DIETARY POLYUNSATURATED FATTY ACIDS MODIFY PLASMA LIPIDS AND RED BLOOD CELL MEMBRANE COMPOSITION BUT DO NOT INDUCE Δ6 DESATURASE MEDIATED CONVERSIONS IN THE DOMESTIC FELINE

A Thesis

by

MELENA KATHLEEN MCCLURE

Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

May 2008

Major Subject: Nutrition

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Approved by:

Chair of Committee,	John E. Bauer
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ABSTRACT

Dietary Polyunsaturated Fatty Acids Modify Plasma Lipids and Red Blood Cell Membrane Composition but Do Not Induce Δ6 Desaturase Mediated Conversions in the Domestic Feline.

(May 2008)

Melena Kathleen McClure, B.S., The University of Texas at Austin

Chair of Advisory Committee: Dr. John E. Bauer

This study explored the effects of dietary unsaturated fatty acids on feline lipid metabolism. It was hypothesized that high dietary linoleic acid (18:2n-6, LA) would enhance conversion to arachidonic acid (20:4n-6), enrichment of dietary long chain n-3 FA (LCn-3FA) would affect lipid parameters, and n-3 FAs incorporation may blunt n-6 FA incorporation. Twenty-nine cats were randomized into groups (n = 9, 10, 10), and fed for 28 days with blood collections on days 0, 14, and 28. Experimental diets consisted of a commercial diet, supplemented with 8g oil/100g kibble. Oil supplements and subsequent diets were: high-oleic sunflower (H diet) with 82% oleic acid (18:1n-9), Menhaden fish (M diet) with LCn-3FA, and safflower (S diet) with 75% 18:2n-6. Dietary 20:4n-6 content was: 0.03 for H and S, and 0.09 for M (g FA/kg diet). Non-esterified fatty acid (NEFA), triacylglycerol (TG), total cholesterol (TC), lipoprotein-cholesterol (LP-C), plasma phospholipid (PL) FAs, red blood cell membrane (RBC) FAs, and Δ5 and Δ6 desaturase indices were measured. Statistical analyses were performed with SAS PROC MIXED with $p < 0.05$ determining significance. Neither TC

nor NEFA showed significant effects. Diet M resulted in significant TG lowering, despite typically low feline TGs. Similarly, pre-β LP-C (i.e. TG-rich VLDL) was decreased in diet M. Plasma PL FAs revealed significant accumulations of the following: 18:1n-9 in diet H, 18:2n-6 in diet S, and LCn-3FA in diet M. Despite high dietary 18:2n-6, plasma PL 20:4n-6 was not increased in diet S over diets H or M. Increased docosadienoic acid (20:2n-6) in diet S demonstrated that 18:2n-6 chain elongation occurred in deference to its Δ6 desaturation further substantiating low feline Δ6 desaturase activity. Interestingly, no diet M blunting of 20:4n-6 incorporation occurred because fish oil supplementation provided additional 20:4n-6. Tissue 20:4n-6 content appears to be diet-dependent. Accumulation of eicosapentaenoic acid (20:5n-3), but low affinity for docosahexaenoic acid (22:6n-3) occurred in diet M RBC membranes. After 28 days, plasma PLs reflect dietary intake more readily than RBC membranes. Fish oil supplementation resulted in plasma PL LCn-3FA enrichment and lowered plasma TG concentrations, both of which may have physiological significance in cats.

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CHAPTER I

INTRODUCTION

Previous research has elucidated some distinctive aspects of feline lipid metabolism; however, much is yet to be revealed about this unique species. The domestic feline, an obligate carnivore, relies on consumption of prey tissues to provide the essential fatty acids required for growth, maintenance, gestation, and lactation [1]. Like many other mammals, cats lack the ability to synthesize adequate 18:2n-6, an EFA [1]. Where they differ from other mammals, is in their apparent limited capacity to synthesize other longer chain EFAs from dietary precursors such as 18:2n-6. Thus, the domestic feline requires a dietary source of these longer chain EFAs, however small, and may be able to conserve tissue levels of these EFAs.

Since the 1970's, it has been understood that the domestic feline requires dietary long chain polyunsaturated fatty acids (LCPUFAs). Rivers et al. demonstrated that the feline liver lacks the Δ6 desaturase enzyme activity required to synthesize 20:4n-6 from 18:2n-6 [2]. Both Hassam et al. and Sinclair et al. confirmed this by using radiolabeled ^{14}C-18:2n-6, which demonstrated that less than 0.5% of the radioactivity from 18:2n-6 was incorporated into liver lipids containing four double bonds such as 20:4n-6 [3, 4]. As described by MacDonald et al., various studies demonstrated EFA deficiencies

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This thesis follows the style of Lipids.

stemming from the lack of Δ6 desaturase, even when sufficient 18:2n-6 was fed [5]. In one study, a purified diet with safflower oil as its sole fat source resulted in no net increase in 20:4n-6 in plasma, RBC, or liver lipids [6]. Reproduction was used as an indicator of 20:4n-6 deficiency in another study, such that when cats were fed diets containing 18:2n-6, but lacking 20:4n-6, they exhibited 20:4n-6 deficiency in the form of reproductive failure [5]. These same authors sought the minimum dietary requirement of 20:4n-6 to allow reproductive success, and found normal reproduction when the diet contained 4.8% 18:2n-6 and 0.04% 20:4n-6 (% dietary energy) [5]. Based on these findings, 20:4n-6 was considered to be essential for all feline life stages. Despite the essential nature of 20:4n-6, research using diets containing 18:2n-6 and deficient in 20:4n-6 determined that the feline does have low levels of Δ6 desaturase [5, 7, 8]. This finding was initially discovered due to the production of Mead's acid (20:3n-9) from 18:1n-9, presumably via Δ6 desaturase [7]. In 1994, the low, but present Δ6 desaturase activity of felines was confirmed using stable isotope techniques coupled with gas chromatography-mass spectrometry (GC-MS) [9]. Even though cats do have some Δ6 desaturase activity, it does not appear to be high enough to result in the synthesis of adequate amounts of 20:4n-6. Consequently, 20:4n-6 is a necessary component of all commercial feline diets approved by the Association of American Feed Control Officials (AAFCO). However, the recently published Nutrient Requirements of Dogs and Cats (NRDC) indicates that while 20:4n-6 is conditionally essential for growth and reproduction, some adult cats may synthesize adequate amounts for maintenance [10].

Because the activity of Δ6 desaturase is low in felines, feline lipid metabolism is unique among mammalian species. Hence, research performed in dogs, rats, or humans with regard to lipid metabolism does not necessarily reflect feline metabolism, so additional research in the feline lipid metabolism field is needed. The present study was performed to explore the effects of different sources of dietary fat on various parameters of feline lipid metabolism. Non-esterified fatty acids, TG, TC, LP-C distributions (i.e. LDL, VLDL, HDL), plasma PL and RBC membrane FA compositions, as well as desaturase indices were evaluated as indicators of feline lipid metabolism. Based on precedents established by previous studies in other species, the diets in the present study were enhanced with one of three oils: high-oleic sunflower oil, Menhaden fish oil, and safflower oil. Safflower oil provides large amounts of EFAs in the form of 18:2n-6. Menhaden fish oil is a rich source of LCn-3FAs. High-oleic sunflower oil is enriched in monounsaturated fatty acids (MUFA). One hypothesis of the present study was that high dietary 18:2n-6 (S diet) will enhance conversion to 20:4n-6 in comparison to cats fed a diet low in 18:2n-6 (H diet). Additional hypotheses were that high dietary LCn-3FA (M diet) will affect plasma lipid parameters, and n-3 FA incorporation may compete with n-6 FA incorporation. Potential health benefits from fish oil feeding are n-3 fatty acid suppression of 20:4n-6 derived eicosanoids, which may impart anti-inflammatory and anti-carcinogenic properties to n-3 fatty acids [11].

An n-6 rich diet with high 18:2n-6 should provide excess substrate to overcome the Michaelis-Menten constant (Km) for Δ6 desaturase, allowing for maximal product formation. Compared to the high MUFA diet, high dietary 18:2n-6 should provide some

insight into the ability of increased substrate to modulate Δ6 desaturase activity. Such a diet would be expected to result in the conversion of 18:2n-6 to its metabolites: γ-linolenic acid (18:3n-6), dihomo-γ-linolenic acid (20:3n-6, DGLA), 20:4n-6, docosatetraenoic acid (22:4n-6), and docosapentaenoic acid (22:5n-6). Also, diets enriched with 18:2n-6 lower cholesterol and LDL-cholesterol in humans; however, cats have not been studied in this regard [12].

Feeding an n-3 rich diet, such as with fish oil supplementation, one expects to see accumulation of the n-3 FAs with blunting of the n-6 series, as has been seen with dogs and humans [13, 14]. The effects of n-3 FAs or fish oil on TG in humans, rats, and to a lesser extent dogs have been documented [14, 15, 16]. Numerous studies have found fish oil supplementation to have a plasma TG lowering effect in humans and rats, although this was not the case in dogs [14, 15]. Stone et al. proposed that n-3 feeding in humans affects lipoprotein metabolism by inhibiting VLDL TG synthesis, enhancing VLDL turnover, and depressing LDL synthesis [16]. In humans, 22:6n-3 supplementation slightly enhanced TG clearance, and a reduction of n-6 long chain fatty acid (LCFA) metabolism from 18:2n-6 was found [17]. Lang et al. showed that fish oil feeding in rats impaired VLDL assembly, and subsequently VLDL cholesterol was lowered [18]. Feline plasma FA incorporation may behave similarly to that of humans, where 20:5n-3 is preferentially incorporated into cholesterol ester and PL fractions, and 22:6n-3 into PL and TG fractions [19].

Because LCPUFAs are important FAs for nervous and retinal tissue, the use of RBC membrane FAs, specifically 22:6n-3, as a marker for the composition of these

neural FAs is of great significance [20]. Makrides et al. demonstrated that in human infants over a 48 week feeding period, RBC 22:6n-3 may be a valid marker of neural 22:6n-3 [20]. A recent study by Arteburn et al. demonstrated that LCn-3FA incorporation into RBC does not reach equilibrium in a 4 week study, although plasma PL incorporation does so after feeding only 2-3 weeks [21].

Diets supplemented with high MUFA are expected to show significant incorporation of 18:1n-9 into the PL and RBC fractions. Due to high MUFA feeding, chain elongation metabolites of 18:1n-9 such as gondoic acid (20:1n-9), erucic acid (22:1n-9), and nervonic acid (24:1n-9) should also be increased. Other metabolites such as eicosadecenoic acid (20:2n-9), 20:3n-9, or docosatrienoic acid (22:3n-9) would require Δ6 desaturase for synthesis, so significant accumulation of these latter FAs would not be expected. Although plasma NEFA may not be affected by this diet, plasma TG might be increased similar to effects seen with high MUFA diets in rats [22]. In the present study, the H diet, because of its lower 18:2n-6 content, was used for comparison to the S diet, including its effects on resultant Δ6 and Δ5 desaturase indices.

In summary, the objectives of this study were twofold. First, a diet enriched in 18:2n-6 was fed in an effort to enhance conversion via Δ6 desaturation to 20:4n-6. Also, comparative aspects of feline lipid metabolism were investigated using a diet enriched in LCn-3FA to determine whether its feeding competes with n-6FA incorporation into plasma PL and RBCs. As such, novel data on feline RBC membrane FA compositions and the response of fish oil feeding on lipid parameters were obtained.

CHAPTER II

MATERIALS AND METHODS

Experimental Design. Thirty clinically-normal, sexually-intact, female cats aged 7-9 months were randomized into three groups of ten prior to the study. The protocol for this study was approved by the Texas A&M University Laboratory Institutional Animal Use Committee. The cats were housed at the Laboratory Animal Research and Resources (LARR) facility with veterinary care provided by its resident veterinarian and staff. During the study, the cats were fed by members of the Companion Animal Nutrition lab in the College of Veterinary Medicine and Biomedical Sciences. Prior to the study, the cats were fed Hill's Science Diet Kitten Original™ dry extruded diet with a caloric intake per the manufacturer's suggestion for adolescent growth. The fatty acid profile of this pre-experimental diet was determined (Appendix A, Table A-1). Body weight and body condition scores (BCS) were recorded weekly (Appendix A, Table A-2, A-3). The cats were individually housed during the study in order to monitor daily caloric intake (Appendix A, Table A-5). The three experimental diets were comprised of Nestle-Purina's Kit'n'Kaboodle™ dry extruded-type kibble, supplemented with one of three oils to an average total fat content of 16.8% as is (Appendix A, Table A-4). All diets were complete and balanced for all life stages by AAFCO standards. Protein, carbohydrate, fiber, vitamin, mineral, and moisture content of the diets remained consistent, as each diet group received these nutrients from the same kibble, Kit'n'Kaboodle™. The only nutrient in which these three diets differed

was fat type. High-oleic sunflower (Clear Valley®, Cargill, Minneapolis, MN), Menhaden fish (Virginia Prime Gold®, Omega Protein, Houston, TX), and safflower (Oilseeds International, San Francisco, CA) oils were supplemented to provide variation in the fat sources. To prepare the diets, the body weight of each cat was measured weekly. The following equation was used to determine daily caloric needs: Metabolizable energy (kcal) = 134*[Body Weight (kg)]$^{0.67}$. This equation is a derivation of the NRDC's recommended intake for maintenance, with the assumption that these juvenile cats were 80% grown [10]. Once the required caloric intake was determined, the amount of dry kibble plus oil was calculated at a rate of 8g oil/100g dry kibble diet. The cats were fed every morning at 8am, and were provided a fresh water source at all times. A five day transition period was used to gradually change the diet from Hill's Science Diet Kitten Original™ to Kit'n'Kaboodle™. On day zero, all cats were eating the Kit'n'Kaboodle™ diet. The experimental diets were fed for 28 days with blood collection occurring on days 0, 14, and 28.

