EFFECT OF DIETARY POLYUNSATURATED FATTY ACIDS AND RELATED NUTRIENTS ON SEBUM LIPIDS, AND SKIN AND HAIR COAT CONDITION IN CANINES

A Thesis

by

NAOMI ANNE KIRBY

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

December 2004

Major Subject: Nutrition

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ABSTRACT

Effect of Dietary Polyunsaturated Fatty Acids and Related Nutrients on Sebum Lipids, and Skin and Hair Coat Condition in Canines. (December 2004)

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A study was performed to investigate the effect of diets rich in polyunsaturated fatty acids and other related nutrients, in the effort to improve skin and hair coat conditions in canines. The study included 24 dogs fed a baseline diet (Ol'Roy®), with an acclimation period of 12 weeks (Phase I). Nine female beagles and 15 male hound mixed-bred dogs were used. For the next 12 weeks (Phase II) the dogs were randomly divided into three groups and fed one of three specially formulated complete and balanced diets. Differences among the three diets were as follows: Diet A contained lower but adequate amounts of dietary zinc and linoleic acid than Diet B. Diet C was similar to Diet B with respect to zinc but contained less linoleic acid and more alpha-linolenic acid.

In the beginning, a preliminary study assessing skin lipids showed that hair is a suitable material to analyze. This study lead to the subsequent investigation, using plucked and shed hair samples obtained on weeks 3, 7, and 11 (Phase I) and again on weeks 1, 3, 7, and 11 (Phase II). One objective of this research was to investigate the dietary effects of the combination of linoleic acid in combination with zinc on canine

sebum lipids during a 12 week diet phase. Another objective was to investigate if any correlation existed between the hair cycle anagen and telogen phase of the hair follicle and the lipid constituents present in the sebum during the 12 week acclimation phase and the 12 week feeding trial.

Two hypotheses were tested. First, the lipid content of hair can be altered by fat and fatty acid composition of the diet. Second, the modification of dietary fat and polyunsaturated fatty acids lead to changes in hair lipids that may be related to improved skin and hair coat scores. Over the 12 week feeding period of Phase II, test results revealed statistically significant increases in both CE and CE/WD, and a prolonged growth of hair follicles in the anagen phase of diet B dogs, which are both consistent with improved skin and hair coat scores.

ACKNOWLEDGEMENTS

The completion of this thesis would not have been possible without the guidance and support of some very essential people. First and foremost I would like to acknowledge the members of my graduate committee. A sincere amount of gratitude goes to my committee chair, Dr. John E. Bauer, for teaching me to think like a scientist and opening my eyes to a whole new world. I have learned and come to appreciate the scientist's rule: When faced with several competing hypotheses, prefer the simplest one. I would also like to express a sincere thanks to Karen Bigley for her invaluable knowledge and assistance with performing my assays on this project and my preceding ones.

Extended acknowledgement goes to Dr. Christine Rees and Dr. Robert Kennis for taking time out of their busy schedules to serve as members on my committee. I would like to give an additional thanks to Dr. Robert Kennis for his support and encouragement throughout my veterinary school admission interviews. Words cannot express the appreciation I have for the faith and support he provided me in those trying weeks. Furthermore, this rewarding, challenging, and hopefully fruitful experience could not have been complete without the knowledge and wisdom my classroom instructors have bestowed upon me, and that I will carry on into my future endeavors.

Finally, the utmost expression of gratitude is reserved for my family, my mom, Susan, my stepfather, Andy, and my brother, Josef, who have all shown considerable love, tolerance, and encouragement during the writing of this thesis. My mother is an embodiment of sophistication, brilliance, drive, and ambition. She always encouraged

me to pursue my passion and always told me when one door closes another one opens. To her I give thanks for believing in me, when at times it was difficult to believe in myself. One last thanks goes to Robbie, Mandy, Simon, and Hannah who were always waiting for me when I got home from my many all-nighters at the research laboratory. It is to them I own a great deal of thanks for sticking by me through the tough times and bringing laughter, love, and joy into my life.

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CHAPTER I

INTRODUCTION

Understanding what role nutrition plays in the development of the canine hair coat sheen and healthy appearance is a question that plagues many dog food companies and dog owners. Dog breeders have long claimed that changing the diet will, in a period of weeks, result in a change in the appearance of the hair coat. This change is not due to the quantity or thickness of the hair (which would require months to be visible) but rather to sheen (1). The first question that must be addressed is if nutrition can influence sebum secrection, then by what mechanism is it inducing glossiness of a healthy coat? Due to the lack of knowledge about the function of sebum, its biochemistry remains a topic of great interest to lipid chemists. The overall objective of the present study is to investigate the possible relationship between nutrition and skin surface lipid content and its constituents.

One method to assess overall canine health is to observe the appearance of skin and coat condition. It has long been recognized that deficiencies of various nutrients manifest themselves as changes in the structure, function, and color of canine skin and hair coat. Thus it is not surprising that many dog owners believe that proper dietary management is the best way to help their pets achieve the finest skin and coat condition.

This thesis follows the style and format of *Lipids*.

Skin Physiology - Holocrine Process

Mammalian skin consists of a thin layer of epidermis and a thicker inner layer of dermis. The sebaceous glands are located in the dermis and are actually appendages of the epidermis (2). Most of the lipids of the skin surface originate from the sebaceous glands, which secrete an oily, waxy substance known as sebum (3). This secretion of sebum by the sebaceous glands is referred to as the holocrine process. The sebaceous gland cells retain the sebum that they synthesize until it is released by degradation of these entire cells. Holocrine secretion involves; continuous cell division in the germinative epithelium of the gland, gradual accumulation of the product in each cell as it grows and differentiates, and eventual complete disruption of the cell as it releases its product (2). Sebum is a complex of various lipids that are thought to act as an epidermal and/or follicular lubricant. Most of the remaining lipids originate in the stratum corneum cells of the epidermis. The stratum corneum contains a complex mixture of high-melting polar lipids produced by the epidermal cells and has a surface film of fluid nonpolar lipids secreted by the sebaceous glands. Constituents of the stratum corneum lipids included a variety of ceramides, cholesterol, free fatty acids, free sphingosines, and cholesterol sulfate, but negligible amounts of phospholipids and glycolipids (4).

Sebaceous glands are alveolar glands attached by a short duct to the opening of primary hair follicles (which are also epidermal appendages) (Figure 1.1 and 1.2) (1, 5). The location of the sebaceous gland and its duct suggests that sebum may play a major role as an infundibular lubricant and/or sealant for the opening of the hair follicle. Microscopically, sebaceous glands consist of an outer layer of germinative cells and

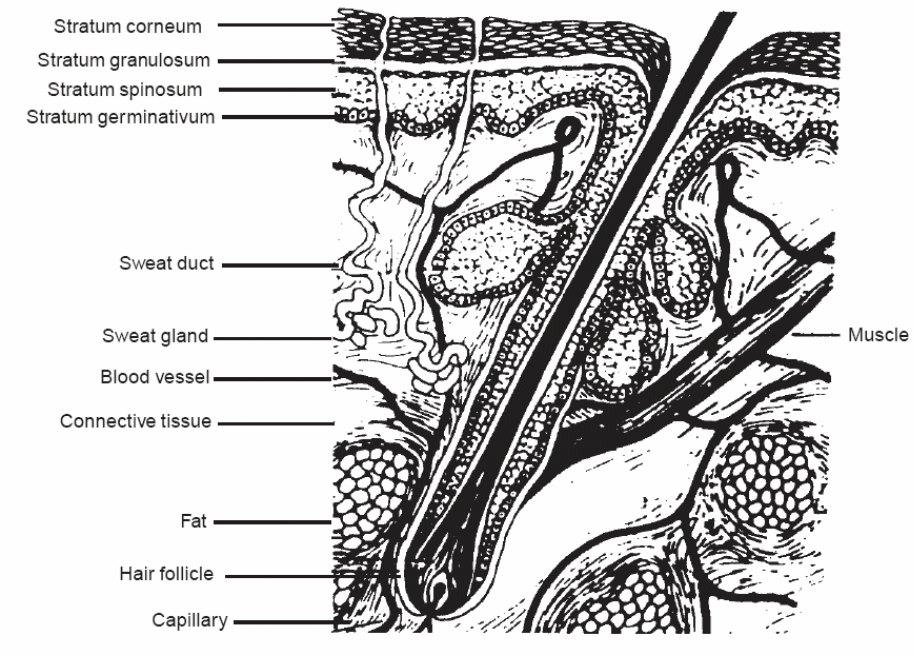

Figure 1.1 Diagrammatic representation of the skin of the human being. The three layers include, the epidermis, dermis, and fat layer (also called the subcutaneous layer). Beneath of the surface of the skin are nerves, nerve endings, glands, hair follicles, and blood vessels (6). (Reprinted figure with permission from Paramjit Singh, V. Sihorkar, Vikas Jaitely, P. Kanaujia, S. P. Vyas. Pilosebaceous Unit: Anatomical Considerations and Drug Delivery Opportunities. *Indian Journal of Pharmacology* 2000; 32 269-281)

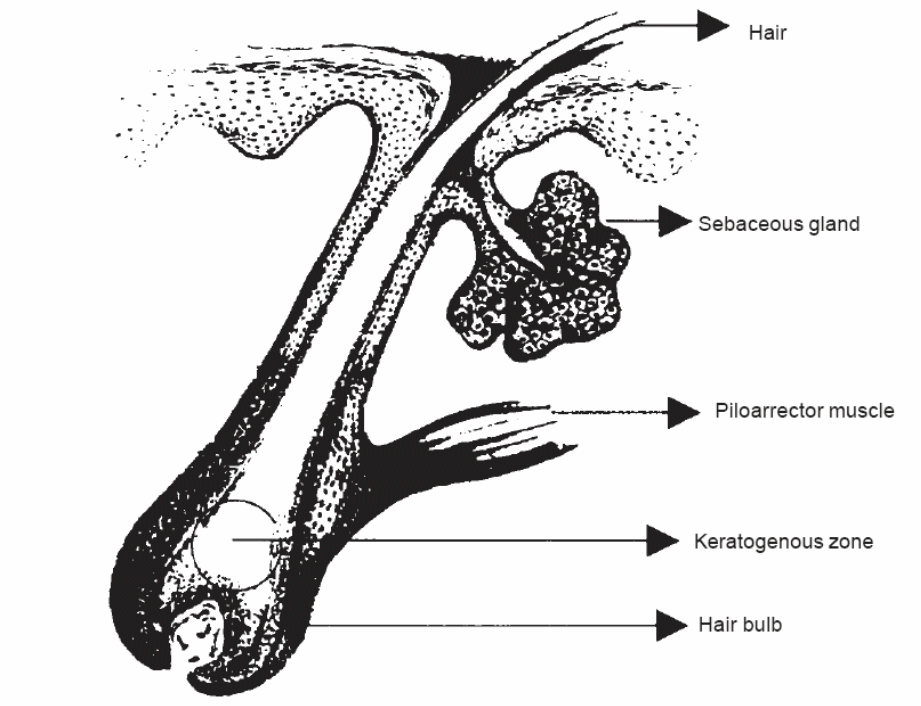

Figure 1.2 Diagrammatic representation of the anatomy of the pilosebaceous gland of the human being (6). The canine is similar in structure, with the exception of the sebaceous gland being positioned in closer proximity to the hair bulb. (Reprinted figure with permission from Paramjit Singh, V. Sihorkar, Vikas Jaitely, P. Kanaujia, S. P. Vyas. Pilosebaceous Unit: Anatomical Considerations and Drug Delivery Opportunities. *Indian Journal of Pharmacology* 2000; 32 269-281)

inner layers of cells that become increasingly large and lipid-filled as they progress toward the sebaceous duct. The germinative cells resemble undifferentiated epidermal cells in structure, having large nuclei, mitochondria, tonofilaments, and other intracellular inclusions (2). Only neutral lipids reach the skin surface. Proteins, nucleic acids, and the membrane phospholipids are digested and recycled during the disintegration of the cells (4). In dogs, the largest sebaceous glands are on the chin and around the mucous membranes of the oral cavity. They are also large and numerous in interdigital spaces, on the dorsal neck and rump, and on the dorsal tail in a region known as the tail gland (supracaudal organ) (1, 5). Only the palms and soles in humans and the footpads and leather of the nose in dogs, which have no hair follicles, are totally devoid of sebaceous glands (1-5).

Lipid Constituents of Sebum

Generally sebum is deposited on hairs inside the follicles and is brought up to the surface of the skin along the hair shaft. One of the most intriguing and unexplained aspects of the sebaceous glands is the variability of both the lipid classes present in sebum and the structures of the sterols and fatty acids among species (1). Each species produces a specific and unique lipid composition of sebum, thus knowledge of the weight percentage of mammalian skin surface lipids and their biochemical composition may be used as a molecular blueprint for the identification of different species. For example, lactones have been identified only in members of the family Equidae, while wax triesters are found only in bovine species (1). Intermediates in the biosynthetic pathway to cholesterol, which do not accumulate in other tissues, are fairly common

sebum constituents. However, only humans produce sebum having significant amounts of triacylglycerol and squalene, a cholesterol precursor (Table 1.1) (1- 3, 7-18). In a survey of 46 mammals, Lindholm et al. (1981) also found profound differences in surface lipid composition between species, but showed that closely related species may have similar sebum composition (2, 14).

The component lipids of sebum are best separated by thin-layer chromatography (TLC). Previous studies have demonstrated that the skin surface lipids of many mammals contain compounds that migrate on TLC. In fact, skin surface lipids of the dog have been reported to contain a high proportion of diol diesters having a lower mobility on thin layer chromatography than diesters from other species in spite of containing similar fatty acid and diol components. In a study performed by the Dermatology and Biochemistry departments of the Boston University Medical Center, dog skin surface lipids were separated by preparative thin layer chromatography into sterol ester (42%), wax diester (32%), free sterol (9%), polar lipid (7%), and unidentified components (10%) (14). These results differ from studies performed on human sebum, whose major lipid classes are triacylglycerol (TAG) (40-60%), wax ester (19-26%), and squalene (11-15%) (11, 15-16, 19-22).

Although recognizably complex, when reduced to least common denominators, the lipids of sebum are either derived from sterols or fatty acids. A sterol is any 4-ringed molecule, which also has a hydroxyl group in the C_3 position and a branched side chain of 8 to 10 carbon atoms at C_{17}. The most abundant sterols observed in sebum are cholesterol, its precursor squalene, and cholesteryl esters – cholesterol molecules to

Table 1.1 Composition (wt%) of skin surface lipids in selected mammals. Although skin surface lipids have been analyzed in over 70 mammalian species, only data from humans and some domestic animals are presented here. Note the marked differences in both class and relative quantity of surface lipids generally believed to be of sebaceous gland origin in these species (1-3, 7-18).

Lipid	Human	Cat	Cow	Horse	Dog*	Dog†
Squalene	12					
Sterol esters	3	12	3	38	42	39
Cholesterol		3	4	14	9	3
Wax esters	25					
Wax diesters		8	8	1	32	45
Wax triesters			30			
Hydroxyacid diesters		66	38			
Lactones				47		
Triglycerides	41		4		7	6
Free fatty acids	16		1			
Free fatty alcohols	1					
Unidentified	2	12	10		10	7

*Sharaf, 1977 †(Poodle) Burton, 1992

 Originally published in Current Research in Canine Dermatology, Proceedings from a Symposium on August 30, 2000 at the Fourth World Congress of Veterinary Dermatology.

which a long-chain fatty acid has been added at the 3' position (1, 4, 7, 16). Free sterols are ubiquitous in mammalian surface lipids, but are usually present in low concentration consisting predominantly of cholesterol, some or all of which may originate in the epidermis (2). Another constituent of sebum are wax esters, consisting of long-chain fatty acids esterified with long-chain alcohols. Wax esters were once thought to be a lipid found on the skin surface, but actually are present in less than half of the species that have been examined, and only in one specie, (i.e. mink) do these esters exceed 25% of the surface lipid (2, 23).

Two types of wax diesters have been identified in sebum. The Type I molecule consists of a hydroxy fatty acid having the carboxyl group esterified with a fatty alcohol and the hydroxyl group esterified with a fatty acid. In Type II, each molecule consists of a diol bearing two esterified fatty acids. In addition, there are at least two kinds of alkane diols: 1,2-diols (Type IIa) and 2,3-diols (Type IIb) (Figure 1.3). In a previous study, both types have been identified as major constituents in a few species and have even been found together in some species (2). Presently, new data has shown that cows, rabbits, and cats produce vast amounts of Type I, whereas the dogs, mice, guinea pigs, gerbils, and baboons produce vast amounts of Type IIa (6). In birds, the uropygial gland excretes diesters of Type IIb as the major lipid, and in some birds, as the sole lipid (3). The diol diesters present on the surface lipids of domestic dogs (Sharaf et al.,1977) are unusual in that they contain one long-chain fatty acid and one isovaleric acid moiety in each molecule (2, 15). In dog skin lipids, another major component is found on thin layer chromatography (TLC) with mobility intermediate between Type IIa diesters and

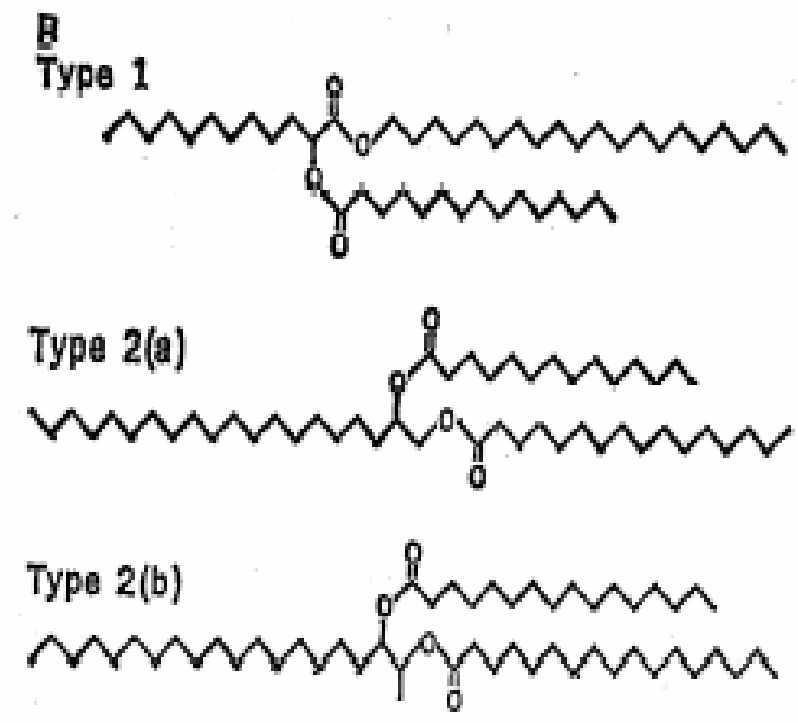

Figure 1.3 Molecular representation of the two types of wax diesters. Type I are α-hydroxy fatty acids esterified to another group of fatty alcohols and a third group of fatty acids. Type II may be either (a) 1,2-alkane diols esterified to two groups of fatty acids or (b) 2,3-alkane diols esterified to two groups of fatty acids (3). (Reprinted figure with legend with permission from Nicolaides, N. Skin Lipids: Their Biochemical Uniqueness, *Science 186*, 19-26. Copyright 1975 American Association of the Advancement of Science).

glyceryl ether diesters. Although the hydrolysis products were reported to contain only long chain diols and long chain fatty acids, it is now known that each diol moiety is esterified with one long chain and one short chain fatty acid, isovaleric acid (15, 24-26). From this data it can be concluded that the wax ester composed of alkane-1,2-diols is unique to the family *Canidae*.

Essential Fatty Acids and Diet

The nutritional significance of specific lipid molecules, particularly polyunsaturated fatty acids (PUFA) was revolutionized through the pioneering work of Burr and Burr in 1929 (27-29). They observed rats maintained on a fat-free diet over a long period developed external abnormalities characterized by growth retardation, severe scaly skin, and extensive water loss through the skin. They discovered that these symptoms were reversed by certain dietary PUFA's designated "essential fatty acids" (EFAs), specifically either linoleic acid (LA, 18:2 n-6) or α-linolenic acid (ALA, 18:3 n-3). These fatty acids were termed "essential" because they are not synthesized in the animal organism and have to be supplemented in the diet for normal physiological and pathophysiological function.

