ABSTRACT

HESS, HOLLY ALINE. Prophylactic enrichment of enterocyte phospholipids with polyunsaturated fatty acids fed to suckling piglets. (Under the direction of Dr. Jack Odle and Dr. Robert J. Harrell.)

Infant formula companies began fortifying formulas with long-chain polyunsaturated fatty acids (PUFA) in 2002, including arachidonic acid (AA) at ~ 0.5% of total fatty acids. This study is the first in a series that will examine effects of supra-physiological supplementation of AA on intestinal health. The objective was to determine the time specific effects of dietary AA on fatty acid composition of enterocyte phospholipids and on the mRNA abundance of hepatic Δ^6- and Δ^5-desaturases. One d old pigs (N=96) were fed a milk-based formula for 4, 8, or 16 d. Diets contained either no PUFA (0%AA, negative control), 0.5%AA, 2.5%AA, 5%AA, or 5% eicosapentaenoic acid (EPA) of total fatty acids. Growth (299 +/- 21 g/d) was unaffected by treatment (P>0.1). By day 16, ileal enterocyte phospholipid concentrations of AA were enriched 176%, 280%, and 355% in pigs fed the 0.5%AA, 2.5%AA, and 5%AA diets, respectively, compared with the 0%AA control (P<0.05). Concentration of AA within enterocytes of the 5% EPA fed group was similar to that of the 0%AA fed pigs, while EPA concentration increased by >8 fold. As pigs aged from birth to 16 d of age, there were no differences in the desaturase mRNA abundance (P>0.1), measured in pigs fed 0.5% or 5%AA. Abundance also was similar among AA-supplemented pigs, but was elevated by 60-80% in the 0%AA-fed pigs compared to those fed 2.5% (P<0.05). These data demonstrate a dose-dependent response in enterocyte AA

concentration to dietary AA and show that supra-physiologic supplementation of AA is not detrimental to growth or desaturase gene expression.

PROPHYLACTIC ENRICHMENT OF ENTEROCYTE PHOSPHOLIPIDS WITH POLYUNSATURATED FATTY ACIDS FED TO SUCKLING PIGLETS

By

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DEDICATION

To Mom, Missy and Dad for being there every step of the way

BIOGRAPHY

Holly Aline Hess was born January 8, 1979 in Pinehurst, North Carolina to Bob and Paula Hess. She graduated from Pinecrest High School in 1997 and later attained her Bachelor of Science degree at North Carolina State University in May of 2002. Following graduation, she worked as a veterinary assistant at Hayes-Barton Animal Hospital for one year. She was then fortunate enough to receive an Intramural Research Training Awards Fellowship that allowed her to work as a lab assistant at the National Institute of Environmental Health Sciences under the direction of Drs. Masahiko Negishi and Yukio Yamamoto. During that time, she applied to graduate school at North Carolina State University and began her Master of Animal Science degree in August of 2004. Upon completion of her degree, she plans to pursue a career in biological science research.

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CHAPTER 1

LITERATURE REVIEW

BIOACTIVE FOOD COMPONENTS

Epidemiologically, a clear correlation between diet and health has been observed (1). Foods providing health benefits beyond their nutritive value are considered "functional foods," while a food's constituents responsible for such health effects are termed "bioactive food components (2)." Giving scientific credence to the belief that many food components promote health, an *ad hoc* Federal working group was organized in September of 2004 to establish a definition for such health proponents, or "bioactive food components." The Federal working group assigned the task includes representatives from the Departments of Health and Human Services, Defense, and Agriculture, as well as numerous agencies within those departments (3). Great interest, expressed by the scientific community, government, and consumers, has brought about the need to define "bioactive food components" (3) Scientists are anxious to have bioactive food components defined as a necessary step in determining ways to assess their health effects (3); only extensive, reliable research can confirm the benefits of a food or its components for a food label's approval by the FDA (4). Both the general public's mounting interest in health through diet and the government's changes in food laws, affecting label and product claims, have provided the impetus for scientists to assess the healthful benefits of foods and their specific components (4).

However, great controversy, over how to define both functional foods and their bioactive constituents, exists among academics, scientists, business analysts, and policy experts alike, all aware of the potential to obscure the line between food and medicine (5). Supporting that potential is the tenet of Hippocrates (400 B.C.), the Father of Medicine, 'Let food be your medicine and medicine be your food' (6).

While scientists have yet to universally agree on a definition for bioactive food molecules, the current proposed definition states that bioactive food components are "constituents in foods or dietary supplements, other than those needed to meet basic human nutritional needs, that are responsible for changes in health status" (3). It was suggested almost unanimously by the proposed definition's reviewers that the definition be modified to include essential nutrients as bioactive food components "when they have physiological effects at levels beyond those required to meet essential nutritional requirements" (7).

Chemical compounds such as carotenoids, flavonoids, plant sterols, allyl and diallyl sulfides, indoles, phenolic acids, ω-3 (n-3) and ω-6 fatty acids (n-6) are all examples of bioactive food components (2). Bioactive food components may deliver health benefits by acting as antioxidants, activating liver detoxification enzymes, attenuating the activity of bacterial or viral toxins, inhibiting cholesterol absorption, decreasing platelet aggregation, destroying harmful gastrointestinal bacteria, etc (2).

Essential Fatty Acids

Normal mammalian cell function depends, in part, on two families of essential fatty acids (EFA), the omega-6 (n-6) and omega-3 (n-3) series fatty acids (8,9). Two such fatty acids are considered essential nutrients (8,10,11) in the human diet, linoleic (LA, 18:2n-6) and α-linolenic (ALA, 18:3n-3) acid (9). LA and ALA cannot be synthesized endogenously from other lipids or from carbohydrates and amino acids (12), so it is essential that they be provided in the diet (10,12). Long chain polyunsaturated fatty acids (LCPUFA) of the n-6 and n-3 series can be obtained directly from the diet or derived metabolically from LA and ALA, respectively (12), by their sequential alternating desaturation and elongation to eventually include 20 or more carbons and 3 or more double bonds (9). High concentrations of LCPUFA, particularly arachidonic acid (20:4n-6), docosatetraenoic acid (22:4n-6) and docosahexanoic acid (22:6n-3), are found in structural lipids of many tissues, including the central nervous system and retina, where they play an important role in many membrane associated functions (9).

Common nomenclature of fatty acids calls for the number of carbon atoms to be given first, followed by a colon and the number of unsaturated bonds (9). As proposed by Holman in 1964 (13), fatty acid families are now referred to by the position of the first double bond from the methyl-n (ω) terminus of the fatty acid carbon chain (9). For example, linoleic acid, written 18:2n-6, has two double bonds with the first double bond situated between the 6th and 7th carbons from the methyl terminal (14). α-Linolenic acid, written 18:3n-3, has three double bonds with the first double bond situated between the 3rd and 4th carbons from

the methyl terminal (14). Confusion created by the previously used numbering system for unsaturation, the abbreviated Geneva chemical terminology, necessitated Holman's numbering system of referring to the unchanging, terminal structure of polyunsaturated fatty acids (13). It is the terminal carbon chains of membrane lipids that influence the membrane's physical properties (13)

Burr and Burr (15,16) noted the essentiality of LA and ALA in 1930 after feeding rats a fat-free diet over a period of several months (9). The EFA deficiency that was created resulted in growth retardation, scaly dermatitis, and reproductive failure (9,13).

PUFA and their oxygenated metabolites, or eicosanoids, are potent bioactive components that help to regulate biological functions (17), such as the inflammatory response, fetal growth and development, and retinal function, and brain development (18). Research has demonstrated that PUFA essentiality is strongly correlated with their ability to incorporate into lipids (19) and to be converted to eicosanoids (12).

PUFA Sources

PUFAs comprise ~7% of total energy intake and 19-22% of energy intake from fat in the adult American's diet (20). LA is the most abundant dietary PUFA, accounting for 84-89% of total PUFA energy, while ALA accounts for merely 9-11% of total PUFA energy (20). The n-6 LCPUFAs, 18:4n-6 and 20:4n-6 (AA), provide ≤0.1% of energy intake, while the n-3 LCPUFAs, DHA and EPA together, provide ≤0.1-0.2% of energy intake (20). It can therefore be inferred that LCPUFAs aren't a substantial portion of fat intake (20).

The parent essential fatty acids, 18:2n-6 and 18:3n-3, of n-6 and n-3 LCPUFA are primarily derived from vegetable oils as higher plants are their major source (21,22), but their LCPUFA metabolites can be found preformed in foods of animal origin (9) such as components of meat, fish, and other animal tissue lipids (23). AA and LA are the predominant n-6 fatty acids found in the diet (24). AA is found primarily in animal meat, while vegetable oils, nuts and seeds (safflower oil, corn oil, peanuts, and soybeans specifically) are the predominate suppliers of LA (24).

The major sources of n-3 fatty acids are fish (20), fish oils, canola oil, flaxseed oil, walnuts and green leafy vegetables (21,24). To be more specific, vegetable oils, walnuts and green leafy vegetables provide ALA, which can be converted endogenously to EPA and DHA, and fish provide both EPA and DHA, with oily fish, such as menhaden fish, serving as their richest source (24,25).

PUFA Metabolism

Although mammalian cells can naturally synthesize saturated, and n-9 and n-7 series unsaturated fatty acids endogenously from acetyl CoA, by lacking the Δ-12 and Δ-15 desaturase enzymes (excepting transgenic animals) that insert double bonds at the positions of fatty acid carbon chains, they don’t have the ability to synthesize n-6 and n-3 series fatty acids (9,23). So, once the essential fatty acid precursors, LA and ALA, are supplied in the diet, all other ω-6 and ω-3 FAs can then be synthesized endogenously by alternating desaturation and elongation via the same microsomal enzyme system resulting in two cascades **(Figure 1)** of FA metabolites up to 22 or more carbons long (9,13,

21,23). All reactions in the desaturase elongase cascade, with the exception of the final reaction producing docosahexaenoic acid (DHA), occur in the endoplasmic reticulum (25). The cascades begin with the rate limiting reaction (9,25,26) by Δ-6 desaturase which inserts a double bond at the Δ-6 carbon in both LA and ALA to form octadecatetraenoic (OTA; 18:4 $\Delta^{6,9,12,15}$) and γ-linolenic acid (GLA; 18:3 $\Delta^{6,9,12}$), respectively. Δ^{6}-desaturase is considered the rate-limiting enzyme, because it controls the entry of the 18C FAs into the LCPUFA synthesis pathway (9). Following this step is a 2 carbon elongation at the carboxyl ends resulting in 20:3 $\Delta^{8,11,14}$ and 20:4 $\Delta^{8,11,14,17}$, which are then desaturated by Δ^{5}- desaturase to yield arachidonic acid (AA, 20:4 $\Delta^{5,8,11,14}$) and eicosapentaenoic acid (EPA, 20:5 $\Delta^{5,8,11,14,17}$) (21). EPA is metabolized to docosapentaenoic acid (DPA) by addition of C2 (21,25). Previous thought suggested that docosahexaenoic acid (DHA) is synthesized from DPA via desaturation by Δ^{4}- desaturase. However, it is currently accepted that DPA is elongated to form 24:5n-3, desaturated by Δ^{6}-desaturase, and then β-oxidized in mitochondria or peroxisomes (23,25) to form DHA (22:6n-3) (25,27,28).

Dietary LA and ALA can by taken up by tissues and subsequently desaturated and elongated to form LCPUFAs, readily undergo mitochondrial β-oxidation for energy, or be acylated into tissue lipids (9,23). Studies involving the brain and retina have provided evidence of the uptake of LA, ALA and 20 carbon chain LCPUFA followed by desaturation and elongation, and evidence of the rapid uptake and acylation of preformed n-6 and n-3 LCPUFA (9,29). However, the biological events regulating the different fates of the n-6 and n-3 parent EFAs

in the body need further investigating (23). Research has shown that dietary LCPUFA precursors are quantitatively less effective than dietary LCPUFA themselves at enriching membrane lipids (9,23). It is known that when adequate LA and ALA are provided in the diet, further supplementation of LA and ALA does not lead to a corresponding increase in tissue levels of AA or DHA (23,30) (31,32), suggesting that desaturation of the parent EFAs is regulated to meet the body's need for AA and DHA for phospholipid synthesis and FA turnover and not to match the body's substrate supply (23).

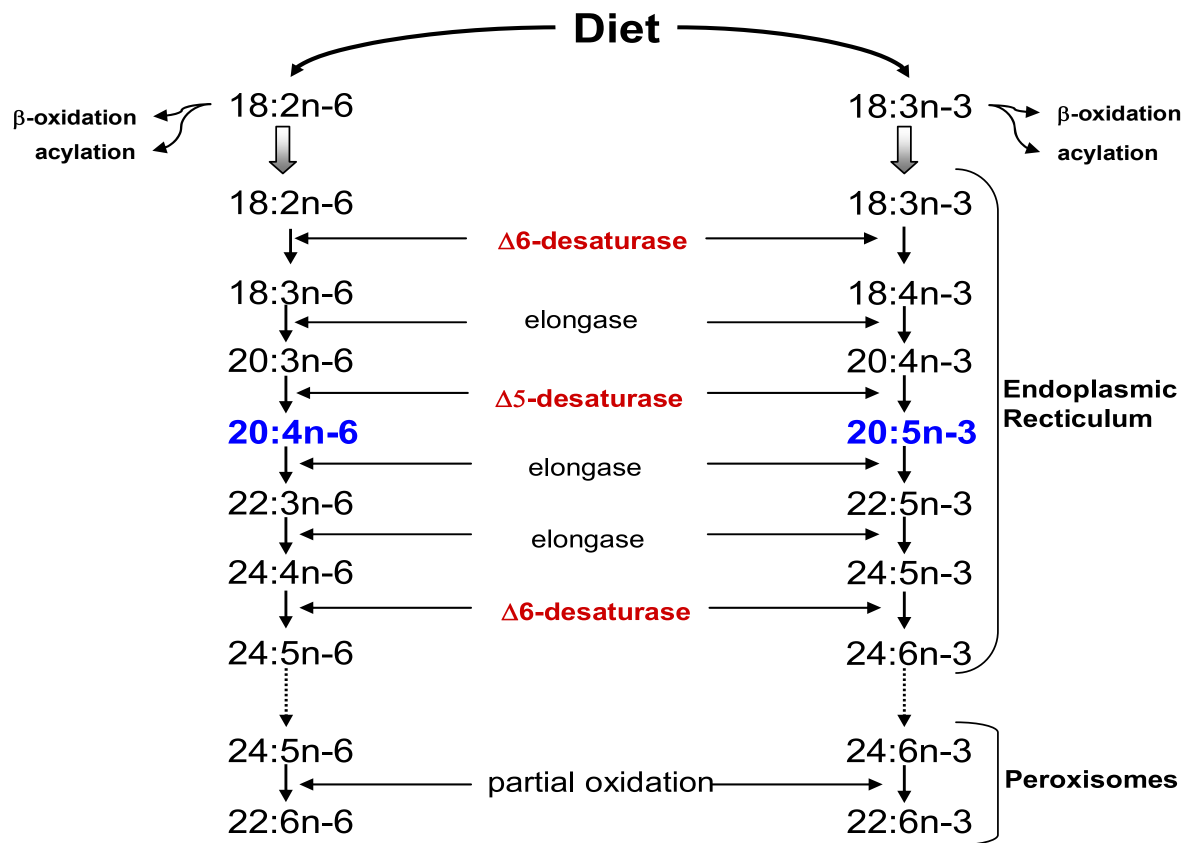

Figure 1: Schematic of n-6 and n-3 fatty acid desaturation and elongation (Innis, 2003).

Competition between ω-6 and ω-3 EFAs

Holman and Mohrhauer hypothesized in 1963 that all FAs (n-3, n-6, and n-9) compete with the EFAs at all steps of the desaturase elongase cascades for metabolism of EFAs. Their hypothesis was based on the finding that when dietary LA is held constant, increasing the level of dietary ALA suppresses the content of ω-6 metabolites (13,33), and inversely, when holding ALA constant and increasing the level of dietary LA, ω-3 metabolites are suppressed (13,34). As one cascade of EFAs is suppressed, that cascade's parent FA increases in liver lipids (13). This competition over the enzymes also results in inhibition of the enzyme pathway by products of both the same and the opposing series of FAs, which is an interaction between substrate and metabolic product (9,26) (35,36). One such case results in decreased tissue AA and reduced LA derived eicosanoids due to the increased consumption of EPA or DHA (35,36,37).

