

**ENRICHMENT OF CANINE GESTATION AND LACTATION DIETS  
WITH N-3 POLYUNSATURATED FATTY ACIDS TO SUPPORT  
NEUROLOGIC DEVELOPMENT**

A Dissertation

by

**KIMBERLY MICHELE HEINEMANN**

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

**DOCTOR OF PHILOSOPHY**

August 2004

Major Subject: Nutrition

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## ABSTRACT

Enrichment of Canine Gestation and Lactation Diets with n-3 Polyunsaturated Fatty Acids to Support Neurologic Development. (August 2004)

Kimberly Michele Heinemann, B.S., Texas A&M University

Chair of Advisory Committee: Dr. John E. Bauer

Long-chain polyunsaturated fatty acids (LCPUFA) are essential for proper neural and retinal development in many mammalian species. One objective of this research was to investigate the effects of dietary  $\alpha$ -linolenic acid (ALA) and LCPUFA on the fatty acid composition of canine plasma phospholipids (PL) and milk during the gestation and lactation periods. The fatty acid composition of plasma PL and the retinal development of puppies reared on the same experimental diets as their mothers were also investigated.

Enriching the canine gestation/lactation diet with ALA (6.8% DM) does not result in enrichment of docosahexaenoic acid (DHA) in the milk. From this data it can be inferred that peroxisomal elongation and desaturation of LCPUFA does not occur in canine mammary tissue. Dose responses of linoleic acid (LA), ALA and DHA were observed in the plasma of adult dogs during gestation and lactation and in puppies during both the suckling and post-weaning periods. Plasma PL fatty acid data from puppies indicate that canine neonates are capable of synthesizing LCPUFA from ALA, but that plasma enrichment of the newly-synthesized DHA does not compare with that obtained from preformed DHA in the diet.

Visual function was assessed via electroretinography (ERG) in 12-wk old canines. One-way ANOVA revealed significantly better visual performance in dogs fed the highest amounts of n-3 LCPUFA. Puppies in this group demonstrated the greatest rod response as measured by the amplitude and implicit time of the a-wave. Neonates reared on the lowest dietary levels of both ALA and n-3 LCPUFA exhibited the poorest visual function. A novel parameter devised in this study was the threshold intensity, which was the initial intensity at which the a-wave was detectable. Again, puppies consuming the greatest concentrations of n-3 LCPUFA responded significantly sooner, i.e. exhibited greater rod sensitivity, than other diet groups.

The findings of this research underscore the importance of preformed n-3 LCPUFA in the diet, rather than ALA, as a means of enriching neural tissues in DHA during the developmental period. Moreover, dietary DHA appears to be related to improved visual performance in developing canines.

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## CHAPTER I

### INTRODUCTION

#### Essential Fatty Acids

The n-3 and n-6 families of polyunsaturated fatty acids (PUFA) contain many biologically important lipids that are essential for proper growth, development and general adult health maintenance. The parent fatty acids, alpha-linolenic acid (ALA, 18:3 n-3) and linoleic acid (LA, 18:2 n-6), are not synthesized in mammals and, thus, are deemed essential. Since LA and ALA are not synthesized endogenously, they must be obtained from the diet. Both fatty acids are of plant origin and can be found in a variety of plant-derived oils. Canola, sunflower, and safflower oils are but a few sources rich in LA while flaxseed oil is highly enriched in ALA. These parent essential fatty acids (EFA) give rise, via elongation and desaturation pathways, to longer chain polyunsaturates that are essential for numerous physiological functions. Moreover, the n-3 and n-6 fatty acids are functionally and metabolically distinct, and no interconversion occurs between the two.

Essential fatty acids were considered of little importance in humans until the early 1960s when indications of clinical deficiency became apparent in infants fed skim milk-based formula and those administered lipid-free parenteral nutrition (1-2). These infants experienced dryness, desquamation and thickening of the skin, growth faltering, and poor wound healing, which are typical manifestations of LA deficiency (3).

Clinical deficiencies of all EFA are manifested in poor growth, skin lesions, degenerative changes in numerous organs, impaired water balance, increased fragility and permeability of cell membranes and increased susceptibility to infection (4).

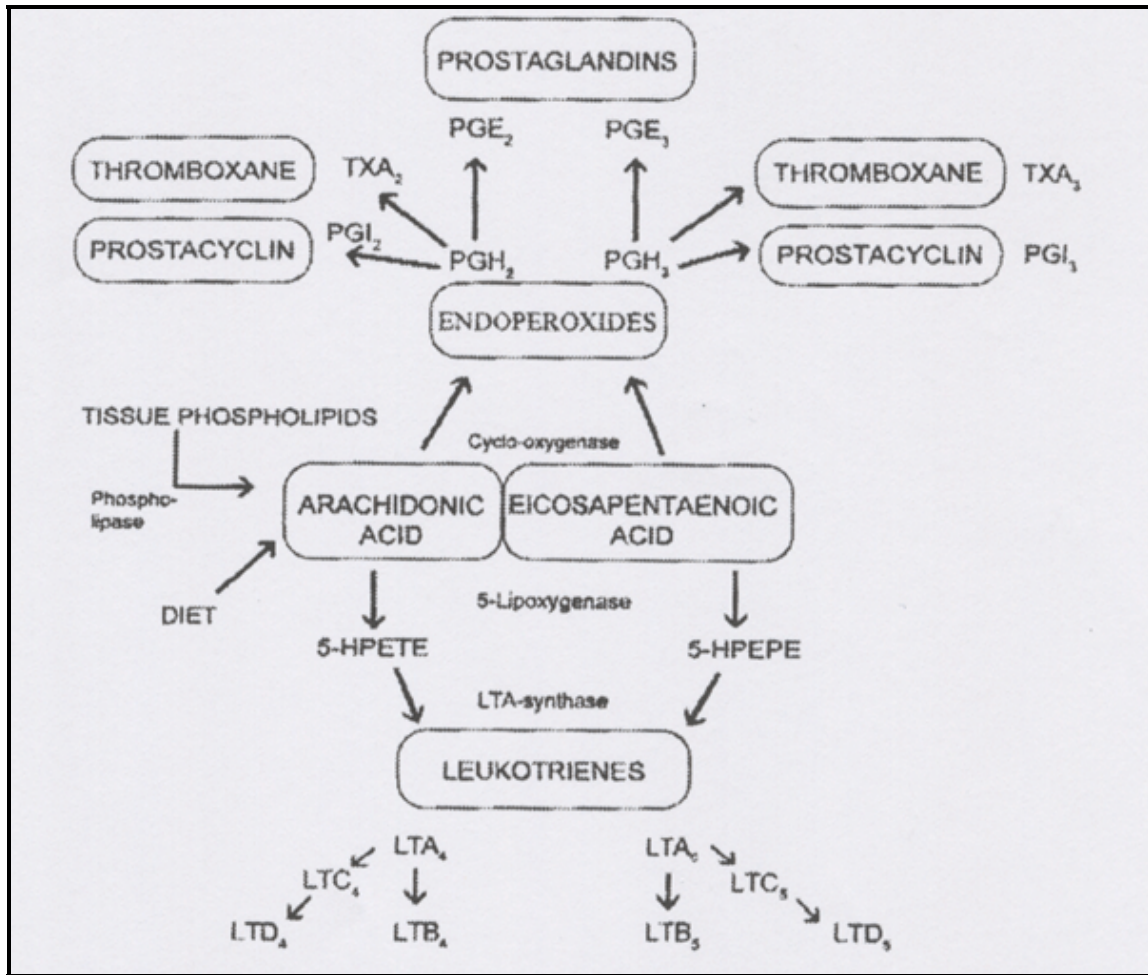
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This dissertation is in the style and format of *Lipids*.

Administration of diets containing 2% or more of total calories as LA relieves these clinical signs (1). While general EFA deficiency may be easily recognized, those of n-3 fatty acid deficiency alone are subtler and include decreased visual acuity and electroretinogram amplitudes, peripheral neuropathy and reduced learning ability (5). Thus, nervous system signs associated with EFA deficiency are most likely the result of an absence of the long chain metabolic derivative of ALA, namely DHA. At times the measurements of neurologic functions are difficult to ascertain; instead, concentrations of docosahexaenoic acid (22:5 n-3, DHA) can be analyzed in serum lipids, erythrocyte membranes, and platelets. These measures often are regarded as indices of DHA status.

Essential fatty acids serve as substrates for a series of enzymatic reactions that convert them to 20-carbon fatty acids, which in turn are precursors for eicosanoid synthesis. Eicosanoids are a group of potent, short-lived biological compounds that are derived from the 20-carbon fatty acids arachidonic acid (20:4 n-6, AA) and eicosapentaenoic acid (20:5 n-3, EPA) (Figure 1.1). Eicosanoids act locally as hormones and include prostaglandins, leukotrienes and thromboxanes (6). Generally, those eicosanoids derived from AA tend to mediate inflammatory and immune responses such as platelet aggregation, vasoconstriction, and increased blood viscosity. On the other hand, EPA-derived eicosanoids tend to be anti-inflammatory in nature, and as a whole, less potent than their n-6 analogs. The general effects of n-3 eicosanoids include decreased platelet aggregation, vasodilation, and reduced blood viscosity.

Both n-6 and n-3 fatty acids utilize the same set of enzymes for conversion to eicosanoids; thus, the synthesis of one group relative to the other depends upon the relative



**Figure 1.1** Schematic diagram of eicosanoid synthesis from AA and EPA. The two classes of eicosanoids exert properties that are antagonistic to one another. Eicosanoids derived from AA exert potent pro-inflammatory effects whereas those derived from EPA exert less potent inflammatory and thrombotic effects and are therefore considered anti-inflammatory in nature (7).

amounts of precursors present. A diet high in n-6 fatty acids will generally increase production of pro-inflammatory compounds and shift the physiological state to one that is pro-thrombotic and pro-aggregatory (7). As a result of such a shift, blood viscosity increases and bleeding time decreases (7). Conversely, a diet rich in fish oil, an excellent source of long chain n-3 fatty acids, mediates an overall anti-inflammatory response manifested by the production of weak platelet aggregators and weak vasoconstrictors that are, in effect, biologically inactive (7). Whereas the n-6 series eicosanoids directly influence inflammation, the n-3 series does so in a somewhat indirect manner. Rather than exerting direct inhibitory effects, they produce an overall anti-inflammatory effect via the decreased synthesis of n-6 eicosanoids and the increased synthesis of essentially inactive compounds.

Further elongation and desaturation of EPA yields the neurologically important docosahexaenoic acid (22:6 n-3, DHA). In recent years, evidence has amassed which asserts the essentiality of DHA for optimal neural and retinal development in neonates.

### **Fatty Acid Nomenclature**

Fatty acids are identified by their chain length and by the numbers and positions of their double bonds. For example in ALA, an n-3 fatty acid with 3 double bonds, the first double bond occurs at the third carbon from the methyl terminus, hence the term n-3 fatty acid. Similarly, in n-6 fatty acids, the position of the first double bond is 6 carbons from the methyl terminus.

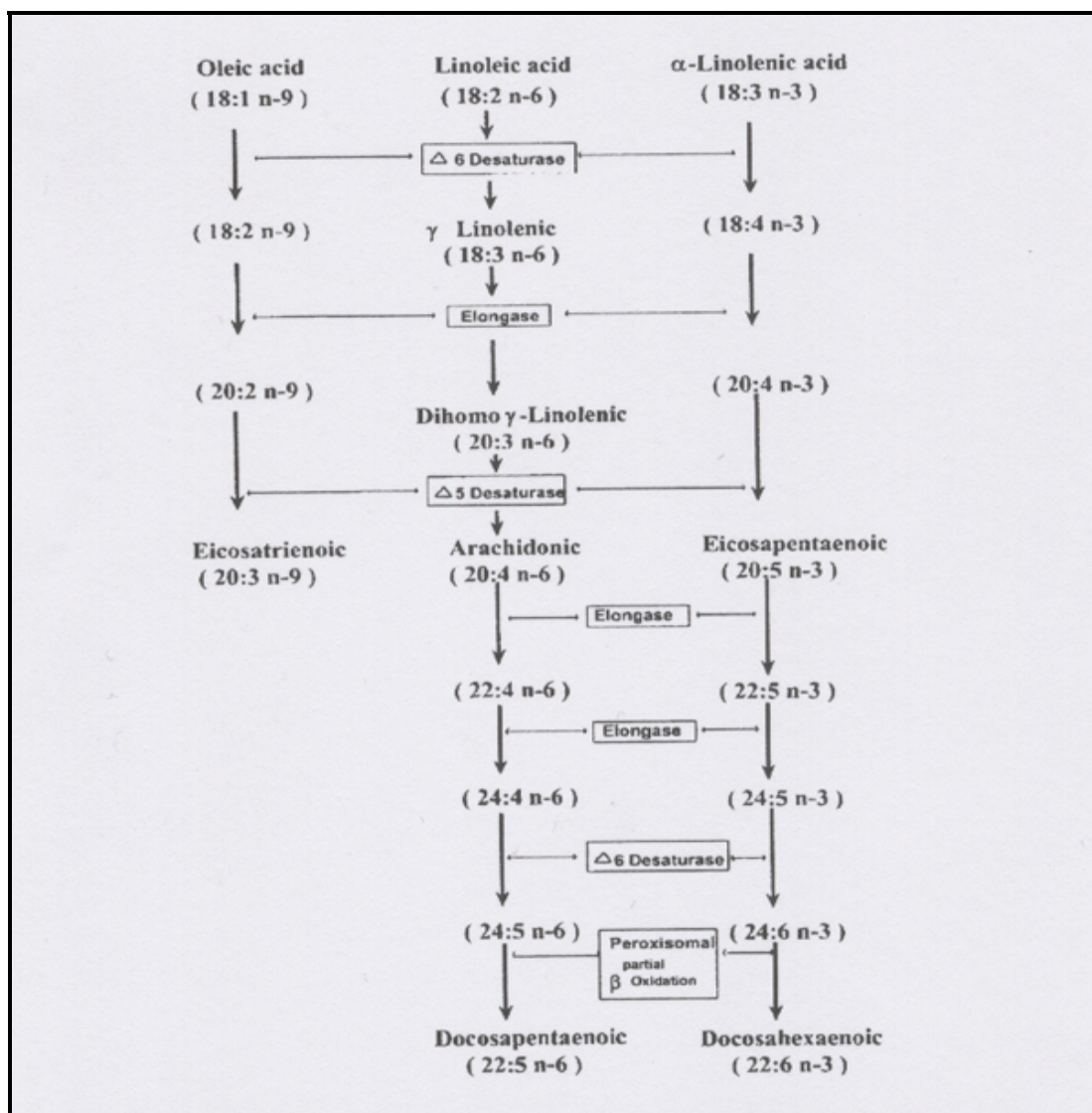
Fatty acids with 2 or more double bonds are considered polyunsaturated fatty acids (PUFA), and fatty acids 20 carbons or more in length are considered long chain polyunsaturated fatty acids (LCPUFA). The double bonds in fatty acids of mammalian

origin are 1, 4-conjugated dienes, and all double bonds are in the *cis* conformation.

Insertion of subsequent double bonds proceeds through the action of one of the 3 mammalian desaturases— $\Delta^9$ ,  $\Delta^6$ , or  $\Delta^5$ —and maintains the methylene-interrupted pattern of double bonds. Beginning with the first double bond from the methyl terminus, one can deduce the positions of all other double bonds by following this pattern.

The liver is the primary site of LCPUFA synthesis, which occurs on the endoplasmic reticulum via an alternating sequence of desaturation and elongation reactions (Figure 1.2). If LA or ALA is the substrate for desaturation, the  $\Delta^6$  desaturase enzyme inserts a double bond 6 carbons from the carboxyl terminus, thus generating 18:3 n-6 or 18:4 n-3, respectively. This is followed by elongation and subsequent desaturation to yield the 20-carbon derivatives AA and EPA. Until recently, the synthesis of DHA from EPA, and docosapentaenoic acid (22:5 n-6, DPA) from AA, was thought to occur via elongation and the action of a  $\Delta^4$  desaturase. No evidence, however, for such a pathway in mammals presently exists (reviewed in 8). Instead, Sprecher (8) proposed that conversion beyond EPA takes place by way of 2 consecutive elongation reactions to yield 24:5 n-3, which becomes the substrate for a second  $\Delta^6$  desaturase. Some evidence suggests that this  $\Delta^6$  desaturase is different from the one that catalyzes the first desaturation reaction in the pathway (9). The resultant 24:6 n-3 then undergoes retroconversion to DHA. This retroconversion involves a partial beta-oxidation, which takes place within the peroxisome rather than in the mitochondria (11) (Figure 1.2). Additionally, some studies indicate that the elongation, and subsequent desaturation and retroconversion, of 22:5 n-3 (DPA n-3) is the rate-limiting or regulatory step in the synthesis of DHA (12). An analogous pathway exists for the conversion of AA to DPA n-6 (13).





**Figure 1.2** Metabolic conversion of essential fatty acids (EFA) to form LCPUFA. Parent EFA of both the n-3 and n-6 series are derived from dietary sources. Elongation occurs two carbons at a time and desaturases introduce double bonds from the carboxyl terminus of the original fatty acid. The final step in the formation of n-3 and n-6 end products is catalyzed by a peroxisomal partial beta-oxidation (figure modified from reference 10).

In order to elucidate the mechanism of DHA synthesis, Sprecher (8) first incubated 1-<sup>14</sup>C labeled 22:5 n-3 with rat liver microsomes and did not observe any radiolabeled DHA products. However, the elongation products 24:5 n-3 and 24:6 n-3 were observed when malonyl Co-A and NADPH were added to the incubation. It was presumed that this elongation occurred by a  $\Delta^6$  desaturase. Then, the same labeled substrate was incubated with rat liver hepatocytes and produced not only labeled DHA, but small amounts of 24:5 n-3 and 24:6 n-3 as well. In an additional study, fibroblasts that lacked peroxisomes, a condition of Zellweger's disease, were incubated with 3-<sup>14</sup>C labeled 24:5 n-3 (8). Interestingly, the desaturation product, 24:6 n-3, was present, but DHA was not. This was in contrast to data from control fibroblasts in which labeled DHA was found, thus indicating that the final conversion to DHA occurred by way of a peroxisomal beta-oxidation pathway (14).

Initially the proposed pathway of Sprecher was met with much controversy and skepticism. However, no experimental evidence to date supports a conclusion contrary to that proposed by Sprecher and colleagues (8). In fact, results from newer studies further reinforce the concept of peroxisomal beta-oxidation (15-17).