Within the diet, fat has two primary roles: to provide a source of energy, and to supply essential fatty acids (30). There are two series of EFAs, those with the n-6 configuration (first double bond at the sixth carbon from the methyl terminal) and those with the n-3 configuration (first double bond at the third carbon from the methyl terminal). These two series are derived respectively from linoleic acid and α-linolenic acids. These parent fatty acids cannot be synthesized *in vivo*, and are therefore

considered essential (Figure 1.4) (31, 32). All mammals need essential fatty acids (EFA) for manufacturing cell membranes. Essential fatty acids are involved in many aspects of health, including the skin and coat, kidney function and reproduction. Much interest has been aroused by the therapeutic value of polyunsaturated fatty acids in the management of dermatologic conditions. In dogs, linoleic acid, found mainly in vegetable oils, is the major source of EFA. EFA deficiency in dogs results in coarse, dry, brittle hair, poor wound healing, and scaly skin. In most animals, including dogs, γ-linolenic acid (GLA, 18:3 n-6) and arachidonic acid (AA, 20:4 n-6) can be synthesized from linoleic acid. Thus if adequate amounts of linoleic acid are provided in canine diet, supplemental amounts of GLA and AA are not required (33). In skin, LA is needed to help maintain the skin's permeability barrier, thereby controlling excessive water loss, preventing dryness and scaliness and maintaining its softness and elasticity (34). Other longer chain polyunsaturated fatty acids of the omega-6 series (such as AA) can be synthesized from linoleic acid through a series of elongation and desaturation steps, which are important regulators of epidermal proliferation via prostaglandin E_2 (34).

Polyunsaturated n-3 and n-6 fatty acids compete for insertion into cellular lipids and for the same enzymes involved in their metabolism (35). The critical enzyme in these reactions is delta-6-desaturase (D6D) for which the greatest affinity is bestowed upon the fatty acid with the greatest number of double bonds in C_{18} substrate. Thus ALA containing three double bonds is desaturated at the highest rate followed by linoleic acid and oleic acid (28, 36). At equal concentrations α-linolenate effectively inhibits the desaturation of linoleate. In a study performed on rats, Hwang et al.

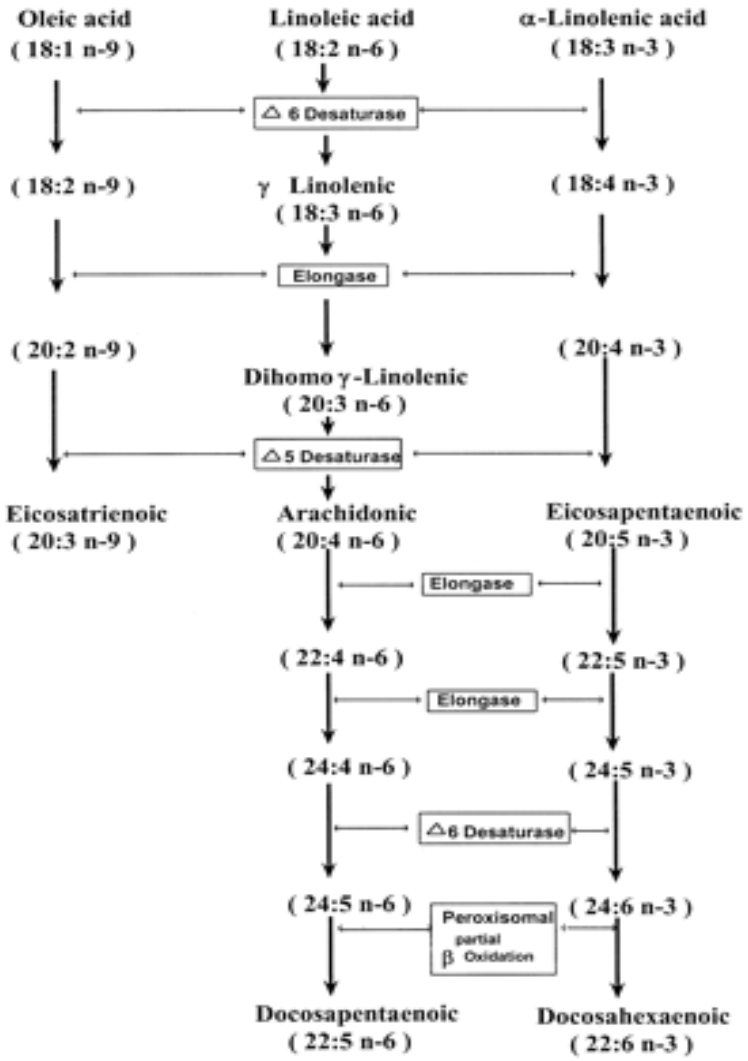

Figure 1.4 Metabolic transformation of essential fatty acids (EFA) to form long-chain PUFA (LCPUFA). Parent EFA are derived from dietary sources for both (n-6) linoleic acid (LA, 18:2 n-6) and (n-3) series α-linolenic acids (ALA, 18:3 n-3). Elongation occurs 2 carbons at a time and delta desaturases (Δ-9, Δ-6, and Δ-5) introduce double bonds at 9, 6 and 5 carbons from the carboxylic moiety. The final step in the formation of (n-3) and (n-6) end-products is catalyzed by a peroxisomal partial beta-oxidation (32).

investigated the effects of diets with varying n-3:n-6 acid ratios from both fish (menhaden fish oil) and plant (linseed oil) sources on platelet eicosanoid formation in rats. The n-3:n-6 ratio varied slightly and it was observed that the plasma phospholipid eicosapentaenoic acid (20:5 n-3; EPA) concentrations were significantly greater, and AA was significantly less, in the menhaden fish oil groups than in the linseed oil groups. Also, EPA can act as a competitive inhibitor of AA conversion to proinflammatory eicosanoids prostaglandin E_2 (PGE_2) and leukotriene B_4, a potent inflammatory mediator. Decreased synthesis of one or both of these eicosanoids has been observed after inclusion of flaxseed oil or fish oil in the diet (Figure 1.5) (37). The authors concluded that at similar n-3:n-6 fatty acid ratios, longer chain n-3 fatty acids from fish oil are more effective in reducing AA concentrations and inhibiting platelet production of pro-inflammatory thromboxanes and prostaglandins. Moreover, not only in humans and rats do long chain n-3 fatty acids reduce AA content in neutrophils, with a corresponding decrease in the production of pro-inflammatory mediators, but these pathways appear functional in dogs as well (38). Canines supplemented with n-3 fatty acids appear to experience a decrease in AA metabolites and superoxide and LTB_4 production, with a subsequent increase in neutrophil phagocytosis (38). This could inevitably lead to a treatment to manage the inflammatory component of many canine diseases.

Importance of Dietary Zinc

Dietary zinc is an essential component of many enzyme systems, including those involved in protein and carbohydrate metabolism necessary for maintaining healthy skin

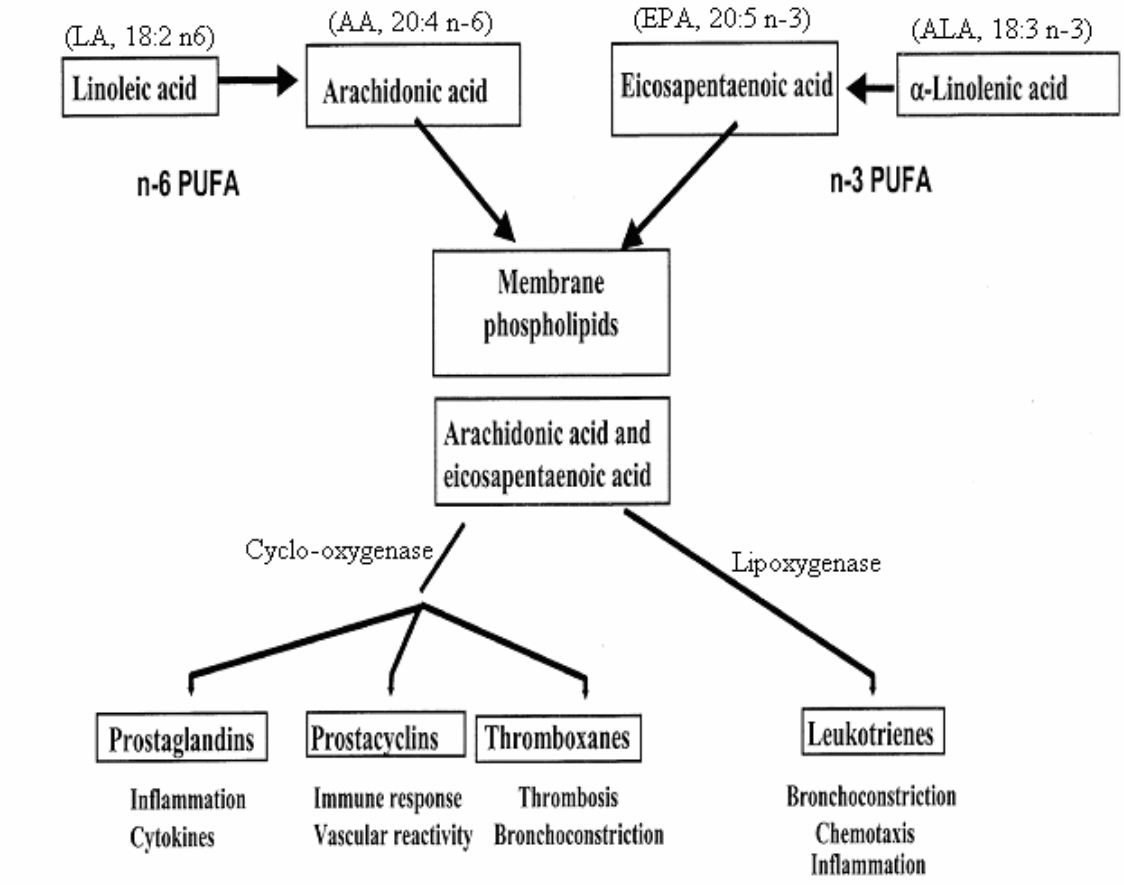

Figure 1.5 Schematic representation of the role of (n-6)/(n-3) fatty acid balance in determining membrane phospholipid composition and eicosanoid production. Excess (n-6) favors arachidonic acid–derived series 2 eicosanoids whereas EPA generates series 3 eicosanoids that antagonize the former.

and coat. It must be continuously supplied in the diet because animals have limiting amounts of readily available zinc stored in the body. This condition exists even though the body contains a relatively large amount of zinc, particularly in bones, skin, and hair (39, 40). Zinc is an essential nutrient that is used to help prevent dry flaky skin and hair loss. Dogs have been shown to have an unusually high requirement for zinc. When fed at 20 to 30 mg/kg zinc in the diet for 6 weeks, severe deficiency symptoms were observed which were only prevented with 120 to 130 mg/kg diet (41, 42). Dogs deficient in zinc can display some of the following symptoms; dull coat, dry scaly skin, skin lesions, depigmentation of hair, impaired growth, reproductive failure, and a compromised immune system (31, 41). Other signs of zinc deficiency include hair loss, thickening of the skin ("parakeratosis") and loss of hair pigmentation ("achromotrichia"). Scientific research conducted by WALTHAM**,** has shown that the addition of zinc to an already complete and balanced diet reduces transepidermal water loss (43). Furthermore, the addition of zinc within a certain range, helps to maintain skin moisture, preventing dryness and improving the barrier function of the skin. Zinc is also important in the dog's natural anti-oxidation system and it has been suggested to decrease lipid peroxidation of the membrane.

Zinc also has a beneficial effectiveness in dermatological conditions of human beings, such as acne, which might be attributed to its effect in reducing sebum secretions (41, 44). This may be related to zinc inhibition of bacterial lipase, thus reducing free fatty acids in sebum and on the skin surface (41, 45). Other studies using dogs have speculated that sebum viscosity may be altered with a combination of zinc and linoleic

acid, which decrease the amount of sebum and its lower viscosity (46). Sebum viscosity has been shown to increase with EFA deficiency (46, 47). However, given an appropriate combination of minerals and EFAs, a decrease in the amount of sebum may reduce greasiness and a decrease in viscosity may result in increased sebum distribution over the hair and therefore increased uniform reflectance. Consequently an overall appearance of sheen and glossiness will occur (46).

Zinc also plays a critical role in regulating many aspects of cellular metabolism and is an integral component of a wide range of metalloenzymes and a cofactor for RNA and DNA polymerases. Its presence is of particular importance in rapidly dividing cells including those of the epidermis. The metabolic steps in the EFA pathway which appear to be most susceptible to the effects of zinc deficiency are the conversion of linoleic acid to longer-chain metabolites, a reaction involving desaturation of linoleic acid (Δ^6) and dihomo-γ-linoleic acid (Δ^5). However, the exact mechanism by which zinc might affect desaturation is unknown. Zinc is present in relatively high amounts in liver microsomes, the cellular component also responsible for fatty acid desaturation. Further observations supporting the statement that the enzyme delta-6-desaturase (D6D) is regulated by zinc, are that most biological effects of zinc deficiency are corrected by GLA (which bypasses delta-6-desaturase) but not by LA (28, 48). Human beings with skin disorders such as eczema, atopic dermatitis and psoriasis show increased levels of linoleic acid with simultaneous decrease in GLA, which suggests a reduction in D6D activity. Studies indicate that GLA, taken orally, increases PGE_1 levels in the skin and suppresses chronic

inflammation. In a study of patients given GLA supplements for skin disorders, not only the skin disorder, but the overall condition of the skin improved (49).

The objectives of the present study were to investigate effects of dietary LA in combination with zinc on canine sebum lipids. A diet adequate in LA and zinc was compared to a diet with enriched amounts of both nutrients and to another diet enriched with not only LA and zinc, but also ALA. A preliminary skin and lipid analysis using Sebutape was performed prior to this dietary skin lipid study in order to establish methodology employed. The dietary study employed thin layer chromatography (TLC), gas chromatography (GC), photodensitometery, lipid composition, and trichography, which including hair cycle phase assessment, (i.e. anagen and telogen cycles). The effect of the diets on hair sebum CE fatty acid profiles were also evaluated. In this latter instance it was hypothesized that a diet containing modified amounts of dietary fat and polyunsaturated fatty acids can alter lipid composition consistent with improvement in skin and hair coat condition scores.

CHAPTER II

SKIN LIPID ANALYSIS: PRELIMINARY STUDY

Introduction

Mammalian skin consists of a thin layer of epidermis and a thicker inner layer of dermis. The sebaceous glands are located in the dermis and are actually appendages of the epidermis (2). Most of the lipids of the skin surface originate from the sebaceous glands, which secrete an oily, waxy substance known as sebum (3). Techniques for the collection of sebum vary widely, and include the use of solvent-saturated swabs, chloroform extraction of hair or fur, and the use of a gel prepared from bentonite clay, ethanol, and carboxymethylcellulose. Other measurement techniques for the collection of skin surface lipids include: the Sebutape, the Sebufix, and the Sebumeter (50). The Sebumeter (Courage & Khazaka, Cologne, Germany) collects sebum by pressing a matte plastic film against the skin, and then is measured photometrically by the increase or decrease in transparency. However, these techniques do not give a rate of sebum secretion or any information about the follicular reservoir (4, 51). To obtain rates of sebum excretion, it is necessary to absorb sebum from a defined site for a specific length of time. Sebutape is commonly used for this latter purpose. Sebutape is a simple and non-invasive absorption technique that appears to be a reliable and fast procedure to obtain numerical values concerning the amount of skin surface lipids. It is best described as a microporous, lipophilic, polymeric film with an adhesive layer. The Sebutape technique is more time consuming compared to the Sebufix and the Sebumeter.

However; it has the advantage to examine a greater surface of the skin, to protect the evaluated region, and the possibility for further quantitative lipid determination.

Sebutape strips are commonly used in human trials because they are a non-invasive absorption method for the recovery of inflammatory mediators to differentiate normal from compromised skin conditions. The Sebutape strips seem to have no or only a minor influence on skin temperature and transepidermal water loss (TEWL). However, the hydration state of the stratum corneum increases significantly during Sebutape application (50).

Materials and Methods

Experimental Design. This preliminary study included three dogs, all Labrador Retrievers, that were identified as V846, V852, and V853, and were property of Nestle-Purina Pet Care® (St. Louis, MO). The study lasted 7 weeks, beginning in mid-December continuing until mid-January with hair (for sebum analysis) and skin sebum samples being collected every 2 weeks. Week 1 was when the first sample was collected and week 7 was when the last sample was collected. Specific information concerning diets and length of feeding are unknown and regarded as irrelevant for this particular methodologic study. The canine sebum samples were acquired with the use of a non-invasive tape (Sebutape^{TM}). The Sebutape strips were placed in 8 dram vials with a mixture of chloroform and methanol (2:1). Each sample was purged with nitrogen gas (N_2), capped with a Teflon-lined screw cap, and stored at -20^{o}C until lipids were extracted. Total lipid extraction of the canine plucked and shed hair samples were performed according to Folch et al (52) (see following chapter for details).

Thin Layer Chromatography (TLC) was performed according to Downing et al. (53), using conventional 20x20 cm silica gel 60 TLC plastic backed plates (EM SCIENCE, Gibbstown, NJ). The plates were cut down to 18x18 cm to fit onto the hot plate for subsequent charring and lipid visualization. The lipids were fractionated using commercially prepared plates and successive development with hexane (to 17 cm), benzene (to 17 cm) and hexane:ether:acetic acid (70:30:1) to 9 cm. A stock solution containing a mixture of the reference standards was prepared in chloroform. Two working standards at concentrations ranging from 25-50 mg/ml were prepared by dilution of the stock solution. Standards used were cholesterol linoleate (cholesterol ester) and palmitoleyl linolenate (wax ester). Standard curves were constructed to determine the concentration of CE/WD that was present in the each of these hair and skin sebum samples. The CE standard curve line had a curve fit line with the equation $y = 53.8x + 15866.9$ and a correlation coefficient of 0.929. The WD standard curve line had a curve fit line with the equation $y = 80.1x + 9564$ and a correlation coefficient of 0.995 (see figure on page 33).

The plates were dipped in a 10% $CuSO_4$ + 85% H_3PO_4 solution and charred at 220^{o}C on a Thermoline Type 1900 Hot Plate (Analtech, Deerfield, IL) for visualization. The lipids were detected as charred spots, each spot being characteristic of a different lipid class. The lipid of interest classes were then quantified using a Model GS-700 Imaging Densitometer (Adobe Systems Incorporated, San Jose, CA) and analyzed with BIO-RAD Laboratories, Quantity One, The Discovery Series^{TM} software. Statistical

analysis was performed by repeated measures ANOVA with a Tukey's comparison of means performed at $p < 0.05$.

Results

Thin layer chromatography data confirmed the major hair and surface epidermal lipids to be cholesterol esters (CE) and wax diesters (WD). Cholesterol (CH), triglycerides (TG), and free fatty acids (FFA) were also identified. Because all dogs had been fed the same diet, CE to WD ratios (CE/WD) were calculated and average values calculated (Table 2.1). Repeated measures ANOVA revealed no time effects or time*lipid interactions when tested at $p < 0.05$.

When lipid extracts from plucked hair were compared with those from sebum (Sebutape collection) a statistically significant difference in CE/WD was found ($p = 0.0257$). Tukey's comparison of means identified the hair follicle in comparison to the skin surface, as having a greater CE/WD ratio, this is represented graphically in Figure 2.1. At the start of the study, the mean hair CE/WD was 0.87 ± 0.35 SEM compared with 0.56 ± 0.20 SEM for the mean skin CE/WD. The greater hair CE/WD compared with skin CE/WD was observed throughout the 7 week collection period. One-way ANOVA was also performed to compare hair vs. skin lipids at each time period. It should be noted, that similar to the findings of Downing et al., there was a notable absence of wax monoesters and of intermediates of cholesterol biosynthesis, both of which have come to be regarded as characteristic components of mammalian surface lipids (15).

Table 2.1 Comparison of mean cholesterol ester to wax diester ratio between hair sebum and skin surface sebum in labrador retrievers

	Week 1		**Week 3**		**Week 5**		**Week 7**	
	Hair Sebum	Skin Sebum	Hair Sebum	Skin Sebum	Hair Sebum	Skin Sebum	Hair Sebum	Skin Sebum
V846 CE/WD	0.26	0.99	1.7	0.94	1.9	0.81	1.08	0.81
V852 CE/WD	1.7	0.15	1.7	1.4	1.4	0.26	1.3	0.78
V853 CE/WD	0.64	0.53	2.3	0.73	0.77	1.02	0.95	0.67
Mean CE/WD	0.87±0.43	0.56±0.24	1.9±0.2[a]	1.02±0.2[b]	1.36±0.33	0.70±0.23	1.11±0.10[a]	0.75±0.04[b]

Values are presented as mean ± S.E.M.
Superscript letters not in common for each specific week are significantly different ($p < 0.05$) by one-way ANOVA.