The affinity of Δ^6-desaturase is highest for the n-3 series fatty acids (13), with the preferential substrate affinity as follows: 18:3n-3>18:2n-6>18:1n-9 (9). Therefore, 18:1n-9 is competitively inhibited by both LA and ALA (9,38) with significant synthesis of 20:3n-9 and 22:3n-9 occurring only during concurrent deficiency of both n-6 and n-3 fatty acids (9). Evidence of this competition has been demonstrated in numerous *in vivo* (9,39) and *in vitro* studies (9,40).

Research has shown that strong suppression of ω-6 metabolism is possible when <2% of calories are provided as 18:3n-3, but that for equal suppression of ω-3 metabolism to occur, nearly 10 times as many calories must be provided by 18:2n-6 (13,33). For equal competition to take place between

parent n-6 and n-3 EFA, LA and ALA should be in the ratio of 14:1 (13). However, it was concluded in cognition studies employing rats that the optimum functional ratio of ω-6/ω-3 is 4:1 (13,41).

EFA Deficiency

Burr and Burr discovered the essentiality of dietary fat in 1930 after having fed young rats a fat-free diet for several months (9,13,15,16). Deliterious effects of the diet included growth retardation, dermatitis, necrosis of the tail, increased transepidermal water loss and reproductive failure, which have all been successfully reproduced in other studies (9,13,42). Burr and Burr concluded that the deleterious effects of the fat free diet were not attributable to the strain of long continued fat synthesis as Krogh and Lindhard had previously postulated (15,16,43) but instead to the lack of dietary linoleic acid and possibly other fatty acids, including linolenic acid (16). Both ALA and LA have been shown to improve growth failure and the dermatitis of EFA deficiency (13).

Essential fatty acid deficiency describes the combined deficiency of the two essential fatty acids, 18:2n-6 and 18:3n-3. An isolated deficiency of one of the two series of fatty acids would have reference made to that specific series (9). Although oleic acid has the least substrate affinity of Δ^6-desaturase, during EFA deficiency when substrate competition is not an issue, it is metabolized to eicosatrienoic acid (C20:3n-9) in significant quantities (9,26). Thus, the quantity/percent of 20:3n-9 relative to 20:4n-6 (triene:tetraene ratio) in plasma or tissue lipids is often used as the biochemical measure of EFA deficiency (9,44). In treating EFA deficiency, arachidonic acid has shown to have greater potency

than LA or ALA, but research has shown all three FA to be effective at treating EFA deficiency and promoting normal growth (45).

EFA deficiency has been documented on numerous occasions in adults (9,46), infants, and children (9,47,48) maintained on fat free or minimal fat parenteral nutrition. Adults maintained on fat free parenteral nutrition who developed EFA deficiency, despite having tissue reserves of n-6 fatty acids, did so because circulating insulin levels prevented lipolisis (9,45). EFA deficiency appeared in 1970 in an infant maintained on a fat-free total parenteral nutrition (TPN) diet, following a volvulus related duodenal-colon anastomosis. Skin lesions, typical of EFA deficiency, appeared after 3 months of TPN and were shown to be related to EFA deficiency (13). During the infant's autopsy, plasma PL were analyzed and compared to the PL of many other tissue specimens, and it was concluded that the plasma PL FA status reflected that of other tissues in the body, demonstrating that the profile of plasma PL is an accurate measure of EFA status of tissue PL (13). Similarly, a report by Rieckehoff et al. (49), that found chemical evidence of EFA deficiency in liver, kidney, heart, brain, muscle, skin, and fat depots of the brain, demonstrated that EFA deficiency affects all tissues (13).

Experimental animal studies have shown that there is a greater susceptibility of the very young to develop EFA deficiency than for adults when fed diets low in fat (4,50). Not surprisingly, EFA tissue reserves are low in low birth-weight infants (45,51) and LA concentration is found to increase in fetal muscle PL with increased gestational age (45,52). Low tissue EFA is therefore

likely what makes the infant more susceptible to EFA deficiency and is what causes EFA deficiency to occur more rapidly in the very young (45).

In a study examining the effect of an EFA deficient diet on the translocation of intestinal bacteria in mice, it was found that an EFA deficient diet appears to increase the incidence of bacterial translocation independent of intraperitoneal lipopolysaccharide (LPS) administration (53). Treatment groups were fed either an EFA-replete (EFA-R) diet or an EFA-deficient (EFA-D) diet for 8 days with the EFA oil source being the only difference between the two diets. Treatment groups consisted of those given diet alone, diet and intraperitoneal LPS, or diet and intramuscular metronidazole (MET) three times a day. Of those groups treated with diet alone, the EFA-D fed group had significantly more bacterial translocation to the mesenteric lymph nodes occur that was independent of an effect on ileal flora and ileal histological conditions (53). The authors of the study raised two possible explanations for the increased bacterial translocation with the EFA-D diet: 1) “depressed levels of AA and its metabolites may impair the cellular immune response to invading bacteria within the intestinal epithelium,” or 2) “EFA deficiency results in structural changes within the intestinal epithelium that in turn lead to a failure of mechanical barrier function” (53). In reference to the second explanation the authors noted that pig brush-border membrane studies have shown that the composition of membrane PL markedly changes in response to EFA deficiency, subsequently affecting membrane fluidity (53,54,55).

Desaturase Enzymes

There are three desaturase enzymes identified in the human. These enzymes, Δ^{9-}desaturase, Δ^{6}-desaturase, and Δ^{5}-desaturase, are membrane-bound, non-heme iron-containing enzymes that introduce double bonds aerobically at fixed carbons from the carboxyl, or delta carbon, end of fatty acids (21,56). All known mammalian desaturases are of the acyl-coenyzme A (CoA) subgroup of membrane-bound desaturases. The acyl-CoA desaturases use fatty acyl-CoAs as substrates and are located in endoplasmic reticulum membrane (21,56).

Δ^{9}-desaturase enzymes catalyze the synthesis of monounsaturated fatty acids from saturated fatty acids, and Δ^{6} and Δ^{5}-desaturases catalyze the synthesis of long chain polyunsaturated fatty acids (56). Desaturase activity was shown not to be evenly distributed throughout all organs (22). It is, however, evident in many human tissues, including placenta, central nervous system, glial tissue, and choroid plexus vasculature (26), but is most abundant in the liver (26) {2A-10}. Consequently, the liver is a valuable source of LCPUFA for many of the body's organs, including the brain (22).

Studies have indicated that the activities of desaturase enzymes are lower in the developing human liver than in the adult liver, raising the question of whether or not the fetus, preterm and term neonate can meet the needs of LCP by conversion from their precursors (25). Studies have also indicated that dietary n-6 and n-3 LCPUFA supplementation in preterm infants improves their growth and cognitive development (56). It is not concretely known yet whether or not the

fetal or neonatal liver desaturase activity can meet the needs of the developing human following an abrupt end to placental supply of LCP as in premature births or when lipid nutrition is provided after birth without LCP supplementation (9). Tracer studies have demonstrated that preterm and term human infants are capable of converting LA and ALA to AA and DHA (**35**,**57**,**58**), respectively, but some tracer studies in developing rats have indicated that only a small percentage of dietary precursor fatty acids are converted to their LCP metabolites (59,60,61).

Desaturase enzymes are regulated by several factors, such as nutritional factors, hormonal factors and physiologic factors (21). One such factor, dietary PUFAs, can inhibit desaturase activity through feedback inhibition (21,56) Cho et al. (26,62) found that dietary PUFAs significantly reduce hepatic Δ^6-desaturase expression (26) and enzymatic activity (63).

Δ^6 desaturation of LA and ALA is induced with low concentrations of both reaction substrate and product and alternately inhibited with high concentrations of reaction substrate or product (44). Studies of rat liver microsomes show that high concentrations of either LA or its n-6 LCP inhibit Δ^6-desaturation and that low concentrations of either will induce Δ^6-desaturation (9,40,64,65). Δ^5-desaturase has been found to respond similarly to both n-6 and n-3 PUFA (9) (40). A study by Marcel et al (21,66) found that rats fed a fat-free diet had a 2-fold higher level of microsomal desaturase activity than chow-fed rats (21).

Two transcription factors are known to be important in the regulation of desaturases by PUFAs, sterol regulatory element binding protein-1c (SREBP-1c)

and peroxisome proliferator activated receptor-α (PPARα) (56). SREBP-1c activates fatty acid synthesis genes, including all three mammalian desaturases, and is suppressed by LCPUFA (56). A study using PPARα-null mice and wild type mice demonstrated that when LCPUFAs are low, PPARα is necessary to mediate Δ^6-desaturase feedback induction (56). LCPUFAs are more potent suppressors of SREBP-1c (56,67,68), but both LCPUFA and their precursors act to suppress SREBP-1c (56). Further research is required to determine if LCPUFA precursors must be converted to their LCP metabolites in order for SREBP-1c suppression to occur (56).

It is also possible that the amount of fat in the diet may affect desaturase activity (21). Kurata and Privett (69) found that liver fatty acid composition was affected by the level of dietary fat in rats; they noted hepatic AA content levels of 11 and 21% in rats fed 5 and 20% coconut oil , respectively for 33 weeks . Their findings may, however, be attributable to differences in AA utilization (21).

Roles of LCPUFA

Unsaturated fatty acids help to maintain normal cellular functions and the fluidity of biological membranes composed of a PI bilayers (56). Among many other biological functions, LCPUFA and there derived eicosanoids regulate cell growth and differentiation by modulating gene expression (26). While MUFAs and the precursor LCPUFAs, LA and ALA, are readily stored in adipose TGs, LCUPFAs are instead mainly incorporated into PLs (56). There they are required for many functions, such as eicosanoid signaling (56,70), pinocytosis (53,71), ion channel modulation (53,72), and gene expression regulation (53, 73).

Eicosanoid Production. The two major LCPUFAs found in high concentrations in membrane structural lipids (53), especially in the CNS, are AA and DHA (9,74). AA is often esterified to the *sn*-2 position of membrane PLs for use in eicosanoid mediated signaling for specialized cell functions (53,75). Before esterified AA can be enzymatically converted to eicosanoids via cyclooxygenase and lipoxygenase enymes, it must be freed from PLs by phospholipases (53). Eicosanoids function as autocrine/ paracrine hormones and locally mediate reactions that include inflammation (53,70) and protection of digestive tract epithelium (53,76).

Omega-3 versus Omega-6

Not only are n-6 and n-3 PUFA structurally and metabolically distinct, but they elicit opposing immune responses in the body (77,78,79,80,81) relative to inflammation. Thus, it is possible that there exists some ratio of the two that would be necessary in maintaining immunological homeostasis in the body (80,82,83). A physiologic ratio of omega-3:omega-6 fatty acids would be approximately 2:1 (81). The interest of n-6:n-3 ratio is due to the competition that occurs between these two fatty acids as substrates for the formation of eicosanoids in cyclooxygenase and 5-lipoxygenase pathways (77). This is important in maintaining the balance of their incorporation into eicosanoids (81) Should there be a higher concentration of n-6 PUFA in relation to n-3 PUFA the result would be a higher proportion of n-6-derived eicosanoids.

Eicosanoids are a family of bioactive mediators synthesized from 20-carbon atom PUFAs, such as eicosatrienoic acid (ETA) (20:3 n-6), arachidonic

acid (AA) (20:4 n-6), and eicosapentaenoic acid (EPA) (20:5 n-3) (77,84). Eicosanoids have short life spans, are involved in the degree and duration of inflammatory responses, and function locally in both autocrine and paracrine ways (13,77). Eicosanoids produced by n-3 and n-6 PUFA are prostaglandins (PG), thromboxanes (TX), leukotrienes (LT), and hydroxy-eicosatetraenoic acids etc. (77,81). Cyclo-oxygenase (COX) enzymes and lipoxygenase enzymes metabolize n-3 and n-6 fatty acids into the various eicosanoids. The three COX isozymes that have been isolated are the constitutively active COX-1, the cytokine inducible COX-2 (77,81) and the COX-3 isozyme {Chandrasekharan 2002}.

Blood and tissue samples indicate that eicosanoids derived from arachidonic acid (an n-6 PUFA) are produced at an increased rate in patients afflicted with inflammatory disorders just as occurs during inflammatory conditions (77). Contrarily, when humans ingest sources high in n-3 fatty acids such as fish or fish oil, they exhibit a marked decrease in the production of n-6 derived eicosanoids, such as leukotriene B4 and thromboxane A2, mediators of inflammation, thus exhibiting anti-inflammatory properties (81).

PUFA Accretion in Infants

EFA and LCPUFA requirements are high in both the fetus and the mother during pregnancy. While the mother obtains LCPUFA either directly through her diet or through their endogenous synthesis from EFA, the fetus's LCPUFA requirements are met primarily through placental transfer (85,86). DHA and AA, specifically, are crucial not only as functional and structural components of all

cellular membranes, but for fetal neural, visual and vascular development (85,87). Fetal LCPUFA accretion occurs exponentially during the last trimester of pregnancy (88) raising the question of whether or not those babies born preterm have the same LCPUFA status advantage as those born at term (85,89,90). Studies have shown that an infant's later LCPUFA status is reflective of its LCPUFA status at birth and with the post-natal diet (91,92,93). Such studies demonstrated that the DHA and AA contents of plasma and erythrocyte lipids of breast-fed infants are higher than those in formula-fed infants fed formula lacking DHA and AA (14). Research has also shown that the fetus and the preterm infant are both capable of synthesizing DHA from its precursor, however at a limited capacity, with preformed DHA being associated with a more efficient accretion of DHA in developing tissues than ALA (94).

The transfer of AA and DHA across the placenta is believed to occur through a multi step process of uptake and intracellular translocation that is facilitated by several membrane-associated and cytosolic FA binding proteins that favor n-6 and n-3 FA over non-essential fatty acids and LCPUFA over their precursors (94,95).

Breast Milk LCPUFA

Bovine milk is known to contain negligible quantities of LCPUFA that, when fed to infants, results in corresponding lower levels of n-6 and n-3 plasma fatty acids than is found in breast fed infants (9,96,97). Pig's milk contains a comparable, if not higher, percentage of n-6 and n-3 fatty acids as does human milk (9). Mammalian milk's fatty acid composition is dependent primarily on the

maternal diet fat content and composition (9,98). However other factors influencing the fat content and fatty acid composition of breast milk include the stage of lactation, parity, maternal age, diurnal rhythm, and time within feeding (9). Regarding premature infants, gestational age at parturition does not seem to influence the n-6 and n-3 fatty acid composition of breast milk (9,99).

Tryacylglycerols represent approximately 98% of total lipids in breast milk. Helping to ensheath the tryacylglycerols within milk fat globules are the glycerophospholipids, the lipid class representing only 1%, or less, of total milk lipid yet containing 50% of the milk n-6 and n-3 LCPUFA (9,100,101). Research has found that, on average, mature breast milk of well nourished mothers contains 8-12% LA, 0.5-1% ALA, and approximately 1%n-6 LCPUFA and 1% n-3 LCPUFA (9,102). The concentration of DHA in milk of women consuming a typical North American diet is generally in the range of 0.1-0.3% of total fatty acids and the level of AA ranges from 0.4-0.6% of total fatty acids (103) while median worldwide amounts reported are approximately 0.3% DHA and 0.6% AA of total fatty acids (104,105,106).

Animal Studies and Clinical Trials

Approaches used to study possible infant fatty acid requirements are extrapolation from the fatty acid composition of human milk, fetal and infant autopsy tissue analysis, and plasma and red blood cell (RBC) fatty acid comparison of infants fed various milk or formula diets (107). Animal studies are advantageous for studying infant fatty acid requirements as they allow for the "rigid control of fatty acids and other nutrient intakes and the degree, timing, and

duration of deficiency or excess, the absence of confounding environmental and clinical variables, and the tissue analysis and testing procedures that cannot be performed in human studies (108)." However, there are limitations to the extrapolations that can be made to humans considering species differences (108). The piglet is advantageous over other species in studying lipid nutrition of the full term infant with similarities to the human infant such as development of the intestine, fat digestion and absorption, pathways of lipid and lipoprotein metabolism, essential nutrient requirements and milk fatty acid composition (107). The piglet is also a more affordable animal model than the non-human primate. Animal studies offer the opportunity to explore the roles of LCPUFA, such as AA, on the unknown aspects of tissue functions (108).