### **Fatty Acid Metabolism in the Neonate**

It is well known that the human fetus and neonate can desaturate and chain-elongate 18-carbon precursors to their respective LCPUFAs (18-22). The uncertainty, however, lies in whether the rate of synthesis is enough to meet the demands for optimal growth and development. A number of studies have reported insufficient desaturase activities in human fetuses, especially during the last 2 weeks of gestation (19,23-25). This information raises the additional concern of whether pre-term infants are at greater risk for

LCPUFA insufficiency than are term infants since, at birth, preterm infants are deprived of their intrauterine EFA supply (26). Studies by Farquharson (27) and Innis (28) indicate that no difference exists between the preterm and term infant in their ability to convert LA and ALA to their long-chain metabolites. Moreover, Uauy (29), in agreement with Salem and others, suggests that LCPUFA formation, in fact, may be more active at earlier gestational ages.

Because the placenta itself does not desaturate LA or ALA, the human fetus must acquire most of its fatty acids via placental transfer. Thus, the primary determinant of fatty acid delivery to the fetus is the concentration in the maternal circulation, which is closely related to maternal fatty acid intake (30). At the fetal-maternal interface, there is a three-fold difference in the concentrations of plasma non-esterified DHA (31). Haggerty *et al.* (32) demonstrated the preferential transfer of DHA relative to other LCPUFA in utero. In their study, DHA was transferred at a rate 3 times higher than that of AA. This same group attributed the specificity of the uptake system to a selective retention of AA in the placental tissue.

A number of studies have attempted to explain the discrepancy in the rates of placental LCPUFA transfer. It is believed that the mechanism involves the preferential binding of AA to a placental plasma membrane fatty acid binding protein (p-FABP<sub>pm</sub>) (32-35). The p-FABP<sub>pm</sub> is located on the maternal-facing placental membrane, which accounts for the unidirectional flow of LCPUFA from the mother to the fetus (33,36). This notion is supported by evidence obtained in vitro, which shows a strong preference of p-FABP<sub>pm</sub> for AA and DHA (37). Additionally, results from studies involving human placental cell lines indicate that DHA is incorporated into triacylglycerols (TAGs) and is then liberated by the

action of lipoprotein lipase before being transferred to the fetus (38). Alternatively, AA primarily is acylated into phospholipids that accumulate within the placental tissue (37,39).

The liver, which accumulates DHA during fetal life, also plays an integral role in the accretion of DHA in the neural tissues both in utero and postnatally. Postmortem studies of human fetuses of varying gestational ages indicate that the parabolic rise of fetal liver DHA is paralleled by an increase in acylated DHA in the brain (40). After birth, the levels of acylated DHA in the liver and adipose tissue decrease, concomitant with a continued increase of acylated DHA in the brain and retina (40-41). Thus, adipose and liver tissues act as “sinks” that sequester DHA in utero in order to provide a reservoir of DHA during early postnatal life.

### **DHA and Neural Development**

In humans and in animals, brain and retinal functions are dependent upon the n-3 polyunsaturate DHA not only during development in utero, but during postnatal life as well. The period of maximum brain growth begins in the third trimester of gestation, peaks at birth, and continues throughout the first eighteen months of neonatal life (42-45). It is during this crucial period that the brain undergoes a ten-fold increase in size (46), and selective accumulation of AA and DHA within the brain and retina occurs. During this significant accrual of DHA, termed the “DHA accretion spurt,” the accretion of AA and DHA in brain and retinal phospholipids proceeds at a rate ten times faster than incorporation via chain elongation and desaturation of their respective precursors, LA and ALA (47-48).

In human retina, DHA accumulation begins around the start of the third fetal trimester and peaks at the 40<sup>th</sup> gestational week (49-50). The occurrence of n-3 fatty acid

deficiency during this developmental accretion phase results in decreased retinal DHA content (51-53), which subsequently manifests itself in decreased visual and neural function (43,51,54). Once neural development is complete, a deficiency of n-3 fatty acids does not significantly affect the DHA content of the retina (52-53). It is important, however, to note that although n-3-deficient subjects are able to obtain normal DHA levels upon repletion, the functional abnormalities resulting from early n-3 deficiency are not corrected upon repletion (5).

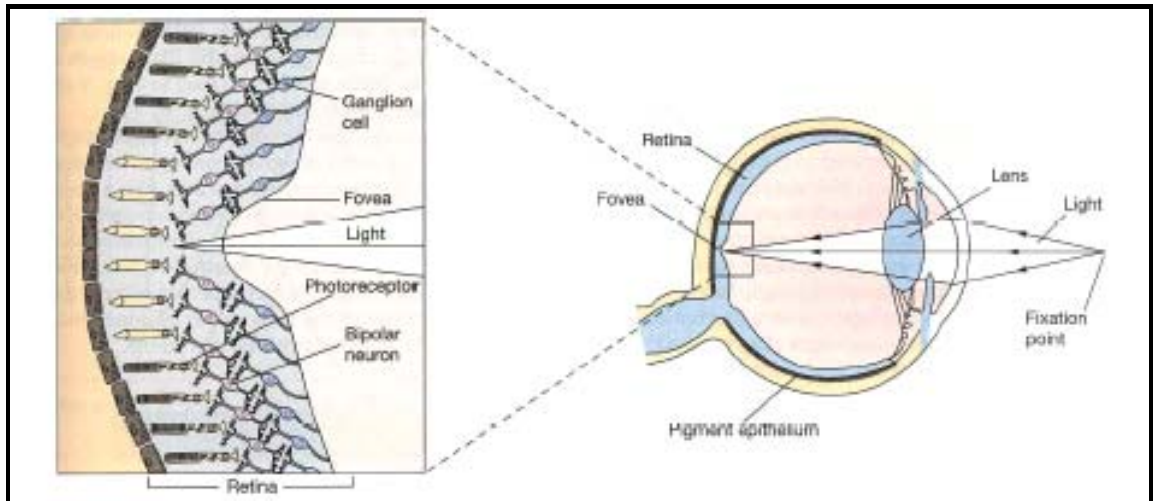
The primary site of DHA accretion in neural tissues is the phospholipid (PL) fraction of brain and retinal cells (55-60). The rod photoreceptor outer segment (ROS) contains the highest concentration of DHA within the body; approximately 50% of the fatty acids in the ROS plasma membrane phospholipids are DHA (58,61). Within the phospholipid subfraction, DHA is usually esterified at the sn-2 position, but supraenoic molecules, in which DHA is esterified at both the sn-1 and sn-2 positions, also exist (57-60,62-64). Phosphatidylserine (PS) and phosphatidylethanolamine (PE) typically contain high levels of di-polyenoic species whereas phosphatidylcholine (PC) contains more saturated, monoenoic- or dienoic species than dipolyenoic species (59,63,65,66). Aveldaño (67) reported that the PL species most closely associated with the visual pigment rhodopsin, namely PC and PS, have greater amounts of DHA than other PL fractions. Furthermore, the interaction of rhodopsin with retinal PL is key in the control of visual function (68-70).

The ROS is a dynamic structure consisting of thousands of plasma membrane invaginations that form disks of phospholipid bilayers and that contain rhodopsin and other enzymes of the visual cascade. Adjacent to the ROS is the retinal pigment epithelium

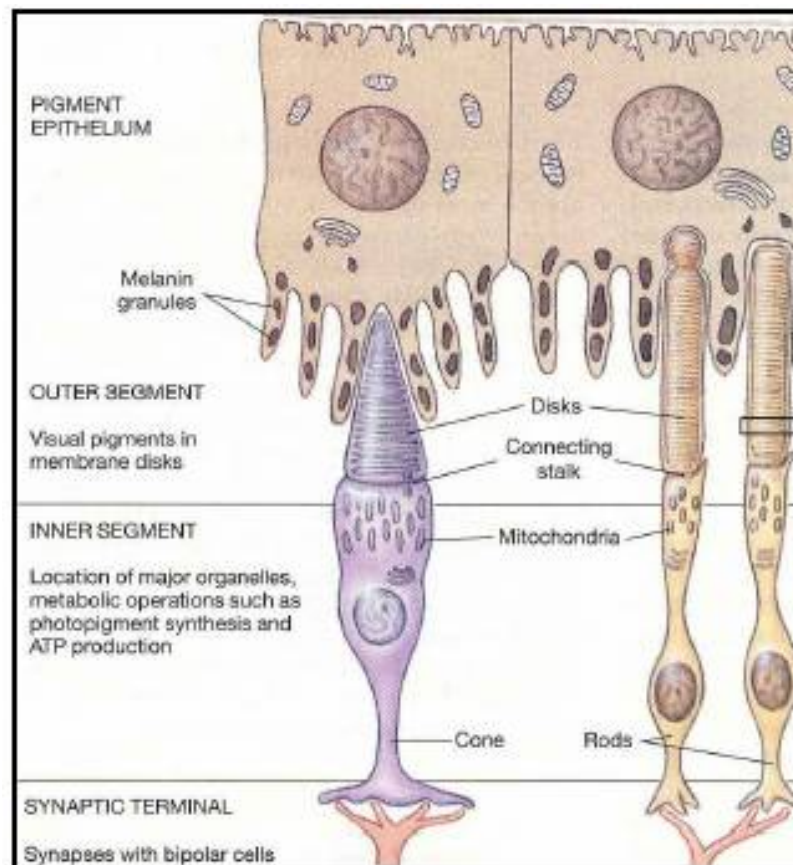
(RPE), which provides the metabolic support for the photoreceptors (71,72). Additionally, the RPE plays an integral role in the regeneration of visual pigments after photoreceptor bleaching, a process in which 11-*cis*-retinal is photo-isomerized to all-*trans*-retinal (73). Furthermore, and perhaps most importantly, the RPE maintains the supply of DHA to the ROS and aids in the retinal conservation of DHA (74-78) (Figures 1.3 and 1.4).

Two possibilities exist for the mechanism by which the ROS becomes enriched with DHA: 1) there is a selective uptake of pre-formed DHA from the plasma or 2) the retina itself synthesizes DHA via the elongation and desaturation of ALA. The conversion of ALA to DHA has been reported in RPE, but not in retina, of frogs (79). Li *et al.* demonstrated that orally-administered, radiolabeled DHA, but not ALA was taken up by the rat retina (80). Other studies have confirmed that plasma DHA is the preferred substrate for the retina during early development (81-83). Anderson and Wang demonstrated that this enrichment occurs at both the plasma-RPE and RPE-photoreceptor boundaries (84). In this study EPA was incorporated into PL in the RPE, but not in the ROS, which suggested that the RPE-photoreceptor boundary is one site of exclusion of 20-carbon PUFA from the ROS. Furthermore, they showed that the enrichment of 22-carbon PUFA within the retina is specific not only for chain length, but also for the number of double bonds (85).

Given the extreme importance of DHA in retinal function, a mechanism must be in place to preserve its integrity within the neural tissues. Indeed, this task is accomplished, in part, by the RPE (75,78). Circulating lipoproteins also play a role in establishing and maintaining high levels of DHA within the photoreceptors (86). New photoreceptor disks are continually being assembled at the base of the ROS, which results in a lengthening of



**Figure 1.3** Schematic diagram of the eye. Inset shows the structural organization of the retina (87).



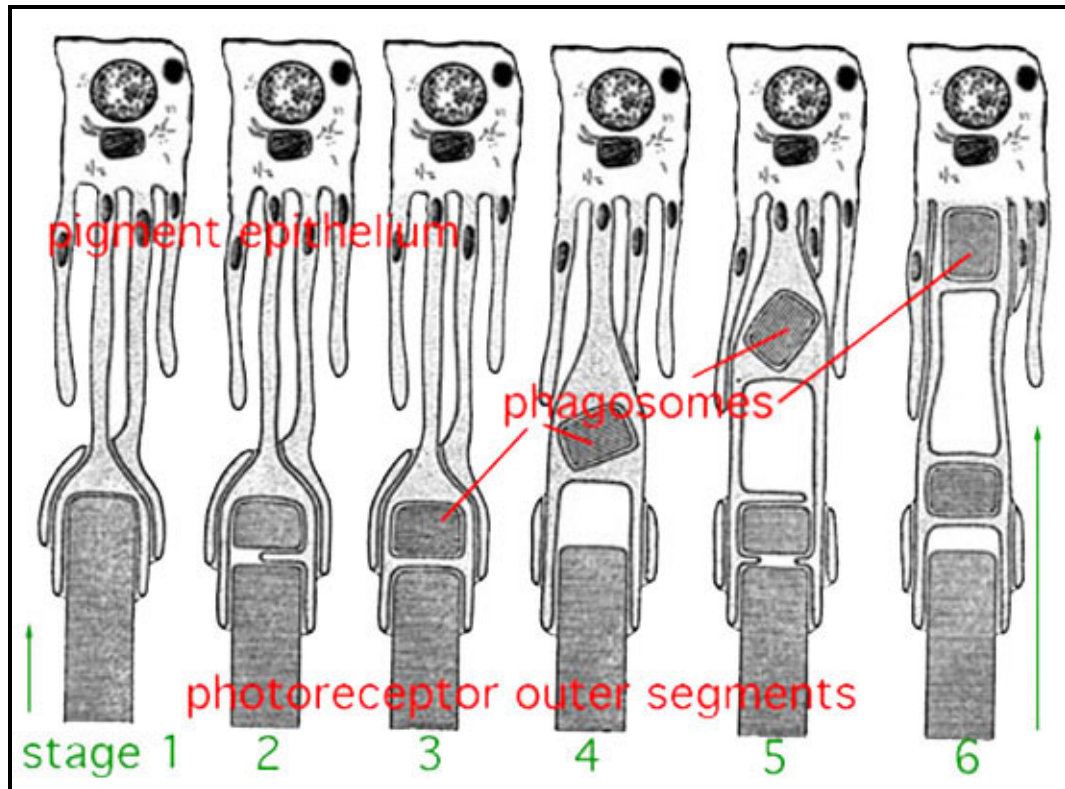
**Figure 1.4** Schematic diagram of the photoreceptors (87).

the ROS as older disks are pushed apically (75). To compensate for this continual elongation, the distal portion of the ROS is shed and phagocytized daily by the RPE, and DHA then is shuttled back to the ROS in an efficient recycling loop (88) (Figure 1.5). Specifically, Bazan *et al.* (75) demonstrated that the RPE cells are directly involved in a short-loop recycling of DHA from the phagosomes within the RPE back to the rod inner segments via the interphotoreceptor matrix. In later studies, this same group reported that phagocytosis triggered the expression of peroxisome proliferator-activated receptor- $\gamma$  (PPAR $\gamma$ ) (89,90), a fatty-acid activated transcription factor that contributes to lipid accumulation (91,92) and that DHA was stored transiently in TAGs within the RPE before being shunted back to the ROS (93). Taken together, these data are evidence for a mechanism that provides for the strict conservation of DHA within the retina, even during periods of extreme n-3 fatty acid deficiency (56,94).

### **The Visual Process**

The process of visual signal transduction involves a member of the biochemically ubiquitous G-protein-coupled motifs. Receptors in the superfamily of G-proteins are integral membrane proteins consisting of seven transmembrane helices and their respective connecting loops. The G-protein and effector proteins are, on the other hand, peripheral proteins, which are bound to the membrane via isoprenoid chain-lipid bilayer interactions (95,96) and electrostatic forces (97). Because the ligand-binding site on the receptor is formed by transmembrane helices, the conformational changes accompanying receptor activation would be, expectedly, dependent upon the physical properties of the membrane lipid bilayers (98). Indeed, high concentrations of DHA-derived n-3 PUFA have been observed in membranes with significant amounts of G-protein-coupled activity (64,100).





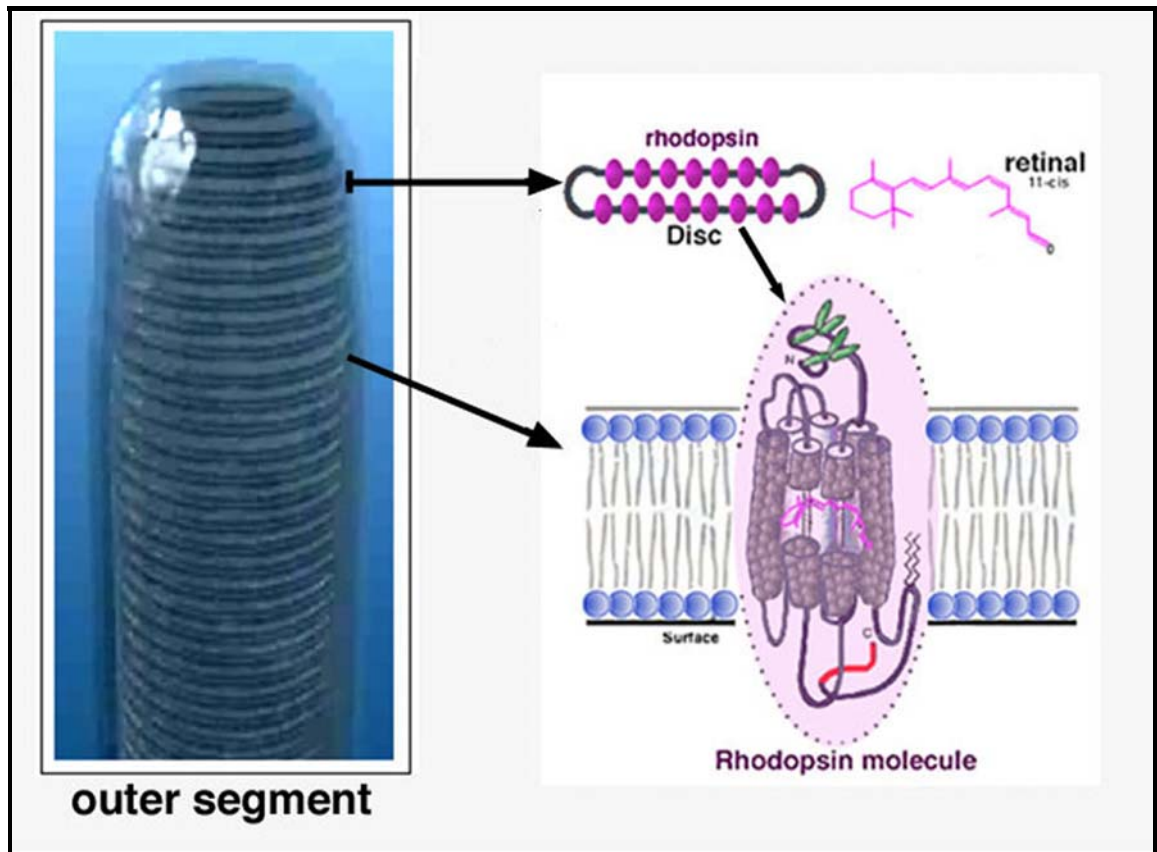
**Figure 1.5** Diagrammatic representation of ROS shedding and phagocytosis by RPE. New photoreceptor discs are continually assembled at the base of the ROS as older discs are pushed apically (stages 1-3). The distal portion of the ROS is shed and phagocytized daily by the RPE (stages 4-6) as DHA is shuttled back to the ROS. (Figure modified from reference 99).