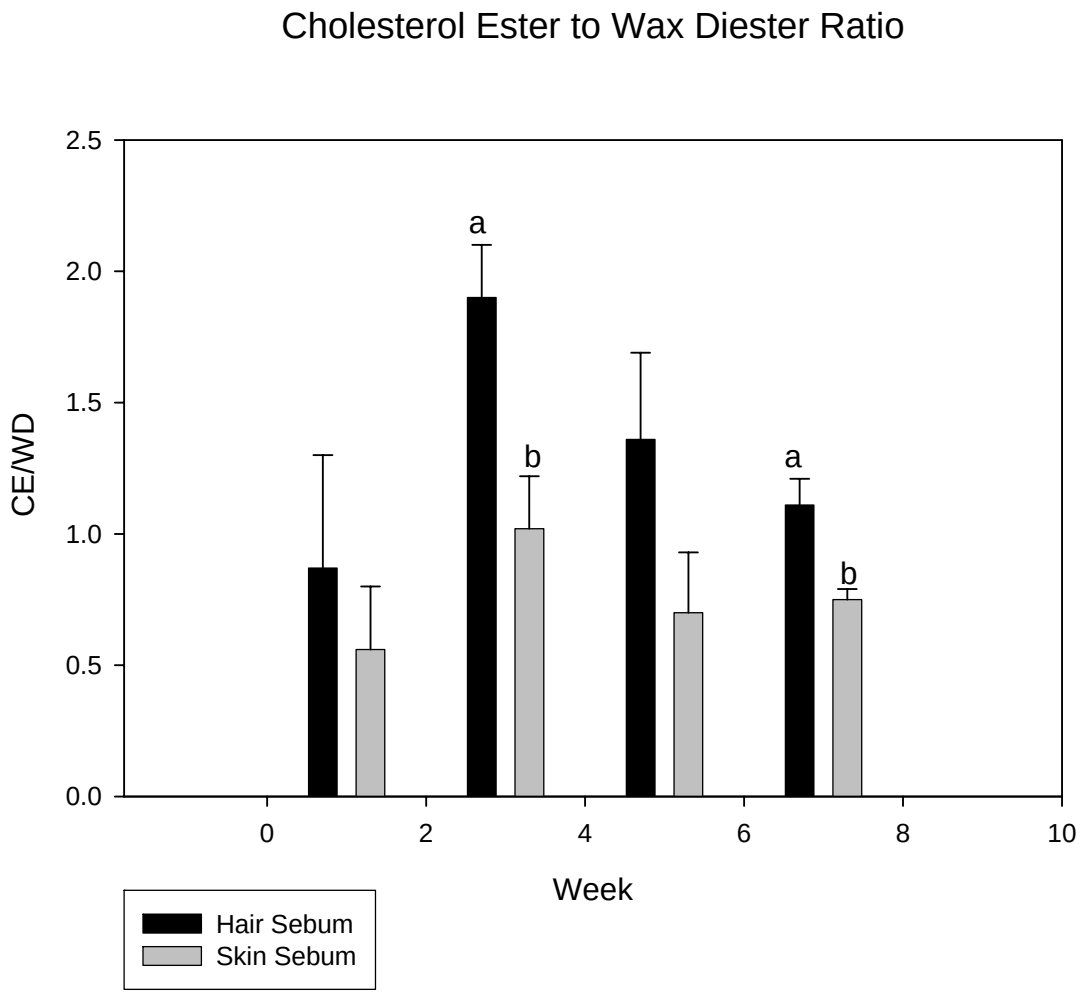

Figure 2.1 Comparison of mean cholesterol ester to wax diester ratio between hair sebum and skin sebum. Letters not in common for each week indicate significant differences by repeated measures ANOVA ($p < 0.05$). Error bars indicate standard error of the mean.

Discussion

Skin surface lipids consist of two different sources: the sebaceous glands and the epidermis. The oily, waxy substance secreted from the sebaceous gland is known as sebum, and is considered the predominant lipid on the skin surface (3). In the current study a simple non-invasive tape absorption method was utilized to remove skin surface lipids and compared to the lipids extracted from the hair samples collected by plucking them using forceps. The Sebutape method has been shown to be useful for determining the composition of the epidermal layer and to distinguish normal from diseased skin conditions. To obtain a rate of sebum secretion studies with human models have deemed it necessary to absorb sebum from a defined site for a specific length of time. Using human subjects, skin lipid data has been obtained by a method that involved placing the solvent-extracted collection papers (Sebutape) over a studied site outlined with adhesive tape. The tapes were left in place for three hours and the collected lipid was then extracted and weighed. In an attempt to shorten collection times, two 15 min. and one 30 min. preliminary trials were executed. However, it was found that these preliminary collections were not nearly long enough to deplete the reservoir and effect a reliable measurement (4, 51).

Although Sebutape as a skin surface sebum collection method has been effectively utilized in human studies, this technique can be less successful in animal studies due to application times needed and the need to protect the site from licking or tape removal and alteration. Unlike humans, animals will not reliably allow tapes to be placed on their skin for the 3 hour period typically needed to collect skin surface lipid

samples. Given the similarities between the lipid distribution on hair and skin surfaces found in this preliminary study, it appears that plucked hair samples are a useful alternative for collection and characterization of sebum lipid fractions under various experimental conditions. Future efforts to understand sebum lipids may thus successfully employ this non-invasive method and the potential for hair to be a suitable material to analyze for evaluation of canine sebum.

This present investigation compared hair sebum to skin surface sebum and found CE and WD to be the major hair and surface lipids. CH, TG, and FFA were also identified; however they were not quantified in this preliminary study. Plucked hair samples taken from the three canines were found to have higher CE/WD ratios although the significance of this observation is presently unknown. These data also suggest that sebum secreted by sebaceous gland do not stop migrating at the skin surface, but continues to spread onto the hair and hair follicle. As such it may be that the presence of this surface lipid on the hair shaft is important in imparting the sheen and glossy appearance to canine hair coats which are interpreted as an index of overall health of pet and show animals. The present investigation also confirms and extends the observation that the canine species skin lipids possess a relatively high amount of cholesterol and wax diesters.

Dogs have a cyclic hair growth with three stages; anagen (active stage), telogen (resting stage), and catagen (transition between anagen and telogen). The amount of shedding that an individual dog shows is dependent on age, sex, nutrition status, physiological status, housing conditions, and season of the year. Al-Bagdadi et al. (54)

reported the appearance of biannual peaks for follicles in both anagen and telogen. Peaks for the follicles in anagen appeared during the summer and winter months, and the peaks for follicles in telogen appeared during the spring and fall months. In this present study the hair and skin sebum samples were collected during December and January, which coincide with the periodicity of when anagen peaks first appear. This observation suggests that the hair cycle anagen phase may correlate with increases in CE/WD that were observed. In total, this data indicates the potential for hair to be a suitable material to analyze for evaluation of canines. Further studies on this topic will provide better understanding of the relationship between diet, health, canine hair lipid composition, and coat evaluation.

CHAPTER III

EFFECT OF DIETARY PUFA AND RELATED NUTRIENTS ON CANINE SKIN LIPIDS AND CE FATTY ACID PROFILES

Introduction

The major surface epidermal lipids in dogs, reported by Dunstan et al., are cholesterol esters, wax diesters, and cholesterol (1). Skin surface lipids of dogs have been reported to contain a high proportion of diol diesters having a lower mobility on thin layer chromatography than diesters from other species in spite of containing similar fatty acid and diol components (15).

In animal tissues, especially in the liver, adrenal glands, and plasma lipids, cholesterol is esterified by a variety of fatty acids and frequently by essential fatty acids, thus forming cholesterol esters (CE) with long-chain fatty acids linked to the hydroxyl group (55). The majority of sterols in cellular membranes when in intimate association with phospholipid molecules, are frequently found esterified to fatty acids. Cholesterol esters are much less polar than free cholesterol and appear to be the preferred form for transport and storage. CEs are largely distributed in body fluids of animals (plasma lipoproteins) and may be also found in vessel walls as fatty streaks in atherosclerosis. Those in plasma are synthesized mainly by transfer of fatty acids from position sn-2 of phosphatidylcholine (PC) by the enzyme lecithin-cholesterol acyl transferase (LCAT). In other animal tissues, the synthesis of cholesterol esters occurs by the enzyme acyl-CoA:cholesterol acyltransferase (ACAT) (55). This present study was conducted, in

part, to characterize sebum cholesterol ester content and fatty acid compositions in dogs fed three different diets varying in their fatty acid and zinc concentrations.

Materials and Methods

Experimental Design. The study included 24 dogs fed a baseline diet, with an acclimation period of 12 weeks (Phase I). The dogs consisted of 9 female beagles and 15 male hound mixed-breed dogs that are property of Texas A&M University, Veterinary Teaching Hospital. All dogs were housed in the Laboratory Animal Resources and Research (LARR) facilities, Texas A&M University. They were individually housed and cared for in kennels according to the American Physiological Society Guidelines for Animal Research and according to the guidelines set forth by Texas A&M University Care and Use Committee. The dogs ranged in age from 1.5 years to 6.5 years with a median age of 4 years, with body weights ranging from 15 to 40 kg. The exact ages of the dogs can be found in Appendix A, Table A-I. Physical assessment, including blood sample collection, of all the dogs before the start of the study showed each one to be clinically healthy.

The dogs were fed a discount store type dry extruded, complete and balanced dog food product during Phase I (Ol'Roy Premium Formula, Wal-Mart Inc. Bentonville, AR, USA). Feed was provided daily to maintain each dog's adult body weight and water was available *ad libitum*. At the end of Phase I they were divided equally and randomly into three groups (n=8). For the next 12 weeks (Phase II) the dogs were fed one of three specially formulated complete and balanced diets (Diets A, B, and C). The diets were formulated with common ingredients and nutrient profiles, however they contained

comparative differences. All diets contained sufficient amounts of LA, ALA, and dietary zinc. LA was 11 g/kg (Diet A) and 33 g/kg (Diet B and C), ALA was 1.5 g/kg (Diet A and B) and 12 g/kg (Diet C), and dietary zinc contents were 120 mg/kg (Diet A) and 350 mg/kg (Diet B and C). Each diet consisted of approximately 12% total dietary fat and contained one of the following as its primary fat source: beef tallow (Diet A), sunflower oil (Diet B), and ground flax seed (Diet C). All diets were isocaloric (based on 3800 kcal/kg and 8.5 kcal fat/g), and met the Association of American Feed Control Officials (AAFCO) standards for protein (amino acid), vitamins, and minerals. All other dietary components were identical. The nutrient composition, list of ingredients, as well as the fatty acid compositions of each diet, can be found in Appendix B.

After the 12-week acclimation period, the 24 dogs were randomly fed one of the three complete and balanced diets (described above) for an additional 12 weeks (Phase II). Plucked and shed hair samples were collected at weeks 1, 3, 7, and 11 of this dietary period and analyzed. Plucked hair samples were collected with a hemostat from the lower back and upper part of the rear legs. To obtain a sufficient amount of hair for analysis, dogs were plucked between 4 to 6 times. Using a plastic template with a rectangular hole cut out of its center, measuring 6 mm by 15.5 mm, shed hair samples were collected from the dorsal rear surface of each dog, approximately 7.5 cm from the base of the tail, using ten strokes of a flea comb (24 teeth per inch) inside the template area. The plucked and shed hair samples were placed in separate envelopes for subsequent analysis. The hair samples were weighed and stored in sealed tubes with Teflon-lined caps to which 2:1 (v/v) chloroform/methanol containing 0.1% glacial acetic

acid was added. The samples were purged with nitrogen gas (N_2) prior to capping the tubes and placed in a -20^{o}C freezer for 3-4 weeks before lipids were extracted. The weights of individual plucked and shed hair samples of the dogs can be found in Appendix A (Table A-II).

Lipid Extraction. Total lipid extraction of the canine plucked and shed hair samples were performed according to Folch et al. (52). All of the chloroform:methanol ($CHCl_3$:CH_3OH, 2:1, v/v) containing 0.1% glacial acetic acid from the above tubes were extracted and placed in clean test tubes to which 2 ml of distilled water (dH_2O) was added. Test tubes were sealed with Teflon-lined caps and placed on a Shaker-in-the-Round shaker (Model S-500; Kraft Apparatus, Inc., Mineola, NY) for 10 minutes at room temperature. Tubes were then centrifuged for 10 minutes at 2500 rpm at 4^{o}C in a Beckman GS-6R tabletop centrifuge to stratify the layers. The infranates were transferred to a clean 12-mL Teflon-lined screw top glass test tube and mixed with 5.0 mL of chloroform:methanol:water ($CHCl_3$:CH_3OH:H_2O, 3:48:47, v/v/v) added to wash the lipid-containing phase. Tubes were shaken for 10 minutes and centrifuged at 3500 rpm for 10 minutes at 4^{o}C. The infranates were transferred, via glass wool filtration, to clean tubes. Each sample was purged with N_2, capped with a Teflon-lined screw cap, and stored at -20^{o}C until further analysis. The lipid yields were determined gravimetrically. This is a simple but most reliable method to measure the weight of total lipids. Organic solvents containing lipids were filtered to remove undissolved material and were evaporated under nitrogen gas flow. The weights of lipids were measured using a microbalance (Mettler, Toledo OH).

The purged lipid samples were resuspended in 1 mL of $CHCl_3$, of which 500 µl was removed and placed in a clean test tube. This sample was again purged with N_2 and resuspended in 50 µl $CHCl_3$ for thin layer chromatography and densitometry analysis. The initial test tube containing the other half of the lipid extraction (500 µl $CHCl_3$) was used for thin layer chromatography and fatty acid analysis.

Thin Layer Chromatography and Densitometry. Most of the lipid classes that are present in sebum can be resolved adequately using TLC on silica gel 60 for quantitative analysis and for preparative separation via a modified procedure originally described by Downing (53). The lipid samples were removed from the freezer, evaporated to dryness under N_2, and resuspended in 50 µl $CHCl_3$ for analysis. Three glass tanks with lids for chromatography were lined with filter paper (Whatman International, Ltd, Maidstone, England). The tanks were filled to a level approximately 2 cm high; the first with hexane; the second with benzene; and the third with a solution of hexane:ether:acetic acid (50:50:1, v/v/v). Lids were placed on top of the tanks for one hour to allow for equilibration of the mobile phase throughout the tank. The thin layer chromatography (TLC) plastic backing plates (20x20 silica gel 60, EM SCIENCE, Gibbstown, NJ) were cut down to 18x18 to fit onto a hot plate for subsequent charring. The lipids were fractionated using commercially prepared plates and successive development with hexane (to 17 cm), benzene (to 17 cm) and hexane:ether:acetic acid (50:50:1, v/v/v) to 9 cm. Lipid fractions were confirmed each time by the development of reference standards of known composition. Working standards were obtained from Sigma Chemical Co. (St. Louis, MO) which included squalene, cholesterol linoleate

(cholesterol ester), palmitoleyl linolenate (wax ester), methyl elaidate (methyl ester), triolein (triglyceride), monolinolein (monoglyceride), stearic acid (free fatty acid), dilinolein (diacylglycerols), cholesterol (5-Cholesten-3β-ol), and phosphatidylcholine (phospholipid). The plates were dipped in a 10% $CuSO_4$ + 85% H_3PO_4 solution and charred at 220^{o}C on a Thermoline Type 1900 Hot Plate (Analtech, Deerfield, IL) for visualization. The lipids were detected as charred spots, each spot being characteristic of different lipid subclasses. The lipid classes were quantified by a Model GS-700 Imaging Densitometer (Adobe Systems Incorporated, San Jose, CA) and analyzed using BIO-RAD Laboratories, Quantity One, The Discovery Series^{TM} software.

Standard curves were constructed to determine the concentration of CE/WD and CH that was present in each of the hair sebum samples. The CE standard curve line had a curve fit line with the equation y = 53.8x + 15866.9 and a correlation coefficient of 0.929. The WD standard curve line had a curve fit line with the equation y = 80.1x + 9564 and a correlation coefficient of 0.995. The CH standard curve line had a curve fit line with the equation y = 246.3x + 5042.4 and a correlation coefficient of 0.977. (Figure 3.1).

Thin Layer Chromatography for Cholesterol Ester Fatty Acid Analysis. Glass tanks with lids for chromatography were again lined with filter paper (Whatman International, Ltd, Maidstone, England). All the tanks were filled to a level approximately 2 cm high, the first with hexane, the second with benzene, and the third with a solution of hexane:ether:acetic acid (50:50:1, v/v/v). Lids were placed on top of the tanks for 1hr to allow for equilibration of the mobile phase throughout the tank. The

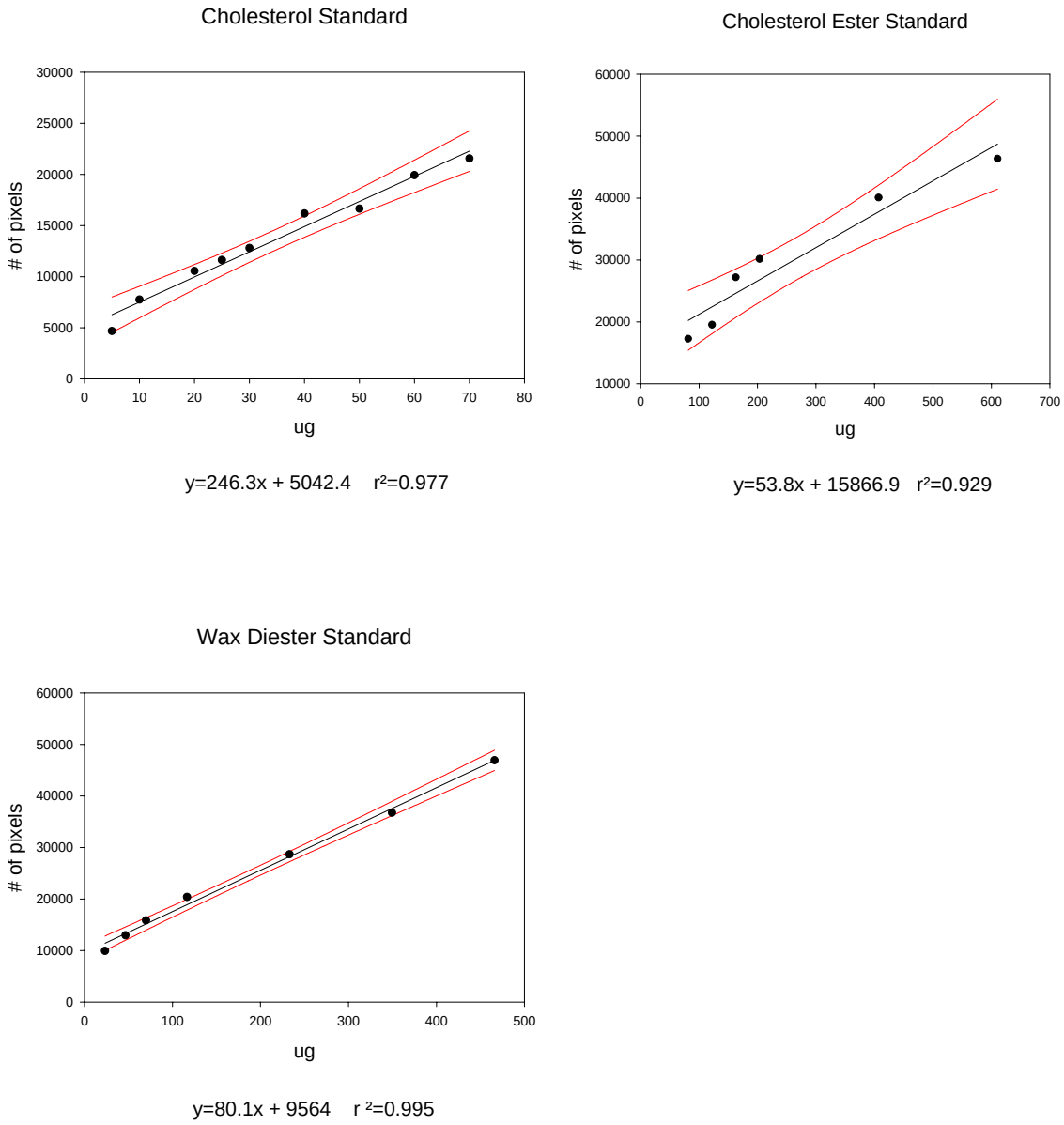

Figure 3.1 Standard curves and linear regression analysis, with 95% confidence interval, of the primary lipid constituents found in hair sebum of beagles and hounds.

thin layer chromatography (TLC) glass plates (20x20 silica gel G coated, 250 μm thickness, Fisher Scientific, Pittsburgh, PA) were washed in chloroform:methanol ($CHCl_3$:CH_3OH, 2:1, v/v) and air dried in a plate box containing silica prior to use. These plates were activated on the day of use by placing them in an 110^oC oven (National Appliance Company Model 5510, Portland, OR) for one hour. The plates were stored in a sealed plate box until needed, but were stored no longer than one week.