Clinical studies in preterm and term infants collectively suggest that LCPUFA are important for the growth and development for preterm infants (109). In 2002, the LSRO noted that large scale studies had suggested that supplementation of preterm infant formulas with both AA and DHA from single-celled sources did not have adverse effects on the health of infants (14,110) and that LCPUFAs' role in visual and cognitive function development may be important on a population basis for later visual/ or cognitive function. Thus, the LSRO set maximum recommendations of AA and DHA in preterm infant formula so as to provide the option of including LCPUFAs in infant formula. The maximum recommendations of AA and DHA were 0.6% and 0.35% of total fatty acids, with no minimum recommendations made. To prevent possible imbalances from occurring in eicosanoids synthesized from n-6 versus n-3 fatty

acids, the LSRO stipulated that infant formulas supplemented contain both AA and DHA in a ratio between 1.5 and 2.0 (14).

Problem of Prematurity

Recent data from the National Center for Health Statistics (111) indicates that the incidence of premature birth has increased by 30% since 1981, impacting 12.3% of newborns overall in 2003 and 17.8% within the Black community. In July of this year the March of Dimes called for passage of proposed federal legislation, known as the PREEMIE Bill (S. 707; HR 2861),"to address the nation's skyrocketing rate of premature birth, which has severe health consequences and extraordinary medical costs associated with it" (112). According to Dr. Jennifer L. Howse, president of the March of Dimes, prematurity should be addressed 'with the same sense of urgency and focus that has been brought to other threats to children's health, including secondhand tobacco smoke and rising rates of obesity' (112). The average hospital charge in 2005 was estimated at $51,600 per infant, with the incidence of premature birth creating a staggering total national economic impact of $26.2 billion (113).

The underdeveloped state of preterm infants makes providing them with optimal nutrition challenging for neonatologists. Additionally, premature infants are born with low stores of EFA in adipose tissue relative to term infants, as their early birth disrupts the placental supply of DHA and AA for structural lipid synthesis (109), suggesting that premature infants may have greater nutritional needs over more mature infants. For example, approximately 1-2% of a 1000g

premature infant's body weight is made up of adipose tissue while 20-25% of a 3500g term infant's body weight is adipose tissue (108).

Enteral nutrition is necessary in promoting the health and development of the intestines of the preterm infant, yet is also believed to play a role in the development of necrotizing intercolitis (NEC) (115). NEC is a gastrointestinal disease affecting up to 5% of infants admitted to neonatal intensive care units, of which 80% are preterm or very low birthweight infants (114). Other factors believed to play a role in the pathogenisis of NEC are mesenteric ischemia, and enteral infection (115). Research indicates that the incidence and mortality, peaking at rates as high as 40%, have not changed substantially over the last three decades (116).

The progressive sloughing of epithelium from vullus tips towards villus crypts is characteristic of ischemic mucosal injury of the small intestine (117), resulting in septic shock as bacteria and their toxins are absorbed across exposed lamina propria (118). Epithelial continuity is reestablished during restitution, the process in which viable epithelial cells adjacent to denuded portions of the mucosa spread out across the denuded basement membrane (119,120). Villus contraction and epithelial restitution are responsible for the initial repair of mucosal injury (120). Using a rat intestinal epithelial cell line, Derek Ruthig and Kelly Meckling-Gill found that the n-3 fatty acids ALA and EPA and the n-6 fatty aids LA, GLA AND AA stimulate the process of intestinal epithelial cell restitution (121). In a subsequent study using the same rat cell line, Ruthig and Meckling-Gill demonstrated that the n-6 fatty acids LA, GLA and AA,

and not the n-3 fatty acids, stimulated intestinal epithelial cell restitution through a COX dependent pathway (122).

Local Interconversion of EFA

Δ-^{6} desaturase is present in both absorptive villus cells and regenerating crypt cells of the small intestine of rats (123,124). Furthermore research has shown that both ^{14}C-labelled LA and ALA are taken up from blood and desaturated-elongated by stomach, small intestine and colon in rats. To investigate the quantitative role of the local desaturation-elongation reactions in these tissues, research was conducted by Zhou, et. al to examine the tissue uptake and interconversion of unesterified ^{14}C-labelled LA in the guinea pig (123). Results showed that the gastrointestinal (GI) tract formed significant amounts of AA and it was thus hypothesized that considerable parts of AA pools of the extrahepatic tissues are formed in situ (123). However, many studies support that dietary LA and ALA are quantitatively less effective than AA and DHA as sources of AA and DHA for incorporation into membrane lipids (9,22).

Campbell, et al. examined changes in colonic PL FA and prostaglandin profiles in growing barrows fed either an ulcerative colitis nutritional formula fortified with n-3 FA or a similar control formula (125). Results showed an elevation of n-3 PUFA ($P<0.05$) of 530% and a reduction of n-6 PUFA ($P<0.05$) of 40% within colonic mucosa PL. As might be expected, prostaglandin E_2 was lowered ($P<0.0001$) by ~60% and 70% in colonic and cecal mucosa, respectively, in those pigs fed the experimental diet verses the control. The study demonstrated that porcine colonic phospholipids can be enriched *in vivo* with

LCP within 7 days of supplementation and that enrichment is maintained when supplementation is continued (125).

Prostanoid Mediated Repair

Prostaglandins derived from AA have been shown to play a critical role in the recovery of intestinal barrier function through the stimulation of epithelial migration and villous contraction, as described earlier, and by triggering the closure of inter-epithelial tight junctions (126). Reductions in PGE_2 were correlated with the failure to fully recover control levels of transepithelial resistance in ischemic-injured porcine tissues treated with the non-selective COX inhibitor indomethacin (126, 127). Also of significance, prostanoids have been shown to enhance intestinal barrier function through cytoprotective mechanisms, such as secretion of mucous and bicarbonate {grant}, maintenance of optimal mucosal blood flow, reduced microvascular neutrophil adhesion, and augmented mucosal repair.

Considering the role of AA derived prostanoids in the recovery of intestinal barrier function within restituting epithelium, a plausible hypothesis is that infant formula enriched with AA will prophylactically alter enterocyte membrane FA composition such that increased concentrations of reparative prostanoids are released by COX following intestinal ischemic injury, thereby enhancing restoration of intestinal barrier function. If proven, nutritional support for intestinal growth and development could be enhanced.

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CHAPTER 2

PROPHYLACTIC ENRICHMENT OF ENTEROCYTE PHOSPHOLIPIDS WITH POLYUNSATURATED FATTY ACIDS FED TO SUCKLING PIGLETS.

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INTRODUCTION

Recent data from the National Center for Health Statistics (1) indicates that the incidence of premature birth has increased by 30% since 1981, impacting 12.3% of newborns overall in 2003 and 17.8% within the Black community. In July of this year the March of Dimes called for passage of proposed federal legislation (S. 707; HR 2861) ”to address the nation's skyrocketing rate of premature birth, which has severe health consequences and extraordinary medical costs associated with it” (2). The average hospital charge in 2005 was estimated at $51,600 per infant, with the incidence of premature birth creating a total national economic impact of $26.2 billion (3).

Provision of optimal nutritional support for the preterm infant poses a major challenge to neonatologists. Among the complicating factors is the underdeveloped state of the digestive system of these infants making gastrointestinal disturbances among the most common health challenges faced by the suckling neonate. While total parenteral nutrition can be used in the short term, it is increasingly clear that some intestinal stimulation via enteral nutrition is needed to promote health and development of the intestine, and yet enteral nutrition is itself a risk factor for the development of necrotizing enterocolitis (NEC). NEC is a gastrointestinal disease affecting up to 5% of infants admitted to neonatal intensive care units, of which 80% are preterm or very low birthweight infants (4). Although the pathogenesis of NEC is not completely understood, mesenteric ischemia, enteral feeding, and enteral infection are all believed to play a role (5). Recent studies indicate that incidence and mortality, which may

reach rates as high as 40%, have not changed appreciably over the last 30 years (6).

Ischemic mucosal injury in the small intestine involves progressive sloughing of epithelium from the tips of villi towards the crypts (7), resulting in septic shock as bacteria and their toxins are absorbed across exposed lamina propria (8). Repair of major breaches in epithelial continuity is achieved by restitution, during which viable epithelial cells adjacent to denuded portions of the mucosa spread out across the denuded basement membrane (9,10). Mechanisms responsible for initial repair of mucosal injury include villus contraction and epithelial restitution (11). Using a rat intestinal epithelial cell line, Ruthig and Meckling-Gill (1988) found that the n-3 fatty acids ALA and EPA and the n-6 fatty aids LA, GLA and AA stimulate the process of intestinal epithelial cell restitution (9). In a subsequent study they further demonstrated that the n-6 fatty acids LA, GLA and AA, and not the n-3 fatty acids, stimulated intestinal epithelial cell restitution through a COX dependent pathway (12). Additionally, Campbell, et al. demonstrated that porcine colonic phospholipids can be enriched *in vivo* with long chain polyunsaturated fatty acids (LCP) within 7 days of supplementation and that enrichment is maintained when supplementation is continued (13).

Proper development of the brain, retina, and other body tissues in infants is dependent on provision of AA and DHA either directly in the diet or through synthesis from linoleic and linolenic acids (14). Human breast milk naturally contains linoleic, linolenic, arachidonic and docosahexaenoic acids (15). The

concentration of DHA in milk of women consuming a typical North American diet is generally in the range of 0.1-0.3% of total fatty acids and the level of AA ranges from 0.4-0.6% of total fatty acids (15). Recently, the Food and Drug Administration approved of the supplementation of AA and DHA into infant formula to help provide the appropriate amounts of AA and DHA for normal growth and development. The recommended concentration of AA (maximum of 0.6% of total FA) and DHA (maximum of 0.35% of total FA) included in infant formula is similar to that observed in breast milk (16).

We have focused on the role of prostanoids (PGs) in the recovery of intestinal barrier function in ischemic-injured porcine ileal mucosa. The piglet has gained much popularity as a pediatric research model (17,18,19). The pig has been used extensively as a model for a number of important human ischemic-related intestinal diseases, including AMI (20,21,22), hemorrhagic shock (23,24), and NEC (25,26). Although these diseases have been modelled using a variety of laboratory animals, the gastrointestinal tract of the pig is more similar to that of the human than other domestic and research animal species (27,28). Previous studies in our laboratory have shown that prostanoids (PG) stimulate rapid recovery of gut barrier function, as measured by transmucosal electrical resistance (TER), and restore baseline levels of permeability after ischemic injury (29). PGs are produced from arachidonic acid by one of three isoforms of cyclooxygenase: COX-1, COX-2, and COX-3 (30). We have accumulated extensive evidence during the past 5 years indicating that PGs orchestrate recovery of paracellular resistance within restituting epithelium (31,32). The

present study was designed to provide more information on the time course of LCP enrichment of intestinal mucosa via dietary supplementation and to determine if there is a dose-response effect of intestinal mucosa phospholipid AA concentration to dietary AA concentration using the pig as a model for the human neonate. The long term goal of this research is to enable the intestine to quickly heal following intestinal ischemic insult.

MATERIALS AND METHODS

Animal care and study design

Animals and Experimental diets. All animals were cared for in accordance with the Institutional Animal Care and Use Committee of North Carolina State University. Colostrum fed piglets (N= 96 across 3 replicates) were acquired within 12-24 hours of age and caged individually in a room under ambient temperature (~30ºC) control. Piglets weighing less than 1kg were not selected. Upon receipt, all pigs were injected with 1mL of Iron-Gard™ 200 (200mg/mL iron dextran complex hematinic, Fermenta Animal Health Co., Kansas City, MO). Piglets also were given 0.5mL gentamycin (10mg/mL) orally on the first and third days. Piglets were randomly allocated to one of five dietary treatments differing in fatty acid (FA) composition and fed by a gravity feeding system adapted (33) that allowed for accurate measurement of milk-based formula consumption (see **Table1**). The dry matter content of the basal liquid diet was15% and the calculated chemical analysis (DM basis) included: crude protein, 31.1%; lactose, 36%; ether extract, 26%; and total energy, 4.6 Mcal/kg (696 kcal/L). Diets contained either no n-6 PUFA (0%AA, negative control), 0.5%AA, 2.5%AA,

5%AA, or 5% eicosapentaenoic acid (EPA) of total fatty acids **Table 2.** The 0.5% AA diet was designed to approximate the AA (n-6) concentration prescribed for preterm infant formulas (16), and arachidonic acid included in the 2.5%AA and the 5%AA diets was supplied at elevated concentrations to prophylactically enrich the small intestine cell membrane with AA.

Animal feeding and management. Diets were provided three times per day (0800, 1600, and 2300), prepared daily and stored under refrigeration until fed. Diets were reconstituted at 180g/L of water ((133.2g dry diet + 46.8g oil)/L water). Pigs were fed formula at ~60% of *ad libitum* to match growth rates found with sow-raising. In order to feed pigs according to the prescribed plan, pig weights and milk intakes were recorded daily. Following that, the average metabolic weight of all pigs (average weight $^{.75}$) was then calculated each day and multiplied by a feeding rate that increased from 100g/day on day 0 of the study to 600g/day by approximately day 7 of the study. The pigs' calculated intake for each day was equally divided across their 3 meals fed approximately every 8 hours. The feeding rate increased by increments of 50-100g/day with no increase on days when many of the pigs (>10%) left considerable weigh back (> ~20%) of their diets. The feeding rate was increased by only 50g/day on days that the pigs left borderline weigh back. Every part of the gravity flow feeding system was cleaned each morning using hot water, dishwashing liquid, and liquid chlorinated detergent disinfectant (DS Liquid: Command, Diversey Corp., Wyandotte, MI).

Pigs, including age-matched, sow reared controls (n=6/timepoint), were euthanized at 0 (n=6), 4, 8, and 16 days of treatment. Pigs were killed by AVMA-approved electrocution and then exsanguinated. Tissue collection occurred immediately following exsanguination.

Sample Collection and Analytical Procedures

Blood Panel Analysis. Blood was collected post-mortem via exsanguination in non-heparinized culture tubes (16 X 100 mm). After collection, samples were covered in Parafilm and stored overnight at 4ºC to allow for clotting. Then, samples were centrifuged using an IEC clinical centrifuge (IEC, MA) at 830 x G for 10 min at 4ºC, and serum aliquots were transferred to 1.5mL centrifuge tubes (Fisher Scientific, GA) and stored at -80ºC for later Superchem panel analysis (Antech Diagnostics, Lake Success, NY). The Superchem panel measured several blood parameters to check for deleterious health effects of feeding supraphysiologic AA.

Tissue Collection. In an effort to avoid RNA degradation, samples were processed immediately after exsanguination. The abdominal cavity was opened and the intestinal tract and liver were removed. The liver was weighed and samples were snap-frozen for mRNA analysis. The duodenum, jejunum and ileum were separated from the rest of the intestinal tract and were flushed with 0.9%NaCl. Each small intestine segment had approximately 3cm sections removed and placed in fixative for histological analysis. Mucosal scrapings were obtained by cutting small intestine segments lengthwise leaving their mucosa facing upward to be scraped by the longest edge of a microscope slide. Mucosal

scrapings were snap frozen from the jejunal and ileal regions of the small intestine for phospholipid FA analysis and a representative mucosal scraping was done of the duodenum, jejunum and ileum by mixing their scrapings together just prior to snap freezing. The representative mucosal scraping was collected for intestinal mRNA analysis and a portion was snap frozen for lactase specific activity.

Histology. Intestinal sections were assessed histologically for gross morphology, villus height and crypt depth measurements. Formalin-fixed segments were embedded in paraffin and 5 μm-thick sections were cut and stained with H&E for examination by light microscopy. Computer-assisted morphometric measurements were conducted with a video-imaging system (Nikon- FXA). All samples were measured by an observer masked to the treatment groups. Six well-oriented villi were measured at 100X magnification to determine mean villus height and width. Crypt depths were measured from 6 sites from the same sections. Villus surface areas for representative villi were calculated as reported previously (34).