Rhodopsin is an integral membrane protein that spans the ROS disk membrane; it is the receptor protein in the visual transduction cascade (Figure 1.6). The essential photoactive unit of rhodopsin consists of a molecule of 11-*cis*-retinal bound to opsin. Transducin, the G-protein, and its effector protein, cGMP phosphodiesterase (PDE), are the activation proteins, and are bound to the membrane by fatty acids (101-103). The absorption of light by 11-*cis*-retinal results in its isomerization to all-*trans*-retinal and its subsequent dissociation from opsin (73). Following this dissociation, rhodopsin exists as an equilibrium mixture of the active conformation of metarhodopsin II (MII) and the inactive conformation, metarhodopsin I (MI) (reviewed in 98). Each MII activates hundreds of transducin ( $G_t$ ) molecules, which in turn activate PDE, a cGMP-dependent effector enzyme. Activated PDE catalyzes the hydrolysis of cGMP, which triggers the closure of cGMP-gated  $\text{Na}^+/\text{Ca}^{+2}$  channels and leads to the hyperpolarization of the ROS plasma membrane and the visual response (Figures 1.7 and 1.8).

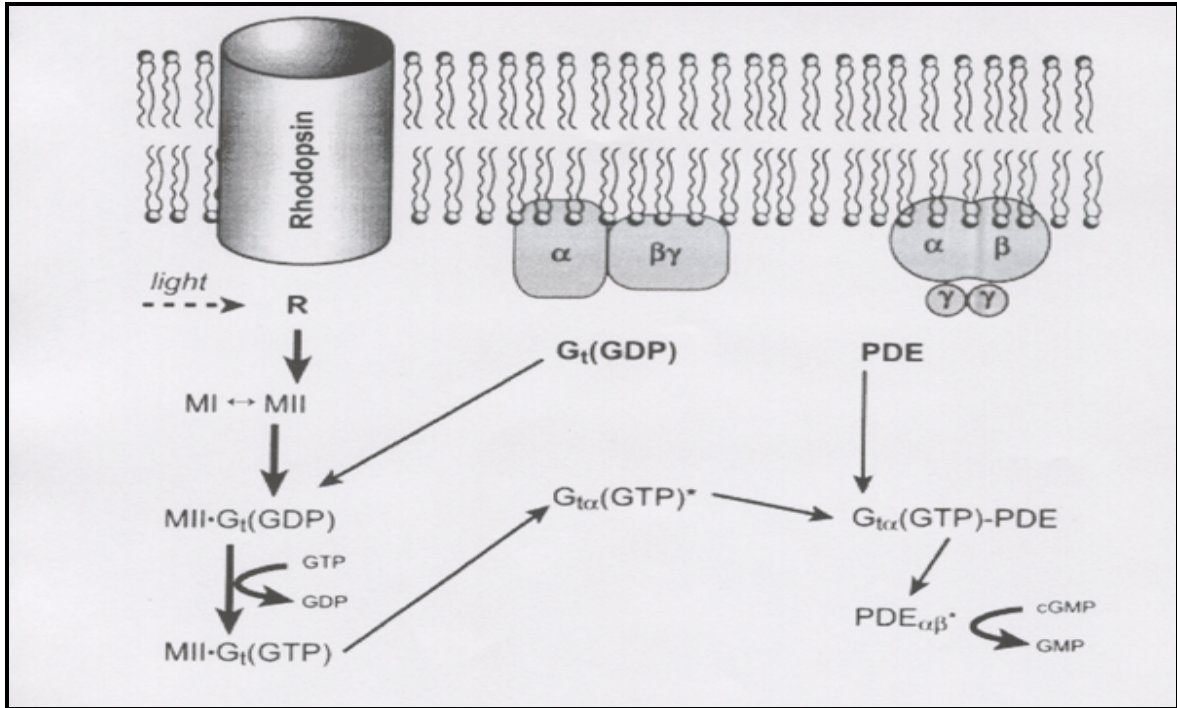
### **The Effect of DHA in the Membrane**

The ease with which signaling proteins can traverse the membrane dictates the efficiency of the visual transduction cascade. In order for phototransduction to occur, MII and  $G_t$  must find each other through lateral diffusion in the surface of the ROS membrane. Clearly, a more fluid membrane would better facilitate this process. Indeed, it has been reported that the rate of rod phototransduction is determined by the lateral diffusion of the visual proteins within the membrane (104). What, then, is the significance of DHA within biological membranes?

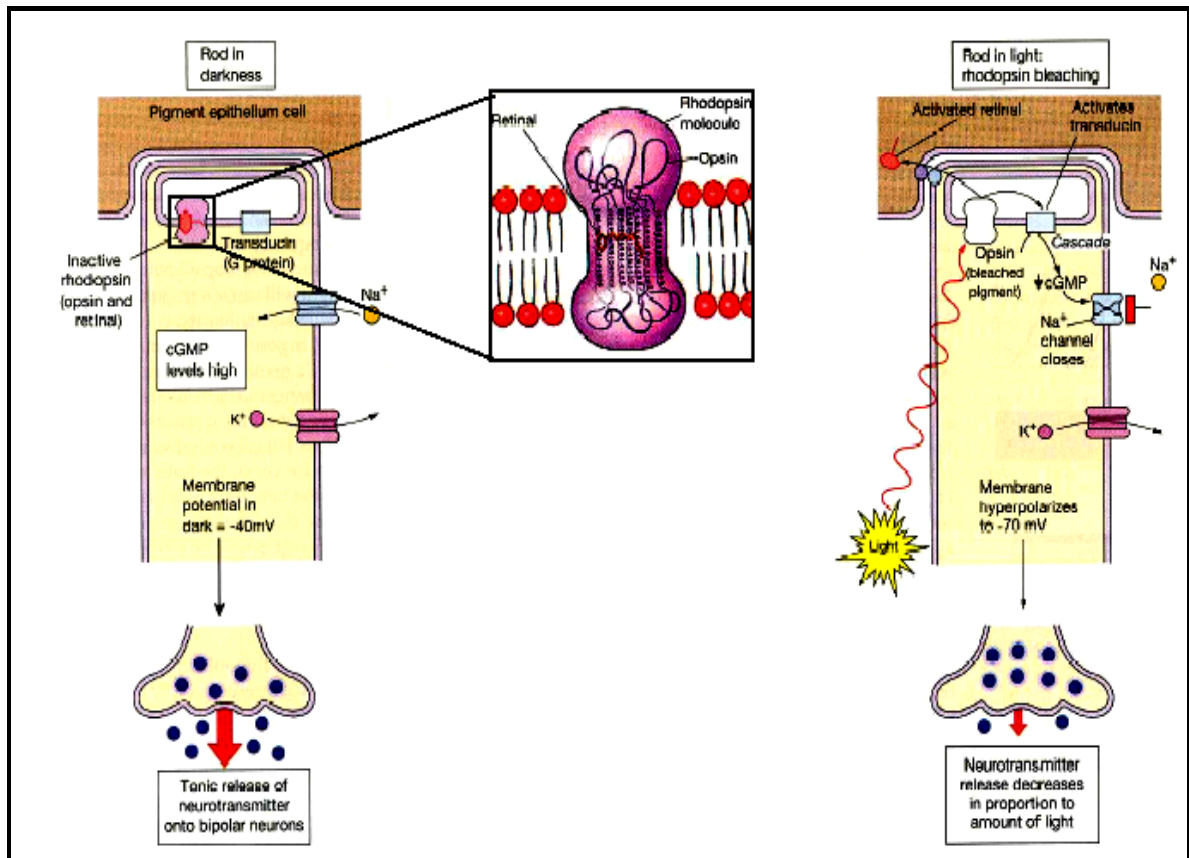
The prevalence of DHA within the PL species of the ROS suggests that a highly fluid membrane permits rapid enzyme action and ion transport (68,105). Studies by



**Figure 1.6** Schematic diagram of rhodopsin within the ROS disc membrane. The characteristic seven transmembrane domains of the G-protein receptor and the site of retinal attachment are shown (99).



**Figure 1.7** Schematic representation of visual transduction cascade. The three proteins involved in the G-protein-coupled signal transduction of the visual system are shown in relation to the ROS disc membrane. Rhodopsin is a transmembrane protein; the G protein, transducin or G<sub>t</sub>, and the effector, cGMP phosphodiesterase or PDE, are bound to the membrane surface. Light converts rhodopsin to an equilibrium mixture of an inactive form, MI and an active form MII. MII binds and activates G<sub>t</sub> (GDP) by catalyzing the exchange of bound GDP for GTP. G<sub>t</sub> (GTP)\* then dissociates and G<sub>tα</sub>(GTP)\* binds to the inactive form of PDE. This complex dissociates to yield the active subunit complex PDE<sub>αβ</sub>\*, which hydrolyzes cGMP. The lowered concentration of c-GMP induces closure of cGMP-gated sodium channels in the plasma membrane, hyperpolarizing the cell (98).



**Figure 1.8** Cartoon representation of the visual phototransduction cascade (87).

Litman and Mitchell (106,107) confirmed this notion and have provided much insight into the relationship between membrane fluidity and signal transduction. They demonstrated, in reconstituted membrane systems, that the highest levels of MII formation occurred in bilayers that contained DHA within the PL. Additionally, in native ROS disks, MII formation is followed rapidly by formation of the MII-G<sub>t</sub> complex, which makes the process of visual signal transduction rather efficient. Also, as the bilayer acyl chains become more saturated, phototransduction is delayed (106, 107). The presence of cholesterol within the membrane provides additional impedance to the formation of MII.

More recent studies indicate that the functional consequences of polyunsaturated PL extend beyond the MI to MII transition to the rapid coupling of receptor and G-protein and to the functional efficiency of the receptor (98). In simulated cellular responses, a membrane with a content of DHA or DPA n-3 PL acyl chain equivalent to that in a healthy rod cell produces a response similar to that recorded in vivo, whereas a membrane in which n-3 is replaced by DPA n-6 produces a much slower response.

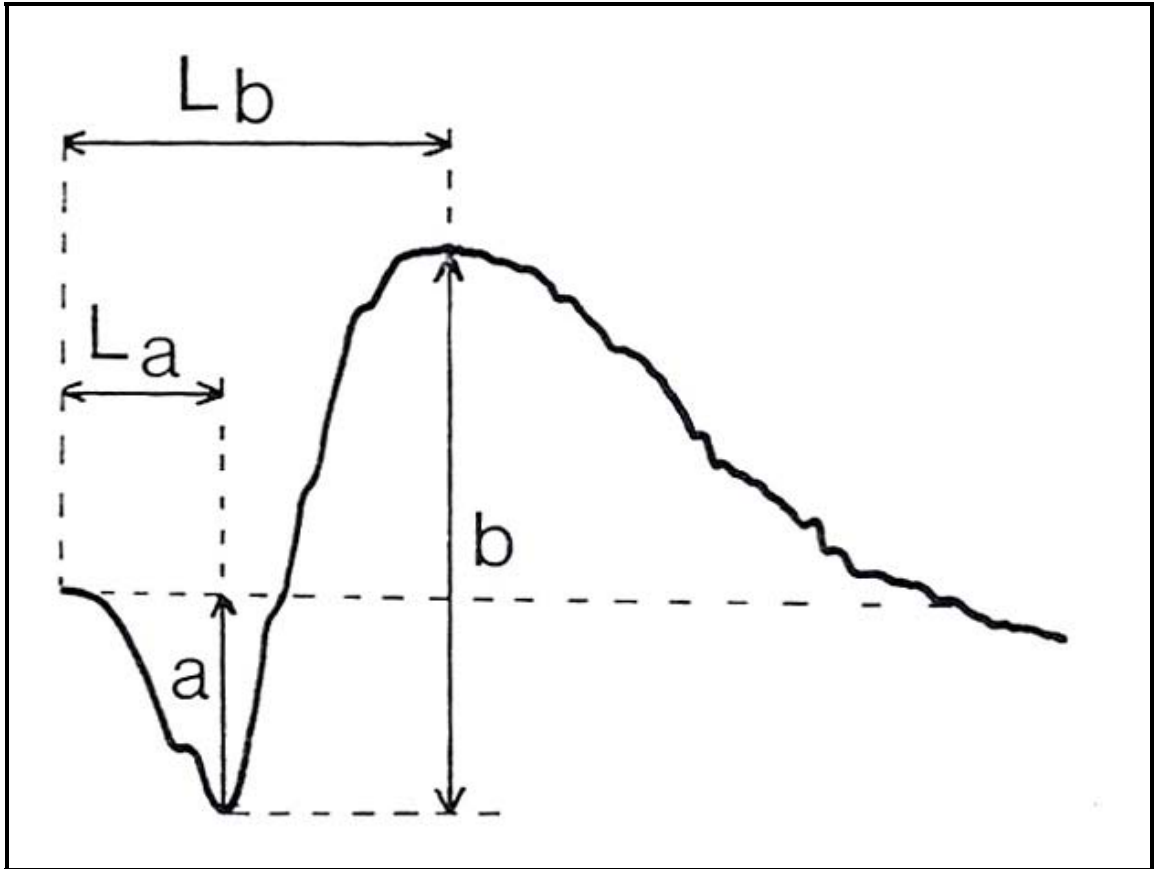
These alterations in PL acyl chains of the ROS offer, at least in part, an explanation for the functional abnormalities observed in the electroretinograms (ERG) of n-3-deficient animals, which will be elaborated upon later in this chapter. The increased lag time in MII-G<sub>t</sub> coupling in less-unsaturated membranes correlates to the lag time in the ERG, and the reduced formation of MII explains the decreased signal amplitude in n-3-deficient retinas (98,108).

### **The Electroretinogram**

Electroretinography is a sensitive and quantitative measure of retinal function in humans and animals. The ERG recording represents the response of the photoreceptors,

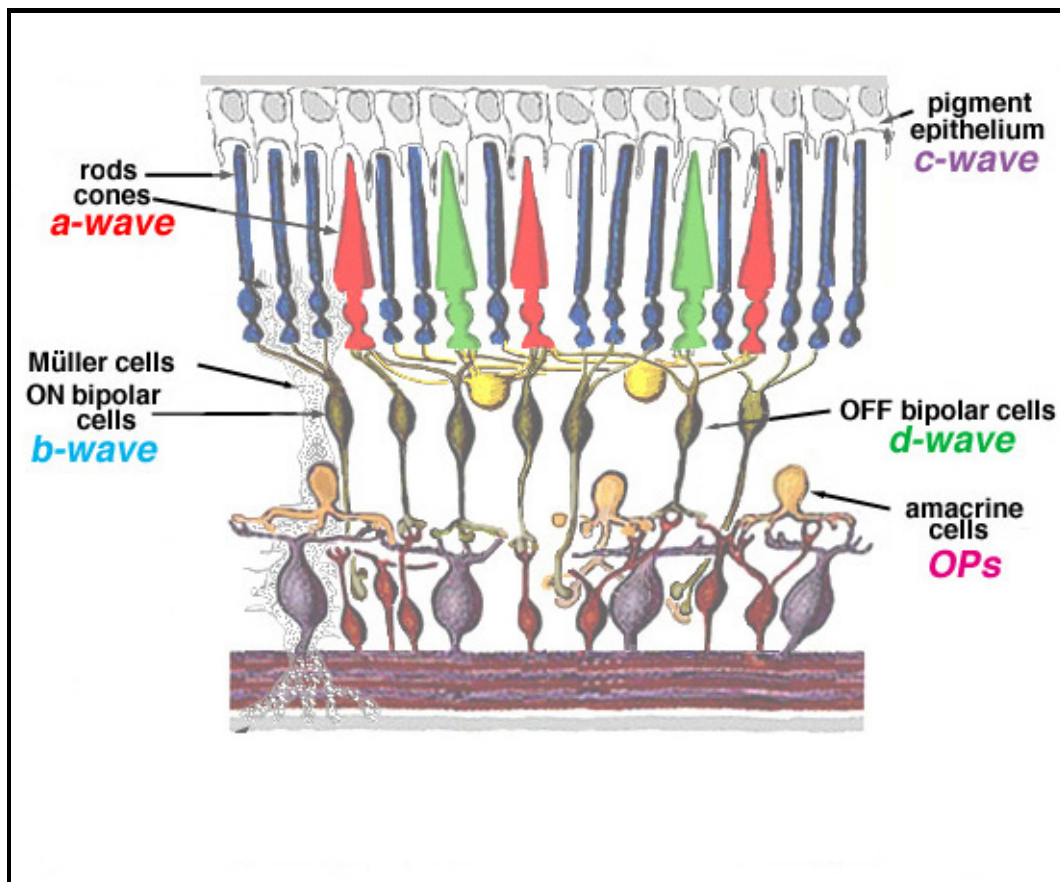
and their subsequent post-synaptic signals, to a series of varying-intensity flash stimuli. It is the sum of responses across the retina and includes the responses of many retinal cell types. The leading edge of the negatively projecting curve is the a-wave; its amplitude is measured from the baseline to the trough of the wave (Figure 1.9). The a-wave is a measure of photoreceptor function and can be further divided into slow and fast components (109). Early studies in rats determined that the photoreceptor layer is the origin of the a-wave, and that it results from extracellular radial current (reviewed in 109) (Figure 1.10). In other words, the a-wave reflects the reduction in 'dark' currents due to light absorption in the photoreceptor outer segments and the closure of c-GMP-gated channels (109). The slow component of the a-wave is indistinguishable in the standard ERG, as it is masked by the large amplitude of the c-wave (for c-wave details, see 109). However, upon isolation of the a-wave, it was determined that the slow response originates from the Müller cells (110). Light absorption by the photoreceptors causes a reduction in the extracellular potassium ion concentration, which results in alterations in the trans-membrane potential of the Müller cells and is visible as the a-wave.