Lipid extracts were suspended in 500 μL chloroform, 150 μL of which was applied in duplicate to the plates, approximately 0.5 cm from the bottom. Four samples were applied to each plate along with 10 μl of the standard lipid mixture described previously. The plates were developed until the solvent front was 2 cm from the top, after which the plates were dried in a N_2 flow-box. The lipid fractions were then visualized by briefly placing the TLC plate in an iodine vapor chamber. The cholesterol ester (CE) fractions were marked using the tip of a clean glass Pasteur pipette, then the plates were again placed in the N_2 flow-box until the iodine had dissipated. Using a spatula, the CE fractions were scraped from the plate into a clean Teflon-lined screw top test tube using a glass funnel. Subsequently, two mL of 4% sulfuric acid in methanol solution was added to each tube. Samples were transmethylated directly, as described below, or were purged with N_2 gas and stored at -20^oC until time of methylation.

Methylation of Lipid Subfraction Fatty Acids. Two milliliters of 4% H_2SO_4 in CH_3OH was added to the silica gel scrapings in each tube and mixed for one minute on a vortex mixer (Vortex-Genie Model K-550-G, Scientific Industries Inc., Bohemia, NY). Air in the tube was displaced with N_2 gas, and the Teflon-lined screw caps were securely

tightened. The samples were placed in a 90°C water bath (Thelco 182, Model 66570; GCA Precision Scientific, Chicago, IL) for one hour. Tube caps were checked frequently to ensure tightness so that the sample was not lost due to evaporation. Samples were removed from the water bath and allowed to cool for five minutes. Three mL of hexane was then added to each tube and the tubes were then again mixed on the vortex mixer for one minute. All tubes were then spun for 10 minutes at 2800 rpm in a Beckman model TJ-6 Centrifuge. The supernatants, which contained the fatty acid methyl esters (FAMEs), were transferred via glass wool filtration into clean tubes. Samples were purged with N_2 gas and stored at -20°C for subsequent analysis by capillary gas chromatography.

Gas Chromatography. The methyl esters of CE were evaporated to dryness under N_2 gas and resuspended in 30 µl of hexane. Two microliters of each sample was injected into the capillary column (FAMEWAX Crossbond®-PEG; Restek, Bellefonte, PA) of a Hewlett Packard Series II 5890 Gas Chromatograph (Hewlett Packard Co., Palo Alto, CA) equipped for split injection. Helium was used as the carrier gas. The oven temperature program was as follows: 150°C, initial temperature held for 10 min; raised to 220°C with 1.5°C/min; 220°C held for 10 min; raised to 250°C with 10°C/min; 250°C held for 10 min. This high temperature used to burn off unwanted fatty acids from the column and cleanse it before the next sample is injected. The total time period from start to finish of one sample was 79.67 minutes. Results were generated from Hewlett Packard's HP Chem Station software package. Authentic fatty acid methyl ester standards (Nu-Check-Prep, Elysian, MN) were used to help identify corresponding fatty

acid peaks via comparison of retention times. Relative area percents for each fatty acid were calculated.

Statistical Analysis. Hester (56) in our lab conducted skin and hair condition evaluations on the dogs in this same study using an original scoring system with a 1-5 scale, 5 being the best. Integer values were assigned to each of the following parameters, glossiness, greasiness, scale, softness, and overall coat quality (Subjective Integer Assessment, SIA). A tick mark scoring system was also applied in which a mark was made on an equidistant 1-5 number line to generate continuous data (Subjective Tick Mark Assessment, STMA).

Statistix 7.0® (Analytical Software, Tallahassee, FL) was used to analyze the data for significant differences of sample means, specifically CH, CE, WD, and CE/WD. Statistical significance was determined by using an analysis of variance (ANOVA) for time, diet, and time*diet. Tukey's multiple comparisons test was then used at $p < 0.05$ when appropriate to identify where significant differences occurred. A one-way ANOVA was used to compare the diet effects at the end of Phase II (week 11).

Results

Initially, the nine female beagles and fifteen male hound mixed-bred dogs plucked and shed hair lipid samples were extracted separately and fractionated into their subclasses via thin-layer chromatography. The lipids from individual dogs, when plucked and shed samples were compared, were found to have virtually identical lipid class compositions (Figure 3.2). For this reason it was decided to combine the plucked and shed hair lipid extracts to obtain larger amounts of lipid for further analysis. In

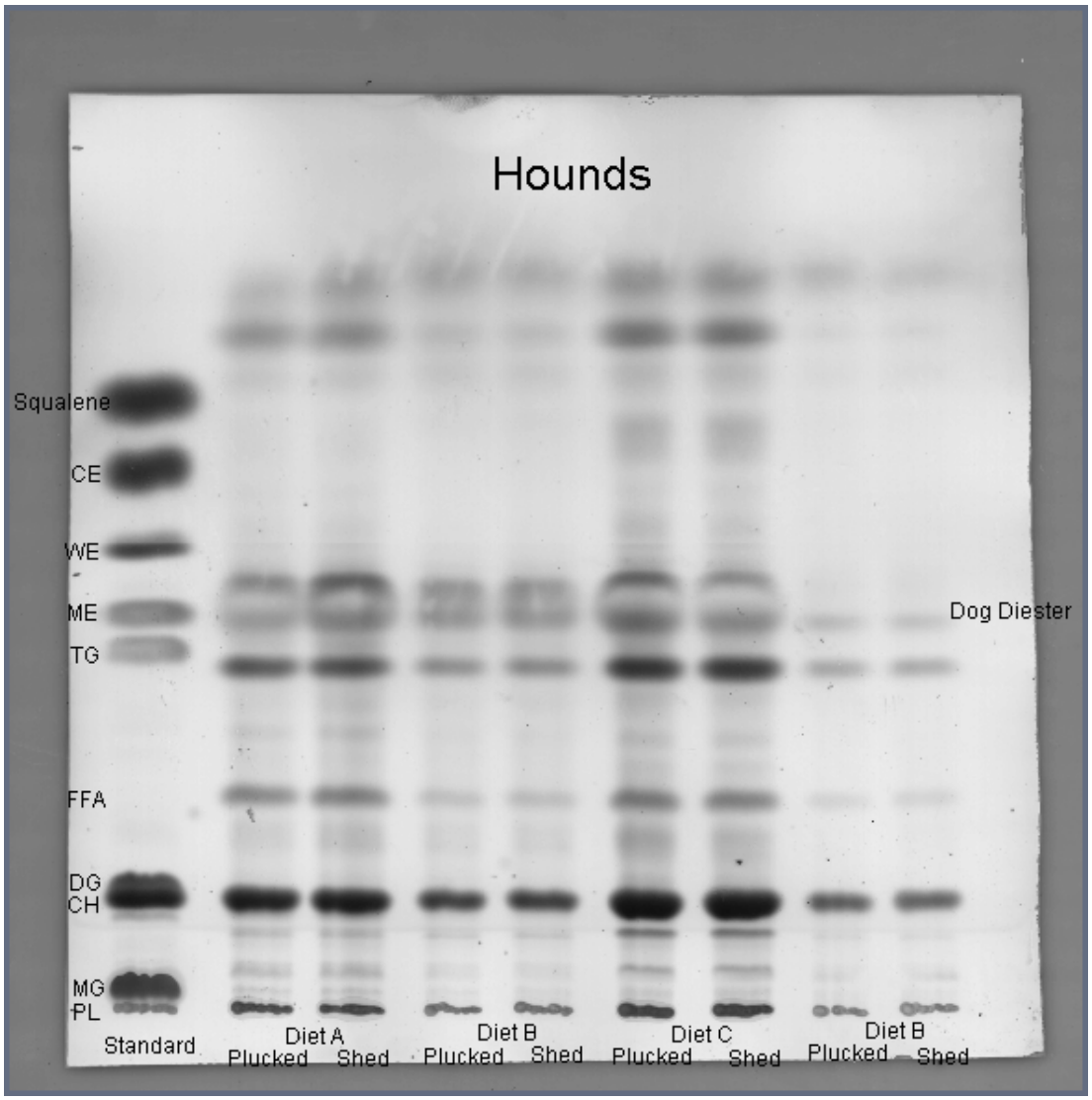

Figure 3.2 Thin layer chromatograms of lipids extracted from the plucked and shed hair samples of individual male hound mixed-breed dogs. Names and abbreviations: cholesterol ester (CE), wax ester (WE), methyl ester (ME), triglyceride (TG), free fatty acid (FFA), diacylglyceride (DG), cholesterol (CH), monoglyceride (MG), and phospholipid (PL).

addition, due to the small quantity of plucked and shed hair samples that were collected, the lipid extracts of beagles and hound mixed-breed dogs that were fed either diet A, B, or C, were pooled to provide better resolution of fatty acids on the TLC plates.

Results obtained found that the major surface epidermal lipids in the dog to be cholesterol esters (CE), wax diesters (WD), and cholesterol (CH). Similar findings have been previously reported by Dunstan et al., Sharaf et al., and Burton et al. (1, 15, 18). Other minor lipid components which were not quantified in this investigation included; triglycerides, free fatty acids, monoglycerides, phospholipids, and some unknown components, which were not identified. The thin layer chromatograms of lipid extracts from hair collected during Phase II (Diets A, B, and C) of both the beagles and hounds, revealed a substantial decrease in CE concentration at week 1 compared to week 11 resulting in a low CE/WD at the earlier time period (Figure 3.3 and 3.4). This change in sebum lipid composition was also seen in the thin layer chromatograms of samples collected at Phase II, weeks 3 and 7 (chromatograms not shown).

Using the equations developed from the standard curves and linear regression analysis (Figure 3.1), CH, WD, and CE concentrations were quantified (Table 3.1). The effects of time, diet, and time*diet on CH, WD, CE, and CE/WD production were also evaluated for Phase II (Diet A, B, and C). Statistically significant time effects ($p < 0.05$) were observed for mean CE and mean CE/WD contents. In addition, mean CH and mean WD were observed to be lower over time, however these results were not significantly different (Table 3.2). Because breed could not be blocked in this experiment for statistical analysis due to concomitant gender differences, graphical

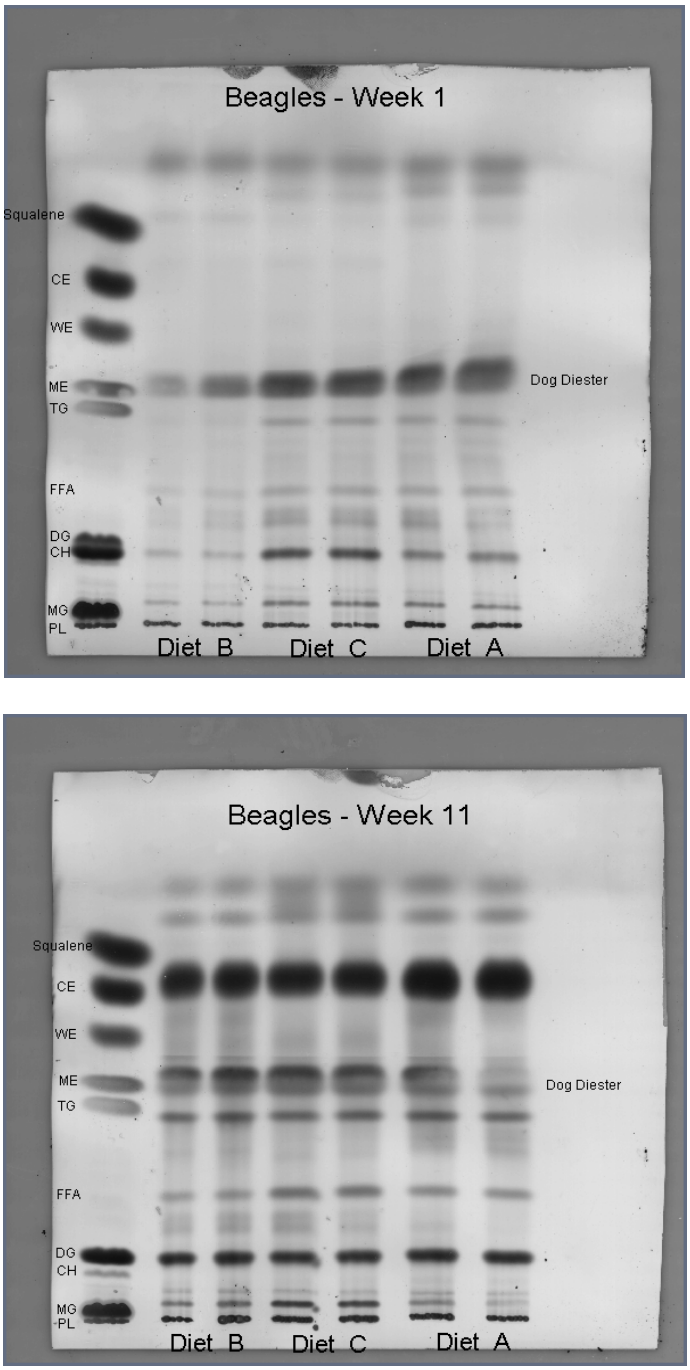

Figure 3.3 Thin layer chromatograms of lipids, ran in dulicates, acquired from the combination of plucked and shed hair samples of the female beagle dogs. Note the lack of any detectible CE at week 1 versus week 11. Names and abbreviations: cholesterol ester (CE), wax ester (WE), methyl ester (ME), triglyceride (TG), free fatty acid (FFA), diacylglyceride (DG), cholesterol (CH), monoglyceride (MG), and phospholipid (PL).

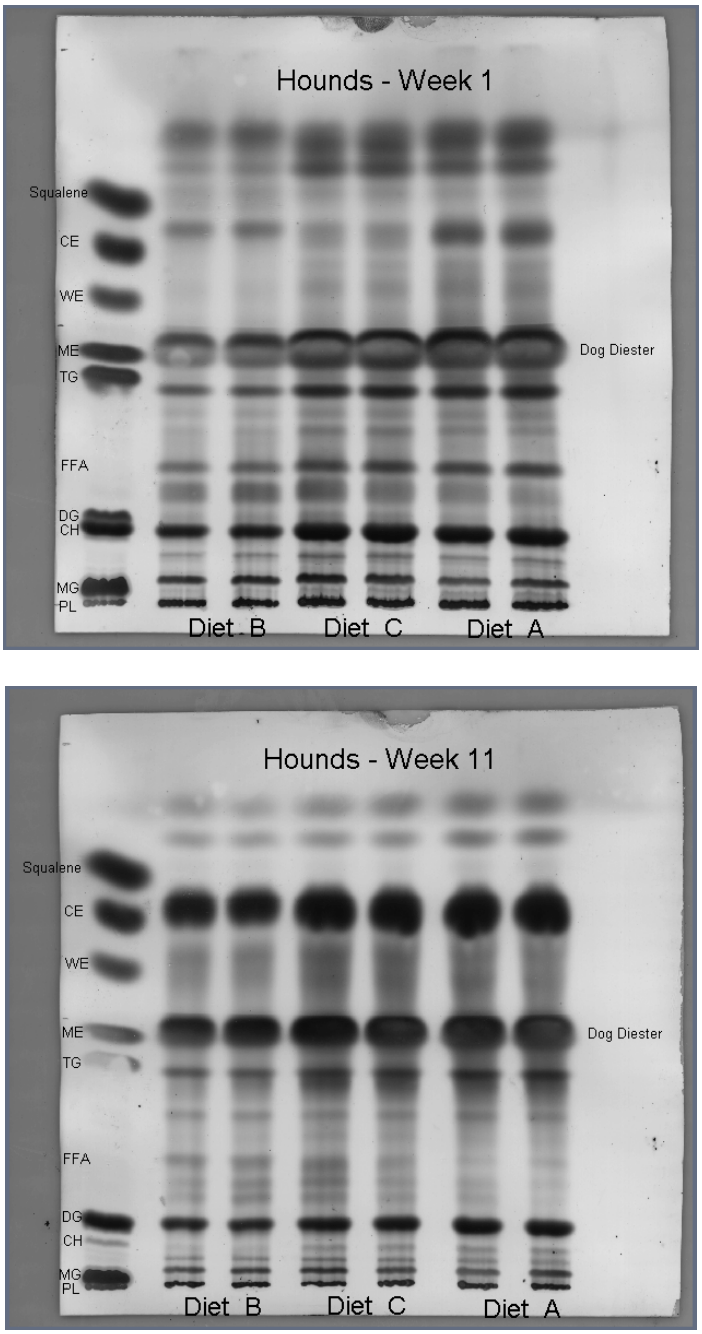

Figure 3.4 Thin layer chromatograms of lipids, ran in duplicates, acquired from the combination of plucked and shed hair samples of the male hound mixed-breed dogs. Note the small amount of CE at week 1 versus week 11. Names and abbreviations: cholesterol ester (CE), wax ester (WE), methyl ester (ME), triglyceride (TG), free fatty acid (FFA), diacylglyceride (DG), cholesterol (CH), monoglyceride (MG), and phospholipid (PL).

Table 3.1 Comparison of the mean values of cholesterol, cholesterol ester, wax diester values, and cholesterol ester to wax diester ratio over time and between diets in beagle and hound mixed-breed dogs

	Beagles				Hounds			
Diet	**CH***	**CE***	**WD***	**CE/WD**	**CH***	**CE***	**WD***	**CE/WD**
A								
1	1.52	0.0[a]	19.39	0.0[a]	0.69	2.86[a]	4.73	0.60[a]
3	2.1	20.5[b]	17.32	1.18[b]	0.74	7.31[b]	7.3	1.0[b]
7	1.22	13.65[b]	11.17	1.22[b]	1.64	13.3[b]	15.64	0.85[b]
11	0.70	8.61[b]	6.37	1.35[b]	0.80	10.89[b]	6.33	1.72[b]
B								
1	2.40	0.0[a]	21.45	0.0[a]	1.13	2.0[a]	9.76	0.21[a]
3	2.90	14.61[b]	19.89	0.73[b]	0.89	6.85[b]	7.49	0.91[b]
7	2.01	16.66[b]	21.64	0.77[b]	1.76	14.95[b]	17.17	0.87[b]
11	0.86	8.73[b]	8.58	1.02[b]	1.17	13.62[b]	9.87	1.38[b]
C								
1	1.73	0.0[a]	16.68	0.0[a]	0.54	1.26[a]	3.64	0.35[a]
3	1.42	13.23[b]	11.36	1.16[b]	0.77	6.31[b]	6.76	0.93[b]
7	2.34	17.64[b]	18.72	0.94[b]	0.74	7.8[b]	5.88	1.33[b]
11	1.13	9.58[b]	9.27	1.03[b]	0.67	9.26[b]	5.17	1.79[b]

Each value is the average of 2 separate determinations.
Superscript letters in a column not in common for each day among the diets are significantly different at $p < 0.05$.
* Mean values are in µg of lipid per mg of hair.

Table 3.2 General ANOVA, P-values for mean cholesterol, mean cholesterol ester, mean wax diester, and mean cholesterol ester to wax diester ratio

	Mean CH	**Mean CE**	**Mean WD**	**Mean CE/WD**
Diet (A, B, C)	0.1631	0.3966	0.1064	0.0947
Week (1, 3, 7, 11)	0.1529	0.0004	0.0729	0.0003

Comparisons between weeks 1 and 11 of Phase II for beagle and hound dogs combined. P-values less than 0.05 are statistically significant.

representations of these results represent the mean values of both beagles and hounds (Figure 3.5). These graphs depict statistically significant increases of CE and CE/WD ratio occurring between week 1 compared with weeks 3, 7, and 11. It should be noted that the increase in CE and CE/WD ratio are consistent with improvement in skin and hair coat scores obtained by Hester (56) in our laboratory during this same experiment. Results from both scoring techniques previously described (SIA and STMA), generally demonstrated significant improvements in all diet groups for all parameters over time. For example, when week 1 data was compared to week 11 of Phase II, statistically significant effects were observed for overall score, glossiness, and softness. The mean values, over time, of dogs fed each of the 3 diets from Hester (56) can be found in Appendix C (Figures C-I and C-II).