*Lactase***.** Mucosal scrapings were homogenized in iced buffer (0.2 M Tris, 0.15 M, KCl, 2.5 mM EDTA, pH 7.4) and immediately frozen in aliquots at -20°C until assayed. Intestinal lactase specific activity was measured by the technique of Dahlqvist et al. (35) as previously described (36). Protein was measured by Biuret Reagent (37,38).

Fatty Acid Analysis. Total fatty acids in phospholipids were determined in the mucosa collected from jejunum and ileum sections. Lipids were extracted

from the scraped mucosa using a chloroform/methanol (2:1, v/v) ratio as described by Folch et al. (39). Phospholipids were separated from other lipid classes using silicic acid columns (Sigma-Aldrich, MO)(40). Fatty acids were methylated (41), and analyzed by a 5890 Hewlett Packard gas chromatograph equipped with a flame ionization detector and a 6890 autosampler (Hewlett Packard PA). A 100-m fused silica capillary column with an i.d of 0.25 um, a 0.2 um film coating and a SP-2380 stationary column (Supelco, PA) was used for separation of the each fatty acid, and each peak was identified by using standard fatty acid methyl esters (Sigma-Aldrich). The operating conditions were as follows: helium was used as the carrier gas, split ratio 1:100, injection temperature 230, detector temperature 230, national oven temperature 140, increased to 225 after two min at rate 3.2, then increased to 135 after 14 min at a rate 2.0, then back to 140 after 20 min at a rate 20. Peak areas were integrated using the Empower Pro system (Waters, MA). Fatty acid concentrations were calculated by the internal standard method using C17:0 as an internal standard and concentrations were expressed as mg/100g total phospholipids.

*mRNA***.** mRNA abundance of Δ^6-desaturase and Δ^5-desaturase was measured in the liver and in a representative sample of the intestinal mucosa. RNA was extracted with TRI-REAGENT and quantified using a Nanodrop™ by absorbance at 260 nm. Primers for pig Δ^6-desaturase and Δ^5-desaturase were used to measure expression of these genes by Real Time RT-PCR using an MJ Research real time RT-PCR workstation and the $\Delta\Delta C_T$ method of quantification (42) with β-actin as the reference standard. Values were normalized to 0.5%AA

on d16. Degenerate primers for Δ^6-desaturase (D6D) and Δ^5-desaturase (D5D) were designed from homologous regions of the aligned human, mouse, and rat published sequences (D6D forward primer: 5' CCCCTCGAGTATGGCAAGAAG 3'; D6D reverse primer: 5' GCCGTAGAAAGGGATGTAGGTG 3'; D5D forward primer: 5' TTGGTGCCCTTCATCCTCTGTG 3'; D5D reverse primer: 5' GCATGTTGATATCGGGGTCTTT 3'; β-Actin forward primer: 5' AGGCCAACCGTGAGAAGATGAC 3'; β-Actin reverse primer: 5' CTCGGCCGTGGTGGTGAAGC 3'). The primers were designed to span introns to avoid genomic DNA contamination and their sequences were verified.

Transepithelial Resistance

In a pilot study, we fed colostrum deprived piglets (n=3/treatment) diets with 0, 0.5, or 5% AA (% dietary fat). Diets were formulated as described in the Animal care and experiment diets (below). After 14 d on trial, pigs were sedated and sections of the ileum subjected to surgical ischemia. Mucosa corresponding to control and ischemic ileal loops was mounted in Ussing chambers for electrophysiological measurements. Mucosa AA concentrations were determined by gas-liquid chromatography using the method described as in fatty acid analysis (above).

Statistical Analysis. Daily gain, feed intake and serum metabolite and enzyme concentrations were analyzed by ANOVA using the GLM procedure of SAS (SAS, NC). Data from lactase, fatty acid and hepatic mRNA analyses were subjected to ANOVA based on a 6 x 3 factorial design (dietary treatments X day of study) using the general linear model (GLM) procedure of SAS. Tissue

differences were evaluated also by multivariate analysis of variance based on a completely randomized design using the GLM procedure. Values were expressed as lsmeans ± SEM, and differences were considered significant when P < 0.05.`

RESULTS

Performance, Growth and Food Intake

Growth (299 +/- 21 g/d) was unaffected by treatment (**Table 3,** P>0.1) Formula-fed pigs gained at a similar rate to sow-reared controls (Sow). Daily feed intake did not differ among treatment groups (**Table 3**).

Serum Metabolite and Enzyme Concentrations

Serum levels of creatinine, biliruben, cholesterol, and triglycerides were all significantly lower in the formula-fed groups than in the Sow group (P < 0.05), but did not differ among formula-fed groups (**Table 4**). Similarly, the BUN/creatinine ratio did not differ among treatments, but on average the treatment groups were higher than the SRCs. Serum levels of urea nitrogen were slightly higher in the 0% AA and 2.5 % AA fed pigs, but did not differ among the 0.5 % AA, 5 % AA, 5 % EPA fed groups and the SRCs. No differences were detected in other blood parameters among any of the groups (P > 0.05).

Small Intestine Morphology

There were no differences in Ileal villus height and area among any of the tested groups (**Table 5**; P > 0.05). Ileal villus width was slightly lower for pigs fed 5% AA and in those nursing the sow. On average the dietary treatment groups did not differ from the time 0 and SRC piglets (P > 0.05). Ileal crypt depths were

greater in the formula-fed pigs than in the time 0 and SRC pigs, but did not differ between time 0 and SRC pigs. The villus:crypt ratio was greater in time 0 pigs than in all of the formula-fed pigs ($P < 0.05$). The villus:crypt ratios were similar among all of the formula-fed pigs.

Lactase Specific Activity

All formula-fed pigs had similar lactase specific activity levels at each time point that did not differ from initial pigs (**Figure 1**). Of the four time points, lactase specific activity was the lowest, on average, on day 16 in formula fed pigs.

Fatty Acid Profile

Jejunal mucosal phospholipid AA concentration increased with increases in dietary AA at all timepoints ($P < 0.05$). The increase progressed with increasing dietary AA concentration from 0% to 2.5%, and plateaued as the dietary AA rose to 5%. The concentrations of AA were enriched 40%, 223% and 367% by day 8 and 159%, 404%, and 409% by day 16 in pigs fed the 0.5% AA, 2.5% AA, and 5% AA diets, respectively, compared with the 0% AA control pigs (Top panel, **Figure 2**). Mucosal AA concentration of the 5% EPA fed pigs was similar to the 0% AA fed pigs at all timepoints in both the ileum and the jejunum. A similar pattern of AA enrichment was observed in ileal mucosal phospholipids of initial pigs and all formula fed pigs at all timepoints (bottom panel, **Figure 2**). AA concentration of mucosal phospholipids in SRC pigs was similar to the 0.5% AA fed pigs in both the jejunum and ileum but was most similar to the 2.5% AA fed pigs in the ileum. Age also had a significant effect on AA concentrations in

intestinal mucosal phopholipids. The concentration of AA in jejunal mucosa increased on average 8 fold by d 8 and 10 fold by day 16 compared with d 0. The concentrations of AA observed in ileal mucosa at day 8 and 16 were 17 times higher than that at day 0.

Jejunal and ileal mucosal phospholipid concentrations of EPA increased significantly in the formula fed group given 5.0 % EPA ($P < 0.05$**; Figure 3**). The increases were more pronounced at day 8 than day 4 and day 16. The concentrations of EPA were enriched on average 34 fold by day 8 and 24 fold by day 16, respectively, compared with day 4. Dietary AA concentration had no effect on EPA concentration in mucosal phospholipids ($P > 0.05$). Intestinal mucosal PL EPA concentrations of all AA supplemented pigs were similar to those of SRC pigs ($P > 0.05$).

Ratios of n6 to n3 fatty acids tended to increase within each time point with increasing amounts of dietary AA in both the ileum and jejunum (**Figure 4**). The EPA fed pigs, on average had a ratio similar to that of 0% AA fed pigs. Age greatly influenced the ratio. The ratios decreased in all groups by day 8 of the study, but the decrease was more pronounced in ileum than in jejunum. excepting in the 0.5% AA fed pigs.

As AA increased in jejunal and ileal phospholipids with increasing concentrations of dietary AA, palmitoleic acid (C16:1) and oleic acid (C18:1) concomitantly decreased (**Figure 5**). In addition, Linoleic acid (C18:2) increased in pigs fed 2.5% AA from those fed 0.5% AA, but decreased in pigs fed 5% dietary AA from those fed 2.5% AA (tables 6 and 7). Summaries of the

phospholipid fatty acid profiles of the jejunum and ileum can be seen in **Tables 6 and 7**.

By day 8, the ratio of saturated:unsaturated fatty acids had decreased in all groups of pigs in both the jejunum and the ileum (**Figure 6**). Within each time point, all groups of pigs had similar jejunal and ileal ratios of saturated:unsaturated fatty acids. There didn't seem to be a diet effect on the saturated:unsaturated fatty acid ratio, although the high dietary AA slightly decreased the ratio on day 4 in ileum.

There were no treatment effects on the percentage of fat in mucosa tissue and the percentage of phospholipids in fat (**Figure 7 & 8**).

Hepatic Δ^6-Desaturase and Δ^5-Desaturase mRNA Abundance

As pigs aged from birth to 16 d of age, there were no differences in the desaturase mRNA abundance (**Figure 9,** P>0.1), measured in pigs fed 0.5% or 5%AA. There were no differences between the initial pigs and the 0.5% and 5%AA treated pigs across time. Abundance also was similar among AA-supplemented pigs, but was elevated by 60-80% on d16 in the 0%AA-fed pigs compared to those fed 2.5% (P<0.05).

Transepithelial Resistance

Intestinal mucosal PL AA content increased proportionally to dietary AA content with the PL AA content of 5% AA fed piglets increasing 86% compared to 0.5% AA piglets and 362% compared to 0% AA fed piglets (*P*<0.01). TER values for mucosa from control loops paralleled increases in mucosal AA content (**Figure 10**); feeding 0.5% and 5% AA increased control loop TER by 74% and

121%, respectively, compared to values for 0% AA (*P*<0.01). TER was reduced by ischemic injury, however, TER values for ischemic mucosa from piglets fed 5% AA were 49% greater (*P*<0.01) than values for piglets fed 0.5% AA signifying greater retention of gut barrier function following injury. These data demonstrate that dietary fatty acid composition influences ileal mucosal fatty acid composition and the resulting changes enhance gut barrier function as measured by TER.

DISCUSSION

Prostanoids (PG) produced from arachidonate (AA) have been implicated in stimulating rapid recovery of gut barrier function, as measured by transmucosal electrical resistance (TER), and in restoring baseline levels of permeability after ischemic injury (29). Prostanoids are produced from AA by one of three isoforms of cyclooxygenase: COX-1, COX-2, and COX-3 (30). With the long term goal of enabling the preterm infant's intestines to quickly heal following intestinal ischemic insult, the objective of this research was to provide more information on the time course of LCP enrichment of intestinal mucosa via dietary supplementation and to determine if there is a dose-response effect of intestinal mucosa phospholipid AA concentration to dietary AA concentration using the pig as a model for the human infant.

AA or EPA (PUFA) supplementation up to 5% dietary FA showed no detrimental effects on piglet growth (299 +/- 21 g/d), feed intake, (**Table 3,** P>0.1), or measured blood metabolites and enzyme concentrations (**Table 4**) after 16 days of supplementation. This was true even for the eicosapenaenoic acid (EPA) supplemented piglets not supplemented with AA. Similar to previous

studies, in which formula containing both AA and docosahexaenoic acid (DHA) was fed to infants (43,44,45,46,47), there were no differences in growth of AA supplemented piglets compared with unsupplemented piglets. However, reduced growth associated with reduced AA status resulted in one early study in which preterm infant formula, supplemented with DHA and eicosapentaenoic acid (EPA) but not AA, was fed (43,48,49) to infants.

Intestinal lactase activity is known to be higher in the newborn pig than in the mature pig (50,51) and to decrease over the first two months of life (50) with little subsequent change in the enzyme. It was observed by Manners et al (50) that the decline in intestinal lactase activity from birth to two weeks of age occurs relatively rapidly when compared to the decline occurring from 2 weeks to 8 weeks of age. Lactase activity in the present study was lower at day 8 and day16 than at day 4, but did not differ between day 8 and 16. These findings are consistent with the previous work of Manners et al (50).

16,16-dimethyl prostaglandin E2 (dmPGE), which AA is a precursor for, is trophic to the small bowel of suckling rats (52,53). When present in high doses, dmPGE has been shown to increase brush border enzyme activities, possibly indicating accelerated mucosal maturation (52). This could explain why the 0%AA supplemented pigs showed reduced levels of lactase activity compared to the SRC pigs. Why then the 0%AA fed pigs had lactase levels not significantly different from the AA supplemented pigs may be that other sow-milk constituents served to increase SRC piglet intestinal lactase activity numerically, but not significantly, over that of the experimental AA supplemented piglets, enabling the

SRC piglets to have lactase levels that were significantly high only relative to the 0%AA fed piglets.

Swine intestinal health is often estimated by small intestine morphology (33,54). The formula fed piglets of this study on average had greater crypt depths and smaller villus to crypt ratios than either the initial piglets or the SRC piglets, but there were no differences in crypt depth or villus:crypt ratios among the formula fed pigs. Villus height and area, which are associated with absorptive area (33), didn't differ among any of the piglets. Overall, morphology measurements showed no deleterious effects of feeding elevated levels of AA or EPA.

Previous research demonstrated that PUFA content of tissue lipids is strongly modulated by dietary PUFA intake (55,56,57,58), noting that when dietary LA was increased in the diet, its metabolite, AA, was increased in tissue lipids. Further research indicated that fatty acids (FA) may be targeted for synthesis of membrane phospholipids in specific tissues (59). In the present study, elevated levels of dietary AA were reflected in intestinal mucosal phospholipids. Both jejunal and ileal mucosal phospholipid concentrations of AA increased with dietary increases in AA concentration at all timepoints in the study ($P<0.05$). Enrichment of jejunal and ileal intestinal mucosa phospholipids plateaued at 8 days of treatment. No further enrichment occurred between day 8 and day 16 in either the jejunum or the ileum. The AA content of ileal and jejunal mucosal phospholipids in the 0%AA fed pigs was similar in the 0.5%AA fed pigs. This was not surprising as we formulated the 0.5%AA treatment to match the

level recommended in infant formula, which is similar to that found in human breast milk (43).

Unexpectedly, the AA concentration within the mucosal phospholipids of the 5% EPA fed group did not differ from the 0%AA fed pigs at any of the time points. This came as a surprise due to the known competition between parent n-6 and n-3 fatty acids for metabolism to their long chain polyunsaturated fatty acid (PUFA) metabolites, such as AA (n-6) and EPA (n-3) (60,61). This indicates that EPA must be fed at a concentration higher than 5% of total FAs to competitively inhibit the desaturation and elongation of lenoleic acid (LA) to AA.

Because n-6 and n-3 fatty acids compete for esterification into PLs, dietary PUFA influence the n-6:n-3 PUFA ratio in PLs (62,63). The ratio of intestinal mucosal n-6:n-3 FAs in the AA supplemented pigs was reflective of the PUFA content of the diets. However, EPA did not enrich intestinal mucosa to the extent that AA did when fed at equal concentrations. There was stronger enrichment of AA. The n-6:n-3 PUFA ratio tended to increase progressively within each time point for the AA supplemented pigs from 0%AA to 5%AA in both the ileum and the jejunum. The ratio of the sow-reared controls was most similar to the 5%AA fed pigs, and the ratio of the 5%EPA fed pigs tended to be lower than the AA supplemented pigs and most similar to the 0%AA fed pigs. These results are consistent with previous studies (9,58) that demonstrated that *in vitro* and *in vivo* supplementation of n-3 PUFA is associated with an increase in rat intestinal cell and mouse hepatic and peritoneal tissue n-3, respectively, and a decrease in n-6 PUFA. The significance of altering the n-6:n-3 FA ratio is that changing the ratio

may modulate physiological processes under the influence or regulation of eicosanoids (58,64).

