Enriching the starter diet in Omega 3 polyunsaturated fatty acids reduces adipocyte size in broiler chicks

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Running Title: Dietary enrichment alters adiposity

i. List of Abbreviations

ACOX1: acyl-coenzyme A oxidase 1
CA: Canola oil
CCL20: chemokine C-C ligand 20
CPT1: carnitine palmitoyltransferase 1
EGR1: early growth response transcription factor 1
FASN: fatty acid synthase
FL: Flaxseed oil
FO: Fish oil
LA: Lard
LC n-3 PUFA: long-chain n-3 polyunsaturated fatty acids

LPL: lipoprotein lipase

LSD: Least significant difference

NEFA: Non-esterified fatty acid

PCK1: phosphoenolpyruvate carboxy-kinase 1

PDK4: pyruvate dehydrogenase kinase 4

PNPLA8: patatin-like phospholipase domain containing protein 8

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Abstract

Epidemiological studies associate perinatal intake of eicosapentaenoic acid (EPA, 20:5, n-3) and docosahexaenoic acid (DHA, 22:6, n-3)) with reduced adiposity in children, suggesting that these fatty acids may alter adipose tissue development. The objective of this study was to determine if enriching the perinatal diet in EPA and DHA reduces fat deposition in juveniles. Cobb 500 broiler chicks were fed isocaloric diets containing fat (8% wt:wt) from fish oil (FO), lard (LA), canola oil (CA), or flaxseed oil (FL) from 7 to 30 d of age. Adiposity (abdominal fat pad wt/body wt) at 30 d was not significantly affected by diet, but FO significantly reduced adipocyte size, increasing the abundance of small adipocytes Plasma non-esterified fatty acid (NEFA) levels suggest that reduced adipocyte size was due in part to enhanced mobilization of fatty acids from adipose tissue. Our work indicates that dietary EPA and DHA effectively reduce the size of developing adipocytes in juveniles, which may limit adipose deposition and provide metabolic benefits.

Keywords: Childhood obesity, Obesity, Fatty acids, Adipose tissue, Model Organism
Introduction

Approximately 27% of children in the U.S. are classified as overweight or obese by age five (1). Obese children are more likely to be obese adults, and both childhood and adult obesity increase the risk of cardiovascular disease, diabetes and other co-morbidities (2). Limiting excess fat accumulation in the first few years of life is therefore therapeutically important for children and for prevention of adult obesity. A plethora of studies have identified factors that influence adiposity in mature animals and humans, in which changes in adipocyte size are the primary basis for differences in fatness. Much less is known about control of adipose mass in juveniles, when both adipocyte hypertrophy and hyperplasia actively contribute to fat deposition.

Polyunsaturated fatty acids of the n-3 and n-6 series differentially regulate preadipocyte proliferation, adipogenesis, and triglyceride storage, all of which contribute to deposition of adipose tissue prior to adolescence. Omega-6 PUFAs tend to be pro-adipogenic, while LC n-3 PUFAs (particularly EPA and DHA) attenuate lipid accumulation and promote an oxidative adipocyte phenotype (rev. in (3)). Large-scale studies in mother-child pairs have associated n-6 PUFA intake with increased adiposity in children, while an inverse relationship has been demonstrated with dietary n-3 PUFA (4, 5). These associations suggest that the types of fatty acids consumed early in life may influence the course of adipose development and subsequently impact the predisposition for obesity.

Avian models are useful for testing the effects of diet on early adipose development because chicks eat independently at hatch, allowing direct manipulation of the diet very early in life. Broiler chickens in particular are a valuable, polygenic model of susceptibility to obesity due to inadvertent consequences of selection for rapid growth (6). Broiler chicks begin to deposit excess abdominal fat compared to other breeds within two weeks of hatch. In contrast, most
rodent models of obesity are monogenic, or are induced by feeding a diet very high in fat. We
used Cobb 500 broiler chicks, one of the most widely-used commercial broiler lines, to
determine if providing EPA and DHA in the diet early in life attenuates adipose tissue deposition
in juveniles. Diets enriched in fish oil (as a source of EPA and DHA) were compared to diets
containing equal amounts of lard, canola oil, or flaxseed oil to evaluate the effects relative to
other types of fatty acids. Experimental diets were provided beginning at seven days of age to
coincide with initial deposition of the abdominal depot, and to focus on a developmental window
in which hypertrophy and hyperplasia contribute comparably to adipose growth.
Materials and Methods

