

then added to each container, sealed and analyzed as above. Samples were measured in duplicate with random samples measured in triplicate (as above). The average CV % for samples measured in triplicate was 11% and 10% for calcium and phosphorus, respectively.

4.8f Prostaglandin E_2 Immunoassay

The purpose of this assay was to determine the amount of PGE_2 released from tibia bone samples into Hanks bone organ culture. This was accomplished as a result of competitive binding, as PGE_2 present in the samples (samples were diluted two-fold in Hanks' Balanced Salt Solution, 100 µl) competed with alkaline phosphatase-labeled PGE_2 (50 µl) for binding sites on a mouse monoclonal antibody (50 µl). Goat anti-mouse antibody was fixed onto the microplate, and during incubation, the mouse monoclonal antibody attaches to it. This was followed by a wash buffer solution (30X; 3 washes, 200 µl) containing a buffered surfactant designed to remove excess conjugate and any unbound sample. A substrate solution of buffered p-nitrophenyl (200 µl) was then added to the well of the microplate, followed by one hour of incubation, to determine the bound enzyme activity level. Stop solution of trisodium phosphate (50 µl) was then added. Following color development, the absorbance was read at 405nm (SPECTRA MAX 340, Molecular Devices, Sunnyvale, California). By observing the resulting color intensity, the concentration of PGE_2 in the samples could be determined via an inverse relationship. The procedure was followed as outlined in Prostaglandin E_2 Immunoassay Kit (R & D Systems, Minneapolis, MN). A standard curve was generated using 50,000 pg/ml PGE_2 standard stock and assay buffer to produce a series of dilutions.

5,000 pg/ml standard was the high standard, and the assay buffer was the zero standard (0pg/ml). A quadratic curve was constructed using Soft Max Pro software (Version 1.1, Molecular Devices Corporation, Sunnyvale, California). Samples were measured in duplicate.

4.8g Creatinine

All urine samples were corrected for creatinine to account for variation in urine concentration among piglets. Creatinine concentration in spot urine samples was determined colorimetrically according to Jaffe method (procedure number 555-A: Sigma Diagnostics, Inc., St. Louis, MO). Urine samples were diluted by a factor of 10, 15 or 20 for piglets in Diet Groups 1 – 4, and urine samples in the sow-fed group were diluted 50 times, all to achieve a CV % of 20 or less. Briefly, 20 µl of diluted urine sample or standard was added to wells of microplate, followed by 200 µl of alkaline picrate solution (creatinine color reagent + sodium hydroxide). The plate was then mixed and allowed to stand for 10 minutes at room temperature. Following incubation, the initial absorbance was read at 500 nm (Spectra max 340; Molecular Devices, Sunnyvale, CA). Then to each well, 7 µl of acid reagent (sulfuric and acetic acid mixture) was added, mixed and allowed to stand for 5 minutes at room temperature to destroy the color derived from creatinine. Final absorbance reading was then taken at 500 nm. Creatinine values were calculated as follows: [initial absorbance – final absorbance/initial absorbance of standard – final absorbance of standard] x 3 (as standard contained 3.0 mg/dl creatinine) x 88.4 (molecular weight of creatinine) ÷ 1000 = mmol/L. Creatinine concentration is

proportional to the difference in color intensity measured before and after addition of the acid reagent. The average CV % for all samples measured in triplicate was 4.9 %.

4.9 STATISTICAL ANALYSIS

Values are expressed as mean ± one standard deviation (SD) unless otherwise stated. Statistical significance was accepted for p values ≤ 0.05. Graph Pad Prism was used, and differences in outcome measurements were detected by one-way ANOVA and post hoc analyses using Student Neuman Keuls all pairwise comparisons test. Simple regression analysis using Pearson Product correlation coefficients were performed to detect relationships between dietary ratio of AA to DHA and effect on bone metabolism and mineralization. Sample size was originally 9 per group, however, one litter was eliminated making final sample size 8 per group.

The sample size, n = 8 per group, was calculated based on a change of 25 g $\pm$ SD = 13 in WB BMC as reported in the same species of animal, fed a formula with a similar ratio of AA:DHA (5.0:1.0: Weiler, 2000) as in this study. The power was set at 0.80 and alpha set at 0.05. In this study n = 9 per group to allow for morbidity.

5.0 RESULTS

Litter number 5 was removed from all results, as one piglet died on study day 1 (due to shock following a jugular stab to remove a blood sample) and two were sick, leaving only two healthy piglets in the litter. Therefore, n = 8 throughout the study (or less in the event of insufficient sample).

5.1 GROWTH

Growth of piglets was measured daily to determine if differences existed between all groups (treatment and sow-fed). The average piglet weights were equal between all groups at baseline, and after 15 days of treatment. No significant differences occurred between groups with respect to weight gain throughout the study. Piglets in formula-fed and sow-fed groups did not differ in whole body length after 15 days of treatment.

No differences were observed in formula intake between treatment groups (Sow-fed group intake was immeasurable). The means and standard deviations of initial weight, final weight, final length, average weight gain, and average formula intake are presented in Table 5-1. Over 15 days, piglets almost tripled in weight (2.1 – 6.0 kg). Organ tissue weights were not significantly different (Table 5-2).

TABLE 5-1. ***Initial and final weight of piglets, average weight gain over 15 days, length after 15 days and average formula intake over 15 days, of piglets fed formula supplemented with varying ratios of AA:DHA or sows' milk.***[1]

	Diet 1[2] (3.0:1.0)	Diet 2 (4.5:1.0)	Diet 3 (6.0:1.0)	Diet 4 (7.5:1.0)	Sow-fed
Initial Weight (kg)	2.1 ± 0.3	2.1 ± 0.4	2.1 ± 0.4	2.1 ± 0.2	2.4 ± 0.5
Final Weight (kg)	5.7 ± 0.8	5.7 ± 0.8	6.0 ± 0.7	5.9 ± 0.7	6.3 ± 1.2
Average Weight Gain (g/kg/d)	62.1 ± 4.9	62.9 ± 4.8	63.8 ± 9.1	64.1 ± 7.7	60.5 ± 6.7
Final Length (cm)	55.4 ± 2.7	54.3 ± 2.2	56.1 ± 2.3	54.9 ± 1.4	56.3 ± 2.6
Average Formula Intake (ml/kg)	357.3 ± 22.4	347.3 ± 23.2	350.1 ± 13.7	350.4 ± 13.2	N/A[3]

[1] Values are mean ± SD
[2] n = 8 per group

TABLE 5-2. ***Weights of heart, liver, right kidney, spleen and brain from piglets fed formula supplemented with varying ratios of AA:DHA or sows' milk, following 15 days of treatment.***[1]

	Diet 1 (3.0:1.0)	Diet 2 (4.5:1.0)	Diet 3 (6.0:1.0)	Diet 4 (7.5:1.0)	Sow-fed
Heart (g/kg)	6.2 ± 0.9[2]	5.9 ± 1.1	5.8 ± 0.7	5.9 ± 0.8	6.1 ± 1.0[→4]
Liver (g/kg)	30.9 ± 1.4	30.1 ± 2.2	28.9 ± 5.1	30.8 ± 4.6	26.8 ± 2.2[→]
Kidney (g/kg)	3.3 ± 0.4	3.4 ± 0.5	3.6 ± 0.4	3.6 ± 0.8	3.1 ± 0.3
Spleen (g/kg)	2.2 ± 0.6	2.3 ± 0.7	2.7 ± 0.9	2.5 ± 0.9	2.8 ± 1.2
Brain (g/kg)	6.4 ± 1.3[→]	7.0 ± 1.1[f3]	6.7 ± 1.2[→]	6.1 ± 1.1[→]	6.6 ± 1.7[→]

[1] Values are mean ± SD
[2] n = 8 per group unless otherwise indicated

[3] *f* = 6 per group
[4] →= 7 per group

5.2 FATTY ACID STATUS

5.2a Plasma Fatty Acids

Following 15 days of dietary treatment, plasma was analyzed for total content of fatty acid (FA) in mg per ml of plasma, and for the specific fatty acids linoleic (18:2 n-6), arachidonic (20:4 n-6), alpha-linolenic (18:3 n-3), eicosapentanoic (20:5 n-3) and docosahexaenoic (22:6 n-3). Total mg FA/ml plasma for each of the dietary treatment groups were significantly lower than for the sow-fed group ($p \leq 0.001$), but were not different from each other. Linoleic acid amounts in all four dietary treatment groups were significantly higher than in the sow-fed group at $p \leq 0.001$, but were not different from each other. Alpha-linolenic acid amounts were significantly higher in diet treatment Group 2 (4.5:1.0) and Group 3 (6.0:1.0) than the sow-fed group at $p \leq 0.05$. But there were no significant differences among groups for arachidonic, eicosapentanoic or docosahexaenoic acids. Data are presented in Table 5-3.