PUFA are the main dietary component to regulate Δ^6-desaturase (D6D) and Δ^5-desaturase (D5D) enzymes (62) responsible for synthesis of n-6 and n-3 LCP. Dietary PUFAs suppress D6D and D5D (62,65). Studies of rat liver microsomes show that high concentrations of either LA or its n-6 LCP metabolites inhibit Δ^6-desaturation and that low concentrations of either will induce Δ^6-desaturation (60,66,67,68). Δ^5-desaturase has been found to respond similarly to both n-6 and n-3 PUFA (60,66). It is likely due to this feedback inhibition that hepatic D6D and D5D abundance, in piglets in the current study, was elevated by 60-80% on d16 in the 0%AA-fed pigs compared to those fed 2.5% ($P<0.05$), although it did not differ among AA-supplemented pigs at day 16. Tracer studies have demonstrated that preterm and term human infants are capable of converting LA and ALA to AA and DHA (69), respectively, but some tracer studies in developing rats have indicated that only a small percentage of dietary precursor fatty acids are converted to their LCP metabolites (70,71,72). Therefore, it is not concretely known yet whether or not the fetal or neonatal liver desaturase activity can meet the needs of the developing human following an abrupt end to placental supply of LCP as in premature births or when lipid nutrition is provided after birth without LCP supplementation (2). The current study found that as pigs aged from birth to 16 d of age, there were no differences in the hepatic desaturase mRNA abundance, measured in pigs fed 0.5% or 5% AA. Also, there were no differences between the initial pigs and the 0.5% and

5% AA treated pigs across time. Considering that ileal and jejunal AA and EPA of the SRC pigs in the current study weren't significantly different from the pigs fed the 0.5% AA formula meant to match current infant formula, the lack of change in desaturase mRNA abundance across time seems to indicate that desaturase enzymes can sufficiently meet the needs of the term piglet.

The pilot study in our lab, using porcine Ischemic-injured Ileum as a model of mucosal repair, showed that mucosal transepithelial resistance increases as content of arachidonic acid increased in both control and ischemic tissues. These data demonstrate that dietary fatty acid composition influences ileal mucosal fatty acid composition and the resulting changes might enhance gut barrier function as measured by TER. Further work is needed to substantiate this observation.

In conclusion, this study demonstrated that supra-physiologic supplementation of AA may be safe for neonates as it was not detrimental to piglet growth, clinical blood parameters, or morhphologic and functional development of the small intestine. Hepatic desaturase mRNA abundance measurements and ileal and jejunal FA analysis data also seem to indicate that desaturase enzymes can sufficiently meet the needs of the term neonate. Mucosal intestinal FA analysis showed a dose-response relationship of enterocyte phospholipid AA concentration and dietary AA concentration, with an enrichment plateau at 2.5% dietary AA at 8 d of feeding. Given the findings of the pilot study and the current study, the goal of further research in this lab will be to further demonstrate the effects of prophylactically altering intestinal fatty acid

composition, through dietary manipulation, on enhancing acute *in vivo* recovery from ischemic injury and to confirm the role of prostanoids in the mechanism of gut recovery from ischemic injury induced when intestinal fatty acid composition is altered by dietary means.

Table 1. Basal Diet Composition

Ingredient	% of dry powder
Whey Protein Concentrate[1]	5.50
Non-Fat Dry Milk[2]	52.35
Fat Blend (detailed in table 2)	26.00
Lactose[3]	3.57
Sodium Caseinate[4]	8.30
L-Arginine•HCL[5]	0.44
L-Histidine•HCL[5]	0.04
Xanthan Gum[5]	1.00
Calcium Carbonate[5]	0.38
Dicalcium Phosphate[5]	1.29
Minerals[6]	0.50
Vitamins[7]	0.13
Salt	0.50
Total	100.00

[1]Hilmar Ingredients (Hilmar, CA)
[2] Milk Specialties (Dundee, IL)
[3]Akey, Inc. (Lewisburg, OH)
[4]International Ing. (St. Louis, MO)
[5]Dyets, Inc. (Bethlehem, PA)
[6]Mineral premix (Merricks, Inc., Middleton, WI) contained 1.002 g/100 g Ca, 0.549 g/100g P, 0.284 g/100g Na, 0.04 g/100g Cl, 2.024 g/100g K, 0.102 g /100g Mg, 20,000 ug/g Fe, 200 ug /100g Co, 1,850 ug/g Cu, 400 ug/g I, 5,000 ug/g Mn, 60 ug/g Se, 23,500 ug/g Zn.
[7]Vitamin premix (Merricks, Inc., Middleton, WI) contained 33,000,000 IU/kg Vitamin A, 6,600,000 IU/kg Cholecalciferol, 55,000 IU/kg α-tocopherol, 257,400 ug/g Ascorbic acid, 29,983 ug/g D-Pantothenic Acid, 33,069 ug/g Niacin, 8378 ug/g Riboflavin, 5,115 ug/g Menadione, 66 ug/g Biotin, 4,4000 ug/g Vyamin B^{12}, 2,038 ug/g Thiamine, 3,996 ug/g Vitamin B^{6}, 2,756 ug/g Folic Acid.

Table 2. Analyzed Oil Blend Fatty Acid Content

		Diet				
FA	0%AA	0.5%AA	2.5%AA	5%AA	5%EPA	Sow
		Ingredient, % of dietary fat				
Coconut oil	61.63	19.23	19.23	19.23	19.23	
Tallow	0.00	9.33	5.10	0.00	1.00	
Soy oil	0.00	27.40	27.21	26.83	26.83	
ARASCO[2]	0.00	1.06	5.48	10.96	0.00	
DHASCO[2]	0.670	0.67	0.67	0.67	0.00	
MCT[4]	33.85	38.46	38.46	38.46	38.46	
EPA oil[3]	0.00	0.00	0.00	0.00	10.66	
Lysolecithin	3.85	3.85	3.85	3.85	3.85	
Total	100.00	100.00	100.00	100.00	100.00	
		*g/ 100 g fatty acid**				
C8:0	9.34	10.58	13.05	10.76	8.06	0
C10:0	18.73	19.68	18.65	19.65	17.56	0.37
C12:0	43.75	15.77	14.44	15.47	15.25	0.73
C14:0	13.68	5.12	4.86	5.05	5.17	5.87
C16:0	5.98	8.85	8.27	7.70	7.28	30.34
C18:0	5.80	5.24	4.94	4.54	4.64	2.31
C20:0	0	0.14	0.16	0.15	0.20	0.45
C22:0	0	0.13	0.24	0.29	0.22	0.09
C24:0	0	0.05	0.14	0.25	0.09	0.00
C16:1	0	0.49	0.32	0.07	0.18	13.17
C18:1	0.67	9.77	8.88	7.64	8.16	29.26
C18:2n6	1.50	20.68	20.05	19.51	20.98	13.82
C18:3n6	0.07	0.27	0.59	0.83	0.46	0.12
C18:3n3	0	0.11	0.12	0.14	0.48	0.00
C20:1	0.17	2.32	2.22	2.17	2.51	0.77
C20:2	0	0	0.04	0.05	0.04	0.48
C20:3n6	0	0	0.15	0.44	0.029	0.21
C20:3n3	0	0	0.05	0	0.71	0.07
C20:4n6	0	0.46	2.54	4.90	0.36	0.00
C20:5n3	0	0	0	0.06	5.4	0.00
C22:6n3	0.28	0.27	0.24	0.27	1.26	0.27
C24:1	0.01	0	0.018	0.014	0.13	0.13
Total	100.00	100.00	100.00	100.00	100.00	100.00

[1] AA = arachidonic acid, EPA = eicosapentaenoic acid
[2]Martek Biosciences Corporation (Columbia, MD)
[3]Epax 4510 TG from Pronova Biocare (Aalesund, Norway)
[4]MCT= medium train tryglycerides
*Fatty acid analyzed as described in Materials and Methods.

Table 3. Daily gain and feed intake of piglets after consuming formula enriched with arachidonic acid (AA) or eicosapentaenoic acid (EPA) in comparison to sow milk (SOW)

		Diet						
Day	**0 AA**	**0.5 AA**	**2.5 AA**	**5 AA**	**5 EPA**	**SOW**	**SEM**	**P > F**
				Daily gain (g/d)				
0-4	190	212	211	207	192	203	11	0.43
0-8	261	257	247	243	216	251	16	0.47
0-16	306	322	308	293	290	272	21	0.64
				Intake (g/d)				
0-4	586	598	597	595	580	N/A	24	0.98
0-8	904	906	886	891	835	N/A	29	0.43
0-16	1412	1538	1428	1467	1325	N/A	58	0.15

Table 4. Serum metabolite and enzyme concentrations in piglets after 16 d of consuming formula enriched with arachidonic acid (AA) or eicosapentaenoic acid (EPA) in comparison to sow milk (SOW)

	Dietary Treatments[1]						
	O% AA	0.5% AA	2.5% AA	5% AA	5% EPA	SOW	SEM
Blood Parameter							
Urea nitrogen, *mg/dL*	24.2[c]	14.2[ab]	16.9[bc]	14.6[ab]	15.3[abc]	7.7[a]	0.04
Creatinine, *mg/dL*	0.7[a]	0.8[a]	0.7[a]	0.7[a]	0.7[a]	1.1[b]	0.15
Total protein, *g/dL*	4.9	5.1	4.9	4.9	4.9	4.9	0.14
Albumin, *g/dL*	3.1	3.4	3.4	3.2	3.2	3.3	0.14
Total biliruben, *mg/dL*	0.1[a]	0.1[a]	0.1[a]	0.1[a]	0.1[a]	0.5[b]	0.05
Alkaline phosphatase, *U/L*	558	532	533	429	448	1745	117
ALT (SGPT)[2], *U/L*	16.2	10.7	9.1	12.5	7.7	19.7	3.37
AST (SGOT)[3], *U/L*	38.2	66.7	43.7	43.7	60.5	59.5	11.46
Cholesterol, *mg/dL*	104.2[a]	96.2[a]	84.2[a]	81.1[a]	73.7[a]	219.0[b]	15.71
Calcium, *mg/dL*	11.2	11.5	11.4	11.1	10.9	11.9	0.22
Phosphorus, *mg/dL*	14.0	16.7	15.6	16.1	15.1	15.3	0.64
Sodium, *mEq/L*	136.8	135.5	134.5	140.0	133.7	136.0	1.26
Potassium, *mEq/L*	11.8	14.5	13.7	13.1	13.3	10.9	1.08
Chloride, *mEq/L*	102.8	98.8	100.0	102.6	98.8	98.2	1.42
Albumin/globulin, *ratio*	1.8	2.1	2.4	1.9	2.2	2.2	0.24
BUN/creatinine, *ratio*	34.2[a]	18.7[ab]	23.1[a]	22.6[a]	22.5[a]	7.0 [b]	4.62
globulin, *g/dL*	1.7	1.5	1.5	1.7	1.7	1.6	0.13
Lipase, *U/L*	20.3	18.3	19.5	18.5	20.0	25.5	2.84
Amylase, *U/L*	907	1025	1081	648	1052	1152	134
Triglycerides, *mg/dL*	95.0[a]	73.2[a]	70.5[a]	80.2[a]	62.2[a]	145.0[b]	0.2
CPK[4], *U/L*	1934	3288	2278	2985	2732	2306	673
GGTP[5], *U/L*	32.3	29.2	32.4	19.2	26.7	27.5	5.80
Magnesium, *mEq/L*	3.2	4.0	3.5	3.6	3.4	4.0	0.25
Calculated osmolality, *mOsm/L*	295.3	299.6	293.9	293.3	289.8	294.8	3.80

[1]Values are lsmeans, n=6 per treatment. SEM = standard error of the mean.
[2] ALT=Alanine Aminotransferase (liver enzyme)
[3] AST=Aspartate Aminotransferase (liver enzyme)

[4]CPK= creatine phosphokinase (an enzyme found predominantly in the heart, brain
and skeletal muscle)
[5]GGTP= Gamma-glutamyl transpeptidase (a serum biliary enzyme)
[abc] Means within a row lacking a common superscript differ, $P < 0.05$.

Table 5. Ileal morphology of piglets fed dietary polyunsaturated fatty acids[1].

Item	Time 0	0% AA	0.5% AA	2.5%AA	5%AA	5 % EPA	SOW	SEM	P-value
Villus height, um	637	463	418	359	546	459	396	66	.12
Villus width, um	115[ab]	109[ab]	130[a]	127[a]	94[b]	109[ab]	106[b]	6	.04
Villus area, mm^2	0.49	0.34	0.37	0.31	0.34	0.33	0.29	0.06	0.32
Crypt depth, um	113[b]	135[ac]	132[ac]	141[ac]	172[a]	136[ac]	90[b]	11.3	0.02
Villus:crypt ratio	5.7[a]	3.8[bc]	3.2[bc]	2.6[b]	3.2[bc]	3.4[bc]	4.5[ac]	0.49	0.01

[1] Values are means for n=6 pigs/treatment.
[abc] Means within a row lacking a common superscript differ, $P < 0.05$.

Table 6. Effect of dietary arachidonic acid (AA) and eicosapenaenoic acid (EPA) on the fatty acid composition of neonatal pig jejunal mucosa phospholipids*