The positively projecting component of the ERG is the b-wave; its amplitude is measured from the trough of the a-wave to the peak of the b-wave. The b-wave has been the focus of most ERG research since it is the major parameter of interest when assessing human retinal function. Early research pinpointed the retinal cells that are post-synaptic to the photoreceptors as the origin of the b-wave, although the exact cellular source remains disputed (109). Consequently, the b-wave can be eliminated by any procedure or substance that blocks synaptic transmission from the photoreceptors. A more extensive



**Figure 1.9** Example of ERG recording. The amplitudes of the a- and b-waves are indicated by 'a' and 'b,' respectively. The implicit times of respective waves are denoted  $L_a$  and  $L_b$  (109).





**Figure 1.10** Cartoon of the retina showing the origin of the major ERG waves. The a-wave originates in the photoreceptors, and the b-wave originates in the post-synaptic neurons. The c- and d-wave and oscillatory potentials (OPs) are minor components and are not of interest in this research (109).

discussion of the b-wave and related studies can be found elsewhere and is beyond the scope of this review (109).

For a more thorough examination of rod function, multiple recordings can be obtained in response to a series of flashes of increasing intensity that covers the range from rod threshold to b-wave saturation (111). Additional parameters can also be evaluated. These include the a- and b-wave implicit times and the a-wave to b-wave ratio. The implicit times are measured from the time of stimulus onset to the peak, or trough, of the corresponding wave (Figure 1.9). The c-wave, which is of little use clinically, is discussed in detail elsewhere (109).

### **Studies of DHA and Visual Development**

In the last twenty years, the importance of n-3 fatty acids in brain and retinal development has become increasingly evident. The question still remains, however, whether ALA provided as a precursor is sufficient for optimal development or if preformed DHA is required. The greatest concern is for premature infants, whose intrauterine development is cut short, often by several weeks (26).

As mentioned previously, neural development in primates begins in the third trimester of gestation, peaks about the time of birth, and continues for about 18-24 months after parturition (42-45). During this developmental period, AA and DHA are rapidly incorporated into the neural tissues (46-48). The high levels of DHA in the brain and especially in the retina suggest a functional role in these tissues (108).

### **Term Infants**

Numerous studies have attempted to evaluate the effects of human milk LCPUFA in breast-fed term infants on visual acuity and other developmental and functional tests.

The results are, at best, equivocal, and the debate continues as to whether or not LCPUFA supplementation of term-infant formula is necessary.

In one study term infants were fed a formula containing 2.1 wt% of total fatty acids as ALA and then outcomes of visual acuity tests were compared to those of breast-fed infants (112). At 3 months of age, no differences in visual acuity, as measured by Teller forced-choice preferential looking, were found between groups. Several studies by Auestad and coworkers confirmed these findings. Infants who were breast fed or fed formula supplemented with DHA and AA displayed no developmental advantage over those infants who were fed standard formula (113-115). Infants in the supplemented formula and breast fed groups did have similar levels of DHA and AA in their plasma and RBC, which were higher than non-supplemented infants, but there were no differences among all groups in any of the developmental parameters assessed (115).

Contrary to these findings are the results of numerous other studies. Makrides *et al.* reported marked functional differences between infants who were breast-fed and those who were fed standard, non-supplemented formula. In one study, they found increased visual evoked potential (VEP) acuity in breast-fed compared to formula-fed infants and noted a correlation between VEP and erythrocyte DHA content (116). In a separate experiment, they randomized infants to non-supplemented formula or to formula containing 0.36 wt% of total fatty acids as DHA and compared them to breast-fed infants. They again found an increased VEP acuity at 15 and 30 weeks of age in breast-fed and the DHA-formula fed groups compared to the non-supplemented group (117).

Similarly, another study reported significantly higher visual acuity at 2 months and again at 4 months in breast-fed infants compared with those who received a formula

containing 1.7 wt% of fatty acids as ALA (118). Carlson and coworkers reported a difference in visual acuity at 2, but not at 4, months in infants fed either a non-supplemented formula or a formula containing 0.1% DHA (119). Birch *et al.*, however, reported superior visual acuity, as measured by both VEP and forced-choice preferential looking, in breast-fed compared with corn oil-based formula-fed infants (120).

Makrides and coworkers reported conflicting results between 2 trials in which the effects of dietary fatty acids on visual acuity were assessed (121,122). One study reported no effect of dietary intervention, in the form of varied LA/ALA ratios, in 16-week old infants. Contrary to this report was one in which they found improved visual acuity in LCPUFA supplemented infants compared to non-supplemented infants. In light of these results, they conducted a retrospective analysis in an attempt to pinpoint some of the indicator variables of visual acuity in healthy, term infants at 16 weeks of age (123). They reported no effects of DHA status near birth on VEP acuity; they suggested that infants born with higher DHA levels may be less responsive to alterations in dietary fatty acid supply. Their data do not support the notion that n-3 LCPUFA status at birth is a significant predictor of visual outcome. Moreover, this analysis reported that numerous other factors, including birth weight, gender and social and demographic characteristics should be considered when evaluating VEP acuity.

### **Pre-term Infants**

The case for the LCPUFA supplementation of premature-infant formula seems to be much stronger than the case for term-infant formula supplementation. Most studies to date report a positive effect of n-3 LCPUFA supplementation on measures of DHA status and on visual acuity and function.

Hoffman *et al.* evaluated the effects of n-3 LCPUFA on retinal and cortical development in premature infants (124). Infants in their study received breast milk, either a corn-oil- or soy-oil-based formula or a marine-oil-supplemented formula from 10 days of life until 57 weeks post-conceptional age (PCA). The breast milk and marine-oil-supplemented groups had similar erythrocyte LCPUFA profiles, which were superior to those of the corn-oil group at 36 and 57 weeks PCA. The marine-oil and breast milk groups had significantly higher levels of DHA in erythrocyte PL than did the corn-oil group. Additionally, the corn-oil group had impaired visual acuity, as measured by VEP and preferential-looking, at these same time points. They also had less mature rod function at 36 weeks PCA, which was manifested in higher rod thresholds and lower ERG a-wave amplitudes compared to the breast-fed and marine oil groups.

In a 1993 study conducted by Carlson *et al.* (125), preterm infants who received an EPA-supplemented formula containing 0.2% DHA and 0.3% EPA had better visual acuity at 2 and 4 months of age. Beyond 4 months of age, the level of visual function in supplemented infants was matched by the control infants. In a separate set of later studies, preterm infants were given a standard preterm infant formula containing 3% ALA or the same formula supplemented with 0.02% DHA and 0.06% EPA for up to 2 months (126) or up to 9 months (127). After the supplementation period, all infants were given the standard formula with 3.0% ALA as the sole source of n-3 fatty acids. The DHA-supplemented infants in both studies had shorter look durations than infants on the control formula. Shorter look duration and visual acuity are both positive neural outcomes of DHA supplementation in premature infants (127). Interestingly, the DHA group also displayed a 10-point advantage in IQ at 12 months post-supplementation, indicating a

long-term effect of short-term supplementation of dietary DHA during infancy. The standard formula in this study contained 3.0% ALA, and the infants assigned to it had the poorest DHA status, which further strengthens the argument that DHA is conditionally essential for neural development in preterm infants.

In the studies mentioned thus far (125-127), the low levels of EPA did not adversely affect AA status or infant growth. However, a study in which preterm infants received standard formula or a supplemented formula with a DHA/EPA ratio of 2/3, the supplemented infants exhibited lower growth parameters (128). More recent data from Rodriguez *et al.* (129) suggests that preterm infant formula containing DHA and EPA in a ratio of 5:1 in combination with 0.6% energy as ALA is compatible with growth and proper n-3 fatty acid metabolism. In this study, supplemented infants maintained the same erythrocyte DHA and AA status as breast-fed infants, and infant weight, height and head circumferences were not different between groups.

### **Animal Studies**

Many non-human species have been used as models for studying the effects of dietary n-3 content on retinal function. In particular, rhesus monkeys are an ideal model for human retinal function because of the many similarities between the structure, function and development in the retina (130-134). Only higher primates have a fovea that enables high visual acuity in addition to 3 classes of cones that enable trichromatic vision (130).

Neuringer and colleagues have conducted numerous studies involving the DHA status of n-3-deficient rhesus monkeys. They showed that monkeys that were born to n-3-deficient mothers and then were reared on an n-3-deficient diet after birth developed low amounts of n-3 fatty acids in brain and retina and displayed impaired visual function

compared to controls (5,51,135). Specifically, they noted a sharp decline in cerebral cortex DHA along with a compensatory increase of 22:5 n-6. The sum total of n-6 and n-3 PUFA in the PL was relatively unchanged, indicating a mechanism exists within the brain that is aimed to conserve the polyunsaturation of the membrane PL despite the state of n-3 deficiency. In the n-3-deficient animals, the impairment of visual function was indicated behaviorally by decreased visual acuity and in the ERG by delayed recovery of a dark-adapted response to a saturating flash. Interestingly, the a-wave and b-wave amplitudes were unaffected by n-3 deficiency, but the implicit times of both the a- and b-waves were significantly increased in n-3-deficient monkeys (136). An additional study reported that, even when DHA was replete, the abnormalities in the ERG persisted (137). These results further underscore the importance of n-3 PUFA, and particularly DHA, during the perinatal period.

In follow-up studies of juvenile rhesus monkeys, Connor *et al.* (138) reported a rapid repletion of DHA in the cerebral cortex to control levels or above control levels when animals deficient since intrauterine life were fed a fish oil diet high in EPA and DHA. The recovery of neural DHA began within a week of fish oil feeding and was complete within 12 weeks. Again, the sum total of n-3 and n-6 fatty acids remained quite similar, and the increase in n-3 PUFA was accompanied by a reciprocal decline in n-6 PUFA.

Jeffrey *et al.* (139) investigated the effects of LCPUFA supplementation on visual acuity and retinal function in infant rhesus monkeys as well. They formulated the experimental diets to contain both DHA and AA in markedly greater quantities, 1.0% each of total fatty acids, than those used in previous human studies. They measured 25 parameters of visual function and found a significant diet effect in only 2. The failure to

detect significant differences between subject groups suggested that an upper limit exists for the developmental and functional benefits of LCPUFA supplementation and that the high concentrations of DHA and AA neither harm nor benefit the development of visual function at 4 months of age.

A recent study in preterm baboons reported a close, linear correlation between retinal DHA status and a-wave parameters (140). Traces from 4-week old premature baboons fed either non-supplemented formula or formula supplemented with 0.6% energy AA and 0.3% energy DHA showed significantly smaller a-wave amplitudes and longer a-wave implicit times than full term, breast-fed neonates. Although the supplemented premature infants performed better than their non-supplemented counterparts, the level of retinal function did not match that of the breast-fed term group. Moreover, b-wave amplitudes only slightly responded to increased retinal DHA in the formula groups. In addition, retinal AA concentrations were unaffected by either prematurity or supplementation.

Rats and guinea pigs have also been used as models for human retinal function. Early studies of retinal function in rats showed that, contrary to data from monkeys, ERG amplitudes were indeed affected by dietary n-3 fatty acids (141,142). Rats that were fed ALA had larger a-wave amplitudes than did deficient rats. However, Leat *et al.* (143) raised rats and guinea pigs to a third generation on n-3-deficient diets and reported no differences in b-wave amplitudes between deficient animals and those reared on commercial diets. Further support for these results came from Bourre *et al.* (54) who observed diminished a- and b-wave amplitudes in young rats fed ALA-deficient diets. They also suggested that the effects on the b-wave were transient while the effects on the



a-wave persisted into adult life. These data are, again, in contrast with those reported in monkeys by Connor and Neuringer (137), which indicate that n-3 repleted monkeys do not regain retinal functionality as measured by ERG. To further confound the issue, data from n-3-deficient guinea pigs show complete functional recovery in ERG parameters after ten weeks on an n-3 fatty acid-replete diet (144). The ERG of the guinea pig is biphasic, rather than the roughly sigmoidal curve observed in rat and human ERG (143). Furthermore, the guinea pig retina can be made nearly devoid of any DHA, whereas rats and primates tenaciously retain retinal DHA (74-78). Thus, species differences may account for the discrepancies among studies in primates and rodents. Consequently, data from such studies should be considered cautiously before making extrapolations to humans.

### **LCPUFA Supply in Maternal Milk**

After parturition, maternal milk serves as the sole exogenous source of LCPUFA for the newborn. The concentration of DHA in human milk varies more than 10-fold and is dependent upon the mother's diet (145). DHA levels in the breast milk of most Western populations reach about 0.1-0.4% of total milk fatty acids (146), whereas in regions with a high fish consumption that amount can be up to 1.4% (147-149). Jørgensen *et al.* (150) reported that, within the Danish population all, milk DHA levels varied by more than a factor of 10, and 55% of that variation could be explained by the differences in maternal fish intake. Thus, since 30% of the individual fatty acids in maternal milk are derived directly from the diet, dietary intake appears to be the most significant determinant of the fatty acid composition of the milk (151,152).

It has been well documented that maternal dietary fat intake is reflected in the fatty acid composition of the breast milk (145,151,153-156). This was evident in a study by Harris *et al.* (154), in which a dose-dependent increase in breast milk DHA was reported in women who consumed fish oil supplements for 1 to 4 weeks. Additionally, Francois *et al.* (151) observed the effects of 6 dietary fats, including menhaden and herring oils, on breast milk fatty acids for 6 days following a single fatty meal. In the menhaden fish oil group, DHA appeared within 6 hours after the meal, peaked within 24 hours, and remained elevated for up to 3 days.

More recently, Francois *et al.* (157) conducted a study in which lactating women were supplemented with 20 grams of flaxseed oil (10.7 g ALA) for 4 weeks. There was an increase in breast milk EPA and DPA, but not DHA. This was a surprising finding and perhaps can be attributed to the strict regulation of DHA synthesis at the level of DPA. An alternative possibility is that the Sprecher pathway does not exist in mammary tissue.

Gibson *et al.* (158) provided the first dose-tissue response curves for dietary DHA in human infants fed breast milk. They showed that the level of DHA in infant plasma and erythrocytes is related to the level of DHA in breast milk in a curvilinear fashion. In their study, the curve reached a plateau when DHA was 0.8% of the total fatty acids, thus indicating that DHA incorporation is saturable. Additionally, they reported that the increased DHA in blood phospholipids was more closely correlated with the decline in total n-6 fatty acids than with the decline in AA all.

In 2 studies that measured the fatty acid composition of breast milk following maternal fish oil supplementation, a several-fold increase in breast milk EPA was observed in addition to the rise in milk DHA (153,154). While many studies provide data to support

the supplementation of commercial infant formula with n-3 LCPUFA, some evidence suggests that EPA may exert detrimental effects in infants. Lower growth rates have been associated with low plasma and RBC concentrations of AA, and it is possible that EPA may compete with AA for incorporation into tissue PL and/or the conversion to eicosanoids or may inhibit the conversion of LA to AA (159-161). Supplementation of preterm formula with fish oil conferred a transient beneficial effect on visual function, negatively affected growth and some indices of neural development (128,162). This potential side effect of n-3 LCPUFA supplementation appears to be offset by the addition of AA to formulas already supplemented with DHA (163,164).

### **Recommendations for Safety and Adequacy**

Despite the abundance of studies claiming benefits of n-3 LCPUFA supplementation in infants, relatively few pinpoint what might be an adequate intake (AI) of either ALA or n-3 LCPUFA. Recent recommendations for infants suggest that at least 3-4.5% of total energy should be in the form of LA and at least 0.5% in the form of ALA to meet minimum EFA requirements (165-170) (Table 1.1). Intake of LA and other n-6 fatty acids should be limited to <10% energy and the total PUFA should be limited to <15% energy. When considering the intakes as a percentage of the total fatty acids, LA and ALA should account for 10% and 1.5% of the total fatty acids, respectively (171). Recommendations for AA and DHA intakes are 0.5% and 0.35% of total fatty acids. Additionally, EPA should not be present in amounts greater than 0.10% of the total fatty acids. To date, there is no evidence to support the need for preformed LCPUFA beyond 6 months of age in infants who were fully breast fed for the first 6 months of life and who receive a variety of lipid-rich foods (10). For adults, the suggested AI for LA is 2.0%

**Table 1.1** Fatty acid AI\* for infant formula/diet

Fatty acid	Percentage of total fatty acids
LA	10.00
ALA	1.50
AA	0.50
DHA	0.35
EPA ( <i>Upper Limit</i> )	<0.10

\* AI = adequate intake. If sufficient evidence is not available to calculate an estimated average requirement, a reference intake called an AI is used instead of a recommended dietary allowance. The AI is a value based on experimentally derived intake levels or approximations of healthy people. The AI for children and adults is expected to meet or exceed the amount needed to maintain a defined nutritional state or criterion of adequacy in essentially all members of a specific healthy population; LA = linoleic acid; ALA = alpha-linolenic acid; AA = arachidonic acid; DHA = docosahexaenoic acid; EPA = eicosapentaenoic acid (table modified from reference 7).

energy and 1.0% energy for ALA 7). Pregnant and lactating women require an additional 300 mg/day of DHA (Table 1.2).

The benefits of n-3 LCPUFA supplementation are not linearly correlated with the dose consumed. Rather, the maximum benefits achieved reach a plateau, after which, additional n-3 LCPUFA consumption ceases to convey additional benefits. In fact, it should be noted that excessive essential fatty acid intakes could have potentially deleterious effects at the cellular level (10).

One such negative effect is the increased susceptibility to oxidative stress. The high degree of unsaturation observed in DHA, combined with the high aerobic capacity of neural tissues sets the stage for potential tissue damage via lipid peroxidation. The products of cellular peroxidation, collectively called reactive oxygen species, include superoxide anion, hydrogen peroxide, and hydroxyl radicals (172). In the last decade or so, a number of neurological pathologies, such as ischemic brain damage, neurodegenerative disorder, aging and Alzheimer disease, have been attributed to excessive oxidation (reviewed in 172).

The notion that high concentrations of LCPUFA accompanied by excessive oxygen consumption and free iron may render the brain more susceptible than other organs to oxidative damage has been widely accepted (172). Moreover, products of lipid peroxidation retain their toxic properties for a longer period of time than do free radicals (173). A study by Brand and Yavin (174) showed that DHA added to oligodendroglia-like cells caused apoptosis following a genotoxic stress.