5.2b Adipose Fatty Acids

Adipose tissue was analyzed for fatty acid content. Fatty acids of interest were linoleic (18:2 n-6), arachidonic (20:4 n-6), alpha-linoleic (18:3 n-3) and docosahexaenoic acids (22:6 n-3). Following 15 days of dietary treatment, both linoleic and alpha-linolenic acids were found in significantly higher amounts in dietary treatment groups compared to sow-fed groups ($p \leq 0.001$) (Table 6-4 displays results). However, there were no differences found among groups for either AA or DHA (Table 5-4 displays results).

5.2c Liver Fatty Acids

Following 15 days of dietary treatment, liver tissues were analyzed for fatty acid content. Fatty acids analyzed included total mg FA/mg liver, linoleic (18:2 n-6), arachidonic (20:4 n-6), alpha-linoleic (18:3 n-3), eicosapentanoic (20:5 n-3), and docosahexaenoic (22:6 n-3) acids. Total mg/FA were significantly higher in the sow-fed group than in the dietary treatment groups ($p \leq 0.05$), however dietary treatment groups were not different from each other. Linoleic acid was significantly higher in dietary treatment groups 3 and 4 than the sow-fed group at $p \leq 0.05$, as well as in treatment groups 1 and 2, but at $p \leq 0.01$. Arachidonic acid was significantly higher in treatment groups 1 to 4 compared to the sow-fed group at $p \leq 0.001$. Eicosapentanoic acid levels were significantly higher in the sow-fed group than treatment group 1 ($p \leq 0.05$), treatment groups 2 and 3 ($p \leq 0.01$) and treatment group 4 ($p \leq 0.001$). There were no differences found among groups for either alpha-linolenic or docosahexaenoic acids. Results are shown in Table 5-5.

TABLE 5-3. ***Select fatty acids in plasma of piglets fed formula supplemented with varying ratios of AA:DHA or sows' milk following 15 days of treatment.***[1]

Fatty Acid	Diet 1[2] (3.0:1.0)	Diet 2 (4.5:1.0)	Diet 3 (6.0:1.0)	Diet 4 (7.5:1.0)	Sow-fed
Total mg FA/ml[3] (%wt/wt)	1.54 ± 0.40[a*4]	1.48 ± 0.34[a*]	1.51 ± 0.28[a*]	1.40 ± 0.21[a*]	2.35 ± 0.66[b]
18:2 n-6 (%wt/wt)	28.22 ± 2.52[a*]	28.10 ± 1.88[a*]	28.00 ± 2.06[a*]	27.18 ± 2.22[a*]	21.39 ± 1.76[b]
20:4n-6 (%wt/wt)	9.06 ± 0.69	9.38 ± 1.33	9.10 ± 1.61	10.79 ± 1.40	9.87 ± 1.13
18:3 n-3 (%wt/wt)	0.94 ± 0.15[ab]	1.05 ± 0.20[a]	1.02 ± 0.21[a]	0.83 ± 0.21[ab]	0.75 ± 0.05[b]
20:5 n-3 (%wt/wt)	0.27 ± 0.19	0.36 ± 0.12	0.36 ± 0.25	0.22 ± 0.05	0.17 ± 0.10
22:6 n-3 (%wt/wt)	2.48 ± 0.48	2.35 ± 0.55	2.25 ± 0.65	2.43 ± 0.45	2.48 ± 0.44

[1] Values are mean ± SD
[2] n = 8 per group
[3] In rows, values with different superscripts are statistically different at $p \leq 0.05$
[4] * Values are different at $p \leq 0.01$

TABLE 5-4. ***Select fatty acids in adipose of piglets fed formula supplemented with varying ratios of AA:DHA or sows' following 15 days of treatment.***[1]

Fatty Acid	Diet 1[2] (3.0:1.0)	Diet 2 (4.5:1.0)	Diet 3 (6.0:1.0)	Diet 4 (7.5:1.0)	Sow-fed
18:2 n-6[3] **(%wt/wt)**	20.72 ± 1.40[a*4]	20.71 ± 0.78[a*]	20.60 ± 0.72[a*]	19.19 ± 3.77[a*]	12.34 ± 3.51[b]
20:4 n-6 (%wt/wt)	0.29 ± 0.05	0.31 ± 0.04	0.35 ± 0.05	0.37 ± 0.02	0.37 ± 0.09
18:3 n-3 (%wt/wt)	1.83 ± 0.11[a*]	1.80 ± 0.10[a*]	1.84 ± 0.12[a*]	1.65 ± 0.24[a*]	1.21 ± 0.24[b]
22:6 n-3 (%wt/wt)	0.08 ± 0.03	0.06 ± 0.04	0.06 ± 0.04	0.05 ± 0.04	0.06 ± 0.05

[1] Values are mean ± SD
[2] n = 8 per group
[3] In rows, values with different superscripts are significantly different at $p \leq 0.05$
[4] *Values are different at $p \leq 0.01$.

TABLE 5-5. ***Select fatty acids in liver of piglets fed formula supplemented with varying ratios of AA:DHA or sows' milk following 15 days of treatment.***[1]

	Diet 1[2] (3.0:1.0)	Diet 2 (4.5:1.0)	Diet 3 (6.0:1.0)	Diet 4 (7.5:1.0)	Sow-fed
Total mg FA/ml (%wt/wt)	65.68 ± 11.51[a]	73.15 ± 9.79[a]	67.23 ± 16.79[a]	71.34 ± 13.48[a]	93.29 ± 24.17[b]
18:2 n-6 (%wt/wt)	16.04 ± 3.26[a*4]	17.16 ± 1.27[a*]	16.37 ± 1.48[a]	16.16 ± 1.79[a]	13.36 ± 0.60[b]
20:4 n-6 (%wt/wt)	17.24 ± 1.48[a*]	17.41 ± 1.44[a*]	17.14 ± 2.04[a*]	18.08 ± 2.12[a*]	12.89 ± 2.97[b]
18:3 n-3 (%wt/wt)	0.38 ± 0.17	0.46 ± 0.12	0.46 ± 0.18	0.48 ± 0.21	0.58 ± 0.29
20:5 n-3 (%wt/wt)	0.28 ± 0.07[a]	0.29 ± 0.06[a*]	0.26 ± 0.10[a*]	0.23 ± 0.09[a*]	0.39 ± 0.06[b]
22:6 n-3 (%wt/wt)	5.51 ± 0.85	5.74 ± 0.41	5.01 ± 1.72	5.58 ± 0.85	4.08 ± 1.72

[1] Values are mean ± SD
[2] n = 8 per group

5.3 BONE MINERAL STATUS

5.3a Bone Mineral Content

After 15 days of treatment, whole body BMC was significantly lower in the dietary treatment groups than in the sow-fed group ($p \leq 0.01$). Whole body BMC remained significantly different between fatty acid supplemented piglets and sow-fed piglets, but not among supplemented formula-fed groups themselves, when corrected for both weight and length. Excised femurs free of soft tissue were significantly different in BMC between fatty acid supplement formula-fed groups and sow-fed groups, but not among treatment groups alone. The same results were observed when excised femurs were corrected to weight and length of the femur bone. Results of BMC for all groups are presented in Table 5-6.

Simple regression analysis was conducted to determine if any relationship existed between excised femur BMC and dietary ratio of AA. No significant relationship was found (g, $r = 0.30$, $p = 0.40$).

Simple regression analysis was conducted to determine if any relationship existed between lumbar spine 1-4 and dietary ratio of AA. No significant relationship was found (g, $r = 0.30$, $p = 0.10$).

5.3b Bone Mineral Density

Following 15 days of treatment, whole body BMD was significantly different only between formula supplemented groups to sow-fed group. The same was observed for lumbar spine and femur BMD. However, femurs in vivo had higher BMD than ex

vivo femurs in all but the sow-fed group, but results were not different significantly (Table 5-7).

5.3c Whole Body Bone Area

Following 15 days of treatment, whole body bone area was significantly different between Diet 1 and Sow-fed group (113.1 ± 14.7 vs 143.0 ± 23.2 cm^2; $p \leq 0.01$); and Diet 2 and Sow-fed group (112.7 ± 11.2 vs 143.0 ± 23.2 cm^2; $p < 0.05$). Diet treatment groups 3 and 4 did not differ significantly from other groups. Results are shown in Table 6-8.

Simple regression analysis was conducted to determine if any relationships existed between treatment groups and dietary ratio of AA. A positive correlation was found with increasing amounts of dietary AA and whole body BMC (g, $r = 0.37$, $p = 0.03$), whole body BMC to length (g/cm, $r = 0.37$, $p = 0.03$) and whole body bone area (cm^2, $r = 0.03$, $p = 0.04$) (see Figure 5-1).

5.3d Femur Bone Area

Following 15 days of treatment, femur bone area both ex vivo and in vivo, were significantly lower in the dietary treatment groups compared to the sow-fed group, but were not different among treatment groups. Femur ex vivo values were lower in dietary treatment groups than in vivo values, but were not significantly lower. Results of femur bone area shown in Table 5-8.

5.3e Lumbar Bone Area

Following 15 days of treatment, lumbar spine (1-4) bone area did not differ significantly among groups. Results are shown in Table 5-8.