	Day 0	Day 4						Day 8						Day 16						P > F		
FA		**0aa**	**0.5aa**	**2.5aa**	**5aa**	**5EPA**	**SOW**	**0aa**	**0.5aa**	**2.5aa**	**5aa**	**5EPA**	**SOW**	**0aa**	**0.5aa**	**2.5aa**	**5aa**	**5EPA**	**SOW**	**D[1]**	**T[2]**	**D*T[3]**
C14:0	1111	10.1^{c}	7.1^{cd}	5.5^{cd}	10.2^{c}	6.3^{cd}	2.4^{d}	26.3^{a}	5.8^{cd}	3.7^{d}	5.2^{cd}	6.4^{cd}	2.9^{d}	16.1^{b}	3.6^{d}	4.9^{cd}	5.4^{cd}	5.0^{cd}	2.8^{d}	0.117	<0.001	<0.001
C16:0		73.2	109.8	79.6	116.8	59.7	85.9	81.3	99.0	77.8	83.4	99.5	99.4	89.7	91.2	93.9	93.4	89.4	100.2	0.82	0.47	0.57
C18:0		90.7	90.0	64.0	91.2	41.8	73.9	112.7	109.6	104.2	95.2	107.9	86.0	80.6	78.9	91.8	79.0	80.5	93.6	<0.001	0.53	0.21
C20:0		0.8	8.0	3.5	4.0	0.4	0.7	0.9	1.4	0.9	2.1	0.9	0.4	0.7	0.7	1.1	0.8	0.9	0.6	0.112	0.396	0.647
C14:1		1.7^{cde}	1.0^{de}	2.0^{cde}	2.4^{cde}	1.1^{de}	1.4^{cde}	2.2^{cde}	2.7^{cde}	2.4^{cde}	2.6^{cde}	2.9^{cde}	3.5^{cd}	7.8^{a}	6.9^{a}	6.2^{ab}	4.2^{bc}	4.2^{bc}	0.2^{c}	<0.001	0.043	<0.001
C16:1		7.7^{c}	3.2^{de}	1.6^{e}	1.9^{e}	1.8^{e}	6.0^{cd}	16.9^{b}	1.8^{e}	1.1^{e}	0.93^{e}	1.2^{e}	8.1^{c}	20.8^{a}	2.3^{cd}	3.2^{de}	2.8^{de}	1.7^{e}	8.0^{c}	<0.001	<0.001	<0.001
C18:1t		2.6	1.0	0.5	0.2	0.9	0.9	2.3	1.0	0.7	0.9	0.9	1.7	3.2	1.4	1.5	1.2	1.3	1.4	0.023	<0.001	0.884
C18:1c		62.6	47.5	32.7	41.6	24.5	31.1	66.4	41.8	32.3	30.4	38.5	50.0	85.0	43.5	42.1	38.1	42.3	54.3	0.015	<0.001	0.284
C18:2t		1.3	2.3	1.3	0.3	0.1	1.2	0.8	0.3	0.2	0.2	0.4	1.2	0.6	0.5	0.5	0.6	0.5	3.8	0.295	0.023	0.156
C18:2c		43.3^{j}	61.4^{ghij}	49.9^{hij}	54.6^{hij}	29.8^{j}	48.0^{ij}	93.2^{defg}	121.8^{bcd}	95.1^{def}	82.9^{efgh}	112.9^{cdef}	115.6^{cde}	80.1^{fghi}	151.5^{ab}	131.3^{bc}	101.6^{cdef}	118.7^{bcd}	177.2^{a}	<0.001	<0.001	0.002
C18:3n6		0.6^{b}	5.9^{a}	1.1^{b}	0.4^{b}	0.0^{b}	0.2^{b}	1.1^{b}	0.8^{b}	0.8^{b}	1.0^{b}	0.9^{b}	0.8^{b}	0.8^{b}	0.8^{b}	1.1^{b}	1.3^{b}	0.4^{b}	0.4^{b}	0.531	0.094	0.042
C18:3n3		1.1^{bc}	0.0^{c}	0.5^{bc}	0.5^{bc}	0.1	0.2	0.6^{bc}	0.3^{bc}	0.2^{c}	0.3^{c}	0.3^{c}	0.5^{bc}	5.2^{a}	4.7^{a}	4.8^{a}	4.6^{a}	5.5^{a}	1.7^{b}	<0.001	0.002	0.001
C20:1		1.6^{c}	0.8	0.9^{c}	0.1^{c}	0.9^{c}	0.7^{c}	6.6^{a}	4.4^{b}	4.2^{b}	3.9^{b}	5.2^{ab}	1.4^{c}	0.3^{c}	0.2^{c}	0.2^{c}	0.2^{c}	0.2^{c}	1.0^{c}	<0.001	<0.001	<0.001
C20:2		2.1	3.3	2.3	2.1	0.9	2.1	2.3	3.3	2.9	2.5	2.6	2.8	0.3	2.0	1.8	1.8	1.8	1.7	<0.001	0.027	0.489
C20:3n6		1.2	0.0	2.5	3.3	0.1	0.2	1.9	2.0	2.5	3.5	1.5	1.9	1.7	1.4	2.7	1.6	2.1	1.2	0.201	0.101	0.833
C20:3n3		0.7	1.3	0.4	0.0	0.0	0.0	3.1	0.3	0.3	0.2	0.1	0.9	1.5	0.3	0.3	0.2	0.2	0.1	0.506	0.011	0.610
C20:4		2.0^{fg}	4.4^{fg}	4.3^{fg}	9.3^{efg}	1.3^{g}	4.3^{fg}	14.0^{efg}	19.7^{de}	45.2^{b}	65.4^{a}	14.0^{efg}	31.7^{cd}	14.4^{efg}	37.3^{bc}	72.6^{a}	73.3^{a}	16.3^{ef}	47.5^{b}	<0.001	<0.001	<0.001
C20:5		0.1^{c}	0.2^{c}	0.7^{c}	0.1^{c}	2.2^{c}	0.3^{c}	1.9^{c}	0.9^{c}	0.8^{c}	0.6^{c}	33.2^{a}	0.7^{c}	1.2^{c}	0.3^{c}	0.5^{c}	0.6^{c}	12.0^{b}	0.3^{c}	<0.001	<0.001	<0.001
C22:1		1.6	0.3	4.7	6.9	0.0	0.8	0.7	0.4	0.3	0.4	0.3	0.5	1.1	2.6	1.1	3.4	1.4	2.8	0.164	0.445	0.652
C22:2		0.2	1.1	0.5	1.9	0.0	0.03	0.06	0.08	0.2	0.1	0.2	0.1	0.2	0.1	0.05	0.03	0.8	0.1	0.251	0.788	0.540
C22:6		0.7^{d}	5.6^{bcd}	1.6^{cd}	6.0^{bcd}	0.3^{d}	0.05^{d}	7.7^{bc}	3.7^{cd}	4.2^{cd}	4.5^{bcd}	7.4^{bc}	2.2^{cd}	10.4^{b}	20.1^{a}	6.9^{bc}	4.6^{bcd}	15.6^{a}	5.5^{bcd}	<0.001	<0.001	<0.001

[8]Pigs were fed sow milk or experimental diets containing either 0, 0.5, 2.5, 5.0% AA or 5% EPA for 16 days. Jejunal mucosa was collected on day 4, 8 and 16 as described in Material and Methods. Values are the least square means ± SEM of 6 individual pigs for each diet group. Values without a common superscript on each row are significantly different (P<.05).

[1]Day effect

[2]Treatment effect

[3]Day * Treatment interaction

Table 7. Effect of dietary arachidonic acid (AA) and eicosapentaenoic acid (EPA) on the fatty acid composition of neonatal pig ileal mucosa phospholipids*

	Day 0		Day 4				Day 8						Day 16						P > F		
FA	**0aa**	**0.5aa**	**2.5aa**	**5aa**	**5EPA**	**SOW**	**0aa**	**0.5aa**	**2.5aa**	**5aa**	**5EPA**	**SOW**	**0aa**	**0.5aa**	**2.5aa**	**5aa**	**5EPA**	**SOW**	**D**	**T**	**D*T**
C14:0 1111	8.1	7.5	6.7	2.8	5.8	6.2	9.3	4.6	5.3	5.9	3.8	1.3	15.7	3.9	3.3	4.7	4.2	2.0	0.623	<0.001	0.097
C16:0	98.4	94.1	77.6	85.1	48.6	72.8	77.6	108.4	107.0	103.3	77.2	92.4	88.1	102.6	88.2	91.1	83.1	107.4	0.068	0.054	0.493
C18:0	86.5	92.2	74.7	68.5	45.0	53.2	73.3	101.0	92.6	88.9	67.1	128.3	68.6	71.8	61.3	61.7	71.0	74.1	0.007	0.239	0.195
C20:0	-	-	-	-	0.7	0.5	0.6	0.9	1.1	1.7	1.4	3.9	0.4	0.6	0.6	0.5	0.5	0.6	0.169	0.791	0.736
C14:1	-	-	-	-	75.6	67.6	5.3	9.3	5.6	9.7	4.1	5.3	1.2	2.7	4.3	5.6	1.7	4.6	<0.001	0.248	0.529
C16:1	2.2	1.3	0.5	1.3	10.5	15.5	9.1	3.0	2.9	2.0	2.3	9.3	17.7	2.9	2.1	2.7	2.0	6.9	0.908	0.009	0.161
C18:1t	2.4	2.4	1.2	2.1	10.8	10.6	3.4	3.5	4.1	3.3	3.0	1.6	2.7	1.5	1.6	1.6	1.4	0.4	0.123	0.788	0.410
C18:1c	43.2	51.3	37.5	44.0	21.7	35.7	78.9	80.6	71.5	66.6	52.2	123.0	89.7	61.2	51.6	54.6	44.4	59.8	<0.001	0.107	0.468
C18:2t	2.6	1.7	0.9	0.8	6.0	8.1	2.0	4.5	6.1	3.9	4.1	3.2	0.8	1.8	1.6	1.6	1.7	0.9	0.165	0.669	0.693
C18:2c	38.7	41.6	34.9	39.7	26.6	40.5	71.8	160.1	133.1	108.3	76.1	163.9	87.6	175.1	119.8	119.0	127.1	109.6	<0.001	0.032	0.309
C18:3n6	1.0	1.2	0.8	0.8	0.6	0.9	2.1	2.4	4.5	1.7	0.4	0.4	1.1	1.3	1.3	2.5	1.6	0.6	0.228	0.452	0.540
C18:3n3	1.3	0.6	0.3	0.4	0.5	0.6	3.0	6.6	6.9	6.2	5.0	1.0	6.3	8.0	6.3	6.8	7.4	1.6	<0.001	<0.001	0.074
C20:1	0.8	0.6	0.6	0.4	0.4	0.6	1.7	0.4	0.6	0.6	1.1	0.7	0.5	0.7	0.4	0.3	0.5	0.8	0.217	0.512	0.837
C20:2	1.5	1.3	1.3	1.0	0.8	1.0	0.9	4.4	9.2	3.5	2.5	2.3	0.5	3.5	2.8	2.9	1.8	2.2	0.0162	0.111	0.384
C20:3n6	-	-	-	-	-	-	4.5	5.1	5.5	5.9	5.2	3.4	2.7	4.9	4.6	5.7	2.7	4.6	0.273	0.384	0.659
C20:3n3	-	-	-	-	2.8	3.4	2.0	0.9	2.6	3.4	5.9	0.4	2.7	2.8	2.4	2.1	2.0	0.6	0.870	0.933	0.675
C20:4	0.0[c]	4.0[c]	5.4[c]	7.5[c]	6.4[c]	7.1[c]	35.5[c]	78.6[b]	125.1[a]b	131.1[a]	22.7[c]	126.2[ab]	29.7[c]	82.0[b]	112.7[ab]	135.0[a]	26.6[c]	117.4[ab]	<0.001	<0.001	0.006
C20:5	0.4	1.2	1.1	0.3	0.4	0.2	6.4	4.6	5.5	3.9	56.6	4.3	6.8	2.4	2.5	2.0	50.4	2.9	<0.001	0.019	0.068
C22:1	-	-	-	-	-	-	1.9	0.3	2.3	0.0	0.3	29.9	1.1	0.1	0.1	0.03	0.08	0.2	0.342	0.598	0.596
C22:2	-	-	-	-	0.2	0.4	1.1	0.1	0.2	0.3	0.8	0.1	0.3	0.2	0.1	0.1	0.9	0.2	0.579	0.214	0.649
C22:6	0.3	1.0	0.6	0.2	8.6	13.1	15.9	11.5	13.2	6.7	10.4	9.8	26.3	17.9	11.4	8.7	22.0	17.2	<0.001	0.025	0.119

*Pigs were fed sow milk or experimental diets containing either 0, 0.5, 2.5, 5.0% AA or 5% EPA for 16 days. Jejunal mucosa was collected on day 4, 8 and 16 as described in Material and Methods. Values are the least square means ± SEM of 6 individual pigs for each diet group. Values without a common superscript on each row are significantly different (P<.05).
[1]Treatment effect
[2]Day effect
[3]Day * Treatment interaction

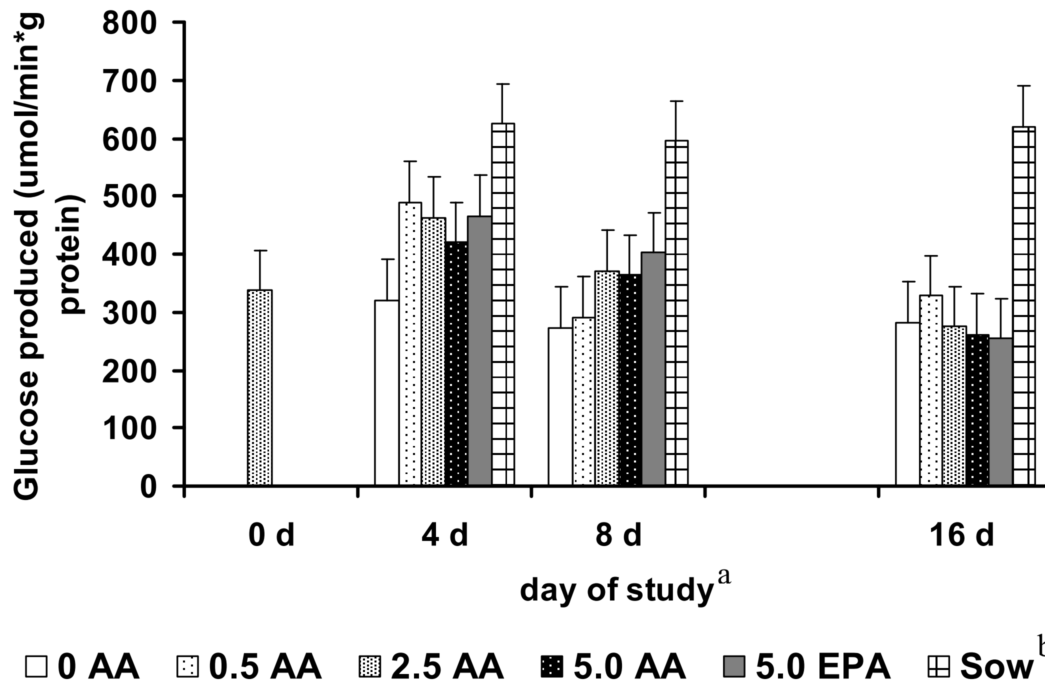

Figure 1. Lactase specific activity in the small intestinal mucosa of neonatal pigs fed diets with different levels of AA and EPA or control sows. Values illustrated are the least square means ± SEM of 6 mucosa samples in each diet group (n = 6). Enzyme specific activity was measured as described in Materials and Methods.

[a] $P < 0.05$, day 4 > day 8 and day 4 > day 16.

[b] $P < 0.05$, 0 AA < SOW.

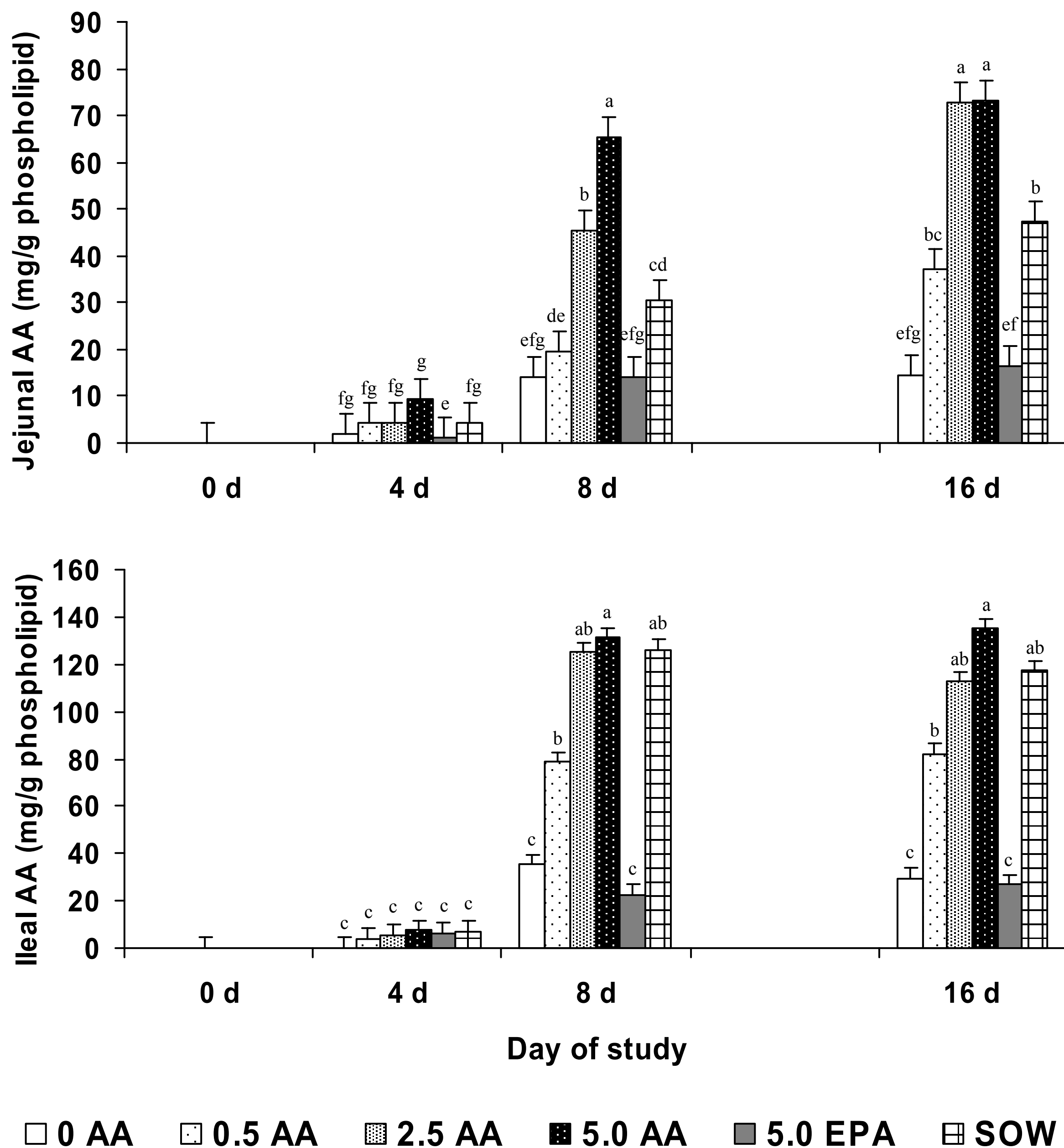

Figure 2. AA contents in isolated mucosa phospholipids in pigs consuming formula enriched with arachidonic acid (AA) or eicosapentaenoic acid (EPA) in comparison to sow milk (SOW). Values illustrated are the least square means ± SEM of 6 mucosa samples in each diet group (n = 6). Bars lacking a common superscript differ, $P < 0.05$.