To the contrary, evidence has surfaced that suggests a possible protective role of DHA in regard to oxidative stress within neural tissues. Hossain *et al.* (175) reported

**Table 1.2** Fatty acid AI\* for adults

Fatty Acid	g/d (2000 kcal diet)	% Energy
LA	4.44	2.0
<i>Upper Limit</i>	6.67	3.0
ALA	2.22	1.0
DHA + EPA	0.65	0.3
DHA <i>to be at least</i>	0.22	0.1
EPA <i>to be at least</i>	0.22	0.1

\* AI = adequate intake. If sufficient evidence is not available to calculate an estimated average requirement, a reference intake called an AI is used instead of a recommended dietary allowance. The AI is a value based on experimentally derived intake levels or approximations of healthy people. The AI for children and adults is expected to meet or exceed the amount needed to maintain a defined nutritional state or criterion of adequacy in essentially all members of a specific healthy population; LA = linoleic acid; ALA = alpha-linolenic acid; AA = arachidonic acid; DHA = docosahexaenoic acid; EPA = eicosapentaenoic acid (table modified from reference 7).

lesser quantities of lipid peroxides in areas of rat brain that experienced an increased DHA uptake following DHA feeding. In areas where the level of DHA remained unchanged, no decrease in lipid peroxides was observed.

Several reports also suggest a role for DHA as a survival factor in photoreceptor cells (176-178). In these studies, DHA was able to rescue photoreceptors from apoptosis, the most common pathway of programmed cell death, during the early stages of their development *in vitro*. Also, this protective effect was specific only to photoreceptor cells, as apoptotic events in developing amacrine cells were unaffected by the addition of DHA (179).

An additional safety concern regarding n-3 PUFA supplementation is the possible interference with n-6 eicosanoid synthesis. Since n-3 and n-6 eicosanoids are synthesized by the same enzymes, an increase in n-3 PUFA, particularly EPA, could negatively shift the physiological balance between n-6 and n-3 derived prostaglandins and thromboxanes. One consequence of such a shift is an increase in bleeding time (increased time to clotting). While a decreased risk of spontaneous clotting is a positive effect of n-3 supplementation, there is some concern that extremely high doses may result in abnormally long bleeding times. Studies in Inuit Eskimos, a population with a high marine fish oil intake, indeed report an increase in bleeding time as a result of the imbalance between pro- and anti-aggregatory eicosanoids (180). Similar studies of bleeding times in fish oil-supplemented pregnant women have yielded mixed results. One study reported a slight, but significant increase in blood loss at delivery, whereas a larger study found no difference between supplemented and non-supplemented mothers (181,182).

Earlier in this chapter, it was mentioned that some studies have reported an adverse effect of high EPA intake on infant growth. It is possible that the increased levels of EPA suppress conversion of AA to prostaglandin E<sub>2</sub>, which has been shown to stimulate cell proliferation in vitro (183-185). This evidence underscores the importance of limiting an infant's EPA consumption to <0.1% of total fatty acids.

Obviously the potential adverse side effects of unusually high concentrations of LCPUFA within biological systems and membranes remain unclear. Until the possible long-term effects of n-3 LCPUFA supplementation are more thoroughly identified, it is advisable to avoid excess intakes.

### **Canine Neurologic Development**

Only limited information regarding canine DHA metabolism is available, but studies to date suggest that DHA is required in canine nervous system and neonatal development. Anderson, *et al.* demonstrated that canine retina is capable of synthesizing DHA from its 22-carbon precursor, DPA, that DHA is highly conserved in the retina, and that it has a role in neurological function in this tissue (83,186). Alternatively, our laboratory has reported the accumulation of DPA, but not DHA, in plasma PL when ALA is fed (187). Although accumulation of DHA was not observed in the plasma fraction of adult dogs, the possibility exists that the noted increase in DPA is an important regulatory step prior to nervous tissue uptake and subsequent retinal DHA synthesis. This notion is consistent with Alvarez, *et al.* (186) who found that canine nervous tissue could convert DPA to DHA. Therefore, it is likely that canine retina, and presumably other nervous tissues, synthesize and utilize DHA in a manner similar to other mammalian species and that plasma DPA provides a likely substrate for such synthesis. It also is possible that a dietary



source of DHA, whether as the 18-carbon precursor or as a preformed long-chain fatty acid, may be necessary during gestation and suckling for normal neural development in dogs.

ALA may be sufficient as a dietary precursor for the synthesis of requisite amounts of DHA during pre- and postnatal development. However, what quantity of ALA may be needed to optimize neural development in companion animals currently is not known. Additionally, because both n-6 and n-3 fatty acids compete for the same enzyme systems, it also is unclear what role the dietary LA/ALA ratio has on subsequent n-3 synthesis. An alternate approach to using dietary ALA as a DHA precursor would be to supply pre-formed amounts of DHA in canine gestational and lactational diets. Regardless of the source of DHA, data on the competitive metabolism of either the 18-carbon or the 20/22-carbon n-3 and n-6 fatty acids and their effects on neurological tissue will be needed in order to better understand what dietary amounts of PUFA are necessary.

In theory, the DHA requirement of neural tissues may be met in one of 4 possible ways: desaturation and elongation of ALA within the brain and retina; uptake of DPA following hepatic conversion from ALA and further conversion within the brain and retinal tissue to DHA; uptake of circulating DHA previously synthesized in tissues such as liver; and uptake of DHA directly from dietary sources. The studies presented here provide important new information as to which of these possibilities most likely exists in dogs.

### **Canine Milk Composition**

Many of the earlier studies of canine milk composition were limited to macronutrient analysis, i.e. carbohydrate, protein and total fat content, and fatty acid analyses were not performed. To our knowledge only three published reports exist

regarding the fatty acid composition of canine milk (188-190). Iverson, *et al.* (188) observed no PUFA with chain length greater than 20 carbons (predominately AA), although they routinely identified longer chain fatty acids, including n-3 PUFA, both in analytical standards and in marine oil. This finding is in contrast to a later study by Lepine in which small amounts of DHA were found in canine milk (190).

Our laboratory recently conducted a preliminary study on canine milk composition (189). In this study, females were fed Hill's Science Diet<sup>®</sup> Canine Growth<sup>®</sup> formula prior to breeding, during gestation and throughout lactation. Because different batches of this commercial diet were fed over time, the n-3 fatty acid content could only be estimated based on the analysis of a single batch. Nonetheless, it was determined that the n-3 content of the diet was low. Values on a dry matter basis were as follows: 0.3% ALA; 0.0% EPA; 0.03% DPA (n-3); and 0.01% DHA. The n-6 fatty acids in the diet were: 4.6% LA and 0.1% AA. Similarly, milk from the dogs fed these diets contained low amounts of n-3 fatty acids and high amounts of n-6 fatty acids. Perhaps most striking was the finding that, compared with human breast milk of various populations, canine milk contained about seven times less DHA and about 2 times more AA. LA content of canine milk was slightly higher than human milk, and ALA was about the same as human milk. These preliminary observations are of special interest because canine neurological tissues such as retina and brain contain the same high proportions of long-chain n-3 and n-6 PUFAs as do the same tissues in humans. Therefore, the emergent question is whether canine milk containing low DHA and high AA is the most appropriate to assure proper neural development for suckling neonates. One objective of this research was to address this question.

## Canine Vision

Although details on the visual anatomy and physiology of canines have yet to be fully established, a few studies have provided data which allow for the comparison of human and canine retina. The visual system of the dog has evolved for survival in an arrhythmic photic existence rather than being adapted strictly for diurnal or nocturnal vision, as are humans (191). That is to say, canine vision can adapt to enhance visual performance in dim light while retaining the ability to function under bright light conditions.

In both dogs and humans, the rod photoreceptors are employed for vision in dim light. Whereas in humans the central 25° of retina consists primarily of cones, in canids this region predominantly contains rod photoreceptors (192). Furthermore, the rhodopsin molecule varies slightly between both species. Canine rhodopsin has a peak sensitivity between 506 and 510 nm, which is crucial to a dog's ability to function in dim light. Moreover, the regeneration of canine rhodopsin following extensive bright light exposure can take well over an hour (192-194). Human rhodopsin, on the other hand, exhibits maximal sensitivity at 496 nm and regenerates much more quickly following photoreceptor bleaching (195).

Flicker fusion, the point at which rapidly flickering light appears to fuse into constant illuminated light, has provided an inroad toward the understanding of the functional characteristics of the canine photoreceptors (195). Electroretinographic data from anesthetized dogs suggest that canine rods can detect flickering up to a maximum of 20 Hz, which is similar to the maximum reported in human rods (196-199). However,

maximal flicker frequency of the cone photoreceptors is somewhat higher in dogs than in humans (196,197,200).

### **Visual Acuity**

Although visual acuity is not of primary interest in this research, it has been employed in numerous studies as a measure of neural development in primate infants. Visual acuity refers to the ability to see the details of an object separately and unblurred (201). It is this ability that allows the visual system of the dog to function well in low light situations. Odom and colleagues suggested that canine visual acuity is limited by the retina and not by postretinal neural processing or the optical properties of the eye itself (202).

Generally, enhanced nocturnal vision necessarily requires that a large number of rod photoreceptors synaptically converge upon a single ganglion cell (195). Such a structural arrangement usually results in decreased visual acuity. To the contrary, retinas with superior resolving power have a high ratio of ganglion cells to photoreceptors, a large number of ganglion cells and optic nerve fibers, and a high density of photoreceptors (191). The visual acuity of dogs is believed to be intermediate to that of felines and primates (202). Reportedly, human optic nerve contains  $1.2 \times 10^6$  fibers compared to 167,000 and between 116,000 and 165,000 respectively, in canine and feline optic nerves (203-207). Although the ratio of photoreceptors to ganglion cells has not been determined in dogs, it is believed to be similar to that reported in cats—4 cones/ganglion cell—which compares with a ratio of one cone/ganglion cell in the primate fovea (195,208).

Instead of a fovea, as is found in primate retina, the area of highest visual acuity in dogs is the visual streak, which is an ovate region located superior and temporal to the

optic nerve (209-211). Work by Kemp and Jacobson (192) provided detailed maps of photopigment concentrations that showed the visual streak to contain high concentrations of rhodopsin. Additionally, the binocular vision of dogs most likely is enhanced by the temporal region of the visual streak (209,210). Finally, it should be noted that retinal ganglion cell topography, and thus visual acuity, can vary greatly amongst breeds (209).

## **CHAPTER II**

### **MATERIALS AND METHODS**

#### **Experimental Design**

##### **Animals**

Dogs for this study were approved for use by the Texas A&M University Laboratory Animal Care Committee (ULACC AUP #2001-110). Animals were housed at the veterinary small animal clinic during the study and were returned to the Laboratory Animal Research Resources (LARR) facility after they had completed their role in the study. Prior to entering the study, complete blood counts were obtained to ensure overall health of the animals. All dogs used in the study appeared to be clinically and physiologically normal.

Twelve clinically normal, sexually intact adult female hound/Labrador retriever crossbred dogs, ages 2 to 4 yr old, and their subsequent puppy litters were used in the study. The puppies were evaluated from birth until 12 wk of age. The females were bred to the same sire, a yellow Labrador retriever, via artificial insemination. The body weights of the mothers and the puppies were recorded throughout the study and are included in the Appendix A, Tables A-I and A-II.

Before entering the study, all animals were housed at the LARR facility where they were monitored for signs of proestrus. Upon the onset of proestrus, the dogs were brought to the Texas A&M University Veterinary Clinic for confirmation of proestrus and for subsequent artificial insemination. At this time, each dog was randomly assigned to one of the four experimental diets, and an EDTA blood sample was taken.

This pre-experimental diet sample, designated as d -3, was also used as a basis for comparison of plasma fatty acid composition between the maintenance and experimental diets. Each animal was inseminated 3 to 5 times, depending upon the individual stage of proestrus at the time of breeding, to increase the likelihood of conception. The third insemination day was marked as d 0 of gestation for the purpose of this study.

### **Diets and Feeding**

The diets for the study were manufactured by Nestlé-Purina Pet Care® (St. Louis, MO) and were complete and balanced for all canine life stages. All diets contained sufficient amounts of LA ranging from 1.8-3.5% dry matter (DM), and differed in their fatty acid compositions. Each diet consisted of approximately 15% total fat and contained one of the following as its primary fat source: beef tallow, linseed oil, “low” amounts of Menhaden fish oil or “high” amounts of Menhaden fish oil. Each diet was designed to have a unique ratio of its ALA to n-3 LCPUFA content. Based on these relative amounts, the diets were designated as low ALA/low n-3 LCPUFA (Lo/Lo, tallow); high ALA/low n-3 LCPUFA (Hi/Lo, linseed oil); low ALA/moderate n-3 LCPUFA (Lo/Mod, low Menhaden fish oil); and low/high (Lo/Hi, high Menhaden fish oil). All other components of the diets, including total protein, nitrogen-free extract, vitamins and minerals, were identical. The nutrient compositions, list of ingredients, and the fatty acid compositions of each diet can be found in the appendix (Tables B-I to B-III).

Dogs were fed their respective diets at the time of first insemination and was maintained on that diet throughout their subsequent gestation, parturition, and lactation

periods. Sufficient quantities of the diets were fed to maintain weight gain during the latter stages of gestation by adjusting the amounts fed as necessary. Body weights were monitored weekly in order to determine adequate quantities of food. At 21 d postpartum, a gruel consisting of the mothers' respective diets and water was offered to the puppies three times a day, in addition to suckling. Gradually, the time the puppies spent suckling was decreased until they were completely weaned by d 28. Upon weaning, puppies were continued on the same diets as their mothers until 12 wk of age. The puppies were weighed daily until 6 wk of age and every other day thereafter to ensure proper growth and development. If any failure-to-thrive issues arose, the puppy was removed from the study and supplemented to ensure proper nutrition. Generally this occurred only when litter size was large; small puppies were the most likely to be removed.

Veterinary student workers fed and provided clean water for the dogs daily. The mothers were fed once a day during early gestation, up to three times a day during late gestation, and twice a day during lactation. The puppies were fed their respective dry diet and water three times a day after weaning. Prior to this study, the adult dogs had been fed Hill's Science Diet<sup>®</sup> Canine Maintenance<sup>®</sup> (Topeka, KS) diet twice a day.

At 12 wk of age, retinal development of the puppies was assessed via electroretinography (ERG). The ERG procedure is outlined later in this chapter. The completion of the ERGs marked the end of the study, at which time suitable homes were found for the puppies.



## **Blood Samples**

Blood samples were collected from mothers and puppies via venipuncture and transferred into tubes containing 7.5% EDTA.

Blood was collected from the mothers prior to artificial insemination (d -3) and at d 3, 7, 14, 28, 42 and 56 during gestation and d 10 and 28 during lactation. Food was withheld from each dog overnight prior to taking the blood samples. Seven milliliters of blood was collected on all days except for d 14 and 42, when 14 mL was taken.

Blood was taken from the puppies at 4, 10, 16, 28, 70 and 84 d of age. The puppies were removed from their mothers for 3 h prior to blood collection on d 4, 10, 16 and 28, and food was withheld overnight on d 70 and 84. Two milliliters of blood was taken each sample day through d 16. On d 28, 4 mL were collected, and on d 70 and 84, 7 mL were obtained.

Plasma from each sample and red blood cells (RBC) from selected sample days were harvested immediately following collection. The samples were centrifuged for 15 min at 2800 rpm in a Centra-7 centrifuge (International Equipment Company, Needham, MA). Plasma was separated from the RBC for subsequent lipid extraction. Red blood cells, collected from adult dogs on d 3, 7, 42 and 56 of gestation, d 10 and 28 of lactation and from puppies on d 4, 28, 70 and 84, were diluted with isotonic saline (0.9% NaCl, 1:1 (v/v) ratio). Both the plasma and RBC fractions were extracted immediately or were stored at -20°C until the time of extraction. Erythrocyte samples were stored no longer than 2 wk and plasma no longer than 4 wk prior to analysis.

**Lipid extraction.** Plasma, erythrocyte, and milk samples were subjected to lipid extraction via a modified Folch procedure (212). Five hundred microliters of each adult plasma, adult RBC and neonate RBC sample and 300  $\mu$ L of each milk and neonate plasma sample was transferred to a 12-mL teflon-lined screw top glass test tube. To each sample was added 9.0 mL of chloroform:methanol (2:1, v/v) with 0.2% glacial acetic acid. The samples were shaken in a tube shaker (Shaker-in-the-Round Model S-500; Kraft Apparatus, Inc., Mineola, NY) for 20 min at room temperature (27 °C). After shaking, 2.0 mL of distilled water was added to each sample. Samples were then shaken for an additional 10 min and were centrifuged for 15 min at 2800 rpm. The infranates were transferred to clean test tubes and washed with 5.0 mL of chloroform:methanol:water (3:48:47, v/v/v). The samples were shaken again for 10 min and spun for 15 min. The infranates were transferred, via glass wool filtration, to clean tubes. Each sample was purged with nitrogen gas and stored in tightly sealed screw-capped glass tubes at -20°C until further analysis as described below.

**Thin layer chromatography.** Plasma lipid subclasses were separated using thin layer chromatography (TLC). Lipid samples were evaporated under nitrogen gas and resuspended in 300  $\mu$ L chloroform, 150  $\mu$ L of which was applied to a 20x20 cm, 250- $\mu$ m thickness silica gel G coated plate (Fisher Scientific, Suwanee, GA). Prior to use, plates were washed in chloroform:methanol (2:1, v/v), air-dried briefly, and then activated in a 110°C oven (National Appliance Company Model 5510, Portland, OR) for 1 h. The plates were stored in a sealed plate box until needed, but were stored no longer than 1 wk. The lipid extracts were developed in a filter paper (Whatman International,

Ltd., Maidstone, England) lined, covered glass tank. A hexane:ether:acetic acid solution (80:20:1, v/v/v) was used as the mobile phase. The tank was equilibrated for no less than 1 h prior to developing a plate with samples. Plates were loaded with 4-5 samples, and at least one plate contained a standard lipid mixture (TLC Standard #18-5-A; Nu-Check Prep, Elysian, MN). Plates were developed in the tank until the solvent front reached 1-2 cm from the top of the plate. The plate was removed, dried briefly in a fume hood, and lipid subfractions were visualized in an iodine vapor chamber. Phospholipid subfractions were removed by scraping prior to visualization in the iodine tank, but the TG and CE subfractions were marked after being subjected to iodine vapor. The iodine was allowed to dissipate under nitrogen gas and the subfractions then were scraped into a clean teflon-lined screw top test tube to which was added 2.0 mL of a 4% sulfuric acid in methanol solution. Samples were transmethylated directly, as described below, or were purged with nitrogen gas and stored at -20°C until the time of methylation.

**Red blood cell phospholipid separation.** Red blood cell extracts were evaporated under nitrogen gas and resuspended in 150  $\mu$ L chloroform, all of which was applied to an activated TLC plate. The plates were developed in the same manner and solvent system as the plasma samples. Red blood cell total PL were scraped without visualization in iodine vapor; no other lipid subclasses were collected. The total PL subsequently were prepared for transmethylation as previously described.