TABLE 5-6. ***Bone mineral content of whole body, femur (ex vivo), femur (in vivo) and lumbar spine (1-4) of piglets fed formula supplemented with varying ratios of AA:DHA or sows' milk following 15 days of treatment.***[1]

	Diet 1[2] (3.0:1.0)	Diet 2 (4.5:1.0)	Diet 3 (6.0:1.0)	Diet 4 (7.5:1.0)	Sow-fed
Whole Body BMC (g)[3]	52.4 ± 7.4[a]	52.9 ± 5.3[a]	61.7 ± 6.1[a]	57.8 ± 8.9[a]	77.5 ± 13.5[b]
Whole Body BMC/weight (g/kg)	9.3 ± 1.7[a]	9.5 ± 1.2[a]	10.4 ± 1.6[a]	9.8 ± 1.2[a]	12.5 ± 1.5[b]
Whole Body BMC/length (g/cm)	0.9 ± 0.1[a]	1.0 ± 0.1[a]	1.1 ± 0.1[a]	1.1 ± 0.2[a]	1.4 ± 0.2[b]
Femur BMC (g) (ex vivo)	1.2 ± 0.6[a]	2.1 ± 0.7[a]	2.2 ± 0.4[a]	1.3 ± 0.2[a]	3.7 ± 0.9[b]
Femur BMC (g) (in vivo)	1.7 ± 0.3[a]	1.8 ± 0.5[a]	1.9 ± 0.4[a]	1.9 ± 0.3[a]	3.1 ± 1.0[b]
Femur BMC/weight (g/kg)	0.06 ± 0.01[a]	0.07 ± 0.02[a]	0.07 ± 0.01[a]	0.07 ± 0.01[a]	0.10 ± 0.02[b]
Femur BMC/length (g/cm)	0.21 ± 0.04[a]	0.24 ± 0.06[a]	0.24 ± 0.04[a]	0.24 ± 0.04[a]	0.38 ± 0.11[b]
Lumbar Spine 1-4 BMC (g)	1.9 ± 0.5[a]	1.7 ± 0.6[a]	1.7 ± 0.3[a]	2.1 ± 0.5[a]	2.9 ± 0.6[b]

[1] Values are mean ± SD
[2] n = 8 per group
[3] In rows, values with different superscripts are statistically different at $p \leq 0.01$.

TABLE 5-7. ***Bone mineral density of whole body, femur (ex vivo), femur (in vivo) and lumbar spine (1-4) of piglets fed formula supplemented with varying ratios of AA and DHA or sows' milk following 15 days of treatment.***[1]

	Diet 1[2] **(3.0:1.0)**	**Diet 2 (4.5:1.0)**	**Diet 3 (6.0:1.0)**	**Diet 4 (7.5:1.0)**	**Sow-fed**
Whole Body BMD (g/cm^2)	0.46 ± 0.05	0.47 ± 0.03	0.47 ± 0.04	0.47 ± 0.04	0.55 ± 0.08
Femur BMD[4] **(ex vivo) (g/cm^2)**	0.25 ± 0.02^{a*f3}	0.26 ± 0.03^{a*5}	0.25 ± 0.02^{a*}	0.27 ± 0.01^{a*}	0.43 ± 0.07^{b}
Femur BMD (in vivo) (g/cm^2)	0.33 ± 0.04^{a*f}	0.36 ± 0.05^{a}	0.37 ± 0.06^{a}	0.34 ± 0.03^{a*}	0.43 ± 0.07^{b}
Lumbar Spine 1-4 BMD (g/cm^2)	0.21 ± 0.04	0.21 ± 0.04	0.20 ± 0.03	0.22 ± 0.04	0.26 ± 0.03

[1] Values are mean ± SD
[2] n = 8 per group
[3] *f* = 7 per group
[4] In rows, values with different superscripts are statistically different at $p \leq 0.05$ unless otherwise indicate

[5] *Statistically different at $p \leq 0.01$

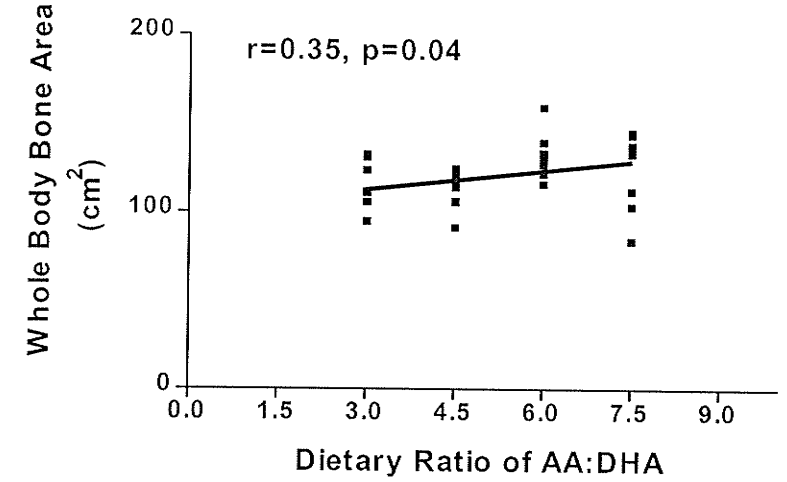
TABLE 5-8. ***Whole body bone area, femur bone area (ex vivo and in vivo) and lumbar (1-4) bone area of piglets fed formula supplemented with varying ratios of AA:DHA or sows' milk, following 15 days of treatment.***[1]

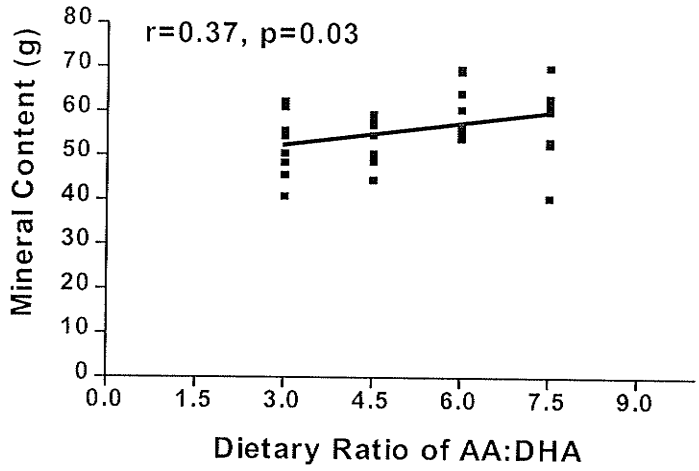
	Diet 1[2] **(3.0:1.0)**	**Diet 2 (4.5:1.0)**	**Diet 3 (6.0:1.0)**	**Diet 4 (7.5:1.0)**	**Sow-fed**
Whole Body Bone Area[4] **(cm^2)**	113.1 ± 14.7^{a*}	112.7 ± 11.2^{a}	132.0 ± 13.0^{ab}	124.4 ± 22.1^{ab}	143.0 ± 23.2^{b}
Femur Bone Area (ex vivo) (cm^2)	8.0 ± 1.2^{a*f3}	8.2 ± 1.6^{a*5}	8.6 ± 1.2^{a*}	9.1 ± 0.6^{a*}	11.0 ± 1.6^{b}
Femur Bone Area (in vivo) (cm^2)	5.2 ± 0.9^{af}	5.2 ± 0.9^{a}	5.2 ± 1.2^{a*}	5.6 ± 1.2^{a}	7.3 ± 1.9^{b}
Lumbar Spine Bone Area (1-4) (cm^2)	9.3 ± 2.4	8.1 ± 1.5	8.7 ± 2.0	9.3 ± 1.3	10.8 ± 1.8

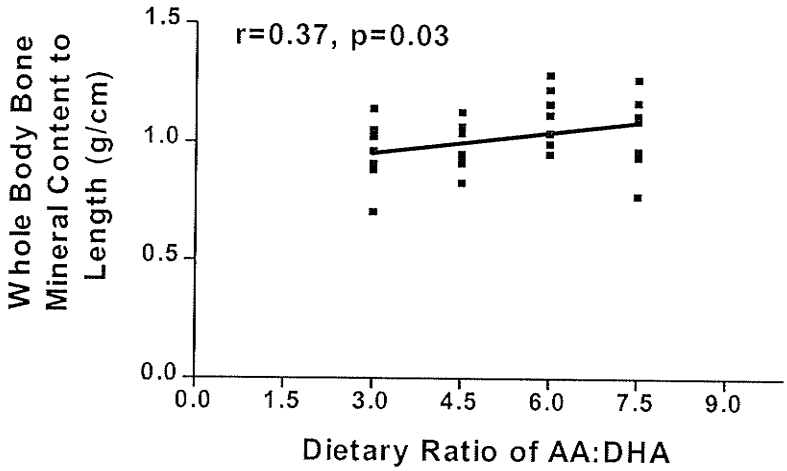
[1] Values are mean ± SD
[2] n = 8 per group unless otherwise indicated
[3] *f* = 7 per group
[4] In rows, values with different superscripts are statistically different at $p \leq 0.05$ unless otherwise indicated

[5] *Statistically different at $p \leq 0.01$

FIGURE 5-1. ***Relationships between diet and piglet whole body bone area, mineral content and mineral content corrected to length following 15 days.***





