ABSTRACT

MATHEWS OLIVER, SUSAN ASHLEY. Investigating the potential effects that long chain polyunsaturated fatty acids have on lipid metabolism in the piglet as a model for the human infant. (Under the direction of Dr. Robert J. Harrell and Dr. Jack Odle.)

The essential fatty acids (EFA) linoleic acid (LA) and linolenic acid (LN) are necessary for growth and development. Tissues of the central nervous system and the retina depend on the conversion of LA to arachidonic acid (AA) and LN to docosahexaenoic acid (DHA). Research in human infants has indicated that supplementation of infant formulas with AA and DHA promotes visual and neural development. The objective of the first study was to determine the utilization as well as the safety and efficacy of two sources of LCPUFA. Piglets (n=10/group) had *ad libitum* access from day 1 to 16 of age to a skim milk-based formula with different fat sources added to provide 50% of the energy. Treatments included: control with no added LCPUFA (CNTL), single cell oil triglyceride (TG), TG with phospholipid and cholesterol added to match phospholipid content in the PL diet (TG+PL), egg phospholipid (PL), and an essential fatty acid deficient group (EFAD). Formulas with LCPUFA provided 0.6% of fatty acids as AA and 0.3% as DHA. Total plasma AA and DHA concentrations (expressed as weight % of total lipid fatty acids) were greater in the TG compared to the CNTL ($P<0.05$), but there were no differences among the TG, TG+PL or PL ($P>0.2$). Apparent dry matter digestibility was 10% greater in the CNTL, TG, and TG+PL compared to the PL ($P<0.002$). Total body accretion of essential fatty acids (EFA) were lower in EFAD compared to all other groups ($P<0.01$). Accretion of AA and DHA was greatest in the TG compared to

CNTL ($P<0.02$), but surprisingly, EFAD had similar accretion of AA as TG. CNTL had 40% longer ileal villi than the PL ($P<0.03$), but the TG and TG+PL were similar to CNTL. These data demonstrate that the TG source of AA and DHA may be a more efficacious supplement for infant formulas.

Over the last decade, the prevalence of childhood obesity has increased significantly in the US. Conjugated linoleic acid (CLA) has been shown to reduce body fat in many species, but little is known about the metabolic interactions between CLA and EFA. Two replicates of 12, 1 d old pigs were fed a milk-based formula *ad libitum* for 17 d that contained 25%(HF) or 3% (LF) fat with either 1% CLA (+CLA) or 1% sunflower oil (-CLA). LF fed pigs consumed 10% more dry formula than HF fed pigs ($P<0.05$), but 19% less metabolizable energy ($P<0.01$). In vitro β-oxidation of ^{14}C-arachidonate, linoleate, and palmitate was not affected b CLA ($P>0.2$) or level of dietary fat ($P>0.1$) in liver, brain, or muscle tissue. Accumulation of body lipid and protein was reduced by 34% and 14%, respectively in pigs fed supplemental CLA ($P<0.05$). CLA was only detected in pigs fed CLA, with more accumulation found in the LF fed pigs than the HF fed pigs ($P<0.01$). Total body accretion of LA, AA and DHA were reduced by fat level ($P<0.0001$) and both LA and LN were reduced by CLA ($P<0.0003$). These data suggest that CLA in conjunction with a low fat diet reduced body fat while not affecting in vitro oxidation of essential fatty acids.

INVESTIGATING THE POTENTIAL EFFECTS THAT LONG CHAIN POLYUNSATURATED FATTY ACIDS HAVE ON LIPID METABOLISM IN THE PIGLET AS A MODEL FOR THE HUMAN INFANT

by

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For my grandparents.

BIOGRAPHY

Susan Ashley Mathews Oliver was born May 17, 1974 in New Bern, North Carolina, to Howard and Helen Mathews. She graduated from New Bern High School in 1992. Susan attended Meredith College and completed her Bachelors of Science degree in Foods and Nutrition, with a concentration in Human Nutrition in May 1996. Upon graduation, Susan moved to Johnson City, TN, to attend East Tennessee State University where she completed a dietetic internship and obtained her Master of Science degree in Clinical Nutrition under the direction of Dr. William L. Stone. During her Masters program, Susan was accepted into and completed the Neonatal and Pediatric Nutrition Fellowship program at James Whitcomb Riley Hosptial for Children and Indiana University School of Medicine. Also, during the fellowship, she sat for and passed the Registered Dietitian Examination. After completion of the fellowship, she successfully defended her Master of Science degree in December 1998. In January 1999, Susan moved back to Raleigh, NC, and began her Doctor of Philosophy degree in Nutrition under the direction of Drs. Bob Harrell and Jack Odle at N.C. State University. It was at N.C. State that she met her husband, William, also a doctoral student at the time. They were married on September 28, 2002 in New Bern, North Carolina. Upon completion of their degrees, she and William will move to Houston, TX, where she has accepted a position with Memorial Hermann Children's Hospital as a Neonatal Dietitian and Researcher, and William will be a Post-doctoral fellow at the Children's Nutrition Research Center at Baylor College of Medicine.

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CHAPTER 1

LITERATURE REVIEW

HISTORY OF ESSENTIAL FATTY ACIDS

The essentiality of polyunsaturated fatty acids (PUFA) was first documented in 1929 by Burr and Burr, who after feeding rats a fat free diet noted growth retardation and dermatitis (1). Both linoleic acid (LA, 18:2 n-6) and alpha-linolenic acid (LN, 18:3 n-3) are known to be essential, but the understanding of the essentiality is not equal between the two fatty acids. Essential fatty acid deficiency disrupts normal growth and can cause infertility as well as skin lesions; however, it is widely known that supplementation with LA alone, will completely ameliorate these symptoms primarily due to the fact that the symptoms relate to the biological functions of n-6 fatty acids (2). The essentiality of the n-3 fatty acid, LN has not been investigated until recently and it is now thought that the n-3 fatty acids have a distinct role apart from the n-6 fatty acids. However, this role deals primarily with the n-3 fatty acid, docosahexaenoic acid which has a specific role in membrane function, especially in the retina and tissues of the central nervous system (3).

Documented cases of human essential fatty acid deficiency are rare. The use of total parenteral nutrition without the use of intravenous lipid has typically been the culprit in both children and adults. In both cases, upon examination of plasma fatty acid profiles an increase in triene to tetraene ratio existed, which demonstrates that arachidonic acid is limiting and instead, the fatty acid known as Mead's acid (20:3 n-

9) is biosynthesized (1). Normally, in a well nourished state, plasma concentrations of the n-9 fatty acid are minimal.

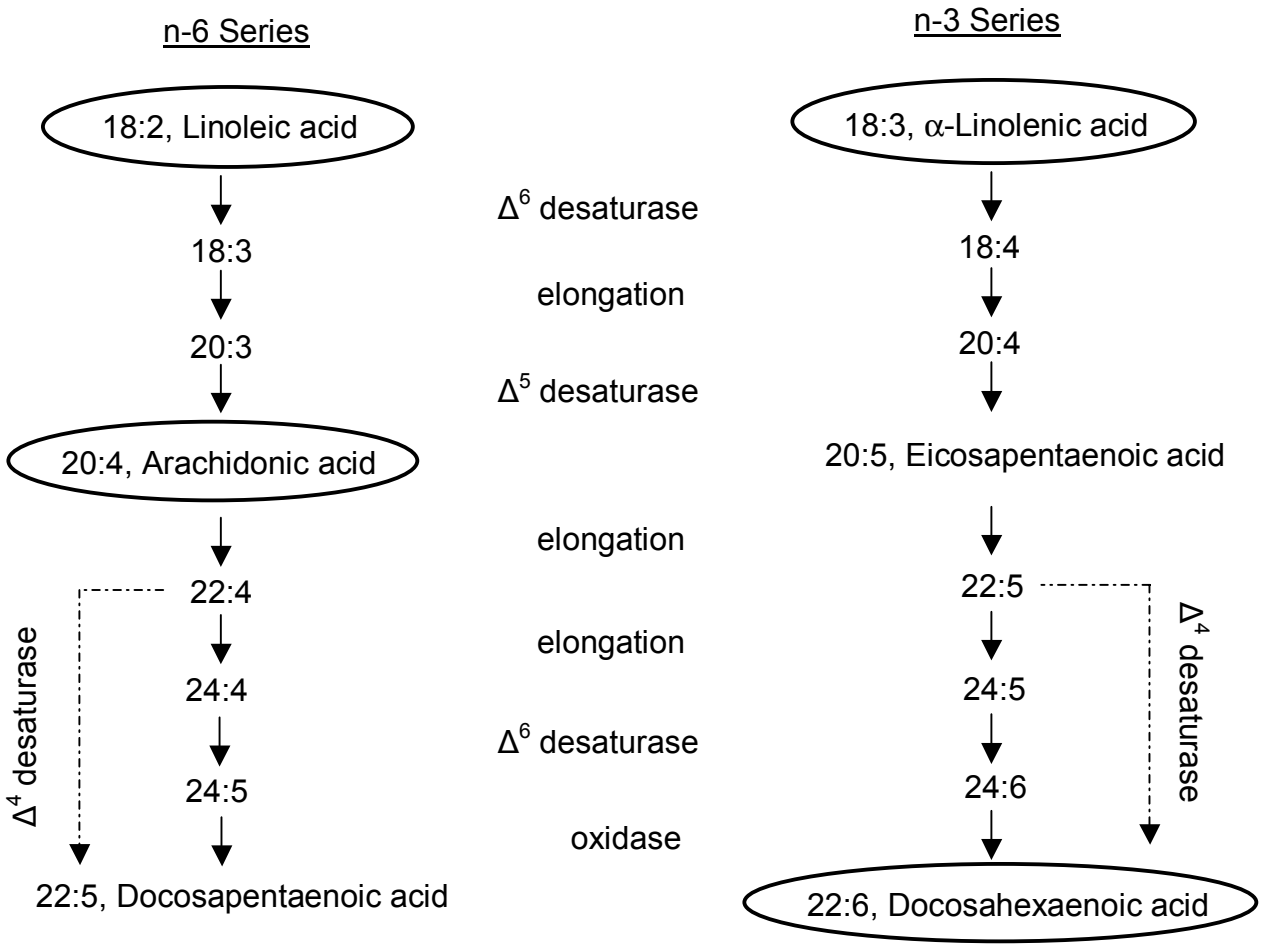

Figure 1. The biosynthetic pathway for the production of arachidonic acid and docosahexaenoic acid from their precursors, LA and LN.

The biosynthetic pathway for the de novo synthesis of essential fatty acids incorporates a series of elongation and desaturation reactions involving both Δ5 and Δ6 desaturase enzymes, with Δ6 desaturase being the rate limiting step. For the n-3 series, the final conversion to 22:6 n-3 is currently believed to require a retroconversion step, involving peroxisomal β-oxidation, known as the "Sprecher pathway" (2, 4). Figure 1 depicts the biosynthesis of both AA and DHA showing two alternative pathways for each, however, most research has elucidated the pathway involving the second Δ6 desaturase for both fatty acids and peroxisomal β-oxidation to be the most precise one. Because of the specific roles that both arachidonic acid (AA; 20:4n-6) and docosahexaenoic acid (DHA; 22:6n-3) encompass in the retinal and central nervous system (CNS) tissue, it is important to look at the growth and development of these tissues, as well as the accretion of these fatty acids in the human neonate.

LONG CHAIN POLYUNSATURATED FATTY ACIDS

The long chain polyunsaturated fatty acids (LCPUFA), LA and LN, are essential fatty acids (EFA) that are necessary for the growth and development of the neonate. Proper development of the brain, retina and other body tissues is dependent upon provision of arachidonic acid (AA) and docosahexaenoic acid (DHA) either directly in the diet or through synthesis from LA and LN, respectively (5). Linoleic acid and LN are found primarily in plasma transport or storage lipids in the body while the EFA metabolites, AA and DHA, are found as major components

within the phospholipid membrane of cells, and are also precursors for the synthesis of eicosaniods (6).

As mentioned previously, the two families of EFA, n-3 and n-6, follow similar metabolic pathways and LA, LN, and their metabolites compete as substrates for the elongation-desaturation pathway. Synthesis rates of AA and DHA are thought to be rate-limited by Δ6-desaturase activity, the initial enzyme in the desaturation-elongation pathway. The newborn may have a physiological immaturity of the enzymes involved in the conversion of EFA to their metabolites; therefore, formula-fed infants may not be able to synthesize the levels of AA and DHA that breastfed infants would normally consume (7). Brain tissue from postmortem infants in the United Kingdom and Australia fed formulas lacking AA and DHA had lower levels of DHA compared to infants fed breast milk (8-10).

Human breast milk naturally contains both LA (8-17% of total fatty acids) and LN (0.5-1.0% of total fatty acids), as well as varying concentrations of preformed AA and DHA which ultimately depend on the maternal diet (11). Commercial infant formulas that are available in the US contain a ratio of LA to LN that is similar to breast milk, and until recently did not contain any preformed AA or DHA. Thus, the recent FDA approval for the incorporation of the EFA metabolites, AA and DHA into infant formula will help to provide the appropriate amounts of the LCPUFA for normal growth and development (6). The approval of the FDA did not come easily, however. The decision to supplement infant formula with the LCPUFA was only

made after strenuous and complex testing involving both animal experiments and human clinical trials.

LCPUFA SUPPLEMENTATION IN THE NEONATE

As mentioned previously, accretion of the LCPUFA in the brain and other tissues of the CNS occurs during the 3rd trimester and also during the first 18 months of life (12, 13). The acquisition of AA and DHA *in utero* is due to the transport of these fatty acids across the placenta, and then after birth, infants who receive human milk continue to obtain these LCPUFA (14). Therefore, premature birth or feeding a formula without any supplemental AA and DHA will reduce the amount of AA and DHA acquired under normal physiologic conditions. Due to the lack of accumulation of LCPUFA in premature or formula-fed infants, it was questioned whether or not these infants could achieve optimal LCPUFA status through biosynthesis and it was this concern that led to the first clinical studies.

Over the past 20 years, many studies have been conducted not only to answer the above question, but also to expand the current knowledge base on LCPUFA supplementation in the neonate. Both animal studies and human clinical trials have presented interesting results that have led to the incorporation of supplemental LCPUFA in infant formula in the US. Animal studies were the first to support the hypothesis that both retinal and brain DHA could alter neural function. This was later followed up by human descriptive studies that infants fed human milk had improved DHA status compared to infants fed an unsupplemented formula (15,

16). More recent trials, in both animals and humans further support the hypothesis that AA and DHA status is reduced in preterm or term infants receiving formula without any preformed AA and DHA (8, 10, 17).

The majority of studies conducted to date have utilized non-invasive measures in both premature or very-low-birth-weight infants and term infants, which include plasma fatty acid profiles, growth as measured by length and head circumference, and cognitive and visual development (18-22). Without exception, all infants in these studies who received a LCPUFA supplemented formula had improved plasma fatty acid profiles, similar to human milk fed infants. Visual function has also been improved, but the results on cognitive development are somewhat mixed with there being no conclusive study to date. Growth data are also mixed and based on a recent meta-analysis by Lapillonne and Carlson (2001) (23) it appears that growth is not affected to any physiological relevance by LCPUFA supplementation. Earlier studies in preterm infants who were fed fish oil as the LCPUFA supplement had reduced length (24, 25), but the experimental formulas containing fish oil had high levels of n-3 fatty acids with low n-6 fatty acids. However others have reported improved growth in both preterm and term infants fed supplemental LCPUFA especially when both AA and DHA were provided at optimal amounts (26).

Through the use of animal models, particularly the neonatal piglet, more invasive procedures can be investigated with regard to LCPUFA supplementation. The effects of AA and DHA from a variety of sources including fish oil, egg

phospholipids or single-cell triglycerides have been investigated (27). Earlier studies using fish oil determined that caution must be taken due to the high levels of 20:5 (n-3) which can have deleterious effects in the absence or low quantity of fatty acids from the n-6 family (27). However, supplementation with fish oil did increase brain DHA, but reduced liver phospholipids AA content, which as mentioned previously is not only important in retinal function, but also an important precursor to eicosanoids. Studies using the single-cell triglyceride source concluded that both AA and DHA were increased in retinal phospholipids with no interference on eicosaniod biosynthesis compared to unsupplemented piglets (28). However, when the triglyceride source was supplemented above levels normally found in sow milk or human milk, lung eicosaniod production was increased, which could compromise lung function (29). Other investigators also using the triglyceride source of AA or DHA supplementation noted that the brain fatty acid composition was resistant to change, and that other organs showed opposing effects when either AA or DHA was incorporated into the diet (30).

The use of egg phospholipids as a source of LCPUFA has been utilized in countries all over the world for more than 10 years (31). Comparison of egg phospholipids to fish oils for DHA supplementation found that piglets had similar blood lipid profiles to sow-reared piglets when fed the egg phospholipids source, but that both supplied DHA equally to the retina (32). More recently, most investigators focused on either the egg phospholipid or single-cell triglyceride source of LCPUFA supplementation because these two sources provided the most benefits without the

potential deleterious effects that fish oils resulted in especially regarding reduced growth. However, only two studies have compared the two sources, egg phospholipid vs. triglyceride (33, 34). Amate et al (33, 34) found that within plasma lipoproteins, the egg phospholipid increased AA and DHA in HDL cholesterol, while the triglyceride source increased AA and DHA in LDL cholesterol. Mathews et al (33, 34) found that the triglyceride source was more available compared to the phospholipid source based on plasma fatty acid profiles, digestibility and total body accretion of AA and DHA.

The recent FDA decision to include LCPUFA supplementation as triglyceride in both term and preterm infant formulas was the result of extensive research. Both positive and negative effects have been reported over the past 20 years of research, but ultimately, after determining the most appropriate source and level of supplementation, infant formulas are now more closely aligned with the composition of human milk. Levels of inclusion of the single-cell triglycreide LCPUFA are not consistent among formula manufacturers; however, the provision of this supplementation makes formula more closely resemble human milk, which is the ultimate goal.

TRIGLYCERIDE VS. PHOSPHOLIPID

To date, less research has focused on the bioavailability and metabolism of the 'conditionally essential' LCPUFA in ingredients that would be of practical use in infant formulas. Until recently, the primary sources of AA and DHA used in infant

formulas had come from either from fish oil or egg phospholipids, but another source, from the triacylglycerols of algae, has received much attention. The absorption of AA and DHA from fish oil, egg phospholipids or single cell source differs from the absorption of AA and DHA from human breast milk. Carnielli et al. (1998) (35) observed no differences in absorption between the algal source of AA and DHA compared to breast milk, but found the egg phospholipids source to have greater absorption of DHA and n-3 LCPUFA compared to both the algal source and breast milk. Birch et al. (1998) (36)conducted a trial using breast milk, formula supplemented with algal forms of AA and DHA, and unsupplemented formula, and measured sweep visual evoked potential (VEP) acuity and RBC lipid composition. Infants who received AA and DHA supplemented formula or breast milk had better VEP acuity and similar RBC lipid composition when compared to infants on the unsupplemented formula. From the results of these studies, it can be concluded that supplementing infant formula with preformed AA and DHA from either egg phospholipids or algae sources provides results that are similar to infants who are breast fed.

NUTRITIONAL PROGRAMMING

The greatest rates of growth and development in an animal's life occur during the fetal and neonatal periods. Appropriate nutritional intake during this phase of life is imperative to support proper growth and development of all organ systems. Numerous studies support the hypothesis of nutritional programming, which

suggests that nutrition early in life can ultimately affect the onset of various chronic metabolic diseases later in life. This hypothesis suggests that nutrition during fetal and neonatal life has multi-faceted consequences possibly influencing the development of chronic diseases such as obesity, diabetes, hypertension, osteoporosis and cardiovascular disease (37-39). Many studies looking at maternal nutrition in relation to birth weight and size have correlated prenatal nutrition with altered risks for developing chronic diseases in adulthood. Obesity is of particular interest because of the increases in body weight seen in the US population over the past decade, especially in the pediatric age group.

Management or treatment of obesity in society today is a problem that leads to much debate. The development of adipose tissue occurs during fetal growth and its expansion can be influenced by a variety of factors including growth factors and gene transcription. Nutrition is an influential component that affects these regulators of adipose development and can affect the level of pre- and postnatal fat deposition. Therefore, the maternal diet during gestation and lactation may alter adipose tissue development in the offspring, which may have a permanent effect on the growth of this tissue. If there is incorrect 'programming' that occurs because of maternal intake, there is the possibility that this could lead to the development of obesity. Understanding the nutritional factors that are involved in this process could have substantial implications for both the human and animal world. Therefore, the next obvious step is to determine which dietary manipulations can be made to alter adipose development and potentially decrease the incidence of obesity.

CHILDHOOD OBESITY

Childhood obesity has increased three-fold in the last 20 years. Approximately 4% of children aged 6-11 were considered obese in the NHANES II (1976-1980), but the NHANES III (1988-1994) reported this age group of children to contain 13% as obese (40, 41). There are many factors that contribute to this significant and troubling increase in childhood obesity. Physical activity has decreased overall, but especially among adolescent girls (42, 43). Children spend approximately six hours a day watching television or sitting at a computer (44). Physical education in the schools is not seen as a vital part of the day, and trends towards eliminating physical education programs in schools are rising (44). The increase in the availability of foods that are high in fat and sugar also plays a role. School lunches and snack foods are more likely to be of the fast food variety, instead of being a fresh fruit or vegetable (44). Also, parental behaviors weigh heavily on the eating and physical activity pattern of children (45, 46). Therefore, an approach or intervention must be multi-faceted to gain the attention of both children and parents alike.

There are conceptually four critical periods for the development of adipose tissue in the human life span (41, 47, 48). A critical period is defined as a developmental stage in which physiological alterations increase the risk of later obesity (48). The four critical periods are: 1) gestation or fetal life, 2) early infant life, from months one to four, 3) the period of adiposity rebound, beginning around age five, and 4) adolescence or puberty (41, 47). It is during one or more of these

periods that obesity is potentially programmed; however, there is much debate over which of the four periods plays the most significant role on adult obesity.

Maternal nutrition during the three trimesters of pregnancy has an effect on the development of fatness later in life. Over- versus under-nutrition during any of the three trimesters can affect growth. Previous studies of mothers who were food restricted due to famine showed that under-nutrition during the first two trimesters lead to an increased prevalence of obesity (49). However, if under-nutrition occurred during the third trimester there was a decrease in the prevalence of obesity at age 18 (49). To further explain these differences, one must look at the developmental signals ongoing during the various stages of development. During the first two trimesters, the hypothalamus begins to develop, so sensitivity to caloric clues or caloric intake may be set by the sensitivity of the hypothalamus and sympathetic nervous system to the substrate availability in the intrauterine environment (48). The last trimester is the crucial period for the development of adipose tissue, but if caloric restriction occurs, there may be reduced fetal fat deposition and consequently, leanness in later life. If over-nutrition occurs during the 3rd trimester, this may influence adipose tissue cellularity and promote obesity (47).

Once born, the infant begins a period of time where rapid growth and development occur. Many factors affect the growth pattern including, but not limited to high birth weight (50, 51), mode of infant feeding (bottle versus breast) (52), introduction of solid foods (53), and overfeeding (54, 55). Stettler et al (2002) (41)

assessed the association between the rate of weight gain from birth to four months of age and the prevalence of overweight status in childhood in a large cohort study consisting of 19,397 participants. It was determined that a more rapid rate of weight gain during the first four months of life coincided with an increased risk for childhood overweight status at age 7. As mentioned earlier, rate of gain varies depending on the mode of infant feeding, and typically breast-fed infants weigh less than bottle-fed infants at 1 year of age (56, 57); however, Stettler et al. (2002) (41) did not include this variable in the analysis because infant feeding data was not available.

The period of adiposity rebound occurs in early childhood beginning at about age 5 (47, 48, 58). It is important to note that during the first year of life, the body mass index (BMI) of the infant increases rapidly and then declines to a minimum at age 5 or 6 at which point a gradual increase begins and remains as such into adolescence and most of adulthood (48, 58). The period when the BMI is at its lowest is considered the adiposity rebound (AR). The early onset of AR is related to a higher BMI in adolescence (59). Whitaker et al (1998) (58) determined that the earlier the onset of AR, the greater the risk of adult obesity and that this was independent of either the child's parents weight status or the child's BMI at the onset of AR.

The final critical period for the development of obesity is adolescence. Obesity in adolescence is associated with a greater risk of adult obesity and the related complications compared to all other critical periods (47, 48). However, the mechanism by which this occurs is not known. It is during this time that a gender

difference is seen; females are more likely to become obese during adolescence and remain that way into adulthood. An estimated 30% of all obese women were obese in early adolescence, but only 10% of obese males were obese as teenagers (60). The gender difference may be due to the changes that occur in fat deposition between males and females, the location of adipocytes, the differential effects of insulin and glucagon signaling, as well as lipolysis (47). Another reason for this gender difference is activity level; girls tend to be less active during adolescence than boys (42).

Determining which of these four critical periods is most important in the development of obesity is difficult. From the epidemiological evidence thus far, the most appropriate measure to result in a decrease in overweight status in the pediatric population is to start early in life and follow through all critical periods. Discovering dietary modifiers such as fats and/or certain fatty acids that can affect body composition early in life may help to reduce the incidence of obesity, and possibly lead to a treatment for this worldwide metabolic disease. Throughout the lifespan, fat is an essential nutrient and early in life, constitutes about 50% of the infant's caloric intake regardless of mode of feeding. Fat is an energy dense substrate that along with providing a major structural component for growth and development, may, if overfed, lead to metabolic consequences that could potentiate the development of obesity.

OBESITY RELATED PROTECTION OF HUMAN MILK

Human breast milk, infant formulas and sow's milk contain approximately 50% of calories from fat, which suggests that the neonate requires a high amount of dietary fat in order to achieve appropriate weight gains. Fat is the most energetically dense macronutrient and is the primary source of energy for the neonate during the early phase of growth, be it the first four to six months of life in the human, or the first 21 days in the piglet. Current data in piglets suggest that formulating milk replacers similar to the composition of sow's milk may not be the optimum approach for growth. For example, growth performance of piglets was maximized when the supply of lysine per unit of energy was approximately 50% higher than found in sow's milk (61). Furthermore, diets that were utilized in artificial rearing studies conducted in our lab, supplied approximately 50% greater amino acid content per unit of energy than sow's milk and resulted in heavier piglet weight gains (62, 63). Also, the dietary inclusion of conjugated linoleic acid reduced sow milk fat content by approximately 35%, but growth performance of the nursing litters was not altered (64). These data suggest that utilizing the pattern of nutrients and energy found in sows milk limits preweaning pig growth. However, to our knowledge, research with varying levels of fat in an infant formula, i.e. high fat versus low fat, has not been conducted because infant formula has always been formulated to emulate human milk.

Breast milk is considered the 'gold standard' for all formula composition. Therefore inclusion of preformed AA and DHA into infant formula has become a

major research focus in infant nutrition. As mentioned earlier, studies demonstrate that infants fed formulas supplemented with AA and DHA show improved visual acuity, better neurodevelopment, and a lower incidence of necrotizing enterocolitis (65-67). Therefore, intake of these LCPUFA plays a vital role not only in infant development, but also in protection of diseases that are limited to the neonatal period. Along with these mechanisms, dietary intake of LCPUFA has also been linked with the suppression of lipogenesis by increasing lipid oxidation, which may lead to a decrease in fat deposition (68). This is thought to occur through the activation of the peroxisome proliferator activated receptor-alpha (PPARα), and may help to regulate adipose development by establishing non-obese "programming" of this tissue during a critical phase of growth. However, in a PPARα-null rodent model, it has been determined that a reduction in body fat and increase in body protein may be independent of this nuclear receptor (69). These LCPUFA are also responsible for the suppression of fatty acid synthase and both Δ-6 and Δ -5 desaturases, which are involved in de novo fatty acid synthesis and formation of very long chain PUFA, respectively (70-72). Therefore, it appears that a balance mechanism exists between the synthesis of AA and DHA and the oxidation of the fatty acids responsible for their biosynthesis.

PEROXISOME PROLIFERATOR ACTIVATED RECEPTORS

Polyunsaturated fatty acids, especially LA, are ligands and hence activators of the peroxisome proliferator activated receptors (PPARs) (73, 74). It has been

shown that n-3 fatty acids may be a more potent ligand than n-6 fatty acids, but that the metabolites of both n-3 and n-6 fatty acids are even stronger activators of the PPARs (75-77). The PPARs encompass a nuclear hormone receptor superfamily that is linked with various biological pathways including lipid metabolism and adipocyte differentiation (78). There are several PPARs that have been identified thus far and include: α, γ, and δ, with there being at least 3 isoforms for the PPARγ. PPARα is abundantly expressed in tissues that have high rates of β-oxidation including liver and skeletal muscle (79-82). PPARγ is expressed primarily in adipose tissue as the isoform PPARγ2, which is associated with adipocyte differentiation (83), has a role in activating the transcription of genes involved in lipogenesis and fatty acid esterification (79, 84, 85), and expression is attenuated by feed deprivation (86).

PPARα as mentioned earlier is localized in the liver and skeletal muscle (79), but there may be species differences in regards to its mechanisms of action. In the rodent model, PPARα maintains both heart and liver β-oxidation pathways, is responsible for the metabolic responses associated with starvation and in the absence of the nuclear receptor, uncoupling of overall lipid metabolism occurs (87, 88). The role of PPARα in the human is not as clearly defined. PPARα activating pharmaceuticals are used to treat hyperlipidemia and may show effectiveness for the treatment of obesity and insulin resistance (89). Skeletal muscle appears to be the primary site for expression of PPARα in the human (90), and until recently its role in this tissue was unknown. The PPARα that is localized in skeletal muscle

appears to have a role in muscle lipid homeostasis as shown by Muoio et al (2002) (81) in a recent study using muscle cell cultures. The role of PPARα in the liver is to promote lipid oxidation by the regulation of several enzymes that are pivotal regulators of lipid metabolism such as acyl Co-A oxidase and carnitine palmitoyl transferase (82).

PPARγ2 appears to be regulated by the type of fat in the diet. Spurlock et al (2000) (78) showed that diets rich in LA, primarily from safflower oil, caused a significant increase in the amount of PPARγ2 mRNA in adipose tissue of pigs. In a more recent study by Spurlock et al (2002) (82), the effects of a prolonged milk feeding versus a typical dry diet on body composition were investigated. The prolonged feeding of a milk diet only transiently increased body fat of pigs, without any effects on the PPARγ2. The milk-replacer was 14.3% fat whereas the dry diet was only 8.6% fat. This difference in dietary fat may have accounted for the transient difference in body fat, because once all pigs were placed on the same diet, body fat differences disappeared. Interestingly, it was noted that pigs fed the prolonged milk diet were leaner once market weight was achieved. This may be due to a difference in the number of adipocytes in the milk fed pigs, but the authors did not measure adipocyte cell number. PPARα was unaffected as well regardless of nutritional regime or body fat content. Both PPARs have important roles in their respective tissues as found in various species, and elucidating their specific mechanisms on lipid metabolism may provide new insights into the treatment of obesity.