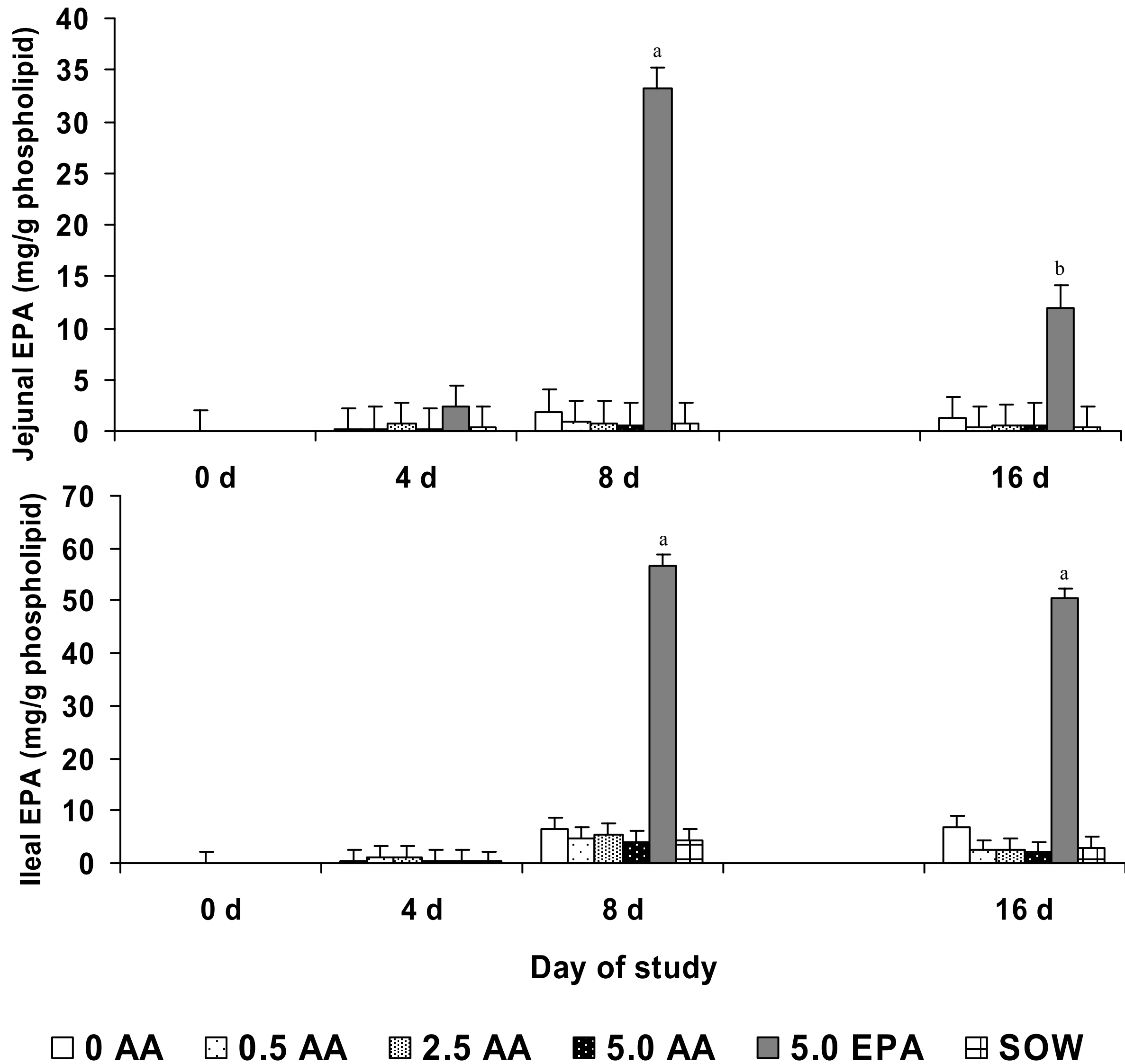

Figure 3. EPA contents in isolated mucosa phospholipids in pigs consuming formula enriched with arachidonic acid (AA) or eicosapentaenoic acid (EPA) in comparison to sow milk (SOW). Values illustrated are the least square means ± SEM of 6 mucosa samples in each diet group (n = 6). Fatty acid was determined as described in Materials and Methods. Bars lacking a common superscript differ, $P < 0.05$.

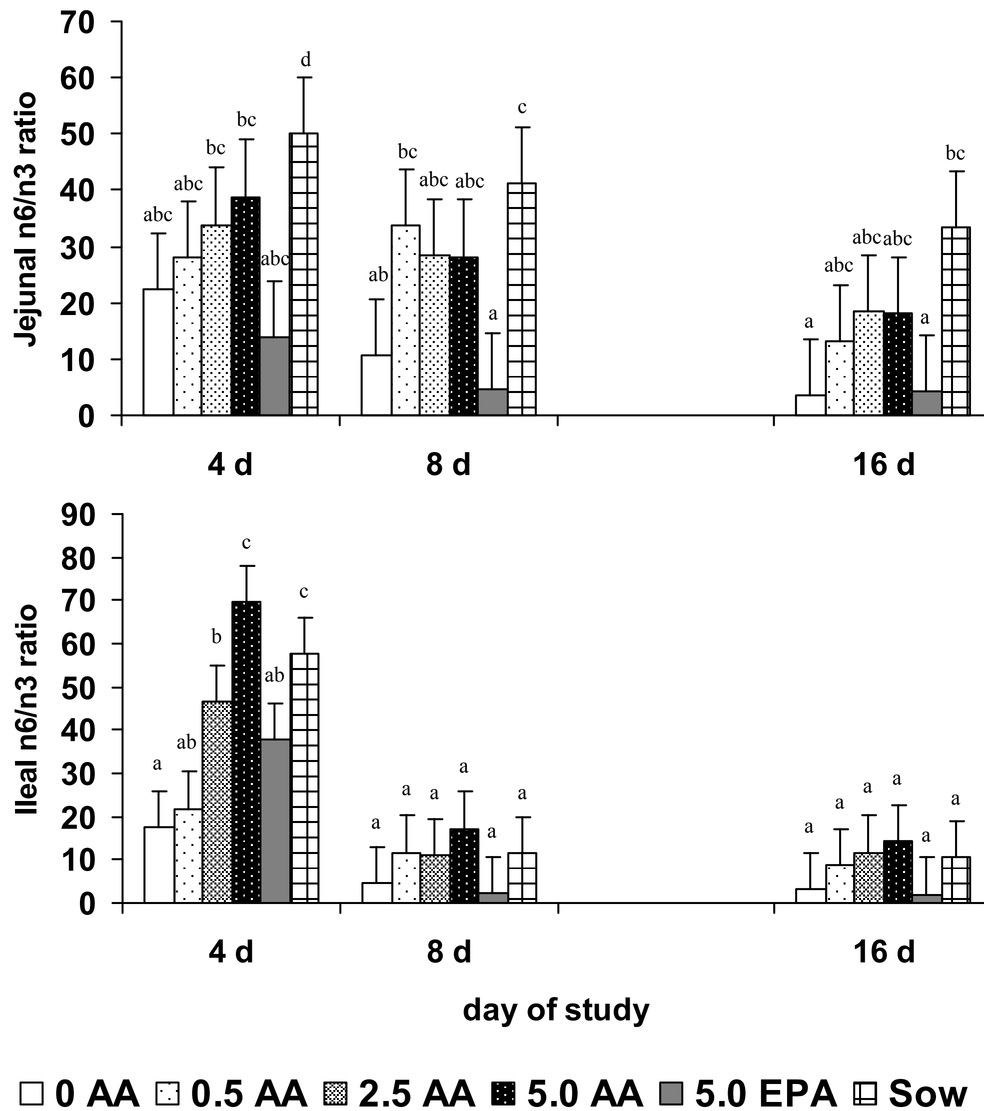

Figure 4. Ratio of n6 and n3 (n6/n3) in isolated mucosa phospholipid in pigs consuming formula enriched with arachidonic acid (AA) or eicosapentaenoic acid (EPA) in comparison to sow milk (SOW). Values illustrated are the least square means ± SEM of 6 mucosa samples in each diet group (n = 6). Fatty acid ratio was calculated as described in Materials and Methods. Bars lacking a common superscript differ, $P < 0.05$.

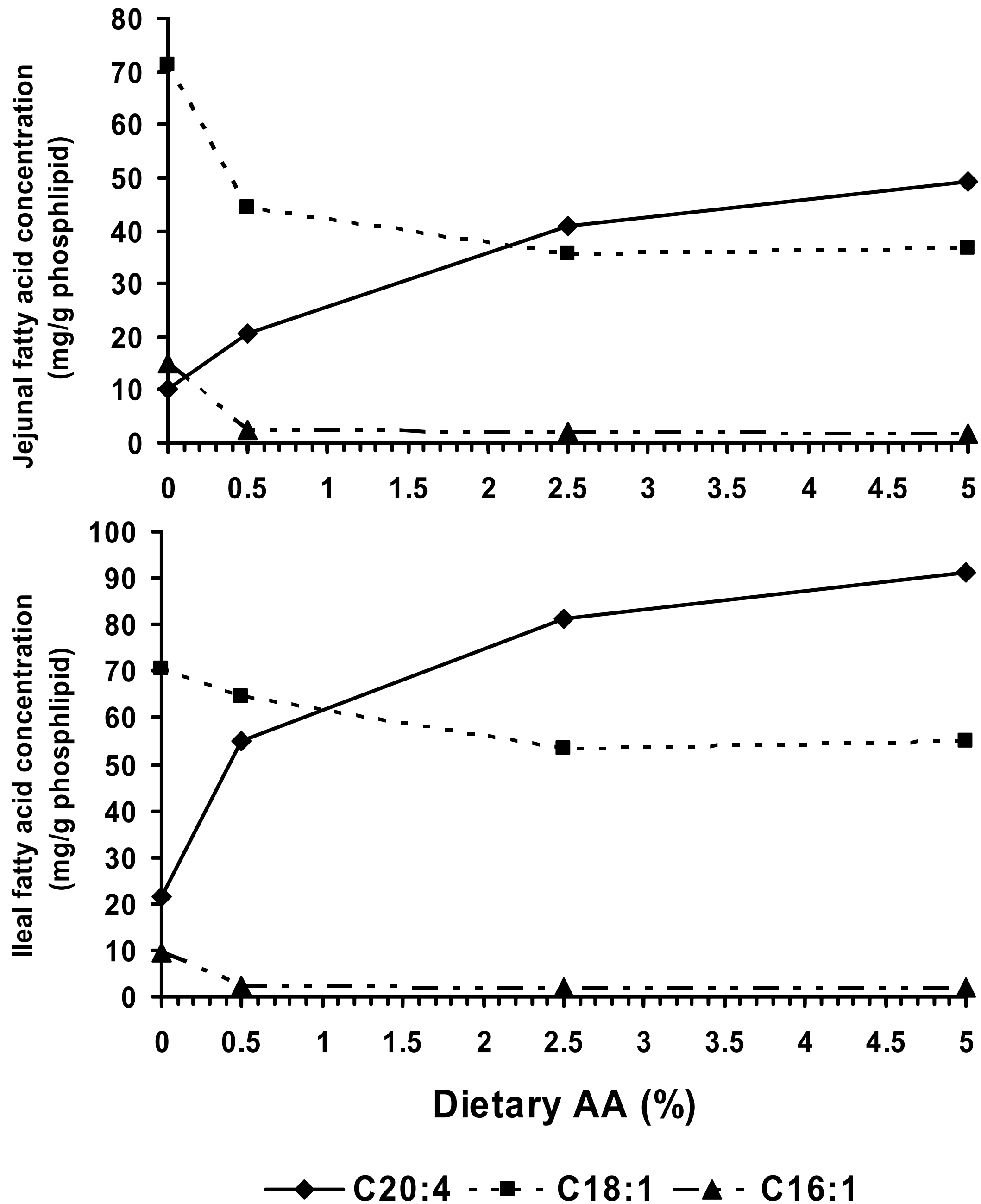

Figure 5. Changes of C18:1, C16:1 and C20:4 concentrations in isolated mucosa phospholipids in pigs consuming formula enriched with arachidonic acid (AA). Values illustrated are the least square means ± SEM of 6 mucosa samples in each diet group. Means within a row lacking a common superscript differ, P < 0.05.

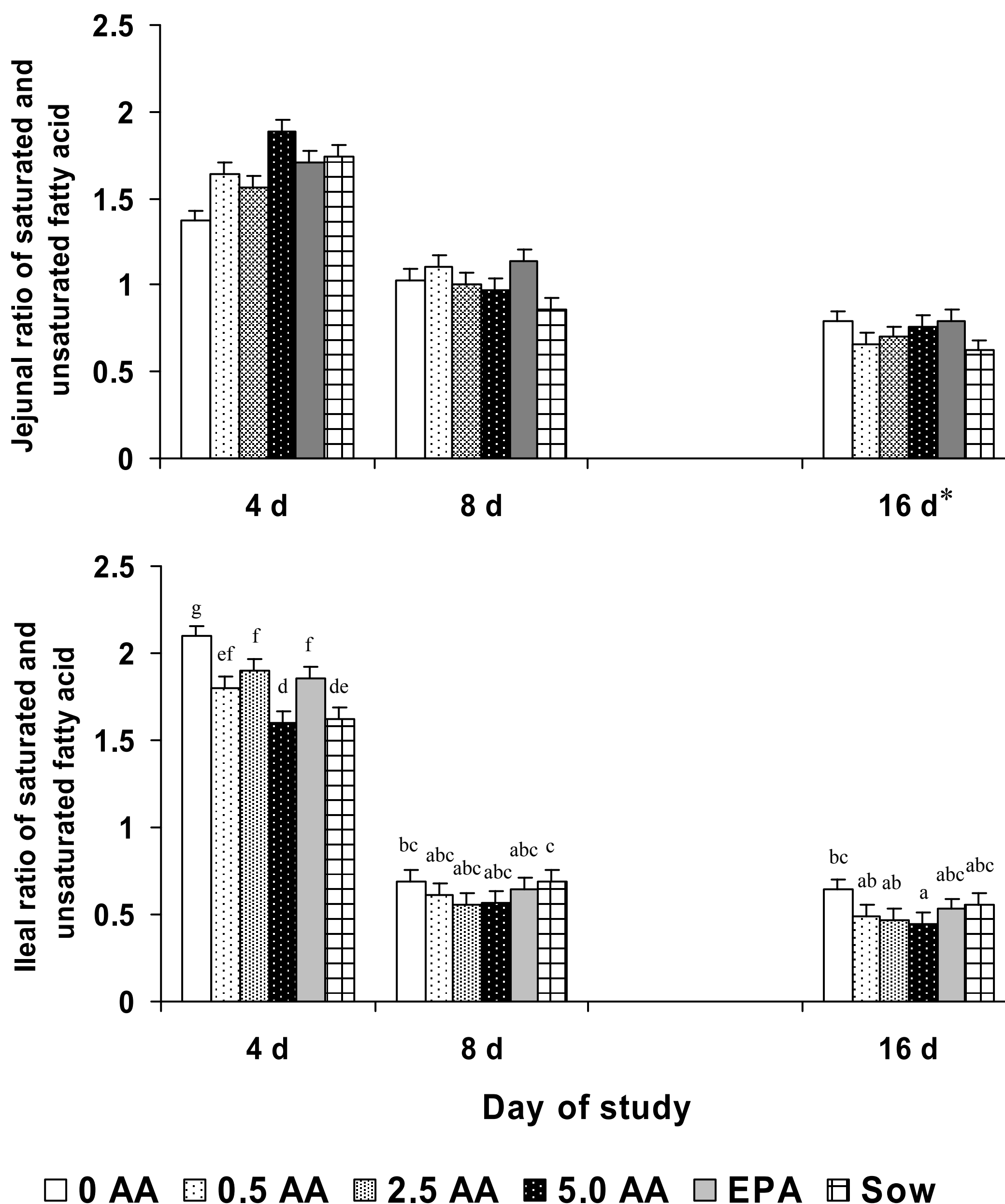

Figure 6. Ratios of saturated and unsaturated fatty acid in isolated mucosa phospholipid from pigs consuming formula enriched with arachidonic acid (AA) or eicosapentaenic acid (EPA) in comparison to sow milk (SOW). Values illustrated are the least square means ± SEM of 6 mucosa samples in each diet group (n = 6). Fatty acid ratio was calculated as described in Materials and Methods. Bars lacking a common superscript differ, $P < 0.05$.
*Different age differ. $P < 0.05$ (means for age of d4, d8 and d16 are 1.67, 1.02 and 16 0.72 in jejunum respectively).