The polyunsaturated fatty acid profile obtained from gas chromatography analysis of hair sebum CE revealed no remarkable differences in fatty acid composition of dogs fed the various diets (Tables 3.3 and 3.4). However, there appeared to be a large percentage of a fatty acid that has been tentatively identified as having 19 carbon atoms and 2 double bonds (19:2). Identification of this fatty acid as 19:2 is based upon its chromatographic retention time coinciding with the authentic 19:2 fatty acid standard and a plot of the log (retention time) vs. carbon number (Figure 3.6). This latter relationship is known to be linear for each series of fatty acids containing the same number of double bonds. A fatty acid methyl ester standards chromatogram and one comparison chromatogram from hair CE fatty acids of beagles collected after being fed diet B for 11 weeks is shown for comparison purposes (Appendix D).

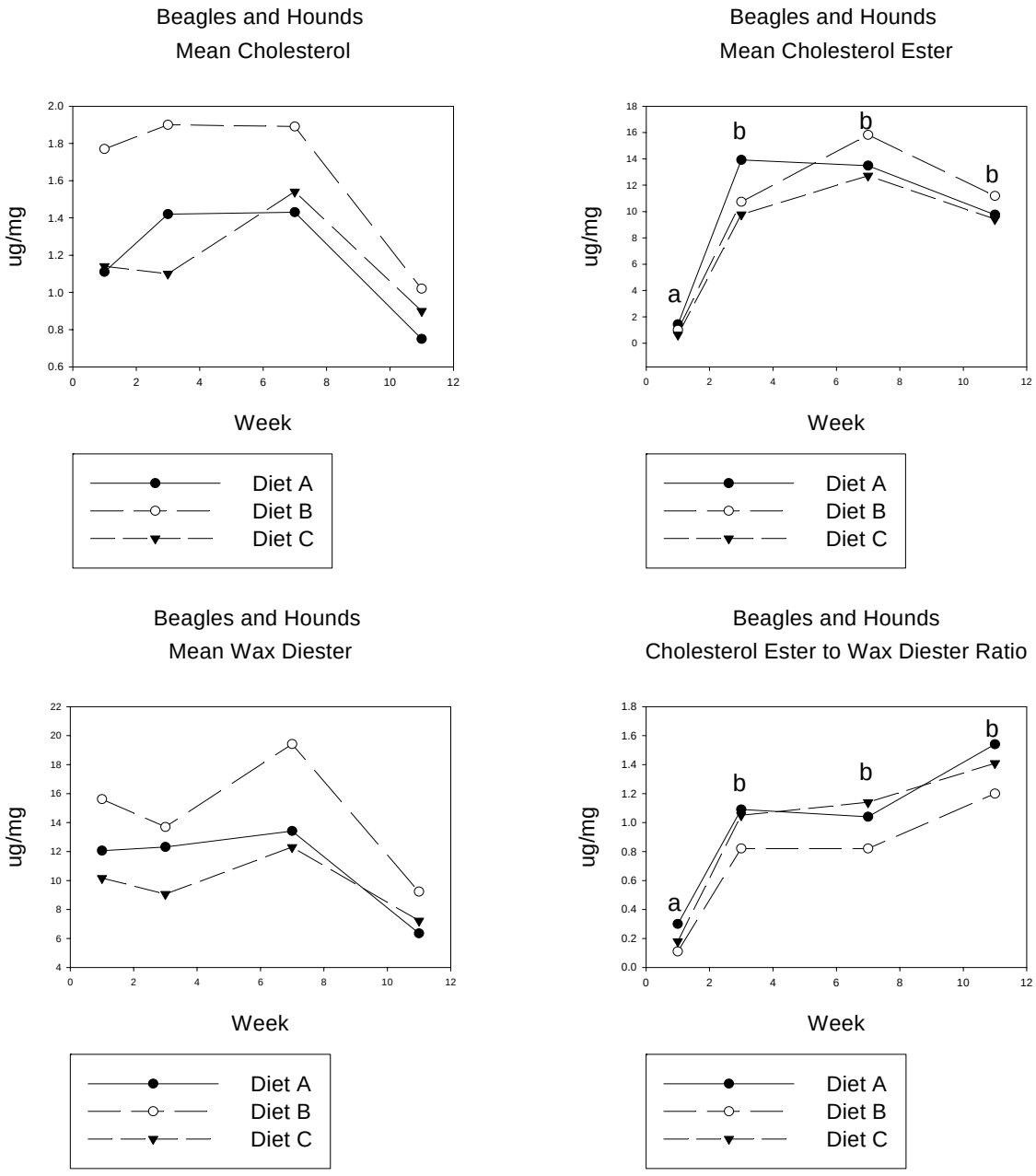

Figure 3.5 Comparison of Phase II (Diets A, B, C) mean values for hair sebum lipids over time in beagle and hound dogs combined. Mean values for CH, CE, and WD are in μg of lipid per mg of hair. Each data point is the average of 2 determinations. Letters not in common for each graph indicate significant difference over time for all diets combined by repeated measures ANOVA ($p < 0.05$).

Table 3.3 Comparison of hair sebum CE fatty acid analysis over time and between diets in pooled beagle samples

Beagles												
	Diet A				Diet B				Diet C			
Week	**1**	**3**	**7**	**11**	**1**	**3**	**7**	**11**	**1**	**3**	**7**	**11**
15:0	5.06	5.51	8.68	6.34	5.03	6.08	6.90	6.84	7.80	10.21	7.25	7.36
16:0	3.39	2.07	2.45	0.68	8.96	3.77	2.05	1.81	2.90	2.95	1.59	0.69
18:0	5.67	4.02	6.22	3.27	11.30	4.88	4.51	2.98	4.47	3.77	3.57	2.18
18:1n9	0.74	0.40	0.83	0.27	1.08	0.34	0.38	0.082	0.24	0.29	0.19	0.39
18:1n7	0.54	0.28	0.27	0.27	0.74	0.38	0.42	0.21	0.53	0.51	0.40	0.18
18:2n6	10.23	10.31	12.66	11.45	10.77	9.64	10.05	10.83	10.81	10.87	8.74	10.80
18:3n6	1.88	2.16	2.27	2.65	1.67	1.97	1.94	2.15	2.20	2.40	2.58	3.53
18:3n3	0.36	0.45	0.45	0.44	0.79	0.60	0.46	0.39	0.42	0.42	0.45	0.30
19:2 ?	7.93	12.28	9.82	11.40	6.58	11.60	12.16	13.15	7.43	8.99	12.41	14.76
20:0	3.73	5.91	4.56	5.35	3.51	5.63	5.15	5.01	5.21	4.50	4.50	2.82
20:1 ?	0.45	ND	ND	ND	2.88	ND	0.37	ND	0.53	0.39	0.42	ND
20:2n6	10.41	15.09	13.67	14.24	9.77	14.25	13.77	15.01	14.18	14.07	14.14	11.74
20:3n6	5.02	7.19	6.48	8.09	5.06	6.92	6.53	7.02	7.81	7.90	10.37	10.46
20:4n6	3.27	ND	1.59	1.45	4.77	0.60	1.80	2.06	1.10	0.86	1.29	1.16
20:3n3	ND	ND	ND	0.57	0.80	ND	1.11	0.64	0.34	ND	1.00	1.26
20:5n3	4.49	7.55	5.88	6.82	3.81	7.02	7.67	7.45	5.46	5.50	8.62	6.99
22:0	2.48	2.91	2.27	2.60	1.81	2.64	2.71	2.51	2.82	2.14	2.38	1.39
22:4n6	0.91	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
23:0	3.70	4.96	4.79	4.98	4.07	4.67	4.39	4.77	4.94	4.37	4.50	3.04
23:1	1.72	2.54	2.59	2.87	1.80	2.43	2.46	2.48	2.93	2.66	4.07	3.07
22:5n3	2.05	2.74	2.45	3.12	1.64	2.37	2.85	3.21	2.25	2.07	2.69	2.04
22:6n3	2.75	4.09	4.11	0.84	1.12	5.12	0.79	0.47	4.51	3.06	0.41	1.13
24:0	1.47	1.70	1.51	2.14	1.72	1.29	1.40	1.86	1.45	1.00	ND	1.71
24:1	0.30	0.75	0.30	0.35	0.91	0.42	ND	0.21	ND	0.45	ND	0.30

ND = not detected.

Each value represents the relative % composition of CE fatty acid content of duplicate TLC analysis pooled after the methylation step prior to GC analysis.

Table 3.4 Comparison of hair sebum CE fatty acid analysis over time and between diets in pooled hound samples

Hounds												
	Diet A				Diet B				Diet C			
Week	**1**	**3**	**7**	**11**	**1**	**3**	**7**	**11**	**1**	**3**	**7**	**11**
15:0	3.33	4.78	4.60	4.64	3.05	6.16	5.19	3.53	3.42	5.89	5.76	3.03
16:0	1.90	1.74	1.36	0.97	0.93	2.09	1.27	0.82	0.82	0.95	1.11	0.66
18:0	4.67	5.65	5.02	4.64	3.12	4.12	4.53	4.03	3.58	3.53	3.99	3.89
18:1n9	0.57	0.33	0.14	0.65	0.43	0.39	0.15	0.32	ND	0.23	0.67	0.51
18:1n7	0.10	0.43	0.30	0.12	ND	0.34	0.33	0.10	ND	0.48	0.16	0.19
18:2n6	5.58	6.69	8.21	8.55	7.39	10.47	9.26	9.04	9.09	9.09	7.69	8.15
18:3n6	1.47	1.58	2.23	2.22	2.10	2.85	2.48	2.38	1.85	2.67	1.86	2.04
18:3n3	0.41	0.48	0.57	0.53	0.38	0.42	0.50	0.50	0.49	0.53	0.46	0.51
19:2 ?	12.80	11.48	13.56	14.22	11.99	13.08	14.68	13.73	12.24	14.97	13.36	14.29
20:0	9.67	9.01	8.54	8.06	7.70	5.04	7.40	7.93	6.36	5.31	5.84	7.27
20:1 ?	ND	ND	ND	ND	ND	0.20	ND	ND	0.97	ND	ND	ND
20:2n6	12.12	12.35	12.79	13.38	13.07	12.38	12.57	14.27	13.61	11.64	10.59	12.78
20:3n6	7.09	6.90	8.03	8.20	8.62	7.36	7.61	8.39	5.87	7.64	5.95	6.74
20:4n6	0.60	1.60	0.77	0.72	1.31	0.69	1.27	1.47	3.42	1.74	1.48	1.00
20:3n3	0.29	0.44	0.30	0.57	1.62	0.90	0.39	0.36	1.62	0.47	1.47	1.10
20:5n3	10.44	10.35	8.97	9.39	8.80	7.22	8.92	8.89	7.54	8.73	9.07	9.82
22:0	4.73	4.48	3.52	3.64	3.45	2.33	3.03	3.66	2.47	2.61	2.54	3.45
22:4n6	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
23:0	4.27	4.12	3.54	3.99	4.29	3.53	3.46	4.08	3.80	3.55	3.16	4.15
23:1	3.26	3.62	2.80	2.97	3.36	2.44	2.53	2.77	1.87	2.53	2.18	2.67
22:5n3	3.70	2.17	2.76	2.60	3.19	2.28	2.64	2.62	2.26	2.62	2.88	3.67
22:6n3	1.94	0.68	1.06	1.26	2.33	0.44	1.12	0.66	2.85	0.89	1.56	1.87
24:0	2.51	2.24	1.37	1.45	2.79	1.28	1.05	1.71	3.39	1.90	2.26	2.69
24:1	1.02	0.70	0.35	0.48	0.84	0.39	0.31	0.38	0.22	0.46	0.77	0.83

ND = not detected

Each value represents the relative % composition of CE fatty acid content of duplicate TLC analysis pooled after the methylation step prior to GC analysis.

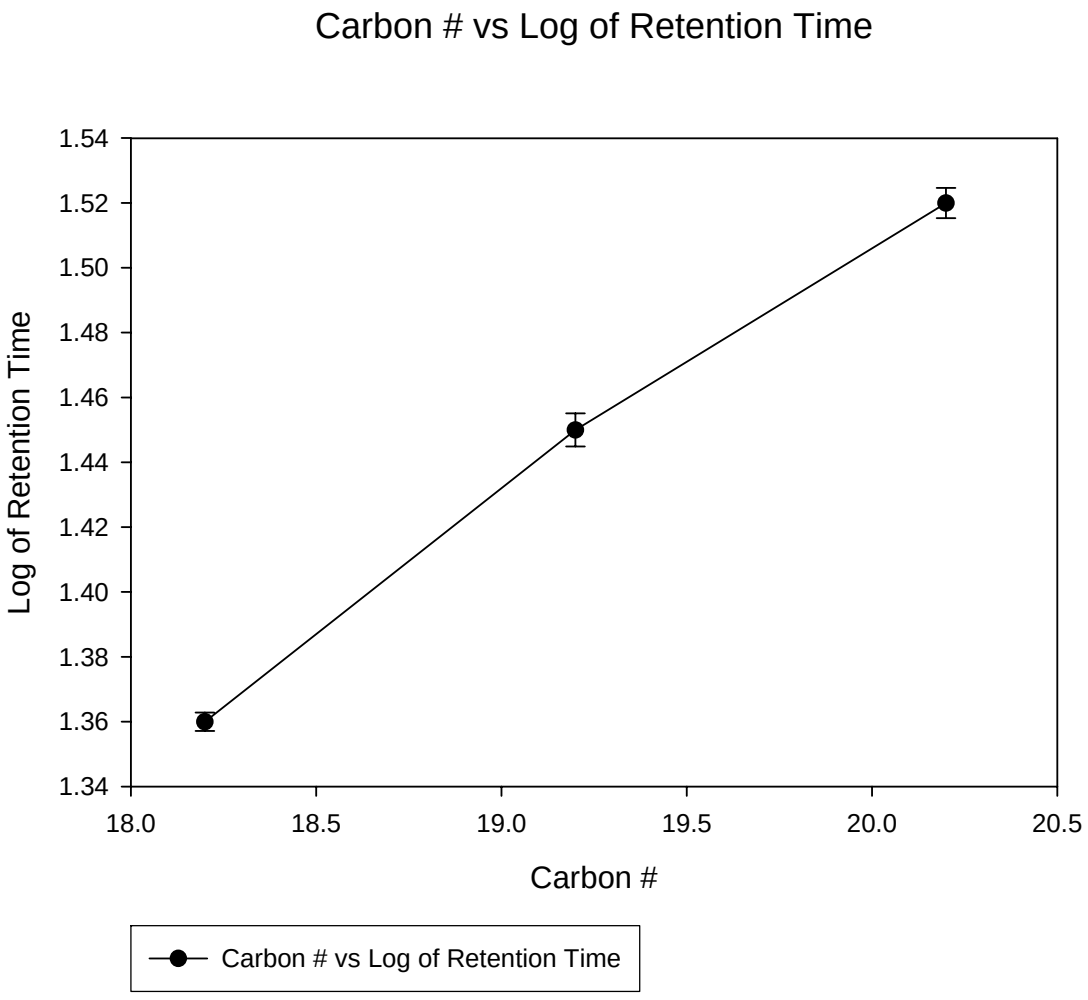

Figure 3.6 Carbon # versus log of retention time of mean values of 18:2, 19:2, and 20:2 of beagle and hound chromatographs combined. Error bars indicate standard deviation.

Discussion

Non-invasive techniques for the objective assessment of skin and hair condition are of considerable interest in veterinary dermatopathology and dermatological research. Although sebum measurements have been more commonly used in assessing human skin disorders, their potential application to veterinary medicine is worthy of investigation. Questions regarding variability, age, body location, gender, and breed effects have yet to be completely answered, but are important factors influencing the volume and class composition of surface lipids.

The technique of thin layer chromatography (TLC) in this study made it possible to fractionate and identified the lipids of interest found on the hair of dogs. The present investigation analyzed the effects of diet, with modified dietary fat and polyunsaturated fatty acids, and time on lipid content of hair that may be related to changes in skin and hair coat scores. From the use of TLC and photodensitometery, initial observations revealed increased amounts of CE and a modest decrease in WD over the 12 week feeding period of Phase II. Even though the decreases in CH and WD were not statistically significantly different, there were statistically significant increases in CE and CE/WD. These increases in CE and CE/WD may be related with the increased dietary fat the dogs had been fed. During the acclimation period (Phase I) the dogs were fed Ol'Roy, which has 9% fat (As-Is). When they were switched to one of the three diets (A, B, or C) in Phase II, their fat composition increased to 13% (As-Is), which was greater than a 30% overall total fat increase. A high fat concentration has been shown to increase CH and CE in plasma neutrophils and presumably other tissues (38, 57). Thus

it is conceivable that under these conditions CE will also be incorporated and found on the hair follicle and sebum of the hair shaft. Such an increase may result in the improvement of skin and hair coat scores observed by Hester (56) in a companion study using these same dogs and diets from our lab. However, because only small amounts of hair and their lipid extracts were available for the present study, it was necessary to pool the samples for analysis. Thus it was not possible to perform regression analysis on the skin and coat scores from Hester (56) with the hair CE concentrations. Further studies will be needed to examine this possibility.

One variable that was not controlled and may have contributed to the observed increase in CE and CE/WD is periodicity of canine hair cycles. Al-Bagdadi et al. (54) reported the appearance of biannual peaks for follicles in both anagen and telogen phases. The anagen phase is the hair follicle growth stage and the telogen phase is the hair follicle resting stage. Peaks for the follicles in anagen have been found to appear during the summer and winter months, while peak values for follicles in telogen appear during the spring and summer months (54). In the present study Phase II diet hair samples were collected from April thru June, coinciding with the periodicity of when the first telogen peak appears (April) and the second anagen peak appears (June). Thus the possibility exists that the hair cycle telogen phase may be associated with low CE and CE/WD values as was observed at the beginning of the study (week 1). By contrast the hair cycle anagen phase may be associated with high CE and CE/WD amounts observed at the end of the study (week 12).

The appearance of a unique fatty acid, tentatively identified as 19:2 from the hair CE polyunsaturated fatty acid profile must be considered speculative until further identification of this fatty acid can be made. Currently, there is not enough information to accurately conclude that this was indeed a straight chain 19 carbon fatty acid with 2 double bonds. Further investigative studies are required, such as the separation, collection, and hydrogenation of this material with resultant gas chromatographic analysis of the resultant 19:0 fatty acid. In addition, collection and identification of its molecular weight via mass spectrometry may also help identify this material. Finally re-chromatographing this fatty acid on a different gas-chromatographic solid phase under different conditions will also help to more specifically identify it. At present, it can only be tentatively identified as 19:2. The possibility exists that it may be a hydroxy fatty acid or one containing a branched chain because of the presence of numerous hyroxylated long chain species found on skin lipids (i.e. wax esters) or its potential synthesis by various bacterial species. Efforts to further identify this fatty acid should be first made by acquiring a significantly larger hair sample size and using techniques for analysis such as those described. In any case the present data indicate that hair is a suitable material to be used for lipid analysis in canine species and for studies designed to better understand the biology and chemistry of fur and its relationship to animal health and welfare. Further studies on this topic will provide better understanding of the relationship between diet, health, canine hair lipid composition, and coat evaluation.

CHAPTER IV

EFFECT OF DIETARY POLYUNSATURATED FATTY ACIDS AND RELATED NUTRIENTS ON CANINE ANAGEN AND TELOGEN PHASES

Introduction

Animal hairs are composed primarily of the protein keratin and can be defined as slender outgrowths of mammalian skin. Each species of animal possesses hair with characteristic length, color, shape, root appearance, and internal microscopic features that distinguish one animal from another. Considerable variability also exists in the types of hairs that are found on the body of an animal. The pattern of mammalian hair cycle can be separated into two types: mosaic and wave. Mammals that demonstrate a mosaic pattern hair cycle are dogs, cats, guinea pigs, and humans. Animals with a wave pattern consist of the mouse, rat, hamster, chinchilla, and rabbit (58-60).