INFANT NUTRITION AND *TRANS* FATTY ACIDS

Another group of dietary components that may affect lipid metabolism are *trans* fatty acids. *Trans* fatty acids (TFA) are unsaturated fatty acids that contain at least one double bond in the *trans* configuration (91). The double bond can be located anywhere along the molecule; so many positional isomers may exist. TFAs are produced during mechanical hydrogenation of oils and fats to produce dietary fats with improved texture, but are also present in meat and milk from ruminant animals (92). Since the late 1950's, many studies, including human and animal, have investigated the effects of TFA on a mother and her offspring. However, after almost 50 years there is no clear evidence suggesting that exposure to TFAs has a negative effect on the infant (93).

Reports on the TFA content of both human milk and formula have been published (94, 95). Human milk TFA concentration is reflective of the maternal diet, as expected, and levels of TFA in human milk are highest in North America (95). Estimates of TFA intake among US women range from 4.2 to 8.0% total fatty acids or 3.2 to 13.3 g/person/day (95). Infant formulas contain TFA in the range of 0.1 to 3.1% of total fatty acids, and infant foods contain anywhere from 0.2 to 7.6 % TFA of total fatty acids (95). However, the effects of TFA in human milk on the metabolism of EFA in the infant have not been closely examined.

Several studies have documented the relationship between maternal and infant plasma levels of TFA as well as TFA levels in mother's milk. Innis and King (1999) (96) reported that the percent of TFA in the mother's milk paralleled the TFA

content of the infant's plasma lipid profile, with inclusion being similar in both the triglycerides and phospholipids. It was also determined that the percent of TFA in mother's milk was inversely associated with the plasma levels of linoleic and linolenic acids, but not so with arachidonic (AA) or docosahexaenoic acids (DHA). Larqué et al (2000) (97), using the rodent model fed 2 levels of a TFA diet, reported an inhibitory effect of TFA on D6D activity in the liver, but there were no effects on the brain. A more recent study, Decsi et al (2001) (98) observed an inverse correlation between the concentration of TFA and total LCPUFA content in the umbilical cord blood lipids of full term infants. It was also reported that maternal TFA intake may be inversely associated with the infant's LCPUFA status at birth. The TFA levels in mother's milk were inversely associated with both DHA and AA, while the earlier study reported an inverse relationship with the precursors to AA and DHA, LA and LN, respectively.

The metabolic effects of TFA on the infant nutrition and health are not well understood. What is known about TFA is that because there is a structural similarity to the essential fatty acid linoleic acid (LA), a competition exists between LA and TFAs for the enzyme Δ-6 desaturase (D6D). This enzyme is responsible for desaturating LA in a series of reactions to produce arachidonic acid (AA), which is essential for proper growth and development of the infant. The competition for D6D has been shown in the presence of an essential fatty acid deficiency and in isolated tissue preparations (94) but what occurs under *in vivo* conditions is not known.

There is limited knowledge about the influence of TFA on the metabolism of very LCPUFA, including the Sprecher pathway to produce docosahexaenoic acid (DHA). There is however, considerable information about the interaction of TFA with the initial altering sequence of Δ6 desaturation, chain elongation, and Δ5 desaturation in the formation of AA and ecosapentanoic acid (20:5n-3). Researchers have shown that both Δ5 and Δ6 desaturases are inhibited by some *trans* 18:1 positional isomers (99). Also, TFA are more likely to inhibit n-6 than n-3 fatty acids (100) and the metabolites of TFA may have greater inhibition of essential fatty acid metabolism than do TFA themselves (94). However, Larque at al (2000) (92) determined that feeding TFA to rats had no effect on the LCPUFA content of their milk. There was a dose-dependent effect of the TFA on the milk, and rats fed TFA had greater LA content in the milk; therefore, they speculate that as long as adequate amounts of EFA are present, the metabolism of LA and LN will be unaffected.

Over the past several decades, there has been an increase in the amount of research conducted on the effects of TFAs. However, no definitive answer on how TFAs, either as a group of compounds or as individual fatty acids, affect the neonate. One TFA of particular interest is conjugated linoleic acid. It is a possible nutritional modifier of body fat, but similar to other TFAs, its effects on neonatal lipid metabolism are unknown.

CONJUGATED LINOLEIC ACID

Conjugated linoleic acid (CLA) is an unsaturated fatty acid that has conjugated diene double bonds and a combination of *cis* and/or *trans* spatial configurations (101). There are many different isomers of CLA that are found in ruminant meats and dairy products. Much research investigating CLA has reported many biological effects including anti-carcinogenic properties (102), anti-athlerogenic effects (103), and anti-diabetogenic effects (104) especially in animal models. Along with these effects of CLA, it has also been seen to affect lipid metabolism (105, 106) and reduce fat mass in both pigs and humans (107-109).

Nutritional research focusing on CLA has increased greatly over the last several years, with the research focus being the use of CLA in various animal models, but less research being done directly with the human, particularly excluding the infant. However, this is not an area to be overlooked in infant nutrition because it has been determined that CLA is in human milk (110, 111). CLA fed to lactating mammals reduced milk fat (64, 112-115), but it was not until recently that the effects of CLA on human milk were known. Innis and King (1999) (96) measured CLA concentrations in both breast milk and infant plasma lipids. A relationship between CLA in the milk and in the plasma lipid of the infant was observed and demonstrated that CLA is preferentially incorporated into the phospholipid fraction. Elias and Innis (2001) (116) determined that the concentration of both CLA and other TFAs were related to the maternal plasma concentration. The distribution of CLA and TFAs in the different plasma lipids differed between mothers and their infants. TFA

concentrations were higher in maternal triglycerides and phospholipids compared to neonatal levels, but for the cholesteryl esters TFA concentration, infants had higher levels. With respect to CLA, AA and DHA, infant plasma lipid concentrations were ≥ 1.5 times the maternal values. Therefore, it was determined for the first time that CLA crosses the human placenta. It was also noted that for every 1% increase in CLA in the infant's plasma triglyceride, that gestational length decreased by 0.5 day ($P < 0.01$) and that birth length decreased by 1 cm ($P < 0.01$). Most importantly, a 1% increase in CLA in the cholesterol ester portion of the infant's plasma lipid caused a decrease in birth weight by 310 g ($P < 0.05$), which accounts for 9% of the infant's birth weight. It was concluded that further research is needed to determine how maternal intake of CLA affects the developing fetus (116).

More recently, a study was performed with lactating mother's who were supplemented with ~1% CLA (114). The supplementation caused a decrease milk fat by ~24%, and also increased the CLA content of the milk, which would in turn increase the infant's consumption of CLA. The authors suggest that lactating mothers not consume CLA supplements due to the risk of decreasing the caloric density of their milk. The authors did not report infant consumption of milk during the CLA supplementation period; therefore it is unclear if the infants increased their volume of milk consumed to make up for the decrease in caloric density (114). Data from our lab have shown that piglets will consume a greater volume of a milk-replacer that is lower in calories (117). If the infants did increase total consumption, and growth rates were unaffected, how would the CLA affect the infant? CLA has

been shown to reduce lipid filling and adipocyte differentiation, so could it play a role in the battle against childhood obesity?

Infant formulas are formulated to emulate human milk, but there are still pieces that are missing from the formula ingredient list. As mentioned previously, studies focused on the effects of TFA have been conducted and have shown TFA to be inhibitory of D6D, which is involved in the elongation/desaturation pathway for both of the essential fatty acids, linoleic and linolenic acids. There have been no studies evaluating the direct effects of CLA on infant nutrition and health. Discretion must be taken when this assumption is made because if LCPUFA are decreased in the infant due to a competition with CLA, will this cause a problem for growth and development of the visual and neurological systems? However, because of the advantageous effects that have been seen with CLA thus far including the effects on body composition, it seems appropriate to determine if these same effects are found in the neonate, especially during a critical period for the development of obesity. The molecular actions of CLA are not known, but it has been determined that CLA is a potent ligand for the PPARα, which as mentioned previously, is a regulator of lipid oxidation. Therefore, could CLA be a means of reducing obesity?

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CHAPTER 2

COMPARISON OF TRIGLYCERIDES AND PHOSPHOLIPIDS AS SUPPLEMENTAL SOURCES OF DIETARY LONG CHAIN POLYUNSATURATED FATTY ACIDS IN PIGLETS[1, 2]

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ABSTRACT

Addition of arachidonic acid (AA) and docosahexaenoic acid (DHA) to infant formula promotes visual and neural development. This study was designed to determine if the source of dietary long chain polyunsaturated fatty acids (LCPUFA) affected overall animal health and safety. Piglets consumed *ad libitum* from 1 to 16 d of age a skim milk-based formula with different fat sources added to provide 50% of the metabolizable energy. Treatment groups were: control, no added LCPUFA (CNTL), egg phospholipid (PL), algal/fungal triglyceride oils (TG), TG plus phospholipid (soy lecithin source) added to match phospholipid treatment (TG+PL), and essential fatty acid deficient (EFAD). Formulas with LCPUFA provided 0.6 and 0.3 g/100 g total fatty acids as AA and DHA, respectively. CNTL piglets had 40% longer ileal villi than PL piglets ($P<0.03$), but the TG group was not different from the CNTL group. Gross liver histology did not differ among any of the formula-fed groups ($P>0.1$). Apparent dry matter digestibility was 10% greater in CNTL, TG, and TG+PL groups compared to PL piglets ($P<0.002$). No differences in alanine aminotransferase were detected among treatments, but aspartate aminotransferase was elevated ($P<0.03$) in PL piglets compared to TG+PL piglets. Total plasma AA concentration was greater in the TG group compared to CNTL piglets ($P<0.05$). Total plasma DHA concentrations were greater in TG piglets compared to PL ($P<0.06$) or CNTL piglets ($P<0.02$). These data demonstrate that the algal/fungal TG sources of DHA and AA may be a more appropriate supplement for infant

formulas than egg PL source based on piglet plasma fatty acid profiles and apparent dry matter digestibilities.

INTRODUCTION

The essential fatty acids (EFA)[4], linoleic acid, [LA; 18:2(n-6)] and α-linolenic acid [LN; 18:3(n-3)], are necessary for the growth and development of human infants. Proper development of the brain, retina and other body tissues depends upon provision of AA and DHA either directly in the diet or through synthesis from LA and LN (1). The precursors, LA and LN, are primarily in plasma transport or storage lipids in the body, while AA and DHA, the EFA metabolites, are major components within the phospholipid membrane of cells (2). Intrauterine accretion of AA and DHA occurs largely during the third trimester of pregnancy; therefore, premature infants may be at increased risk for a deficiency (3).

Human breast milk naturally contains LA and LN as well as varying concentrations of preformed AA and DHA, all of which depend on the maternal diet. Concentrations of LA range from 11 to 21 g/100 g total fatty acids and LN from 0.3 to 1.9 g/100 g total fatty acids in human milk samples from the US, Japan and Germany (4-6). These lipids are found as about 98% triglyceride and 0.8% phospholipid (6). Commercial infant formulas that are available in the US contain a ratio of LA to LN and levels of these fatty acids that are similar to breast milk, but most do not contain any preformed AA or DHA. Data indicate that conversion of LA

and LN to AA and DHA, respectively, by the desaturation-elongation pathway may not be sufficient to support the needs of growing infants (7-9). Cunnane et al. (7) estimated that formula-fed infants accumulate only half of the DHA that breast-fed infants accrete over the first six months of life; therefore, supplementation with DHA in conjunction with AA is considered necessary to support proper growth and development during this early and rapid growth phase of life. Brain tissue from postmortem infants in the United Kingdom and Australia fed formulas lacking AA and DHA had lower levels of DHA than infants fed breast milk (10-12).

Breast milk is considered the 'gold standard' for formula composition. Therefore, inclusion of preformed AA and DHA into infant formula has become a major research focus in infant nutrition. Some studies demonstrate that infants fed formulas supplemented with AA and DHA show improved visual acuity, better neurodevelopment, and a lower incidence of necrotizing enterocolitis (13-15). Less research has focused on the bioavailability and metabolism of these LCPUFA in ingredients that would be of practical use in infant formulas.

Presently there are two primary types of pre-formed AA and DHA that may be used as supplemental sources of LCPUFA in infant formula; a triglyceride source that is produced from single cell microorganisms (AA from fungi and DHA from microalgae) and a phospholipid source that is extracted from egg yolk oil. The differences in absorption and metabolism of these two sources have received little attention to date. Recently, Amate et al. (16) conducted a trial comparing the two sources of LCPUFA, egg phospholipid vs. triglyceride, in piglets and reported

plasma concentrations of the lipoproteins, as well as the composition of the intestinal mucosa. The lipid composition of the jejunal mucosa was not affected by the triglyceride and phospholipid sources, but the sources had different effects on the high versus low density lipoproteins. The egg phospholipid diet increased AA and DHA in the high density lipoprotein phospholipid, while the triglyceride diet increased AA and DHA in the low density lipoprotein phospholipid. The piglet has proven to be a suitable model for comparison to the human infant when studying lipid nutrition. The piglet has many similarities with human infants including a likeness in the development of the intestine, similar fat digestion and absorption, and also many of the pathways of lipid metabolism (17). The purpose of this study was to examine the utilization of algal/fungal triglyceride oils in comparison to an egg phospholipid source, and conduct a stringent assessment of the safety and efficacy of these lipids as delivery sources of LCPUFA.

MATERIALS AND METHODS

Animal Care

General. The Institutional Animal Care and Use Committee of North Carolina State University (NCSU) approved all procedures. A total of 48 piglets from 13 sows were obtained from the NCSU Swine Educational Facility, Raleigh, NC, and moved to the Grinnells Intensive Swine Research Laboratory at approximately one d of age. Pigs were placed in individual cages in an environmentally controlled room (32° C) and trained to consume liquid diet from a gravity flow feeding system adapted from

McClead et al. (18). The feeding system consisted of bottles suspended above the cages with tubing connecting the bottle to the permanently affixed nipple. All pigs were routinely consuming the liquid diet after 12 to 16 h of training and were then randomly assigned to one of five dietary treatments. Treatments groups were as follows (**Table 1**) (19): 1) piglet formula without any preformed AA or DHA added, but adequate amounts of LA and LN (CNTL, n=10), 2) piglet formula plus AA and DHA from egg phospholipids (Ovothin, Lucas Meyer, Inc, Decatur, IL) (PL, n=10), 3) piglet formula plus AA and DHA from the fungal and algal triglyceride oils (Martek Biosciences Corp., Columbia, MD) (TG, n=10), 4) piglet formula plus AA and DHA from the fungal and algal triglyceride oils (Martek Biosciences Corp., Columbia, MD) with additional choline, cholesterol and soy lecithin phospholipids (American Lecithin Co., Oxford, CT) to match the phospholipid formula (TG+PL, n=10), 5) piglet formula deficient in essential fatty acids (< 2 g/100 g total fat as LA and devoid of LN), (EFAD, n=8). Formulas with LCPUFA provided 0.6 g/100 g of fatty acids as AA and 0.3 g/100 g as DHA. Fatty acid composition of the diets is presented in **Table 2**. Another 13 piglets from 2 litters remained with the sows for the duration of the study (Sow). At the end of the study, piglets were killed with an AMVA-approved electrocution devise followed by exsanguination (laceration of the brachiocephalic arteries) and tissues collected. An initial group of 10 piglets from 5 litters was also used to detect any changes that occurred due to treatments.

Animal Feeding and Diets. Diets were reconstituted at 150 g/L of water (approximately 11 g/100 g dry matter). Formula was added four times daily (0800,

1300, 1800, and 2300) to ensure freshness and to provide pigs free access. All components of the feeding system were cleaned thoroughly each day prior to the first feeding (0800h) with a liquid chlorinated detergent (DS Liquid: Command, Diversey Corp., Wyandotte, MI). Formula was reconstituted on a daily basis and stored at 4° C until fed.

Cobalt EDTA was prepared as described by Uden et al. (20) and was added to diets (0.1 g/100 g of dry diet) approximately 36 h prior to removal of pigs from the experiment as an inert marker of dry matter digestibility.

Rationale. The objective of this study was to determine the efficacy and safety of the algal/fungal triglyceride source of supplemental AA and DHA in neonatal piglets. The TG source also was compared to the currently utilized egg PL source of AA and DHA. The TG+PL group was included to determine if the potential deleterious effects of elevated phospholipids on intestinal health were due to phospholipids in general or specific to the PL source of AA and DHA. The initial piglets served as a beginning reference point for comparison with all treatments. The sow-reared piglets, which remained on the sow for the entire study and received the ideal source of nutrients for the pig, were used as a comparison for all formula fed groups. The EFAD diet was included to serve as a negative control to illustrate the responsiveness of the model to poor EFA status.

Sample Collection and Analytical Procedures

Performance and Blood Collection. Formula intake was determined gravimetrically on a daily basis. Pigs were weighed daily and blood was collected

via jugular venipuncture on d 0, 8, and 16 of the study at 0900h after all piglets had been fed. After collection, blood samples were centrifuged (Sorvall, model 64000, Newtown, CT) at 825 x g for 10 min at 4°C. Plasma was collected and aliquots were frozen at –80° C until fatty acid analysis. On d 16, an additional blood sample was taken and 17 blood metabolites were measured by a VetScreen (Antech® Diagnostics, Farmingdale, NY). These variables were measured to investigate the clinical safety of the LCPUFA sources. The VetScreen 17 measured: glucose, blood urea nitrogen (BUN), creatinine, total protein, albumin, total bilirubin, alkaline phosphatase, alanine aminotransferase (ALT), aspartate aminotransferase (AST), cholesterol, calcium, phosphorus, sodium, potassium, chloride, albumin/globulin ratio, BUN/creatinine ratio, and globulin.

Fatty Acid Analysis. Plasma lipids were extracted using the method of Bligh and Dyer and fatty acid methyl esters were produced using the method of Morrsion and Smith (21, 22). Fatty acid methyl esters were analyzed by gas-liquid chromatography using a Hewlett Packard Agilent 5890-Plus (Delaware) equipped with a flame ionization detector. The fatty acid methyl esters were separated on a 30 m FAMEWAX capillary column (Restek, Bellefonte, PA; 0.25 mm diameter, 0.25 μm coating thickness) using helium at a flow rate of 2.1 mL/min with a split ratio of 48:1. The chromatographic run parameters included an oven starting temperature of 130°C that was increased at 6°C/min to 225°C, where it was held for 20 min before increasing to 250°C at 15°C/min, with a final hold of 5 min. The injector and detector temperatures were constant at 220°C and 230°C, respectively. Peaks were identified

by comparison of retention times with external fatty acid methyl ester standard mixtures from NuCheck Prep (Elysian, MN). The fatty acid profiles were expressed as g/100 g total fatty acids.

Tissue Collection and Analytical Procedures. Immediately after exsanguination, the abdomen was opened and the gastrointestinal tract was removed from the gastroesophageal junction to the distal end of the rectum. The jejunum and ileum were separated from the duodenum, stomach, and mesentery from the peritoneal inflection to the ileocecal junction. The anterior and posterior ends of the removed small intestinal segment were noted and the jejunum and ileum were laid in 60-cm serpentine loops. The midpoint was marked, and intestine proximal and distal to this midpoint was considered jejunum and ileum, respectively. At approximately mid-jejunum and mid-ileum, two adjacent segments, one 3-cm and another one slightly larger than 10-cm in length, were removed. Digesta contents were taken from the distal ileum and frozen (-20° C) for dry matter digestibility analysis (23). Lactase specific activity was measured using the method of Dahlqvist (24) as modified by Oliver et al. (25). The 3-cm intestinal segments were processed, embedded, and stained according to procedures described by Luna (26) as reported in Oliver et al. (25) for measurement of villi height and width as well as crypt depth. Invasive measures were included because a reduction in villi height and lactase specific activity and an increase in crypt depths would indicate deleterious effects on the intestine.

Both the liver and the spleen were removed from the abdomen and weighed. Two pieces of liver were collected from the same two liver lobes in each piglet. Samples were fixed in 10% neutral buffered formalin for at least 48 h. Fixed liver was routinely processed, embedded in paraffin, sectioned at 6 μm thickness and stained with hematoxylin and eosin for histologic review.

Liver sections were evaluated and graded using a subjective scale by a Board Certified Veterinary Pathologist at North Carolina State University, College of Veterinary Medicine, Raleigh, NC, who was unaware of the treatment groups. Cytoplasmic vacuoles were interpreted as either glycogen containing or lipid containing based on their histologic appearance and then scored on a 4-point scale of scant, mild, moderate, or extensive. Other features noted were inflammation, extramedullary hematopoiesis and hemosiderin deposition. Again, these invasive procedures allowed for the further investigation of the safety of the supplemental sources for AA and DHA.

Statistical Analysis

Values in the text are means ± standard error of the mean. SAS (SAS Inst. Inc., Cary, NC) Proc GLM procedure was used for statistical analysis appropriate for a completely randomized design. Treatment differences were evaluated using a protected LSD which provided all pair-wise comparisons. Differences were deemed significant when $P < 0.05$.

Liver histology data were analyzed using StatXact software (version 3.1, Cytel Software Corporation, Cambridge, MA). The Kruskal-Wallis test was used to

examine vacuolization liver data and differences were deemed significant when $P < 0.05$.

RESULTS

Performance, Growth and Food Intake

Over the treatment period, there were no differences in piglet body weights (data not shown) except for on d 16 when the TG piglets were heavier than the EFAD piglets (7599 $\pm$ 304 g vs. 6562 $\pm$ 340 g, $P < 0.05$). Formula-fed piglets gained 324 $\pm$ 17 g/d throughout the study period, with the TG piglets having a greater gain than the EFAD piglets (346 $\pm$ 17 vs. 293 $\pm$ 18 g/d, $P < 0.05$). The sow-reared piglets gained 297 $\pm$ 29 g/d. Daily feed intake and feed efficiency (g gain/g feed) did not differ among groups for the 16 day period and were 1953 $\pm$ 68 g/d and 1.58 $\pm$ 0.06, respectively.

Small Intestinal Morphology

Jejunal and ileal villi height decreased from d 0 to d 16 in all treatments as assessed by comparison with the initial piglets (Figure 1, $P < 0.0001$). There were no differences in jejunal villi height among any of the formula fed groups nor were they different from the sow-reared piglets on d 16. CNTL, TG, TG+PL and EFAD piglets all had similar ileal villi height, while PL piglets had lower ileal villi heights than the CNTL piglets ($P < 0.03$). TG+PL, PL and EFAD piglets had lower ileal villi heights compared to the sow-reared piglets ($P < 0.05$), but did not differ from CNTL or TG piglets. Jejunal and ileal crypt depths were greater in all formula-fed groups at

d 16 compared to the initial piglets ($P < 0.03$). Jejunal villi width was greater in the TG, TG+PL and PL piglets compared to the sow-reared piglets ($P < 0.05$) and both TG+PL and PL piglets had greater widths than the initial piglets ($P < 0.02$, data not presented). Ileal villi width was greater in CNTL, TG, TG+PL and PL piglets compared to the initial piglets ($P < 0.03$), and both TG+PL and PL piglets had greater villi width than the sow-reared piglets ($P < 0.03$, data not presented).

Enzyme Specific Activity

The addition of either triglyceride or phospholipid sources of AA and DHA did not affect jejunal or ileal lactase specific activity in any of the formula-fed piglets (Figure 2). Both initial and sow-reared piglets had greater lactase specific activity than the formula-fed groups ($P < 0.0001$) but they did not differ from one another.

Dry Matter Digestibility and Digesta Dry Matter Content

Dry matter content of the digesta from the distal rectum was 22.5 ± 1.3 % and did not differ among dietary treatments (data not shown). Ileal and rectal apparent dry matter digestibilities of the diets were 81.3 ± 2.5 % and 89.7 ± 2.0 %, respectively (Figure 3). Ileal apparent dry matter digestibility did not differ among the CNTL, TG, TG+PL, and EFAD piglets, but the PL piglets had a lower ileal apparent dry matter digestibility than the CNTL and TG piglets ($P < 0.01$).

Liver Biochemistry

Plasma alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were used as indicators of potential liver damage. All formula-fed piglets and the sow-reared piglets had similar ALT activities that were lower than those of the

initial piglets (Figure 4, $P < 0.01$). AST was higher in the initial piglets compared to the CNTL, TG, TG+PL, and EFAD piglets ($P < 0.003$), but in initial piglets, this activity did not differ from PL piglets. PL piglets had higher AST activity than the TG+PL piglets and also the sow-reared piglets ($P < 0.02$). Relative liver weights were not different in CNTL, TG, TG+PL, and PL piglets (3.2 ± 0.1g/100 g body weight; data not shown). EFAD piglets had greater relative liver weights than all other groups (3.5 ± 0.1g/100 g body weight, $P < 0.03$), while the sow-reared piglets had the lower relative weights than all other treatments (2.5 ± 0.1g/100 g body weight, $P < 0.002$). Gross liver histology (**Table 3**) showed that sow-reared piglets had moderate lipid and glycogen containing vacuoles compared to essentially none found in the formula-fed piglets ($P < 0.01$). All treatments had signs of both extramedullary hematopoiesis and hemosiderin, but differences were not detected between treatments ($P = 0.6$). Crude protein percentage of the liver (**Table 4**) did not differ among treatments ($P > 0.06$). Liver lipid did not differ among the CNTL piglets and the LCPUFA supplemented groups. The percentage of liver lipid was higher in the EFAD and initial piglets than in the CNTL, TG and PL piglets ($P < 0.005$). Piglets in the TG+PL group had similar percentages of liver lipid with the CNTL, TG and PL, sow-reared and initial piglets, but had lower liver lipid compared with the EFAD piglets ($P < 0.005$).

Plasma Fatty Acids (Table 5)

Piglets that were fed the LCPUFA triglyceride source alone (TG piglets) had higher plasma levels of both AA and DHA than the CNTL piglets ($P < 0.05$, Figure

5). All three groups that were fed the preformed AA (TG, TG+PL, and PL) had similar plasma AA concentrations that were much higher than those of the EFAD piglets ($P < 0.0001$), but not different from the sow-reared piglets. The TG, TG+PL and sow-reared piglets did not differ from the initial piglets in plasma AA levels. Plasma DHA levels were higher in the TG and TG+PL piglets than in the sow-reared piglets ($P < 0.03$), but did not differ among piglets given LCPUFA supplemented formulas and initial piglets. Although the groups differed in several plasma fatty acid levels, (Table 5), especially the EFAD group, the focus of this study concerned efficacy of different sources of LCPUFA.

Blood Biochemistry

Blood urea nitrogen (BUN) concentrations did not differ among of the formula-fed groups. Sow-reared piglets had lower BUN (5.5 ± 1 mol/L) and initial piglets had much higher BUN (23.0 ± 1 mol/L) than the formula-fed piglets (12.3 ± 1 mol/L, $P < 0.0002$, data not shown). Plasma cholesterol levels were higher in the sow-reared piglets than in all other groups ($P < 0.03$) while the initial piglets had the lowest plasma cholesterol levels ($P < 0.03$). The CNTL, TG, TG+PL and PL piglets did not differ in plasma cholesterol (data not shown). Plasma glucose did not differ among the CNTL, EFAD, TG and TG+PL piglets ($P > 0.08$), but was lower in the PL piglets than in the CNTL, TG and TG+PL piglets ($P < 0.04$, data not shown).

DISCUSSION

Over the last decade, much research has focused on the need for inclusion of LCPUFA, AA and DHA into infant formulas in the United States using both animal models such as the piglet (13, 15, 16, 27-29), as well as human clinical trials (13, 15, 16, 27, 28). The piglet model has proven to be an appropriate and useful tool when making comparisons to the human infant. The interest in supplementation stems from these LCPUFAs being important to perinatal retinal and central nervous system growth and development. As stated previously, the composition of human milk has a triglyceride content of about 98% of total lipid, while only about 0.8% is phospholipid (6). To date there have been few comparisons of the two sources, triglyceride and phospholipid, especially with regard to the gastrointestinal tract and how the sources are digested and absorbed.

In this study, our primary objective was to investigate the utilization of novel algal and fungal triglyceride sources for the supplementation of the LCPUFA, AA and DHA in infant formulas, and to perform a rigorous evaluation of the safety and efficacy of these substances, as well as a comparison with the phospholipid source. After 16 days of supplementation we determined that neither source of LCPUFA affected piglet growth rate, formula intake, plasma cholesterol, or BUN. Our data are similar to previously reported studies in that growth rates and intakes were consistent for both the control and the LCPUFA-supplemented groups (16, 28), and plasma cholesterol and BUN levels were similar to the results published by Huang et al. (30).

To date, no other study evaluating either the triglyceride or phospholipid source of LCPUFA together or separately has investigated their effects on small intestine health. Small intestinal morphology has typically been used as an estimate of intestinal health in pigs (31-34). Lopez-Pedrosa et al. (35) used dietary phospholipids to speed repair of the small intestine in malnourished piglets. It is difficult, however, to compare these results to ours because the piglets were undernourished and had different jejunal and ileal morphology than the piglets in our study. Also, the levels of AA and DHA were lower than used in the present study, and the phospholipid load was 2.5 % of the formula, which is slightly less than the 2.7 % of the formula that was used in the present study. Amate et al. (16) measured the lipid composition of the jejunum mucosa and found no differences between the TG and PL sources of LCPUFA. In the current study, we found no differences in jejunal villi height across any of the formula-fed pigs compared to the sow-reared piglets. However, ileal villi height in the PL-supplemented piglets was 40 % shorter than in the CNTL piglets, whereas the TG and TG+PL piglets did not differ from the CNTL piglets. This villi shortening is associated with a decreased absorptive area for the PL piglets. The sow-reared piglets had greater ileal villi height than the PL, TG+PL, and EFAD piglets. Jejunal crypt depths showed similar results as the sow-reared piglets had less crypt depth than the PL or TG+PL piglets; however, these changes were small and probably not physiologically important. Due to the limited research that has been conducted on intestinal morphology when comparing the TG and PL sources of LCPUFA, there are no data for comparison with these results.

However, values reported for the CNTL, TG and sow-reared piglets are similar to other research conducted in our laboratory evaluating intestinal morphology in piglets that ate *ad libitum* (25).