Blood Sample Collection. Whole blood samples (7mL) were collected primarily from the saphenous vein via 21 or 23 gauge vacutainer, although in some cases jugular venipuncture with an 18 or 21 gauge needle was used. All blood samples were collected into tubes with EDTA (1.5 mg/ml) as the anti-coagulant. All samples were collected after withholding food for 12 hours. Blood samples were collected at 8am, and the cats were then fed between 9 and 10am on blood collection days. Fresh blood samples were immediately fractionated by slow speed centrifugation at 100 x g for 10 minutes. Lipoprotein electrophoresis was performed immediately on fresh plasma.

Plasma aliquots were stored at -20°C for TG, TC, plasma PL extraction, and NEFA analyses. Packed RBC's were kept on ice for immediate RBC membrane ghost preparation.

Plasma Non-Esterified Fatty Acids. The NEFA C method (ACS-ACOD Method) by Wako Chemicals USA, inc. was used as an in vitro enzymatic colorimetric assay to quantitatively measure the NEFA in the plasma samples. Four μL of deionized water was pipetted into the first well as a blank. A linear calibration curve was constructed by adding 4μL of each standard to 3 consecutive wells. The standard concentrations used were 0.50, 1.00, and 1.97 mEq/L. Four μL of sample was added to each well, and samples were measured in triplicate. Eighty μL of Color Reagent A was added to all wells. The plate was mixed using a microplate reader (Molecular Devices Corporation, Menlo Park, CA), and incubated at 37°C for 10 minutes after which, 160 μL of Color Reagent B was added and mixed. The samples were incubated for 10 minutes at 37°C. The plate was removed from the incubator and allowed to equilibrate to room temperature. The absorbance was measured at 550nm. Using the aforementioned calibration curve, the measured absorbance was used to calculate the concentration in mEq/L for each sample.

Plasma Total Cholesterol. Following the procedure described by Warnick, plasma TC concentrations were measured enzymatically using a microplate reader (Molecular Devices Corporation, Menlo Park, CA) and its software (SoftMax® Pro, Molecular Devices Corporation, Sunnyvale, CA) [23]. Six μL of sample plasma and 200 μL of TC reagent (described below) were pipetted into microplate wells and mixed by

the microplate reader. The plate was incubated at 37°C for 30 minutes, and then at room temperature for 30 minutes. The absorbance was read at 490 nm. A standard curve was created by combining 6 μL of calibrator standards (100, 200, 400 mg/dL) (Sigma Diagnostics, St. Louis, MO) with 200 μL of the TC reagent. All samples and standards were measured in triplicate, and deionized water was used as a blank.

Reagents were prepared as described by the following steps. PIPES buffer (50mM, pH 6.9) was prepared by mixing 17.3 g of disodium salt (Sigma-Aldrich, St. Louis, MO), 1.292 g of sodium cholate, 1 mL of Triton X-100 (Sigma-Aldrich, St. Louis, MO), and deionized water up to a total volume of 1 L. To make reagent A, 100 mL of PIPES buffer was mixed with 0.102 g of 4-aminoantipyrine and 1.492 g of KCl (Sigma-Aldrich, St. Louis, MO). Reagent B was prepared by adding 0.08 g of 2-hydroxy-3,5-dichlorobenzenesulfonic acid, sodium salt (Research Organics, Inc., Cleveland, OH) to 100 mL of PIPES buffer. Equal volumes reagent A and B were mixed with cholesterol esterase (0.5 U/ml), cholesterol oxidase (0.5 U/ml), and horseradish peroxidase (10 U/ml) (Sigma-Aldrich, St. Louis, MO) enzymes to form the TC reagent.

The TC reagent catalyzed the following reactions: the hydrolysis of EC to UC and FAs by cholesterol esterase; the oxidation of UC into cholest-4-en-3-one and H_2O_2 by cholesterol oxidase; and the synthesis of quinoneimine dye from 4-aminoantipyrine, 2-hydroxy-3,5-dichlorobenzensulfonic acid, and H_2O_2 by horseradish peroxidase. The red color produced by quinoneimine dye when measured at 490 nm allows for quantification of TC (mg/dL).

Plasma Triacylglycerol. Plasma TG concentrations were measured enzymatically using a microplate reader (Molecular Devices Corporation, Menlo Park, CA) and its software (SoftMax® Pro, Molecular Devices Corporation, Sunnyvale, CA). Six µL of sample plasma and 200 µL of triacylglycerol GPO reagent were pipetted into microplate wells and mixed by the microplate reader. The plate was incubated at 37°C for 20 minutes, and then at room temperature for 20 minutes. The absorbance was read at 490 nm. A standard curve was generated by combining 6 µL of calibrator standards (250, 500 mg/dL) (Sigma Diagnostics, St. Louis, MO) with 200 µL of the TG GPO reagent. All samples and standards were measured in triplicate, and deionized water was used as a blank.

Lipoprotein Electrophoresis. Lipoprotein distributions were determined by agarose gel electrophoresis, which separates plasma lipoproteins into α (high-density lipoproteins, HDL), pre-β (very-low-density lipoproteins, VLDL), and β (low-density, LDL) fractions. Titan Gel Lipoprotein Electrophoresis System (Helena Labs cat#3045) was used for the separation of the feline plasma. Thirty mL of diluted Titan Gel Lipoprotein Buffer was poured into the inner sections of the gel chamber. The chamber was then covered for 30 minutes until equilibrated. The gel was then blotted dry in the application area. The applicator template was placed on the blotted gel, and slits aligned to be even with the minus symbols on the sides of the gel. Two µL of sample plasma was added to the wells. All samples were run in duplicate. The excess sample was blotted away from the gel after 7 minutes, and the applicator template was removed. The gel was run in the electrophoresis chamber for 40 minutes at 90 volts. After

electrophoresis, the gel was dried in a convection oven at 70°C for 40-50 minutes. To visualize the bands, the gel was stained with Fat Red 7B for 2 minutes and then destained in 1:1 (v/v) MeOH:H_2O. The gel was dried at room temperature and kept away from light until analysis. Each gel was analyzed via densitometry on a Bio-Rad GS-700 Imaging Densitometer (Bio-Rad Laboratories, Inc., Hercules, California) with Quantity One software (Bio-Rad Laboratories, Inc., Hercules, California). Using the "stain all" setting for reflective light, the bands were evaluated for % adjusted volume and standard deviation. A calculation was made to transform lipoprotein percentage data into lipoprotein-cholesterol (LP-C) data using the TC data (Appendix B).

Plasma Phospholipid Fatty Acid Profile. Plasma samples were lipid-extracted by a modified Folch method, and the PL subfraction was isolated using thin-layer chromatography (TLC) [24]. The PL fraction was prepared for gas chromatography analysis by methylation in order to create PL fatty acid methyl esters (FAMEs).

Lipid Extraction. Five hundred μL plasma and 9 mL chloroform:methanol (2:1, v/v) with 0.2% acetic acid were combined into a large screw-top test tube. The samples were shaken for 20 minutes, and then 2 mL deionized water was added. Samples were shaken again for 10 minutes, and then centrifuged for 10 minutes at 1400 x g to separate the lipid fraction into the bottom layer. The bottom layer was transferred to a clean screw-top test tube. This bottom layer was then washed by adding 5 mL chloroform:methanol:water (3:48:47, v/v/v) and then shaken for 10 minutes. The samples were centrifuged for 10 minutes at 1400 x g. The bottom lipid-containing layer

was transferred to a clean screw-top test tube. Nitrogen gas was blown into the test tube to protect the samples from oxidation, and they were stored at -20°C overnight.

Thin-Layer Chromatography. Lipid-extracted plasma samples were evaporated to dryness under N_2 gas. The samples were reconstituted with 100 µL chloroform and mixed. The 20cm × 20cm silica gel G TLC plates (Analtech, 15 Newark, DE) were washed with chloroform:methanol (2:1, v/v) with 0.2% acetic acid and dried in a glassware dryer overnight. Fifty µL of sample was added in a continuous narrow band to the washed silica plates. Three samples were added to each individual plate. A 20 µL standard (#18-5A, Nu-Check Prep, Inc., Elysian, MN) was added to each plate for identification of the lipid subfractions. All plates were developed in a chamber containing hexane:ether:glacial acetic acid (80:20:1, v/v/v) until the solvent front reached 1 cm below the top of the plate. Once the plates were dry, the PL fraction at the origin was scraped into a small screw-top test tube. Two mL of 4% sulfuric acid in methanol was added to each tube and mixed.

FAME Preparation. The lipid-extracted plasma PL samples, in a 4% sulfuric acid with methanol solution, were then incubated in a 90°C water bath for 1 hour to allow formation of FAMEs. The samples were removed from the incubator and cooled to room temperature. Three mL of hexane was added to each sample and mixed. The samples were centrifuged at 1400 x g for 15 minutes. The top layer, which contained the FAMEs, was transferred to a clean test tube. Until analysis, the samples were stored under N_2 at -20°C.

Gas Chromatography. The FAMEs were evaporated under N_2 and reconstituted with 40 µL hexane. Twenty µL of sample was inserted into a gas chromatography (GC) vial. Samples were loaded on a Hewlett Packard 6890 Series Autosampler. Two µL of each sample was injected onto a FAMEWAX™ (Restek, Bellefonte, PA) fused silica capillary column (30 m long, 0.25 µm thickness, and 0.32 mmID) in a Hewlett Packard 5890 Series II Gas Chromatograph (Hewlett Packard Co., Palo Alto, CA) with a flame ionization detector. Helium was the carrier gas at an initial velocity of 28.3 cm/sec and flow of 1.25 ml/min. Oven temperature was initially held at 170°C for 10 min, then increased at a rate of 1.0°C/min until a temperature of 220°C and held at 220°C for 5 minutes. To remove remaining FAMEs and sample debris from the column, the temperature was increased 10°C/min to 250°C and held for 10 minutes after each run. Hewlett Packard ChemStation software package was used to produce results. Authentic FAMEs standards (#68-A, plus 17:0, 18:1n-7, 20:5n-3, and 22:4n-6, Nu-Check Prep, Inc., Elysian, MN) were used to identify individual FA peaks based on retention times.

Red Blood Cell Membrane Fatty Acid Profile. Red blood cell membranes were prepared using saline wash, lysis, and ultracentrifugation. They were then lipid-extracted and methylated to create the FAMEs for analysis via gas chromatography.

RBC Membrane Preparation. Whole blood samples were centrifuged at 1400 x g for 15 minutes to separate plasma from RBC. Plasma and the buffy coat, which contains neutrophils, were removed from the top of the packed RBCs. A 0.9% NaCl solution was mixed into the RBCs to wash them. The RBCs were centrifuged for 15 minutes at 1400 x g. The supernatant was removed and discarded. The RBC wash was repeated until the

supernatant was clear, which occurred after 2-3 wash cycles. The washed RBC's were transferred to a 5/8" x 3" thick-walled ultracentrifuge tube. Ice-cold deionized water was added to the tube until it was 2/3 full and then gently mixed to achieve RBC lyses. The samples were loaded into a Sorvall T-1270 rotor and centrifuged at 20,609 x g for 30 minutes at 4°C. The supernatant was removed and ice-cold deionized water was again added until the tube was 2/3 full. The samples were gently mixed and centrifuged again at 20,609 x g at 4°C for 30 minutes. The supernatant was clear after the samples were mixed with ice-cold deionized water and centrifuged at 20,609 x g after 4 repetitions. Supernatants were removed and discarded. Seven hundred μL of phosphate buffer, pH 7.2, was then added to the membrane pellet and mixed. Sample aliquots were separated and stored at -80°C until analysis.

Lipid Extraction. Lipid extraction of the RBC membranes was performed using the method previously described. Sample volumes of 400 μL were extracted for the RBC membranes.

FAME Preparation. RBC membrane total lipid extracts were methylated as described above.