Animals and diets

All animal procedures were approved by the University of Tennessee Institutional Animal Care and Use Committee. Forty mixed-sex Cobb 500 broiler chicks were fed ad libitum a commercial starter diet from hatch until 7 d, then switched to one of four experimental diets. Experimental diets were produced by adding fat (8% wt:wt) from lard (LA), canola oil (CA), flaxseed oil (FL) or fish oil (FO) into a commercially-formulated starter diet base. Final energy content of each diet was 3018 Kcal/kg. Chicks were fed experimental diets from 7 to 30 d. At 30 d, chicks were weighed, then euthanized by CO2 asphyxiation, and tissues and blood were collected for experimental procedures. Complete experimental details are provided in Supplemental Information.

Statistical analysis

Data were analyzed for effects of diet using ANOVA, implemented in SAS (version 9.4, SAS Institute Inc., Cary, NC) with P < 0.05 as the criterion for statistical significance. A significant F-test was followed by post-hoc comparisons using Fischer’s least significant difference (LSD) to identify pairwise differences between diet groups.
Results

Effects on body composition and growth rate

Fatty acid composition of the diet significantly affected final body weight (P = 0.045), with FO birds weighing less than those in the LA group (Table 1, P = 0.02). Neither absolute nor relative (adjusted for body weight) weights of the abdominal adipose depot or of breast muscle differed significantly between diet groups (P > 0.05). Plasma levels of NEFA (P = 0.002), but not glucose (P = 0.130) were affected by diet, with increased NEFA in FO chicks compared to each of the other diet groups. As expected, the fatty acid profile of the abdominal fat reflected the dietary fatty acid composition (Supplemental Table 1). Feed intake did not differ across diets (data not shown).

Despite similarities in fat pad weight, diet significantly affected abdominal adipocyte volume (P = 0.020). Average adipocyte size was smallest in FO chicks, differing significantly from chicks fed LA or CA diets (Table 1). Adipocyte number varied with diet (Table 1), but differences were not statistically significant (P = 0.093). Fish oil promoted a shift in adipocyte size, favoring the abundance of relatively small adipocytes compared to diets enriched in LA or CA (Figure 1A). The frequency of very small (< 2000 μm³) adipocytes was significantly increased in FO vs. either LA or CA (P < 0.05). Conversely, frequencies of cells in each size bin beyond 4000 μm³ were lower (P < 0.05) in FO vs. CA, and in FO vs. LA for most bins.

Adipocyte volumes tended to be smaller in chicks fed the FL diet, with frequencies intermediate between those of FO and LA or CA in most size bins.

Effects on Relative mRNA Expression in Visceral White Adipose Tissue and Liver

Dietary fat source significantly influenced expression of PPAR gamma (PPARG), early growth response transcription factor 1 (EGR1), patatin-like phospholipase domain containing
protein 8 (PNPLA8), and pyruvate dehydrogenase kinase 4 (PDK4) in abdominal adipose tissue (Figure 1B). Both PPARG and EGR1 were expressed at significantly lower levels in FO and CA chicks relative to LA. Expression of PNPLA8 was significantly reduced in FO, CA and FL compared to LA. Genes associated with fatty acid oxidation (acyl-coenzyme A oxidase 1 (ACOX1) and carnitine palmitoyl acyltransferase 1 (CPT1)), lipogenesis (fatty acid synthase (FASN)), lipid storage (lipoprotein lipase (LPL)), gluconeogenesis (phosphoenolpyruvate carboxykinase 1 (PCK1)), and inflammation (chemokine C-C ligand 20 (CCL20), colony stimulating factor 1 receptor (CSF1R)) were not significantly affected by dietary fat type. In liver, expression of ACOX1, but not CPT1 or FASN, was significantly affected by diet (Figure 1C). Expression of ACOX1 was higher in FO vs. all other diet groups.