Intestinal lactase activity is high at birth and reaches maximum activity at approximately one week of age in piglets (36-38). In the current study, the supplementation of LCPUFA in the form of triglyceride or phospholipid did not affect lactase specific activity. Comparatively, the sow-reared piglets had 1-2 fold higher lactase specific activity compared to all other formula-fed treatments. The inability of diet to affect lactase activity is well documented (39-42). However, the decrease in lactase specific activity seen in the formula-fed piglets compared to the sow-reared piglets could be due to environmental factors and/or differences between sow milk and milk ingredients derived from bovine sources.

The apparent ileal and rectal dry matter digestibilities of the diets was greater in the TG piglets (84.7 $\pm$ 2 %) compared to the PL piglets (76.5 $\pm$ 2 %). No other studies have evaluated the dry matter digestibility of diets containing LCPUFA, but we found that the addition of the phospholipid source of AA and DHA decreased the rectal apparent dry matter digestibility compared to all other treatments ($P < 0.04$). The TG+PL piglets had similar ileal dry matter digestibility to the PL piglets, but rectal dry matter digestibility of the TG+PL piglets was similar to the CNTL and TG piglets in that it was higher than in the PL piglets. Amate et al. (43) measured fat apparent absorption in rats after feeding two phospholipid LCPUFA sources. Compared with a fish oil triglyceride source, the pig brain phospholipid source was

absorbed less, but when both LCPUFA sources were from egg, the phospholipid did not differ from the triglyceride source. Thus, they concluded that the absorption of a phospholipid or triglyceride source depends on the characteristics of the individual fat source. Carnielli et al. (44) measured absorption of triglyceride and phospholipid LCPUFA in supplemented formulas as well as in preterm breast milk in preterm infants. They found better absorption of the DHA from the phospholipid source compared with either the triglyceride source or preterm breast milk, but no difference in AA absorption among the three groups. The discrepancy between the difference in digestibility and/or absorption of the two different sources of LCPUFA may be due in part to a difference in the gastrointestinal tract maturity, which is much less in the premature infant compared to the term piglet (45). Gastrointestinal tract maturity of pre-term and term piglets, however, was not examined in the current study.

Total plasma lipid AA and DHA concentrations were reflective of the inclusion of LCPUFA in the diets of the TG, TG+PL and PL piglets. The rise in plasma AA and DHA after supplementation is similar to what has been seen previously in preterm infants fed formulas with increasing levels of added AA and DHA from the algal/fungal triglyceride sources (46). In our study, there were no differences in plasma DHA or AA detected among any of the LCPUFA-supplemented groups. The TG piglets had a greater percentage of plasma AA and DHA compared to both the CNTL and the EFAD piglets. However, the TG+PL and the PL piglets did not differ from the CNTL piglets, suggesting that the dietary phospholipid load in the PL and TG+PL formulas decreased the absorption of the LCPUFA. Amate et al. (16) found

that in piglets, the triglyceride source of LCPUFA increased the AA and DHA in LDL phospholipids, but that the phospholipid source increased the AA and DHA in HDL phospholipids, suggesting that the two sources might follow different transport pathways. Other studies in human infants that examined either triglyceride (13) or phospholipid (15, 47) sources found an increase in plasma and/or erythrocyte AA and DHA compared to a non-supplemented group, but few studies have compared the two sources to one another (16, 44).

The liver is a metabolically sensitive organ that may be examined to determine potential side effects of the metabolism of different sources of LCPUFA. Limited research has focused on the liver with the exception of Huang et al. (30) who measured liver weights, both absolute and relative to individual body weight, and also liver histology in piglets fed increasing levels of the algal and fungal triglyceride sources of LCPUFA. As seen in the Huang et al. (30) study, LCPUFA-supplemented piglets had similar liver weights compared to the CNTL piglets. The sow-reared piglets had the lowest liver weights, which may be due to their lower intakes compared to the formula-fed groups. Liver histology did not differ between the LCPUFA-supplemented piglets or when compared to the CNTL piglets, again similar to the results of Huang et al. (30). The sow-reared piglets had a greater frequency of both lipid and glycogen containing vacuoles in the liver compared with all other treatments, which could be indicative of the high level of fat in sow milk. The proportion of liver crude protein did not differ among treatments, but lipid content varied. The CNTL, TG and PL piglets had the lowest lipid percentage, while

the initial piglets and the EFAD piglets had the highest lipid percentage. All treatments were fed similar levels of fat, but the metabolism of short and medium chain saturated fatty acids is quite different than that of LCPUFA and thus might explain the increase in liver lipid in the EFAD piglets. Other effects of essential fatty acid deficiency, such as a change in lipid transport out of the liver, also may have altered liver fat content. Two other markers of putative cellular damage are the enzymes ALT and AST. There were no differences in ALT; however, AST was greater in PL piglets than in TG+PL piglets. There are several factors that could explain this increase in AST, such as intestinal injury; PL pigs experienced villi shortening during the study period. The AST levels in the PL piglets did not differ from that found in the CNTL or the TG piglets, but AST was higher than the sow-reared piglets. Huang et al. (30) also measured these enzymes, and reported no difference between treatment groups, but the data only represent the algal/fungal TG sources of the LCPUFA. Therefore, further study of the liver enzymes is needed to determine the reason for the elevated AST in the PL group.

In conclusion, the results of this study show that the TG form of the LCPUFA, AA and DHA may be more efficacious than the PL source based on increases in both plasma AA and DHA concentrations compared with CNTL piglets. The TG sources may also be more appropriate based on effects of the PL source on small intestinal morphology, apparent dry matter digestibility, and elevated AST levels. The purpose of this study was to investigate these novel sources of supplemental AA and DHA and from the variables measured, the TG sources appear to be safe

and efficacious for use in human neonates as demonstrated using the piglet model. Supplementation of infant formula with the triglyceride source of AA and DHA also helps to maintain the overall balance of triglyceride and phospholipid in formula that is typically found in human milk.

1

Table 1. Composition and calculated analysis of the formula diets fed to piglets, comparing triglyceride and phospholipid sources of AA and DHA.[1]

	Diet[2]				
Ingredient	CNTL	TG	TG+PL	PL	EFAD
			g/kg		
Mead Johnson Oil Blend[3]	180	182	182	97	0
Martek ARASCO (AA)[4]	0	4.4	4.4	0	0
Martek DHASCO (DHA)[4]	0	2.1	2.1	0	0
Ovothin 120 (AA & DHA)[5]	0	0	0	85	0
Soybean Oil	110	101.5	69	105	0
Flaxseed Oil	0	0	0.8	3	0
Coconut Oil (non-hydrogenated)	0	0	0	0	290
Powdered Lecithin[6]	0	0	34	0	0
Choline Chloride	0	0	10.6	0	0
Cholesterol	0	0	4.5	0	0
Sodium Caseinate[7]	128	131	131	128	128
Whey Protein Concentrate[8]	86	89	89	86	86
Skim Milk[9]	388	373	373	388	388
Lactose	50	59	37	55	50
$CaCO_3$	5	5	8.7	8.5	5
Dicalcium Phosphate	27	27	27	19	27
Mineral Premix[10]	5	5	5	5	5
Vitamins Premix[11]	1.3	1.3	1.3	1.3	1.3

Table 1, continued

Lysolecithin	10	10	10	10	10
Xanthan Gum	10	10	10	10	10
Calculated Analysis[12]	CNTL	TG	TG+PL	PL	EFAD
ME, kJ/kg[13]	5053	5053	4977	5067	4783
Fat, g/100 g	30	30	30	30	30
Crude Protein, g/100 g	32	32	32	32	32
Lactose, g/100 g	26	26	24	25	26
Calcium:Phosphorus	1.4	1.4	1.4	1.4	1.4
Phospholipid, g/100 g	0.2	0.2	2.7	2.7	0
Triglyceride, g/100 g	29.2	29.2	26.2	26.1	29.4
Cholesterol, g/100 g	0	0	0.43	0.43	0
Phosphotidylcholine, g/100 g	0	0	1.9	1.9	0
Phosphatidylethanolamine, g/100 g	0	0	0.7	0.5	0
Ratio of LA:LN	7.7	7.6	7.7	7.7	--
Calculated g/100 g of Total Fat					
LA	30.3	29.0	29.9	29.9	1.3
LN	3.9	3.8	3.9	3.9	0
AA	0	0.6	0.6	0.6	0
DHA	0	0.3	0.3	0.3	0

[1] Expressed on an air-dry weight basis

[2] Diet groups are: control, CNTL; triglyceride, TG; triglyceride with added phospholipid, TG+PL;

phospholipid, PL; essential fatty acid deficient, EFAD.

[3]Mead Johnson Oil Blend of palm olein, soy, coconut and high oleic sunflower oils (Mead Johnson Nutritionals, Evansville, IN 47721)

[4]Martek ARASCO® and DHASCO® (Martek Biosciences Corporation, Columbia, MD 21045)

[5]Ovothin 120 (Lucas Meyer, Inc, Decatur, IL 62524)

[6]Powdered Soy Lecithin (Alcolec F100, American Lecithin Company, Oxford, CT 06478)

[7]Sodium Caseinate (International Ingredient Co., St. Louis, MO, 63116)

[8]Whey Protein Concentrate (AMP 80, Proliant, Ames, IA 50010)

[9]Skim Milk (Milk Specialties Corp., Dundee, IL 60118)

[10]Mineral premix (Milk Specialties Corp., Dundee, IL 60118) contained 1.002 g/100 g Ca, 0.549 g/100 g P, 0.284 g/100 g Na, 0.040 g/100 g Cl, 2.024 g/100 g K, 0.102 g/100 g Mg, 20,000 µg/g Fe, 200 µg/g Co, 1,850 µg/g Cu, 400 µg/g I, 5,000µg/g Mn, 60 µg/g Se, 23,500 µg/g Zn

[11]Vitamin premix (Milk Specialties Corp., Dundee, IL 60118) contained 33,000,000 IU/kg Vitamin A, 6,600,000 IU/kg Cholecalciferol, 55,000 IU/kg α-tocopherol, 257,400 µg/g Ascorbic acid, 29,983 µg/g D-Pantothenic Acid, 33,069 µg/g Niacin, 8378 µg/g Riboflavin, 5,115 µg/g Menadione, 66 µg/g Biotin, 44,000 µg/g Vitamin $B_{12,}$ 2,038 µg/g Thiamine, 3,996 µg/g Vitamin $B_{6,}$ 2,756 µg/g Folic Acid

[12]Calculated analysis based on analysis provided by companies furnishing product and standard feed tables. (19)

[13]ME, metabolizable energy as estimated from book values and information provided by companies supplying ingredients.

Table 2. Calculated fatty acid composition of diets[1].

Fatty Acid	CNTL	TG	TG+PL	PL	EFAD
			g/100 g diet		
8:0	0.07	0.07	0.07	0.07	0.07
10:0	0.00	0.00	0.00	0.00	1.57
12:0	0.00	0.01	0.01	0.00	13.78
14:0	0.00	0.04	0.04	0.00	5.63
14.1	0.04	0.04	0.04	0.04	0.04
16:0	5.08	5.12	5.48	5.38	2.93
17:1	0.18	0.18	0.18	0.18	0.18
18:0	0.50	0.51	0.54	1.46	0.73
18:1	9.34	9.31	8.92	9.30	2.23
18:2(n-6)	9.09	8.69	8.94	8.98	0.38
18:3(n-3)	1.18	1.13	1.17	1.16	0.00
20:3	0.00	0.02	0.02	0.00	0.00
20:4(n-6)	0.00	0.18	0.18	0.18	0.00
22:0	0.00	0.01	0.01	0.00	0.00
22:6(n-3)	0.00	0.09	0.09	0.09	0.00
24:0	0.00	0.01	0.01	0.00	0.00

[1] Diet groups are defined in Table 1.

1

Table 3. Gross liver histology in neonatal piglets fed supplemental LCPUFA of AA and DHA in the form of either triglyceride or phospholipid.[1, 2]

Diet Group		Lipid in Vacuoles[3]				
	n	None	Scant	Mild	Moderate	Marked
CNTL[a]	10	9	1	0	0	0
TG[a]	10	10	0	0	0	0
TG+PL[a]	10	10	0	0	0	0
PL[a]	10	10	0	0	0	0
EFAD[a]	8	8	0	0	0	0
Sow[b]	13	5	1	0	5	2
Initial[ab]	10	6	1	3	0	0
		Glycogen in Vacuoles[4]				
		None	Scant	Mild	Moderate	Marked
CNTL[a]	10	0	2	7	1	0
TG[ab]	10	0	5	3	2	0
TG+PL[ab]	10	0	3	6	1	0
PL[ab]	10	0	7	2	1	0
EFAD[b]	8	1	5	2	0	0
Sow[c]	13	0	1	4	6	2
Initial[ab]	10	0	3	6	1	0

Table 3, continued

[1] Diet groups are: control, CNTL; triglyceride, TG; triglyceride with added phospholipid, TG+PL; phospholipid, PL; essential fatty acid deficient, EFAD; sow-reared, Sow; initial reference piglets, Initial.

[2] Values are means. Within the first column, treatments lacking common superscripts differ, $p < 0.05$.

[3] Vacuoles containing lipid in the liver.

[4] Vacuoles containing glycogen in the liver.

Table 4. Liver crude protein and fat composition of neonatal piglets fed supplemental LCPUFA of AA and DHA in the form of either triglyceride or phospholipid.[1]

Treatment[2]	n	Crude Protein[3], g/100 g	Fat, g/100 g
CNTL	10	71.5 ± 1.5	12.6 ± 0.8[a]
TG	10	73.5 ± 1.7	12.0 ± 0.8[a]
TG+PL	10	70.2 ± 1.5	13.3 ± 0.8[ac]
PL	10	71.7 ± 1.5	12.0 ± 0.8[a]
EFAD	8	76.1 ± 1.9	16.7 ± 0.9[b]
Sow	13	68.2 ± 1.7	13.3 ± 0.8[ac]
Initial	10	69.8 ± 1.5	15.2 ± 0.8[bc]

[1] Tabulated values are least square means ± SEM. Within a column, means without a common letter differ, $p < 0.05$.

[2] Treatment groups are defined in the footnote to Table 3.

[3] Nitrogen x 6.25

1

Table 5. Plasma fatty acids in neonatal piglets fed supplemental LCPUFA of AA and DHA in the form of either triacylglycerol or phospholipid.

Fatty Acid	CNTL[1]	TG	TG+PL	PL	EFAD	Sow	Initial	Pooled SEM
	g/100 g total fatty acids							
14:0	0.26^{ac}	0.28^{a}	0.21^{ac}	0.16^{ac}	1.53^{b}	0.31^{ac}	0.08^{c}	0.08
16:0	22.34^{ab}	23.23^{ab}	23.61^{ab}	23.59^{ab}	22.02^{a}	24.11^{b}	15.63^{c}	0.8
16:1	0.14^{a}	0.14^{a}	0.11^{a}	0.22^{a}	1.16^{b}	1.04^{c}	0.46^{d}	0.04
16:3	0.37^{a}	0.43^{ab}	0.31^{a}	0.38^{a}	0.53^{b}	0.36^{a}	0.35^{a}	0.04
18:0	20.87^{acd}	19.87^{cd}	21.86^{abc}	22.39^{ab}	23.36^{b}	19.50^{d}	23.62^{b}	0.9
18:1(n-9)	13.47^{a}	13.40^{a}	12.31^{ac}	11.91^{ac}	20.18^{b}	11.63^{c}	19.58^{b}	0.6
18:1(n-7)	1.48^{a}	1.47^{a}	1.70^{ab}	1.89^{bc}	1.65^{a}	1.97^{c}	2.99^{d}	0.09
18:2	28.24^{a}	24.58^{c}	24.09^{c}	25.22^{c}	19.78^{b}	24.90^{c}	17.12^{d}	0.8
18:3(n-6)	0.09^{a}	0.07^{a}	0.08^{a}	0.10^{a}	0.20^{b}	0.04^{a}	0.18^{b}	0.03
18:3(n-3)	0.72^{a}	0.68^{a}	0.54^{bd}	0.65^{ad}	0.47^{b}	0.32^{c}	0.56^{d}	0.04
20:0	0.08^{a}	0.10^{a}	0.12^{a}	0.10^{a}	0.10^{a}	0^{b}	0^{b}	0.03
20:1(n-9)	0.05^{a}	0.1^{ab}	0.05^{a}	0.07^{a}	0.08^{a}	0.06^{a}	0.14^{b}	0.02
20:2	0.34^{a}	0.36^{a}	0.30^{a}	0.37^{a}	2.34^{b}	0.31^{a}	0.31^{a}	0.06
20:3	0.30^{a}	0.35^{a}	0.32^{a}	0.28^{a}	0.72^{b}	0.58^{c}	1.48^{d}	0.04
20:4(n-6)	7.49^{a}	9.85^{b}	9.72^{ab}	8.56^{ab}	3.44^{c}	9.56^{ab}	11.91^{d}	0.8
20:5	0.21^{ad}	0.24^{ab}	0.24^{ab}	0.30^{b}	0.51^{c}	0.14^{d}	0.28^{a}	0.03
22:0	0.09^{ad}	0.12^{ab}	0.15^{b}	0.03^{c}	0.08^{ad}	0.07^{ac}	0.05^{cd}	0.02

Table 5, continued

22:4	0.25[a]	0.31[a]	0.25[a]	0.25[a]	0.19[a]	0.85[b]	0.58[c]	0.05
22:5	1.00[ac]	0.77[a]	0.85[a]	0.84[a]	0.32[b]	1.20[c]	0.72[a]	0.1
22:6(n-3)	1.76[ad]	3.02[b]	2.72[ab]	2.13[abd]	0.58[c]	1.67[d]	2.37[abd]	0.3
24:0	0.07[a]	0.08[ab]	0.06[a]	0.04[a]	0.06[a]	0.18[b]	0.05[a]	0.03
24:1	0[a]	0.06[abd]	0.03[abd]	0.09[bd]	0.02[abd]	0.19[c]	0.08[d]	0.03
Other[2]	0.06	0.11	0.07	0.06	0.27	0.63	0.94	0.02

[1] Treatment groups are defined in the footnote to table 3. Within a row, means without a common letter differ, $p < 0.05$.

[2] Trace amounts of 14:1, 15:0, 15:1, 20:3n3, 22:1, 22:2, 22:3 were also detected in some of the samples.

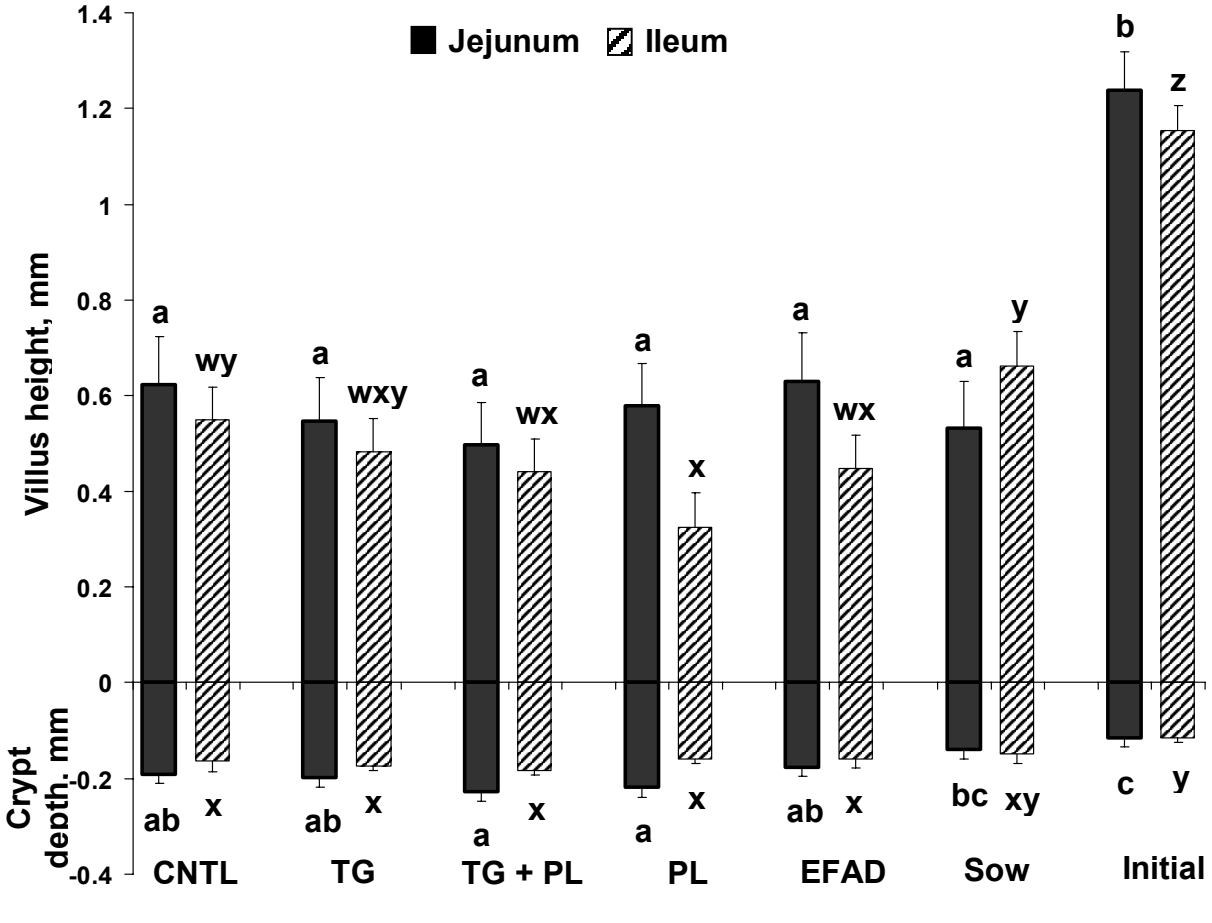

Figure 1. Villus heights and crypt depths of pigs fed either triglyceride or phospholipid sources of AA and DHA for 16 d. Treatment abbreviations are defined in the footnote to Table 3. Values are means ± SEM, n = 8 to 13, Initial piglets were killed prior to treatment initiation at 1 d of age. Bars lacking common letters differ ($P < 0.05$).

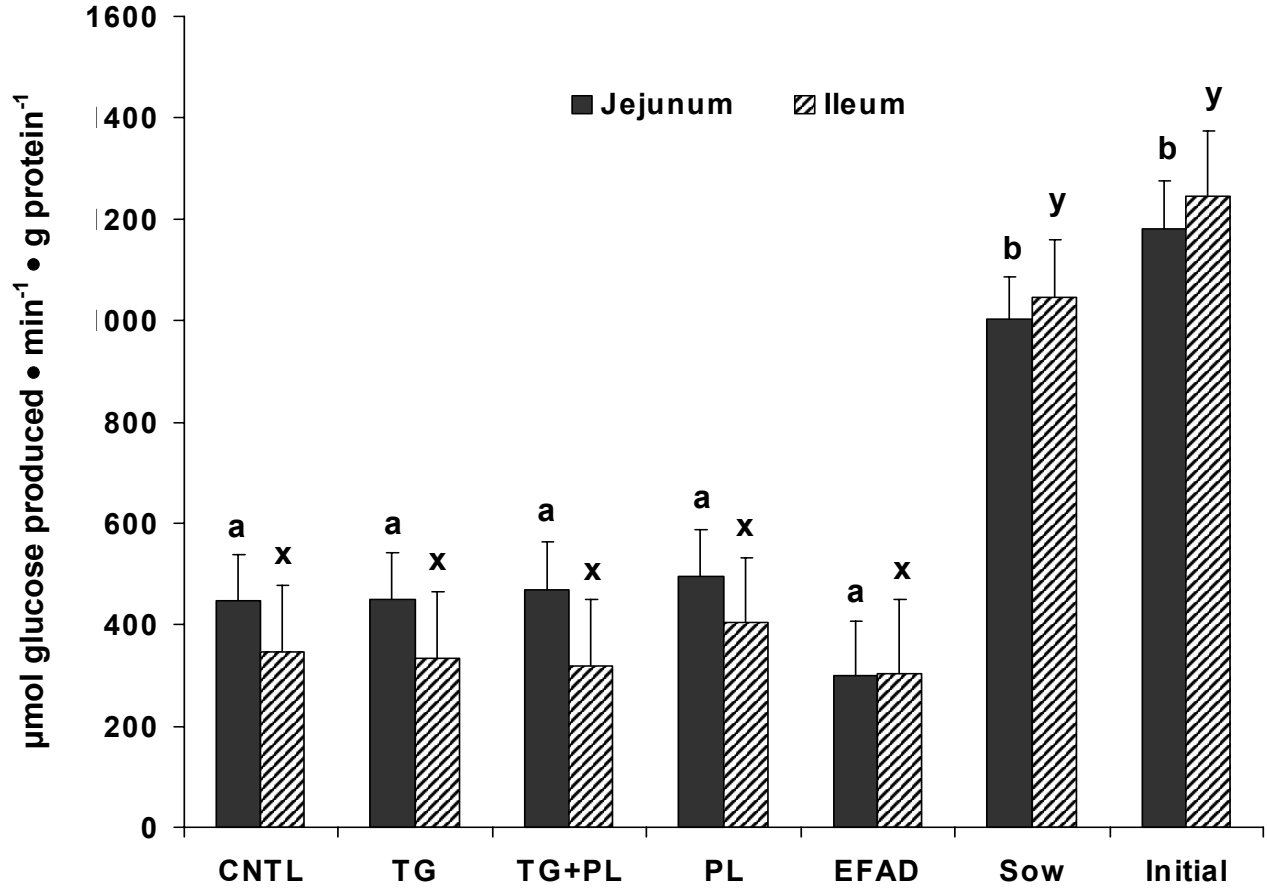

Figure 2. Lactase specific activity in the jejunum and ileum of neonatal pigs fed either triglyceride or phospholipid sources of AA and DHA for 16 d. Treatment abbreviations are defined in the footnote to Table 3. Values are means ± SEM, n=8 to 13. Initial piglets were killed prior to treatment initiation at 1 d of age. Bars lacking common letters differ ($P < 0.05$).

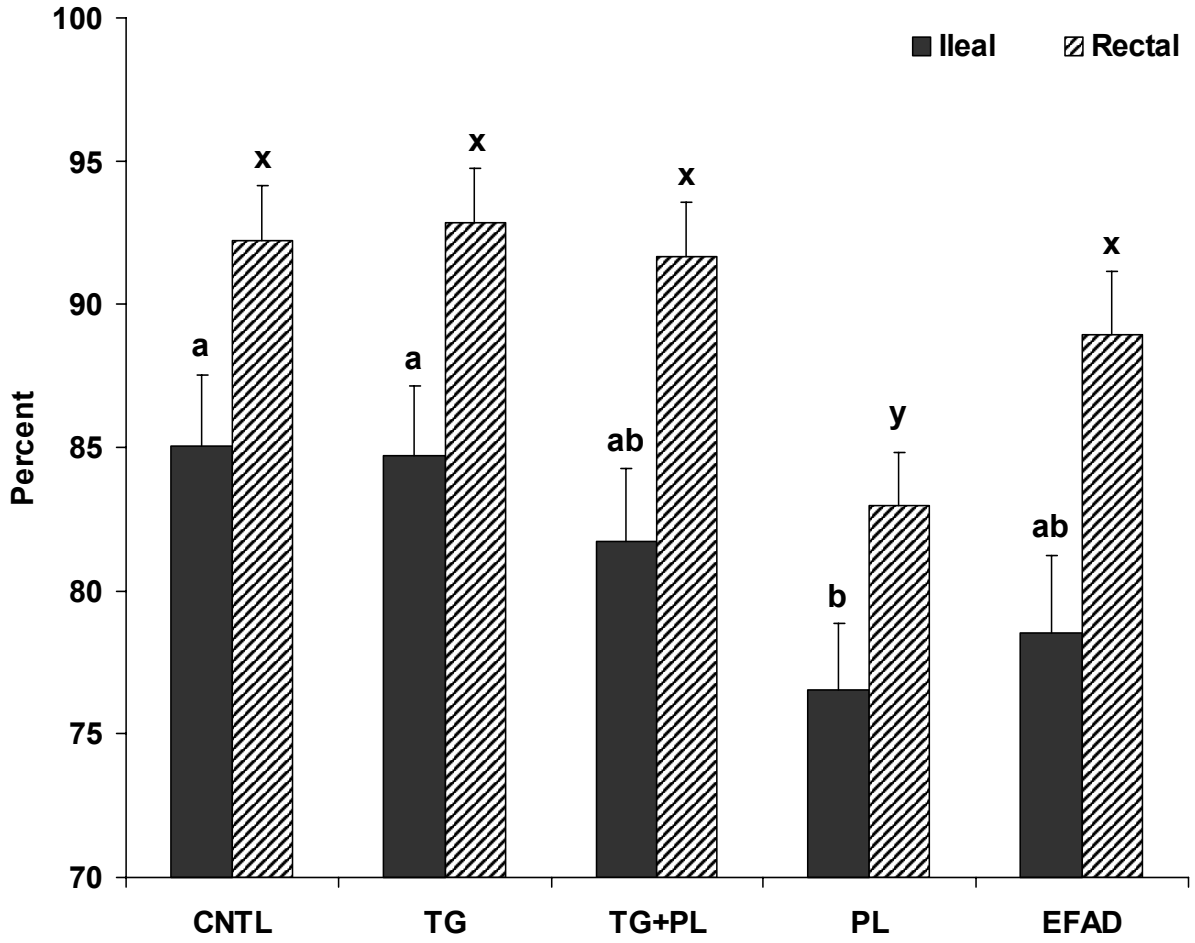

Figure 3. Ileal and rectal apparent dry matter digestibilities of neonatal pigs fed either triglyceride or phospholipid sources of AA and DHA for 16 d. Treatment abbreviations are defined in the footnote to Table 1. Values are means ± SEM, n=8 to 10. Bars lacking common letters differ ($P < 0.05$).