**Methylation of lipid subfraction fatty acids.** Previously separated lipid subfractions in 4% sulfuric acid in methanol were heated in a 90°C water bath (Thelco 182, Model 66570; GCA Precision Scientific, Chicago, IL) for 50 min. Samples were

removed from the water bath and allowed to cool for 5 min. Three milliliters of hexane was then added to each tube, and the tubes were mixed for 30 seconds each on a Vortex-Genie Model K-550-G (Scientific Industries, Inc., Bohemia, NY). All tubes were centrifuged for 15 min at 2800 rpm. The supernates, which contained the fatty acid methyl esters (FAMES), were transferred to clean test tubes, purged with nitrogen gas, and stored at -20°C for subsequent analysis by capillary gas chromatography.

**Gas chromatography.** The transmethylated samples were evaporated to dryness under nitrogen gas, and the FAMES were resuspended in a known volume of hexane. Two microliters of each sample was injected onto the capillary column (FAMEWAX Crossbond<sup>®</sup>-PEG; Restek, Bellefonte, PA) of a Hewlett Packard Series II 5890 Gas Chromatograph. Helium was used as the carrier gas, and the oven temperature was ramped from 170°C to 220°C over a period of 90 min. In all runs, 24:1n-9 is the last possible identifiable fatty acid peak in this system. Hewlett Packard HP ChemStation software package was used to generate results from the analyses. Authentic fatty acid methyl ester standards (Nu-Check-Prep, Elysian, MN) were used to identify the individual fatty acid peaks by comparing retention times.

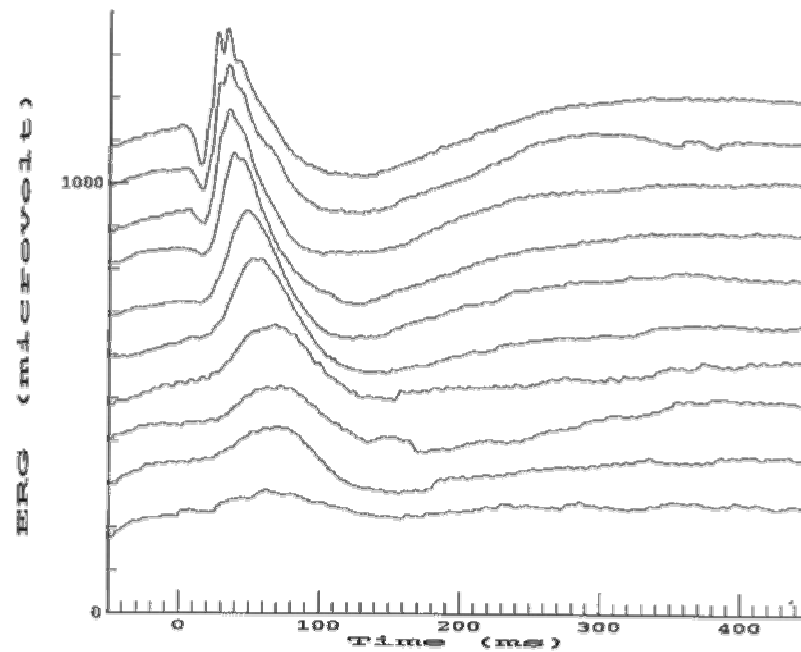
### **Electroretinography**

Electroretinograms were recorded using the Cornell ERG System (Cornell University, Ithaca, NY), a custom-built, computer-based ERG acquisition system (Windows-based software; Microsoft, Redmond, WA) was acquired from colleagues at the Cornell University veterinary hospital (Dr. Ellis Loew).

On the day ERGs were performed, 12-wk-old puppies had been dark adapted for

2 h prior to measurement. Before the procedure was begun, 0.04 mg/kg atropine sulfate (Sparhawk Laboratories, Inc., Lenexa, KS) was administered subcutaneously as a preanesthetic. This was followed by a subcutaneous injection of 20 mg/kg ketamine hydrochloride (Ketaset<sup>®</sup>; Fort Dodge Animal Health, Overland Park, KS) and 2.0 mg/kg xylazine (Vedo, Inc., St. Joseph, MO). When the puppies were sufficiently sedated, they were positioned in lateral recumbancy on the examination table, and subdermal platinum-iridium needle electrodes were placed on the muzzle (indifferent) and in an ear flap (ground). The eye to be tested was then exposed using a lens speculum inserted underneath the lids and the nictitating membrane. Local anesthesia was achieved using a 0.5% solution of proparacaine hydrochloride (Ophthetic<sup>®</sup>; Allergan America, Hormigueros, Puerto Rico) applied to the cornea, and pupillary mydriasis was induced by the addition of one drop of 1.0% tropicamide (Mydriacyl<sup>®</sup>; Alcon Laboratories, Ft. Worth, TX) before placing the active contact lens electrode (ERG-Jet; LKC Technology, Gaithersburg, MD) on the eye.

Each eye was tested separately using a series of square-wave flash stimuli 50 msec in duration, with an inter-flash interval of 5 s, from a white-light emitting diode (LED) placed approximately 1 cm from the cornea. The ERGs were obtained at 10 increasing 0.5 log unit intensity steps up to b-wave saturation. The highest intensity setting has been found to saturate the rod response in canines (213). The parameters used to assess ERG characteristics were a- and b-wave amplitudes, a- and b-wave implicit times. A typical ERG series obtained with the equipment used in this study can be found in Figure 2.1.



**Figure 2.1** Typical ERG trace obtained in this experiment. This series shows the rod photoreceptor response at 10 increasing 0.5 log unit intensity steps up to b-wave saturation. The highest intensity setting has been shown to saturate the rod response in canines (Loew, personal communications).

Following the completion of the ERGs, veterinary technicians monitored the puppies until they fully recovered from anesthesia. The animals then were removed from the study and gradually were weaned from the experimental diet to Hill's Science Diet<sup>®</sup> Canine Growth<sup>®</sup> formula.

### **Statistical Analyses**

The effect of diet and time on the individual plasma phospholipid fatty acid data from adult dogs was evaluated using a multivariate model that accounted for the random variance of dog. Repeated measures analysis of variance (ANOVA) with multiple comparisons where appropriate for main effects of diet, time and diet\*time interactions were performed at  $p < 0.05$  (Statistix 7.0; Analytical Software, Tallahassee, FL). Where there was a significant interaction of group and time, contrasts were made for each plasma phospholipid fatty acid using Bonferroni's test to determine where the difference occurred. An experiment-wide type I error of 0.05 was maintained. Milk fatty acid data were similarly analyzed via repeated measures ANOVA with Bonferroni multiple comparisons. One-way ANOVA was performed on the adult plasma phospholipid data comparing gestation d 56 and lactation d 28 to investigate statistically significant differences of plasma phospholipid fatty acids at the end of gestation with the end of lactation.

Puppy plasma phospholipid fatty acid data were analyzed by repeated measures ANOVA using litter as experimental unit with Bonferroni's multiple comparisons of main effects of diet, time and time\*diet interactions at  $p < 0.05$ . Puppy plasma PL data from d 28 and 84 were evaluated via one-way ANOVA as were the ERG parameters.

When ANOVA was significant, pair-wise comparisons were made using Bonferroni's test at  $p < 0.05$ .



## CHAPTER III

# EFFECTS OF DIETARY ALA AND N-3 LCPUFA ON MATERNAL PLASMA PHOSPHOLIPIDS DURING GESTATION IN CANINES

### Introduction

Both the n-3 and n-6 classes of long-chain polyunsaturated fatty acids are important in perinatal growth and development. Brain and retinal functions depend on the n-3 polyunsaturate docosahexaenoic acid (22:6 n-3, DHA) not only during gestational development, but during postnatal life as well. Maximal brain growth begins in the third trimester of gestation and continues throughout the first few months of neonatal life (42-44). During this crucial period, there is a select accumulation of both arachidonic acid (20:4 n-6, AA) and DHA in brain and retina which occurs ten times faster than incorporation of their respective precursors, linoleic acid (18:2 n-6, LA) and alpha-linolenic acid (18:3 n-3, ALA) via chain elongation (47, 48). In accordance with this knowledge, several authors have demonstrated that plasma DHA is the preferred substrate for retinal uptake in early developmental stages when the demand for DHA is greatest (78, 81, 83).

### Canine Neurologic Development

The tenacious conservation of DHA within the retina suggests it has a role in neurologic function (83). Anderson *et al.* (58) demonstrated that canine retina is capable of synthesizing DHA from its 22-carbon precursor, docosapentaenoic acid (22:5 n-3, DPA). Bauer *et al.* (187) reported the accumulation of DPA, but not DHA, in canine plasma phospholipids when the precursor ALA is fed. It, therefore, is likely that canine retina, and presumably other nervous tissues, synthesize and utilize DHA in a manner

similar to other mammalian species and that plasma DPA provides a likely substrate for such synthesis. Thus, a dietary source of pre-formed DHA or one of its precursors may be necessary during gestation and suckling for normal neural development in dogs.

It is possible that ALA may be sufficient as a dietary precursor for the synthesis of requisite amounts of DHA during pre- and postnatal development. However, what quantity of ALA may be needed to optimize neural development in companion animals currently is not known. Additionally, because both n-6 and n-3 fatty acids compete for the same enzyme systems, it also is unclear what relative amounts may be needed.

### **Materials and Methods**

An existing breeding colony of dogs provided bred hound/Labrador retriever dogs and their puppies for this study. The colony contains a kindred of dogs with hereditary nephritis (214) but all dogs used in this study were clinically healthy. Twelve dogs (three each per diet group) were fed one of four complete and balanced, extruded-type diets from the time of insemination and throughout gestation, parturition, and lactation. Sufficient quantities of the diets were fed to maintain weight gain in the latter stages of gestation by adjusting the amount fed as necessary. The diets were similarly formulated, but differed in their fatty acid composition and were sufficient in linoleic acid (Appendix B, Table B-I). They contained approximately 15% total fat using either beef tallow, linseed oil, or higher and lower amounts of menhaden fish oil as primary fat source (Nestle-Purina PetCare, St. Louis). Using these dietary fat sources, diets were formulated using typical pet food ingredients to contain adequate linoleic acid ranging from 1.8 to 3.5 % dry matter (DM) and were designated based on their ALA/n-3 LCPUFA contents as follows: Lo/Lo, Lo/

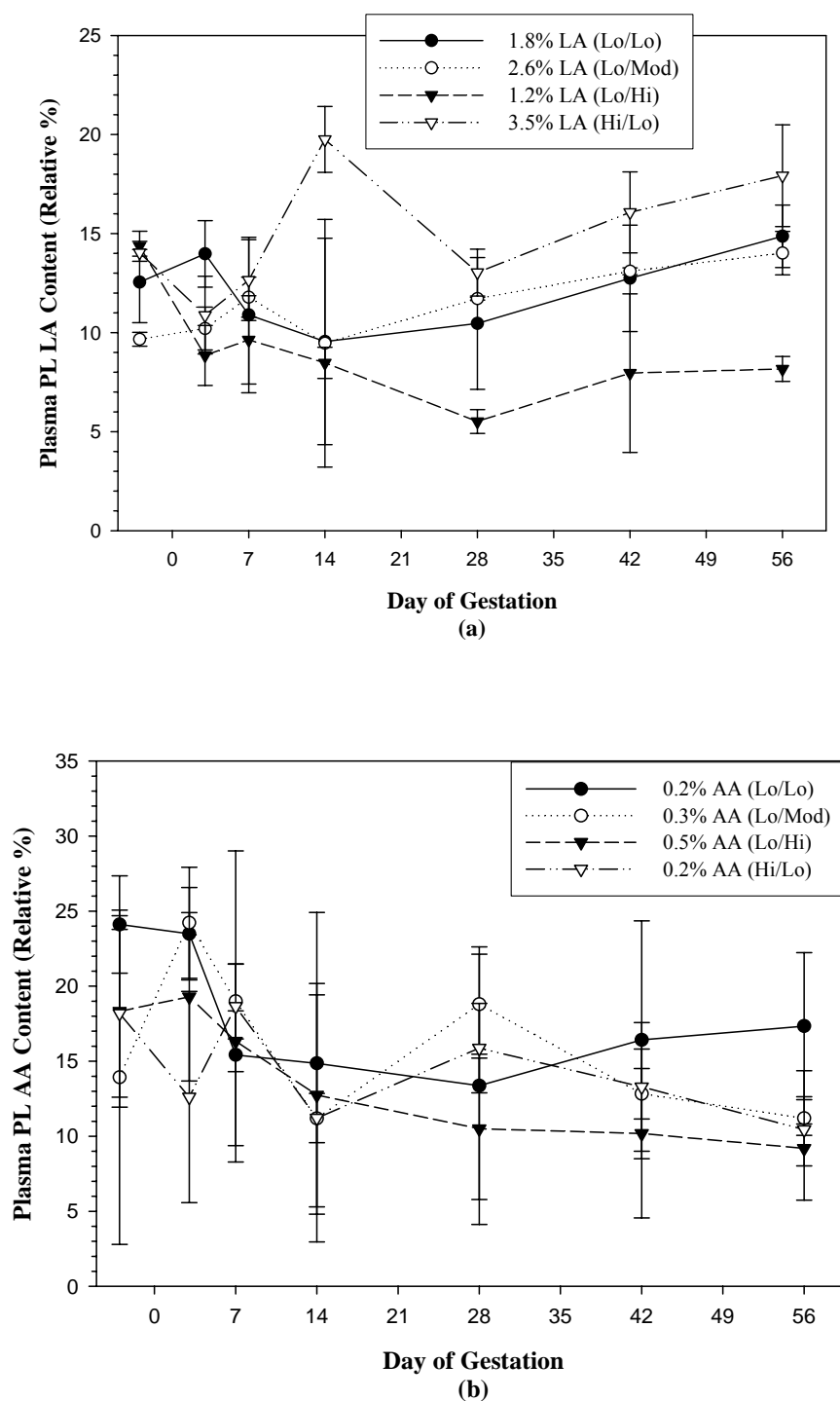
Mod, Lo/Hi, and Hi/Lo. All other dietary ingredients including total protein, nitrogen-free extract, vitamins, and minerals were identical in all diets (Appendix B, Table B-II).

EDTA blood samples were collected on d 3, 7, 14, 28, 42, and 56 post breeding. As described previously, plasma total lipids were extracted using chloroform:methanol (2:1, v/v) and subsequently separated into subclasses via thin-layer chromatography. Fatty acid methyl esters were prepared, using the total phospholipid subfraction (plasma PL), and fatty acid profiles were determined via gas chromatography (187). Statistical analyses were performed by repeated measures ANOVA with Bonferroni comparisons performed at  $p < 0.05$  for plasma PL fatty acids. One-way ANOVA was also performed on plasma PL data from day 56 of gestation to compare statistically significant differences between the beginning and end of gestation.

## Results

Initially, all dogs had similar plasma phospholipid (PL) fatty acid profiles before being placed on their respective experimental diets; no significant pre-experimental diet differences in any plasma PL fatty acid existed among groups. No widespread time effects or time\*diet interactions during gestation were detected at  $p < 0.05$ .

A dose response for LA in plasma PL fatty acid profiles was apparent by d 14, and persisted through d 56, of gestation (Figure 3.1a). The Hi/Lo diet contained 3.5% LA on a DM basis, which was more than twice that contained in the Lo/Hi diet. Consequently, the plasma PL LA of both these groups mirrored these dietary differences. At the end of gestation, plasma PL LA was  $17.9 \pm 2.6\%$  S.D. in the Hi/Lo group compared with  $8.2 \pm 0.6\%$  S.D. in the Lo/Hi group. Plasma PL LA concentrations in the Lo/Lo and Lo/Mod groups were intermediate to these values. Surprisingly, plasma PL AA remained

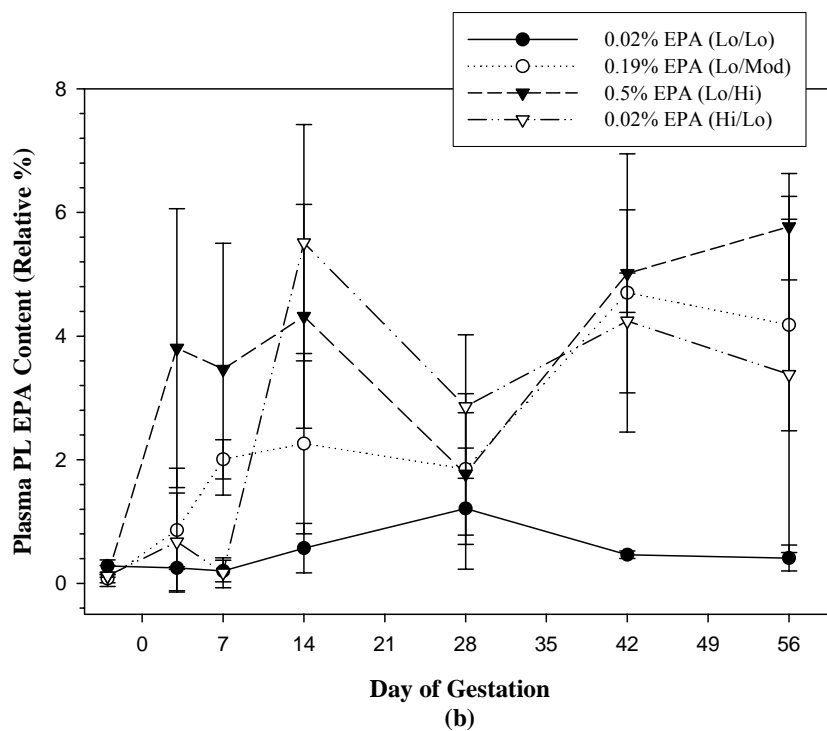
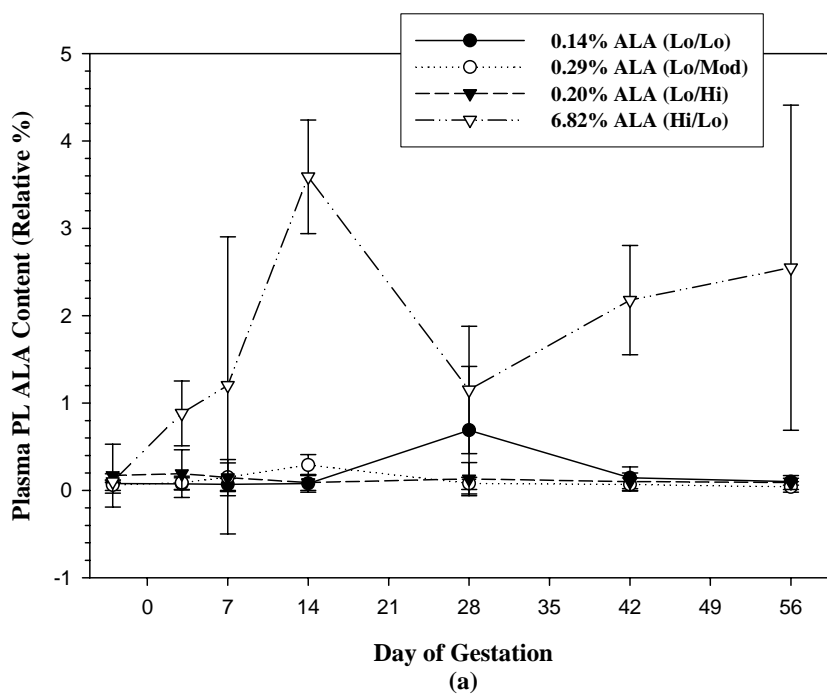


**Figure 3.1** Mean  $\pm$  S.D. plasma PL n-6 PUFA during gestation in dogs fed the experimental diets. Pre-experimental diet fatty acid profiles, shown as d -3, were not significantly different among dogs at  $p < 0.05$  ( $n = 3$  per group). Dietary LA (a) and AA (b) contents are indicated on a % DM basis in each respective figure legend. Plasma AA was not significantly altered from pre-diet levels in any group at  $p < 0.05$ .