Gas Chromatography. RBC membrane FAMEs were analyzed by gas chromatography as described above, but with 3μL of sample injected and a slower method. The oven temperature was initially held at 165°C for 12 min, then increased at a rate of 0.9°C/min to a temperature of 220°C and held for 5 minutes. To remove remaining FAMEs and sample debris from the column, the temperature was increased 8°C/min to 250°C and held for 10 minutes after each sample.

Highly-Unsaturated Fatty Acids (HUFA). HUFA are FAs with ≥ 20 carbons, and ≥ 3 double bonds. Plasma PL and RBC membrane FAME data were used to calculate n-3 and n-6 %HUFA scores (Appendix B).

Desaturase Indices. Δ6 and Δ5 desaturase indices were calculated using both the plasma PL and RBC membrane FAME data as detailed in Appendix B [1]. The ratio of 20:3n-6/18:2n-6 (DGLA/LA) was also calculated for comparison (Appendix B).

Statistical Analysis. Shapiro-Wilks tests were performed in SPSS version 13 (SPSS Inc, Chicago, Illinois) to determine if the data were normally distributed. Non-normal data was transformed using square root for weakly skewed data, logarithm for strongly skewed data, and reciprocal for exponential data. In cases with one extreme outlier, transformations were made to Winsorize that outlier so as to achieve a normal distribution. Non-normal data were analyzed with Kruskal-Wallis one-way ANOVA. Once normally distributed, the data were statistically analyzed by repeated measures ANOVA using SAS PROC MIXED, to test for main effects of time and diet as well as the interaction of time with diet (SAS Institute Inc, Cary, NC). Tukey-Kramer adjusted p-values of < 0.05 were considered significant for main effects of time, diet, and time x diet interactions. For data with significant interactions, differences of least square means were evaluated to locate where the significance occurred. For data where a significant interaction was not found but a time or diet effect was significant, differences of least means squares were used to determine where significant main effects of time and/or diet occurred. All data with a Tukey-Kramer adjusted p-value of < 0.05 were considered significant.

CHAPTER III

RESULTS

Animals and Diets. Food intake was monitored daily to ensure the experimental diets were well tolerated. Weekly body weights and body condition scores were recorded in order to maintain consistent body weights and body conditions throughout the study (Appendix A-3, A-4). During the study, one of the cats assigned to the H diet group developed symptoms consistent with calici virus and was subsequently quarantined and removed from the study.

Plasma Non-Esterified Fatty Acids. No time, diet, or time x diet interactions were seen with plasma NEFA concentrations (Table 1).

Table 1 Repeated measures ANOVA of feline lipid parameters

Parameter	Effect		
	Diet	Time	Interaction
NEFA	0.2428	0.2957	0.6692
TG	0.0012	0.0798	0.0012
α LP-C	0.7866	0.0075	0.4836
β LP-C	0.6666	0.7713	0.1748
pre-β LP-C	0.0499	<.0001	0.1840

Values given are p-values of lipid parameters from statistical analysis by PROC MIXED. $p < 0.05$ was considered significant.

Plasma Total Cholesterol. No significant time, diet, or time x diet interactions were found in plasma TC concentrations (Appendix, Table A-5).

Plasma Triacylglycerol. Plasma TG concentrations showed statistically significant time x diet interactions as well as a main effect of diet (Table 1). These interactions included a statistically significant TG lowering effect with diet M compared to diets H and S on both day 14 and day 28 (Table 2). It should be noted that diet H group cats had significantly higher TG concentrations on day 0 compared with the cats fed either diet M or diet S. Also, diet H TG concentrations were significantly lower on day 14 than day 0, but returned to the initial values on day 28. While Diet M resulted in TG lowering, diet S TG concentrations significantly increased between days 0 and 28.

Plasma Lipoprotein-Cholesterol. Concentrations of α LP-C exhibited a main time effect with the concentration increased on day 14 but decreased on day 28 (Table 1). This was the only effect found for α LP-C, as the main effects of diet and time x diet interactions were not significant (Table 1). Also, β LP-C concentrations showed no differences due to time, diet, or time x diet interaction (Table 1). Although α LP-C and β LP-C showed little or no significant effects, pre-β LP-C showed several significant differences. Significant main effects of time and diet were both found in pre-β LP-C (Table 1). The time effect for pre-β LP-C showed that day 14 concentrations were decreased relative to concentrations on days 0 and 28. In addition, a diet effect for pre-β LP-C revealed that the M diet group concentrations were decreased relative to the H diet group.

Table 2 Concentration of feline lipid parameters

	Diet H (n=9)			Diet M (n=10)			Diet S (n=10)		
	Day 0	Day 14	Day 28	Day 0	Day 14	Day 28	Day 0	Day 14	Day 28
NEFA (mEq/L)	0.56±0.10	0.56±0.05	0.67±0.06	0.61±0.08	0.46±0.08	0.56±0.05	0.65±0.08	0.63±0.05	0.66±0.06
TG (mg/dL)	26.65±1.37^{a1}	22.39±1.35^{b1}	25.54±1.70^{ab1}	21.34±1.77^{a2}	14.04±0.75^{b2}	17.06±1.22^{b2}	20.09±1.45^{a2}	23.40±1.50^{ab1}	24.94±2.04^{b1}
α LP-C (mg/dL)	72.39±11.69	80.73±11.06	68.13±9.70	58.70±3.95	81.86±2.75	70.35±3.01	63.62±6.00	73.52±4.27	68.63±4.36
β LP-C (mg/dL)	28.94±2.80	23.07±2.46	24.06±3.41	24.40±2.59	27.88±1.27	27.27±1.00	24.63±2.69	23.57±1.23	25.41±2.02
pre-β LP-C (mg/dL)	27.35±3.07	19.98±3.59	25.17±3.35	22.25±1.53	8.26±3.39	17.40±2.07	23.24±1.53	17.15±3.39	25.26±2.07

Values given are mean ± SEM.

Superscript letters not in common in a row within a diet group indicate a significant difference between days by time x diet from PROC MIXED, $p < 0.05$.

Superscript numbers not in common in a row within a day indicate a significant difference between diets by time x diet from PROC MIXED, $p < 0.05$.

Plasma Phospholipid Fatty Acid Profile. All fatty acids were individually tested for effects of time, diet, and the time x diet interaction with a significance level set at $p < 0.05$ (Table 3).

Only one difference was observed between groups in the baseline relative percentages of individual FAs in the plasma PL fraction (Table 4). The one FA that was not similar between diet groups on day 0 was docosatetraenoic acid (22:4n-6). On day 0, the M diet group 22:4n-6 was unexplainably higher than the relative percentage of that FA in diets H or M.

Time x Diet Interactions. As expected, many diet effects were seen within subsequent time points for plasma PL FAs (Table 4). On day 14, stearic acid (18:0) in the S diet was significantly increased relative to diet M, and diet H 18:0 was similar to both. Also on day 14, diet H 18:1n-9 was increased relative to both diets M and S. Similarly, 20:5n-3, 22:6n-3, and 24:1n-9 relative percentages were increased in diet M relative to both diets H and S. Conversely, on day 14, 18:2n-6 was decreased in the M diet relative to diet S, and diet H was similar to both. Also, 20:3n-6 was decreased in the S diet relative to diet H, with the M diet group similar to both diets H and S.

Further differences were seen between diet groups on day 28 (Table 4). Similar to the change seen on day 14, 18:1n-9 was increased in diet H compared with both diets M and S on day 28. Also, margaric acid (17:0), 20:5n-3, and 22:6n-3 were increased on day 28 in the M diet group relative to the H and S diets. Another FA, 20:2n-6, increased on day 28 in the S diet group relative to the M and S diets. On day 28, 18:2n-6 relative percentages were significantly different across all three diets, described by S > H > M.

Table 3 Repeated measures ANOVA of feline plasma phospholipid fatty acid composition

Fatty Acid	Effect		
	Diet	Time	Interaction
14:0	0.6052	0.0044	0.8929
16:0	0.9347	<.0001	0.2562
16:1n-7	0.326	0.0146	0.5983
17:0	0.0005	<.0001	0.0054
17:1	0.005	0.0007	0.1771
18:0	0.3901	<.0001	0.0401
18:1n-9	<.0001	0.0108	0.0014
18:1n-7	0.0051	0.422	0.9837
18:2n-6	<.0001	0.0005	<.0001
18:3n-3	0.2647	0.0197	0.2438
20:1n-9	0.7553	0.0194	0.8151
20:2n-6	0.0048	0.4603	0.0002
20:3n-6	0.0734	<.0001	0.003
20:4n-6	0.1774	<.0001	0.0073
20:5n-3	<.0001	<.0001	<.0001
22:1n-9	0.4097	0.0326	0.4103
22:4n-6	0.0063	0.0478	0.0144
22:5n-3	0.0245	<.0001	0.1006
22:6n-3	<.0001	0.1365	<.0001
24:0	0.1835	0.0073	0.6806
24:1n-9	<.0001	<.0001	0.0075

Values given are p-values of fatty acids with at least one significant effect.

$p < 0.05$ was considered significant.

Numerous time effects within each diet group were found in the plasma PL fraction (Table 4). In the diet H group, 18:1n-9 was increased on day 28, and on day 14 was similar to both days 0 and 28. Similarly, 24:1n-9 in diet H was increased on day 14, and day 28 was similar to both days 0 and 14. Also, diet M exhibited increased 17:0, 20:5n-3, and 22:6n-3 on day 14, and on day 28 values remained elevated. Conversely, both 18:2n-6 and 22:4n-6 decreased significantly on day 14, and day 28 values were similar to both days 0 and 14. In the diet S group, 18:0 was increased on day 14, and

Table 4 Feline plasma phospholipid fatty acid composition (relative %)

Fatty Acid	Diet H (n=9)			Diet M (n=10)			Diet S (n=10)		
	Day 0	Day 14	Day 28	Day 0	Day 14	Day 28	Day 0	Day 14	Day 28
14:0	0.10±0.02	0.31±0.18	0.10±0.01	0.08±0.01	0.41±0.17	0.15±0.01	0.08±0.01	0.24±0.12	0.09±0.01
14:1n-5	0.16±0.16	0.18±0.18	ND	ND	0.14±0.09	ND	ND	ND	ND
16:0	11.94±0.74	14.55±1.08	11.98±1.11	11.78±0.63	13.75±1.03	12.93±0.28	10.68±0.31	15.80±1.39	11.37±0.47
16:1n-7	0.21±0.05	0.40±.0.16	0.22±0.04	0.21±0.02	0.59±0.18	0.32±0.02	0.21±0.02	0.30±0.15	0.22±0.02
17:0	0.60±0.02	0.75±0.08	0.60±0.06^{1}	0.60±0.02^{a}	0.97±0.12^{b}	1.04±0.05^{b2}	0.53±0.02	0.74±0.08	0.59±0.04^{1}
17:1n-9	0.26±0.05	ND	0.05±0.04	0.27±0.05	0.23±0.07	0.17±0.05	0.31±0.05	0.12±0.04	0.23±0.05
18:0	29.28±0.62	35.92±2.07^{12}	30.17±1.76	30.74±1.53	32.55±1.20^{2}	32.54±0.95	30.23±0.57^{a}	39.65±2.95^{b1}	30.21±0.49^{a}
18:1n-9	9.65±0.49^{a}	10.76±1.32^{ab1}	12.78±1.05^{b1}	8.60±0.50	5.87±0.24^{2}	6.41±0.16^{2}	8.06±0.35	5.22±0.63^{2}	6.90±0.21^{2}
18:1n-7	2.34±0.15	2.52±0.15	2.71±0.43	2.40±0.22	2.48±0.19	2.56±0.07	1.96±0.18	1.96±0.18	2.11±0.12
18:2n-6	20.15±0.88	16.79±2.08^{12}	21.94±1.62^{1}	18.18±1.42^{a}	9.72±0.87^{b1}	10.09±0.24^{ab2}	19.31±0.56^{a}	18.23±3.58^{a2}	30.61±0.70^{b3}
18:3n-3	0.27±0.04	0.34±0.15	0.18±.0.02	0.26±0.03	0.29±0.10	0.08±0.03	0.25±0.02	0.12±0.04	0.14±0.02
20:0	0.75±0.03	1.08±0.18	0.78±0.07	0.94±0.08	1.05±0.11	0.96±0.04	0.83±0.05	0.82±0.10	0.85±0.03
20:1n-9	0.20±0.04	0.45±0.14	0.29±0.05	0.20±0.04	0.72±0.32	0.27±0.04	0.21±0.01	0.46±0.27	0.24±0.02
20:2n-6	0.57±0.08	0.57±0.13	0.49±0.07^{1}	0.57±0.05	0.41±0.09	0.27±0.01^{1}	0.58±0.02^{a}	0.53±0.08^{a}	0.90±0.08^{b2}
20:3n-6	0.82±0.06	0.73±0.11^{1}	0.66±0.04	0.82±0.07^{a}	0.56±0.09^{ab12}	0.45±0.02^{b}	0.95±0.04^{a}	0.38±0.07^{b2}	0.46±0.04^{b}
20:4n-6	8.35±0.40	5.70±0.76	6.32±0.51	8.91±0.64	6.94±0.72	7.13±0.23	10.21±0.72^{a}	4.50±0.94^{b}	5.08±0.36^{b}
20:3n-3	ND	0.16±0.00	ND	ND	0.10±0.00	ND	ND	ND	ND
20:5n-3	1.80±0.66	1.15±0.30^{1}	1.00±0.24^{1}	1.93±0.65^{a}	8.51±0.30^{b2}	9.12±0.24^{b2}	1.94±0.64	0.90±0.31^{1}	0.69±0.22^{1}
22:0	0.75±0.30	0.97±0.24	0.80±0.21	0.90±0.27	0.83±0.22	0.63±0.20	0.87±0.28	0.74±0.24	0.63±0.20
22:1n-9	ND	0.17±0.00	ND	ND	0.20±0.00	ND	0.01±0.00	ND	ND
22:4n-6	0.52±0.21^{1}	0.49±0.20	0.74±0.25	1.10±0.18^{a2}	0.66±0.18^{b}	0.74±0.24^{ab}	0.64±0.19^{1}	0.61±0.21	0.73±0.24
22:5n-3	1.06±0.46	0.60±0.17	0.69±0.25	1.27±0.43	0.98±0.14	0.97±0.24	1.35±0.42	0.51±0.14	0.74±0.23
22:6n-3	3.87±1.69	2.00±0.43^{1}	2.94±0.27^{1}	4.30±1.61^{a}	7.30±0.79^{b2}	7.70±0.59^{b2}	5.12±1.61^{a}	2.27±0.39^{b1}	2.83±0.16^{b1}
24:0	1.00±0.10	0.69±0.35	0.89±0.04	1.25±0.09	0.93±0.20	0.85±0.02	1.14±0.06	0.53±0.17	0.76±0.03
24:1n-9	1.78±0.19^{a}	0.66±0.20^{b1}	1.17±0.06^{ab1}	2.21±0.13	1.87±0.18^{2}	2.27±0.04^{2}	2.15±0.13^{a}	0.81±0.21^{b1}	1.48±0.09^{c1}
Unidentified	3.57	2.05	2.51	2.52	1.95	2.36	2.37	4.57	2.12
SFA	44.42±1.11	54.27±3.47	45.32±2.75	46.29±2.12	50.49±2.38	49.10±1.16	44.36±0.78	58.52±4.47	44.50±0.37
MUFA	14.60±0.52	15.14±1.01^{1}	17.22±0.73^{1}	13.89±0.67	12.10±0.53^{2}	12.00±0.24^{2}	12.91±0.44^{a}	8.87±0.63^{b3}	11.18±0.35^{ab2}
PUFA	37.41±1.58	28.54±2.58	34.95±2.40	37.30±2.23	35.46±2.55	36.54±1.09	40.36±0.97	28.04±4.75	42.20±0.32