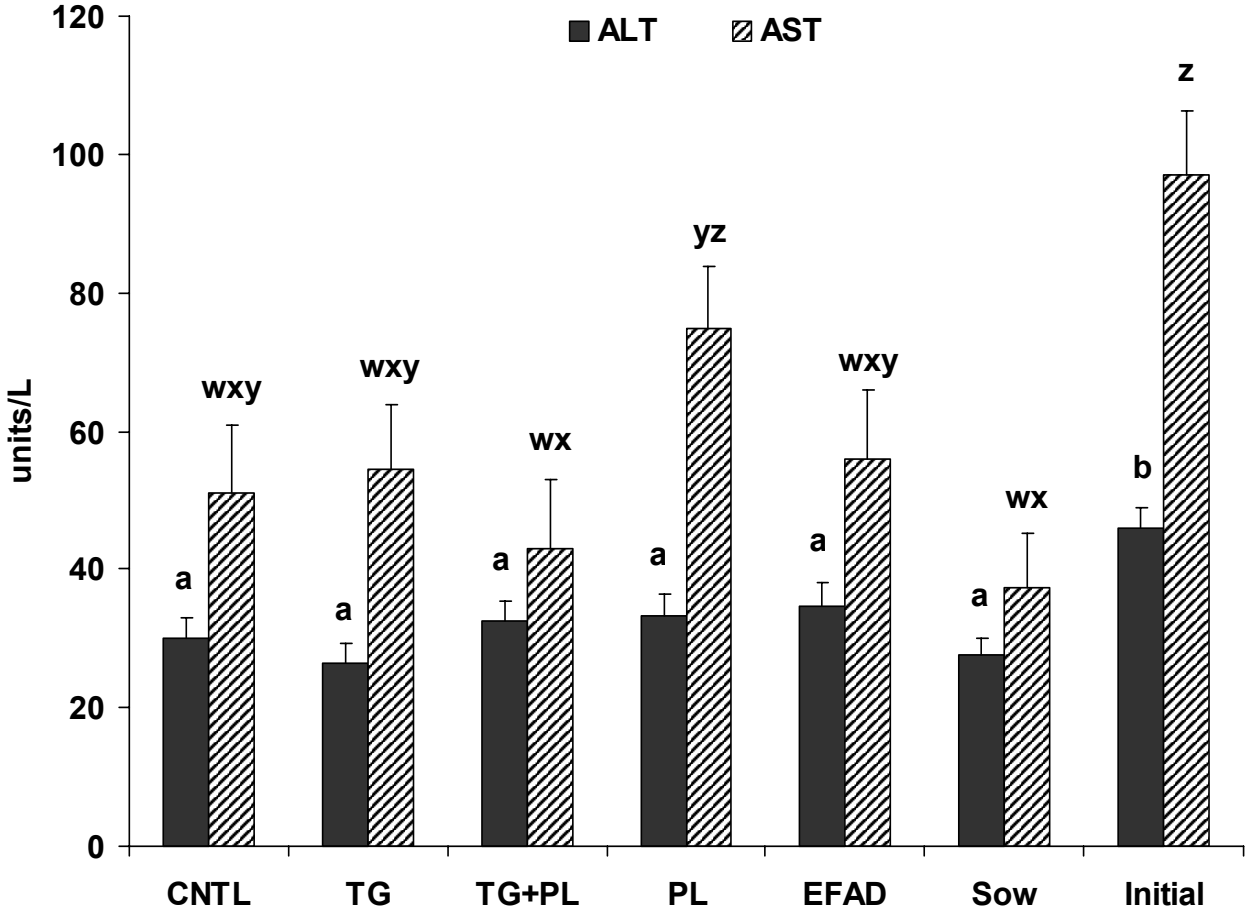

Figure 4. Alanine aminotransferase (ALT) and aspartate aminotransferase (AST) values in neonatal pigs fed either triglyceride or phospholipid sources of AA and DHA for 16 d. Treatment abbreviations are defined in the footnote to Table 3. Values are means ± SEM, n=8 to 13. Initial piglets were killed prior to treatment initiation at 1 d of age. Bars lacking common letters differ ($P < 0.05$).

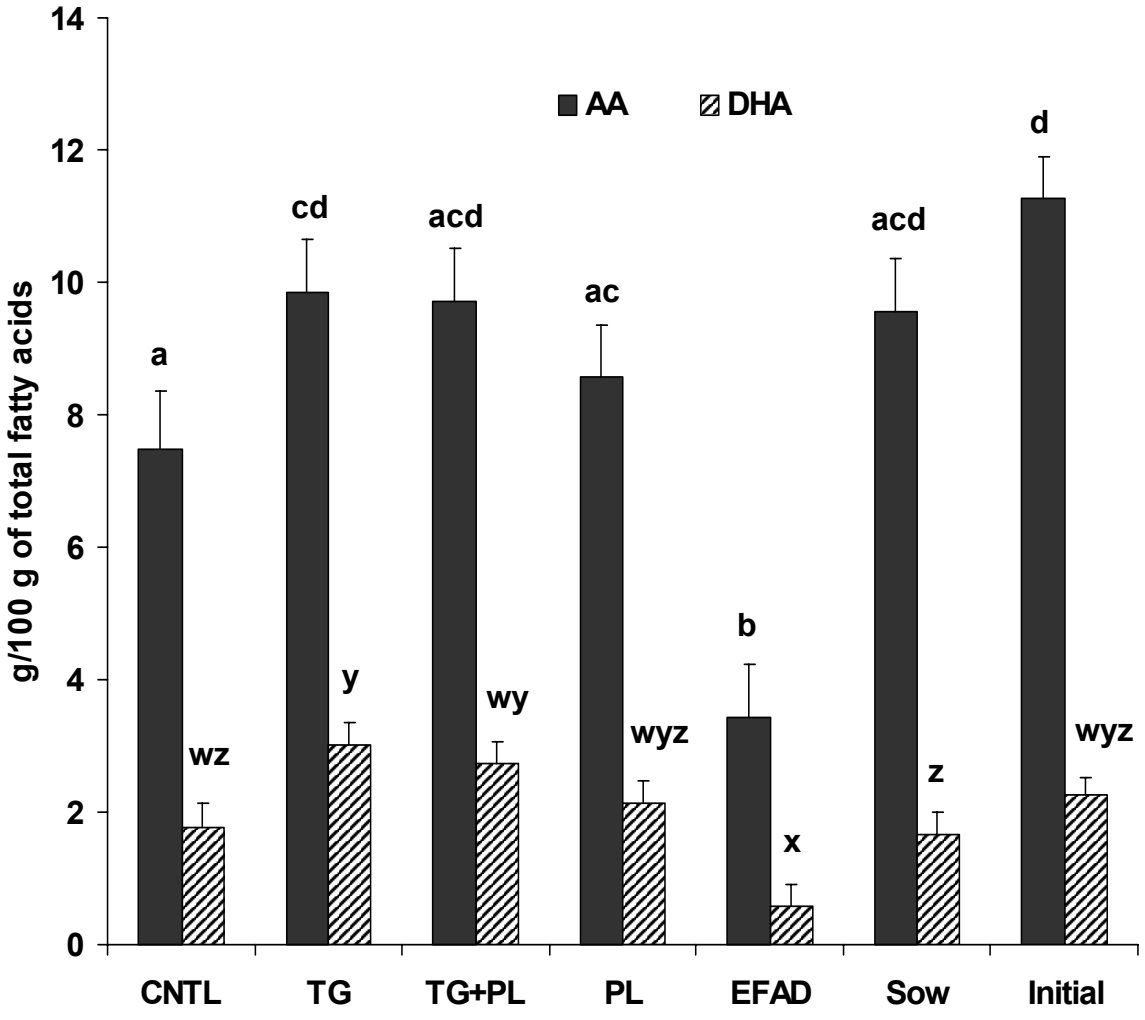

Figure 5. Arachidonic acid (AA) and docosahexaenoic acid (DHA) concentrations expressed as g/100 g total plasma lipid fatty acids in neonatal pigs fed either triglyceride or phospholipid sources of AA and DHA for 16 d. Treatment abbreviations are defined in the footnote to Table 3. Values are means ± SEM, n=8 to 13. Initial piglets were killed prior to treatment initiation at 1 d of age. Bars lacking common letters differ ($P < 0.05$)

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CHAPTER 3

THE EFFECT OF LONG-CHAIN POLYUNSATURATED FATTY ACIDS (LCPUFA) SOURCE ON BODY COMPOSITION AND TISSUE ACCRETION RATES IN THE NEONATAL PIG[1,2]

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ABSTRACT

Supplementation of infant formulas with AA and DHA may promote visual and neural development. However, the effects of the LCPUFA, arachidonic acid (AA) and docosahexaenoic acid (DHA) on body composition and tissue accretion rates are not well known. This study was designed to investigate such effects in the neonatal pig. Piglets had *ad libitum* access from d 1 to 16 of age to a skim milk-based formula with different fat sources added to provide 50% of the energy. Treatment groups were: control, no added LCPUFA (CNTL), algal/fungal triglyceride oils (TG), egg phospholipid (PL), TG plus phospholipid (soy lecithin source) added to match phospholipid treatment (TG+PL), and essential fatty acid deficient (EFAD). Formulas with LCPUFA provided 0.6 and 0.3 g/100 g total fatty acids as AA and DHA, respectively. A group of 10 piglets remained on the sow for the duration of the study (Sow). Growth did not differ between any of the treatments except for on d 16 when TG was heavier than the EFAD fed piglets ($P<0.05$). Accretion of fat was greatest in the sow-reared piglets ($P<0.0001$). TG fed piglets had greater fat accretion than the PL or EFAD fed piglets ($P<0.02$). Protein accretion was greater in TG, TG+PL and PL fed piglets compared to EFAD and Sow piglets ($P<0.03$). Water accretion was greater in pigs fed TG, TG+PL, PL and CNTL compared to Sow and EFAD piglets ($P<0.05$). TG fed piglets had a higher accretion rate of AA and DHA compared to the CNTL ($P<0.001$), but rates of accretion of AA and DHA did not differ between the TG, PL or TG+PL fed piglets ($P<0.09$). These data demonstrate that the TG source of LCPUFA may be more efficacious due to the increased

retention of AA and DHA over the CNTL group, whereas the PL source remained similar to the CNTL fed piglets.

INTRODUCTION

Linoleic acid [LA; 18:2(n-6)][4] and α-linolenic acid [LN; 18:3(n-3)] are essential fatty acids (EFA) required for normal growth and development. Maturity of the retinal and central nervous system tissue in the infant are dependent upon the provision of AA and DHA either directly in the diet or through synthesis from LA and LN (1). The EFAs, LA and LN, are primarily found in plasma or as storage lipids in the body, while AA and DHA, the EFA metabolites, are major components within the phospholipid membrane of cells (2). Premature infants may be at increased risk for a deficiency of AA and DHA because intrauterine accretion of these fatty acids occurs largely during the third trimester of pregnancy (3).

Commercial infant formulas that are available in the US have a pattern and content of LA and LN that are similar to breast milk. However, until recently, most formulas did not contain any preformed AA or DHA. Data from infants given labeled LA and LN, indicate that conversion of LA and LN to AA and DHA, respectively, by the desaturation-elongation pathway may not be sufficient to support the needs of growing infants (4). Presently there are two primary types of pre-formed AA and DHA that may be used as supplemental sources of LCPUFA in infant formulas; a triglyceride source that is produced from single cell microorganisms (AA from fungi and DHA from microalgae) and a phospholipid source that is extracted from egg yolk

oil. The differences in absorption and metabolism of these two sources have received little attention to date. Recently published data from our laboratory indicates that the triglyceride source may be a more efficacious form of supplementation in infant formula due to the increase in plasma AA and DHA and the similarity of high digestibility to the control formula (5). However, the PL source resulted in reduced ileal villi length and a lower digestibility than the control or the triglyceride source (5). Amate et al. (2001) (6) compared the two sources of LCPUFA, egg phospholipid vs. triglyceride, in piglets and reported no differences in lipid composition of the jejunal mucosa between the triglyceride and phospholipid sources. However, the egg phospholipid source increased AA and DHA in the high density lipoprotein phospholipid, while the triglyceride source increased AA and DHA in the low density lipoprotein phospholipid.

The metabolism of the long chain polyunsaturated fatty acids (LCPUFA) within the whole body and specific tissues such as the brain is an area of research that has not been expanded fully. Due to the limitations of both practical and ethical considerations, conducting experiments to determine whole body accretion rates of essential fatty acids is not available in the human infant. However, through the use of animal models predictions of requirements for the human infant can be inferred. Cunnane et al. (2000) (7) who published a commentary from the existing literature that focused on the accumulation of DHA in both brain and whole body tissue, estimated that formula-fed infants accumulate only half of the DHA that breast-fed infants accrete over the first six months of life. Therefore, supplementation with

DHA in conjunction with AA is thought to be necessary to support proper growth and development during this early and rapid growth phase of life. Brain tissue from postmortem infants in the United Kingdom and Australia fed formulas lacking AA and DHA had lower levels of DHA than infants fed breast milk (8-10). Formulas supplemented with AA and DHA fed to infants results in improved visual acuity, better neurodevelopment, and a lower incidence of necrotizing enterocolitis (11-13).

The piglet has proven to be a suitable model for comparison to the human infant when studying lipid nutrition. The piglet has many similarities with human infants including development of the intestine, fat digestion and absorption, and also many of the pathways of lipid metabolism (14). Neonatal growth in the piglets is much more rapid compared to the human infant and therefore provides a sensitive model to better understand and investigate the effects of LCPUFA on whole body composition and tissue accretion of specific fatty acids. The purpose of this study was to examine the effects of algal/fungal triglyceride oils in comparison to an egg phospholipid source on whole body composition and essential fatty acid tissue accretion rates, and to determine the efficiency with which piglets utilize these LCPUFA.

MATERIALS AND METHODS

Animal Care

General. The Institutional Animal Care and Use Committee of North Carolina State University (NCSU) approved all procedures. All animal care and animal

feeding procedures were described previously, and dietary treatment groups were the same as described previously in Chapter 2, Table 1 (5). Formulas with LCPUFA (TG, TG+PL, PL) provided 0.6 g/100 g of fatty acid as AA and 0.3 g/100 g as DHA. Analyzed fatty acid composition of the diets is presented in **Table 1.** Another 13 piglets from 2 litters remained with the sows for the duration of the study (Sow). At the end of the study, piglets were killed with an AMVA-approved electrocution followed by exsanguination (laceration of the brachiocephalic arteries) and tissues collected. An initial group of 10 piglets from 5 litters was also used to establish a baseline for fatty acid composition so that individual fatty acid accretion could be computed.

Sample Collection and Analytical Procedures

Performance. Formula intake was determined gravimetrically on a daily basis and all pigs were weighed daily.

Body Composition and Fatty acid analysis. A total of 6 pigs / treatment were used to determine body and fatty acid composition. The whole body was ground and thoroughly mixed (TorRey model M22-R-2 using a 15.9 mm plate (TorRey, TOR 12P 5/8) once and then a 4.8 mm plate (TorRey, TOR 12P 3/16) twice more). Subsamples were taken and stored at -20˚C for proximate analysis. Water content was calculated by weight loss after drying at 100˚C for 24 h in a forced-air oven (15). Total body crude protein was determined using the Kjeldahl procedure (15). Total fat was assayed using the Folch procedure (16) and an internal standard (C17:0) was added to each sample prior to tissue homogenization. After fat extraction was

complete, all samples were transmethylated to fatty acid methyl esters (FAME) (17). Fatty acid methyl esters were quantitatively analyzed by gas-liquid chromatography using a Hewlett Packard Agilent 5890-Series II (Delaware) equipped with a flame ionization detector and 6890 Series injector. The FAME were separated on a 100m SP-2380 Fused Silica capillary column (Supelco, Bellefonte, PA: 0.25mm diameter, 0.2μm film thickness) using helium at a flow rate of 2.1 mL/min with a split ratio of 50:1. The chromatographic run parameters included an oven starting temperature of 140°C that was increased at 3.2°C/min to 225°C, and then held for 14 min before increasing to 230°C at 2°C/min, with a final hold of 22 min. The injector and detector temperatures were both constant at 220°C. Peaks were identified by comparison of retention times with external FAME standard mixtures from Supelco (Bellefonte, PA; F.A.M.E. Mix C_4-C_{24}). The fatty acid concentrations were calculated, adjusting for the recovery of the internal standard.

Statistical Analysis

Values presented are means ± standard error of the mean. SAS (SAS Inst. Inc., Cary, NC) Proc GLM procedure was used for statistical analysis appropriate for a completely randomized design. Treatment differences were evaluated using a protected LSD which provided all pair-wise comparisons. Differences were deemed significant when $P < 0.05$.

RESULTS

Performance, Growth and Food Intake

Over the treatment period, there were no differences in piglet body weights among the formula fed groups (data not shown) except for on d 16 when the TG fed piglets were heavier than the EFAD piglets (7599 ± 304 g vs. 6562 ± 340 g, $P < 0.05$). Overall, formula-fed piglets gained 324 ± 17 g/d throughout the study period. The TG fed piglets had a greater ADG than the EFAD and Sow piglets ($P < 0.01$, **Table 2**). Daily feed intake and feed efficiency (g gain/g feed) did not differ among groups for the 16 d period.

Body Composition and Tissue Accretion Rates

Supplementation of LCPUFA as TG did not affect overall whole body protein or water content compared to CNTL piglets ($P>0.1$, **Table 3**). Piglets supplemented with the PL source of LCPUFA had lower body lipid content compared to TG, TG+PL, and CNTL piglets ($P<0.05$) while the sow-reared piglets had higher total body lipid content ($P<0.001$) compared to all other formula fed piglets. All formula fed pigs differed from initial piglets with respect to total body lipid, ash and water content ($P<0.001$). Protein content was lower in the sow-reared piglets compared to the initial piglets ($P<0.001$).

TG fed piglets had greater lipid accretion than the PL or EFAD fed piglets ($P<0.02$), but no differences were detected between the PL and EFAD fed piglets ($P<0.9$). Lipid accretion was greatest in the sow-reared piglets ($P<0.0001$) compared to all other treatments. The supplementation of LCPUFA from either the

TG or PL source increased protein accretion compared to EFAD fed and sow-reared piglets (P<0.03), but were not different from CNTL (P > 0.1). There was a trend for CNTL piglets to have greater protein accretion compared to the EFAD or Sow piglets (P<0.08). Water accretion was greater in TG, TG+PL, PL and CNTL compared to sow-reared and EFAD piglets (P<0.05). Ash accretion was greater in the TG fed piglets compared to the EFAD fed pigs (P < 0.02), but similar to all other groups (P > 0.1).

Accretion of Essential Fatty Acids

Daily accretion rates (**Table 4**) and total accretion of LA (Figure 1) and LN (Figure 2) were similar between CNTL, TG, TG+PL, PL and Sow-reared piglets (P>0.1). Piglets fed the EFAD diet had lower total accretion rates and total accretion of both LA (Figure 1) and LN (Figure 2) compared to all other treatment groups (P<0.001). Total accretion of AA (Figure 3) and DHA (Figure 4) was variable among treatment groups.

The source of LCPUFA supplementation did not alter AA or DHA accretion. TG fed piglets had greater AA accretion than CNTL and Sow-reared piglets, but surprisingly EFAD fed piglets had similar AA accretion as TG fed piglets. CNTL and Sow-reared piglets had lower DHA accretion than LCPUFA supplemented groups, but surprisingly EFAD fed piglets accreted an intermediate amount of DHA and were not different from any other treatment group.

Because of the fat source (coconut oil) that was used in the EFAD diet, piglets in this group had higher accretion rates of 16:1 compared to all other formula

fed groups (P<0.01). Sow-reared piglets had much greater accretion rates of 16:0, 16:1, 18:0 and 18:1 than all other formula fed piglets (P < 0.01).

Accretion Efficiency

Efficiency of dietary LA/18:2 utilization for LA/18:2 accretion averaged 0.24 ± 0.04 and was not different among the formula fed pigs (P>0.1, **Table 5**). However, the minimal LN/18:3n3 intake of the EFAD fed piglets was more efficient compared to all other formula fed groups (P<0.05). Within the LCPUFA supplemented groups, no differences were detected in the efficiency of use of either AA/20:4 (P>0.2) or DHA/22:6 (P>0.4).

DISCUSSION

Due to the importance of LCPUFA in early human development, much research has examined the need for inclusion of AA and DHA into infant formulas in the United States using both animal models such as the piglet (6, 18, 19), as well as human clinical trials (11, 13, 20). The interest in supplementation stems from these LCPUFAs being important to perinatal retinal and central nervous system growth and development. The piglet model has proven to be an appropriate and useful tool when making comparisons to the human infant. Previously published piglet data suggested that the TG source of LCPUFA is more efficacious than the PL source based on increased plasma fatty acid concentrations of AA and DHA, and increased digestibility of the TG source (5). During 16 days of supplementation, the source of LCPUFA did not affect piglet growth rate or formula intake (5). Our data are similar

to previously reported studies in that growth rates and intakes were similar for control and the LCPUFA-supplemented groups (6, 18).

A study involving body composition and the determination of total body accretion and the efficiency of accretion of LCPUFA has limitations in a clinical setting. Some of the first data on the chemical composition of the human infant was published in the mid 20th century, but provided data based only from small sample sizes (21-25). In an effort to establish a point of reference for others to compare body composition data, Ziegler (1976) (26) published mathematically generated body component values for a "reference fetus". However, fatty acid accretion has not been determined by any of these previous researchers, but has previously been examined in the rodent model. Early studies in rodents fed essential fatty acid deficient diets and reported fatty acid accretion data (27, 28). Since then, studies focusing on the accretion of LCPUFA have been conducted primarily on the brain or retinal tissue of neonatal cadavers (8-10), and not included data on whole body fatty acid accumulation. Neonatal pig carcass chemical composition values have been published by several investigators (29-32), but again, data on total body fatty acid composition of the neonatal pig is limited, and most published data on LCPUFA accretion is for the brain and retinal tissues only (33, 34).

With respect to chemical body composition, values obtained for the initial group of piglets are similar to values reported by Ziegler (1976) (26) for the reference fetus at 40 wk gestation for protein, water and ash, but total body fat was quite different. The human infant accretes a significant amount of body fat *in utero*

during the third trimester (35), but the piglet is born with relatively low body fat stores and accretes lipid rapidly during the first few weeks after birth (36-38). At the conclusion of the trial, total body protein and protein accretion rates were similar in CNTL and all LCPUFA supplemented groups, but less in the sow-reared pigs. Total body lipid and lipid accretion were greatest in the sow-reared pigs compared to the all formula-fed pigs, except the EFAD fed piglets. The high energy/fat content and relatively low protein content in sow's milk accounts for the high rates of lipid gains. It is well known that the sow limits the piglet's growth as found when reared independently of the sow (32, 39, 40). Overall, the higher fat content and accretion of the sow-reared piglets was accounted for by the inherently low amount of amino acid supply compared to the energy content of sow milk (41). Piglets fed the PL diet had lower total body lipid and lipid accretion than the TG fed piglets. The PL source of LCPUFA is less digestible than the TG source which is a potential reason of the lower accretion of LN in the PL fed piglets (5).

When comparing the body content and accretion of the essential fatty acids, LA and LN, all formula-fed groups, except the EFAD fed group accumulated both of the EFA in similar amounts. Sow-reared piglets had similar rates of LA content and accretion as the PL fed piglets, but greater amounts of LN than the PL fed piglets. The lower digestibility of the PL fat source may account for the difference in LN content and accretion. Piglets fed the EFAD diet had lower accretion of both LA and LN compared to all other treatments; however, these piglets did not show any

clinical signs or symptoms of EFA deficiency such as growth retardation or skin lesions (42).

Dietary efficiency of fatty acids differed primarily between the EFAD and all other formula-fed groups. With respect to the LCPUFA, the overall accumulation was surprising in that EFAD fed pigs had higher total body AA content than the CNTL, TG+PL, PL and sow-reared groups without receiving any performed AA. Other published studies investigating LCPUFA metabolism have not included an essential fatty acid deficient group. Due to the fact that these pigs were fed an EFAD diet, conservation of LA and LN appeared to occur which was also seen in a follow-up study that was conducted in our laboratory (see Appendix A). No differences were detected between any of the groups in efficiency of LA accretion, but EFAD fed pigs utilized LN more efficiently than all other formula-fed pigs. The TG source of LCPUFA resulted in greater total accretion of AA compared to the CNTL piglets, but neither the PL source nor the TG+PL resulted in levels of AA above the CNTL pigs. Both LCPUFA sources resulted in DHA accumulation, and accretion of the DHA was greater in the LCPUFA supplemented groups compared to the CNTL and sow-reared groups. Cunnane et al. (2000) (7) estimated that an intake of 20 mg/d of DHA is needed to achieve an accretion rate similar to breast fed infants (10.3 mg/d). The LCPUFA supplemented pigs in the current experiment had intakes of 140 to 300 mg/d which exceeded the estimated intake, and accreted 10 to 15 mg/d of DHA for the study period.

In the current experiment, supplementation of AA and DHA were fed at levels that fell within the range as previously published (6, 18, 43, 44). Supplementation with the TG LCPUFA source increased total body accumulation of both AA and DHA over that of CNTL animals. The CNTL group served as a reference for the unsupplemented formula fed infant, and as expected, there were no increases found in the total body levels of LCPUFA. Conversion rates of LA and LN to AA and DHA, respectively, suggests that the CNTL piglets metabolized all LCPUFA produced from the elongation-desaturation pathway. EFAD fed piglets provided surprising results converting LA and LN in the diet to AA and DHA, and retained these fatty acids; however, the mechanism is unknown. In a follow-up study, it was determined that oxidation of both essential and non-essential fatty acids were reduced in the EFAD fed piglet (see Appendix A).

In conclusion, the results of this study support previously published data from our laboratory that the TG form of the LCPUFA, AA and DHA may be more efficacious than the PL source. LCPUFA supplementation as TG resulted in greater lipid accretion and a tendency to have a greater rate of AA accretion compared to the PL source. Supplementation of infant formula with the TG source of AA and DHA maintains the overall balance of triglyceride and phospholipid in formula that is typically found in human milk.

1

Table 1. Analyzed fatty acid composition of diets.

Fatty Acid	CNTL	TG	TG+PL	PL	EFAD
			g/100 g diet		
8:0	0.02	0.004	0.013	0.02	0.05
10:0	0.15	0.13	0.16	0.10	1.0
12:0	1.65	1.61	1.72	0.94	13.88
14:0	0.79	0.81	0.90	0.52	5.62
14.1	0.01	0.02	0.02	0.03	0.02
16:0	13.93	13.86	16.26	15.78	10.53
16:1	0.042	0.048	0.055	0.186	0.019
18:0	1.10	1.09	1.26	1.52	1.29
18:1	7.27	8.58	8.41	8.09	2.45
18:2(n-6)	7.06	8.11	8.04	7.55	1.30
18:3(n-3)	0.72	0.90	0.99	0.74	0.15
20:0	0.08	0.08	0.09	0.08	0.25
20:1	0.75	0.94	1.03	0.77	0.00
20:2	6.15	3.53	3.31	4.44	0.94
20:3	0.00	0.02	0.02	0.01	0.00
20:4(n-6)	0.00	0.19	0.24	0.12	0.00
20:5	0.00	0.03	0.03	0.02	0.00
22:0	0.09	0.1	0.1	0.1	0.00
22:6(n-3)	0.00	0.09	0.09	0.09	0.00
24:1	0.00	0.00	0.02	0.01	0.00

Table 2. Average daily gain, average daily feed intake and feed efficiency of Piglets fed supplemental LCPUFA of AA and DHA in the form of either triglyceride or phospholipid.[1, 2]

	CNTL	TG	TG+PL	PL	EFAD	Sow	SEM
ADG[3], g	326[ab]	358[b]	333[ab]	326[ab]	293[a]	297[a]	19
ADFI[3], g	201[ab]	223[a]	210[ab]	216[ab]	197[b]	ND[4]	9
Feed Efficiency	1.6	1.7	1.6	1.5	1.5	ND[4]	0.1

[1] Treatment groups are: control, CNTL; triglyceride, TG; triglyceride with added phospholipid, TG+PL; phospholipid, PL; essential fatty acid deficient, EFAD; sow-reared, Sow

[2] Values presented are means ± SEM. Within columns, treatments lacking a common superscript differ, $P < 0.05$.

[3] Average daily gain (ADG); Average daily feed intake (ADFI) on a dry matter basis; Feed efficiency is defined as grams of weight gain per gram of dry feed consumed

[4] ND = not determined

Table 3. Effect of LCPUFA on the composition of the empty body and tissue accretion rates of neonatal pigs.[1, 2]

	Initial	CNTL	TG	TG+PL	PL	EFAD	Sow	SEM
Body Composition, %								
Protein	13.2^{ab}	12.7^{ab}	13.2^{ab}	13.0^{ab}	13.4^{a}	12.2^{bc}	11.5^{c}	0.4
Lipid	2.7^{a}	7.3^{b}	7.6^{b}	7.7^{b}	6.4^{c}	6.9^{bc}	12.8^{d}	0.3
Ash	3.6^{a}	2.6^{b}	2.7^{b}	2.7^{b}	2.7^{b}	2.5^{b}	2.7^{b}	0.1
Water	79.4^{a}	71.5^{b}	71.6^{b}	72.1^{bc}	72.7^{bc}	73.7^{c}	64.7^{d}	0.8
Accretion Rates[3], g/d								
Protein	--	37.9^{ab}	44.9^{a}	39.9^{a}	39.8^{a}	29.4^{b}	29.6^{b}	3.6
Lipid	--	28.4^{acd}	32.6^{d}	30.6^{acd}	24.7^{ac}	24.5^{c}	47.6^{b}	2.6
Ash	--	6.4^{ab}	7.5^{a}	7.3^{a}	6.4^{ab}	5.4^{b}	6.4^{ab}	0.7
Water	--	208.3^{a}	229.5^{a}	215.1^{a}	201.8^{a}	178.1^{b}	161.1^{b}	13.8

[1] Treatment groups are: initial reference piglets, Initial; control, CNTL; triglyceride, TG; triglyceride with added phospholipid, TG+PL; phospholipid, PL; essential fatty acid deficient, EFAD; sow-reared, Sow.

[2] Values presented are means ± SEM, n = 6. Within columns, treatments lacking a common superscript differ, $P < 0.05$.