The pattern of growth and rest and the rate of growth vary from species to species and sometimes in the same animal from one part of the body to another. The entire body of the dog, the sheep, the cow, and the horse is covered with a hairy coat. The whale and the hippopotamus have only a few hairs. In humans, hair is not found on palms of the hands or the soles of the feet. The skin, which is considered the largest organ of any species is 24% body weight of puppies and 12% body weight of adult dogs (61-62). Canine skin is an important mechanical and biological protector for the dog's body. It is also an important sensory structure, conveying information about touch, pressure, pain, and environmental temperature. The canine skin is thickest over the

neck, back, and thinnest in the axilla, groin, and pinna. Below the skin is the hair root, which is enclosed by a sack-like structure, called the hair follicle (61). Tiny blood vessels at the base of the follicle provide nourishment. A nearby gland, i.e. sebaceous gland, arises from the walls of hair follicles and produces an oily substance called sebum that lubricates the skin and hair. The presence of sebum is what gives dogs hair coat a glossy sheen appearance.

In dogs the growth of hair is a cyclic process. The amount of shedding that an individual dog shows is dependent on age, nutritional status, physiological status (estrus), stress, housing conditions, and season of the year (30). However, there are two periods of maximum replacement in the year – spring and autumn (63). Apparently, when compared to environmental temperatures (seasonality), photoperiod has a stronger influence on hair growth and shedding of dogs (61). This is because the dog cyclic phases can be manipulated similarly whether natural or artificial light is used. As day length shortens, the rate of growth decreases and hairs complete their entire growth cycle in a longer time period. As a result, shedding decreases and the dog develops its "winter" coat. As day length increases, rate of hair growth increases and shedding occurs at a faster rate (i.e. the dog loses its winter coat) (Figure 4.1) (30). For example, up to 50 per cent of hair follicles may be in telogen in the summer and may increase to 90 per cent in winter. When dogs are kept indoors under artificial light, however, shedding often occurs throughout the year, and dogs never develop a heavy winter coat. In fact, what is observed in house dogs and cats is a coarser coat and a decrease in hair

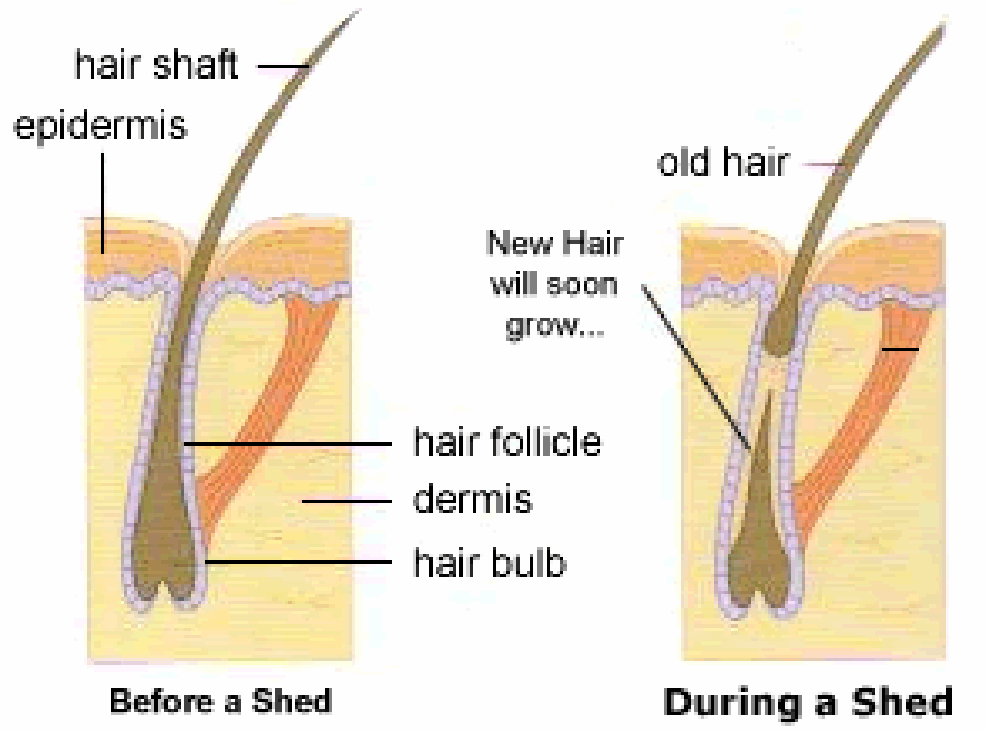

Figure 4.1 Schematic diagram of the human being hair follicle before and after a shed (64).

density (61). The growth cycle is the basis for the cyclic shedding of hair, which provides an advantage in terms of climatic and habitat adaptation.

Besides the environmental factors described, the intrinsic rhythm of the hair cycle is particularly influenced by systemic factors (65-67). The most important being hormones such as, testosterone, estradiol, adrenal steroids, and thyroid hormones (65, 68-72). The pituitary gland controls seasonal pelage changes in molting species. In general, anagen is retarded by estrogen, testosterone, and adrenocorticotropic hormone (ACTH). Conversely, thyroid hormones, initiate anagen and hair growth rate is accelerated (61).

The process of repetitive shedding and replacement of hair involves a three-phase 'hair cycle' denominated: anagen, catagen, telogen (ACT). The anagen phase is considered the growing stage where there is an intact inner sheath and hairs are described as having a blunted (straight across) end. It is also characterized as the rapid mitosis of cells by the dermal papilla, which together create the hair shaft and its surrounding anchoring membrane, the epithelial sheath. Approximately 80-90% of hair follicles will be in the anagen phase at any given time (73). When the hair follicle is passing from the anagen phase into the catagen phase, the melanocytes in the papilla stop synthesizing melanin. The middle part of the bulb becomes constricted, with the base of the hair becoming club shaped and keratinized (63). The hair follicle has now entered the catagen phase.

The catagen phase is a short transitional stage where mitosis ceases and the follicle commences to shut down. In other words, this stage is one of quiescence and

cessation of new hair cell production. This is also the stage where the inner sheath is being actively replaced by trichilemmal cornification. As the lower end of the hair follicle below the club becomes thickened and corrugated, the upper end containing the club hair is pulled upwards in the dermis toward the surface (63). The catagen hairs appear more rounded and smooth than the anagen hairs and come across being tapered with a slight bulbed appearance to it. This stage in dogs is very rare and transient and approximately only 3% (never exceeding 7%) of healthy follicles will be entering this shutting down phase at any given time (54). Because this percentage is small and difficult to determine, the catagen phase is not typically examined in clinical conditions.

The telogen phase is the follicle resting stage, in which the hairshafts are free to vacate. In this stage the trichilemmal cornification has completely replaced the inner sheath, and the hairs have a large bulbed structure on the end. Finally, the telogen stage may last for several weeks or months, until eventually a new anagen phase is initiated. Approximately 12-20% of hair follicles are in this amitotic or resting (telogen) phase at any given time. The entire growth cycle of the hair follicle can be observed in Figure 4.2.

Anagen and telogen percentages and ratios can be useful for looking for trends in the hair cycle. This information can also be compared to gross hair weights of shed hair from each of the dogs. Any significant changes in the ratio and/or amount of shedding would suggest that something different has occurred or that some factor may be affecting the hair cycle. A predominance of one stage of the hair cycle over the other (effluvium) results in hair loss (alopecia). Two forms of effluvium, anagen effluvium and telogen

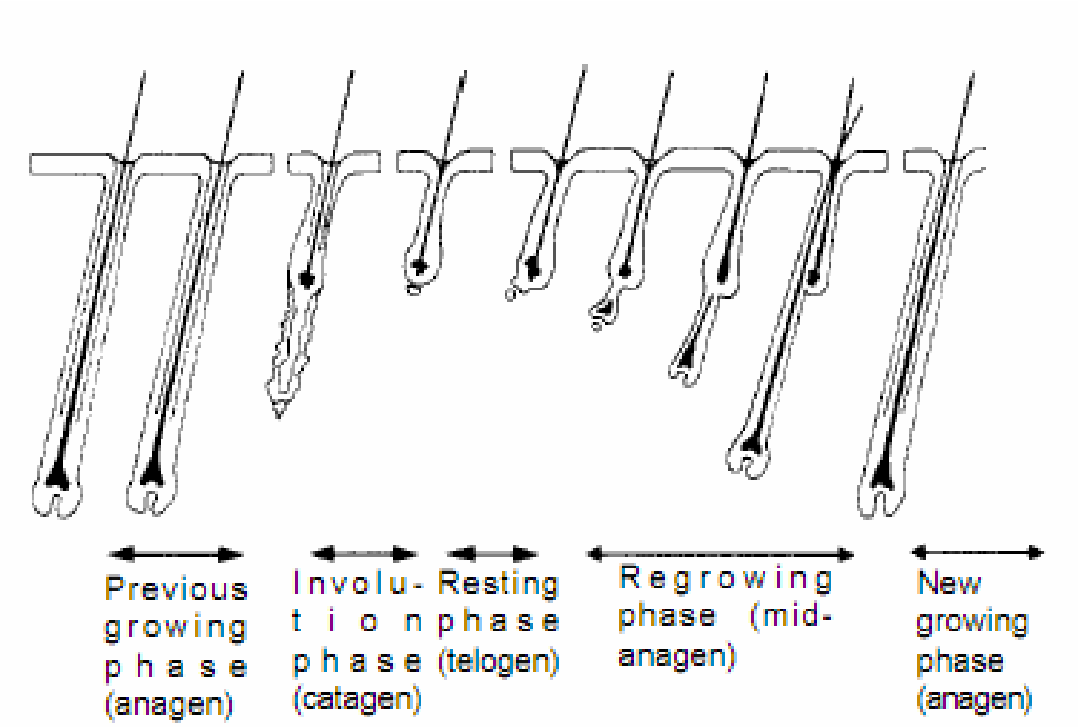

Figure 4.2 Illustrated representation of the human being hair follicle undergoing a growth cycle of alternating active growth and rest stages. Regression of a mature anagen hair follicle occurs upon entering the catagen phase. The dermal papilla condenses as the cells become inactive. With a lack of dermal papilla cell stimulation, the hair fiber and root sheaths stop growing. In telogen the dermal papilla can become isolated in the dermis and the hair fiber can easily be pulled out (by combing, shampooing, or brushing). Upon returning from resting telogen to growing anagen, if the old fiber has not already fallen out it is pushed out by the new hair fiber growing underneath (6). (Reprinted figure with permission from Paramjit Singh, V. Sihorkar, Vikas Jaitely, P. Kanaujia, S. P. Vyas. Pilosebaceous Unit: Anatomical Considerations and Drug Delivery Opportunities. *Indian Journal of Pharmacology* 2000; 32 269-281).

effluvium, have been described in the human and veterinary literature. The only type of effluvium in humans that has been specifically linked to problems in the diet is telogen effluvium. Humans on inadequate or crash diets, with severe protein-caloric restriction have been found to develop telogen effluvium (74-76). If predominance toward the telogen stage of the hair cycle occurs in dogs being fed a particular diet, dogs on that diet would shed and lose hair excessively. An objective in this study is to determine, through hair analysis, whether predominance toward the anagen or telogen phase has occurred in dogs fed various diets with modified polyunsaturated fatty acids and dietary zinc. Another objective is to observe if there is any correlation between the hair cycle stage of the hair follicle and the lipid constituents present in the sebum. Overall, this study will presumably establish any link these two objectives have with the improved in skin and hair coat condition that was observed by Hester (56).

Materials and Methods

Twenty-four dogs, consisting of nine female beagles and fifteen male hound crosses, ranging in age from 1.5 years to 6.5 years, were randomly assigned to one of four diet groups. The diets were formulated to contain the following different amounts of 18 carbon essential fatty acids and zinc: LA contents were 11 g/kg (Diet A) and 33 g/kg (Diet B and C), ALA contents were 1.5 g/kg (Diet A and B) and 12 g/kg (Diet C), and dietary zinc concentrations were 120 mg/kg (Diet A) and 350 mg/kg (Diet B and C). All other dietary components were identical. After having been fed an acclimation diet (Ol'Roy®) for 12 weeks, the dogs were randomly divided and fed one of these three complete and balanced diets for 12 weeks. The dogs were fed daily to maintain adult

body weights. Plucked and shed hair samples were obtained on weeks 1, 3, 7, and 11 (Phase II), as previously described. Hester (56) in our laboratory conducted skin and hair condition evaluations on the dogs using an original scoring system with a 1-5 scale, 5 being the best. Integer values were assigned to each of the following parameters, glossiness, greasiness, scale, softness, and overall coat quality (Subjective Integer Assessment, SIA).

Hair Sample Collection Procedure. Hair samples for the hair sample analysis (trichogram) were collected according to established methods in veterinary dermatological practice. This technique allowed for evaluation of follicular unit morphology and follicular growth dynamics (i.e. anagen-telogen). Hair samples were collected by plucking hair using a hemostat from the lower back and upper part of the rear legs. For our analysis an excess of 100 hairs per dog were needed. Therefore, to obtain a sufficient hair sample size, dogs were plucked between 4 to 6 times. These hairs were placed in labeled envelopes for subsequent analysis. The analysis of the trichogram consisted of placing the plucked hair samples on a slide along with some mineral oil and a cover slip and examining the hairs under the microscope using low power (4X) magnification. The mineral oil was used to help the hairs adhere to the slide. One hundred hairs for each dog at each sample collection period were counted and categorized according to the hair cycle stages – anagen or telogen. Microscopic evaluations of each hair sample for anagen and telogen cycles of all the dogs during the 12 week of sampling of Phase II were performed. This data was used for statistical analysis via repeated measures ANOVA and Tukey's multiple comparisons performed at

$p < 0.05$. A one-way ANOVA was used to compare the diet effects at the end of Phase II (week 11).

Results

On the basis of distinguishing morphologic features, counts of the hair follicle stages were made and recorded. In the anagen stage, the hairs have a blunted (straight across) end, which are smooth, skinny, glistening, and often pigmented (Figure 4.3 (a)). In the telogen stage, the hairs have bulbs that are still attached, which are club- or spear-shaped, rough-surfaced, and nonpigmented (Figure 4.3 (b)). The mean relative percentages of hairs in anagen or telogen, and the anagen to telogen ratio from beagles and hounds of Phase I (Acclimation) and Phase II (Diet A, B, and C) were determined (Table 4.1 and 4.2). Analyses were performed on the effects of diet, time, and diet*time. Using repeated measures ANOVA and significant changes noted (either increase or decrease) when $p < 0.05$ (Table 4.3). Table 4.3 shows a highly significant diet effect on anagen and telogen and statistically significant effect of time on these values. The one-way ANOVA was used to compare the diet effects at the end of Phase II (week 11); however these results were not significant.

Diet B was shown to have a significantly higher mean anagen to telogen ratio, when compared to diets A and C (Figure 4.4 and 4.5). The effect time had on the hair cyclic phases of Phase II are as follows: week 1, when compared to week 11, was observed to have a statistically significantly higher percentage of hairs in the anagen phase; week 1 and 3, when compared to week 11, were observed to have a statistically

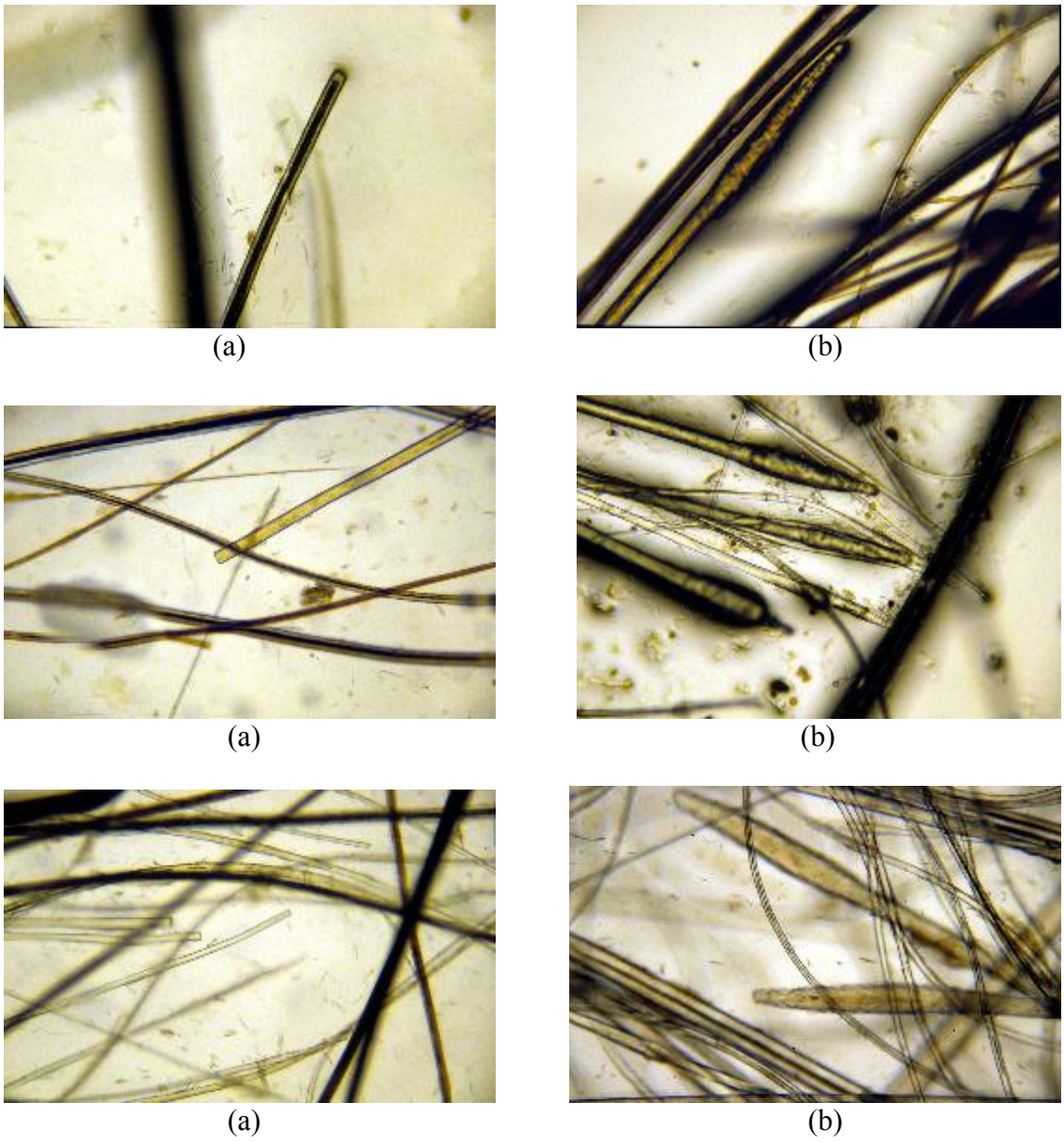

Figure 4.3 Trichograms illustrating the differences between hairs in (a) anagen and (b) telogen phases.

Table 4.1 Comparison of mean anagen, mean telogen, and mean anagen to telogen ratio over time and among diets in beagles

Beagles									
	Diet A			Diet B			Diet C		
Week	Mean Anagen (%)	Mean Telogen (%)	Mean A/T (%)	Mean Anagen (%)	Mean Telogen (%)	Mean A/T (%)	Mean Anagen (%)	Mean Telogen (%)	Mean A/T (%)
Phase I									
3	48.0±2.4	52.2±2.4	1.1±0.09	70.2±1.8	29.8±1.8	3.0±0.38	46.5±0.65	53.8±0.65	0.91±0.02
7	28.7±2.1	71.3±2.1	0.44±0.05	51.0±2.0	49.2±2.0	2.6±0.48	32.5±1.9	67.6±1.9	0.53±0.05
11	35.7±3.0	64.3±3.0	0.81±0.08	49.3±2.5	51.0±2.5	1.7±0.35	27.2±2.7	73.0±2.7	0.41±0.05
Phase II									
1	26.3 ± 1.4^{a}	73.7 ± 1.4^{a}	0.46 ± 0.04^{a}	67 ± 2.03^{a}	33.3 ± 2.0^{a}	8.9 ± 1.7^{a}	26.5 ± 2.0^{a}	73.8 ± 2.0^{a}	0.54 ± 0.08^{a}
3	30.0 ± 1.9^{ab}	70.2 ± 1.9^{a}	0.46 ± 0.04^{ab}	51.7 ± 3.3^{ab}	48.3 ± 3.3^{a}	2.0 ± 0.39^{ab}	22.0 ± 1.7^{ab}	78.2 ± 1.7^{a}	0.35 ± 0.04^{ab}
7	17.7 ± 2.5^{ab}	82.3 ± 2.5^{ab}	0.24 ± 0.03^{b}	29.8 ± 1.4^{ab}	70.3 ± 1.7^{ab}	0.53 ± 0.09^{b}	27.0 ± 1.9^{ab}	73.3 ± 1.9^{ab}	0.42 ± 0.04^{b}
11	17.3 ± 1.1^{b}	82.8 ± 1.1^{b}	0.22 ± 0.02^{b}	36.0 ± 2.3^{b}	64.0 ± 2.3^{b}	0.75 ± 0.06^{b}	17.3 ± 2.9^{b}	82.8 ± 2.9^{b}	0.23 ± 0.07^{b}

Values are presented as mean ± S.D.
Superscript letters not in common in a given column are significantly different at $p < 0.05$.