Values given are mean ± SEM

ND = not detected.

Superscript letters not in common in a row within a diet group indicate a significant difference between days by time x diet from PROC MIXED, $p < 0.05$.

Superscript numbers not in common in a row within a day indicate a significant difference between diets by time x diet from PROC MIXED, $p < 0.05$.

decreased on day 28 such that days 0 and 28 were similar. Both 18:2n-6 and 20:2n-6 in the S diet were significantly increased on day 28. Also, 20:3n-6, 20:4n-6, and 22:6n-3 in diet S were all decreased on day 14 and remained low on day 28. For diet S, 24:1n-9 was decreased on day 14 and increased on day 28 relative to day 0, with values described by day 0 > day 28 > day 14.

Main Time Effects. For plasma PL FAs in which significant time x diet interactions were not found, many main effects of time were found (Table 3). Day 0 heptadecenoic acid (17:1) was significantly higher than both days 14 and 28. Similarly, linolenic acid (18:3n-3) on day 0 was increased relative to day 28, and day 14 was similar to both days 0 and 28. Day 0 docosapentaenoic acid (22:5n-3) was significantly higher than both other days. Also, lignoceric acid (24:0) on day 0 was increased relative to day 14 with day 28 similar to both days 0 and 14. On day 14, relative percentages of myristic acid (14:0) and palmitic acid (16:0) were significantly increased over days 0 and 28. Also, day 14 values of palmitoleic acid (16:1n-7) were significantly increased relative to day 0, and day 28 was similar to both days 0 and 14. Similarly, on day 14, 20:1n-9 and 22:1n-9 were increased, and 20:1n-9 on day 28 was similar to both days 0 and 14, but 22:1n-9 on day 14 was significantly higher than both other days.

Main Diet Effects. A few significant main effects of diet were found in those FAs without significant time x diet interactions (Table 3). Diet H was significantly depressed relative to diets M and S for 17:1. Similarly, the S diet showed lower relative percentages than diets H or M for vaccenic acid (18:1n-7). Also, 22:5n-3 was increased in diet M relative to diet H, and diet S was similar to both.

Red Blood Cell Membrane Fatty Acid Profile. All fatty acids were individually tested for effects of time, diet, and the time x diet interaction with a significance level set as $p < 0.05$ (Table 5). No differences were found in the relative percentages of individual FAs in the RBC membrane fraction between diet groups at baseline (Table 6).

Table 5 Repeated measures ANOVA of feline red blood cell membrane fatty acid composition

Fatty Acid	Effect		
	Diet	Time	Interaction
14:0	0.0147	0.0286	0.8423
16:1n-7	0.0060	0.0058	0.9947
17:0	0.2399	0.0007	0.9418
17:1	0.9494	<.0001	0.2732
18:1isomers	<.0001	0.0016	<.0001
18:2n-6	0.0018	0.1991	0.0578
18:3n-3	0.7758	0.0241	0.4006
20:2n-6	0.0001	0.0228	0.0001
20:4n-6	0.3144	0.0163	0.6090
20:3n-3	0.2835	0.0063	0.8767
20:5n-3	<.0001	0.0345	0.0004
22:0	0.9482	0.0013	0.3499
22:1n-9	0.3084	0.0021	0.3398
22:4n-6	0.8593	0.0159	0.6310
22:5n-3	0.1062	0.1354	0.0115
22:6n-3	0.3147	0.0124	0.2576
24:0	0.4485	0.0045	0.0512

Values given are p-values of fatty acids with at least one significant effect.

$p < 0.05$ was considered significant.

Time x Diet Interactions. Diet effects within subsequent time points were found for several of the RBC membrane FAs (Table 6). As early as day 14, 18:1 isomers

Table 6 Feline red blood cell membrane fatty acid composition (relative %)

Fatty Acid	Diet H (n=9)			Diet M (n=10)			Diet S (n=10)		
	Day 0	Day 14	Day 28	Day 0	Day 14	Day 28	Day 0	Day 14	Day 28
14:0	0.12±0.04	0.08±0.04	0.07±0.05	0.27±0.08	0.15±0.05	0.10±0.04	0.13±0.03	0.06±0.03	0.03±0.03
14:1n-5	0.02±0.02	ND	ND	0.04±0.03	ND	0.01±0.01	ND	0.01±0.01	ND
16:0	18.20±1.17	18.22±2.23	18.60±1.68	22.00±2.12	17.12±1.72	18.77±1.50	18.96±1.09	17.32±1.28	17.41±1.99
16:1n-7	0.24±0.05	0.10±0.04	0.10±0.05	0.32±0.08	0.21±0.06	0.17±0.06	0.15±0.04	0.06±0.04	0.03±0.03
17:0	0.59±0.05	0.13±0.09	0.09±0.08	1.05±0.40	0.39±0.13	0.16±0.11	1.00±0.41	0.34±0.16	0.20±0.14
17:1n-9	0.31±0.04	0.01±0.01	0.01±0.01	0.21±0.06	0.05±0.03	0.09±0.07	0.26±0.07	0.03±0.02	0.02±0.02
18:0	22.85±1.00	24.86±1.77	26.23±1.61	25.65±1.55	24.45±1.69	28.20±2.01	25.62±1.42	25.05±1.05	26.50±1.79
18:1iso	9.41±0.32^{a}	11.16±0.43^{a1}	13.38±0.58^{b1}	7.94±0.67	8.35±0.56^{2}	8.66±0.40^{2}	9.16±0.40	9.31±0.29^{12}	8.35±0.27^{2}
18:2n-6	16.58±0.60	12.86±1.16	10.48±1.80	10.42±2.04	9.17±1.14	7.83±1.45	12.14±1.04	15.03±1.00	14.54±2.22
18:3n-3	0.30±0.05	0.19±0.07	0.05±0.03	0.19±0.04	0.18±0.06	0.06±0.03	0.14±0.04	0.39±0.23	0.04±0.04
20:0	0.71±0.06	0.86±0.14	0.99±0.15	1.41±0.59	0.83±0.08	0.86±0.17	2.52±1.17	1.27±0.29	1.10±0.18
20:1n-9	0.83±0.09	0.60±0.12	0.64±0.12	1.06±0.26	0.62±0.06	0.33±0.07	0.84±0.27	0.70±0.18	0.70±0.21
20:2n-6	1.04±0.04	0.93±0.14^{12}	0.85±0.09^{1}	0.82±0.09^{a}	0.85±0.07^{a1}	0.38±0.11^{b2}	0.83±0.07^{a}	1.30±0.10^{b2}	1.23±0.10^{b1}
20:3n-6	0.75±0.06	0.55±0.12	0.40±0.11	0.49±0.06	0.55±0.07	0.65±0.17	0.61±0.05	0.58±0.09	0.33±0.15
20:4n-6	14.86±1.05	13.98±1.54	9.99±1.56	10.26±1.93	12.11±1.69	9.71±1.84	12.56±0.82	12.64±1.07	10.11±1.43
20:3n-3	0.05±0.04	0.33±0.23	0.92±0.36	0.25±0.15	0.52±0.32	0.83±0.39	0.10±0.05	0.06±0.05	0.54±0.22
20:5n-3	2.52±0.22	2.64±0.35^{1}	1.81±0.21^{1}	2.27±0.24^{a}	5.63±0.86^{b2}	6.46±1.37^{b2}	1.96±0.17	1.68±0.19^{1}	1.77±0.33^{1}
22:0	1.01±0.06	1.48±0.23	1.38±0.15	1.14±0.14	1.50±0.10	1.34±0.12	0.88±0.17	1.34±0.19	1.68±0.33
22:1n-9	0.17±0.05	0.04±0.04	0.09±0.05	0.45±0.22	0.11±0.06	0.06±0.06	0.20±0.08	0.08±0.03	ND
22:4n-6	1.04±0.13	1.51±0.35	1.06±0.21	1.04±0.18	1.48±0.21	1.06±0.17	1.01±0.07	1.42±0.20	1.44±0.21
22:5n-3	0.42±0.06	0.42±0.10	0.23±0.10^{12}	0.23±0.05^{a}	0.58±0.08^{ab}	0.67±0.18^{b2}	0.31±0.05	0.39±0.08	0.20±0.10^{1}
22:6n-3	2.32±0.37	3.12±0.73	2.49±0.47	2.01±0.11	3.30±0.51	3.86±0.53	2.04±0.15	2.53±0.43	2.63±0.45
24:0	2.06±0.18	3.36±0.62	3.03±0.64	2.60±0.26	3.93±0.66	3.78±0.44	2.63±0.31	2.22±0.61	4.54±0.64
Unidentified	3.60	2.57	7.11	7.88	7.92	5.96	5.95	6.19	6.61
SFA	45.54±1.87	48.99±3.29	50.39±3.12	54.12±3.53	48.37±3.53	53.21±3.43	51.74±2.35	47.60±2.04	51.46±3.53
MUFA	10.98±0.33^{a}	11.91±0.41^{a1}	14.22±0.61^{b1}	10.01±0.52	9.34±0.58^{2}	9.32±0.40^{2}	10.61±0.37	10.19±0.35^{12}	9.10±0.30^{2}
PUFA	39.88±2.01	36.53±3.48	28.28±3.60	27.99±4.29	34.37±3.94	31.50±4.56	31.70±2.12	36.02±2.46	32.83±4.06

Values given are mean ± SEM.

ND = not detected.

Superscript letters not in common in a row within a diet group indicate a significant difference between days by time x diet from PROC MIXED, $p < 0.05$.

Superscript numbers not in common in a row within a day indicate a significant difference between diets by time x diet from PROC MIXED, $p < 0.05$.

(18:1n-7 + 18:1n-9) were increased in diet H relative to diet M, and diet S was similar to both. Also, on day 14, diet S increased relative to diet M, and diet H was similar to both. Similarly, 20:5n-3 was increased in the M diet group relative to both diets H and S.

On day 28, further differences between diet groups were found (Table 6). Relative percentages of 18:1 isomers were increased in the H diet group on day 28 relative to both other diets. Similarly, day 28 20:5n-3 was increased in the M diet relative to the H and S diets. Diet M also increased 22:5n-3 on day 28 relative to diet S, and diet H was similar to both. Conversely, day 28 20:2n-6 was decreased in the M diet relative to both diets H and S.