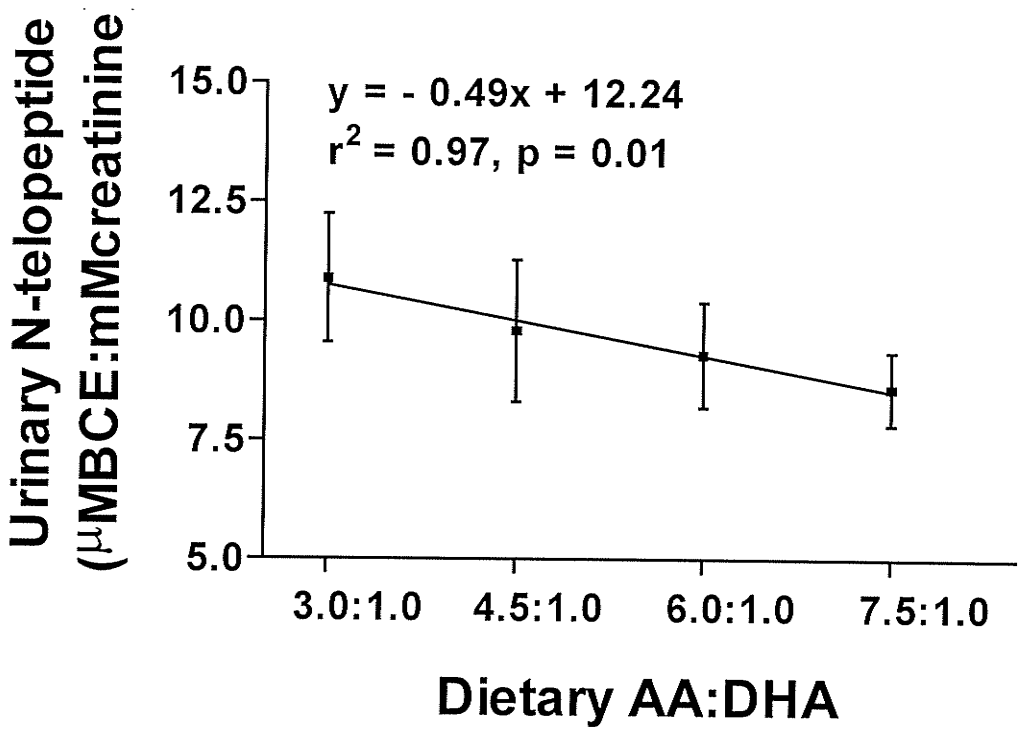


5.4 BONE METABOLISM

5.4a Urinary N-telopeptide of Type I Collagen (NTx)

Modeling and resorption of collagen matrix was determined by analyzing NTx in urine samples following 15 days of treatment. The results were then normalized for urine dilution by urinary creatinine analysis. Final results are reported as micromoles BCE/millimolar creatinine. No significant differences were found among groups. Results of Diet Treatment 1 through Sow-fed respectively were (12.7 ± 5.7, 10.3 ± 4.2, 10.7 ± 4.0, 9.9 ± 3.7, and 10.2 ± 6.0 μM/mM). Figure 5-2.

Figure 5-2. ***Simple regression analysis: correlation between Urinary N-telopeptide (μMBCE:mMcreatinine) and diet.***[1]



[1] n = 8 per group

5.4b Osteocalcin

Following 15 days of treatment, plasma osteocalcin was analyzed to determine osteoblast activity. No significant differences were found among groups. Results of Diet Treatment 1 through Sow-fed respectively were (15.9 ± 5.8, 15.3 ± 5.0, 16.2 ± 5.0, 13.7 ± 4.5 and 15.3 ± 3.0 nmol/L).

Simple regression analysis was conducted to determine if any relationship existed between osteoblast activity and dietary ratio of AA. No significant relationship was found (g, $r = 0.30$, $p = 0.10$).

5.4c Calcium Analysis

On treatment day 1 and 16, plasma and urine samples were analyzed for calcium content (tibia samples were also analyzed on day 16). Values did not differ significantly between groups on either day (values are presented in Table 5-9).

5.4d Phosphorus Analysis

On treatment day 1 and 16, plasma and urine samples were analyzed for phosphorus content (tibia bone samples were also analyzed on day 16). Values obtained on treatment day 1 did not differ significantly between groups. However, following 15 days of treatment, urinary phosphorus values were significantly lower in the dietary treatment groups than in the sow-fed group (Diet 1 to 4 respectively: 0.84 ± 1.27; 0.94 ± 1.36; 0.96 ± 1.17; 1.65 ± 2.02 vs Sow-fed 5.27 ± 2.68 mol:mol) at $p \leq 0.01$ (results presented in Table 5-10).

TABLE 5-9. ***Calcium found in plasma and urine on treatment day 1, and calcium found in plasma, urine and tibia in piglets fed formula supplemented with varying ratios of AA:DHA or sows' milk, following 15 days of treatment.***[1]

	Diet 1[2] (3.0:1.0)	Diet 2 (4.5:1.0)	Diet 3 (6.0:1.0)	Diet 4 (7.5:1.0)	Sow-fed
Day 1					
Plasma Calcium[2] (mmol/L)	1.2 ± 0.8	1.7 ± 0.4	1.3 ± 0.5	1.4 ± 0.2	1.7 ± 0.5
Urinary [3]Calcium/Creatinine (mol:mol)	0.26 ± 0.42	0.44 ± 0.57	0.83 ± 0.64	0.17 ± 0.08	0.15 ± 0.24
Day 16[4]					
Plasma Calcium (mmol/L)	1.7 ± 0.5	1.2 ± 0.4	1.5 ± 0.5	1.6 ± 0.8	1.5 ± 0.4
Urinary Calcium/Creatinine (mol:mol)	0.15 ± 0.18	0.05 ± 0.05	0.06 ± 0.06	0.05 ± 0.05	1.07 ± 1.08
Tibia Calcium (mg/g bone)	160.0 ± 53.7	183.5 ± 61.2	152.1 ± 32.2	174.2 ± 54.6	161.3 ± 36.8

[1] Values are mean ± SD
[2] Plasma calcium samples are as follows: Diet 1 n = 7; Diet 2 n = 6; Diet 3 n = 8; Diet 4 n = 6; Sow-fed n = 7
[3] Urinary calcium samples are as follows: Diet 1 n = 5; Diet 2 n = 6; Diet 3 n = 4; Diet 4 n = 7; Sow-fed n = 1
[4] n=8 per group

TABLE 5-10. ***Phosphorus in plasma and urine on treatment day 1, and phosphorus found in plasma, urine and tibia in piglets fed formula supplemented with varying ratios of AA:DHA or sows' milk, following 15 days of treatment.***[1]

	Diet 1 (3.0:1.0)	Diet 2 (4.5:1.0)	Diet 3 (6.0:1.0)	Diet 4 (7.5:1.0)	Sow-fed
Day 1					
Plasma Phosphorus[2] (mmol/L)	1.7 ± 0.3	1.8 ± 0.4	2.0 ± 0.7	2.0 ± 0.9	2.6 ± 0.9
Urinary Phosphorus/ Creatinine [3](mol:mol)	2.24 ± 3.30	2.13 ± 1.09	5.89 ± 5.08	2.93 ± 6.07	--
Day 16[4]					
Plasma Phosphorus (mmol/L)	2.1 ± 0.6	1.6 ± 0.6	1.9 ± 0.8	2.0 ± 0.9	2.3 ± 0.7
Urinary Phosphorus/ Creatinine (mol:mol)[5]	0.84 ± 1.27[a*6]	0.94 ± 1.34[a*]	0.96 ± 1.17[a*]	1.65 ± 2.02[a*]	5.27 ± 2.68[b]
Tibia Phosphorus (mg/g bone)	86.3 ± 29.6	94.9 ± 34.0	75.4 ± 22.9	91.5 ± 29.1	85.1 ± 21.2

[1] Values are mean ± SD
[2] Plasma phosphorus values are as follows: Diet 1 n=7; Diet 2 n=6; Diet 3 n=8; Diet 4 n=6 Sow-fed n=7
[3] Urinary phosphorus values are as follows: Diet 1 n=6; Diet 2 n=5; Diet 3 n=6; Diet 4 n=7
[4] n=8 per group
[5] In rows, values with different superscripts are significantly different, p≤0.05
[6] *values are different at p≤0.01

5.4e Prostaglandin E_2

Following 15 days of treatment, PGE_2 released from tibia was determined by analyzing the PGE_2 content found in the 10 ml of Hanks Balanced Salt Solution. Results are expressed as nanograms PGE_2 per gram tibia (ng PGE_2/g Tibia). No differences were observed among groups, (n = 8 per group), at $p \leq 0.05$ (5.2 ± 3.5, 10.2 ± 4.6, 6.0 ± 4.2, 5.6 ± 3.1, 5.9 ± 3.1 ng PGE_2/gTibia; Dietary Treatment Groups 1 to 4 and Sow-fed respectively).