**Discussion**

A number of studies in rodents have shown that dietary fish oil can attenuate the obesogenic effects of a high fat diet. These studies have largely used mature animals, in which growth-related adipose expansion has ceased and changes in adipose mass result from effects on adipocyte size. In contrast, we focused on the first few weeks after hatch to capture the period when the abdominal depot develops and rapidly expands through both adipocyte hyperplasia and hypertrophy (7). Evaluating the effects of fish oil on these pathways is important because both contribute to childhood obesity (8, 9). A specific benefit of using an avian model, compared to rodents, is that they lack UCP-1 and thus the capacity for adipose browning. This is valuable because recent studies indicate that at least part of the ant-obesity effects of fish oil in rodents are due to induction of beige adipocytes in white adipose depots (10). Therefore our model enables us to evaluate the specific effects of dietary FO on white adipocytes.
Unlike comparable diet studies in mature chickens (e.g., (11)), we did not find an effect of dietary n-3 PUFA on adiposity. This may be due to the relatively brief period of feeding (23 days), or because fatty acid type was not sufficient to influence the inherent stimulus for rapid adipose deposition during this age window. However, both diets that were enriched in n-3 PUFA (particularly FO) favored the abundance of small adipocytes relative to diets enriched in LA or CA. Although adipocyte hypertrophy is a normal component of adipose development, excessive hypertrophy presents very early in obese children and promotes insulin resistance and adipose inflammation (9). Therefore, the ability of dietary n-3 PUFA to reduce adipocyte size in juveniles, even in the absence of decreased fat mass, may provide metabolic benefits for children prone to obesity.

Adipocyte size results from a balance between fatty acid uptake and mobilization, particularly in species (e.g., avians and humans) in which de novo lipogenesis in adipose tissue is relatively modest. Increased plasma NEFA levels suggest that enhanced fatty acid mobilization may have contributed to reduced adipocyte size in FO chicks. Interestingly, elevated NEFA, reduced adipocyte size, and enrichment of adipose tissue in EPA and DHA are also found in genetically lean lines of chickens relative to obesity-prone broilers (12). Both DHA and EPA have been shown to stimulate lipolysis and reduce adipocyte lipid accumulation in vitro (13, 14). Consumption of FO may therefore have reduced adipocyte size by promoting mobilization of fatty acids that are then oxidized by other tissues. Hepatic expression of ACOX1, the rate-limiting enzyme for oxidation of very long chain fatty acids in peroxisomes, was increased by dietary FO. However, expression of CPT1, which regulates mitochondrial fatty acid oxidation and is often co-regulated with ACOX1 (15), was not significantly affected by diet in liver. Therefore increased expression of ACOX1 in the FO group may reflect a specific response to the
abundance of very long chain PUFA (EPA and DHA), rather than a net increase in hepatic fatty acid catabolism. Dietary EPA has also been shown to enhance fatty acid oxidation within white adipocytes (16). However, our data do not indicate that this pathway contributed to reduced adipocyte size in FO and FL chicks, based on expression of CPT1 and ACOX1. It is also possible that FO, and to some extent FL, increased the abundance of smaller adipocytes by suppressing their progression through differentiation, rather than altering the balance between lipid storage and mobilization. This possibility is supported by a study in which dietary perilla oil (~ 52% ALA) down-regulated the later stages of adipocyte differentiation in rats (17). Additional characterization with stage-specific markers of adipocyte differentiation is necessary to investigate this possibility in our study.

None of the genes that we profiled were specifically affected by the diets (FO and FL) that reduced adipocyte size. However, all three diets enriched in unsaturated fatty acids reduced expression of PPARG, EGR1 and PNPLA8 compared to LA. PPARG is a well-characterized transcriptional regulator of both adipocyte differentiation and maintenance of the mature adipocyte phenotype (18). Increased PPARG expression with dietary SFA vs. PUFA-enriched diets is consistent with comparable studies in mature broilers (19). EGR1 is a pleiotropic transcription factor that has been linked to multiple aspects of adipocyte function (20). Elevated EGR1 expression in adipose tissue is associated with obesity in humans and mice, while loss of EGR1 enhances adipocyte metabolism and confers protection from obesity (21). Decreased expression of this gene in the FL, FO and CA groups relative to LA may therefore reflect beneficial effects of dietary fat quality on adipocyte metabolism. Calcium-independent phospholipase A_2γ (iPLA_2γ, encoded by PNPLA8), is a phospholipase that catalyzes the release of fatty acid side chains from mitochondrial phospholipids to generate production of eicosanoids
and other lipid second messengers that regulate cellular energetics. The specific roles of \textit{iPLA}_\gamma in adipose tissue are not known, but \textit{PNPLA8}-/- mice are resistant to diet-induced obesity, with reduced adipocyte size relative to wild type controls (22). Although expression was not consistently associated with adipocyte size in our study, reduced levels across all PUFA-enriched diets suggest that dietary fatty acids may regulate mitochondrial lipid mediators and subsequently adipocyte metabolism through \textit{PNPLA8}.