Several fatty acids exhibited significant time effects within each diet group (Table 6). In cats fed diet H, relative percentages of 18:1 isomers were significantly increased by day 28. Similarly, diet M exhibited increased 20:5n-3 on day 28. Also, 22:5n-3 in the M diet was increased on day 28, and day 14 values were similar to both days 0 and 28. The opposite effect was found for 20:2n-6 in the M diet. Relative percentages of this FA were decreased by day 28. Also, the S diet showed significantly increased 20:2n-6 by day 14 and the value remained elevated on day 28.

Main Time Effects. As expected with the RBC membrane FAs, many main effects of time were found (Table 5). For those FAs without significant time x diet interaction, significant main effects of time were evaluated. Both 18:3n-3 and 20:4n-6 on day 14 were increased relative to day 28 with day 0 similar to both day 14 and 28. Also on day 14, 22:4n-6 was increased relative to day 0, and day 28 was similar to both. In addition, both eicosatrienoic acid (20:3n-3) and 24:0 were increased on day 28

relative to day 0, and day 14 was similar to both days 0 and 28. The relative percentages of docosanoic acid (22:0) and 22:6n-3 were both increased on days 14 and 28 relative to day 0. On both days 14 and 28, 14:0 was significantly decreased relative to day 0. The relative percentages of 16:1n-7, 17:0, 17:1, and 22:1n-9 were all decreased on days 14 and 28 relative to day 0.

Main Diet Effects. A few main effects of diet were found in RBC membrane FAs (Table 5). Interactions were not significant for these FAs, so the main effect of diet was evaluated. Both 14:0 and 16:1n-7 exhibited increased relative percentages in diet M when compared to diet S, and diet H was similar to both diets M and S. Conversely, diet M was decreased relative to both diet H and S for 18:2n-6.

Gas Chromatography-Mass Spectrometry. Because some of the RBC membrane chromatograms showed possible co-elution of peaks at the 24:1n-9 retention time, those samples were analyzed via GC-MS. Samples were analyzed in the Mass Spectrometry Facility of the Department of Chemistry and Biochemistry at the University of Texas at Austin. Analyzing several RBC membrane samples with the known problem at 24:1n-9, by GC-MS, showed co-elution of both 24:1n-9 FAME and an unknown lipid-soluble contaminant. Therefore, the peak initially identified, by retention time, as 24:1n-9 was excluded from the RBC membrane data set as it was 24:1n-9 mixed with some unknown component.

Plasma Phospholipid HUFA Composition. The HUFAs measured in this study were: 20:3n-6, 20:4n-6, 20:3n-3, 20:5n-3, 22:4n-6, 22:5n-3, and 22:6n-3. These data were individually tested for effects of time, diet, and the time x diet interaction with a

significance level set as $p < 0.05$ (Table 7). For comparison purposes, n-3 and n-6 HUFA as a percentage of total HUFAs were calculated (Table 8).

Diet effects within subsequent time points were found for both %n-3HUFA and %n-6HUFA (Table 8). On both days 14 and 28, the %n-3HUFA increased in diet M relative to diets H and S. Also, the %n-6HUFA showed a significant decrease in the M diet group on day 14 relative to diets H and S.

No time effects within each diet group were found in diet H or diet S for the %n-3HUFA or %n-6HUFAs (Table 8). However, diet M showed a significant increase of %n-3HUFA on day 14 and that increase was maintained on day 28. The %n-6HUFA in the M diet group decreased on day 14 and maintained that decrease on day 28.

Table 7 Repeated measures ANOVA of feline %HUFA

%HUFA	Effect		
	Diet	Time	Interaction
PLn6HUFA	<.0001	0.0020	<.0001
PLn3HUFA	<.0001	0.0020	<.0001
RBCn6HUFA	<.0001	0.0003	0.5045
RBCn3HUFA	<.0001	0.0003	0.5045

Values given are p-values of %HUFA with at least one significant effect.
$p < 0.05$ was considered significant.

Red Blood Cell Membrane HUFA Composition. Previously defined %HUFA calculations were also performed on the RBC membrane FA composition data. The n-3 and n-6 %HUFAs were individually tested for effects of time, diet, and the

Table 8 Plasma PL and RBC membrane n-3 and n-6 HUFA as a percentage of total HUFA (%)

Fatty Acid	Diet H (n=9)			Diet M (n=10)			Diet S (n=10)		
	Day 0	Day 14	Day 28	Day 0	Day 14	Day 28	Day 0	Day 14	Day 28
PLn-3HUFA	40.55±1.62	33.33±5.05[1]	37.44±1.37[1]	41.18±1.07[a]	67.98±1.47[b2]	67.75±1.43[b2]	41.77±1.21	41.72±5.38[1]	40.52±1.30[1]
PLn-6HUFA	59.45±1.62	66.67±5.05[1]	62.46±1.37[1]	58.82±1.07[a]	32.02±1.47[b2]	32.25±1.43[b2]	58.23±1.21	58.28±5.38[1]	59.48±1.30[1]
RBCn-3HUFA	23.95±1.18	29.77±1.87	34.47±5.11	34.82±6.04	43.12±2.60	53.30±3.25	23.81±1.16	23.57±1.91	31.51±2.65
RBCn-6HUFA	76.05±1.18	70.23±1.87	65.53±5.11	65.18±6.04	56.88±2.60	46.70±3.25	76.19±1.16	76.43±1.91	68.49±2.65

Values given are mean ± SEM.

Superscript letters not in common in a row within a diet group indicate a significant difference between days by time x diet from PROC MIXED, $p < 0.05$.

Superscript numbers not in common in a row within a day indicate a significant difference between diets by time x diet from PROC MIXED, $p < 0.05$.

time x diet interaction with a significance level set as $p < 0.05$ (Table 7).

Neither the RBC membrane %n-6HUFA nor %n-3HUFA exhibited significant time x diet interactions (Tables 7, 8). However, both showed significant main effects of time in which the %n-6HUFA was decreased on day 28 and the %n-3HUFA was increased on day 28 relative to both days 0 and 14 (Tables 7, 8). A significant main effect of diet was found in which the M diet exhibited significantly lower values than those seen in diets H and S for %n-6HUFA and higher for %n-3HUFA (Table 7,8).

Plasma Phospholipid Fatty Acid Type Distribution. Fatty acids from the plasma PL fraction were grouped to reflect fatty acid type distributions. Total saturated fatty acids (SFA), MUFA, and polyunsaturated fatty acids (PUFA) were calculated (Table 3).

No significant differences between diets were found on day 0 (Table 9). However, several interactions were found on day 14 with plasma MUFA. On day 14, the plasma MUFA of all three diets were significantly different from one another, and described by H > M > S. By day 28, plasma MUFA in diet H was increased relative to diets M and S. The S diet plasma MUFA was decreased on day 14 relative to day 0, and day 28 was similar to both days 0 and 14.

Some main effects of time for the plasma PL fatty acid type distributions were found (Table 9). Plasma SFA was increased on day 14 relative to both days 0 and 28. Also, on day 14, plasma PUFA were decreased relative to days 0 and 28.

Table 9 Repeated measures ANOVA of feline fatty acid type distribution

FA Type	Effect		
	Diet	Time	Interaction
PL SFA	0.8144	<.0001	0.1482
PL MUFA	<.0001	0.0006	0.0001
PL PUFA	0.1463	0.0006	0.1047
RBC SFA	0.4837	0.3278	0.5412
RBC MUFA	<.0001	0.3822	<.0001
RBC PUFA	0.5738	0.1940	0.1831

Values given are p-values of SFA, MUFA, PUFA for plasma PL or RBC membrane lipids.

$p < 0.05$ was considered significant.

RBC Membrane Fatty Acid Type Distribution. Fatty acids from the RBC membrane were grouped to reflect fatty acid type distributions. Total RBC SFA, RBC MUFA, and RBC PUFA were calculated (Table 6).

No significant differences between diets were found on day 0 (Table 9). However, on day 14, RBC MUFA was increased in diet H relative to diet M, and diet S was similar to both. Similarly, on day 28, diet H RBC MUFA was increased relative to both diets M and S (Table 6).

Plasma Phospholipid and Red Blood Cell Membrane Correlations. Correlation graphs with regression calculations were performed to compare plasma PL to RBC membranes for select FAs and %HUFAs (Table 10). For the following FAs correlations (r^2) were assessed and found not to be significant ($p > 0.05$ and $r^2 < 0.5$): 18:0, 18:2n-6, 18:3n-3, 20:3n-6, 20:4n-6, and 22:4n-6. Across all diets on day 28 the following FAs showed statistically significant correlations: 20:2n-6, 20:5n-3, 22:5n-3, and 22:6n-3. The most significant correlations were found in the S diet on day 28 for

20:2n-6 with an r^2 value of 0.612, and for 22:4n-6 with an r^2 of 0.529. Correlations between plasma PL %HUFA and RBC membrane %HUFA revealed statistically significant correlations with an r^2 of 0.408.

Table 10 Fatty acid and %HUFA correlations of plasma PLs to RBC membranes

FA/Score	r^2	p-value
18:0	0.004	0.734
18:2n6	0.099	0.097
18:3n3	0.045	0.268
20:2n6	0.489	<.0001
S diet 20:2n-6	0.612	0.007
20:3n6	<.0001	0.915
20:4n6	<.0001	0.957
20:5n3	0.548	<.0001
22:4n6	0.024	0.421
S diet 22:4n-6	0.529	0.017
22:5n3	0.215	0.011
22:6n3	0.235	0.008
%n-3HUFA	0.408	<.0001
%n-6HUFA	0.408	<.0001

Values given are r^2 coefficients and p-values for the correlation between plasma PLs and RBC membranes on day 28 across all diets for various FAs and %HUFA scores. Two FAs showed significant effects within their diet group: 20:2n-6, and 22:4n-6.

Desaturase Indices. Desaturase indices were calculated for Δ6 and Δ5 desaturase in both the plasma PL and RBC membrane samples [1]. Diets H and S FA compositions were used to calculate the indices for comparison. Because diet M contained three times more 20:4n-6 than diets H or S, it was not considered for this comparison.

Table 11 Repeated measures ANOVA of feline desaturase indices

Index	Effect		
	Diet	Time	Interaction
Plasma PL Δ6 Desaturase	0.1214	<.0001	0.0004
Plasma PL Δ5 Desaturase	0.0993	0.6569	0.1913
Plasma PL DGLA/LA	0.1129	0.1153	0.1173
RBC Δ6 Desaturase	0.0304	0.4193	0.0055
RBC Δ5 Desaturase	0.3791	0.3855	0.9318
RBC DGLA/LA	0.7574	0.2034	0.6199

Values given are p-values of desaturase indices. $p < 0.05$ was considered significant.

$p < 0.05$ was considered significant.

No differences were found for PL Δ5 desaturase index, PL DGLA/LA ratio, RBC Δ5 desaturase index, or RBC DGLA/LA ratio (Table 11). Significant time x diet interactions were found for PL Δ6, and RBC Δ6 desaturase indices (Table 12). On day 28, diet H RBC Δ6 was increased relative to diet S. The S diet PL Δ6 was significantly decreased on day 14 and 28 relative to day 0. Similarly, RBC Δ6 in the S diet was decreased significantly on day 28 relative to day 0, and day 14 was similar to both.

Table 12 Calculated desaturase indices

Index	Diet H (n=9)			Diet S (n=10)		
	Day 0	Day 14	Day 28	Day 0	Day 14	Day 28
Plasma PL Δ6 Desaturase	0.46±0.02	0.42±0.05	0.32±0.01	0.58±0.05[a]	0.28±0.04[b]	0.18±0.02[b]
Plasma PL Δ5 Desaturase	10.50±0.69	8.74±1.09	9.84±0.93	10.72±0.60	11.39±0.77	11.27±0.58
Plasma PL DGLA/LA	0.04±0.002	0.06±0.03	0.03±0.002	0.05±0.003	0.02±0.01	0.02±0.002
RBC Δ6 Desaturase	0.94±0.06	1.12±0.05	1.10±0.11[1]	1.13±0.09[a]	0.88±0.05[ab]	0.76±0.07[b2]
RBC Δ5 Desaturase	20.27±1.43	20.43±1.47	16.88±1.94	22.60±3.53	21.34±1.79	19.54±3.53
RBC DGLA/LA	0.04±0.003	0.04±0.01	0.04±0.01	0.05±0.003	0.04±0.01	0.03±0.01

Values given are mean ± SEM.

PL = Phospholipid, RBC = Red Blood Cell Membrane, DGLA = 20:3n6, LA = 18:2n6.

Superscript letters not in common in a row within a diet group indicate a significant difference between days by time x diet from PROC MIXED, $p < 0.05$.