6.0 DISCUSSION

The purpose of this thesis was to study the effects of supplementing piglet diets with increasing amounts of AA, while providing a consistent amount of DHA added into formula on bone metabolism and mineralization, as a possible therapy for use in term and premature infants. It was hypothesized that formula supplemented with AA in amounts most similar to those found in sows' milk, would support elevated growth, bone mass and metabolism in piglets compared to formula supplemented with amounts of AA not comparable to sows' milk. The primary objectives of this thesis were to determine: 1) if somatic growth, fatty acid stores and bone mass and metabolism varied as a function of the amounts of AA in formula; 2) if there were differences between the treatment groups; 3) if there were differences between formula-fed piglets in the AA supplemented groups and the sow-fed piglets, with regard to somatic growth, fatty acid storage and bone mass and metabolism; 4) the ratio of dietary AA:DHA in formula, that supports bone metabolism in piglets most similar to that resulting from feeding sows' milk. Thus in this thesis, the target for bone mass and metabolism is that observed in the suckling group of piglets.

Both term and preterm infants are capable of synthesizing LC PUFA from their shorter chain derivatives LA and ALA (Salem et al, 1996; Demmelmair et al, 1995; Szitanyi et al, 1999). It is quite well established that new born infants fed formula devoid of LC PUFAs, specifically AA and DHA, acquire lower concentrations of these fatty acids in plasma and RBC's (Carlson et al, 1991; Clandinin et al, 1992; Kohn et al, 1994; Desci et al, 1995; Koletzko, 1996; Boehm et al, 1996; Bondia-Martinez et al, 1998; Vanderhoof et al, 1999; Ryan et al, 1999; Lapillone et al, 2000), however few studies

have examined the effects of adding both these LC PUFAs into formula on bone metabolism and mineral mass during infancy.

Infant formula excluding AA, but containing only DHA, or high amounts of DHA could compromise endogenous synthesis of PGE_2 and potentially have a detrimental effect on bone mineralization (Watkins et al, 1996). Essential omega-3 (n-3) and omega-6 (n-6) fatty acids may compete for the same enzymes, Δ6 and Δ5 desaturase, required for conversion to long-chain AA and DHA. Even adding preformed LC PUFA can alter activities of these enzymes (Boyle et al, 1998; Ward et al, 1998; Dela Presa-Owens et al, 1998).

EPA may act as an antagonist to AA. An overabundance of dietary n-3 long chain PUFA (ALA, DHA and EPA) decreases the amount of AA in tissue phospholipids, while increasing the levels of n-3 PUFA (Ward et al, 1998; Dela Presa-Owens et al, 1998). This inhibits the production of AA-derived eicosanoids such as PGE_2 and leukotriene B4, and increases the production of EPA-derived eicosanoids such as PGE_3 and leukotriene B5 (Whelan, 1996). The synthesis of n-3 derived eicosanoids is not as effective as synthesis of n-6 and thus the total eicosanoid pool is reduced (Watkins et al, 2001). Therefore n-6 and n-3 fatty acids, particularly AA and DHA must be present in the body in balanced amounts (reviewed by Connor, 1996) to prevent competitive inhibition of n-6:n-3 fatty acid metabolism.

Premature infants are at high risk of developing osteopenia during periods of accelerated growth due to their complex skeletal mineralization and mineral homeostasis. The amount of calcium, phosphorus and magnesium provided in the diet plays a role in the infant's ability to absorb and retain these minerals (Lapillonne et al, 1994), but the

amount and type of dietary fat supplied also plays a role in bone formation (Watkins et al, 1996, Watkins et al, 2000).

To assess whether dietary fatty acids, specifically AA and DHA, have a significant effect on various outcome measurements in the piglet, an appropriate animal model used for infant nutrition analysis, a number of factors were analyzed. Measuring BMC, BMD and bone area in whole body, femur and lumbar spine assessed bone mineral status. Bone metabolism, specifically osteoblast and osteoclast activity was assessed by measuring NTx in urine, and osteocalcin in plasma. Calcium and phosphorus amounts were measured in urine, plasma and tibia to assess mineral status. The ex-vivo release of PGE_2 from cortical bone was measured, as it is linked to bone formation. Measuring weight gain, final weight and final length of the piglets assessed growth. Finally, fatty acids were measured in plasma, liver and adipose to assess whether dietary fatty acids would be reflected in these various tissues.

6.1 BONE MINERAL STATUS

Feeding formula to piglets supplemented with AA and DHA at varying ratios of 3.0:1.0, 4.5:1.0, 6.0:1.0, or 7.5:1.0 resulted in similar BMC, BMD and bone area of whole body, femur and lumbar spine 1-4. Compared to those fed formula at ratios of 3.0:1.0 and 4.5:1:0, piglets fed formula supplemented with AA:DHA at a level of 6.0:1.0 and 7.5:1.0 had values for whole body bone area (cm^2) most similar to values in the sow-fed piglets, however the SD was large making this result questionable.

Weiler and Fitzpatrick-Wong (2002) reported that piglets fed sows' milk for 15 days had the highest BMC of whole body and femur ($p \leq 0.01$) compared to those fed

unsupplemented formula. Piglets fed formula containing AA (0.5% wt/wt total fat) were intermediate for whole body BMC, but similar to suckled piglets for femur BMC, indicating that dietary AA is associated with elevated whole body and femur BMC. Weiler (2000) reported that 14 days of feeding piglets formula supplemented with 0.5% (wt/wt) of the fat as AA and 0.1% (wt/wt) DHA resulted in greater whole body BMC, and BMD of whole body, average lumbar spine 2-4, and femur compared to piglets fed unsupplemented formula.

Similar whole body bone area, but not BMC and BMD of femur, whole body and lumbar spine between piglets fed formula supplemented with 6.0 to 1.0 and 7.5 to 1.0, and those fed sows' milk is difficult to interpret as, to the author's knowledge, this is the first study conducted that examined the effect of various ratios of AA to DHA supplemented into milk formula versus sows' milk on whole body bone area.

Femur bone area, BMC and BMD were higher in sow-fed compared to formula-fed piglets. Diet may have played a role, but exercise may also be considered, as intensity of exercise may have varied between the sow-fed and treatment groups. Piglets housed at the University of Manitoba animal care facility were allowed approximately ½ to 1 hour of exercise per feed, in a pen located outside of their cages. Piglets kept at the Glenlea Research facility were not caged, but remained in a pen with the sow and other piglets. As the femur bone is weight bearing, the sow-fed piglets having been in a different environment may provide one explanation for the elevated femur mineral content, density and bone area seen in this group. However, excised femurs removed of soft tissue were similar between sow and formula-fed piglets in weight and length suggesting no difference in bone growth, in spite of the apparent difference in

mineralization. The femur was measured in the excised state to attempt to eliminate positional error.

The higher BA in the sow-fed piglets as a possible result of exercise is supported by a study in Moyer-Mileur et al (2000). A regime of daily physical activity, consisting of 5 to 10 minute range of motion against passive resistance to all extremities, was performed with 32 preterm infants to evaluate changes in bone mineralization. Despite similar nutrient intake, the daily activity resulted in greater BMC and BA in the active group of infants compared to controls. However, Watkins et al (1997) reported lower values for total and cortical BA in chicks fed a SBO diet (high in n-6 FA), whereas the sow milk in this study contained the lowest amount of n-6 FA compared to the other groups, and piglets in the sow-fed group had higher values for BA.

Recently Weiler et al (in press) reported that the hormone leptin was related to bone area, and piglets that were suckled rather than formula-fed had higher bone area. The suckled piglets in this current study may have had higher leptin levels than the formula-fed piglets that may have affected their bone area, and possibly bone mineral content.

In this research, BMC in lumbar spine was significantly higher in the sow-fed piglets ($p \leq 0.01$), but not different among formula-fed piglets, which would likely not be exercise, but rather diet related, as the lumbar spine is not weight bearing.

In contrast to the elevated BMC in lumbar spine, Weiler and Fitzpatrick-Wong (2002) reported that piglets fed sows' milk for 15 days had elevated BMC of whole body and femur, but not lumbar spine compared to those fed standard formula. Femurs of suckling piglets were also shorter and weighed less compared to those fed standard

formula, but BMC was higher suggesting that both linear and appositional growth was slower in the suckling piglets.

The sow-fed group had higher BMC in all sites measured, and the sow-fed group was similar for BMD in whole body and lumbar spine compared to supplemented formula-fed piglets. It would be expected that because AA is a precursor to PGE_2 which is involved in bone formation, that the piglets in the treatment group containing amounts of AA most similar to the sow-fed group (which was the ratio of AA:DHA of 6.0:1.0) would have similar BMC, which was not seen at a level of significance in this research. However, there was a positive effect of diet shown. As dietary amounts of AA increased, there was a positive relationship to whole body BMC, whole body BMC to length and whole body bone area, ($p \leq 0.05$; shown in Figure 6-1). However, using non-linear regression analysis rather than linear, a curve would likely occur beyond a level of AA supplementation of 0.6% (wt/wt).