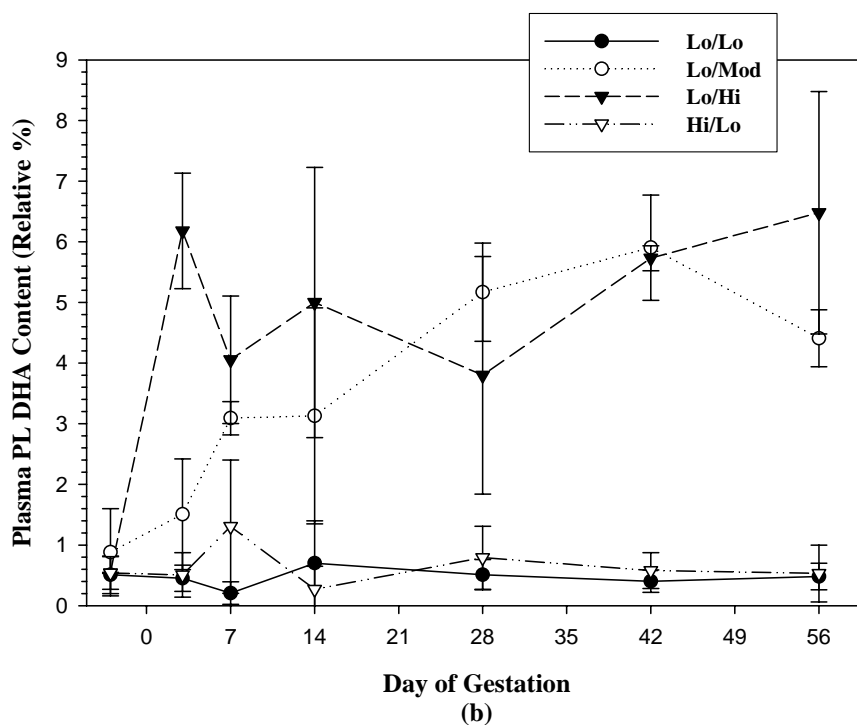
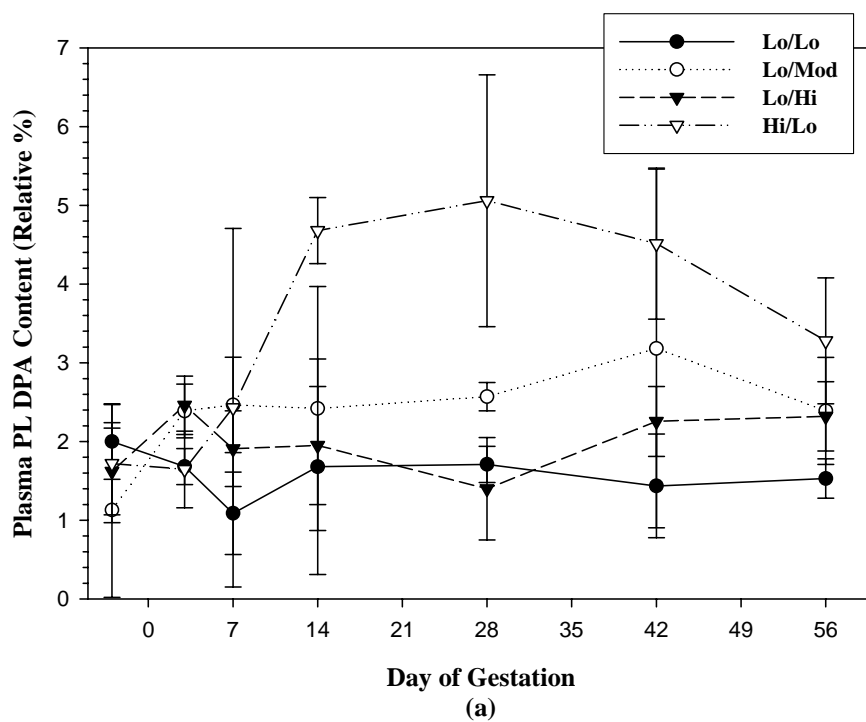
relatively constant within all diet groups throughout gestation (Figure 3.1b). Although the Hi/Lo diet contained a fairly high amount of the precursor LA (3.5% DM), plasma PL arachidonate in this diet group, as in all others, was not significantly different from pre-study values, nor was it different from the other groups.

Noticeable differences in the n-3 fatty acids were observed as well. In the Hi/Lo (high ALA) group, plasma PL ALA was significantly elevated above all other groups for the entirety of gestation (Figure 3.2a). Plasma PL ALA increased modestly by d 3 of gestation and was significantly higher than pre-diet levels by d 14, whereas plasma PL ALA remained unchanged in the other diet groups. This spike in plasma PL ALA occurred concomitantly with a sharp rise in EPA (Figure 3.2b) and DPA (Figure 3.3a) by d 14. Interestingly, plasma PL ALA and EPA values were roughly halved at d 28, but DPA remained steady.

The Lo/Lo and Hi/Lo diets contained equivalent amounts of derived n-3 LCPUFA, yet plasma EPA in the Hi/Lo group was significantly different from plasma in the Lo/Lo group. Curiously though, the plasma PL concentration of DHA (Figure 3.3b) was similar between both groups. Although the Lo/Hi diet contained derived LCPUFA in concentrations two times those of the Lo/Mod diet and roughly 20 times those in the Hi/Lo diet, the differences in plasma PL concentrations of the LCPUFA did not parallel these dietary differences. Mean plasma PL EPA in the Lo/Hi group during gestation was  $3.5 \pm 2.0\%$  S.D. compared to  $2.3 \pm 1.8\%$  S.D. and  $2.6 \pm 2.3\%$  S.D., in the Lo/Mod and Hi/Lo diets, respectively. None of these values were significantly different from each other. Plasma PL DHA became markedly elevated by d 3 in the Lo/Hi group while a less exaggerated increase occurred in the Lo/Mod group. The rise in plasma PL DHA in



**Figure 3.2** Mean  $\pm$  S.D. plasma PL ALA (a) and EPA (b) during gestation in dogs fed experimental diets. Pre-experimental diet fatty acid profiles, shown as d -3, were not significantly different among dogs at  $p < 0.05$  ( $n = 3$  per group). Dietary ALA and EPA contents are indicated on a % DM basis in each respective figure legend.



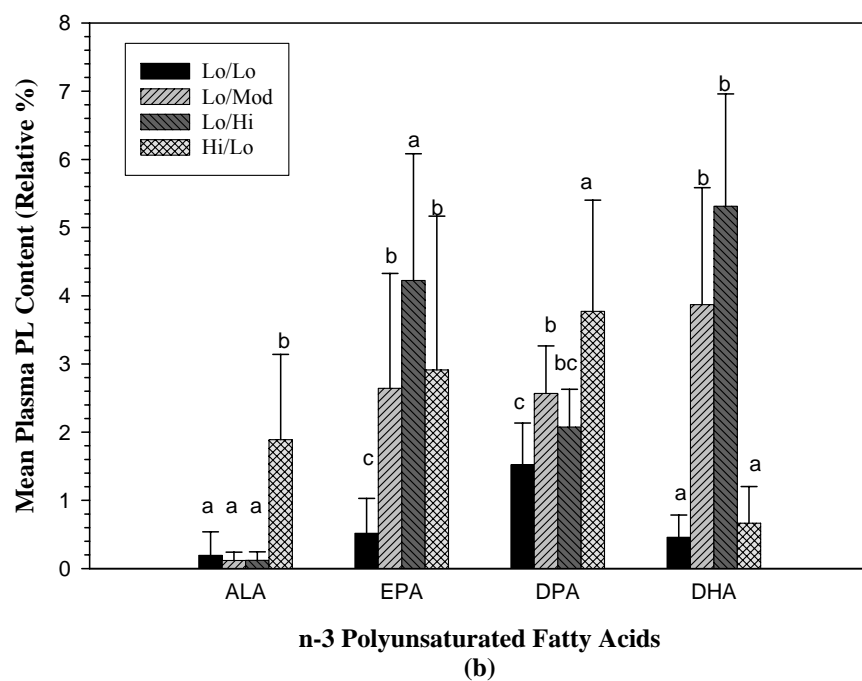
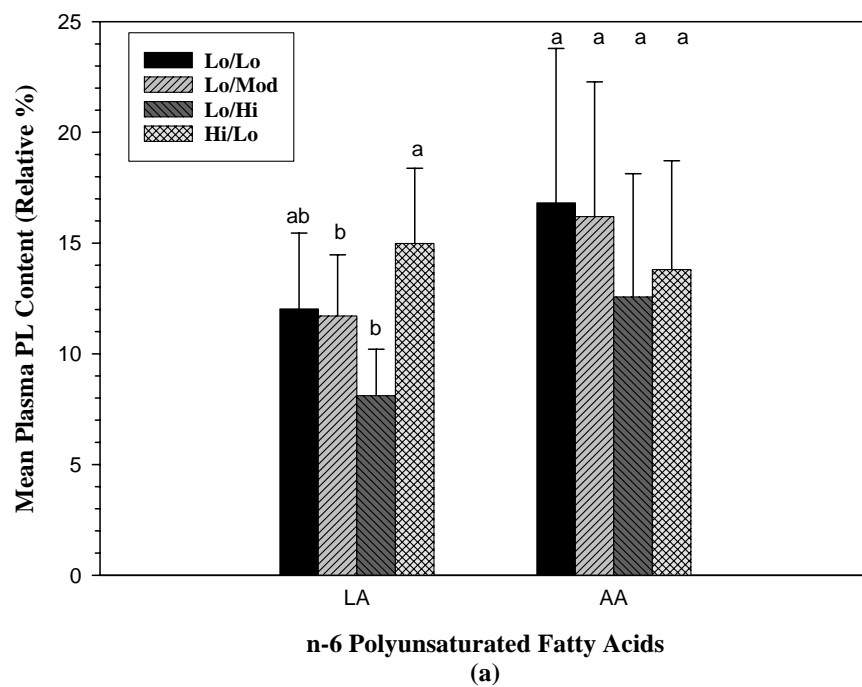
**Figure 3.3** Mean  $\pm$  S.D. plasma PL DPA (a), and DHA (b) during gestation in dogs fed the experimental diets. Pre-experimental diet fatty acid profiles, shown as d -3, were not significantly different among dogs at  $p < 0.05$  ( $n = 3$  per group).

these two groups persisted throughout gestation. Mean plasma PL DHA values in the moderate and high fish-oil diet groups were significantly different from both the Lo/Lo and Hi/Lo groups, but they were not significantly different from each other. Because differences over time did not exist during gestation, data from all sample days, excluding d -3, were pooled. Mean plasma n-6 and n-3 PUFA contents are indicated in Figures 3.4a and 3.4b, respectively.

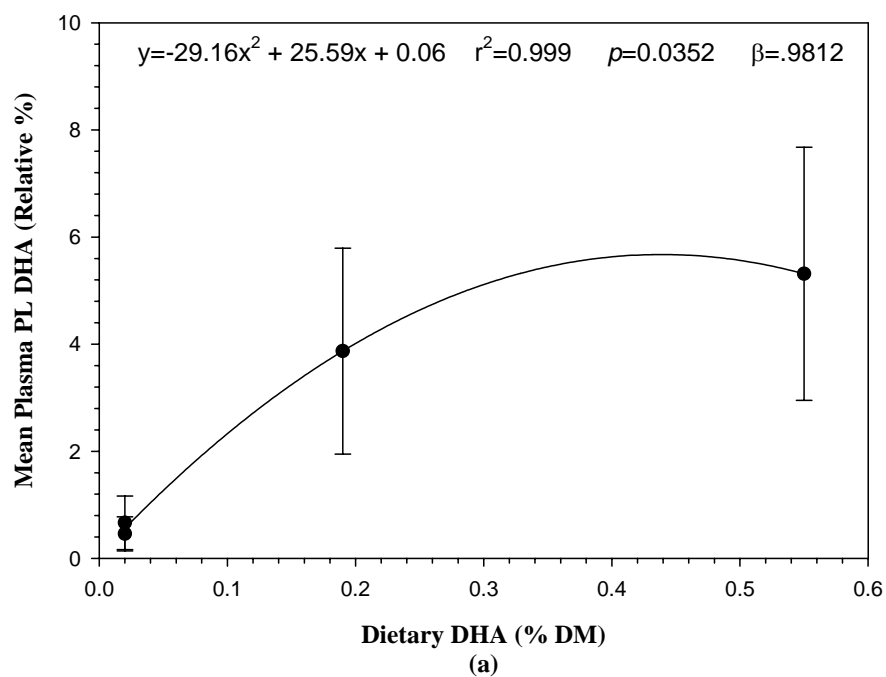
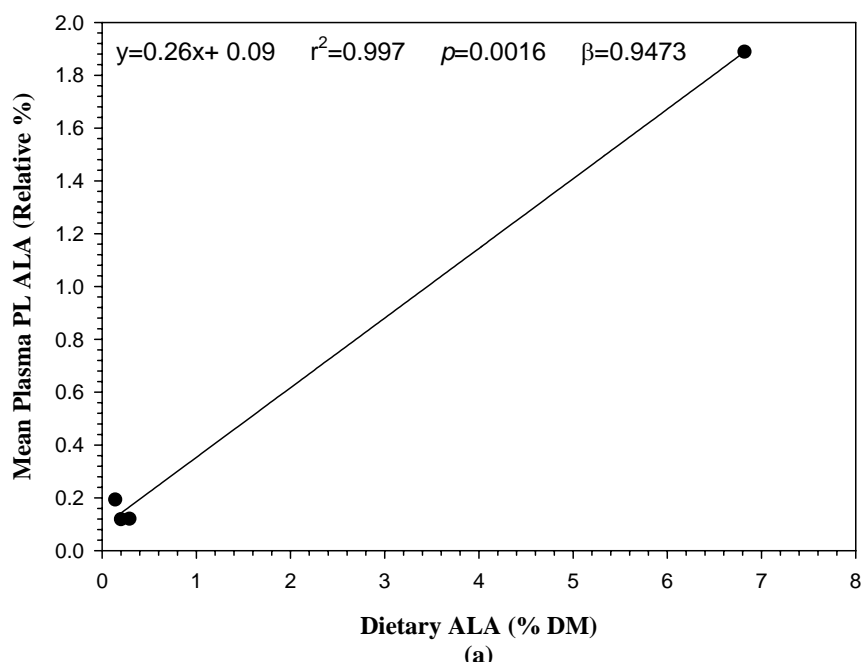
The highest plasma PL value of 22:5 n-3, DPA, was observed in the Hi/Lo group rather than in either of the fish oil-diet groups. Phospholipid DPA in the Hi/Lo group remained elevated well above all other diet groups through d 28 (Figure 3.3a), after which the plasma concentration declined slightly but was still greater than the other three groups. These differences, however, did not attain statistical significance.

Regression analysis of the n-3 PUFA revealed that plasma PL ALA and PL DHA content were highly correlated with dietary concentrations of the respective fatty acids (Figure 3.5). Correlation coefficients ( $r^2$ ) for these analyses were 0.997 and 0.999, respectively. Similarly, plasma PL LA content was closely related to dietary LA content ( $r^2 = 0.841$ , regression not shown). However, this relationship did not hold for AA and the other n-3 PUFA, whose correlation coefficients were less than 0.5. Smaller values of  $r^2$  indicate that a factor or factors other than dietary intake of the given fatty acids contributed to the variation in their plasma concentrations, and are consistent with the data of Bauer *et al.* from which predictive equations based on saturation kinetics have been developed (214). In the case of EPA and DPA, the elongation and desaturation of ALA can account for the curvilinear relationship between dietary and plasma amounts of these fatty acids.





**Figure 3.4** Mean  $\pm$  S.D. plasma PL content of LA and AA (a) and major n-3 PUFA (b) during gestation. The diets are designated Lo/Lo, Lo/Mod, Lo/Hi, and Hi/Lo based on their ALA/n-3 LCPUFA contents. Letters not in common for individual fatty acids are significantly different with respect to diet at  $p < 0.05$ .



**Figure 3.5** Linear regression of plasma ALA vs. dietary ALA (a) and plasma DHA vs. dietary DHA (b). Plasma concentrations of these fatty acids, as well as LA (not shown), are strongly correlated with their concentrations in the diet, as indicated by the high  $r^2$  values.

Finally, a general decline in most PUFA occurred across all experimental groups around d 28 of gestation and then slowly increased by d 56. This trend may reflect the placental transfer of fatty acids in gestating canids.

### **Discussion**

Dietary LA varied more than two-fold in the experimental diets, which resulted in a dose response of LA in the plasma PL. By contrast, however, plasma PL AA amounts were fairly steady across all experimental groups throughout gestation. According to predictive equations devised by Bauer *et al.* (215), tissue LA and AA become saturated at low concentrations of dietary LA. Based on those equations, dietary LA in all groups in this study was adequate to induce tissue saturation. Moreover, both dietary LA and ALA in the Hi/Lo group were unusually high (3.5% DM and 6.8% DM, respectively). Therefore it is also likely that ALA somewhat limited the elongation of LA via competition for  $\Delta^6$  desaturase. Studies of  $\Delta^6$  desaturase enzyme kinetics indeed indicate that LA and ALA compete for access to the enzyme's active site (216). These topics are discussed in further detail in Chapter IV of this dissertation.