Superscript numbers not in common in a row within a day indicate a significant difference between diets by time x diet from PROC MIXED, $p < 0.05$.

CHAPTER IV

DISCUSSION

The present study sought to induce Δ6 desaturase activity by dietary substrate enhancement, examine comparative aspects of feline lipid metabolism when a diet enriched in LCn-3FA was fed, and provide novel data on feline RBC membrane FA composition. In addition to the above mentioned objectives, this study revealed differences between plasma PL and RBC membrane FA incorporation and the possible utility of these measures in determining tissue FA composition. Also, this study raised possible clinical benefits of fish oil supplementation in cats.

It is interesting to note that humans [12] and monkeys [25] both experience TG lowering in response to fish oil feeding. Cats follow suit with the aforementioned mammals, and respond to fish oil feeding with decreased TG. In contrast, fish oil feeding has not been reported to lower TG in dogs [14]. Thus, TG lowering by fish oil supplementation is of special interest, and of possible clinical relevance, especially in cats with hyperlipidemia. As Pazak et al. reports, patients with idiopathic feline hepatic lipidosis have elevated plasma TG concentrations of 155.88 mg/dL (1.76 mmol/L) [26]. Standard treatment for cats with hepatic lipidosis is tube-feeding. Additionally, those patients could be treated with fish oil supplementation to help correct the hyperlipidemia. As some cats are prone to hepatic lipidosis, regular fish oil supplementation could be used as a preventative measure.

Baseline plasma TG concentrations in the present study were, on average, slightly lower than the 31.89 mg/dL (0.36 mmol/L) Butterwick et al. reported for the adolescent cat (age 32 weeks), despite similar methodology [27]. The majority of TG concentrations in the present study, however, fell within Butterwick's reported reference range for adult feline TG of 21.26-38.97 mg/dL (0.24-0.44 mmol/L) [27]. Another study differentiated between the sexes on TG concentrations, and found plasma TGs of 26.57 mg/dL (0.30mmol/L) and 17.71 mg/dL (0.20 mmol/L) for male and female cats respectively [28]. The plasma TG concentration of the M diet group was 17.06 mg/dL on day 28, and thus much lower than the concentrations reported by Butterwick et al. [27], but similar to that of Demacker et al. [28]. As seen in the present study and previously reported by Demacker et al., female cats have low-normal TG concentrations [28]. Despite these low-normal feline TG concentrations, the M diet revealed statistically significant TG lowering. Therefore, the present study provides some insight into the potential benefit of fish oil treatment in feline patients with hepatic lipidosis to most efficiently correct the hyperlipidemia they experience.

Lipoprotein-cholesterol metabolism, specifically pre-β LP-C (i.e. VLDL), reflected the TG lowering effect of the M diet. This lipoprotein primarily targets endogenous TG. Day 14 pre-β LP-C was statistically significant in that all diets showed a decrease at that time point. This effect can most likely be attributed to both the decrease in fat content of the diet from 24.3 % to 16.8% (as is), which caused lowering of VLDL across diets. Further lowering of VLDL can be attributed to the fish oil diet effect. Castillo et al. demonstrated that fish oil feeding in chicks produced a clear

decrease in the amount of total FA in the plasma, which may have contributed to a depression of VLDL synthesis and secretion [29]. Fish oil may also cause rapid conversion of VLDL to IDL or LDL and/or rapid absorption of VLDL into the liver [29]. In rats, fish oil feeding impairs VLDL assembly [18]. Several mechanisms have been suggested for the fish oil lowering effect on VLDL, but it seems the most straightforward explanation of the decrease found in the present study is that decreased TG with the M diet resulted in lowered VLDL synthesis, particle assembly, and secretion [18, 29].

All diets resulted in statistically significant relative increases of α LP-C (i.e. HDL) on day 14 returning to their initial values on day 28. Whether this change is physiologically significant is unknown. It may be that it is a transient effect of a dietary protein source change from a chicken and corn dominant diet (Hill's Science Diet Kitten Original™) to a mixed protein source diet (Kit'n'Kaboodle™) which among others, contains oceanfish meal. Indeed, a study in rabbits found that fish meal increased HDL levels compared to soybean meal [30]. The authors speculated that either enhanced hepatic secretion or reduced catabolism of HDL may have occurred [30]. Interestingly, a study in humans showed that after 120 days of 22:6n-3 supplementation, HDL-C was significantly increased [31]. It is possible that after feeding a fish oil supplement to cats for 120 days, a statistically significant increase in HDL could be achieved similar to that seen in humans.

In humans, high 18:2n-6 feeding has been reported to result in increased clearance of LDL and thus a lower plasma concentration of LDL [12]. This was not

observed in the present study. Lipoprotein-cholesterol data revealed that neither the change in total fat intake nor the different FA composition of the diets had an effect on β LP-C (i.e. LDL).

Although few published reports have characterized feline lipoprotein metabolism, Ibrahim et al. found VLDL concentrations, on average, to be low in comparison to HDL and LDL concentrations [32]. Similarly, Butterwick et al. reported 8.89 mg/dL (0.23mmol/L) VLDL-C, 162.80 mg/dL (4.21 mmol/L) HDL-C, and 61.10 mg/dL (1.58mmol/L) LDL-C [27]. Cats in the present study had 2-3 times more VLDL-C, and half as much HDL-C and LDL-C relative to values reported by Butterwick et al. [27]. This difference could be attributed to the ultracentrifugation density gradient method used in determining LP-C by both Ibrahim and Butterwick et al. [27, 32] compared to the lipoprotein electrophoresis procedure performed in the present study. Another study looked at Iranian Persian cats that were fed commercial diets, in which both α LP-C and β LP-C concentrations were similar to those found in the present study, with β LP-C most similar to those found in male Iranian Persian cats [33].

Despite different methodology, LP-C values found in the present study were comparable to those previously reported [28, 33, 34]. These previous studies agreed that cats are an HDL animal and lack cholesterol ester transfer protein (CETP) [28, 33, 34]. Thus, long-term fish oil feeding may have physiological significance because of lowered VLDL-C and the tendency toward elevated HDL-C.

Non-esterified fatty acids were measured as a result of an unexpected additional faint lipid staining region found on the electrophoretogram, which migrated similarly to

plasma albumin. Because NEFA are bound to plasma albumin, it was speculated that plasma NEFA, in those cats with the additional band, would be elevated. However, the relative staining densities of this region in the electrophoretogram did not correlate with plasma NEFA concentrations. In addition, NEFA concentrations were not significantly altered in any of the cats. Thus, the diets fed and fasting duration before blood collection did not appear to have any impact on NEFA in this study.

Many effects of dietary n-6 FAs on plasma PLs were found in cats after high 18:2n-6 feeding. Dietary enrichment of 18:2n-6 in a species with normal Δ6 desaturase activity would result in accumulation of 20:4n-6. Although feline Δ6 desaturase activity is considered low [5], it was speculated that some 20:4n-6 accumulation would occur. However, despite the excess dietary 18:2n-6, 20:4n-6 did not accumulate in the S diet relative to the H diet, further substantiating claims that Δ6 desaturase activity is limited in cats. It is important to note that Diet H was used for comparison because of its low dietary 18:2n-6, and the similar 20:4n-6 content of both diets S and H, which was 0.03 g FA/kg diet. Low Δ6 desaturase activity, in concert with relatively low dietary 20:4n-6, resulted in modest plasma PL 20:4n-6 relative percentages in the S diet group. Additionally, high dietary 18:2n-6 in diet S resulted in accumulation of 18:2n-6 in the plasma PL. These accumulations were expected, as a dose-dependent relationship between dietary intake and plasma PL FA incorporation is commonly accepted in rats [35] and should be applicable to cats. Also, due to excess 18:2n-6, a significant increase of 20:2n-6 in the S diet was found on day 28. This demonstrated that chain elongation of 18:2n-6 occurred preferentially over its Δ6 desaturation, further emphasizing the low

Δ6 desaturase activity of cats. It is important to note that the significant decrease of 20:4n-6 in the S diet from day 0 to day 28 reflects the decrease in dietary 20:4n-6 from the pre-experimental level of 0.62% to 0.19% (relative %). Despite the relatively low dietary 20:4n-6, this decrease may not be physiologically significant. Previous reports have shown that cats fed diets high in 18:2n-6 and deficient in 20:4n-6 supported reproduction [5]. Those authors speculated that adequate 20:4n-6 was being made available even when dietary 20:4n-6 was low [5].

It is noteworthy to report that the M diet did not result in statistically significant blunting of 20:4n-6 incorporation into the plasma PL fraction. This effect was likely minimized because the fish oil supplement in the M diet provided 3 times more 20:4n-6 to the diet than the vegetable oil supplements of diets H and S. Additionally, blunting of 20:4n-6 into PLs was not seen because tissue 20:4n-6 content in cats is most likely diet-dependent. A similar effect, in which 20:4n-6 was not blunted, occurred in horses where the high 20:4n-6 of the fish oil supplement overwhelmed the fish oil blunting effect on this FA [36]. Minimizing the fish oil blunting on 20:4n-6 is a positive development for the promotion of fish oil feeding in cats, since reduction of this dietary EFA beyond its already lower relative amount might not be physiologically beneficial. The present study contrasts work in humans that show fish oil feeding to blunt 20:4n-6 incorporation into plasma PL [13, 17]. Flaten et al. showed that fish oil feeding in humans decreased 18:2n-6, 20:3n-6, and 20:4n-6 [13]. While the M diet in the present study provided relatively high dietary 20:4n-6, the dietary amounts of 18:2n-6 and 20:3n-6 in this diet were similar to that of diet H. So, in the M diet group 18:2n-6 and 20:3n-6 were

decreased in comparison to the H diet group. Thus, the M diet demonstrated dietary n-3 blunting of n-6 incorporation into plasma PLs for 18:2n-6 and 20:3n-6. Similarly, the decreased 22:4n-6 in diet M on day 14 may be attributed to n-3 substitution for n-6 FAs in the plasma PL fraction.

Many plasma PL FA composition changes occurred as a response to high dietary intake as anticipated. As expected with the high dietary LCn-3FA from diet M, there were significant increases in 20:5n-3 and 22:6n-3. All diets showed relatively elevated 18:3n-3 and 22:5n-3 concentrations at day 0, likely due to the comparatively high dietary level of these FAs in the pre-experimental diet. The washout period for n-3 FAs is approximately 4-8 weeks [37, 38]. So, 18:3n-3 and 22:5n-3 concentrations in plasma of cats fed the H and S diets decreased accordingly over time during this 28 day study. Plasma PL concentrations of 22:5n-3 are highest in the M diet, because 22:5n-3 is present in sufficiently elevated levels in that diet. An interesting effect where n-6 FA incorporation competed against n-3 FA incorporation was described by Cleland et al. in humans [39]. This effect was seen in which the high 18:2n-6 content of the S diet blunted the incorporation of 20:5n-3 into the plasma PL. Also, the significant accumulation of diet M 17:0 seems counterintuitive with fish oil supplementation, as it is best known for increasing n-3 fatty acids; however, fish oil contains elevated levels of many SFAs. In fact, Plantinga et al. found increased concentrations of 14:0, 16:0, and other SFAs in the plasma cholesterol ester of cats fed fish oil compared to cats fed sunflower oil [40]. Also, the M diet was higher in dietary 20:1n-9 relative to diets H or

S, so increased amounts of 24:1n-9 in that diet likely reflect chain elongation of 20:1n-9 to 24:1n-9.

Distribution of dietary fat type is readily reflected in the plasma PL. Relative fatty acid type distributions are inherently linked, such that as one type is increased another must decrease. Because the diets in the present study were designed to significantly change the FA composition of the plasma PLs, statistically significant time effects were expected. Thus, some variation in the relative amounts of SFA, MUFA, and PUFA were expected among diet groups. However, because two of the diets were high in PUFA and the other high in MUFA, only the MUFA changes were statistically significant. Diet H was composed of 26.47% SFA, 58.93% MUFA, and 14.60% PUFA, where the increase of MUFA over time was counteracted by a decrease in PUFA. A significant accumulation of plasma PL MUFA in the H diet on days 14 and 28 was found due to high dietary 18:1n-9.

One recent study showed measuring %n-3HUFA to be a useful biomarker for n-3 tissue status in humans, rats, and pigs [41]. In fact, the authors suggested that %n-3HUFA to be more effective than total n-3 FA evaluation [41]. Stark et al. examined the %n-3HUFA in total lipid extract, and proposed that this measure was a good reflection of the status of PL *sn-2* position [41]. In the present study, %n-3HUFA as well as %n-6HUFA were evaluated in cats. Statistically significant effects were seen with %n-3HUFA and %n-6 HUFA in the M diet group. Not surprisingly, on days 14 and 28 the %n-3HUFA was increased in diet M. This increase reflects the higher relative percentages of n-3 FAs in the plasma PL, namely 20:5n-3 and 22:6n-3. Also, decreased

%n-6HUFA was found in the M diet group on both days 14 and 28. These changes are directly related to the competition for incorporation of the n-3 and n-6 species in the M diet group. Thus, both %n-3HUFA and %n-6HUFA in diet M were significantly different from both diets H or S on days 14 and 28. It is interesting to note that on day 28, diet H %n-3HUFA and %n-6HUFA are almost the exact opposite of diet M n-3 and n-6 %HUFA values.