[3] Accretion rates were calculated as follows for each component: (Ending pig g protein, lipid, ash or water – Initial pig g protein, lipid, ash or water) ÷ 16 days

1

Table 4. Daily accretion of fatty acids from piglets fed supplemental LCPUFA of AA and DHA in the form of either triglyceride or phospholipid.[1, 2]

Fatty Acid	CNTL	TG	TG+PL g/d	PL	EFAD	SOW	SEM
16:0	17.33^{a}	18.03^{a}	17.46^{a}	14.23^{a}	15.73^{a}	32.37^{b}	2.4
16:1 (n-7)	0.22^{a}	0.21^{a}	0.18^{a}	0.26^{a}	0.79^{b}	2.78^{c}	0.14
18:0	1.74^{a}	1.87^{a}	1.83^{a}	1.63^{a}	1.25^{a}	2.81^{b}	0.25
18:1 (n-9)	9.6^{a}	9.97^{a}	8.96^{a}	8.08^{a}	3.64^{b}	14.49^{c}	1.2
18:2 (n-6)	4.41^{a}	5.15^{a}	3.96^{a}	3.99^{a}	0.64^{b}	4.02^{a}	0.6
18:3 (n-6)	0.027^{ab}	0.037^{b}	0.022^{ad}	0.023^{ad}	0.007^{c}	0.012^{cd}	0.004
18:3 (n-3)	0.13^{ac}	0.14^{ac}	0.12^{ac}	0.11^{ac}	0.03^{b}	0.17^{c}	0.02
20:0	0.04	0.01	-0.01	-0.01	0.04	-0.003	0.02
20:1 (n-9)	0.31^{a}	0.38^{a}	0.27^{ac}	0.31^{a}	0.04^{b}	0.16^{bc}	0.05
20:2	3.63^{a}	3.73^{a}	3.88^{a}	3.33^{a}	0.94^{b}	4.16^{a}	0.6
20:3	0.007^{ab}	0.02^{b}	0.017^{ab}	0.005^{a}	0.013^{ab}	0.017^{ab}	0.005
20:4 (n-6)	-0.038^{a}	0.043^{bc}	-0.007^{ac}	-0.005^{ac}	0.06^{b}	-0.018^{a}	0.02
20:5	0.012	0.013	0.008	0.015	0.007	0.012	0.003
22:0	0.02^{abc}	0.023^{ab}	0.027^{a}	0.022^{ab}	0.015^{bc}	0.012^{c}	0.004
22:6 (n-3)	-0.01^{a}	0.015^{b}	0.01^{bc}	0.01^{bc}	0.005^{bc}	-0.005^{ab}	0.005
24:1	0.032^{a}	0.027^{ab}	0.015^{bc}	0.023^{ab}	0.005^{c}	0.020^{abc}	0.006

2 [1] Treatment groups are defined in Table 3.

[2] Values presented are means ± SEM, n = 6. Within columns, treatments lacking a common superscript differ, $P < 0.05$.

[3] Accretion rates were calculated as follows for each component: (Ending pig g fatty acid – Initial pig g fatty acid) ÷ 16 days

1

Table 5. Calculated efficiency of accretion of fatty acids from piglets fed supplemental LCPUFA of AA and DHA in the form of either triglyceride or phospholipid.[1, 2]

Fatty Acid	CNTL	TG	TG+PL	PL	EFAD	SEM
16:0	0.63[a]	0.65[a]	0.53[ab]	0.45[b]	0.82[c]	0.05
16:1 (n-7)	2.68[a]	2.11[a]	1.60[a]	0.70[a]	22.32[b]	0.9
18:0	0.80[a]	0.84[a]	0.71[ab]	0.53[b]	0.53[b]	0.07
18:1 (n-9)	0.67[ac]	0.58[ab]	0.53[**ab**]	0.49[b]	0.81[c]	0.06
18:2 (n-6)	0.32	0.31	0.24	0.26	0.27	0.04
18:3 (n-6)	0.28	0.24	0.17	0.27	--	0.05
18:3 (n-3)	0.09[a]	0.07[ab]	0.06[b]	0.07[ab]	0.13[c]	0.01
20:0	0.24[a]	0.08[ab]	-0.04[ab]	-0.09[b]	0.09[ab]	0.1
20:2	0.30[a]	0.52[bc]	0.57[c]	0.37[ab]	0.55[bc]	0.07
20:3	--	0.59	0.49	0.57	--	0.3
20:4 (n-6)	--	0.10	-0.01	-0.02	--	0.07
20:5	--	0.24	0.15	0.32	--	0.07
22:0	0.12	0.13	0.12	0.10	--	0.02
22:6 (n-3)	--	0.07	0.03	0.06	--	0.03
24:1	--	--	0.38[a]	1.7[b]	--	0.34

2 [1] Treatment groups are defined in Table 3.

[2] Values presented are means ± SEM. Within columns, treatments lacking a common superscript differ, $P < 0.05$.

[3] Efficiency rates were calculated as follows: (Ending amount of fatty acid (g) – Initial amount of fatty acid (g)) / total intake of fatty acid (g)

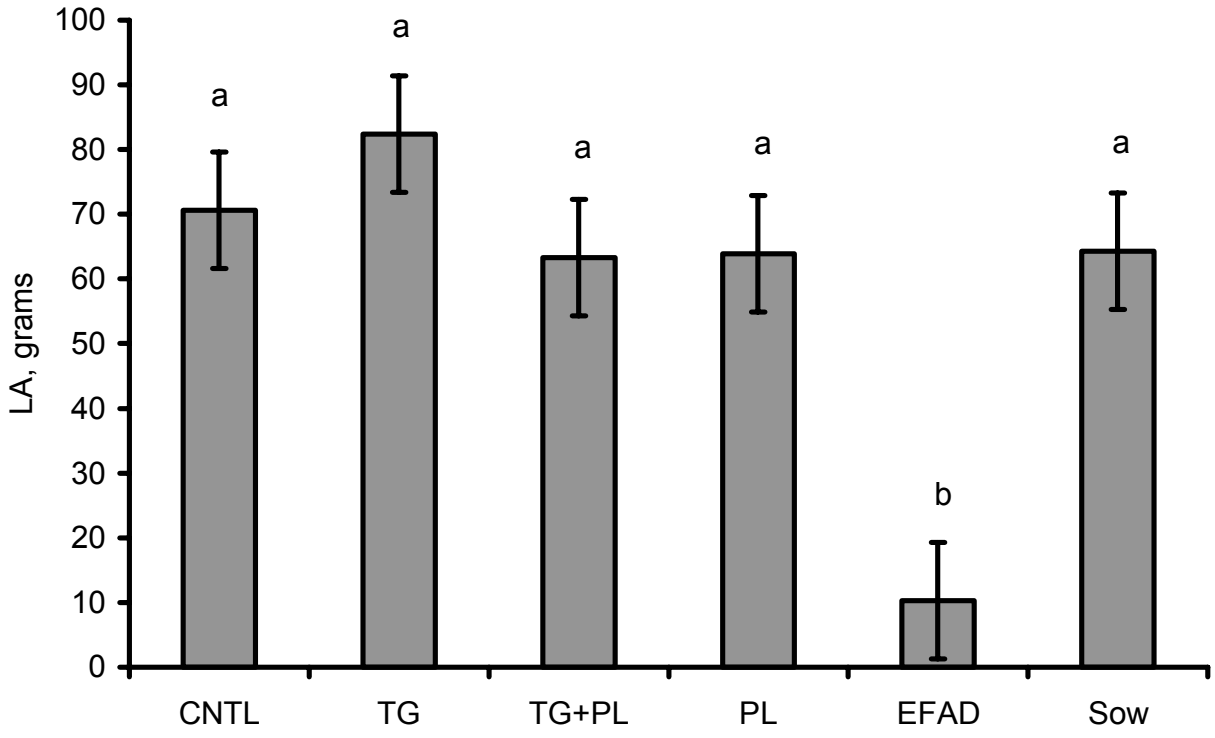

Figure 1. Total body accretion of linoleic acid (LA) over the 16 d treatment period of neonatal pigs fed sources of either triglyceride or phospholipid AA and DHA. Values presented are means ± SEM. Bars lacking a common letter differ ($P < 0.05$); n = 6 for all treatment groups.

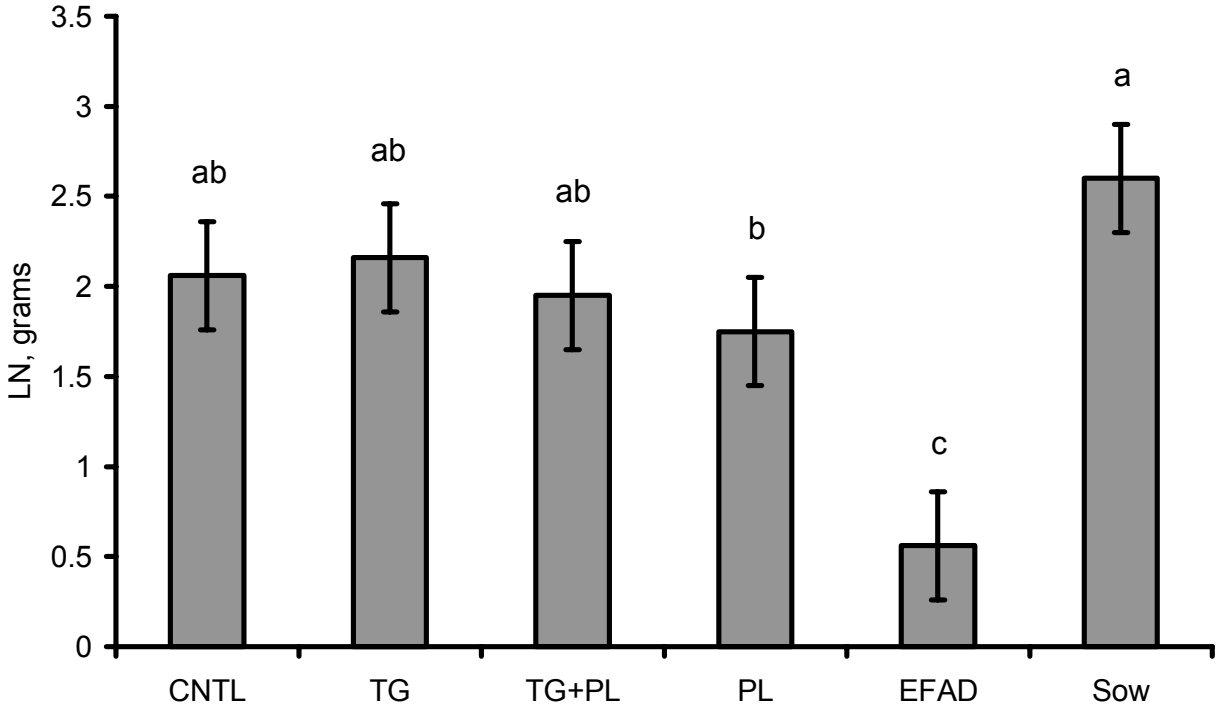

Figure 2. Total body accretion of linolenic acid (LN) over the 16 d treatment period of neonatal pigs fed sources of either triglyceride or phospholipid AA and DHA. Values presented are means ± SEM. Bars lacking common a letter differ ($P < 0.05$); n=6 for all treatment groups.

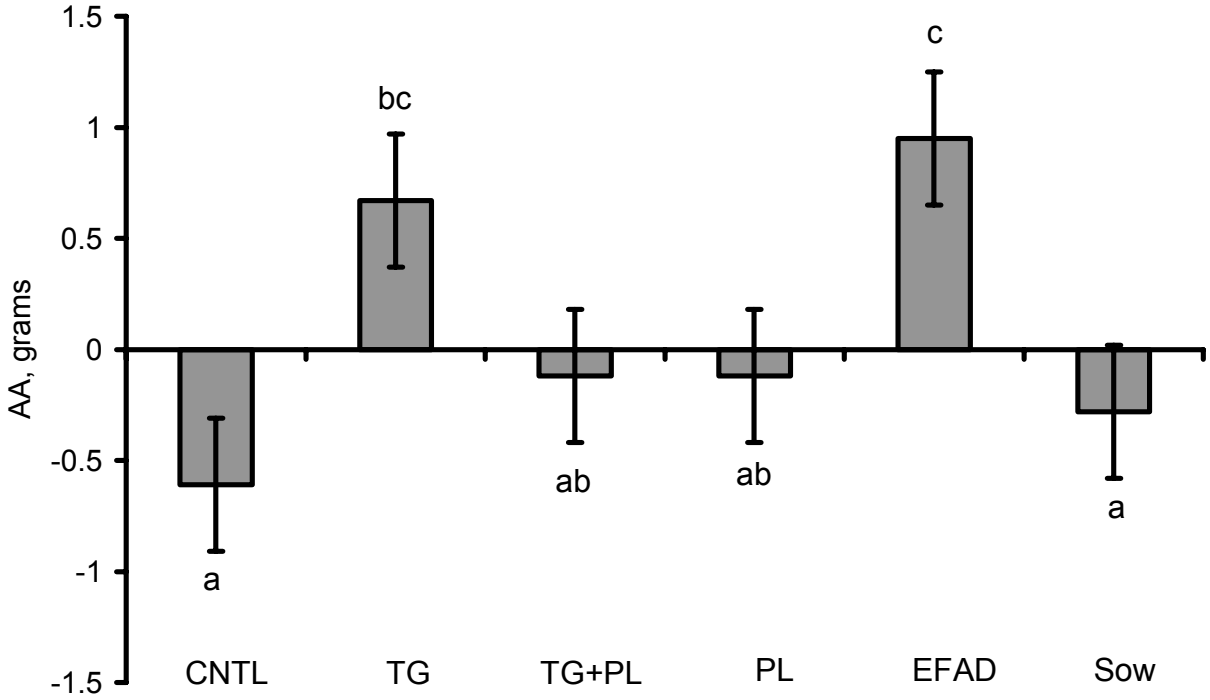

Figure 3. Total body accretion of arachidonic acid (AA) over the 16 d treatment period of neonatal pigs fed sources of either triglyceride or phospholipid AA and DHA. Values presented are means ± SEM. Bars lacking a common letter differ ($P < 0.05$); n=6 for all treatment groups.

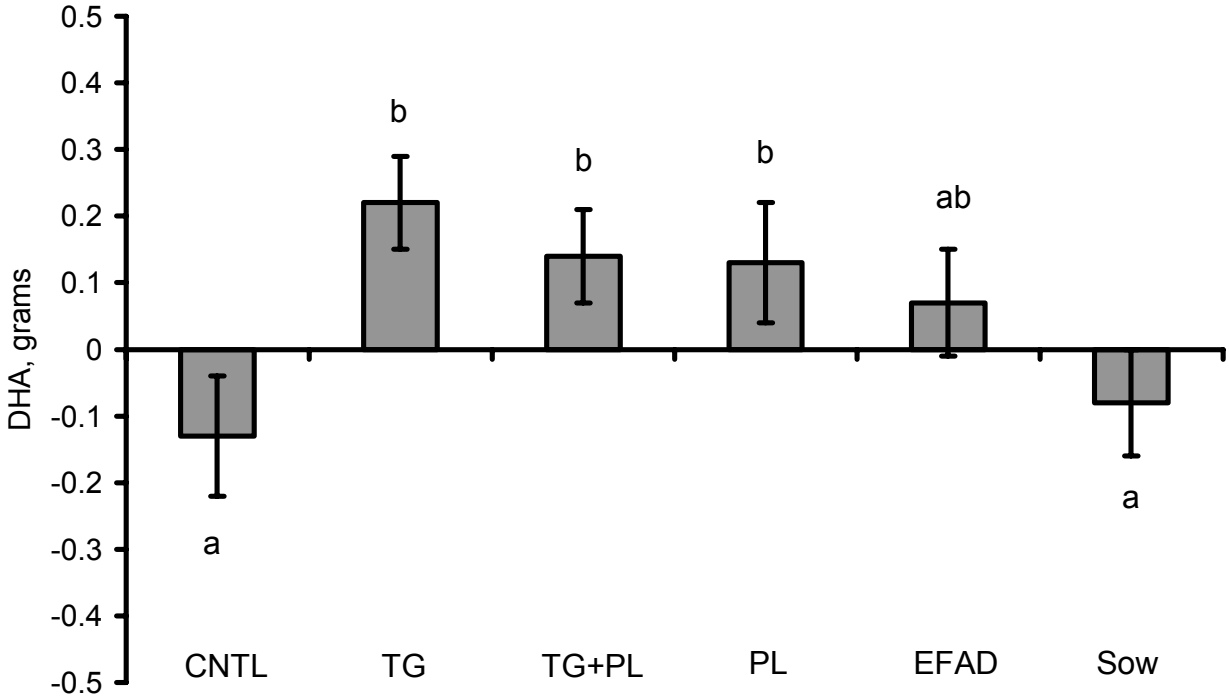

Figure 4. Total body accretion of docosahexaenoic acid (DHA) over the 16 d treatment period of neonatal pigs fed sources of either triglyceride or phospholipid AA and DHA. Values presented are means ± SEM. Bars lacking a common letter differ ($P < 0.05$); n=6 for all treatment groups.

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CHAPTER 4

CONJUGATED LINOLEIC ACID REDUCES TOTAL BODY FAT, WHILE NOT AFFECTING THE OXIDATION OF ESSENTIAL FATTY ACIDS IN THE LIVER, BRAIN OR SKELETAL MUSCLE TISSUE OF NEONATAL PIGS[1]

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ABSTRACT

The prevalence of childhood obesity is a growing problem in the US which poses a potential health threat because of the development of subsequent chronic diseases, such as diabetes and cardiovascular disease. Conjugated linoleic acid (CLA) has been shown to reduce body fat in many species, but little is known about the metabolic interactions between CLA and essential fatty acids. Two replicates of 12, 1 d old pigs were fed a milk-based formula ad libitum for an average of 16.5 d that contained 25%(HF) or 3% (LF) fat with either 1% CLA (+CLA) or 1% sunflower oil (-CLA) as methyl esters. Growth was unaffected by CLA ($P>0.2$). LF fed pigs consumed 10% more formula than HF fed pigs ($P<0.05$), but 19% less metabolizable energy ($P<0.01$). Accumulation of body lipid and protein was reduced by 34% and 14%, respectively in pigs fed CLA compared to pigs that did not receive CLA ($P<0.05$). CLA was only detected in tissues of pigs fed CLA, with more accumulation found in the LF fed pigs than the HF fed pigs ($P<0.01$). In vitro □-oxidation of ^{14}C-arachidonate, linoleate, and palmitate was not affected by CLA ($P>0.2$) or level of dietary fat ($P>0.1$) in liver, brain, or muscle tissue. Total body accretion of LA, AA and DHA were reduced by fat level ($P<0.0001$) and both LA and LN were reduced by CLA ($P<0.0003$). These data suggest that CLA in conjunction with a low fat diet reduced body fat but did not affect in vitro □-oxidation of either essential or non-essential fatty acids.

Keywords: Long chain polyunsaturated fatty acids, conjugated linoleic acid, childhood obesity, piglet

INTRODUCTION

Childhood obesity has increased three-fold in the last 20 years. Approximately 4% of children aged 6-11 were considered obese in the NHANES II (1976-1980), but the NHANES III (1988-1994) reported this age group of children to contain 13% as obese (1, 2). There are many factors that contribute to this significant and troubling increase in childhood obesity. Physical activity has decreased overall, while approximately six hours a day are spent watching television or sitting in front of a computer (3-5). The increase in the availability of foods that are high in fat and sugar also plays a role. School lunches and snack foods are more likely to be of the fast food variety, instead of being a fresh fruit or vegetable (5). Also, parental behaviors weigh heavily on the eating and physical activity pattern of children (6, 7).

There are conceptually four critical periods for the development of adipose tissue in the human life span (2, 8, 9). A critical period is defined as a developmental stage in which physiological alterations increase the risk of later obesity (9). The four critical periods are: 1) gestation or fetal life, 2) early infant life, from months one to four, 3) the period of adiposity rebound, beginning around age five, and 4) adolescence or puberty (2, 8). It is during one or more of these periods

that obesity is potentially programmed; however, there is much debate over which of the four periods plays the most significant role on adult obesity.

Determining which of these four critical periods is most important in the development of obesity is difficult. From the epidemiological evidence thus far, the most appropriate measure to ensure a decrease in overweight status in the pediatric population is to start early in life and follow through all critical periods. Discovering dietary modifiers such as fats and/or certain fatty acids that can affect body composition early in life may help to reduce the incidence of obesity, and possibly lead to a cure for this worldwide metabolic disease. Throughout the lifespan, fat is an essential nutrient and early in life, constitutes about 50% of the infant's caloric intake regardless of mode of feeding. Fat is an energy dense substrate that along with providing a major structural component for growth and development, may, if overfed, lead to metabolic consequences that could potentiate the development of obesity.

Conjugated linoleic acid (CLA) is an unsaturated fatty acid that has conjugated diene bonds and a combination of *cis* and/or *trans* spatial configurations (10). There are many different isomers of CLA that are found in ruminant meats and dairy products (11). The positive biological effects that are linked with CLA include anti-carcinogenic properties (12), anti-athlerogenic effects (13), and anti-diabetogenic effects (14) especially in animal models. Also CLA has been shown to affect lipid metabolism (15, 16) and reduce fat mass in rodents (17, 18), pigs (19, 20) and humans (21).

Nutritional research focusing on CLA has increased greatly over the last several years. Conjugated linoleic acid fed to lactating mammals reduced milk fat, (22-25) and recently it was determined that CLA also reduced milk fat in lactating women (26). Conjugated linoleic acid is also secreted in the milk of several species including humans (27, 28), however, the effects of CLA on infant health are undetermined. Innis and King (1999) (29) found a positive relationship between CLA in breast milk and in the plasma lipid of the infant. Elias and Innis (2001) (30) determined that the concentration of both CLA and other trans fatty acids (TFA) in breast milk were related to the maternal plasma concentration. This suggests that the higher the level of maternal CLA intake, the greater the concentration of CLA the infant is exposed to, both in utero via placental trasnfer and ex utero via breast milk.

Human breast milk, infant formulas and sow's milk contain approximately 50% of calories from fat (31-33) which suggests that the neonate requires a high amount of dietary fat in order to maximize weight gains. Fat is the most energetically dense macronutrient and is the primary source of energy for the suckling neonate in the first four to six months of life in the human, or the first 21 days in the piglet. Current data in piglets suggest that formulating milk replacers similar to the composition of sow's milk may not provide optimum growth. For example, growth performance of piglets was maximized when the supply of lysine per unit of energy was approximately 50% higher than found in sow's milk (34). Furthermore, diets that were utilized in artificial rearing studies conducted in our laboratory, supplied approximately 50% greater amino acid content per unit of

energy than sow's milk and resulted in faster piglet weight gains (35, 36). These data suggest that utilizing the supply and pattern of nutrients found in sows milk limits preweaning pig growth. However, to our knowledge, research with varying levels of fat in an infant formula, i.e. high fat versus low fat, has not been conducted.

Infant formulas are formulated to emulate human milk, but many components are not present. The effects of TFA have shown to inhibit delta-6 desaturase, which is involved in the elongation/desaturation pathway for both of the essential fatty acids, linoleic and linolenic acids. No studies have evaluated the direct effects of CLA on infant nutrition and health. For example, biosynthesis of LCPUFA could be compromised, which is important in growth and development of the visual and neurological systems. However, other effects of CLA could be beneficial for the neonate, especially during a critical period for the development of obesity. The piglet has proven to be a suitable model for comparison to the human infant when studying lipid nutrition. The piglet has many similarities with human infants including the development of the intestine, fat digestion and absorption, and also many of the pathways of lipid metabolism (37). The objectives of this study were: (1) to determine if feeding CLA alters lipid oxidation in the liver, brain and skeletal muscle, and (2) to determine if the dietary fat content (3% vs. 25%) with or without CLA supplementation will alter body composition and/or nutrient accretion rate.

METHODS AND MATERIALS

Animal Care

General. The Institutional Animal Care and Use Committee of North Carolina State University (NCSU) approved all procedures. A total of 24 piglets from 7 sows were obtained from the NCSU Swine Educational Facility, Raleigh, NC, and moved to the Grinnells Intensive Swine Research Laboratory at approximately one d of age. Piglet housing and feeding system was described previously by Mathews et al. (2002) (38). Piglets were randomly assigned to one of the following four dietary treatments (**Table 1**) (39): 1) high fat, containing 25% fat (HF, n = 6), 2) high fat supplemented with 1% CLA (HF+CLA, n = 6), 3) low fat, containing 3% fat (LF, n = 6), 4) low fat supplemented with 1% CLA (LF+CLA, n = 6). Formulas not containing CLA had the addition of 1% sunflower oil methyl esters added to match the methyl ester concentration of the CLA diets. All diets provided an adequate amount of essential fatty acids (**Table 2**) and also contained supplemental LCPUFA which provided 0.6 g/100 g of fatty acids as AA and 0.3 g/100 g as DHA. At the end of the study, piglets were killed via an AMVA-approved electrocution devise followed by exsanguination (laceration of the brachiocephalic arteries) and tissues collected. An initial group of 10 piglets from 5 litters was used as a reference for calculation of nutrient accretion rates.

Animal Feeding and Diets. Diets were reconstituted at 150 g/L of water (approximately 11 g dry matter /100 g) and the feeding was adapted from McClead et al (1990) (40). Formula was re-freshed four times daily (0800, 1300, 1800, and

2300) to provide pigs ad libitum access. All components of the feeding system were cleaned thoroughly each day prior to the first feeding (0800h) with a liquid chlorinated detergent (DS Liquid: Command, Diversey Corp., Wyandotte, MI). Formula was reconstituted on a daily basis and stored at 4° C until fed. Cobalt EDTA was prepared as described by Uden et al. (41) and added to diets (0.1 g/100 g of dry diet) approximately 36 h prior to removal of pigs from the experiment as an inert marker of dry matter digestibility.

Sample Collection and Analytical Procedures

Performance and Blood Collection. Pigs were weighed daily and formula intake was determined gravimetrically on a daily basis. Blood was collected via jugular venipuncture on d 0 and 16 or 17 of the study at 0900 after all piglets had been fed. After collection, blood samples were centrifuged (Sorvall, model 64000, Newtown, CT) at 825 x g for 10 min at 4°C. Plasma was collected and aliquots were frozen at –20° C until plasma urea nitrogen (PUN) and non-esterified fatty acid (NEFA) analyses. Plasma urea nitrogen and NEFA were analyzed in duplicate by enzymatic colorimetric assays (Sigma, St. Louis, MO).

In vitro β-oxidation. Immediately after exsanguination, the liver, brain and semitendonosus muscle were removed and weighed. β-oxidation experiments and determination of ^{14}C in CO_2, ASP and ESP were conducted as previously reported by Odle et al (1991) (42). A section of each tissue was placed in a tissue homogenate buffer that contained: 220 mM mannitol, 70 mM sucrose, 2 mM HEPES, and 0.1 mM EDTA. Tissues were homogenized on ice with a handheld

Pyrex Potter-Elvehjem tissue grinder (Fisher Scientific, Pittsburgh, PA) until tissue was evenly dispersed into buffer. The tissue to buffer ratio was 1 to 7 mL. Homogenates were centrifuged at 750 x g for 15 min and then the supernatant was transferred to a weighed and labeled vial. Protein analysis was conducted by the Biuret method (43).

Tissues were incubated with 1mM concentrations each of palmitate, linoleate, and arachidonate and each of the fatty acid substrates contained 0.5 μCi of carboxyl labeled ^{14}C-palmitate (PA), ^{14}C-linoleate (LA), and ^{14}C-arachidonate (AA), respectively (ARC, St. Louis, MO). Radiolabeled fatty acids were solublized in a 5:1 concentration of bovine serum albumin solution and added to the reaction buffer. The reaction buffer contained: 50 mM sucrose, 150 mM Tris-HCl, 20 mM K_2PO_4, 10 mM $MgCl_2$-$6H_2O$, 2 mM EDTA, 1 mM carnitine, 10 mM ATP, 2 mM NAD, 0.2 mM coenzyme-A, and 0.1 mM malate. Once the isotopic solutions were made, 1.7 mL was placed into a 25 mL Erlenmeyer flask that was fitted with a rubber stopper and a spring apparatus that suspended an Eppendorff tube containing 0.5 mL ethanolamine to capture generated CO_2. One-half of the flasks were incubated with antimycin/rotenone as previously described by Yu et al (1997) (44). Each fatty acid for each tissue was conducted in duplicate. After the addition of 0.3 mL of tissue homogenate to the flask, the rubber stopper was affixed to the flask and was placed into a 37°C shaking water bath for 30 min. To terminate the reaction, all flasks were injected with 0.5 mL of a 3 M $HClO_4$ solution. Flasks were left undisturbed for 2 hr for the complete capture of CO_2. After the 2 hr period, the rubber stoppers were

removed and the Eppendorff tube was rinsed with 0.5 mL methylcellusolve and 20 mL of Scin-Safe cocktail (Fisher Scientific, Pittsburgh, PA). The homogenate/buffer solution remaining in the flask was sampled for acid soluble products and esterified products. ^{14}C accumulation in the CO_2, acid soluble products, and esterified products was measured by counting 20 min in a liquid scintillation counter (LS-6500 IC, Beckman Instruments, Fullerton, CA). Oxygen consumption was measured using the YSI Biological Oxygen Monitor (Model 5300, YSI Incorporated, Yellow Springs, OH, 45387) and as previously described by Odle et al (1991) (42).

Body Composition and Fatty Acid Analysis. The whole body was ground and thoroughly mixed (TorRey model M22-R-2) using a 82.6 mm kidney plate (TorRey model TOR 22KP), then a 15.9 mm plate (TorRey model TOR 12P 5/8) and then a 4.8 mm plate (TorRey model TOR 12P 3/16). Subsamples were taken, freeze-dried, powdered in liquid nitrogen and stored at -20˚C until proximate analysis. Water content was calculated by weight loss after drying at 100˚C for 24 h in a forced-air oven (45). Total body crude protein was determined using the Kjeldahl procedure (45). Total fat was assayed using the Folch procedure (46) and an internal standard (C17:0) was added to each sample prior to tissue homogenization. After fat extraction was complete, all samples were transmethylated to fatty acid methyl esters (FAME) (47). Fatty acid methyl esters were quantitatively analyzed by gas-liquid chromatography using a Hewlett Packard Agilent 5890-Series II (Delaware) equipped with a flame ionization detector and 6890 Series auto-injector. The FAME were separated on a 100 m SP-2380 Fused Silica capillary column (Supelco,

Bellefonte, PA: 0.25 mm diameter, 0.2 µm film thickness) using helium at a flow rate of 2.1 mL /min with a split ratio of 50:1. The chromatographic run parameters included an oven starting temperature of 140°C that was increased at 3.2°C/min to 225°C, where it was held for 14 min before increasing to 230°C at 2°C/min, with a final hold of 22 min. The injector and detector temperatures were both constant at 220°C. Peaks were identified by comparison of retention times with external FAME standard mixtures from Supelco (Bellefonte, PA; F.A.M.E. Mix C_4-C_{24}). The fatty acid concentrations were adjusted to account for recovery of the internal standard. Total fatty acid analysis was conducted on whole body, brain and ileal fat extracts.

Statistical Analysis

Values in the text are least square means ± standard error of the mean. SAS (SAS Inst. Inc., Cary, NC) Proc GLM procedure was used for statistical analysis appropriate for a 2x2 factorial, completely randomized design. Treatment differences were evaluated using the main effect of fat level and CLA, as well as the interaction. Differences were deemed significant when $P < 0.05$.