In conclusion, we have demonstrated that dietary FO attenuates adipocyte hypertrophy in juveniles that are prone to rapid fat accumulation. The mechanisms underlying this effect remain to be determined, but may include increased mobilization of stored fatty acids for oxidation by other tissues or disruption of adipocyte maturation. Although the effect of diet on adipocyte size did not manifest as significantly less adipose mass during the relatively brief period of feeding used herein, it may nonetheless be sufficient to elicit favorable metabolic effects in children who are prone to obesity.

\textbf{Acknowledgments}

ET performed the experiments, analyzed data and drafted the manuscript. SD provided technical assistance. RCB provided technical assistance and edited manuscript. BHV directed research and had primary responsibility for final content. All authors have read and approved the final manuscript. The authors would like to thank Dr. Michael O. Smith for his input into the project. This work was supported by funding to BHV from the Center of Excellence in Livestock Diseases and Human Health, University of Tennessee College of Veterinary Medicine, and by AgResearch, University of Tennessee Institute of Agriculture.
References


Table 1. Effects of dietary LA, CA, FL and FO on body, adipose and muscle weights and on serum metabolites in broiler chicks

<table>
<thead>
<tr>
<th></th>
<th>LA</th>
<th>CA</th>
<th>FL</th>
<th>FO</th>
<th>P-value</th>
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<tbody>
<tr>
<td>Body (g)</td>
<td>1752 ± 46.7^b</td>
<td>1650 ± 54.6^ab</td>
<td>1694 ± 45.1^ab</td>
<td>1562 ± 45.0^a</td>
<td>0.045</td>
</tr>
<tr>
<td>Breast (g)</td>
<td>337.9 ± 13.8</td>
<td>360.2 ± 16.5</td>
<td>354.6 ± 13.6</td>
<td>346.1 ± 7.9</td>
<td>0.573</td>
</tr>
<tr>
<td>Breast (%)^3</td>
<td>19.4 ± 1.0</td>
<td>21.9 ± 0.6</td>
<td>21.0 ± 1.0</td>
<td>22.1 ± 1.7</td>
<td>0.400</td>
</tr>
<tr>
<td>Adipose (g)</td>
<td>26.0 ± 1.9</td>
<td>24.5 ± 1.2</td>
<td>22.3 ± 1.2</td>
<td>24.3 ± 1.8</td>
<td>0.455</td>
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<tr>
<td>Adipose (%)^4</td>
<td>1.48 ± 0.09</td>
<td>1.51 ± 0.07</td>
<td>1.33 ± 0.07</td>
<td>1.56 ± 0.12</td>
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<td>NEFA (mM)</td>
<td>6.25 ± 1.98^b</td>
<td>6.49 ± 0.79^b</td>
<td>6.55 ± 1.41^b</td>
<td>10.04 ± 0.96^a</td>
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<td>Glucose (mg/dL)</td>
<td>175.7 ± 12.8</td>
<td>184.5 ± 10.8</td>
<td>189.4 ± 7.0</td>
<td>168.1 ± 5.7</td>
<td>0.130</td>
</tr>
<tr>
<td>Adipocyte volume</td>
<td>3651 ± 420.7^a</td>
<td>3706 ± 184.1^a</td>
<td>3098 ± 95.2^ab</td>
<td>2546 ± 153.1^b</td>
<td>0.020</td>
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<tr>
<td>Adipocyte number^5</td>
<td>75.4 ± 13.3</td>
<td>70.8 ± 9.5</td>
<td>93.7 ± 9.8</td>
<td>90.3 ± 8.1</td>
<td>0.093</td>
</tr>
</tbody>
</table>