Table 4.2 Comparison of mean anagen, mean telogen, and mean anagen to telogen ratio over time and among diets in hounds

Hounds									
	Diet A			Diet B			Diet C		
Week	Mean Anagen (%)	Mean Telogen (%)	Mean A/T (%)	Mean Anagen (%)	Mean Telogen (%)	Mean A/T (%)	Mean Anagen (%)	Mean Telogen (%)	Mean A/T (%)
Phase I									
3	56.9±1.3	43.4±1.3	2.4±0.15	50.5±1.9	49.5±1.9	1.6±0.10	39.4±1.7	60.7±1.7	0.92±0.08
7	41.0±2.4	59.0±2.4	1.0±0.12	44.2±1.6	56.0±1.6	1.1±0.08	32.5±1.3	67.7±1.3	0.55±0.03
11	31.0±1.3	69.1±1.3	0.86±0.16	24.6±2.0	75.5±2.0	0.42±0.05	15.5±1.5	84.7±1.5	0.19±0.02
Phase II									
1	32.1±2.1^{a}	68.1±2.1^{a}	0.58±0.06^{a}	27.8±1.9^{a}	72.4±1.9^{a}	0.41±0.04^{a}	25.3±2.6^{a}	74.7±2.6^{a}	0.09±0.05^{a}
3	32.7±2.0^{ab}	67.1±1.9^{a}	0.89±0.09^{ab}	21.0±1.1^{ab}	69.7±1.1^{a}	0.29±0.02^{ab}	24.0±1.4^{ab}	76.2±1.4^{a}	0.39±0.03^{ab}
7	40.2±1.1^{ab}	60.1±1.1^{ab}	0.83±0.04^{b}	28.4±1.6^{ab}	71.7±1.6^{ab}	0.53±0.04^{b}	20.4±1.4^{ab}	80.0±1.4^{ab}	0.30±0.04^{b}
11	25.0±1.9^{b}	75.2±1.9^{b}	0.48±0.08^{b}	25.6±1.7^{b}	74.7±1.7^{b}	0.44±0.03^{b}	22.8±2.1^{b}	77.3±2.1^{b}	0.33±0.04^{b}

Values are presented as mean ± S.D.
Superscript letters not in common in a given column are significantly different at $p < 0.05$.

Table 4.3 General ANOVA, P-values for mean anagen, mean telogen, and mean anagen to telogen ratio

	Mean Anagen	**Mean Telogen**	**Mean A/T Ratio**
Diet (A, B, C)	0.0000	0.0000	0.0053
Week (1, 3, 7, 11)	0.0037	0.0039	0.0196

Comparisons between weeks 12 and 24 (1 to 11) of Phase II feeding period.
P-values less than 0.05 are statistically significant.

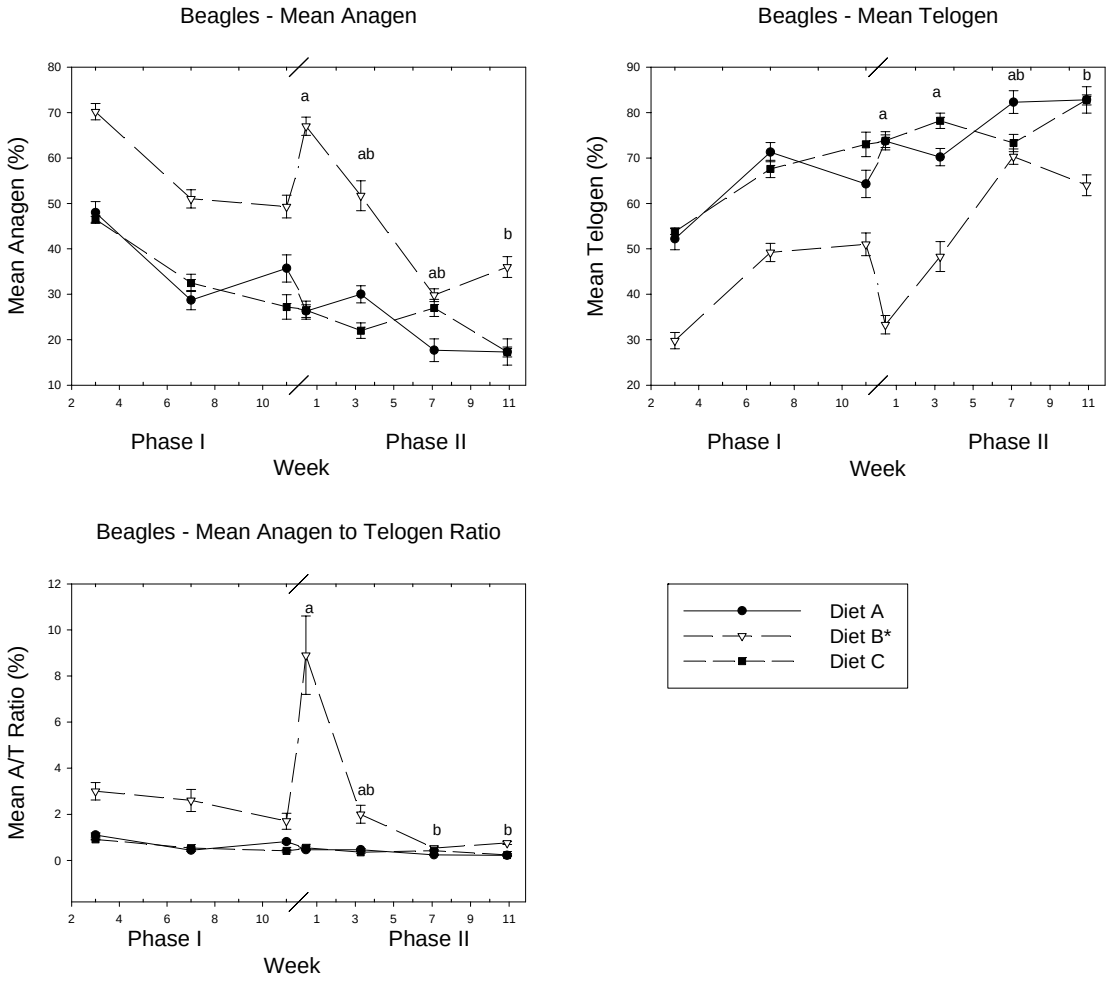

Figure 4.4 Comparison of Phase I (Acclimation) and Phase II (Diets A, B, C) mean values for cyclic hair growth phases over time in beagles. The graphs are based upon the averages of all the beagle dogs, however, the p-values and significant differences are based upon the mean values of each dog (n-9). Letters not in common for each graph indicate significant differences over time for all the diets combined by repeated measures ANOVA ($p < 0.05$). The asterisk (*) denoted by diet B in the legends box indicates significance for all times combined by repeated measures ANOVA ($p < 0.05$) during Phase II.

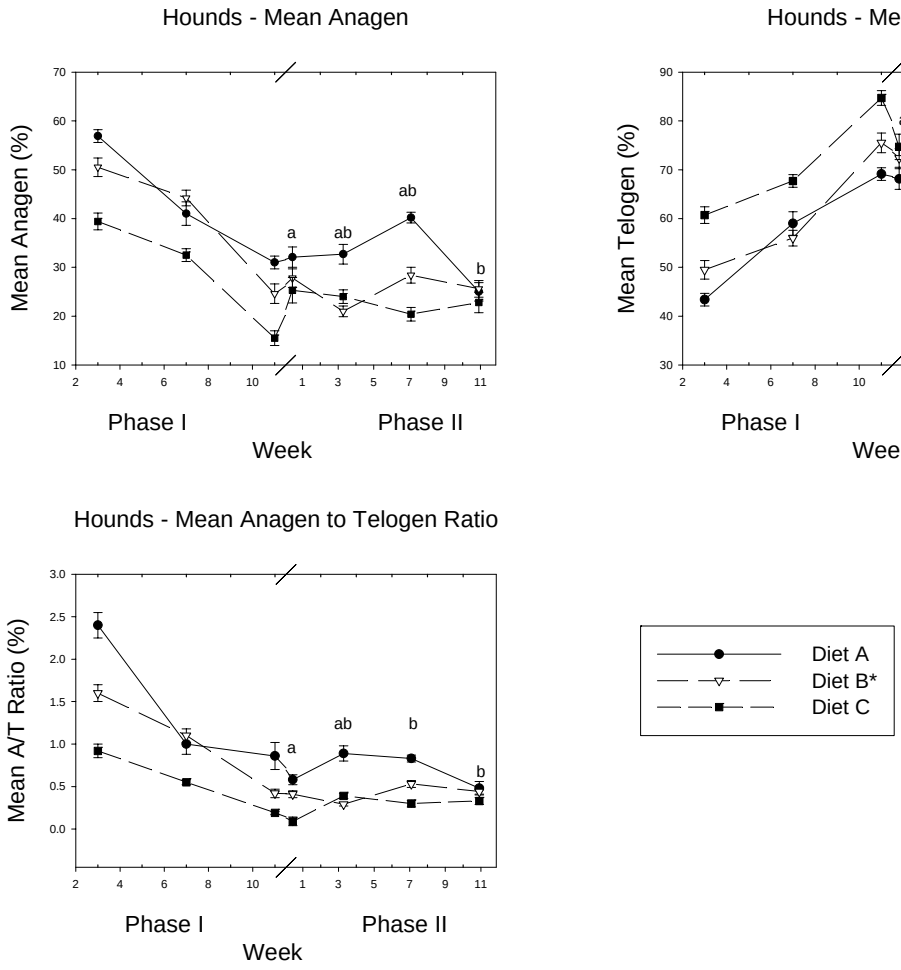

Figure 4.5 Comparison of Phase I (Acclimation) and Phase II (Diets A, B, C) mean values for cyclic hair growth phases over time in hounds. The graphs are based upon the averages of all the hound dogs, however, the p-values and significant differences are based upon the mean values of each dog (n-15). Letters not in common for each graph indicate significant differences over time for all the diets combined by repeated measures ANOVA ($p < 0.05$). The asterisk (*) denoted by diet B in the legends box indicates significance for all time combined by repeated measures ANOVA ($p < 0.05$) during Phase II.

significantly lower percentage of hairs in the telogen phase; week 1, compared to week 7 and 11, was observed to have a statistically significantly higher anagen to telogen ratio.

Discussion

Much attention has been focused on the usefulness of hair analysis as a diagnostic tool, but hair growth is still a wondrous subject that needs much research. Hair, being predominantly made up of protein, can be used as an initial diagnostic tool in determining malnutrition. Poor nutrition may produce a dull, dry, brittle, or thin hair coat, with or without pigmentary disturbances. However, many internal and external variables contribute to its investigative unreliability. These include environmental effects (topical agents, geographic location, occupational exposures), differing hair growth rates (health, drugs, age, gender), and lack of standardization of analysis techniques (60). The hair coat of pet animals is considered a cosmetic or ornamental feature and is disrupted by even the most minimal hair follicle removal procedures; including clipping, shaving, plucking, and combing (61). It has been known for many years that plucking of resting hair from telogen follicles advances the onset of anagen. Thus it has been theorized that the hair cycle is manipulated by locally active inhibitors that accumulate during anagen causing entry into catagen, when present in sufficient concentrations (the Chalone hypothesis) (6, 77-78).

The main feature of the plotted values of the hair cycle was the appearance that all dogs were in the anagen phase at the beginning of Phase II (Diet A, B, and C); however, only diet B dogs stayed in that phase for the entire 12 week diet period, when compared to diets A and C. This finding is consistent with improvements in skin and

hair coat found by Hester (56) and coworkers showing that dogs fed diet B, containing increased amounts of LA and dietary zinc, were observed to have improved in skin and hair coat scores. Inspection of the data in these figures indicate that diet B, maintained hair follicles in the anagen cyclic growth stage for a statistically significant longer period of time. However, dogs fed diet B were also observed to begin the study with a markedly greater number of hair follicles in anagen compared to the other two groups and this difference was statistically significant. Also over time, it appeared that dogs fed diet B also showed increased losses of anagen hair follicles. Thus it cannot be precisely determined whether dogs fed diet B actually remained in anagen for a longer time during the feeding period in spite of the repeated measures ANOVA. The possibility exists that the periodicity of the hair cycle anagen phase of dogs in this study during the period from January through June may have overwhelmed any possibility of a diet effect that may have existed. Studies designed to control for this effect will be needed in order to better identify the role that diet may play in the hair cycle.

CHAPTER V

SUMMARY AND CONCLUSION

Essential fatty acids play a role in every life process in the mammalian body. Linoleic acid, the most abundant PUFA in skin, aids in the maintenance of the skin's permeability barrier, thus controlling excessive water loss, preventing dry, scaly skin and preserving its softness and elasticity. This effect can also be seen with the addition of zinc, an essential nutrient that contributes to the prevention of dry flaky skin and hair loss.

Initially, a preliminary study lasting 7 weeks was performed to assess the differences in lipid subfractions between hair sebum and skin surface sebum. Three Laborador Retrievers were used to compare sebum extracted from Sebutape to sebum extracted from plucked hair samples. Results concluded that the two forms of sebum had identical lipid constituents and that hair is a suitable material to analyze and appears to contain a greater CE/WD amount than epidermal sebum. The skin lipid analysis provided the basis for the present study that investigated the effect of dietary polyunsaturated fatty acids and related nutrients on sebum lipids and skin and hair coat condition in canines.

The objectives of this research were to investigate the effects of diet with varying ratios of LA, ALA, and dietary zinc on the following: 1) hair sebum lipid constituents; 2) sebum CE fatty acid composition of adult canines during a 12 week feeding trial; 3) hair cycle transition stages of follicular hairs during a 12 week acclimation phase and a

12 week feeding trial; 4) the potential improvements on skin and hair coat condition under the above conditions.

The study was divided into two 12 week periods (Phase I and Phase II). In Phase I, the acclimation phase, the dogs were supplemented with a complete and balanced dog food product (Ol'Roy®). In Phase II, the dogs were divided into three groups and fed one of the three specially formulated complete and balanced diets (A, B, or C). Diet A contained adequate AAFCO amounts of LA and dietary zinc. Diet B contained increased amounts of LA and dietary zinc. Diet C was similar to diet B with respect to LA and dietary zinc concentrations being held constant, but it also contained increased amounts of ALA. It was hypothesized that the lipid content of hair can be altered by fat and fatty acid composition of the diet and may be related to improved skin and hair coat condition.

From the use of TLC and photodensitometery, initial observations revealed similar lipid constituents appearing from the plucked and shed hair samples of canines. Results obtained found that the major surface epidermal lipids in the dog to be CE, WD, and CH. Other minor lipid components which were not quantified in this investigation included; triglycerides, free fatty acids, monoglycerides, phospholipids, and some unknown minor constituents.

Results from the 12 week feeding period of Phase II, showed a modest decrease in WD and statistically significant increases in CE and CE/WD, in all three test diets (A, B, and C). The increase in CE and CE/WD may correlate with feeding diets higher in total fat concentration because high fat diets are known to increase cholesterol

concentrations in plasma. Consequently greater amounts of CE may be incorporated and found on the hair follicle and sebum of the hair shaft resulting in improvement of canine skin and hair coat appearance.

Dogs fed diet B, enriched with LA and dietary zinc, maintained high relative amounts of hair follicular growth in the anagen phase for the entire 12 week diet period (Phase II), compared to diets A and C. This finding is also consistent with improvements in skin and hair coat of dogs fed diet B. Even though dogs fed diet B, sustained hair follicles in the anagen cyclic growth stage for a statistically significant longer period of time, at the beginning of the acclimation phase (Phase I) they were already observed to have a high relative percentage of hair follicles in the anagen cycle. Also over time, it appeared that dogs fed diet B also showed increased losses of anagen hair follicles. Thus it cannot be precisely determined whether dogs fed diet B actually remained in anagen for a longer time during the feeding period.

One variable that was not controlled and could have possibly contributed to the observed increase in CE and CE/WD was periodicity (light). The possibility exists this effect on the hair cycle anagen phase of dogs in this study during the period from January through June may have overwhelmed any possibility of a diet effect that may exist.

The findings of this research are speculative but suggest an association between the hair cycle anagen phase and increased CE and CE/WD that were observed during at the end of the Phase II. These findings also suggest that a diet containing high amounts of LA and dietary zinc can increase CE and CE/WD in the hair sebum and promote

longevity of the anagen phase. Thus such a diet can improve sheen, glossiness, and overall skin and hair coat condition of canines. However, no conclusive evidence has been shown in this study because of seasonal or other effects on the hair cycle. Additional controlled studies on the effects of polyunsaturated fatty acids and other nutrients on sebum constituents and hair follicular growth will be needed.

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APPENDIX A

TABLE A-I

AGES OF DOGS

Names	Age in years (March 2003)
Beagles:	
Spin	6.5
Trinity	6
Sadie	6.5
Belle	4.5
Abbey	4
Ally	6.5
Daisey	6
Tiny	6
Princess	5.5
Hounds:	
Dan	2
Winston	2
Duke	1.5
Pluto	4
Gordy	1.5
Gus	1.5
Stanley	4
Jake	2.5
King	3
Red	4
Sherlock	5.5
Wizzer	2.5
Snickers	4
Houser	3
Trailer	2

TABLE A-II

HAIR WEIGHTS OF DOGS

	Week 1		Week 3		Week 7		Week 11	
	Plucked (g)	Shed (g)	Plucked (g)	Shed (g)	Plucked (g)	Shed (g)	Plucked (g)	Shed (g)
Diet A:								
Spin	0.01603	0.02154	0.03000	0.01400	0.02700	0.00607	0.04264	0.00675
Trinity	0.01582	0.00000	0.02750	0.00000	0.03077	0.01678	0.03915	0.15463
Sadie	0.01529	0.01691	0.02170	0.00100	0.01840	0.00000	0.05274	0.03634
Houser	0.02834	0.21282	0.07470	0.08450	0.02060	0.00833	0.02890	0.02915
Trailer	0.13172	0.20048	0.06350	0.07800	0.01915	0.02890	0.03110	0.10610
Dan	0.01305	0.02070	0.02800	0.02600	0.03690	0.01684	0.07036	0.08835
Gus	0.05608	0.18188	0.02260	0.15600	0.02167	0.02120	0.05343	0.01743
Jake	0.01240	0.00000	0.02520	0.00335	0.04342	0.00725	0.03866	0.08626
Diet B:								
Belle	0.00250	0.00000	0.01340	0.00040	0.02074	0.00328	0.01720	0.02602
Abbey	0.00621	0.00000	0.01510	0.00000	0.02954	0.00320	0.03861	0.05362
Daisey	0.01935	0.00300	0.01970	0.00237	0.02129	0.01134	0.01522	0.02214
Stanley	0.01286	0.06665	0.02650	0.02000	0.02700	0.00608	0.05388	0.05240
Winston	0.04156	0.10979	0.03120	0.05150	0.02150	0.00229	0.02964	0.00494
Duke	0.01503	0.02976	0.01570	0.04200	0.02470	0.00293	0.02753	0.04700
Pluto	0.01759	0.04624	0.02900	0.14600	0.01666	0.02642	0.02666	0.01231
Gordy	0.00355	0.00000	0.02590	0.00000	0.02800	0.03170	0.01887	0.00150
Diet C:								
Ally	0.02203	0.01107	0.03520	0.01600	0.01992	0.00300	0.01780	0.02718
Tiny	0.02771	0.00000	0.04330	0.00150	0.01700	0.00449	0.01194	0.00696
Princess	0.0168	0.00000	0.03030	0.00000	0.04543	0.00103	0.02387	0.11652
King	0.00570	0.16916	0.01480	0.10500	0.02180	0.01172	0.01735	0.07116
Red	0.02342	0.04123	0.02610	0.05120	0.01533	0.01174	0.01340	0.02282
Sherlock	0.06330	0.53548	0.04690	0.18400	0.02261	0.04838	0.02674	0.13480
Wizzer	0.01565	0.10881	0.03700	0.04100	0.01966	0.00765	0.03590	0.06892

APPENDIX B

TABLE B-I

DIET COMPOSITION

Linoleic acid, alpha-linolenic acid, and zinc content of Phase II diets (energy basis and g/kg as-is)