RESULTS

Performance, Growth and Food Intake

Over the treatment period, there were no differences in piglet body weights among the treatment groups ($P > 0.2$; data not shown). Metabolizable energy intake was 19% less in the LF fed piglets ($P < 0.01$; data not shown). Piglets in both the LF groups had greater formula intakes than the HF fed groups ($P < 0.007$, **Table 3**).

Feed conversion (gram of feed per gram of gain) tended to be more efficient in the HF fed piglets compared to the LF fed groups ($P < 0.08$). Plasma urea nitrogen and NEFA concentrations were unaffected by CLA (PUN, $P > 0.4$; NEFA $P > 0.2$). However, both PUN and NEFA concentrations were lower in the LF fed piglets compared to the HF fed piglets ($P < 0.0001$, **Table 3**).

Dry Matter Digestibility and Digesta Dry Matter Content

Dry matter content of the digesta from the ileum was greater in HF fed piglets compared to piglets fed LF diets (11.7 ± 0.7 vs. 8.7 ± 0.8, $P < 0.05$). However, dry matter content of the digesta from the distal rectum was 30.1 ± 5.2 % and tended to be reduced by the low fat level ($P < 0.07$), but unaffected by CLA supplementation ($P > 0.6$; data not shown). Ileal apparent dry matter digestibility of the diets was decreased by CLA ($P < 0.01$) with a greater reduction in the digesta of piglets fed a LF diet compared to HF fed piglets ($P < 0.001$). Rectal apparent dry matter digestibility was 83.1 ± 3.4 and was not affected by fat level ($P > 0.1$) or supplemental CLA ($P > 0.5$; data not shown).

Total Ileal Lipid and Fatty Acid Content

The amount of lipid extracted from the ileal contents was affected not only by fat level ($P < 0.0001$), but also by CLA supplementation ($P < 0.01$; data not shown). LF fed piglets had lower ileal lipid content than piglets fed HF diets ($P < 0.0001$). CLA supplementation reduced the amount of total lipid extracted from the ileal contents, with a greater reduction seen in the HF fed piglets than in the LF fed piglets ($P < 0.03$). CLA, however, was not detected in the ileal contents.

In vitro β-Oxidation

Conjugated linoleic acid nor fat level altered fatty acid β-oxidation by liver (fat level $P > 0.3$, CLA $P > 0.4$; **Table 4**), brain (fat level $P > 0.8$, CLA $P > 0.3$; **Table 5**) or skeletal muscle (fat level $P > 0.4$, CLA $P > 0.3$; **Table 6**) in either mitochondrial or peroxisomal fractions (or total β-oxidation) for any of the fatty acids measured. In all tissues, peroxisomal oxidation of AA was greater than oxidation of either PA or LA ($P < 0.03$). Contributions of mitochondrial and peroxisomal to total β-oxidation were similar across all tissues and all in vitro fatty acid treatments ($P > 0.1$). No differences were detected in the accumulation of radiolable in esterified products (ESP) in the liver for either PA ($P > 0.3$) or LA ($P > 0.2$), but AA had a greater amount of ESP in all treatments ($P < 0.05$; data not shown). Liver oxygen consumption rates were also unaffected by either the inclusion of CLA ($P > 0.6$) or fat level of the diet ($P > 0.3$, **Table 4**).

Body Composition and Tissue Accretion Rates

Neither the supplementation with CLA or dietary fat level affected the overall whole body accretion of ash ($P > 0.2$). Total body water accretion tended to be reduced by CLA ($P < 0.08$). CLA reduced total body protein accretion ($P < 0.02$). Total body lipid levels were reduced by CLA supplementation ($P < 0.02$) and pigs fed the LF diet had less fat then those fed the HF diet ($P < 0.05$; Table 7).

Accretion of Whole Body Fatty Acids

Daily total accretion of 16:1 was higher in LF fed piglets ($P < 0.0001$), however, CLA reduced accretion of 16:1 ($P < 0.0002$). Linoleic acid accretion was

reduced in pigs fed LF diets ($P < 0.0001$) and by the inclusion of CLA ($P < 0.0001$; Table 8 and Figure 1). Linolenic acid accretion was reduced by the inclusion of CLA ($P < 0.0001$; Figure 2), but was not altered by fat level ($P < 0.7$). However, CLA reduced LN accretion more in the LF fed piglets than in the HF fed piglets ($P < 0.03$). Accretion of both 20:1 and 20:2 was lowered in piglets fed the LF diets compared to HF diets ($P < 0.0001$), but was not affected by CLA ($P > 0.3$). Total body accretion of both AA (Figure 3) and DHA (Figure 4) were reduced in piglets fed LF compared to HF fed piglets ($P < 0.0001$), but was not altered by CLA ($P < 0.1$). Accretion of 18:1 was lowered in piglets fed LF diets compared to HF fed piglets ($P < 0.0001$) and by CLA ($P < 0.0001$). Also accretion of 18:1 was less in piglets fed CLA in LF fed group than in the HF fed group ($P < 0.0007$). Both the cis-9, trans-11 and the trans-10, cis-12 isomers of CLA were accreted only in pigs supplemented with dietary CLA ($P < 0.0001$), with more CLA accretion in the LF fed piglets than in the HF fed piglets ($P < 0.01$).

Efficiency of Accretion of Fatty Acids

Overall, LF fed piglets had a higher efficiency of utilization for the majority of fatty acids (**Table 9**). Efficiency of dietary LA utilization for LA accretion was reduced by fat level ($P < 0.02$) with the LF fed piglets having lower efficiencies compared to the piglets fed HF diets, but was not altered by CLA ($P > 0.2$). Piglets fed LF diets used dietary LN more efficiently compared to piglets fed HF diets ($P<0.0001$), and CLA reduced the efficiency of LN utilization more in the HF fed piglets compared to the LF fed piglets ($P < 0.03$). Efficiency of dietary AA utilization

was reduced by fat level, being lower in the LF fed piglets ($P < 0.002$) compared to the HF fed piglets, but unaffected by CLA ($P > 0.8$). Dietary DHA efficiency of use not affected by fat level ($P > 0.3$), but there was a trend for CLA to reduce DHA efficiency in LF fed piglets ($P < 0.06$).

Total Brain Lipid and Fatty Acid Accretion (Table 10)

No differences were detected in the amount of brain lipid for any of the treatment groups ($P > 0.9$; data not shown). Overall, there were few differences detected in the accumulation of fatty acids in the brain by either level of fat or CLA supplementation. Neither fat level ($P > 0.1$) nor CLA ($P > 0.8$) altered LA, LN or AA accumulation. However, DHA accumulation was less in the LF fed groups than in the HF fed piglets ($P < 0.0005$), but it was unaffected by CLA ($P > 0.3$). CLA was not detected in the brain of any piglets. Also, brain weights (data not shown) were unaffected by fat level ($P > 0.1$) or CLA supplementation ($P > 0.1$).

DISCUSSION

The prevalence of childhood obesity over the past 20 years has doubled (48). This increase in the number of overweight children may lead to future health problems in our society such as increased prevalence of diabetes and athlerosclerosis. Determining ways to reduce the rate of obesity would help to improve the overall health of the obese population, and also potentially reduce the number of obesity related complications. Defining specific nutritional components that are potential modifiers of adipose development at critical periods in

development are possible strategies to modify body fatness. Currently, both LCPUFA and CLA are agents involved in the modulation of lipid metabolism (15, 16, 49). However, it is unclear if CLA affects essential fatty acid metabolism. Conjugated linoleic acid is a *trans* fatty acid and may have deleterious effects on the elongation/desaturation pathway that produces AA and DHA, both of which are necessary for proper growth and development of the neonate. To date, no studies have investigated how CLA affects the neonate, but it is an important area to investigate because CLA was recently detected in human milk (26, 27), and it was established that CLA crosses the placenta (30). Conjugated linoleic acid has been shown to reduce body fatness in several animal models (15, 16, 18, 50, 51) including the pig (10, 19, 20, 52) and may have beneficial affects in reducing the incidence of obesity. However, CLA may lower the de novo LCPUFA biosynthesis and could potentially endanger the health and well being of infants.

Our primary objective was to determine if feeding CLA altered EFA metabolism in the liver, brain and skeletal muscle and reduced fat deposition in piglets fed a high (25%) or low (3%) fat diet. After approximately 17 days neither fat level in the diet or supplementation with 1% CLA affected overall piglet growth. Piglet performance in the present trial was similar to other trials conducted in our laboratory (35, 38, 53).

Amino acid oxidation, as measured indirectly by plasma urea nitrogen concentrations was higher in the HF fed pigs groups, suggesting that dietary amino acids were not utilized as efficiently for protein deposition. However, protein

accretion was not affected by fat level. Low fat fed groups did have a higher volume intake of milk formula, meaning higher water consumption; therefore the lower PUN observed in the LF fed piglets could have been accounted for by the increased volume of distribution. Also NEFA, an indirect measure of lipolysis and / or the availability of fatty acids for uptake, was lower in the groups fed the LF diets. Chemical composition of the whole body found that LF fed groups had lower total body lipid compared to the HF fed groups. However, there were no effects of CLA on either PUN or NEFA.

Due to the unique structure of CLA, it was hypothesized that potential competition could occur between the EFA, LA and LN, and CLA for the elongation/desaturation pathway. This potential competition may possibly lead to a decrease in the amounts of AA and DHA synthesized de novo, which ultimately could impair proper neurological development. However, in vitro β–oxidation rates of PA, LA or AA were not altered by dietary CLA supplementation in the brain, liver or skeletal muscle. Because CLA was not detected in the brains of piglets fed supplemental CLA, it is not surprising that there was no alteration in brain EFA metabolism. The finding that CLA was not detected in the brain tissue supports previous hypotheses about specific yet protective mechanisms involved in the transport of lipids across the blood-brain barrier (54). Also, gas chromatography-mass spectrophotometer analysis of liver tissue revealed no accumulation of CLA in the liver (not presented), therefore again, no detectable alteration in the metabolism of in vitro fatty acids in the liver was consistent with the results of the brain.

However, we did find an increase in liver weights as a percent of piglet body weight (9% increase; data not shown), similar to previously published data in rodents (15, 55).

Previous studies focusing on the metabolism of CLA have been conducted primarily in the rodent model and have produced mixed results. Most recently, Demizieux et al (2002) (56) found that cis-9, trans-11 and trans-10, cis-12 isomers of CLA were less oxidizable than LA and palmitoleic acid (16:1), and also more likely to interfere with the oxidation of other fatty acids, including the EFA, LA. However, this in vitro study was conducted on liver tissue homogenates from male Wistar rats who received no supplemental CLA, but both CLA isomers were used as fatty acid treatments in the in vitro experiment as were LA and 16:1. Interestingly, a study conducted previously at the same laboratory found that CLA feeding did not alter liver mitochondrial carnitine palmitoyltransferase-I (CPT-I) activity in male Wistar rats, but found a 30% increase in CPT-I activity in adipose tissue, and concluded that the Wistar rat species was poorly responsive to CLA feeding (57). Similarly, a previous study by Clouet et al (2001) (58) reported that carnitine acyltransferase activity was reduced by the cis-9, trans-11 CLA isomer in rat mitochondria. These results are contradictory however to those published by Rahman et al (2001) (59) who concluded that mice fed a mixture of CLA isomers in either the triglyceride or free fatty acid form, had increased β-oxidation in the liver, brown adipose tissue and red gastrocnemius muscle as measured by carnitine palmitoyltransferase activity.

Evaluation of the muscle data in the present trial to that previously mentioned, illustrates that there are differences between the two studies. Aside from the differences in the animal model, there is the possibility that other enzymes involved in the β-oxidation of LA were limiting in the present study. Two additional enzymes are necessary for the complete oxidation of LA, and because CLA shares a similar configuration to LA, it would also require both enoyl-CoA isomerase and 2,4 dienoyl reductase for complete oxidation to CO_2. It has been reported that these enzymes may be a rate limiting step in the oxidation of LA and CLA (58). However, since CLA is a potent activator for the peroxisome proliferators activated receptor-alpha (PPARα) which up regulates β-oxidation (60), it would appear that differences in muscle oxidation of PA, LA, or AA would be neutralized by these two mechanisms and not differ in animals supplemented with CLA. PPARα is abundantly expressed in tissues that have high rates of β-oxidation including liver and skeletal muscle (61-64). Recently published data in the PPARα-null mice provides evidence that while CLA is a potent activator of PPARα, the effects of CLA on body composition are independent of this nuclear receptor (51). However, in the present study we did not measure levels of PPARα in liver or muscle.

Supplemental CLA has been shown to alter energy expenditure and reduce body fat. Terpstra et al (2002) (65) using the Balb-C mouse reported a reduction in body fat, and also a lower digestibility of the CLA diets, as well as an increase in energy expenditure. Similarly, West et al (2000) (66) after feeding mixed CLA isomers to AKR/J mice reported an increase in energy expenditure, but no effect of

CLA on energy intake. In the present study, we did not find an effect of CLA on energy intake, but it appeared that CLA reduced the ileal apparent dry matter digestibility of the diet with a greater reduction in the LF fed animals. Also DeLaney et al (1999) (55) reported an increase in energy expenditure but found no effect of CLA on energy intake. It has been suggested that the reduction in body fat without a difference in intake or body weight may be caused by an increase in energy expenditure. The increase in energy expenditure might be the result of an in increase in basal metabolic rate, an increase due to the thermic effect of absorption, digestion and assimilation of nutrients after a meal, or an increase in physical activity. However, it was not due to a decrease in de novo fatty acid synthesis, or an alteration in the uncoupling proteins associated with thermal regulation as reported by West et al (2000) (66).

At the conclusion of the trial, total body protein content and protein accretion rates were reduced by CLA. However, these data are unlike previously published data in mice which concluded enhanced body protein with the supplementation of trans-10, cis-12 CLA isomer (67). However, more recently Terpstra et al (2002) (65) concluded that in restricted fed mice with supplemental CLA, accretion of whole body protein was reduced, but was not affected in *ad libitum* fed mice. Total body lipid and lipid accretion were lower in the LF fed piglets compared to the piglets fed HF diets. This effect was additive in that both low fat level and CLA together resulted in a greater reduction in body fat. Spurlock et al (2002) (64) investigated the effects of a prolonged milk feeding (high fat, 14.3%) versus a typical dry diet (low

fat, 8.6%) on body composition and reported that the prolonged feeding of a milk diet only transiently increased body fat of pigs. The difference in dietary fat may have accounted for the transient difference in body fat, because once all pigs were placed on the same diet, body fat differences were not detected. Interestingly, it was noted that pigs fed the prolonged milk diet were leaner once market weight was achieved. This may be due to a difference in the number of adipocytes in the milk fed pigs, but the authors did not measure adipocyte cell number. PPARα was unaffected regardless of nutritional regime or body fat content. As mentioned previously, the reduction in body fat in the present study was due not only to low fat level, but also CLA. It is well known that CLA is a modulator of body composition primarily by reducing body fat in numerous species including both pigs (20) and humans (68). In this experiment, we show for the first time that CLA reduced piglet total body lipid as early as 17 d of age.

To date, data on total body fatty acid composition of the neonatal pig is limited, especially with respect to effects of CLA. Currently, no other data on how CLA affects neonatal body composition has been published. Most of the data on fatty acid accretion has been targeted at LCPUFA accretion in the brain and retinal tissues only (69, 70), and has not measured effects on the whole body. Accretion of fatty acids was primarily affected by fat level in the diet in that the LF fed groups had lower accretion of the majority of the fatty acids examined such as 18:1, 18:2 (LA), 20:1, 20:2, 20:4(AA), and 22:6(DHA) compared to the HF fed pigs. CLA reduced the accretion of the EFA, LA and LN in the whole body; however, accretion of these

fatty acids remained unchanged in the brain. Also, CLA was not detected in the brain of any piglets suggesting a protective mechanism for preventing *trans* fatty acids from crossing the blood brain barrier. The daily accretion and overall accumulation of both AA and DHA was unaffected by CLA in all tissues examined. We did however find a decrease in 18:1 in the carcass in the CLA supplemented groups, which suggests that CLA inhibits the □9 desaturase activity as found by others (71, 72).

In conclusion, the results of this study suggest that CLA is a modulator of body lipid in the neonatal period which may potentially reduce the risk of obesity not only in childhood, and also subsequently as an adult. There were no deleterious effects of CLA supplementation noted on essential fatty acid metabolism. LF diets did not affect growth rates and did not effect LCPUFA fatty acid accretion in the brain or in the whole body. However before further recommendations can be made for the use of CLA in reducing body fatness, further investigation on the mechanisms of action of CLA should be conducted.

1

Table 1. Composition and calculated analysis of the formula diets fed to piglets, comparing high (25%) versus low (3%) fat with or without the addition of 1% conjugated linoleic acid (CLA) .[1]

	Diet[2]			
Ingredient, g/kg	HF	HF+CLA	LF	LF+CLA
Non-fat dry milk	533	533	395	395
Mead Johnson Oil Blend[3]	230	230	13	13
CLA[4]	0	10	0	10
Sunflower oil methyl esters[5]	10	0	10	0
Lysolecithin[6]	10	10	10	10
Lactose	0	0	406	406
Sodium Caseinate[7]	100	100	74	74
Whey Protein Concentrate[8]	60	60	44	44
Arginine	7.6	7.6	5.6	5.6
Histidine	2.5	2.5	1.9	1.9
L-Lysine•HCl	2.0	2.0	1.5	1.5
$CaCO_3$	3.2	3.2	1.8	1.8
Dicalcium Phosphate	16.2	16.2	12.0	12.0
Xanthan Gum	10	10	10	10

Mineral Premix[10]	6.3	6.3	6.3	6.3
Vitamins Premix[11]	2.5	2.5	2.5	2.5
Sodium Chloride	6.0	6.0	6.0	6.0
Calculated Analysis[12]				
ME, kJ/kg[13]	4687	4687	3447	3447
Fat, g/100 g	25	25	3.0	3.0
Crude Protein, g/100 g	34.7	34.7	25.6	25.6
Lactose, g/100 g	27.9	27.9	61.3	61.3
Calcium:Phosphorus	1.45	1.45	1.45	1.45
Ratio of LA:LN	9.4	9.4	9.6	9.6

[1] Expressed on an air-dry weight basis

[2] Diet groups are: high fat, HF; high fat plus CLA, HF+CLA; low fat, LF; low fat plus CLA, LF+CLA.

[3]Mead Johnson Oil Blend of palm olein, soy, coconut and high oleic sunflower oils (Mead Johnson Nutritionals, Evansville, IN 47721) plus the addition of Martek ARASCO® (0.6% of total fat) and DHASCO® (0.3% of total fat) oils (Martek Biosciences Corporation, Columbia, MD 21045)

[4]Conjugated linoleic acid (BASF, Mount Olive, NJ, 07828)

[5]Sunflower methyl esters were produced in our lab as previously described by Lang et al (2001) (73)

[6]Lysolecithin (Kemin Industries, Des Moines, Iowa 50301-0070)

[7]Sodium Caseinate (International Ingredient Co., St. Louis, MO, 63116)

Table 1, continued

[8]Whey Protein Concentrate (AMP 80, Proliant, Ames, IA 50010)

[9]Skim Milk (Milk Specialties Corp., Dundee, IL 60118)

[10]Mineral premix (Merrick's Inc., Union Center, WI 53962) contained 9.99 g/100 g Ca, 0.01 g/100 g P, 0.015 g/100 g Na, 0.040 g/100 g Cl, 0.05 g/100 g K, 6.1 g/100 g Mg, 9.3 g/100g S,18,999 µg/g Fe, 1.901 µg/g Co, 32,000 µg/g Cu, 669 µg/g I, 2,799 µg/g Mn, 48 µg/g Se, 19,000 µg/g Zn

[11]Vitamin premix (Merrick's Inc., Union Center, WI 53962) contained 33,000,000 IU/kg Vitamin A, 6,600,000 IU/kg Cholecalciferol, 55,000 IU/kg α-tocopherol, 257,400 µg/g Ascorbic acid, 29,983 µg/g D-Pantothenic Acid, 33,069 µg/g Niacin, 8378 µg/g Riboflavin, 5,115 µg/g Menadione, 66 µg/g Biotin, 44,000 µg/g Vitamin $B_{12,}$ 2,038 µg/g Thiamine, 3,996 µg/g Vitamin $B_{6,}$ 2,756 µg/g Folic Acid

[12]Calculated analysis based on analysis provided by companies furnishing product and standard feed tables. (39)

[13]ME, metabolizable energy as estimated from book values and information provided by companies supplying ingredients.

Table 2. Analyzed fatty acid composition of diets[1].

Fatty Acid	HF	HF+CLA	LF	LF+CLA
		G/100 g diet		
14:0	1.00	1.07	0.10	0.11
16:0	4.53	4.90	0.58	0.64
16:1	0.04	0.04	0.02	0.01
18:0	0.86	0.96	0.19	0.21
18:1	12.57	13.73	1.80	2.01
18:2(n-6)	5.12	5.49	2.36	1.43
18:3(n-3)	0.03	0.02	0.01	0.01
20:0	0.06	0.07	0.01	0.01
20:1	0.20	0.21	0.08	0.09
20:2	0.00	0.02	0.00	0.05
20:4(n-6)	0.03	0.03	0.02	0.02
22:0	0.08	0.06	0.02	0.02
22:6(n-3)	0.08	0.08	0.01	0.01
9c11t CLA[2]	0.00	0.07	0.00	0.17
10t12c CLA[2]	0.00	0.06	0.00	0.15

[1]Diet groups are defined in Table 1.

[2]cis-9, trans-11 and trans-10, cis-12 isomers of CLA

Table 3. Performance and plasma metabolite data from piglets fed either a high (25%) or low (3%) fat diet with or without 1% conjugated linoleic acid (CLA). [1]

	Diet					Significance		
Item	HF	HF+ CLA	LF	LF+ CLA	SEM[2]	FL[3]	CLA	FL x CLA
ADG, g/d	420	397	404	401	12	0.5	0.2	0.3
ADFI[4], g/d	337	329	367	365	12	0.006	0.6	0.7
Feed to Gain	0.71	0.76	0.77	0.89	0.06	0.07	0.1	0.5
PUN, mg/dl	28.8	28.8	19.0	15.2	2.0	0.001	0.4	0.4
NEFA, µEq/L	212.3	198.3	108.0	87.5	14.5	0.001	0.2	0.8

[1] Tabulated values are least square means. Piglets were fed for 16 or 17 days.

[2] Standard error of the difference of the means.

[3] FL = fat level (high vs. low)

[4] ADFI is reported as dry intake

Table 4. β-oxidation of ^{14}C-palmitate, ^{14}C-linoleate, and ^{14}C-arachidonate in the liver of piglets fed a diet containing either high (25%) or low (3%) fat with or without the inclusion of 1% conjugated linoleic acid (CLA).[1]

Item	Diet				SEM[2]
	HF	HF + CLA	LF	LF + CLA	
	nmol/mg protein/hr				
^{14}C-Palmitate					
Mitochondrial	3.72	2.88	2.65	2.21	0.5
Peroxisomal[a]	1.82	2.11	1.91	1.90	0.2
TOTAL (M+P)	5.54	5.04	4.47	4.11	0.5
^{14}C-Linoleate					
Mitochondrial	3.01	3.06	2.49	1.75	0.5
Peroxisomal[a]	1.61	1.87	1.67	1.73	0.2
TOTAL (M+P)	4.95	4.93	4.17	3.78	0.5
^{14}C-Arachidonate					
Mitochondrial	3.87	1.99	3.09	2.68	0.5
Peroxisomal[b]	2.13	2.04	2.41	2.32	0.2
TOTAL (M+P)	5.46	4.01	5.13	4.72	0.5
O_2 Consumption	3.21	3.09	2.88	2.70	0.3

[1] Tabulated values are least square means. Within a column, means without a common letter differ, $P < 0.05$.

[2] Standard error of the difference of the means.

Table 5. β-oxidation of ^{14}C-palmitate, ^{14}C-linoleate, and ^{14}C-arachidonate in the brain of piglets fed a diet containing either high (25%) or low (3%) fat with or without the inclusion of 1% conjugated linoleic acid (CLA).[1]

Item	Diet				SEM[2]
	HF	HF + CLA	LF	LF + CLA	
	nmol/mg protein/hr				
^{14}C-Palmitate					
Mitochondrial	2.63	2.20	2.55	2.43	0.4
Peroxisomal[a]	0.99	1.05	0.96	0.93	0.1
TOTAL(M+P)	3.62	3.25	3.51	3.36	0.3
^{14}C-Linoleate					
Mitochondrial	2.11	2.25	2.83	1.63	0.4
Peroxisomal[ab]	0.97	0.91	0.82	0.91	0.1
TOTAL(M+P)	3.24	3.15	3.42	2.80	0.3
^{14}C-Arachidonate					
Mitochondrial	1.89	1.75	2.08	1.69	0.4
Peroxisomal[b]	0.86	0.75	0.75	0.74	0.1
TOTAL(M+P)	2.69	2.50	2.68	2.43	0.3

[1] Tabulated values are least square means. Within a column, means without a common letter differ, $P < 0.05$.

[2] Standard error of the difference of the means.

Table 6. β -oxidation of ^{14}C-palmitate, ^{14}C-linoleate, and ^{14}C-arachidonate in the skeletal muscle of piglets fed a diet containing either high (25%) or low (3%) fat with or without the inclusion of 1% conjugated linoleic acid (CLA).[1]

Item	Diet				SEM[2]
	HF	HF + CLA	LF	LF + CLA	
	Nmol/mg protein/hr				
^{14}C-Palmitate					
Mitochondrial	1.87	2.30	1.58	0.94	0.9
Peroxisomal[a]	1.38	1.49	1.38	1.21	0.2
TOTAL(M+P)	2.37	3.80	2.64	2.14	0.5
^{14}C-Linoleate					
Mitochondrial	2.61	2.95	2.79	3.03	0.9
Peroxisomal[a]	1.90	2.20	1.20	1.63	0.2
TOTAL(M+P)	3.86	5.57	4.74	4.32	0.5
^{14}C-Arachidonate					
Mitochondrial	1.73	2.03	1.11	0.66	0.9
Peroxisomal[b]	1.31	1.46	1.46	1.52	0.2
TOTAL(M+P)	2.55	3.49	2.54	2.47	0.5

[1] Tabulated values are least square means. Within a column, means without a common letter differ, $P < 0.05$.

[2] Standard error of the difference of the means.

Table 7. Total body accretion (g/d) for piglets fed either a high (25%) or low (3%) fat diet with or without the inclusion of 1% conjugated linoleic acid (CLA).[1]

	Diet					Significance		
Item	HF	HF+ CLA	LF	LF + CLA	SEM	FL[2]	CLA	FLxCLA
Protein, g/d	80.5	74.2	84.1	72.3	4.0	0.8	0.02	0.4
Water, g/d	304.1	287.1	320.6	281.4	16.3	0.7	0.08	0.4
Fat, g/d	48.3	37.9	39.2	19.9	7.2	0.05	0.04	0.5
Ash, g/d	16.2	16.0	15.8	13.3	1.9	0.3	0.4	0.5

[1]Tabulated values are least square means. Piglets were fed for 16 or 17 days.

[2]FL = Fat level

Table 8. Daily accretion of fatty acids from piglets fed either a high (25%) or low (3%) fat diet with or without the inclusion of 1% CLA.[1]

	Diet					Significance		
Fatty Acid	HF	HF+ CLA	LF	LF+ CLA	SEM	FL	CLA	FL x CLA
			g/d[2]					
16:0	6.9	6.6	6.9	5.3	0.6	0.22	0.11	0.28
16:1	0.6	0.3	1.4	0.8	0.1	0.0001	0.0002	0.21
18:0	2.5	3.0	3.7	2.7	0.2	0.11	0.36	0.009
18:1	30.5	24.4	29.1	11.6	1.5	0.0001	0.0001	0.0007
18:2 (n-6)	11.8	10.4	5.39	2.5	0.4	0.0001	0.0001	0.09
18:3 (n-3)	0.2	0.2	0.2	0.1	0.02	0.80	0.0001	0.04
20:0	-0.01	-0.02	-0.01	-0.03	0.02	1.0	0.002	0.47
20:1	0.43	0.43	0.13	0.08	0.02	0.0001	0.34	0.38
20:2	0.31	0.31	0.42	0.35	0.02	0.005	0.9	0.12
20:3	0.05	0.04	0.04	0.03	0.01	0.09	0.56	0.21
20:4	0.14	0.17	0.06	0.04	0.02	0.0001	0.71	0.17
22:6	0.10	0.11	0.001	0.02	0.01	0.0001	0.26	0.76
9c11t CLA[3]	0.0	0.23	0	0.35	0.02	0.0005	0.0001	0.0005
10t12c CLA[3]	0.0	0.12	0	0.16	0.01	0.01	0.0001	0.01

[1] Values are means. Piglets were fed diets for 16 or 17 days.

Table 8, continued

[2]Accretion rates were calculated as follows: (((Ending individual pig g fatty acid/g carcass x carcass weight) – (Average of Initial group g FA/g carcass)) x initial piglet carcass weight) ÷ number of days fed

[3]cis-9, trans-11 and trans-10, cis-12 isomers of CLA

Table 9. Calculated efficiency of accretion of fatty acids from piglets fed either a high (25%) or low (3%) fat diet with or without the inclusion of 1% CLA.[1, 2]

	Diet					Significance		
Fatty Acid	HF	HF+ CLA	LF	LF+ CLA	SEM	FL	CLA	FL x CLA
16:0	0.5	0.5	3.5	2.8	0.1	0.0001	0.008	0.016
16:1	5.9	2.9	25.6	22.3	1.0	0.0001	0.005	0.91
18:0	1.0	1.1	5.7	4.3	0.2	0.0001	0.003	0.0008
18:1	0.8	0.6	4.7	1.9	0.1	0.0001	0.0001	0.0001
18:2 n6	0.73	0.72	0.67	0.58	0.04	0.02	0.24	0.31
18:3 n3	3.1	1.8	7.0	6.6	0.4	0.0001	0.03	0.13
20:0	-0.04	-0.12	-0.21	-0.64	0.1	0.002	0.002	0.01
20:1	0.72	0.72	0.49	0.33	0.04	0.0001	0.07	0.07
20:2	ND[3]	-0.75	ND	-2.5	0.5	0.0001	--	--
20:4	1.7	1.8	0.8	0.6	0.2	0.0002	0.87	0.41
22:6	0.4	0.5	1.0	0.6	0.1	0.38	0.06	0.10
9c11t CLA[4]	ND	1.2	ND	0.7	0.1	0.0001	--	--
10t12c CLA[4]	ND	0.6	ND	0.4	0.1	0.09	--	--

[1] Values are means, $P < 0.05$. Piglets were fed diets for 16 or 17 days.