1 LA= Lard; CA=Canola oil; FL=Flax; FO=Fish oil. Data are means ± SEM for all chicks in each diet group, N=10/group. Means with shared superscripts do not differ significantly; p<0.05.
2 P-value from single factor ANOVA for effect of diet
3 (Breast wt (g)/body wt (g))*100
4 (Abdominal adipose depot wt (g)/body wt (g))*100
5 Calculated from adipocyte volume and adipose depot weight
Figure legends

Figure 1. Effects of dietary FO, FL, CA and LA on abdominal adipocyte size distribution and on gene expression in liver and adipose tissue. (A), Adipocyte area was measured from images of H&E-stained sections of abdominal adipose tissue using Image J (Version 1.48, National Institutes of Health); areas < 500 μm² were removed from analyses. Adipocyte volume (μm³) was calculated from area (μm²). Frequency distributions were produced by grouping adipocytes into bins based on volume and counting the frequency of cells within each bin. ANOVA was used to evaluate the effect of diet within each bin. Values with different superscripts differ significantly (P < 0.05) based on post-hoc testing when ANOVA indicated a significant effect of diet (P < 0.05); (B, C) Relative mRNA expression of genes involved in lipid metabolism, adipogenesis and inflammation in abdominal adipose tissue (B), and in lipid metabolism in liver (C), N=6 birds / diet. Values are group means ± group std. dev.; values with different superscripts differ significantly (P < 0.05) based on post-hoc testing when ANOVA indicated a significant effect of diet (P < 0.05); Lard (LA), Canola Oil (CA), Flaxseed oil (FL), Fish oil (FO).
Supplemental Methods

Animals and experimental diets. Animal husbandry procedures were reviewed and approved by the Institutional Animal Care and Use Committee of the University of Tennessee. Forty 1-d old mixed sex Cobb 500 broiler chicks were utilized in the study. Birds were raised in stacked wire cages under standard management conditions, and had ad libitum access to water and feed. For the first six days, birds were fed a standard commercial broiler starter diet. On day seven, birds were assigned to one of four diets that differed in the type of added fat. Diets were prepared by adding lard (LA) (LA; Refined Lard, Lundy’s, USA), canola oil (CA; Pure Wesson 100% Natural, ConAgra Foods Inc., USA), flaxseed oil (FL), and fish oil (FO) (JEDWARDS International Inc., Quincy Massachusetts) at 8% (wt:wt) to a standard broiler starter base diet formulated primarily from corn and soybean (Table 1). The base diet was mixed in one large batch, and the four sources of fat were added to the same base diet. Final energy content of each diet was 3018 Kcal/kg. Experimental diets (those with added oil) were prepared every five days and stored at 4°C to minimize oxidation. Chicks were maintained on the experimental diets for 23 days (until 30 d of age). Body weight and feed intake were monitored weekly.

Chicks were euthanized by CO₂ asphyxiation. At the time of euthanasia blood was collected by cardiac venipuncture and transferred to 10 ml SST tubes (Fisher Scientific, Pittsburgh, PA). Serum was separated by centrifugation and frozen at -80°C until analyses of circulating metabolites. Abdominal and femoral (subcutaneous) adipose depots were dissected and weighed as indices of adiposity. Breast (pectoralis major) muscle was dissected and weighed to assess muscle deposition. Samples of adipose, breast and liver tissue were subsequently snap-frozen in liquid nitrogen and stored at -80°C. Samples of abdominal adipose tissue were also fixed for 24 h at 4°C in paraformaldehyde (4%) for determination of adipocyte size by histology.
Abdominal fat was removed and submerged in chilled 4% paraformaldehyde in 0.1 molar sodium phosphate buffer at pH = 7.4 for tissue fixation. Tubes were incubated at 4°C for 12 hour, washed in sodium phosphate buffer at 0.1 molar, and then transferred into chilled sodium phosphate buffer for storage.

*Serum metabolites.* Commercially available colorimetric assay kits were used to measure serum glucose (Cayman Chemical, Ann Arbor, MI) and non-esterified fatty acid (NEFA) levels (Wako Chemicals, Neuss, Germany).