Jones et al (2000) compared the effect of either breast-feeding or formula-feeding on bone density in the term and preterm infant both at birth (1988) then again after 8 years (1996). Bone mineral density was measured using DEXA, and it was shown that in term infants who were breast-fed, BMD was higher in the femoral neck (+0.20 SD, $p = 0.07$), lumbar spine (+0.25 SD, $p = 0.03$) and total body (+0.29 SD, $p = 0.007$) than those bottle-fed. These results were seen in breast-fed infants who were born at term, but not preterm, indicating a possible benefit in term infants of breast-feeding in infancy on elevated bone volume pre-puberty. In this research, BMD was similar among piglets fed LC PUFA formula or sows' milk, and as human infants do not exercise substantially

early in life, this research suggests a possible benefit to the infant who is fed supplemented formula or breast milk as opposed to a standard formula.

6.2 BONE METABOLISM

6.2a Urinary NTx

Feeding piglets formula containing varying ratios of AA to DHA from 3.0 to 7.5 to 1, or sow milk did not produce significantly different levels of urinary NTx indicating no difference between groups with respect to bone resorption. In contrast, Weiler and Fitzpatrick-Wong (2002) showed that NTx in piglets fed LC PUFA supplemented formula was higher compared to those fed sows' milk. Also, Lucia (2001) reported that supplementing formula with 0.8% AA and 0.1% DHA suppressed bone resorption as indicated by the lower concentration of NTx in urine of piglets fed the fatty acid supplemented diet.

Kruger and colleagues (1997) reported that after 6 weeks of feeding 11 week old female rats a diet supplemented with GLA:EPA (n-6:n-3) + DHA at 9:1 and 3:1 ratios, RBC GLA and DGLA increased. Red blood cell levels of EPA and DGLA increased in rats fed 1:3 and 1:9 diets, however AA decreased. A positive relationship was reported such that DGLA (n-6 fatty acid) was positively correlated with femur calcium content and negatively correlated with deoxypyridinoline, an indicator of bone collagen degradation indicating a possible anabolic effect on bone.

Although there were no differences in urinary NTx shown in this thesis research, an inverse relationship was seen between dietary LC PUFA and urinary NTx, such that as the ratio of AA:DHA increased, the concentration of urinary NTx decreased, indicating

increasingly suppressed bone resorption with increasing amounts of dietary AA. Therefore we can conclude that both AA and GLA:EPA reduce bone resorption.

6.2b Plasma Osteocalcin

Plasma osteocalcin, an indicator of osteoblast activity was measured in plasma and resulted in no differences among groups. Similarly, no differences were reported in plasma OC concentration between piglets fed 0.8% of total fat as AA (Lucia, 2001) or 0.5% of total fat as AA (Weiler, 2000) and 0.1% of total fat as DHA and those fed unsupplemented formula for 15 days, suggesting osteoblast activity was not affected by diet manipulation at those levels.

In contrast, Weiler and Fitzpatrick-Wong (2002) reported that plasma osteocalcin concentration was lower in piglets fed LC PUFA formula (0.5% AA and 0.1% DHA) compared to those fed unsupplemented formula, however levels were similar in sow and LC PUFA fed piglets. Bone formation rate (BFR) and bone alkaline phosphatase concentration (serum bone formation biomarker) have been reported for chicks (Watkins et al, 1997) and rats (Watkins et al, 2000) fed various ratios of n-6:n-3 (23.8, 9.8, 2.6 or 1.2) or high n-3 (high fish oil) FA supplemented formula. Formation rate and alkaline phosphatase were highest in rats fed a lower dietary ratio (n-6:n-3) or high n-3 FAs. Watkins et al (1997) used histomorphometry measurements to determine BFR in chicks fed diets containing moderate amounts of n-6 FA + saturated FA (butter + corn oil: BC), high amounts of n-6 FA (soybean oil: SBO) or high n-3 FA (menhaden + corn oil: MEC). Histomorphometric data for cortical bone modeling in tibia revealed that BFR was increased in chicks fed the BC diet at 21 days. Values were also higher in the BC group

for periosteal BFR (mm^2/d), total new BFR (mm^2) and intracortical porosity (mm^2) compared to chicks fed the SBO and MEC diets. Chicks fed the BC diet had lower AA concentration than chicks fed the SBO diet with a lower BFR. The higher AA concentration possibly lead to an excess production of PGE_2 in the SBO group, which lead to, depressed bone formation. Also in Watkins et al (2000) bone formation rate was negatively correlated with dietary AA:EPA ratio (n-6:n-3) and PGE_2 in bone, which indicated that bone concentrations of FAs are related to changes in ex vivo PGE_2 production, which in turn is associated with rate of bone formation. The results indicating depressed bone formation in the previous study also suggest that bone formation may also be influenced by eicosanoids other than PGE_2, perhaps PGE_3, an eicosanoid derived from EPA.

Based on the above evidence, lower levels of dietary AA, low ratios of n-6:n-3 fatty acids, or the addition of fish oil to formula, have a positive effect on BFR. This may occur via prostaglandin activity including PGE_2, and possibly PGE_3.

In the human infant, Fleischer and colleagues (1992) found that in healthy infants, those who were fed breast milk had significantly higher serum OC values than infants who were formula-fed.

6.2c Calcium

There were no differences in calcium concentration in plasma, tibia and urine of piglets fed formula supplemented with LC PUFA or sow-fed. These results were similar to those found in Lucia (2001) where the addition of 0.8 % AA + 0.1 % DHA into formula resulted in similar calcium levels in plasma, urine, and bone as well as calcium

absorption (Weiler, 2000) (which was not measured in this thesis research) compared to an unsupplemented formula-fed group of piglets.

In contrast, in the research conducted by Claassen et al (1995) showed that dietary supplementation of EFA in varying ratios had an influence on calcium absorption, calcium excretion, calcium balance and bone calcium content compared to controls. The researchers fed ratios (3:1, 1:1 and 1:3; GLA:EPA + DHA) of n-6:n-3 FA to 5 -12 week old rats for 6 weeks. This resulted in an increase in intestinal calcium absorption (mg/24hr) of 41.5% in the 3:1 group, a reduction of 41.5% in urinary calcium excretion in the 1:3 group, and higher in bone calcium by 24.7% in the 3:1 group, compared to controls (LA + ALA) indicating that GLA and EPA were more effective in modulating calcium metabolism than their precursors LA and ALA, which may have been due to their effect on prostaglandins. Typically, during periods of rapid skeletal growth, urinary calcium excretion is low (Anderson, 2000). The results in this research may indicate that the levels of FA in the diets were too similar to enable sufficient differences to be distinguished between calcium homeostasis in these animals.

6.2d Phosphorus

The sow-fed group had greater values of urinary phosphorus excretion after correction to creatinine, than piglets fed LC PUFA supplemented formula ($p \leq 0.01$). This high value for phosphorus from the sow-fed group may be a reflection of the phosphorus contained in the sows' milk, which was not measured in this research. Alternatively, the higher level of phosphorus may be explained by possible action of PTH in the sow-fed group. PTH may act by stimulating bone cells to resorb mineral and

release them into blood, stimulating intestinal and renal absorption of calcium thereby reducing urinary calcium excretion, which decreases renal absorption of phosphate, thereby increasing phosphate excretion (Fitzpatrick et al, 1999). Similarly, Weiler and Fitzpatrick-Wong (2002) showed a main effect of feeding on urinary phosphorus corrected to creatinine, where piglets fed sows' milk had significantly greater values (3.25 ± 1.63 mM/mM) than piglets fed supplemented formula (0.5% AA wt/wt total fat: 1.94 ± 1.63 mM/mM) or standard formula (1.10 ± 1.99 mM/mM) at $p = 0.005$.

6.2e PGE_2

The ex-vivo release of PGE_2 from cortical bone was similar among all groups of piglets in this study. Similarly in Weiler (2000), although feeding formula supplemented with 0.5% AA and 0.1% DHA resulted in higher AA in liver and cortical bone, urinary PGE_2 concentration was similar between the FA supplemented and unsupplemented groups of piglets. Perhaps the degree of AA supplementation was not sufficient to cause competition for the enzymes that would compete in the formation of AA or EPA, as suggested by the author.

In contrast, Whelan (1993) and Watkins (1996, 1997 and 2000) reported that feeding formula supplemented with n-6 PUFA resulted in elevated levels of PGE_2 found in various tissues in a variety of animals. Including AA in the diet of hamsters (Whelan, 1993) significantly enhanced PGE_2 production in macrophages. Feeding chicks (Watkins, 1996; Watkins, 1997) and rats (Watkins, 2000) diets rich in n-6 PUFAs compared to diets rich in n-3 PUFA resulted in higher ex-vivo PGE_2 biosynthesis in liver homogenates and bone organ culture, however bone formation rate was significantly

lower compared to chicks fed menhaden oil (high in 20:5 n-3 and 22:6 n-3) (Watkins, 1996). Values of ex-vivo release from tibia of PGE_2 were also similar among piglets fed standard formula or sow milk, but were elevated in piglets fed LC PUFA supplemented formula in Weiler and Fitzpatrick-Wong (2002). The above research indicates that dietary n-6 PUFAs including AA fed to animals increases PGE_2 production in various tissues.

In this study, perhaps amounts of dietary AA were not varied enough to cause PGE_2 synthesis to be hindered or enhanced among these groups of piglets.