[2] Efficiency rates were calculated as follows: g of fatty acid accreted / g of fatty acid intake

[3] ND = not detected

Table 9, continued

[4]cis-9, trans-11 and trans-10, cis-12 isomers of CLA

Table 10. Total accumulation of fatty acids in the brain of piglets fed either a high (25%) or low (3%) fat diet with or without the inclusion of 1% CLA.[1]

	Diet					Significance		
mg Fatty Acid	HF	HF+ CLA	LF	LF+ CLA	SEM	FL	CLA	FL x CLA
14:0	3.7	3.6	2.8	3.6	0.3	0.1	0.2	0.1
16:0	114.0	118.4	100.7	110.7	8.0	0.2	0.3	0.7
16:1	5.4	5.0	5.0	5.2	0.4	0.7	0.7	0.4
18:0	111.0	113.8	99.0	103.5	7.1	0.1	0.6	0.9
18:1	184.9	192.2	171.3	180.9	15.0	0.4	0.5	0.9
18:2	69.0	68.2	64.6	67.7	5.0	0.6	0.8	0.6
18:3 n3	3.4	3.5	3.3	3.3	0.4	0.6	0.9	0.9
20:1	2.5	2.2	2.2	2.3	0.3	0.8	0.7	0.5
20:3	3.7	3.3	2.3	3.5	0.4	0.7	0.7	0.4
20:4	55.3	57.4	51.8	51.3	3.0	0.1	0.8	0.6
22:0	2.9	2.9	4.7	4.8	0.3	0.0001	0.8	0.9
22:6	87.8	92.4	63.4	69.9	5.6	0.0005	0.3	0.8
9c11t CLA[2]	0	0	0	0	0			
10t12c CLA[2]	0	0	0	0	0			

[1] Values are means, $P < 0.05$. Piglets were fed diets for 16 or 17 days.

[2] cis-9, trans-11 and trans-10, cis-12 isomers of CLA

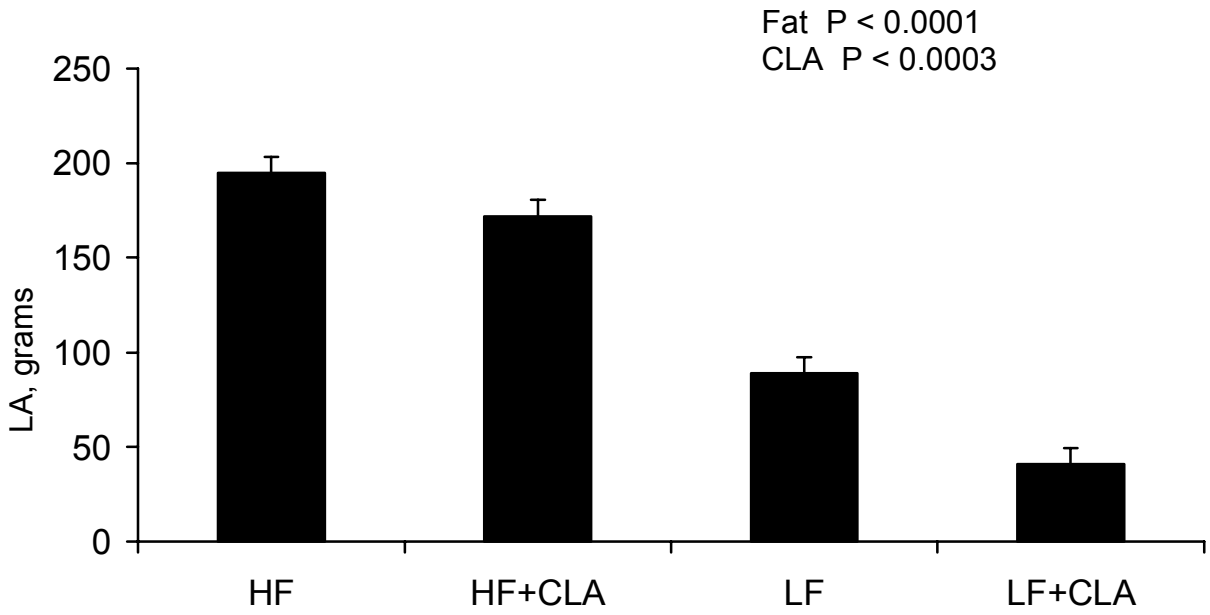

Figure 1. Total body accretion of linoleic acid (LA) of neonatal pigs fed either a high (25%) or low (3%) fat diet with or without the inclusion of 1% CLA. Piglets were fed diets for either 16 or 17 days. Values presented are means ± SEM; n = 6 for all treatment groups.

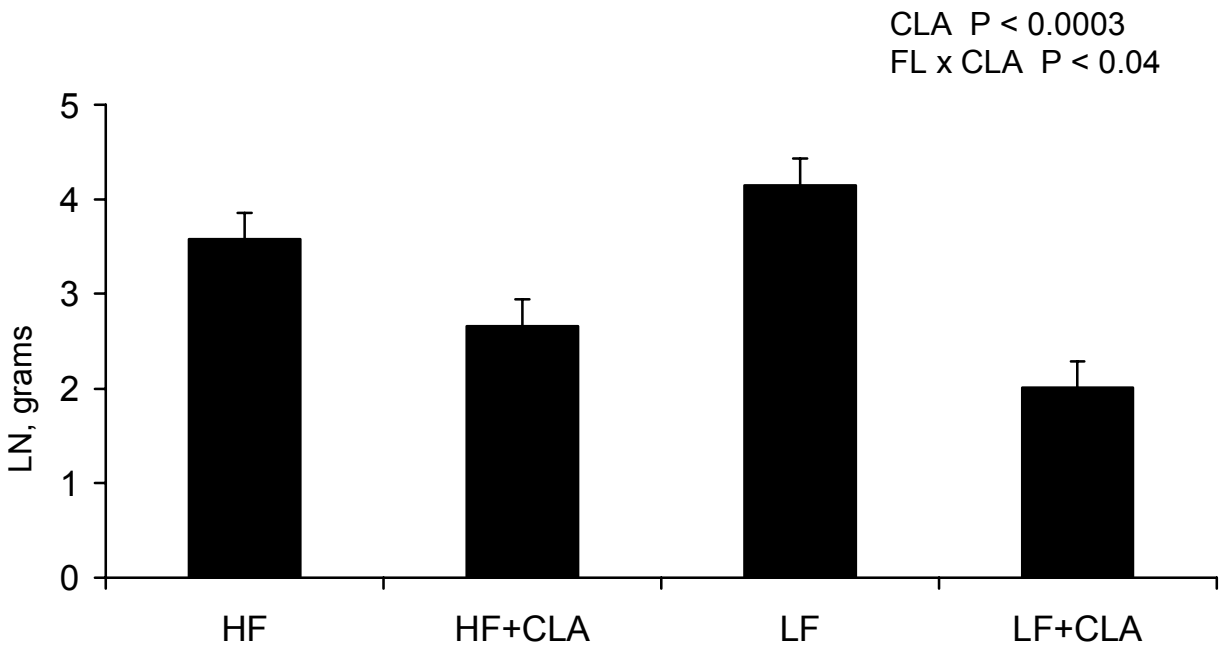

Figure 2. Total body accretion of linolenic acid (LN) of neonatal pigs fed either a high (25%) or low (3%) fat diet with or without the inclusion of 1% CLA. Piglets were fed diets for either 16 or 17 days. Values presented are means ± SEM; n = 6 for all treatment groups.

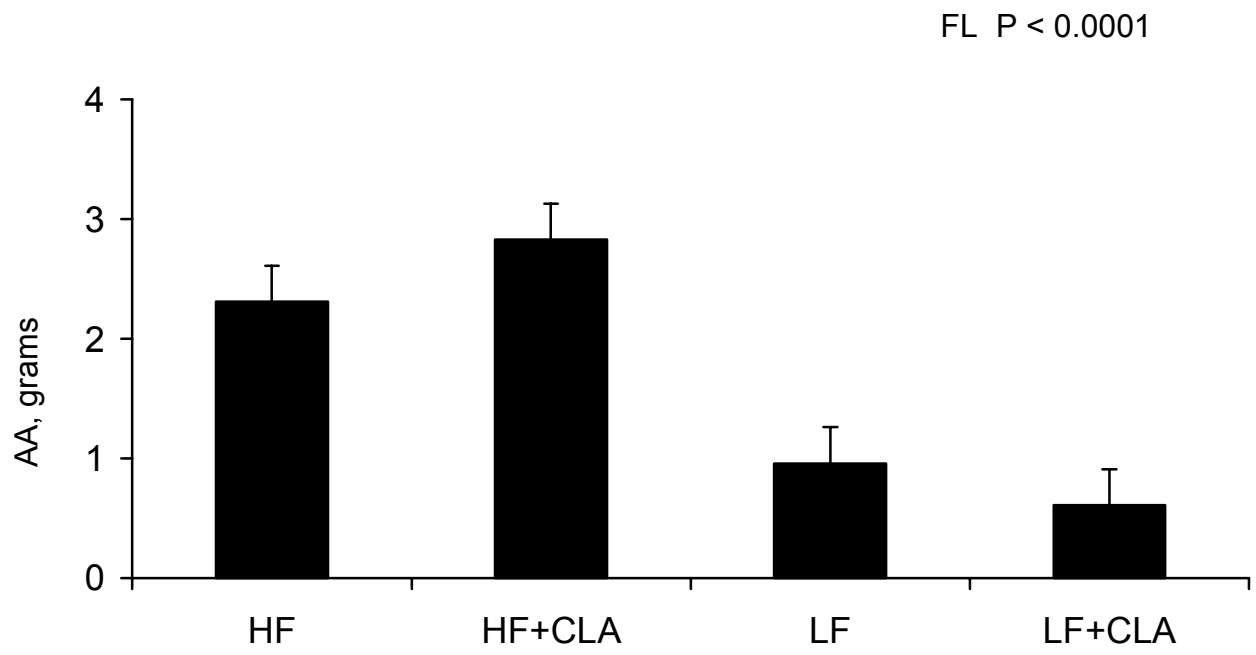

Figure 3. Total body accretion of arachidonic acid (AA) of neonatal pigs fed either a high (25%) or low (3%) fat diet with or without the inclusion of 1% CLA. Piglets were fed diets for either 16 or 17 days. Values presented are means ± SEM; n = 6 for all treatment groups.

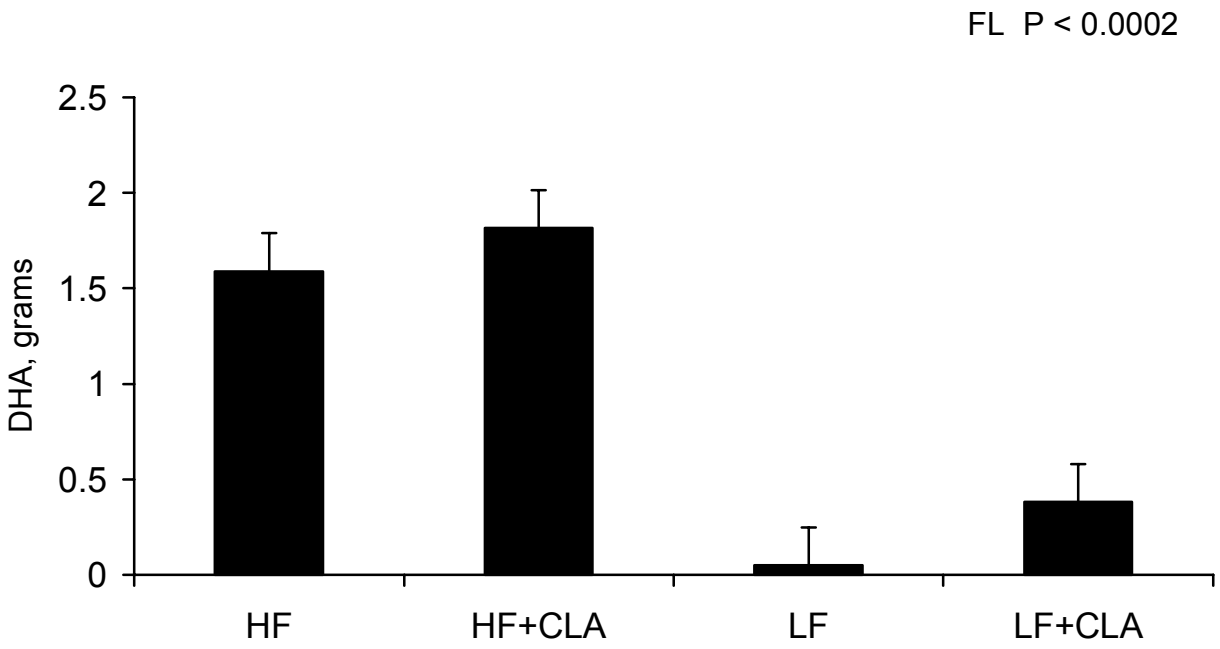

Figure 4. Total body accretion of docosahexaenoic acid (DHA) of neonatal pigs fed either a high (25%) or low (3%) fat diet with or without the inclusion of 1% CLA. Piglets were fed diets for either 16 or 17 days. Values presented are means ± SEM; n = 6 for all treatment groups.

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APPENDIX A.

ESSENTIAL FATTY ACID DEFICIENCY IN PIGLETS DECREASES β-OXIDATION OF ^{14}C-LINOLEATE AND ^{14}C-PALMITATE IN BOTH LIVER AND BRAIN TISSUE

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Abstract

Previous research of pigs fed an essential fatty acid deficient (EFAD) diet had lower tissue levels of linoleic acid (LA) and linolenic acid (LN), but greater levels of arachidonic acid. The present study was conducted to investigate fatty acid metabolism of liver and brain in EFAD pigs. Two replicates of 12, 1 d old pigs were fed a milk-based formula *ad libitum* for 12 d that contained either adequate levels of LA and LN (CNTL), or was devoid of LA and LN (EFAD). Liver and brain homogenates were incubated with ^{14}C-linoleate (^{14}C-LA) or ^{14}C-palmitate (^{14}C-PA). In liver, accumulation (nmol/mg protein/hr) of ^{14}C in CO_2 was reduced by 52% ($P<0.01$) and ^{14}C in acid soluble products (ASP) was decreased by 33% ($P<0.02$) in EFAD compared to CNTL. Correspondingly, in brain, accumulation of ^{14}C in CO_2 and ASP were each reduced by 16% ($P<0.05$). Similarly, liver total β-oxidation rate (CO_2 + ASP) of ^{14}C-LA and ^{14}C-PA was reduced by 33% ($P<0.01$) while brain total

β-oxidation was decreased 16% ($P<0.01$) in the EFAD compared to the CNTL. These data suggest that pigs fed a diet deficient in essential fatty acids show reduced catabolism of both essential and non-essential fatty acids regardless of tissue type.

Introduction

- The essential fatty acids, 18:2 (linoleic acid, LA) and 18:3 (linolenic acid, LN) are necessary for growth and development
- Development of the retina, brain and other neural tissues depends on the conversion of 18:2 → 20:4n-6 (arachidonic acid, AA) and 18:3 → 22:6n-3 (docosahexaenoic acid, DHA)
- Essential fatty acid deficiency results in developmental problems and poor growth performance
- Previous data from our laboratory suggested that piglets who were fed an essential fatty acid deficient diet, retained more AA and DHA compared to controls (Reference figures from Chapter 3).

Objective

To determine if the rate of β-oxidation of linoleic acid (LA) and palmitic acid (PA) in the liver and brain is reduced in piglets fed an EFAD diet

Hypothesis

β-oxidation of LA will be reduced in the liver, but not the brain of EFAD fed piglets, but that β-oxidation of PA will be the same regardless of dietary treatment.

Methods and Materials

Experimental Design

Trial 1: Liver

- 12, 1 d old piglets fed either a CNTL diet or EFAD diet for 12 d
- Measured β –oxidation of ^{14}C-LA and ^{14}C-PA

Trial 2: Brain

- 12, 1 d old piglets fed either a CNTL diet or EFAD diet for 12 d
- Measured β –oxidation of ^{14}C-LA and ^{14}C-PA

- Piglets had *ad libitum* access to their respective diets
- Diets were mixed to contain 150 g/L
- Fresh milk was added 4x/d to ensure freshness
- Feeding apparatus was cleaned daily

β-oxidation experiment

1. Tissue homogenates (0.02g) incubated with 0.5μCi ^{14}C-palmitate and ^{14}C-linoleate and a 1mM unlabeled solution of the respective fatty acid
2. Incubations occurred for 30 min at 37°C, then the reaction was terminated with the addition of 3M $HClO_4$

3. CO_2 collected for 2 hr post-reaction termination
4. ^{14}C accumulation was measured in CO2 and Acid Soluble Products (ASP)

Body composition and Fatty acid analysis

A total of 6 pigs / treatment were used to determine body and fatty acid composition. Except for blood, the contents of the gastrointestinal tract and urinary bladder, the whole body was ground and thoroughly mixed (TorRey model M22-R-2) using a 82.6 mm kidney plate (TorRey, TOR 22KP), then a 15.9 mm plate (TorRey, TOR 12P 5/8) once and finally, a 4.8 mm plate (TorRey, TOR 12P 3/16) twice more). Subsamples were taken and stored at -20˚C for proximate analysis. Water content was calculated by weight loss after drying at 100˚C for 24 h in a forced-air oven (1). Total body crude protein was determined using the Kjeldahl procedure (1). Total fat was assayed using the Folch procedure (2) and an internal standard (C17:0) was added to each sample prior to tissue homogenization. After fat extraction was complete, all samples were transmethylated to fatty acid methyl esters (FAME) (3). Fatty acid methyl esters were quantitatively analyzed by gas-liquid chromatography using a Hewlett Packard Agilent 5890-Series II (Delaware) equipped with a flame ionization detector and 6890 Series injector. The FAME were separated on a 100m SP-2380 Fused Silica capillary column (Supelco, Bellefonte, PA: 0.25mm diameter, 0.2µm film thickness) using helium at a flow rate of 2.1 mL/min with a slpit ratio of 50:1. The chromatographic run parameters included an oven starting temperature of 140°C that was increased at 3.2°C/min to 225°C, and then held for 14 min before

increasing to 230°C at 2°C/min, with a final hold of 22 min. The injector and detector temperatures were both constant at 220°C. Peaks were identified by comparison of retention times with external FAME standard mixtures from Supelco (Bellefonte, PA; F.A.M.E. Mix C_4-C_{24}). The fatty acid concentrations were calculated using the internal standard method.

Statistics

GLM procedure of SAS was used for comparison of ^{14}C accumulation in CO2, acid soluble products and piglet performance data.

1

Table 1. Composition and calculated analysis of the formula diets fed to piglets fed either a control (CNTL) or an essential fatty acid deficient diet for 12 d .[1]

Ingredient	Diet[2]	
	CNTL	EFAD
	g/kg	
Mead Johnson Oil Blend[3]	180	0
Soybean Oil	110	0
Coconut Oil (non-hydrogenated)	0	290
Sodium Caseinate[4]	128	128
Whey Protein Concentrate[5]	86	86
Skim Milk[5]	388	388
Lactose	50	50
$CaCO_3$	5	5
Dicalcium Phosphate	27	27
Mineral Premix[7]	5	5
Vitamins Premix[8]	1.3	1.3
Lysolecithin	10	10
Xanthan Gum	10	10

Table 1, continued

Calculated Analysis[9]	CNTL	EFAD
ME, Kcal/kg[10]	5053	4783
Fat, g/100 g	30	30
Crude Protein, g/100 g	32	32
Lactose, g/100 g	26	26
Calcium:Phosphorus	1.4	1.4
Ratio of LA:LN	7.7	--

[1] Expressed on an air-dry weight basis

[2] Diet groups are: control, CNTL; essential fatty acid deficient, EFAD.

[3]Mead Johnson Oil Blend of palm olein, soy, coconut and high oleic sunflower oils (Mead Johnson Nutritionals, Evansville, IN 47721)

[4]Sodium Caseinate (International Ingredient Co., St. Louis, MO, 63116)

[5]Whey Protein Concentrate (AMP 80, Proliant, Ames, IA 50010)

[6]Skim Milk (Milk Specialties Corp., Dundee, IL 60118)

[7]Mineral premix (Milk Specialties Corp., Dundee, IL 60118) contained 1.002 g/100 g Ca, 0.549 g/100 g P, 0.284 g/100 g Na, 0.040 g/100 g Cl, 2.024 g/100 g K, 0.102 g/100 g Mg, 20,000 µg/g Fe, 200 µg/g Co, 1,850 µg/g Cu, 400 µg/g I, 5,000µg/g Mn, 60 µg/g Se, 23,500 µg/g Zn

[8]Vitamin premix (Milk Specialties Corp., Dundee, IL 60118) contained 33,000,000 IU/kg Vitamin A, 6,600,000 IU/kg Cholecalciferol, 55,000 IU/kg α-tocopherol, 257,400 µg/g Ascorbic acid, 29,983 µg/g D-Pantothenic Acid, 33,069 µg/g Niacin, 8378 µg/g Riboflavin,

Table 1, continued

5,115 µg/g Menadione, 66 µg/g Biotin, 44,000 µg/g Vitamin $B_{12,}$ 2,038 µg/g Thiamine, 3,996 µg/g Vitamin $B_{6,}$ 2,756 µg/g Folic Acid

[9]Calculated analysis based on analysis provided by companies furnishing product and standard feed tables (4).

[10]ME, metabolizable energy as estimated from book values and information provided by companies supplying ingredients.

1

Table 2. Analyzed fatty acid composition of diets[1].

Fatty Acid	CNTL	g/100 g diet	EFAD
8:0	0.02		0.05
10:0	0.15		1.0
12:0	1.65		13.88
14:0	0.79		5.62
14.1	0.01		0.02
16:0	13.93		10.53
16:1	0.042		0.019
18:0	1.10		1.29
18:1	7.27		2.45
18:2(n-6)	7.06		1.30
18:3(n-3)	0.72		0.15
20:0	0.08		0.25
20:1	0.75		0.00
20:2	6.15		0.94
20:3	0.00		0.00
20:4(n-6)	0.00		0.00
20:5	0.00		0.00
22:0	0.09		0.00
22:6(n-3)	0.00		0.00
24:1	0.00		0.00

2 [1] Diet groups are defined in Table 1.

RESULTS

Table 3. Performance data for piglets fed either a CNTL or EFAD diet for 12 days.[1,2]

	CNTL	EFAD	SEM
ADG, g/d	337[a]	297[b]	11
DMI, g/d	198[a]	180[b]	6
Feed to Gain[3]	0.59	0.61	0.2

[1] Treatment groups are: control, CNTL; essential fatty acid deficient, EFAD.

[2] Values presented are means ± SEM. Within rows, treatments lacking a common superscripts differ, $P < 0.05$.

[3] Feed to gain is defined as grams of dry feed consumed per gram of weight gain.

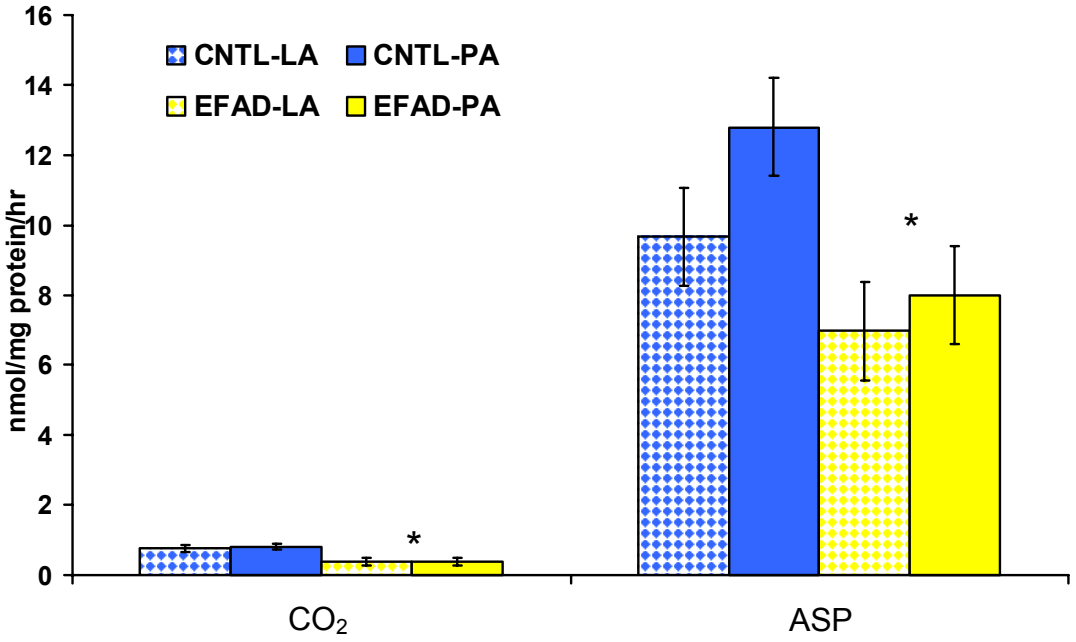

Figure 1. Oxidation of LA and PA in the liver of piglets fed either a CNTL or EFAD diet for 12 days. Values presented are means ± SEM. n = 6 for all treatment groups, * P < 0.01, diet effect

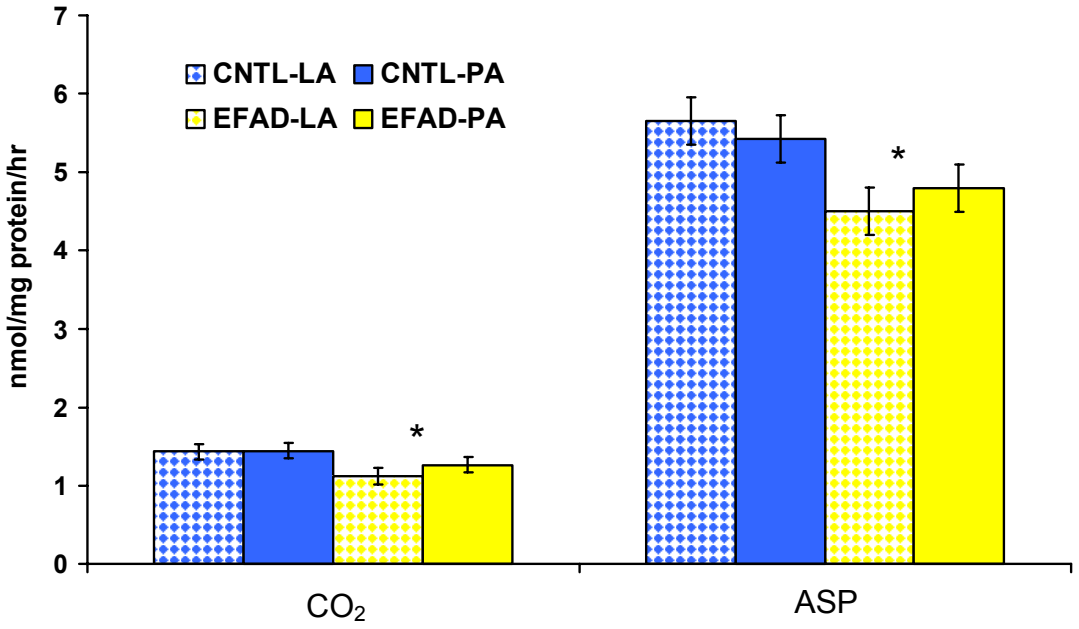

Figure 2. Oxidation of LA and PA in the brain of piglets fed either a CNTL or EFAD diet for 12 days. Values presented are means ± SEM. n = 6 for all treatment groups, * P < 0.05, diet effect

Table 4. Effect of an essential fatty acid deficient diet on the composition of the empty body and tissue accretion rates of neonatal pigs.[1, 2]

	Body Composition[3], %		
	CNTL	EFAD	SEM
Protein	15.5	15.7	0.3
Lipid	6.5[a]	5.1[b]	0.3
Ash	3.0	2.9	0.2
Water	72.6[a]	74.1[b]	0.8
	Accretion Rates[4], g/d		
Protein	49.3[a]	41.7[b]	2.2
Lipid	26.2[a]	17.5[b]	1.9
Ash	8.1	6.0	1.4
Water	200.4[a]	166.6[b]	9.2

[1] Treatment groups are: control, CNTL; essential fatty acid deficient, EFAD.

[2] Values presented are means ± SEM. Within rows, treatments lacking common superscripts differ, $P < 0.05$.

[3] Body composition was calculated as follows for each component: (Ending pig body weight (g) x composition (g/g))

[4] Accretion rates were calculated as follows for each component: (Ending pig g protein, lipid, ash or water – Initial pig g protein, lipid, ash or water) ÷ 12 days

Table 5. Daily accretion of fatty acids from piglets fed either a control or and essential fatty acid deficient diet for 12 d.[1, 2]

Fatty Acid	CNTL	EFAD	SEM
	g/d		
16:0	12.70	10.92	0.6
16:1	0.88[a]	1.99[b]	0.11
18:0	4.31[a]	2.96[b]	0.18
18:1	47.00[a]	16.50[b]	1.5
18:2 (n-6)	31.46[a]	4.97[b]	1.6
18:3 (n-3)	0.32[a]	0.09[b]	0.01
20:0	0.04	-0.02	0.02
20:1	1.32[a]	0.08[b]	0.12
20:2	-0.01[a]	-0.37[b]	0.02
20:3	0.04	0.05	0.03
20:4 (n-6)	0.25	0.30	0.06
22:6 (n-3)	0.07	0.05	0.03

[1] Treatment groups are defined in Table 3.

[2] Values presented are means ± SEM. Within rows, treatments lacking a common superscripts differ, $P < 0.05$.