Red blood cell membrane FA incorporation also reflected dietary n-6 FA effects. Similar to plasma PL results, no additional RBC membrane 20:4n-6 accumulation was found, presumably from the low Δ6 desaturase enzyme's inability to synthesize appreciable amounts of this FA from excess 18:2n-6. In addition, RBC 20:2n-6 in diet S was elevated over time, likely due to chain elongation of excess dietary 18:2n-6. A similar competition between incorporation of n-3 and n-6 FAs occurred in the RBC membranes. The moderate n-6 blunting by the M diet is reflected in the decreased RBC 20:2n-6 on day 28. Also, a trend ($p = 0.0578$) towards 18:2n-6 lowering was found in the M diet on day 28. This trend was supported by statistically significant diet effects differentiating between diets M and S for the 18:2n-6 relative percentage. Also, the statistically significant time effect for 20:4n-6 likely represents the early stages of 20:4n-6 depletion in the RBC membrane. However, a longer feeding time would be necessary to see to what extent the 20:4n-6 decrease in this tissue would occur in response to diets used in this study. Additionally, in spite of many dietary PUFA changes, RBC 20:4n-6 relative percentages remained relatively constant throughout the study. Red blood cell membrane 20:4n-6 was 11.76% on average across all diets and time points. This

conservation of 20:4n-6 by the RBC suggests the recycling of 20:4n-6 back into the membranes.

Fish oil supplementation also had an effect on RBC membrane FA composition. On day 28, RBC 20:5n-3 in the M diet group showed statistically significant accumulation. Though not statistically significant, there appeared to be a slight increase in RBC 22:6n-3 on day 28 indicating that perhaps if given sufficient time, 22:6n-3 would accumulate. Indeed, Flaten et al. showed incorporation of 20:5n-3 > 22:6n-3 into the RBC membrane post fish oil feeding in humans [13]. Vidgren et al. demonstrated that with fish oil feeding in humans, both 20:5n-3 and 22:6n-3 were incorporated into platelets and RBC membranes similarly after 6 weeks [19]. In the same study, 22:6n-3 was accumulated after 14 weeks [19]. An increase in 20:3n-3 occurred on day 28 across all diets in the RBC membrane, but reasons for this effect remain unknown. Additionally, the combination of pre-experimental diet carryover and high M diet n-3 FA resulted in significant accumulation of RBC 22:5n-3.

The function of 22:6n-3 inclusion into the RBC membrane is unknown, as 22:6n-3 may be more important to incorporate into neural, retinal, or cardiovascular FAs [42, 43]. In one human study, fish oil supplementation showed 20:5n-3 and 22:6n-3 incorporation into atrial PL within 7 days of fish oil feeding and the incorporation of 20:5n-3 into RBC membranes correlated to that of atrial PLs [42]. In rats, both 20:4n-6 and 22:6n-3 are incorporated into brain PLs [44]. Makrides et al. confirmed earlier work that RBC membrane 22:6n-3 may be valid as a marker of brain DHA in humans [20]. In

piglets, Blank et al. showed that there is a strong correlation between brain 20:4n-6 and RBC membrane 20:4n-6, and no correlation between brain PL and plasma PL [45]. Thus, 22:6n-3 accumulation in the RBC membrane would be less important, as many other tissues are competing for inclusion of this FA.

In addition to the effects of n-3 and n-6 FAs, other changes were seen in the RBC membrane FA composition. Similar to the effect of the H diet on 18:1n-9 in the plasma PLs, RBC membrane 18:1 isomers were also statistically increased in diet H. Because 22:0 was not a component of the pre-experimental diet, but was present in the experimental diets, it increased across all diets over time. Increased 24:0 across all diets over time was likely due to the amount of 24:0 provided by the diet, and chain elongation of SFAs.

For RBC membranes, the distributions of FA by type were evaluated. Only MUFA was statistically significant for diet H. Similar to plasma PLs, on day 28, MUFA was increased in the H diet relative to diets M and S. This effect is directly related to the high MUFA content of the H diet. The lack of SFA and PUFA changes is possibly a function of the short study time. Also for RBC membranes, %n-3 and %n-6 HUFAs were calculated. Similar to the effects found in plasma %HUFAs, the M diet resulted in increased RBC %n-3HUFA and lower RBC %n-6HUFA. These values again correspond to the n-6 and n-3 FA contents of this diet.

Two complications arose during analysis of the RBC membranes. Due to column age, degradation occurred during RBC membrane sample analysis such that the 18:1n-9 and 18:1n-7 peaks no longer separated consistently. They were subsequently

reported as 18:1 isomers. For samples where the peaks separated properly, 18:1n-7 and 18:1n-9 were mathematically combined and reported as 18:1 isomers. Also, data on RBC membrane FAs does not include 24:1n-9 due to co-elution, in some samples, of an unknown component during GC separation. Using GC-MS, it was discovered that the peak corresponding to 24:1n-9 on the chromatograms was not exclusively 24:1n-9 FAME, but 24:1n-9 plus some other membrane lipid component; possibly a cholesterol derivative. Therefore, 24:1n-9 was culled from the RBC membrane data set, and the relative percentages of all other FAs measured were mathematically adjusted to reflect this removal.

Cell turnover is an important factor in dietary FA enrichment, and is a key consideration when comparing plasma and RBC FA incorporation. Plasma is a dynamic pool and thus readily modified by changes in the diet. The RBC FA incorporation is dependent on its half-life of approximately 120 days. Thus, it is expected to take more time to significantly alter the composition of the RBC membrane compared to plasma PLs. Also, RBCs are synthesized in the bone marrow from circulating plasma PL. Therefore RBCs can only incorporate FAs from the diet or via de novo synthesis in their juvenile form before they undergo organelle loss during maturation. A human study on the kinetics of dietary FA incorporation into different fractions showed that for 20:5n-3, cholesterol ester reflects the past week or two, RBC membrane the past 1-2 months, and adipose tissue the past 2 years of dietary intake [46]. Katan et al. also suggested that the half-life of 20:5n-3 incorporation into RBC membrane was 28 days, and that a steady state in humans could be achieved after 180 days [46]. A recent study suggests that

RBC membranes respond to dietary interventions on a similar time course to that of plasma [41]. This finding was not apparent in the present study because fewer significant changes were seen in RBC membrane FA incorporation compared to plasma PLs. Another factor that contributes to FA incorporation into the RBC membrane is the functional need, or lack thereof, for certain FAs. It is due to these unique aspects of RBC membrane FA incorporation that time x diet interactions were seen to a lesser extent in the RBC membrane than the plasma PL during this 28 day study. Plasma PL responded to this dietary change, and many significant effects were seen on day 14. However, day 14 data appears to represent a transitional metabolic response to the experimental diet prior to achieving a true steady state. In many cases, day 14 values are very different from baseline, and possibly more extreme than would be found under conditions of a metabolic steady state, which in plasma PLs approached on day 28. In humans, plasma PLs are more responsive to dietary alterations than RBC membranes [47]. For these reasons, it is exciting to report significant differences in the RBC membrane FAs in a 28 day time period, because it was unclear before the study began whether or not 28 days would be long enough. Therefore, feline lipid metabolism can be significantly altered in a 28 day study. Further work is needed to determine more precisely whether 28 days is sufficient time to establish a metabolic steady state when dietary FAs are modified especially in the RBC membrane.

In an attempt to establish similarities between plasma PL and RBC membrane FA incorporation, correlation graphs with regression calculations were performed. Correlations were made for select FAs of the n-6 and n-3 categories both within diets

and across all diets. Both 20:2n-6 and 22:4n-6 in the S diet showed correlation coefficient values between 0.5 and 0.7 indicating positive correlations. These are not strong correlations but they do show that plasma PLs and RBC membranes are correlated for some of the n-6 FAs in the S diet. A positive correlation was found for 20:5n-3 across all diets perhaps due to high affinity for this FA by both plasma PL and RBC membranes. Both 22:5n-3 and 22:6n-3 had statistically significant weak positive correlations possibly due to lower RBC membrane affinity for these FAs. Because these correlations are between plasma PL and RBC membrane total lipid extract, they are perhaps less reliable than comparing the same fractions in whole plasma and RBC membrane. However, recent evidence suggests that PL fractions of both plasma and RBC membranes correlate highly to their total lipid extracts [41]. Additionally, day 28 n-3 and n-6 %HUFA from plasma PL and RBC membranes were modestly correlated in cats and an r^2 value of 0.408 each was found. This suggests a modest positive correlation in the present study, which is in contrast to the strong %n-3HUFA correlations found in rats by Stark et al. [41]. The most likely explanation for the weaker correlations in the present study is the 28 day feeding period. Stark et al. fed rats diets enriched in n-3 FAs for 20 weeks [41]. Stronger %HUFA correlations would be expected in cats fed for a longer period of time. Another possible explanation for the higher correlations found in the Stark et al. study is that they incorporated two additional FAs into their %HUFA calculation. They included 20:3n-9 and 4, 7, 10, 13, 16-docosapentaenoic acid (22:5n-6) in their HUFA calculation, neither of which were specifically observed in this study.

Desaturase indices of diets H and S can be compared due to equal amounts of 20:4n-6 provided by the diet. Compared to the H diet, the S diet shows a statistically significant decrease in Δ6 desaturase index over time for both plasma PLs and RBC membranes, primarily due to increased 18:2n-6 and decreased 20:3n-6 and 20:4n-6 relative percentages in diet S. Diets H and S values from plasma PL for Δ5 desaturase were not significantly different from one another. The depressed value of plasma PL calculated Δ5 desaturase in the H diet versus the S diet was due to a modestly higher 20:3n-6 in the H diet group. Due to the change in 20:4n-6 concentration from the pre-experimental diet to the H and S diets, day 28 data was most useful for interpreting the changes in Δ5 and Δ6 desaturase indices.

Implications of this study are multi-faceted. Because feline metabolism of fish oil appears similar to that of humans, potential benefits of fish oil supplementation in cats, in addition to treatment of hyperlipidemia, may include cardiovascular health and anti-carcinogenic benefits. As reported by Rush et al., hypertrophic cardiomyopathy is the most common form of cardiovascular disease in cats, with some clinical symptoms including syncope and arrhythmias [48]. One potential mechanism of cardiovascular health benefit from fish oil supplementation is its antiarrhythmic effects [49]. This mechanism of action could prove to be physiologically significant in cats. Larsson et al. describe possible mechanisms for dietary n-3 PUFA to inhibit promotion and progression of carcinogenesis, including: suppression of 20:4n-6 altering immune response to cancer cells, inflammation modulating effects, decrease of nuclear factor κB, reduced estrogen-stimulated cell growth, and changes in production of free radicals and

reactive oxygen species [11]. Additionally, the present study supports previous research indicating that cats have low Δ6 desaturase activity which appears to be unaffected by excess substrate. The present study suggests that further research be performed on cats with regard to fish oil supplementation, and that %n-3HUFA may be used as a biomarker of feline cardiovascular disease.

CHAPTER V

SUMMARY

This study provides insight into some of the unique features of feline lipid metabolism with regards to fish oil supplementation, Δ6 desaturase activity, and FA incorporation into plasma PL and RBC membranes. Notably, fish oil supplementation in normal cats caused a significant decrease in plasma TG. Also, this is the first study to demonstrate that fish oil supplementation decreases VLDL in cats. This study suggests the possibility of a nutritional prevention and treatment in fish oil supplementation for the hyperlipidemia caused by hepatic lipidosis, which is a common feline condition. Human research has supported fish oil supplementation as a way to prevent cardiovascular disease and possibly cancer. Because cats respond similarly, supplementation of fish oil may have similar positive benefits. These findings provide justification for more research on the potential health effects of fish oil feeding in cats.

Typically, when fish oil is fed, the high n-3 FAs provided by the diet are incorporated preferentially to n-6 FAs, resulting in an n-6 blunting effect. In this study, the M diet showed a slight trend in 20:4n-6 blunting, but this effect was minimized due to the high concentrations of 20:4n-6 in the Menhaden fish oil supplement. However, the favored incorporation of n-3 FAs versus n-6 FAs was seen with the diet M group having decreased 18:2n-6 and 20:2n-6 relative percentages in both plasma PL and RBC membrane.

Fish oil supplementation in cats revealed feline PL affinity for both 20:5n-3 and 22:6n-3. In RBC membranes, the M diet showed an accumulation of 20:5n-3, but not as much affinity for 22:6n-3. This finding suggests RBC membrane FA incorporation is either selective to meet functional demands, or that 22:6n-3 takes longer to accumulate. Retinal, neural, and cardiac tissues require 22:6n-3 for optimal function, so 22:6n-3 from the diet may be accumulating in those tissues rather than in the RBC membrane. Also, since the mature RBC is devoid of organelles, synthesis of 22:6n-3 from 20:5n-3 may not occur.