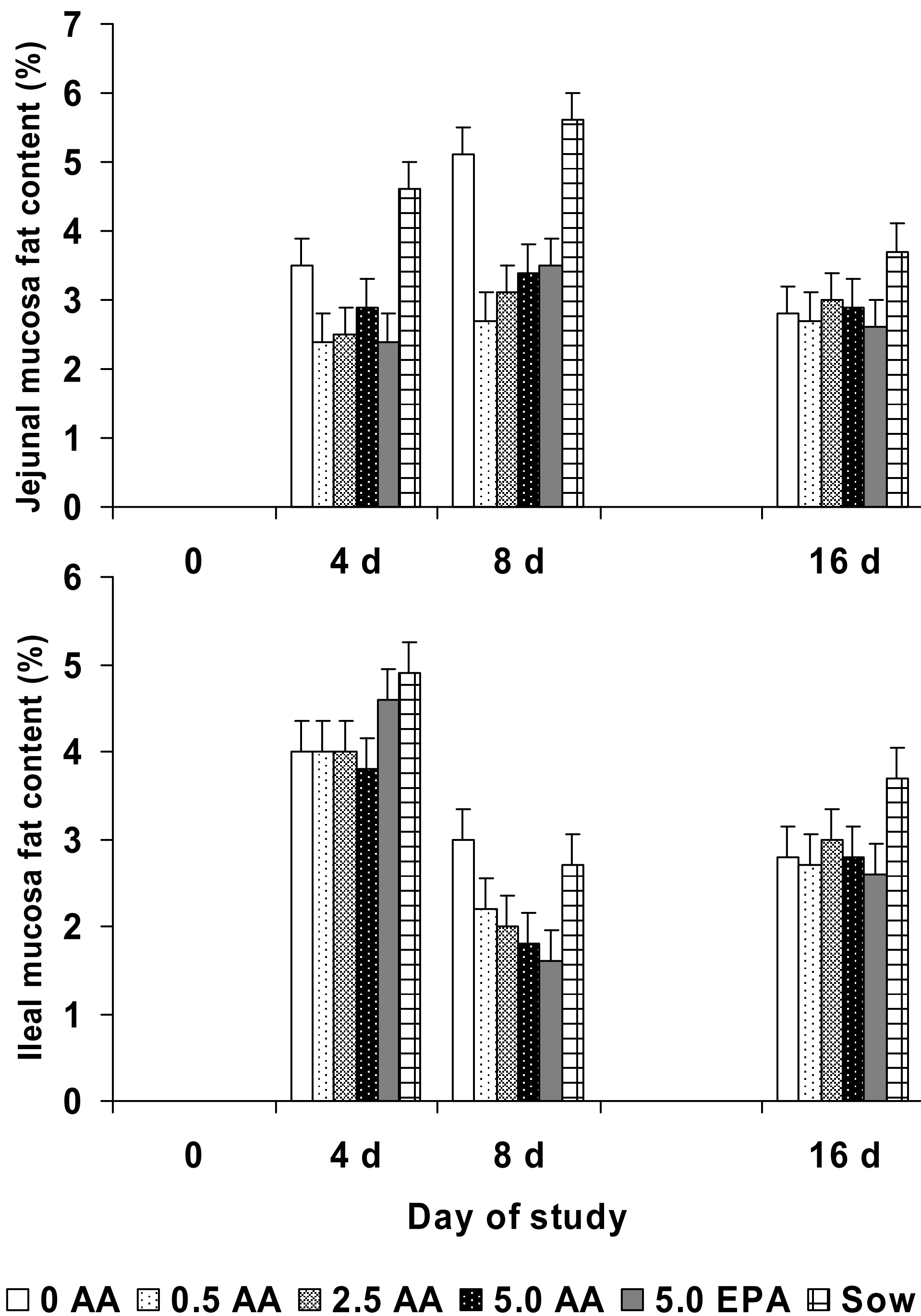

Figure 7. Fat content in isolated mucosa from pigs consuming formula enriched with arachidonic acid (AA) or eicosapentaenic acid (EPA) in comparison to sow milk (SOW). Values illustrated are the least square means ± SEM of 6 mucosa samples in each diet group.

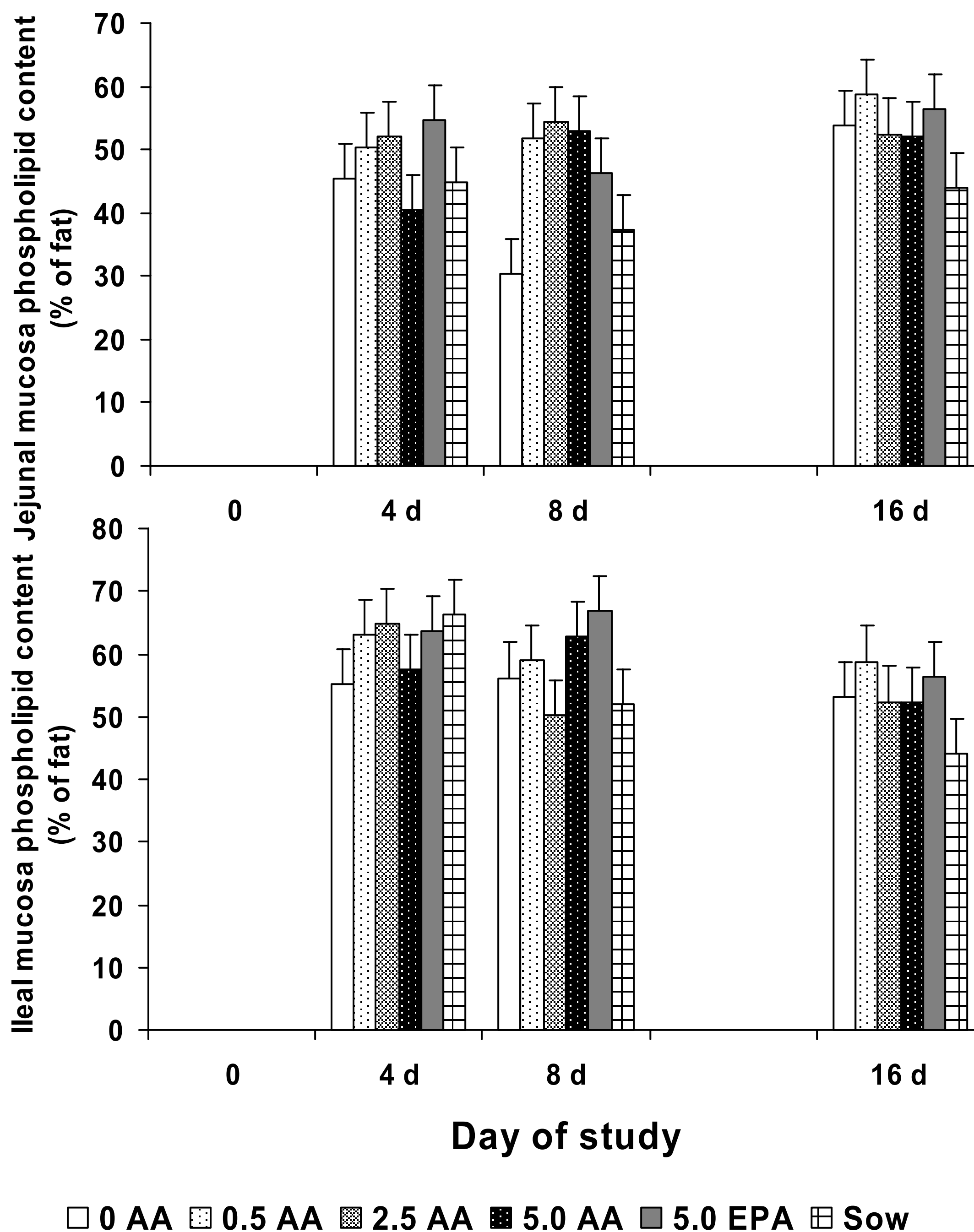

Figure 8. Phospholipid content in isolated mucosa from pigs consuming formula enriched with arachidonic acid (AA) or eicosapentaenic acid (EPA) in comparison to sow milk (SOW). Values illustrated are the least square means ± SEM of 6 mucosa samples in each diet group.

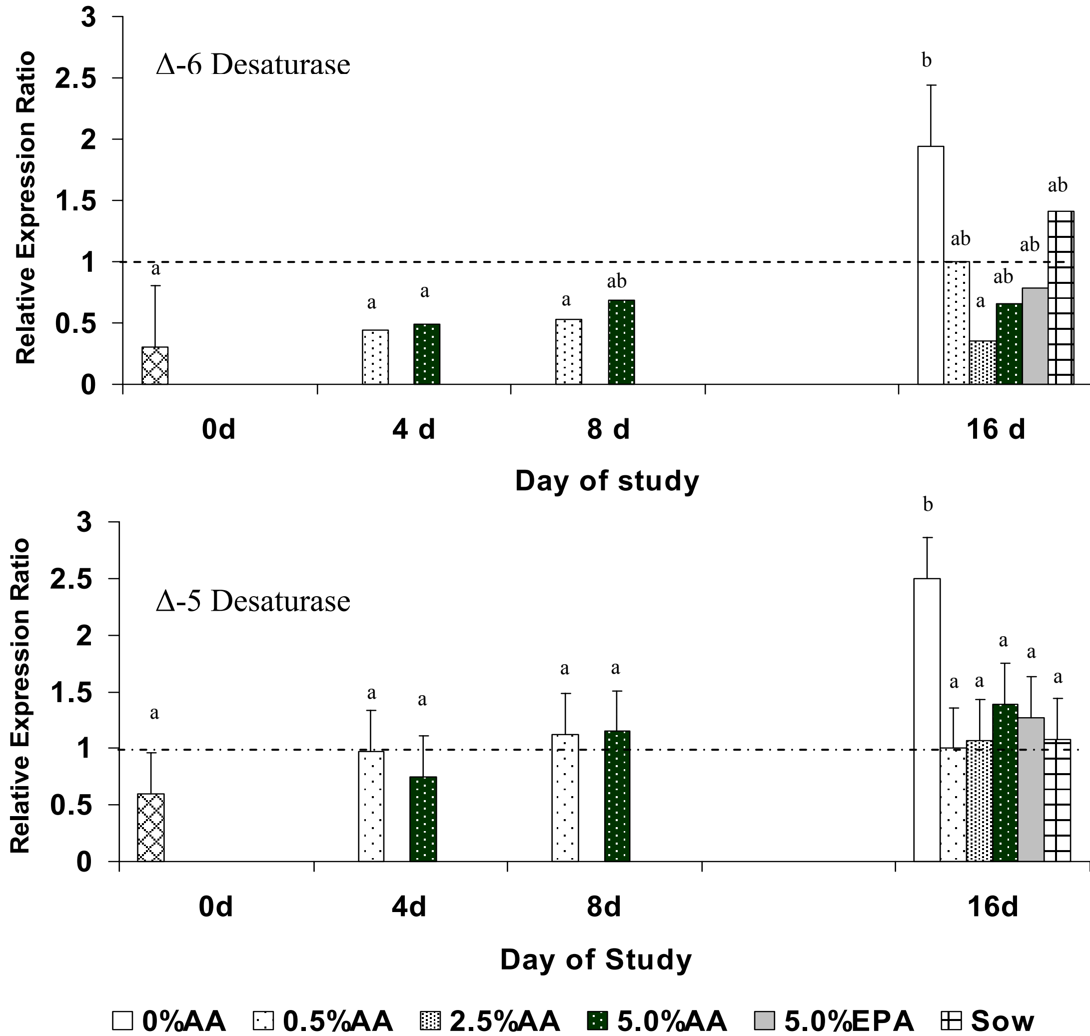

Figure 9. Hepatic desaturase mRNA abundance determined in pigs fed diets with different levels of arachidonic acid (AA) and eicosapentaenoic acid (EPA) or control sows. Values illustrated are the least square means ± SEM of 6 mucosa samples in each diet group. Bars lacking a common superscript differ, $P < 0.05$.

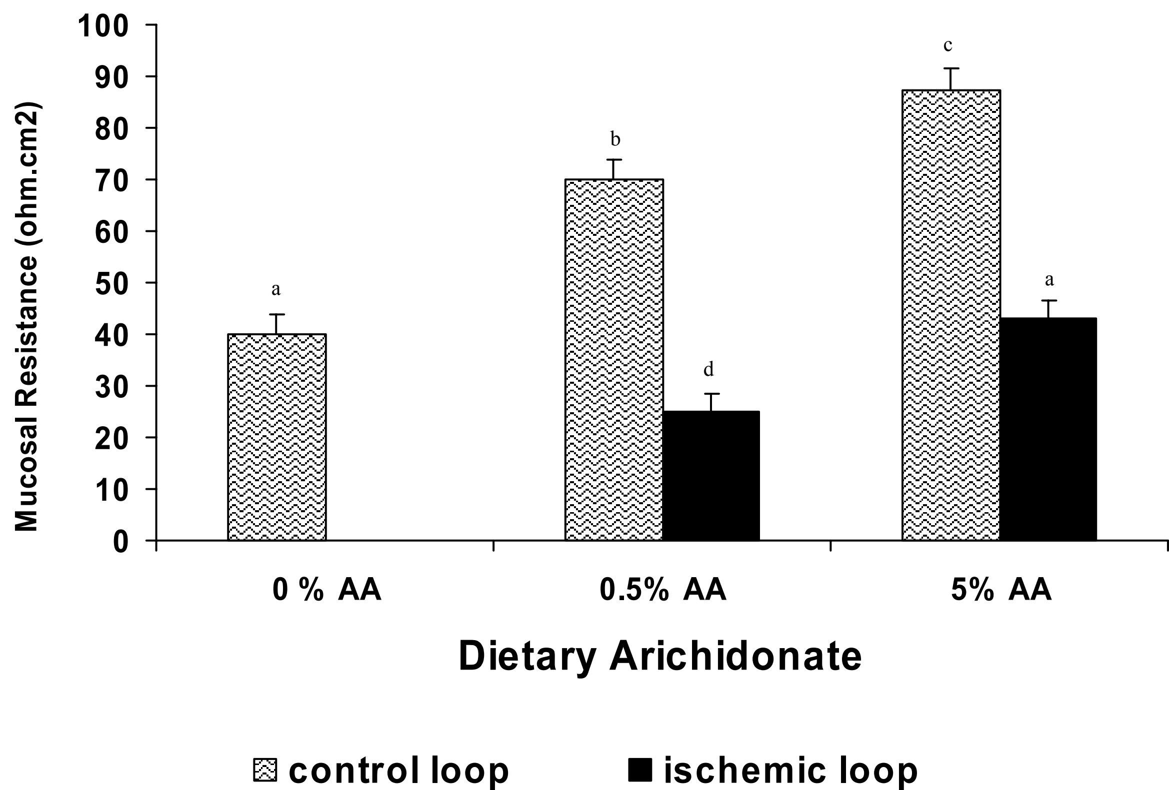

Figure 10. Mucosal transepithelial resistance increases as content of arichidonic acid increased in both of oxygenated (control) and ischemic tissues. Small intestinal mucosa obtained from piglets (n=3/treatment) fed 0, 0.5, or 5% AA (% dietary fat) for 14 d was mounted in Ussing chambers and electrophysiologic measurements were collected. Columns and means lacking a common superscript differ (*P<0.01*).

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