*Fatty acid analysis.* Abdominal fat from one randomly selected bird for each treatment was analyzed for phospholipids (PL), neutral lipids (NL), and total lipids (TL) using gas chromatography. Fatty and acids were extracted using a modified Folch method (Ref). Briefly, lipids were separated using thin layer chromatography (TLC) plates, pre-coated with silica gel 60 (Merck, Darmstadt, Germany). Boron tri-fluoride in methanol and heat were used to saponify lipids into fatty acid methyl esters (FAME). Hexane was used to extract and dissolve the FAME. Fatty acid methyl esters were separated using a Hewlett-Packard 5880 gas chromatograph (Rochester, NY) and a DB23 capillary column (0.25 mm × 30 m) (J and W Chromatography, Folsom, OH) with hydrogen as the carrier gas. Based on the known internal standard (NuChek Prep, Elysian, MN), fatty acids were identified by retention times and fatty acid composition was calculated as a mole percentage relative to total fatty acids.

*Adipose tissue histology.* Abdominal fat samples from three birds in each diet group were embedded, sectioned and stained with hematoxylin and eosin (H&E; two slides/bird) for determination of adipocyte size, as previously described by (12). Briefly, images of three independent fields were captured on each slide under 20x magnification with the Advanced Microscopy Group EVOS XL Core microscope (Fisher Scientific, Pittsburgh, PA). For
consistency, the same person performed all measurements. Image J (Version 1.48, National Institutes of Health) was used to determine adipocyte area, ($\mu m^2$), using microscope settings of 2.8 $\mu m$/pixel, and using the restriction that measurements must exceed 500 $\mu m^2$. Frequency distributions were produced by grouping adipocytes into bins based on area and counting the frequency of cells within each bin. Adipocyte number was estimated based on average adipocyte volume and on weight of the adipose depot.

Real time PCR assay. Total RNA was isolated from approximately 200 mg of abdominal adipose tissue and liver from five chicks in each treatment using Invitrogen$^{TM}$ TRIZOL$^{TM}$ (Invitrogen, Carlsbad, CA). CDNA was synthesized from 500 ng total RNA in 20 $\mu l$ reactions using iScript cDNA Synthesis kit (Bio-Rad Laboratories, Hercules, CA). Predesigned and validated primers for quantitative real-time PCR (QPCR) were purchased from Qiagen (Quantitect; Germantown, MD). QPCR was performed in triplicate for each sample using iQ SYBR Green Master Mix (Bio-Rad Laboratories, Hercules, CA), as previously described (12). Expression levels of genes of interest were normalized to expression of TBC1 domain family, member 8 ($TBC1D8$) used as a housekeeper.
Supplemental Table 1. Base experimental diet composition

<table>
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<th>Ingredient</th>
<th>Amount (%)&lt;sup&gt;1&lt;/sup&gt;</th>
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<td>Corn</td>
<td>56.76</td>
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<tr>
<td>Soybean meal</td>
<td>31.94</td>
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<tr>
<td>Vitamin Premix&lt;sup&gt;2&lt;/sup&gt;</td>
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<tr>
<td>Fat&lt;sup&gt;3&lt;/sup&gt;</td>
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</table>

<sup>1</sup>% in diet, wt:wt

<sup>2</sup>Vitamin/mineral premix mix: vitamin A, 30,800 IU; Vitamin D<sub>3</sub>, 9,250 IU; vitamin E, 153.9 IU; vitamin B<sub>12</sub>, 0.154 mg; riboflavin, 46.2 mg; niacin, 185 mg; pantothenic acid, 84 mg; menadione sodium bisulfite, 16.2 mg; folic acid, 12.3 mg; pyridoxine HCl, 46.2 mg; thiamine HCl, 20.5 mg; biotin, 9.3 mg; choline, 2.944 mg; niacin, 185 mg Cu, 55 mg; I, 7.3 mg; Fe, 366 mg; Mn, 310 mg; Zn, 321 mg; K, 2.23 g; Mg, 1.09 g; Se, 0.48 mg

<sup>3</sup>Supplied as either lard, canola oil, flaxseed oil or fish oil
### Supplemental Table 2. Effects of dietary LA, CA, FL and FO on fatty acid profiles of phospholipids and total lipids in abdominal adipose tissue

<table>
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LA= Lard; CA= Canola oil; FL= Flax; FO= Fish oil; N=1/diet
Supplemental Figure 1. Representative H&E-stained images of abdominal adipocytes; (A) Lard (LA), (B) Canola Oil (CA), (C) Flaxseed oil (FL), (D) Fish oil (FO); 20X magnification.