The present study sought to induce Δ6 desaturase activity by providing large amounts of dietary substrate. The S diet provided excess 18:2n-6, but the derivatives of this FA, namely 20:4n-6, were not increased significantly via the Δ6 desaturase pathway; rather, chain elongation occurred preferentially. This finding substantiates previous research that Δ6 desaturase activity is low in cats despite the presence of adequate substrate. Concentrations of 20:4n-6 in plasma and tissues of cats appear to be directly related to the quantity provided by the diet and its possible conservation, rather than accumulation from Δ6 desaturation of 18:2n-6. Red blood cell 20:4n-6 relative percentages remained constant throughout the study, with an average value of 11.76%. This conservation of 20:4n-6 by the RBC suggests the recycling of 20:4n-6 back into the membranes.

As expected, plasma PL accumulations of 18:2n-6 in the S diet, 20:5n-3 and 22:6n-3 in the M diet, and 18:1n-9 in the H diet occurred, because these FAs were enriched in these diets. The rapid turnover of plasma FAs explains why plasma PLs

changed rapidly and may have reached a steady state by day 28. The accumulation of FAs in the RBC membrane occurs more slowly due, in part, to the 120 day half-life of the RBC. Therefore, it is exciting to note that some of the same statistically significant accumulations were seen in the RBC membrane FA composition as were found in plasma PLs. Measuring plasma PL and RBC membrane FA composition has been utilized in previous studies because these fractions serve as good biomarkers of lipid incorporation into tissues. It appears that plasma PL, due to its rapidly responsive nature, may be a good marker of tissue FA incorporation in short-term feeding scenarios. For long-term feeding, the RBC membrane may serve as a valid biomarker of tissue FA incorporations. Because of the long half-life of RBC membranes, they can likely be utilized as a biomarker for historical dietary intake covering the previous 1-2 months of feeding.

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APPENDIX A

Table A-1 Fatty acid composition of pre-experimental period diet[a] in cat study

Fatty Acid	Relative %
14:0	1.46
14:1n-5	0.06
16:0	21.86
16:1n-7	3.43
17:0	0.30
17:1	ND
18:0	9.80
18:1n-7	3.15
18:1n-9	35.88
18:2n-6	17.25
18:3n-3	1.87
20:0	0.29
20:1n-9	0.73
20:2n-6	0.48
20:3n-3	0.08
20:3n-6	0.15
20:4n-6	0.62
20:5n-3	1.03
22:0	ND
22:1n-9	ND
22:4n-6	ND
22:5n-3	0.17
22:6n-3	ND
24:0	0.68
24:1n-9	ND
Unidentified	0.71
SFA	34.39
MUFA	43.24
PUFA	21.65
n-3	3.15
n-6	18.50

[a] Hill's Science Diet® Kitten Original; 24.8% fat and 4208 kcal/kg as reported by manufacturer.
Values are averages of two representative samples.
ND = not detected;
SFA = saturated fatty acids;
MUFA = monounsaturated fatty acids;
PUFA = polyunsaturated fatty acids.

Table A-2 Fatty acid composition and diet characteristics of experimental kibble, oils, and complete diets in cat study (relative %)

		Oil[a]			Diet (Kibble + Oil)[a]		
Fatty Acid	Kibble[a]	H	M[b]	S	H	M[b]	S
14:0	2.43	0.04	6.89	0.09	1.38	4.19	1.45
14:1n-5	0.42	ND	0.05	ND	0.23	0.28	0.23
16:0	22.65	3.36	14.62	5.98	14.35	19.84	15.39
16:1n-7	2.47	0.09	10.55	0.08	1.38	5.64	1.36
17:0	0.88	ND	0.26	ND	0.57	0.83	0.51
17:1	ND	ND	0.64	ND	ND	0.43	ND
18:0	13.24	3.23	2.75	2.35	9.26	10.14	8.50
18:1n-7	4.19	1.26	3.21	0.80	3.06	4.34	3.16
18:1n-9	32.73	82.37	6.82	13.52	53.98	23.57	24.59
18:2n-6	17.87	7.67	1.52	74.78	13.47	11.36	43.52
18:3n-3	0.92	0.22	1.42	0.28	0.66	1.09	0.69
20:0	0.46	0.29	0.28	0.46	0.34	0.30	0.37
20:1n-9	0.29	0.24	1.43	0.17	0.29	0.78	0.23
20:2n-6	0.10	ND	0.24	ND	0.001	0.01	0.001
20:3n-3	ND	ND	ND	ND	ND	ND	ND
20:3n-6	0.05	ND	0.22	ND	ND	0.14	ND
20:4n-6	0.24	ND	1.83	ND	0.19	0.57	0.002
20:5n-3	0.27	ND	14.09	ND	0.12	5.24	0.001
22:0	0.10	0.68	0.82	0.19	0.39	0.36	0.001
22:1n-9	ND	ND	0.17	ND	ND	0.001	ND
22:4n-6	0.04	ND	0.21	ND	ND	0.002	ND
22:5n-3	ND	0.20	2.62	ND	ND	1.02	ND
22:6n-3	0.32	ND	12.79	ND	0.15	5.13	0.02
24:0	0.14	0.18	0.09	ND	0.17	0.15	0.001
24:1n-9	ND	ND	0.30	0.11	ND	ND	0.001
Unidentified	0.18	0.17	16.17	1.19	0.00	4.59	0.00
SFA	39.91	7.78	25.61	9.14	26.47	35.82	26.23
MUFA	40.10	83.95	23.13	14.69	58.93	35.04	29.57
PUFA	19.72	8.10	34.94	75.07	14.60	24.55	44.21
n-3	1.51	0.42	30.92	0.28	0.94	12.48	0.69
n-6	18.21	7.67	4.03	74.78	13.66	12.07	43.52
% fat (as fed)[c]	11.60	-	-	-	16.60	16.10	17.70
kcal/kg[d]	3442	-	-	-	3816	3816	3816

[a] Values are averages of two representative samples.

[b] Values from one representative sample; according to the manufacturer's specifications, contained significant amounts of fatty acids not present in our standard mixture, such as 3.53% of 18:4n-3.

[c] Determined gravimetrically.

[d] Estimated by calculation from nutrient composition using modified Atwater factors.

ND = not detected; SFA = saturated fatty acids; MUFA = monounsaturated fatty acids; PUFA = polyunsaturated fatty acids.

Table A-3 Cat body weights before and during the study

Cat	Pre-Study	Day 0	Day 7	Day 14	Day 21	Day 28
1	3.50	3.55	3.57	3.57	3.68	3.50
2	2.91	3.09	3.23	3.27	3.27	3.32
3	2.55	2.64	2.50	2.50	2.45	2.55
4	2.95	3.05	3.09	3.00	2.95	2.86
5	3.59	3.64	3.68	3.68	3.68	3.73
6	3.23	3.32	3.32	3.27	3.23	3.32
7	3.18	3.18	3.14	3.05	3.09	3.05
8	2.73	2.77	2.86	2.86	2.95	2.95
9	3.64	3.59	3.59	3.55	3.50	3.55
10	3.45	3.36	3.32	3.23	3.09	3.07
11	3.64	3.73	3.73	3.68	3.77	3.91
12	3.18	3.32	3.27	3.27	3.32	3.36
13	3.86	3.86	3.93	3.95	3.95	4.05
14	3.95	3.95	3.86	3.86	3.86	3.82
15	3.73	3.86	3.95	3.95	4.00	3.86
16	4.00	4.09	4.18	4.18	4.23	4.27
17	3.82	3.82	3.91	3.86	3.82	3.86
18	3.05	3.09	3.18	3.18	3.23	3.18
19	2.86	2.82	2.86	2.86	2.95	2.95
20	3.59	3.59	3.55	3.77	3.45	3.50
21	2.59	2.59	2.55	2.45	2.50	2.64
22	3.59	3.64	3.73	3.64	3.68	3.64
23	3.55	3.55	3.64	3.64	3.64	3.68
24	2.45	2.50	2.55	2.55	2.55	2.55
25	3.32	3.36	3.27	3.18	3.18	2.95
26	3.18	3.27	3.36	3.32	3.36	3.32
27	3.23	3.14	3.05	2.95	2.95	3.05
28	3.68	3.59	3.64	3.64	3.64	3.68
29	3.18	3.27	3.32	3.41	3.55	3.55

Values given are body weights measured in kilograms.

Table A-4 Cat body condition scores (BCS)

Cat	Day 0	Day 7	Day 14	Day 21	Day 28
1	5	6	6	6	6
2	5	5	5	5	5
3	4	5	5	5	5
4	4	5	5	5	5
5	6	6	6	6	5
6	5	5	5	5	5
7	5	5	5	6	6
8	5	5	5	5	5
9	6	5	5	5	5
10	5	5	5	5	5
11	6	6	5	5	6
12	4	5	5	5	5
13	5	6	6	6	6
14	6	6	6	6	6
15	6	6	6	6	5
16	5	6	6	6	6
17	6	6	6	6	6
18	5	5	5	5	5
19	5	5	5	5	5
20	5	5	5	5	5
21	4	4	4	4	4
22	6	6	6	6	6
23	5	5	5	5	6
24	4	4	4	5	4
25	6	6	6	6	6
26	5	5	5	6	5
27	6	6	6	6	6
28	6	6	6	6	6
29	6	6	6	6	6

Values given are body condition scores as measured using a 9 point scale.

Table A-5 Average weekly caloric intake per cat (kcal).

Cat	Pre-Study	Week 1	Week 2	Week 3	Week 4
1	267.11	283.54	259.54	286.32	192.79
2	261.07	279.78	282.38	286.70	288.60
3	226.34	194.70	166.85	206.60	145.29
4	230.47	267.27	246.78	196.52	194.56
5	289.28	309.82	321.32	321.86	321.52
6	257.16	253.98	239.40	229.98	234.58
7	220.95	211.73	190.29	199.56	202.01
8	269.55	228.03	255.12	257.12	245.43
9	282.50	243.05	223.25	212.43	261.34
10	250.56	193.62	203.65	163.60	169.87
11	286.95	308.43	256.95	324.73	326.06
12	262.08	285.43	267.29	293.26	269.80
13	257.09	318.35	300.94	335.92	334.79
14	279.58	304.73	313.47	324.74	272.01
15	294.23	330.38	338.72	338.97	274.22
16	281.07	329.96	349.15	351.18	353.27
17	276.17	275.39	324.65	317.92	298.40
18	247.69	289.16	286.40	290.19	276.87
19	206.58	236.68	178.94	220.71	250.14
20	256.39	302.45	296.90	313.03	293.78
21	222.18	224.16	198.81	222.25	233.58
22	256.48	306.30	277.63	310.21	285.60
23	262.33	273.30	277.74	318.63	254.25
24	193.96	229.26	226.32	239.18	210.48
25	259.91	212.00	209.83	213.95	220.62
26	273.79	292.13	312.78	306.30	303.85
27	218.24	163.38	117.85	154.31	212.23
28	208.45	274.23	262.96	263.97	264.28
29	259.01	247.92	300.24	303.04	310.88

Values given are average weekly caloric intake (kcal) per cat.

Table A-6 Plasma Total Cholesterol in mg/dL

	Day 0	Day 14	Day 28
Diet H (n = 9)	128.68±17.30	123.78±14.66	117.33±16.20
Diet M (n=10)	105.34±7.69	118.00±4.87	115.03±3.88
Diet S (n=10)	111.49±9.87	114.10±6.47	119.43±7.51

Values given are mean ± SEM.

Superscript letters not in common in a row within a diet group indicate a significant difference between days by ANOVA, $p < 0.05$.

Superscript numbers not in common in a row within a day indicate a significant difference between diets by ANOVA, $p < 0.05$.

APPENDIX B

Lipoprotein-Cholesterol Calculation:

LP-C (mg/dL) = (Average % Adjusted Volume * Total Cholesterol) ÷ 100

Desaturase Index Calculations:

Δ6 desaturase index = (20:3n-6 + 20:4n-6)/18:2n-6

Δ5 desaturase index = 20:4n-6/20:3n-6

DGLA/LA = 20:3n-6/18:2n-6

Highly-Unsaturated Fatty Acid Calculations:

% n-3 HUFA = (20:3n-3 + 20:5n-3 + 22:5n-3 + 22:6n-3) ÷ (20:3n-6 + 20:4n-6 + 20:3n-3 + 20:5n-3 + 22:4n-6 + 22:5n-3 + 22:6n-3) x 100

% n-6 HUFA = (20:3n-6 + 20:4n-6 + 22:4n-6) ÷ (20:3n-6 + 20:4n-6 + 20:3n-3 + 20:5n-3 + 22:4n-6 + 22:5n-3 + 22:6n-3) x 100

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