6.3 Growth

Feeding formula to piglets containing 0.1% DHA + either 3.0%, 4.5%, 6.0% or 7.5% AA, or sow milk (ratio of AA:DHA of 7.7:1.0) to piglets for 15 days did not limit or elevate growth as indicated by lack of significant differences between groups for average weight gain, final weight or final length.

Inadequate intake of EFA (Burr and Burr, 1929) has been shown to have a negative influence on growth in humans, and blood concentrations of AA positively correlate with the birth weight of preterm infants (Koletzko et al, 1991). In animals, it has been demonstrated by Weiler (2000) that feeding formula supplemented with 0.5% AA and 0.1% DHA (% wt/wt total fat) to 10-day-old piglets for 14 days, resulted in greater whole body weight (but similar length) compared with piglets fed unsupplemented formula. A main effect of feeding sows' milk to piglets ($p \leq 0.002$) was reported by Weiler and Fitzpatrick-Wong (2002). The sow-fed piglets gained weight

significantly more slowly than piglets fed supplemented formula. Standard formula-fed piglets gained weight significantly more slowly than those fed LC PUFA formula.

Alternatively, also in our lab, Lucia (2001) found no positive effect on growth when feeding formula supplemented with 0.8% AA + 0.1% DHA for 14 days to piglets (as indicated by no differences in weight or length at end of study). Similarly, growth in rats (Watkins, 2000) was not affected by diets varying in ratio of n-6:n-3 fatty acids from 1.2 to 23.8.

Makrides et al (1999) found no differences in growth among infants (weight, length or head circumference) fed formula supplemented with 0.35% DHA, formula supplemented with 0.34% DHA + 0.34% AA (as total fatty acids), unsupplemented formula or breast milk. Also, in a large, multivariate study conducted by Auestad and colleagues (2001), where term infants (n = 239) were fed formula with AA (0.45% fatty acids) + DHA (0.13% FA) or without LC PUFA, with a group of breast-fed infants (n = 165) as a reference for one year, growth was similar within all groups, regardless of diet as indicated by outcome measurements of weight, length and head circumference. Similarly, Simmer (2000) reviewed a number of studies (studies conducted from 1995 to 1997) in which term infants were fed formula with or without LC PUFA for various periods of time to determine the effect of supplementing formula on growth (and other outcomes) and concluded that with current available data, there was no indication that supplementation of LC PUFA had a positive effect on the growth of term infants. A review of literature in the preterm infant was also conducted by Simmer (2000), in which the author concluded that the addition of n-6 and n-3 fatty acids into formula does not

impair the growth of preterm infants. However, no long-term benefits for preterm infants receiving LC PUFA supplemented formula were demonstrated.

Although there was not an unsupplemented formula-fed control group of piglets for comparison, this thesis research indicates that the addition of AA as low as 0.3% + 0.1% DHA (wt/wt) is a safe amount considering growth.

6.4 Fatty Acids in Plasma, Adipose and Liver

It has been generally found by various researchers that dietary lipids fed to animals (Yeh et al, 1998; Weiler, 2000) or infants (Boehm et al, 1996; Simmer, 1996; Clandinin et al, 1997; Xiang et al, 2000) are reflected in tissues and blood, and that as dietary n-6 FA levels are increased, n-3 FA levels in tissues and blood generally decrease. This relationship was not seen in this study.

The following is a summary of the values found for the two main FA of interest in this study, AA and DHA as reported in plasma, adipose and liver. There were no significant differences in values of DHA found in plasma, adipose or liver of piglets fed either supplemented formula or sows' milk. Values for AA were not significantly different in plasma or adipose tissues in piglets receiving the 4 diets or sows' milk. However, AA values were significantly lower in liver of the piglets fed sows' milk, compared to the piglets fed any of the 4 supplemented formulas.

The main differences in FA status following 15 days of treatment were found when the piglets in the sow-fed group were compared to the piglets in the formula-fed groups as a whole (Figure 6-1).

In this research, there were no differences in total FA/ml of plasma or total FA/mg in liver, between formula supplemented groups. However the piglets in the 4 formula supplemented groups had significantly lower amounts of total FA/ml than the sow-fed group ($p \leq 0.001$) in plasma, and significantly higher amounts than the sow-fed group in liver ($p \leq 0.05$), perhaps indicating the source of FA provided in diet results in different rates of transport and utilization of fatty acids. In another study conducted in our lab (Petryk and Weiler, 2002) 10-day-old piglets were either suckled or fed formula devoid of AA or DHA for 15 days. Piglets fed formula had lower AA and DHA ($p \leq 0.008$) in liver and plasma compared to the suckled piglets, which was a main effect of diet.

In plasma, feeding formula containing 0.45% and 0.60% AA and 0.1% DHA (wt/wt) to piglets, resulted in higher ALA (18:3 n-3) compared to plasma of piglets that were suckled, but there were no differences found in the long-chain derivative DHA (22:6 n-3). This is consistent with a study conducted by Weiler and Fitzpatrick-Wong (2002) that reported no differences among feeding groups (sow-fed, standard and LC PUFA formulas) in plasma DHA content.

The sow-fed piglets were also lower in amounts of plasma LA (18:2 n-6) but not AA (20:4 n-6), compared to the formula-fed piglets. Feeding formula supplemented with AA to male hamsters for three weeks (Whelan et al, 1993) or male piglets for two weeks (Weiler, 2000) resulted in higher levels of AA at the expense of LA in plasma and liver (Whelan et al, 1993) and liver and bone (Weiler, 2000). Weiler and Fitzpatrick-Wong (2002) reported that piglets fed formula had greater levels of LA and ALA and lower EPA than piglets fed sows' milk. LC PUFA formula-fed piglets had

greater ALA than standard formula-fed piglets ($p \leq 0.001$). Piglets fed sows' milk had greater levels of AA than those fed standard or LC PUFA supplemented formula, and piglets fed FA supplemented formula had greater AA than those fed the unsupplemented diet.

Preterm infants (< 2.3 kg birth weight) fed formula without AA and DHA had a lower level of AA in RBC phospholipids compared to infants fed formula supplemented with AA ranging from 0.32 to 1.1% AA and 0.24 to 0.75% DHA (Clandinin et al, 1999). The researchers found that feeding supplemented formula at a level of 0.49% AA and 0.35% DHA most closely matched blood levels of infants who were breast-fed.

Feeding formula to piglets with added AA from 0.3% to 0.75% (wt/wt) resulted in similar amounts of AA in liver. However, lower amounts of LA (18:2 n-6) and AA (20:4 n-6), but higher amounts of EPA (20:5 n-3) but not DHA were found in liver of the suckling piglets. The increase in liver EPA rather than DHA may suggest preferential formation of EPA from ALA, which is dissimilar to what often occurs as DHA synthesis is generally preferred due to brain requirements. Chicks fed diets high in soybean oil (rich in 18:2 n-6) had higher amounts of LA and AA in liver and bone, compared to chicks fed diets high in menhaden oil (high in 20:5 n-3 and 22:6 n-3) (Watkins et al, 1996).

Piglets fed formula containing only LA and ALA for 14 days, had lower AA and DHA in plasma and liver compared to piglets that were sow-fed (Petryk and Weiler, 2002), as a main effect of diet. This has also been seen in other piglet studies (Rioux et al, 1997) and human infant studies (Auestad et al, 1997) where AA and DHA found in tissues were reduced or significantly correlated with dietary AA and DHA.

In adipose tissue, storage of LA (18:2 n-6) and ALA (18:3 n-3) was found in significantly lower amounts in the sow-fed piglets than in piglets that were formula-fed, but levels were similar among formula-fed groups. This is a reflection of amounts of LA fed to piglets (17.27, 19.13, 17.06, 17.04, 12.51; % wt/wt total FA in formula Group 1 to sows' milk, respectively). The sow milk contained amounts of AA (0.54 ± 0.15 % FA wt/wt) most similar to the formula in Treatment Group 3 (0.52 ± 0.01 % FA wt/wt). Docosahexaenoic acid (22:6 n-3) values in adipose tissue were not significantly different among any groups, which would be expected considering amounts of DHA were similar in all 4 supplemented formulas and in sows' milk (0.08; 0.10; 0.09; 0.08; 0.07, % wt/wt: groups 1 to 4 and sows' milk respectively).

6.5 CONCLUSION

This research study was designed to assess the effects of feeding formula with various amounts of AA, but consistent levels of DHA to piglets. It was hypothesized that formula supplemented with AA in amounts most similar to those found in sows' milk, would support elevated growth, bone mass and metabolism compared to piglets fed amounts of AA not comparable to sows' milk. Based on the set level of significance, the hypothesis was rejected.