	Zinc	**Linoleic Acid**		**Alpha-Linolenic Acid**	
Diet	mg/kg	Energy %	g/kg	Energy %	g/kg
A (tallow)	120	2.43	11	0.22	1.5
B (sunflower)	350	8.77	33	0.22	1.5
C (flaxseed)	350	8.77	33	1.63	12

TABLE B-II

NUTRIENT ANALYSIS OF DIETS

Nutrient	Ol'Roy	Diet A	Diet B	Diet C
Moisture	12.0%	6.2%	6.6%	6.2%
Protein	21.0%	22.9%	22.3%	22.9%
Fat	9.0%	13.1%	12.9%	13.3%
Ash	8.2%	8.8%	8.6%	8.8%
Fiber	4.0%	2.1%	2.1%	2.7%
Carbohydrate	45.8%	46.9%	47.5%	46.1%
Calories kcal/100g	325	357.9	356.5	356.9

Amounts shown as % as-is basis

TABLE B-III

INGREDIENTS OF DIETS

Ol'Roy	Diet A	Diet B	Diet C
Ground Yellow Corn	Lamb Meal	Lamb Meal	Lamb Meal
Meat and Bone Meal	Ground Rice	Ground Rice	Ground Rice
Soybean Meal	Rice Flour	Rice Flour	Rice Flour
Wheat Middling	Rice Bran	Rice Bran	Rice Bran
Animal Fat	***Beef Tallow***	***Sunflower Oil***	***Ground Flax Seed***
Chicken By-Product Meal	Natural Flavor	Poultry Fat	Poultry Fat
Brewers Rice	Rice Gluten	Natural Flavors	Natural Flavors
Animal Digest	Dried Egg Product	Rice Gluten	Rice Gluten
Salt	Dried Beet Pulp	Dried Egg Product	Dried Egg Product
Calcium Carbonate	Potassium Chloride	Dried Beet Pulp	Dried Beet Pulp
Choline Chloride	L-Lysine	Potassium Chloride	Potassium Chloride
Zinc Sulfate	Dried Kelp	L-Lysine	L-Lysine
Ferrous Sulfate	Salt	Dried Kelp	Dried Kelp
Vitamin E Suppl.	Choline Chloride	Salt	Salt
Zinc Oxide	Zinc Sulfate	Choline Chloride	Choline Chloride
Niacin	Vitamin E Suppl.	Zinc Sulfate	Zinc Sulfate
Copper Sulfate	Taurine	Vitamin E Suppl.	Vitamin E Suppl.
Manganous Oxide	Ferrous Sulfate	Taurine	Taurine
Vitamin A Suppl.	Ascorbic Acid	Ferrous Sulfate	Ferrous Sulfate
Calcium Panthothenate	Biotin	Ascorbic Acid	Ascorbic Acid
Biotin	Copper Proteinate	Biotin	Biotin
Vitamin B12 Suppl.	Niacin	Copper Proteinate	Copper Proteinate
Pyridoxine Hydrochloride	Manganous Oxide	Niacin	Niacin

TABLE B-IV

FATTY ACID PROFILE OF EXPERIMENTAL DIETS

Fatty Acid	Ol' Roy	Diet A	Diet B	Diet C
12:0	0.45	ND	ND	ND
14:0	0.94	2.10	1.00	0.62
14:1	0.20	0.34	0.19	0.11
15:0	0.16	0.35	0.15	0.11
15:1	ND	ND	ND	ND
16:0	23.22	22.52	17.79	14.15
16:1	5.37	2.50	3.37	2.47
17:0	0.35	0.92	0.37	0.32
17:1	0.20	0.54	0.25	0.19
18:0	6.72	14.78	7.54	7.40
18:1(n-9)	34.09	36.15	33.69	34.09
18:1(n-7)	3.44	3.72	2.42	ND
18:2(n-6)	24.71	10.17	30.19	27.82
18:3(n-3)	1.17	0.69	0.86	6.82
20:0	0.40	0.78	0.30	1.25
20:1(n-9)	0.09	0.14	0.12	0.49
20:2(n-6)	0.30	0.11	0.44	0.11
20:4(n-6)	0.25	0.12	0.36	0.04
20:5(n-3)	0.33	0.13	ND	0.36
22:0	ND	ND	ND	ND
22:1(n-9)	ND	ND	ND	ND
22:5(n-3)	0.10	0.19	0.34	0.06
22:6(n-3)	0.24	0.47	0.08	0.31
24:0	0.08	0.09	0.12	0.04

Fatty acids reported as relative percentage composition
ND = not detected

APPENDIX C

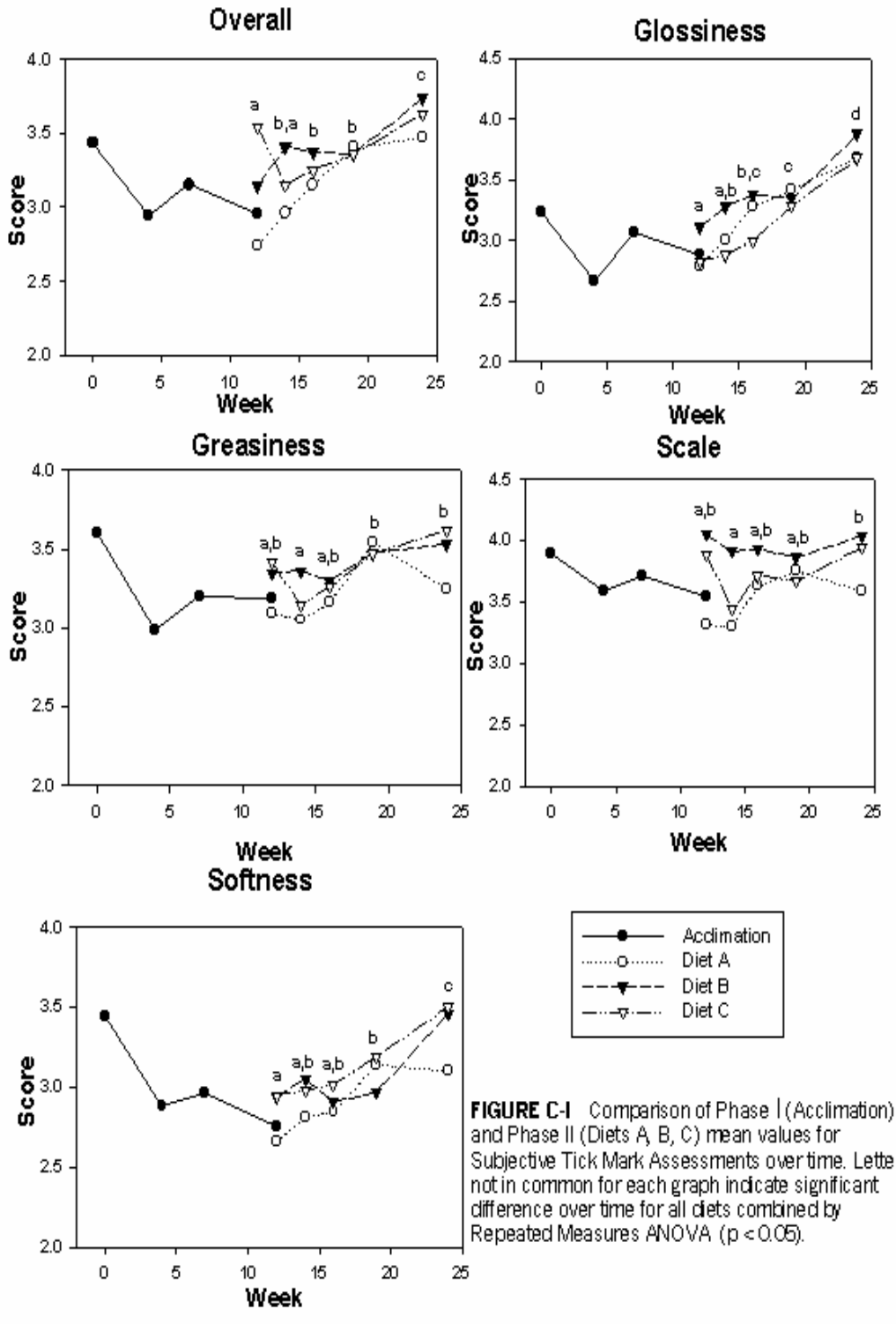
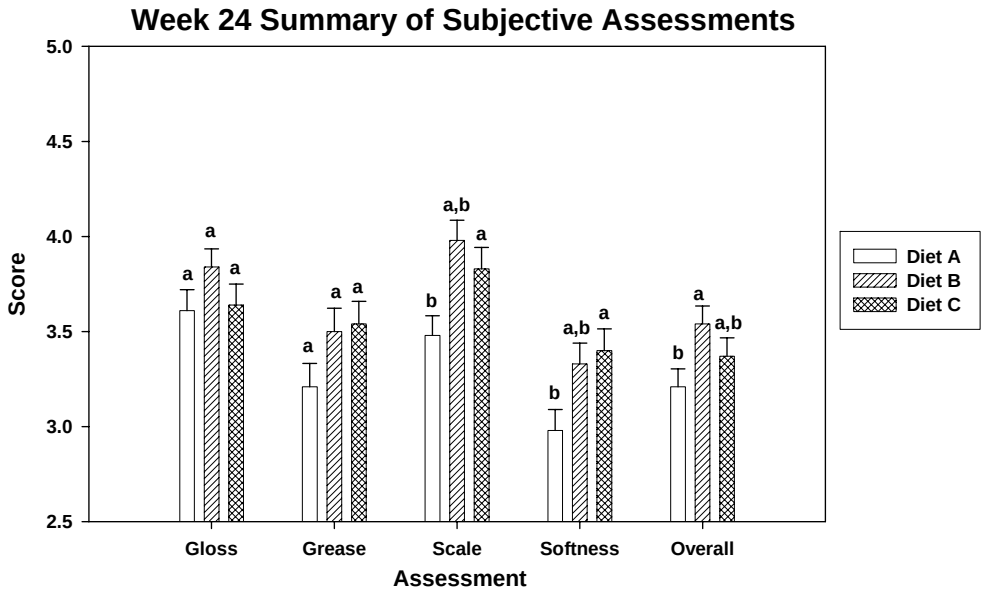
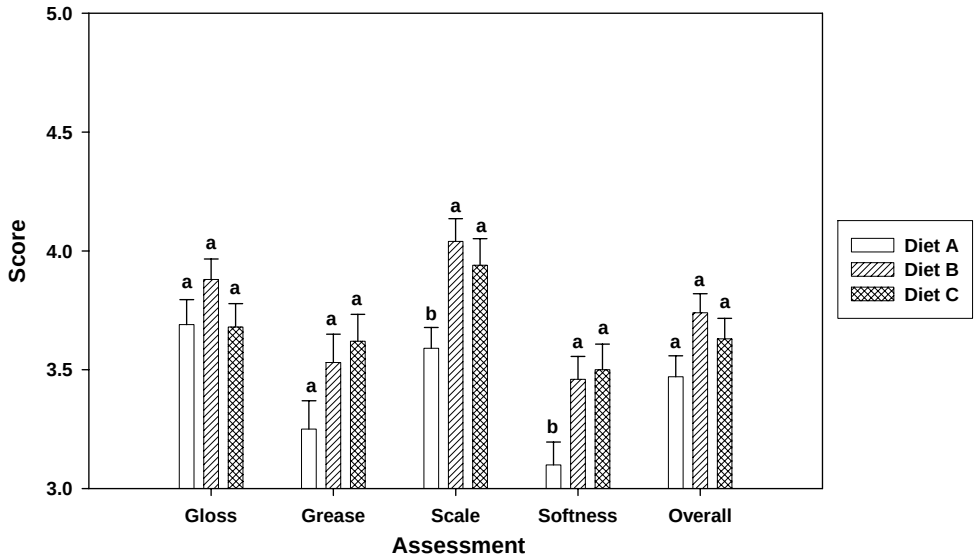


FIGURE C-I Comparison of Phase I (Acclimation) and Phase II (Diets A, B, C) mean values for Subjective Tick Mark Assessments over time. Letters not in common for each graph indicate significant difference over time for all diets combined by Repeated Measures ANOVA ($p < 0.05$).



Skin and Hair coat condition scores (Integer) at end of Phase II feeding period (Week 24). Letters not in common for score category are significantly different at $p < 0.05$. Results are derived from Kruskal-Wallis One-way Nonparametric AOV test. Error bars indicate standard error of the mean.



Skin and Hair coat condition scores (Tick Mark) at end of Phase II feeding period (Week 24). Letters not in common for a score catagory are significantly different at $p < 0.05$. Results derived for Repeated Measures ANOVA. Error bars indicate standard error of the mean.

FIGURE C-II Skin and hair condition scores (integer and tick mark) at end of Phase II week 24.

APPENDIX D

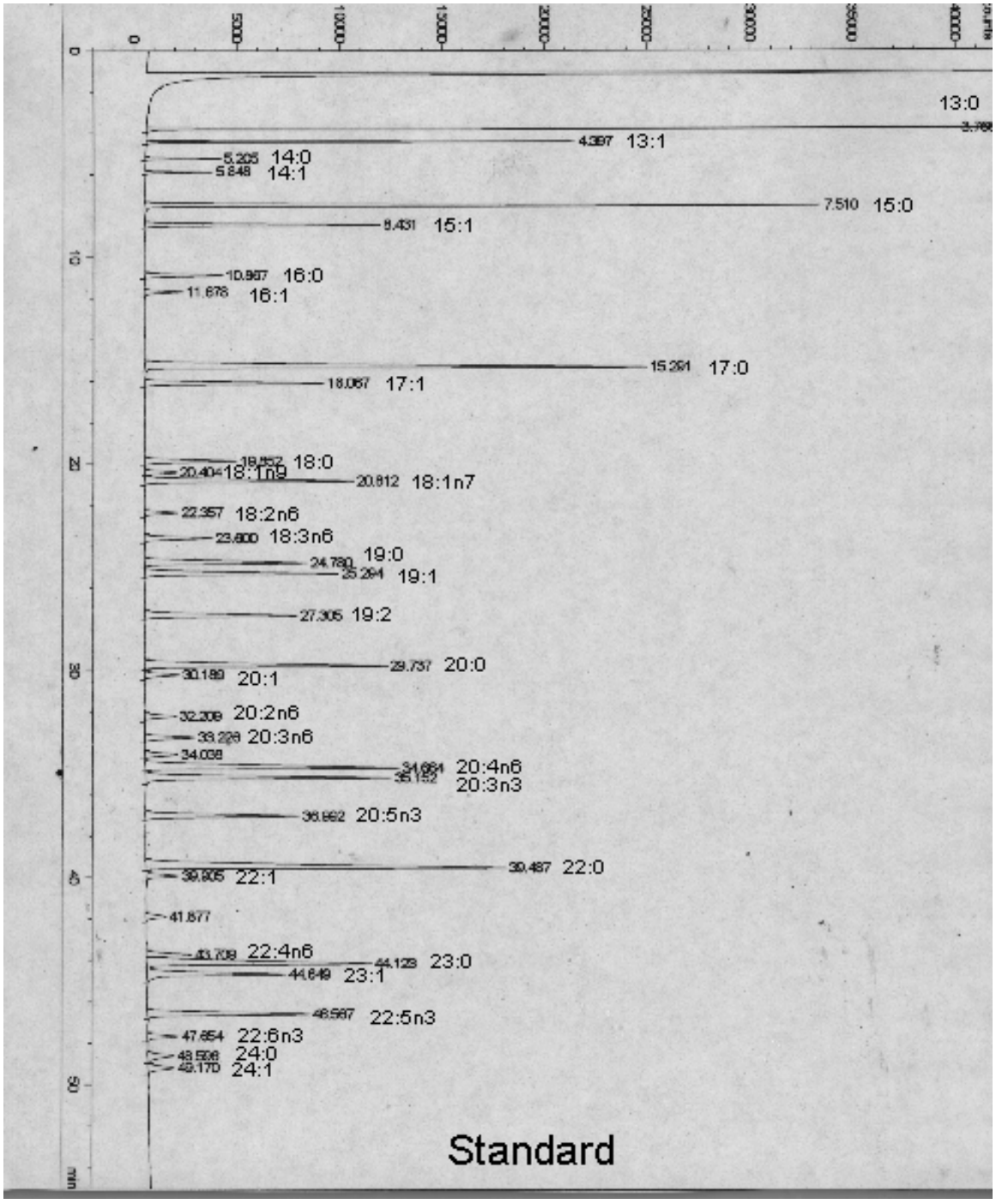


Figure D-I Fatty acid methyl ester standard chromatogram.

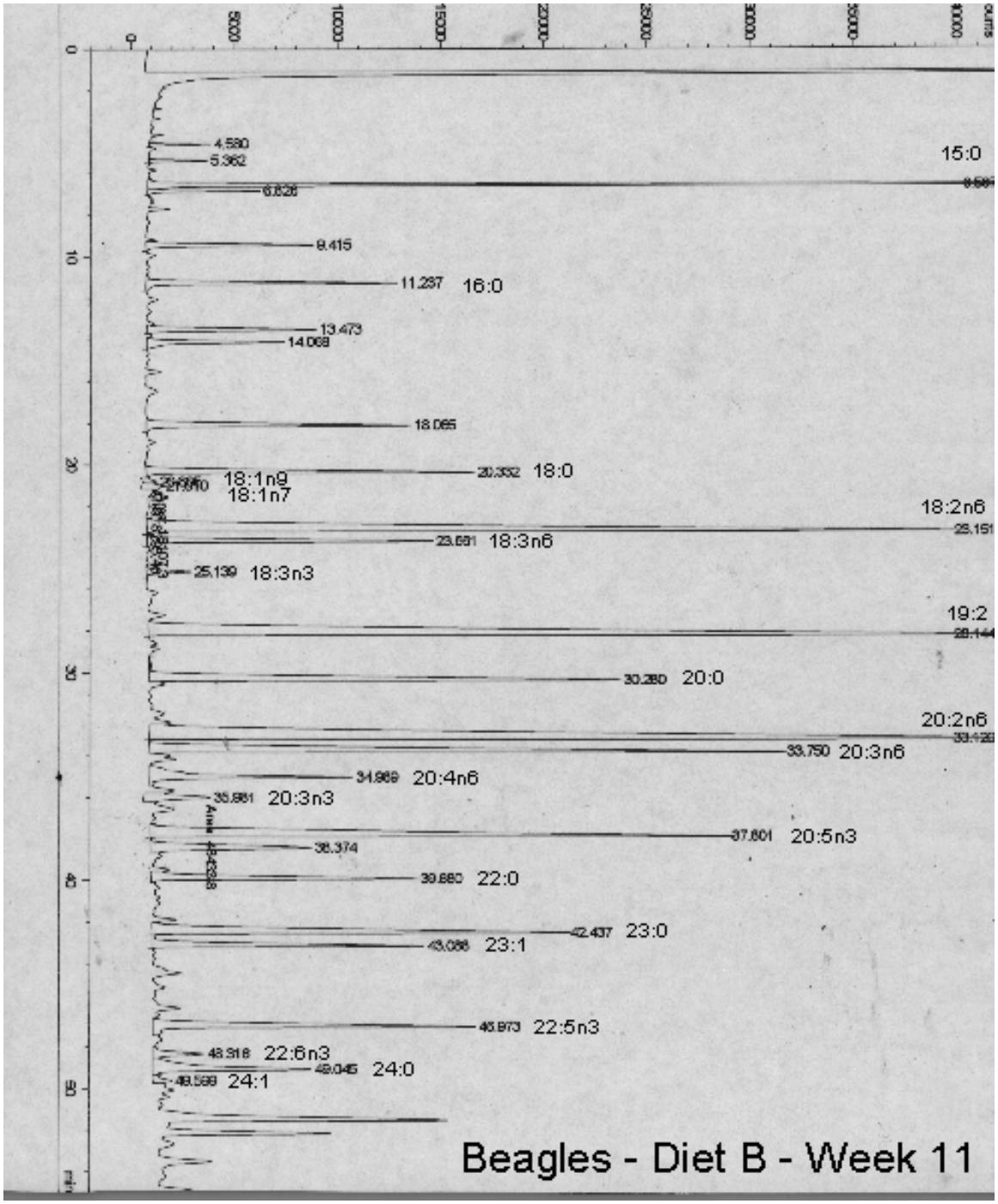


Figure D-II Chromatogram from hair CE fatty acids of beagles collected after being fed diet B for 11 weeks.

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