[3] Accretion rates were calculated as follows for each component: (Ending pig g fatty acid – Initial pig g fatty acid) ÷ 12 days

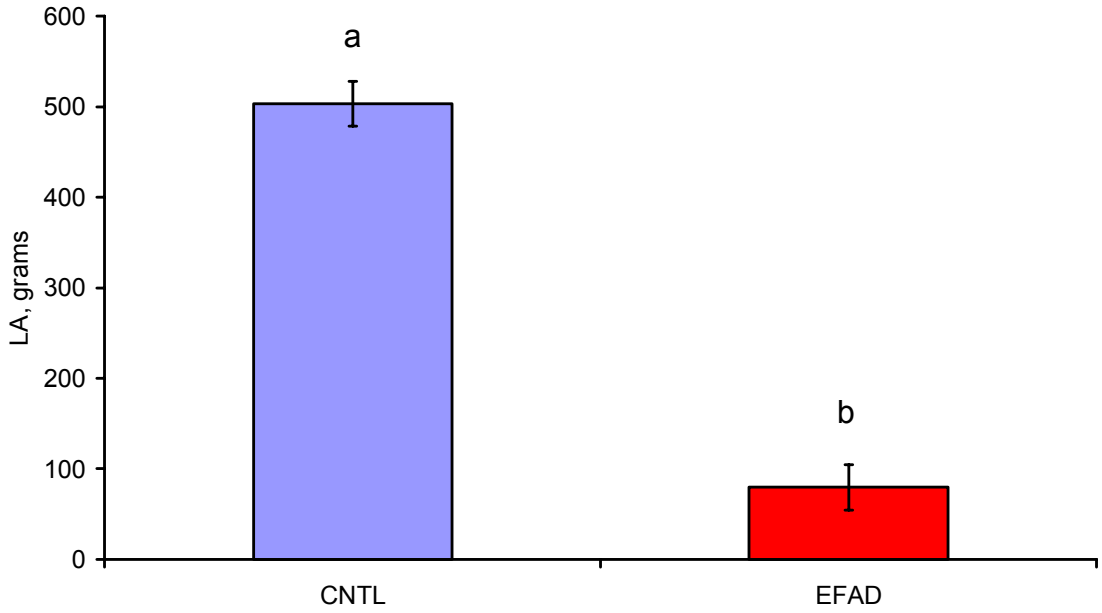

Figure 3. Total body accretion of linoleic acid (LA) over the 12 d treatment period of neonatal pigs fed either a CNTL or essential fatty acid deficient diet. Values presented are means ± SEM. Bars lacking common letters differ ($P < 0.05$); n = 6 for both treatment groups.

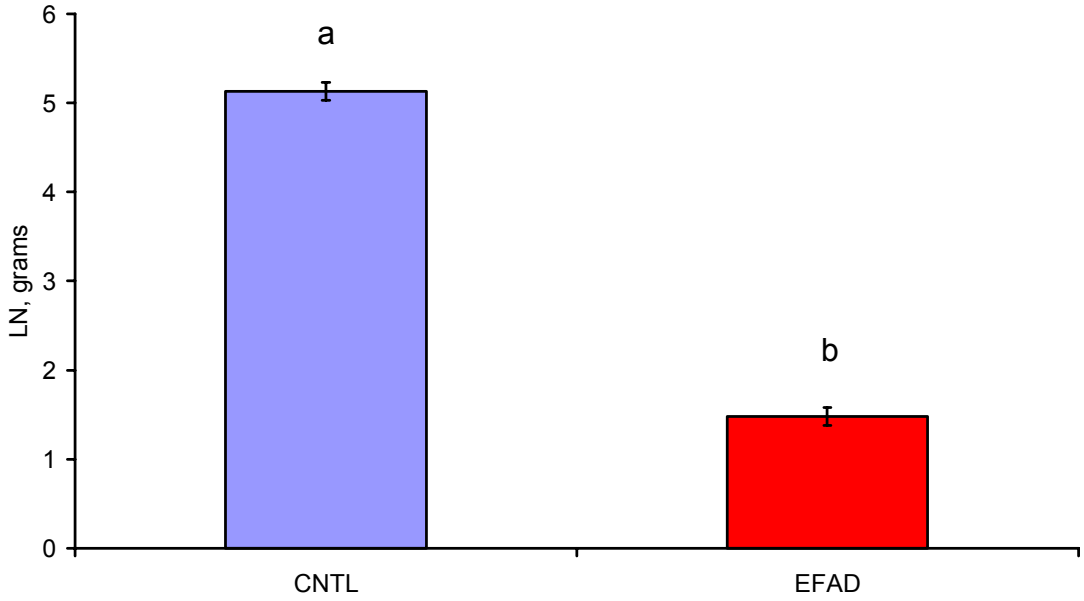

Figure 4. Total body accretion of linolenic acid (LN) over the 12 d treatment period of neonatal pigs fed either a CNTL or essential fatty acid deficient diet. Values presented are means ± SEM. Bars lacking common letters differ ($P < 0.05$); n = 6 for both treatment groups.

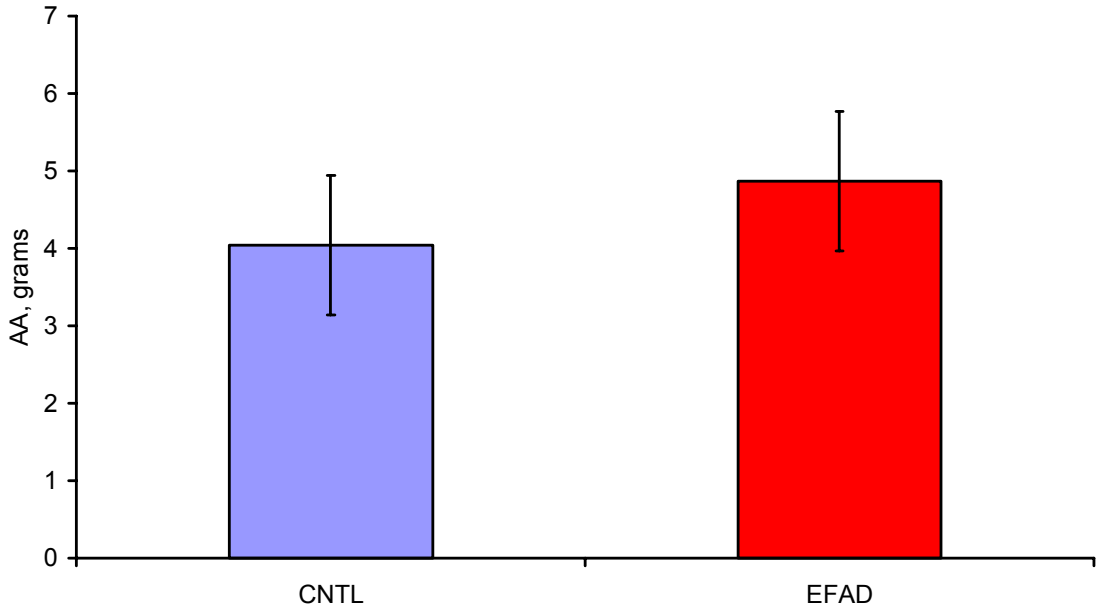

Figure 5. Total body accretion of arachidonic acid (AA) over the 12 d treatment period of neonatal pigs fed either a CNTL or essential fatty acid deficient diet. Values presented are means ± SEM. Bars lacking common letters differ ($P < 0.05$); n = 6 for both treatment groups.

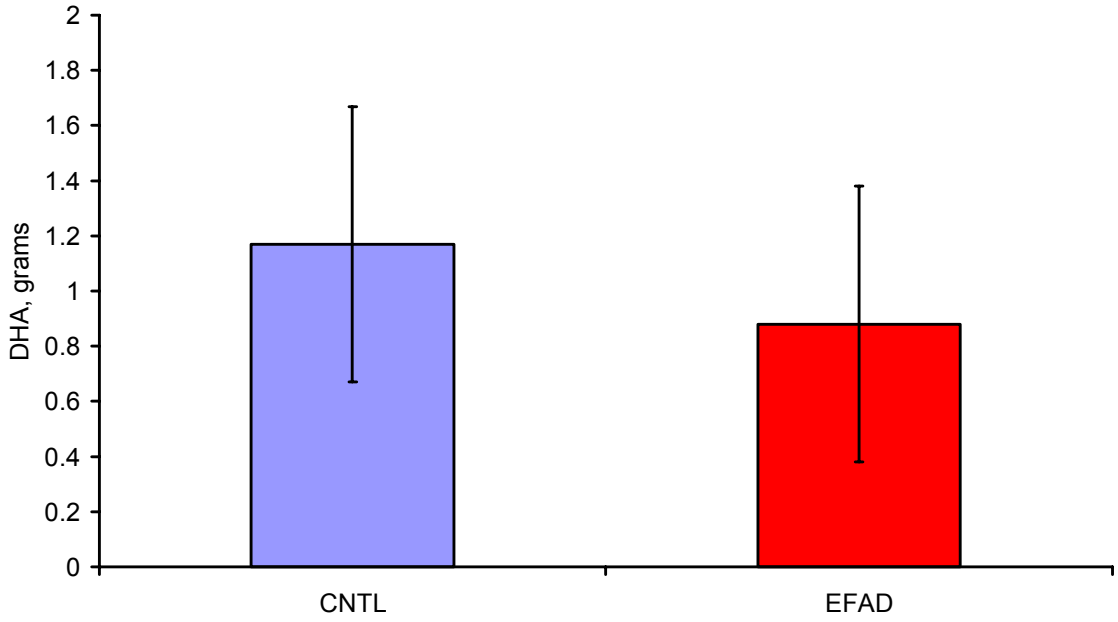

Figure 6. Total body accretion of docosahexaeonic acid (DHA) over the 12 d treatment period of neonatal pigs fed either a CNTL or essential fatty acid deficient diet. Values presented are means ± SEM. Bars lacking common letters differ ($P < 0.05$); n = 6 for both treatment groups

SUMMARY

Performance

- Growth rate and feed intake were reduced in the EFAD fed piglets, however, feed efficiency was unaffected.

β-oxidation

Trial 1: Liver

- Accumulation of ^{14}C in CO_2 was reduced by 52% in EFAD piglets
- Accumulation of ^{14}C in ASP was decreased by 33% in EFAD piglets
- Total liver β-oxidation (CO_2+ ASP) was reduced by 33% in EFAD piglets compared to CNTL piglets

Trial 2: Brain

- Accumulation of ^{14}C in CO_2 and ASP was decreased by 16% in EFAD piglets
- Total brain β-oxidation was reduced by 16% in EFAD piglets compared to CNTL piglets

Body Composition and Fatty acid analysis

- Total body lipid content was reduced by the EFAD diet, but total body water content was increased over the CNTL group. No changes in total body protein or ash content.
- Daily accretion of protein, lipid and water were reduced in the EFAD fed piglets, but ash was not affected.
- Daily accretion of both essential fatty acids, LA and LN were reduced in the EFAD fed piglets.

- Daily accretion of the LCPUFA, AA and DHA was not different between groups.

CONCLUSIONS

- Feeding an EFAD diet reduces the catabolism of both essential and non-essential fatty acids regardless of tissue.
- Overall accumulation of body lipid and daily accretion of protein, lipid and water were reduced by EFAD diet.
- Essential fatty acid accretion was lower in the EFAD fed pigs, but LCPUFA did not vary between groups, similar to previously reported results.

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APPENDIX B.

GLUCAGON-LIKE PEPTIDE-2 (GLP-2) AND ROTAVIRUS INFECTION

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INTRODUCTION

Within the last several years, much research attention has focused on one of members of the proglucagon-derived peptide (PGDP) family, glucagon-like peptide-2 (GLP-2). This intestinotropic hormone is a potent intestinal growth factor as documented in both the rodent and the neonatal pig, and is currently being used in human clinical trials (1). GLP-2 is a 33-amino acid peptide that is located on the carboxyl-terminal of the major proglucagon fragment (2). The peptide is produced by and secreted from the enteroendocrine “L” cells in the lower small intestine and the large intestine (3). After the proglucagon fragment is processed and GLP-2 is produced, it has a relatively short half-life in circulation of about 7 minutes. The 33-amino acid peptide is rendered biologically inactive by the enzyme dipeptidylpeptidase IV (DPP-IV) which cleaves at the N-terminus leaving a 31-amino acid peptide, GLP-2 [3-33] (4, 5). A synthetic GLP-2 analogue, h[Gly-2]-GLP-2, with an amino acid substitution at the 2-position, has been patented and shown to resist

cleavage by DPP-IV. Therefore the half-life has been extended and improves the biologic activities of GLP-2 (6).

The biological properties of GLP-2 are relatively unknown compared to what is known about other PGDPs such as GLP-1. In the rodent model, administration of GLP-2 promotes the stimulation of intestinal growth in both the small and large intestine, up-regulates villus height associated with increased crypt cell proliferation, and inhibits enterocyte apoptosis (7). Also, a positive correlation with GLP-2 administration and decreased intestinal hyperplasia has been well documented in the rodent and the piglet when receiving total parenteral nutrition (1, 8). Other biologic activities associated with GLP-2 include regulation of gastric emptying, stimulation of the hexose transport system, and it is thought to be a component of the 'ileal brake' mechanism which causes the slowing of the upper gastrointestinal tract when undigested nutrients are present in the ileum (1, 9).

Rotavirus is an enterpathogen that burdens the absorptive capacity of the gut causing villous atrophy and secretory or malabsorptive diarrhea (10, 11). This double-stranded RNA virus has been implicated as a diarrhea-provoking agent in a variety of animal species (12). Rotavirus enteritis is the leading cause of diarrhea in infants worldwide (11). Determining ways to reduce the severity of the rotavirus infection or speed recovery of the virus is a way to reduce the incidence of rotavirus in infants and children, but also animals that may die from dehydration caused by secretory diarrhea.

With the advent of the promising effects found with the administration of GLP-2 in TPN fed piglets, the question of potential gut restoration by GLP-2 after gastrointestinal insult has not been investigated. A series of studies was designed to determine the effects of GLP-2 on the gastrointestinal tract during a rotavirus infection challenge in enterally fed piglets. We hypothesized that piglets infected with rotavirus that were provided with a continuous intravenous infusion of GLP-2 would have improved gut integrity.

METHODS AND MATERIALS

Experiment 1. All piglets were delivered by terminal Caesarian section to ensure that piglets remained RV free until time of infection. Each piglet was fitted with a heparinized umbilical arterial catheter that was sutured and wrapped onto the piglet to provide ease of daily blood sampling. All piglets were deprived of sow colostrums due to the potential of RV infection from the sow, but received bovine colostrums, which came from the 2nd milking of heifers at the NC State University Dairy Educational Unit, for first 24 h to provide passive immunity. During this time, piglets were trained to consume milk from an artificial rearing system. Piglets were given *ad libitum* access to a milk based diet (Table 1). Piglets were randomly assigned to one of two treatment groups: (1) control group, infused with a control solution (n=4), (2) GLP-2 group, infused with GLP-2 (n=4). Piglets were weighed daily and feed intake was measured gravimetrically. Body temperature, heart rate and oxygen saturation were measured daily.

GLP-2 infusions began on d 3 of life and were calculated to provide 0.98 mg/kg body weight/d and the dose was adjusted daily for each individual piglet. Infusates were prepared on a daily basis and infused at a rate of 50 ml/kg/d using an Abbott Lifecare Infusion System 5000. Rotavirus infection occurred on d 5 with 1 mL of an inoculum containing 6.8 x 10^6 virus particles mixed with milk formula and given to the piglet by orogastic administration. Diarrhea scores, as assessed visual to determine consistency of the feces, were recorded daily for all piglets and virus shedding was tested daily beginning 24h after infection. Blood was collected daily at 2000 h from umbilical catheter (4-6 mL/pig) and put into tubes containing 0.1 mL of a solution containing 1.7 mg Diprotin A (inhibitor of Dipeptidly peptidase IV), 60 mg disodium EDTA, and 25 mL Trasylol® (protease inhibitor). This solution aided in the stabilization of the GLP-2 molecule to prevent further degradation by the DPP-IV. Blood samples were centrifuged (Sorvall, model 64000, Newtown, CT) at 825 x g for 10 min at 4°C. Plasma was collected and aliquots were frozen at – 20°C.

Peptide solubization and infusate preparations. GLP-2 peptide was ordered from American Peptide Corporation and solublized with: 0.01% Ammonium Hydroxide Solution and H_20 and DMSO. Sterile saline was used to dilute the peptide concentration to 0.49 µg/500 µL. Aliquots were frozen at -20°C until daily infusates were made. Infusates were prepared 2X/day for each piglet and infusate bags were changed at 0800 and 2000 and included 1% serum in each bag. Total volume was 48 mL/12 hr (rate 4 mL/hr) and piglets were weighed at 0700 and infusate calculations were computed daily. This provided GLP-2 and control solution

at 50 µg/kg BW. A control solution contained same amounts of NH_4OH, DMSO, & saline.

Tissue Collection and Preparation. All piglets were given 50 mg/kg 5-bromo-deoxyuridine (BrdU), four hr prior to euthanasia to allow time for incorporation of the BrdU into the intestinal tissue so that crypt cell proliferation could be measured. At the end of the study, piglets were killed with an AMVA-approved electrocution devise followed by exsanguination (laceration of the brachiocephalic arteries) and intestinal tissues collected. The abdomen was opened and the gastrointestinal tract was removed from the gastroesophageal junction to the distal end of the rectum. The jejunum and ileum were separated from the duodenum, stomach, and mesentery from the peritoneal inflection to the ileocecal junction. The anterior and posterior ends of the removed small intestinal segment were noted and the jejunum and ileum were laid in 60-cm serpentine loops. The midpoint was marked, and intestine proximal and distal to this midpoint was considered jejunum and ileum, respectively. At approximately mid-jejunum and mid-ileum, two adjacent segments, one 3-cm and another one slightly larger than 10-cm in length, were removed. The 3-cm intestinal segments were processed, embedded, and stained according to procedures described by Luna (13) as reported in Oliver et al. (14) for measurement of villi height and width as well as crypt depth. Invasive measures were included because a reduction in villi height and lactase specific activity and an increase in crypt depths would indicate deleterious effects on the intestine.

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Table 1. Diet formulation

Ingredient	% Included
Non-Fat Dried Skim Milk	54
AMP 80	5.8
Na Casienate	9
Fatpack 80	18
Lactose	7.6
Arginine	0.5
Histidine	0.25
Xantham Gum	1
CaCO3	0.67
Dical	2.6
Min	0.5
Vit	0.127

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RESULTS

Table 2. Performance data from rotavirus infected piglets with or without a continuous GLP-2 infusion.[1]

	Treatments			
Item	CNTL	GLP-2	SEM[2]	P Value
ADG[3] (g)				
d 0-3	131	137	24	0.8
d 3-5	177	223	45	0.4
d 5-9	50	93	35	0.4
ADFI[3] (g)				
d 0-3	60	55	7	0.6
d 3-5	76	84	4	0.2
d 5-9	80	102	13	0.2
Feed Efficiency[3]				
d 0-3	0.49	0.39	0.1	0.2
d 3-5	0.49	0.41	0.1	0.5
d 5-9	1.51	0.86	0.2	0.2

[1] Tabulated values are least square means, $P < 0.05$.

[2] Standard error of the difference of the means.

[3] ADG = average daily gain, ADFI = average daily feed intake, Feed Efficiency = gram of feed per gram or gain

Table 3. Intestinal parameters in rotavirus infected piglets with or without a continuous GLP-2 infusion.[1]

	Treatments			
Item	Control	GLP-2	SEM[2]	P-value
GI[3] Length (cm)	8.0	8.3	0.6	0.6
Total Intestine Wt (g)	59.2	76.6	12	0.3
Ileal Mucosal Wt (g)	1.2	1.3	0.1	0.4
Jejunal Mucosal Wt (g)	1.4	1.3	0.2	0.8
Villi Height (mm)				
Jejunum	0.34	0.53	0.08	0.1
Ileum	0.35	0.46	0.1	0.5
Villi Width (mm)				
Jejunum	0.13	0.11	0.02	0.6
Ileum	0.12	0.11	0.01	0.6
Crypt Depth (mm)				
Jejunum	0.21	0.17	0.02	0.3
Ileum	0.19	0.19	0.03	0.9

[1] Tabulated values are least square means, $P < 0.05$.

[2] Standard error of the difference of the means.

[3] GI = gastrointestinal length

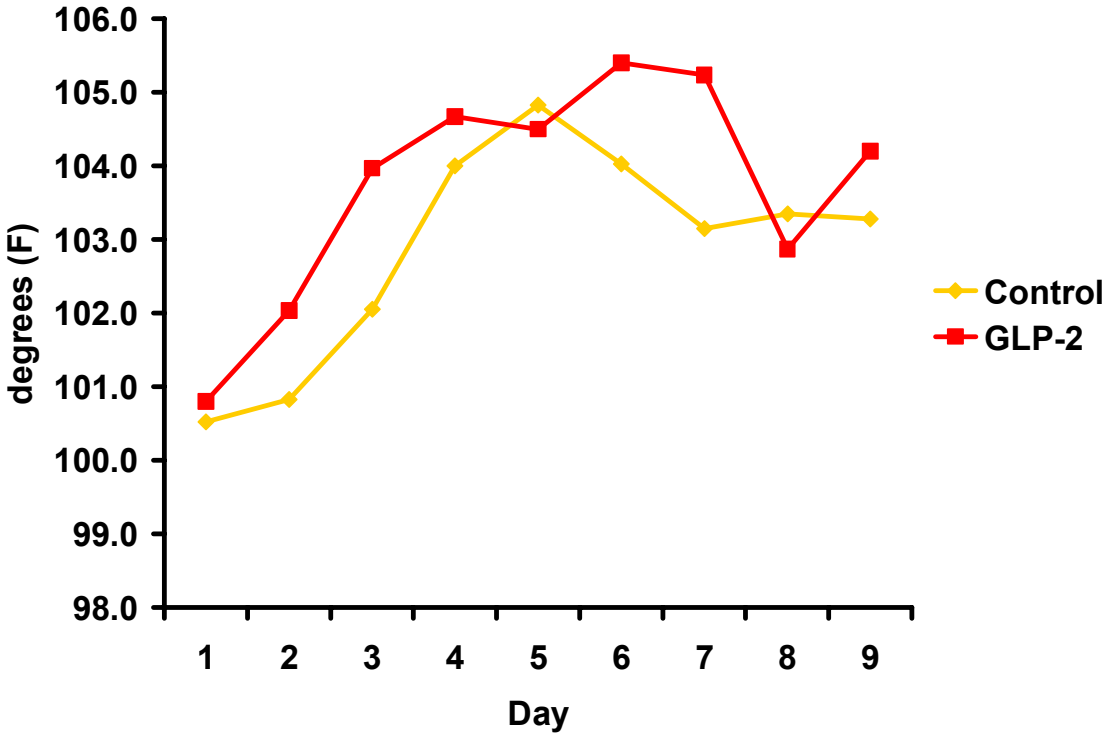

Figure 1. Body temperatures in rotavirus infected piglets with or without a continuous GLP-2 infusion. Values presented are means ± SEM; $P < 0.05$; $n = 4$ for all treatment groups.

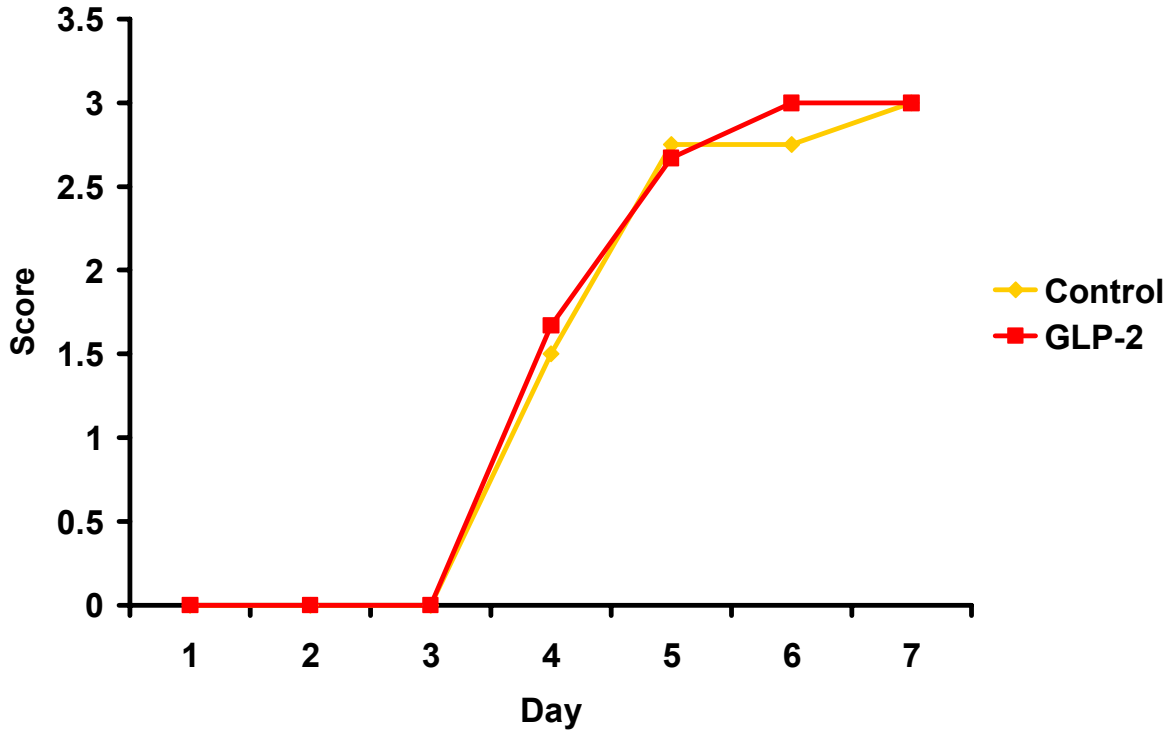

Figure 2. Virus shedding in rotavirus infected piglets with or without a continuous GLP-2 infusion. Values presented are means ± SEM; $P < 0.05$; $n = 4$ for all treatment groups.

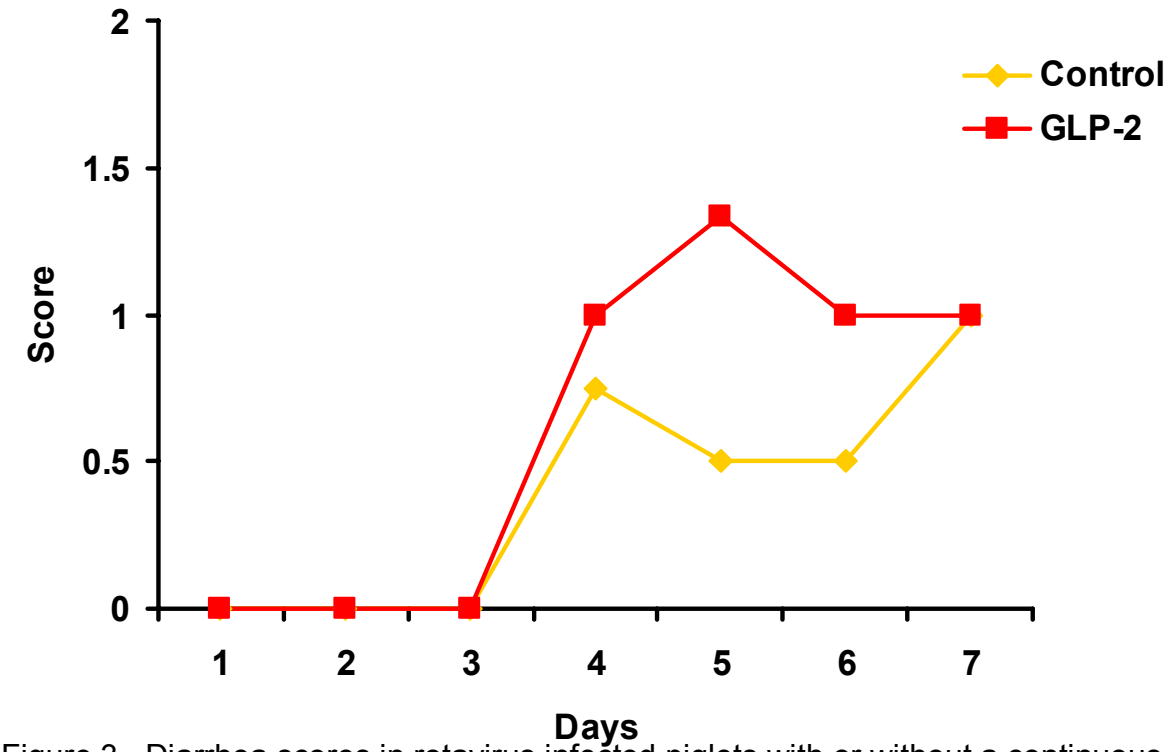

Figure 3. Diarrhea scores in rotavirus infected piglets with or without a continuous GLP-2 infusion. Values presented are means ± SEM; $P < 0.05$; $n = 4$ for all treatment groups.

Experiment 2. The second experiment was conducted to determine the interaction of GLP-2 in healthy vs. rotavirus infected pigs. There were two differences between Experiment 1 and Experiment 2. In Experiment 2, piglets received 2 daily bolus infusions of GLP-2 at 0.48 μg/kg BW every 12 h instead of continuous infusions. Also in Experiment 2, only ½ of the piglets were infected with RV. All other methods and materials were the same.

RESULTS

Table 4. Performance data from piglets (+/- rotavirus, RV) with or without daily bolus GLP-2 infusions.[1]

Item	Treatments				SEM[2]	P Value
	-RV -GLP-2	-RV +GLP-2	+RV -GLP-2	+RV +GLP-2		
ADG[3], g/d						
d 1-4	248	292	272	272	38	0.8
d 4-7	223	216	219	279	44	0.6
Total	236	255	246	276	37	0.8
ADFI[3], g/d						
d 1-4	110	117	108	108	14	0.9
d 4-7	154	160	154	158	24	0.9
Total	130	137	129	131	18	0.9
Feed Efficiency[3]						
d 1-4	0.45	0.40	0.40	0.40	0.03	0.3
d 4-7	0.68	0.77	0.81	0.57	0.2	0.2
Total	0.56	0.54	0.54	0.48	0.02	0.09

[1] Tabulated values are least square means, $P < 0.05$.

[2] Standard error of the difference of the means.

[3] ADG = average daily gain, ADFI = average daily feed intake, Feed Efficiency = gram of feed per gram or gain

SUMMARY OF RESULTS

For both experiments, there appeared to be no protective effect on GLP-2 on piglets during a rotavirus infection.

Experiment 1

No difference in performance data or intestinal data was observed between piglets with or without continuous GLP-2 infusions. No differences were observed in diarrhea scores or in virus shedding. Total intestinal length, ileal and jejunal length and weight were also not different between treatments. No differences were observed in intestinal morphology. There was no difference in body temperature between the groups.

Experiment 2

No differences in ADG or ADFI, but a trend was observed in total feed efficiency, with the +RV/+GLP-2 appearing more efficient compared to all other treatments. Also, in analyzing the data we also looked at the interactions of health and GLP2 infusion, but only found a trend for non-infection ($P < 0.09$) and +GLP-2 infusion ($P < 0.08$) to increase feed efficiency.

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