This study demonstrated that dietary enrichment of increasing amounts of AA but consistent DHA into formula, including an amount most similar to sows' milk (Group 3; formula containing AA at 0.60% wt/wt), did not elevate or compromise growth. Piglets fed formula supplemented with 0.60% and 0.75% AA (wt/wt) had whole body bone area most similar to the sow-fed piglets, but BMC was higher in the sow-fed piglets at all sites

measured. Although BMC was not significantly different among formula-fed groups, the value for BMC in the group fed at a 6.0:1.0 ratio, was 15% higher than the values for BMC found in the group fed at a 3.0:1.0 ratio (AA:DHA). Feeding at a higher ratio of 7.5:1.0 (AA:DHA) resulted in a value only 9.5% higher than the group fed at a 3.0:1.0 ratio. This difference may suggest that feeding AA at a level above 0.6% wt/wt provides no benefit with regard to bone content. The weak, but positive relationship reported between dietary AA and WB BMC demonstrated a possible positive effect of increasing amounts of AA up to 0.6%, on bone mineralization.

BMC values were significantly higher in the sow-fed piglets at all sites measured. Considering diet as a possible explanation for the elevated BMC, unlike the DHA content, which was similar in formula and sows' milk, the amount of AA in sows' milk was most similar to the formula with added AA to DHA at a 6.0:1.0 ratio (Group 3). However, the piglets fed AA:DHA at the 6.0:1.0 ratio were not similar for BMC in lumbar spine compared to the sow-fed piglets. Therefore, the amount of AA and DHA may not have contributed to the differences found. A possible explanation for the elevated BMC in the sow-fed group may have been related to phosphorus. In the sow-fed group, urinary phosphorus was excreted at a significantly higher concentration (from 3 to 6 times higher) than the urinary phosphorus excreted in the urine of the formula-fed piglets. In the formula-fed groups, phosphorus may not have been adequately absorbed from formula, or may have been excreted in the feces of the formula-fed piglets, rather than through their urine. These two factors may have been contributors to the lesser values for BMC reported in the formula-fed groups compared to the values for BMC reported in the sow-fed group.

Alternatively, BMD values in lumbar spine were similar between all groups. This anomaly between BMC and BMD may have been a result of voluminous but undermineralized bone in the formula-fed groups compared to the sow-fed group.

Due to the significantly higher amounts of AA found in liver of the LC PUFA supplemented formula-fed groups compared to the sow-fed group ($p \leq 0.001$), we may be able to say that higher AA alone does not result in elevated somatic and bone growth.

The inverse relationship seen between AA and urinary NTx suggests bone resorption is decelerated as dietary AA is increased.

This study provides evidence that the effect of diet on bone metabolism and mineralization is complex. Differences between the sow-fed versus formula-fed piglets may in part be due to differences in diet during the first few days of life. The piglets left on the farm, received two additional days of sows' milk, compared to the piglets that underwent adaptation to formula that was devoid of AA and DHA, during that time frame. The two days may have been a critical period of development for the piglets, leading to the differences in the values between formula-fed and sow-fed groups. Sow milk may contain factors other than LC PUFAs that play a role. Factors such as parathyroid hormone, calcitonin, and vitamin D status, to name a few, should be investigated to help determine possible confounding factors. Another possible explanation for the differences in values reported for outcome measurements in bone between the formula-fed piglets compared to the sow-fed piglets may have been macronutrients in the sows' milk. Macronutrients such as protein, which is known to affect bone, were not measured in sows' milk in this research and should be considered in future research.

6.6 STRENGTHS AND LIMITATIONS

6.6a Strengths

This study provided a controlled environment in which to observe the effect of dietary treatment outcomes in piglets. The piglet served as a useful model in which to monitor the effects of these outcome parameters in a short period of time. However, the piglet has been shown to have a greater capacity to synthesize fatty acids than human infants (Morris et al, 1999), and to date it is difficult to determine whether similarities exist between piglets and infants with regard to bone in response to diet manipulations. Also, although the volume of formula fed to piglets could be measured, it was not possible to measure the volume of milk consumed by the suckled piglets, thus not allowing a comparison of intake.

6.6b Limitations

Although it would not have been possible with this study design, the existence of a control group fed unsupplemented formula would have allowed the researcher to detect differences and determine the level of supplementation at which these differences begin.

This study investigated differences using one-way ANOVA, whereas two-way ANOVA would have allowed for determination of litter effect. The difference in the housing condition of the animals present potential for type 2 errors between the formula-fed and suckled piglets that may have affected results.

Content of bone was analyzed, but not structure. Including bone morphology in future research may help assess whether higher BMD reflects greater bone mineralization or larger bones with normal mineralization.

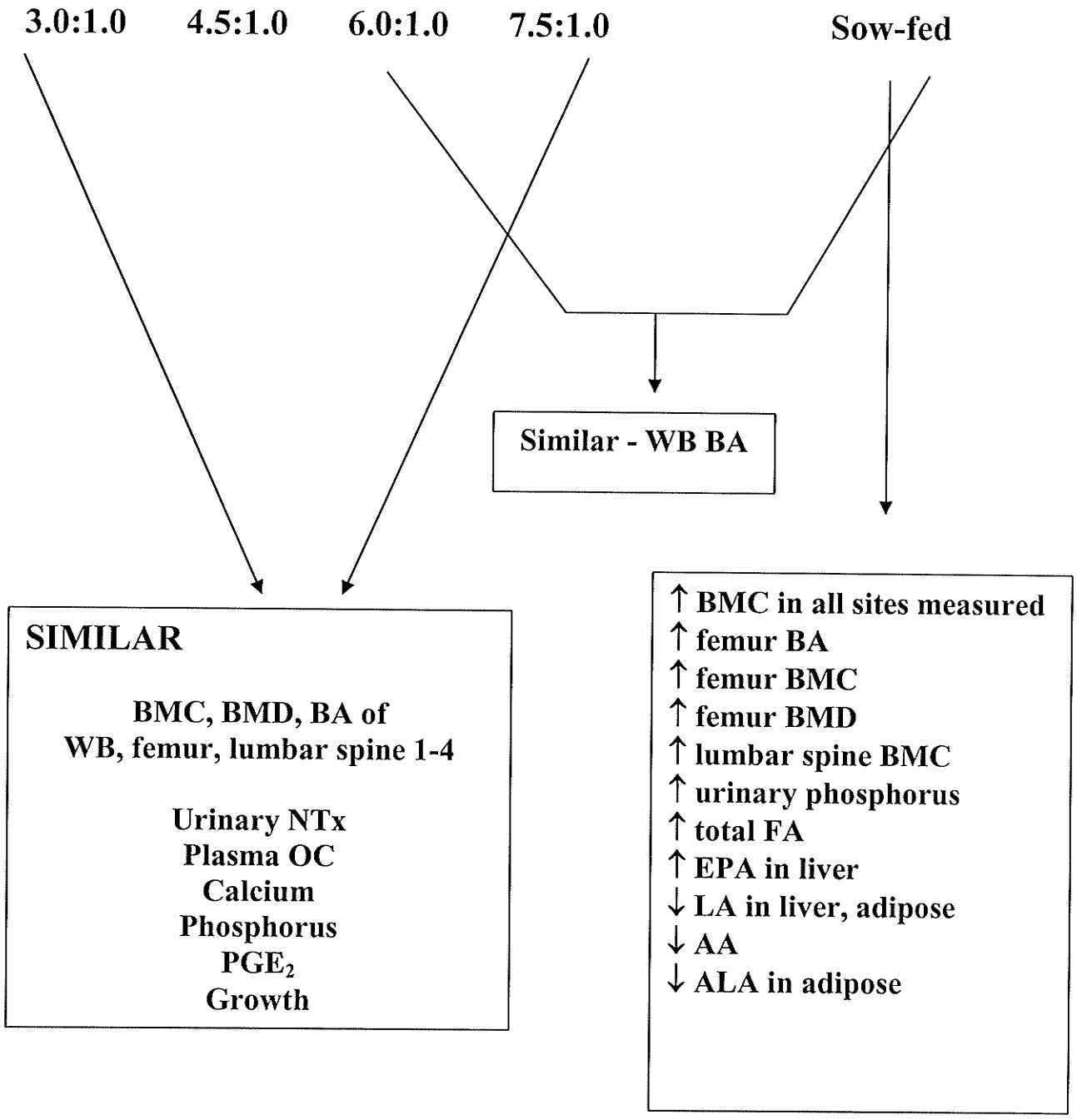
Piglets double or triple their weight within as little as 15 days, however a longer study period would allow further investigation as to whether the differences seen in 15 days continue, or if further differences would occur with time. Piglets were full-term, perhaps small for gestational age piglets would better represent the preterm infant.

6.7 FUTURE RESEARCH

As the piglet serves as a useful model in which to study growth, bone mineralization and metabolism in response to dietary fatty acid manipulation, further research could involve the comparison of a control group with a larger variation in AA and DHA, as well al:

* Effects of dietary manipulation in a younger/small for gestational age piglet
* Intestinal calcium absorption
* Differences from sow-fed piglets, but in the same environment as formula-fed piglets
* Effects on bone fatty acid status of other fatty acids such as n-3 (EPA and higher amounts of DHA)
* Fatty acid composition of cortical and trabecular bone
* Histomorphometry data investing bone formation rate

Figure 6-1. *Effect of dietary arachidonic acid and docosahexaenoic acid versus sows' milk on fatty acid status and bone metabolism and mineralization in piglets.*



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