The observation that the Hi/Lo group had the highest plasma PL concentration of DPA despite the very low dietary DPA content is of particular interest. Undoubtedly, the Hi/Lo group actively converted ALA to its longer-chain derivatives, EPA and DPA. However, further conversion to DHA was minimal in this group when plasma PL fractions are considered. Accumulation of DPA did not occur in the fish oil groups even though derived DPA was present in amounts many times higher than in the high ALA diet. The lack of significant plasma accrual of DPA in the fish oil-diet groups suggests that DPA is probably a rather transient intermediate between EPA retroconversion and DHA synthesis.

The fact that DPA accumulation occurred in the Hi/Lo group indicates that, although conversion of ALA occurs, its final conversion to DHA is limited at some regulatory point. This finding is not unique to gestating dogs; Bauer *et al.* (187) reported similar results when non-gestating dogs were fed high-ALA (10% total fatty acids) diets. In that study, the accumulation of DPA, but not DHA, was observed in plasma PL. Innis *et al.* (12) demonstrated in rat brain astrocytes that synthesis of DHA was limited at the elongation and subsequent desaturation of DPA. Furthermore, Sprecher (217) reported that the rate-limiting step in the final conversion to DHA occurs at the  $\Delta^6$  desaturase. However, the substrate for this reaction is 24:5 n-3, not DPA. Thus, another point of limitation must exist upstream from this  $\Delta^6$  desaturase step. In general, the elongation reactions are believed to be rapid and, thus, not rate-limiting. Therefore, the possibility exists that the limitation is in the mechanism by which DPA gains entry either into the peroxisomes or into the cells themselves. Although the details are unknown, this process is likely carrier-mediated and saturable. Consequently, and regardless of the abundance of DPA, once the transport system is saturated, DPA will begin to accumulate in either the cytoplasm or the plasma. The data presented here further emphasize the importance of dietary derived n-3 LCPUFA, rather than ALA, for gestating mothers. Further studies must be done to assess the exact mechanism of transport and to determine the concentration of DPA at which such a mechanism becomes saturated.

Finally, a general, albeit statistically insignificant, decline in most PUFA was observed around d 28 in all diet groups. In particular, plasma PL concentrations of AA and DHA were noticeably reduced on d 28 and gradually increased again by d 56. This observation likely reflects the increased demand for and placental transfer of fatty acids to

the fetal puppies. In humans, the placental transfer of AA and DHA begins around the onset of the third trimester and continues through the 40<sup>th</sup> wk, and fetal accretion of DHA is known to decrease maternal DHA status (49, 50, 218). Detailed information on the mechanisms of placental transfer of nutrients in gestating canines currently is not known. However, the results of the present study indicate that the transfer of fatty acids may occur somewhat earlier in canines than in humans. The reason for this is that the decrease in plasma PL PUFA occurred near the midpoint of gestation, rather than at the end of the second trimester, as in humans. Further studies must be conducted to ascertain the time course and mechanisms of placental nutrient transfer in gestating dogs.

## **CHAPTER IV**

# **EFFECTS OF DIETARY ALA AND N-3 LCPUFA ON MATERNAL PLASMA PHOSPHOLIPIDS AND MILK DURING LACTATION IN CANINES**

### **Introduction**

The n-3 and n-6 families of polyunsaturated fatty acids (PUFA) contain many biologically important lipids that are essential for proper growth, development, and general health maintenance. Brain and retinal functions depend on the n-3 polyunsaturated docosahexaenoic acid (22:6 n-3, DHA) not only during gestational development, but during postnatal life as well. Maximal brain growth begins in the third trimester of gestation and continues throughout the first few months of neonatal life (42-44). During this crucial period, there is a select accumulation of both arachidonic acid (20:4 n-6, AA) and DHA in brain and retina which occurs ten times faster than incorporation of their respective precursors, linoleic acid (18:2 n-6, LA) and alpha-linolenic acid (18:3 n-3, ALA) via chain elongation (47, 48). In accordance with this knowledge, several authors have demonstrated that plasma DHA is the preferred substrate for retinal uptake in early developmental stages when the demand for DHA is greatest (78, 81, 83).

### **Canine Neurologic Development**

Docosahexaenoic acid is highly conserved in the retina, and it has a role in neurologic function in this tissue (83). Canine retina is capable of synthesizing DHA from its 22-carbon precursor, docosapentaenoic acid (22:5 n-3, DPA) (58). Bauer *et al.* (187) reported the accumulation of DPA, but not DHA, in canine plasma phospholipids when the precursor, ALA, is fed. Therefore, it is likely that canine retina, and presumably other

nervous tissues, synthesize and utilize DHA in a manner similar to other mammalian species and that plasma DPA provides a likely substrate for such synthesis. Thus, a dietary source of pre-formed DHA or one of its precursors may be necessary during gestation and suckling for normal neurodevelopment in dogs.

It is possible that ALA may be sufficient as a dietary precursor for the synthesis of requisite amounts of DHA during pre- and postnatal development. However, what quantity of ALA may be needed to optimize neural development in companion animals currently is unknown. Additionally, because both n-6 and n-3 fatty acids compete for the same enzyme systems, it also is unclear what relative amounts may be needed.

### **Canine Milk Composition**

Early studies of canine milk composition were limited to macronutrient analysis and fatty acid analyses typically were not performed. Thus, few reports exist regarding the fatty acid composition of canine milk (188, 190). An effect of dietary LCPUFA intake during gestation and lactation on milk composition is expected; however, this effect has not been specifically investigated nor has a dose-response relationship been established. The purpose of the present study was to document diet effects of an 18-carbon n-3 precursor, ALA, as well as LCPUFA on canine milk and maternal plasma when these fatty acids are included in gestation/lactation diets and to evaluate the possibility of a dose-response with respect to dietary amounts of these fatty acids.

### **Materials and Methods**

An existing breeding colony of dogs provided bred hound/Labrador retriever dogs and their puppies for this study. The colony contains a kindred of dogs with hereditary nephritis (214) but all dogs used in this study were clinically healthy. Twelve dogs (three

each per diet group) were fed one of four complete and balanced, extruded-type diets from the time of insemination and throughout gestation, parturition, and lactation. Sufficient quantities of the diets were fed to maintain weight gain in the latter stages of gestation by adjusting the amount fed as necessary. The diets differed in their fatty acid composition and were sufficient in linoleic acid (Appendix B, Table B-I). They contained approximately 15% total fat using either beef tallow, linseed oil, or higher and lower amounts of menhaden fish oil as primary fat source (Nestle-Purina PetCare, St. Louis). Using these dietary fat sources, diets were formulated using typical pet food ingredients to contain adequate LA ranging from 1.8 to 3.5 % dry matter (DM) with varying ratios of ALA/n-3 LCPUFA content and were designated as follows: Lo/Lo (Lo ALA/Lo n-3 LCPUFA), Lo/ Mod, Lo/Hi, and Hi/Lo. All other dietary ingredients including total protein, nitrogen-free extract, vitamins, and minerals were identical, resulting in diets with similar nutrient concentrations except for fatty acid types (Appendix B, Table B-II).

After normal parturition, milk samples were collected from each dog by manual expression on lactation d 4, 10, 16, and 28. Additionally, EDTA blood samples were collected on d 3 of gestation and on d 10 and 28 postpartum. Plasma and milk total lipids were extracted using chloroform:methanol (2:1, v/v), fatty acid methyl esters prepared, and fatty acid profiles determined via gas chromatography (187). Statistical analyses were performed by repeated measures ANOVA with Bonferroni multiple comparisons performed at  $p < 0.05$ . However, more conservative p-values ( $p < 0.001$ ) are presented where appropriate to show highly significant differences when they occurred. Plasma PL fatty acid data from d 3 of gestation was included in the statistical analyses in order to



compare statistically significant differences between the end of lactation and the end of gestation.

## **Results**

### **Maternal Plasma Phospholipids**

The fatty acid profiles of maternal plasma PL closely mirrored the fatty acid composition of the diets. The percentages of major PUFA in maternal plasma PL are presented in Table 4.1. Values from d 3 of gestation are included as a reference point for lactation. Neither time nor diet\*time effects were found for any fatty acid during the lactation period; however, there was a statistically significant difference in plasma AA and EPA content between gestation and lactation. Furthermore, statistically significant diet effects were observed for most PUFA.

When the lactation samples were compared to d 3 of gestation (Table 4.1), a notable and statistically significant decrease in plasma PL AA content was observed. Significant increases in plasma PL EPA were also seen, but this is likely a reflection of increased substitution for AA.

For all diet groups, the sample size was n=3 with the exception of the Lo/Mod group on d 10 of lactation. Two of the samples for this time point evaporated during the methylation step, thus leaving only one sample available for analysis. The maternal plasma PL content of major n-6 and n-3 PUFA are represented graphically in Figure 4.1. Because no time effects were found, data from both lactation days presented in these figures and in the text have been pooled.

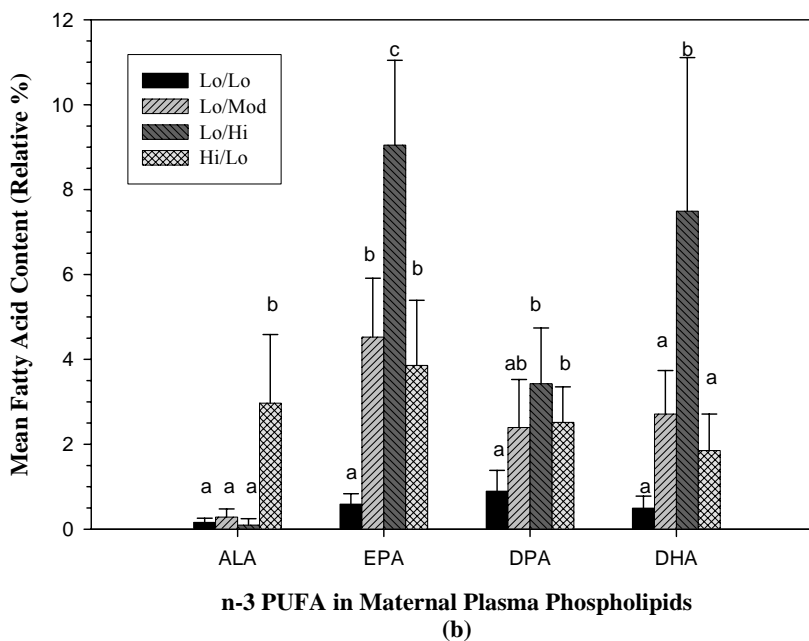
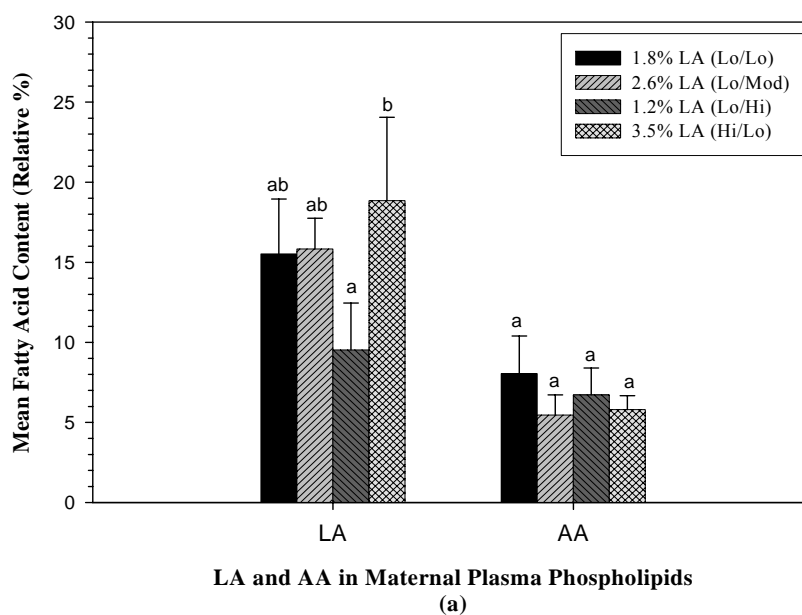
With respect to the n-6 PUFA, a dose response for LA was observed in maternal plasma. The Hi/Lo diet contained 3.5% LA on a DM basis, which was more than twice the

**Table 4.1** Relative percentage (mean  $\pm$  S.D.) of major PUFA in maternal plasma phospholipids during lactation

		FATTY ACID					
DIET	DAY	18:2 n-6	18:3 n-3	20:4 n-6	20:5 n-3	22:5 n-3	22:6 n-3
Lo/Lo	G3	14.0 $\pm$ 1.7	0.1 $\pm$ 0.1	23.5 $\pm$ 3.1 <sup>a</sup>	0.3 $\pm$ 0.0	1.7 $\pm$ 0.2	0.5 $\pm$ 0.2
	L10	16.8 $\pm$ 2.8	0.2 $\pm$ 0.1	8.6 $\pm$ 1.3 <sup>b</sup>	0.6 $\pm$ 0.2	0.7 $\pm$ 0.1	0.5 $\pm$ 0.5
	L28	14.2 $\pm$ 4.1	0.1 $\pm$ 0.1	7.5 $\pm$ 3.4 <sup>b</sup>	0.6 $\pm$ 0.3	1.1 $\pm$ 0.7	0.5 $\pm$ 0.0
Hi/Lo	G3	10.9 $\pm$ 2.0	0.9 $\pm$ 0.4	14.0 $\pm$ 9.0	0.7 $\pm$ 0.8	1.6 $\pm$ 0.5	0.5 $\pm$ 0.4
	L10	16.8 $\pm$ 8.5	3.2 $\pm$ 2.4	5.6 $\pm$ 0.7	3.6 $\pm$ 1.5	1.9 $\pm$ 1.1	1.5 $\pm$ 0.5
	L28	20.2 $\pm$ 3.3	2.8 $\pm$ 1.5	5.9 $\pm$ 1.1	4.0 $\pm$ 1.9	2.9 $\pm$ 0.3	2.1 $\pm$ 1.1
Lo/Mod	G3	10.2 $\pm$ 1.1	0.1 $\pm$ 0.1	24.2 $\pm$ 3.7 <sup>a</sup>	0.9 $\pm$ 1.0 <sup>a</sup>	2.4 $\pm$ 0.3	1.5 $\pm$ 0.9
	L10*	13.6	0.1	6.9 <sup>b</sup>	6.1 <sup>b</sup>	3.7	3.9
	L28	16.9 $\pm$ 0.3	0.2 $\pm$ 0.0	4.7 $\pm$ 0.1 <sup>b</sup>	3.8 $\pm$ 0.6 <sup>ab</sup>	1.8 $\pm$ 0.3	2.1 $\pm$ 0.0
Lo/Hi	G3	8.9 $\pm$ 1.5	0.2 $\pm$ 0.3	19.3 $\pm$ 5.6 <sup>a</sup>	3.8 $\pm$ 2.3 <sup>a</sup>	2.5 $\pm$ 0.4	6.2 $\pm$ 1.0
	L10	10.4 $\pm$ 1.7	0.2 $\pm$ 0.2	7.8 $\pm$ 1.0 <sup>b</sup>	9.2 $\pm$ 0.9 <sup>b</sup>	3.5 $\pm$ 1.0	7.8 $\pm$ 3.4
	L28	8.7 $\pm$ 4.1	0.0 $\pm$ 0.0	5.6 $\pm$ 1.5 <sup>b</sup>	8.9 $\pm$ 3.0 <sup>b</sup>	3.3 $\pm$ 1.8	7.2 $\pm$ 4.6
ANOVA ( <i>p</i> )	time	0.0163	0.0897	0.0000	0.0000	0.4819	0.3107
	diet	0.0023	0.0000	0.0126	0.0000	0.0006	0.0000

<sup>a</sup>Means within a diet group with letters not in common differ with respect to time at  $p < 0.05$ . Differences due to diet are indicated in Figure 4.1. (G3 = d 3 of gestation; L10 and L28 = d 10 and 28 of lactation, respectively).

\*Two of the samples in this group evaporated during methylation, thus leaving only one sample for analysis;  $n = 3$  for all other groups.



**Figure 4.1** Relative concentrations (mean  $\pm$  S.D.) of LA and AA (a) and n-3 PUFA (b) in plasma of dogs fed the experimental diets. Dietary concentrations of LA in the diets are indicated on a % DM basis in (a). The diets are designated as Lo/Lo, Lo/Mod, Lo/Hi, and Hi/Lo based on their ALA/n-3 LCPUFA contents. Letters not in common for each fatty acid are significantly different at  $p < 0.05$  ( $n = 3$  per group).

amount contained in the Lo/Hi diet (Table B-I). Consequently, the plasma PL LA of both these groups differed accordingly—the plasma PL of the Hi/Lo group contained  $18.9 \pm 5.2\%$  S.D. LA and was roughly twice that of the Lo/Hi group. No differences in plasma PL AA were observed during lactation, despite the up to two-fold difference in dietary LA. All diets contained nearly equal amounts of AA on a percent DM basis (Table B-I).

More pronounced differences were observed in the n-3 PUFA. The Hi/Lo diet contained 6.8% DM ALA, which ranged from nearly 25- to 50-fold higher than the other three diets. Likewise, similarly large differences in plasma PL ALA were observed. In the Hi/Lo group, the average plasma PL ALA during lactation was  $2.7 \pm 1.6\%$  S.D. of the total fatty acids, a value that was 10 to 30 times higher than, and significantly different from, all other diet groups.

Plasma PL EPA and DHA were, expectedly, highest in the Lo/Hi diet group, which received the highest concentration of dietary n-3 LCPUFA. Dietary EPA and DHA contents of 0.5% and 0.55% DM, respectively, resulted in much higher plasma PL concentrations of each fatty acid. The mean plasma PL content of EPA was  $9.1 \pm 2.0\%$  S.D., while the mean plasma PL DHA content was  $7.5 \pm 3.6\%$  S.D. In the Lo/Mod and Hi/Lo groups, dietary n-3 LCPUFA concentrations were quite different, yet plasma PL contents of both EPA and DHA were not statistically different between groups. This finding provides evidence for the conversion of dietary ALA to longer-chain metabolites in vivo. Moreover, these data demonstrate a dose-dependent response of plasma PL EPA and DHA to varying dietary amounts of these fatty acids. The Lo/Lo diet group was fed the lowest amounts of ALA, EPA and DHA, which was manifested in the lowest plasma PL contents of EPA, DPA and DHA. The maternal plasma PL concentrations of each of these

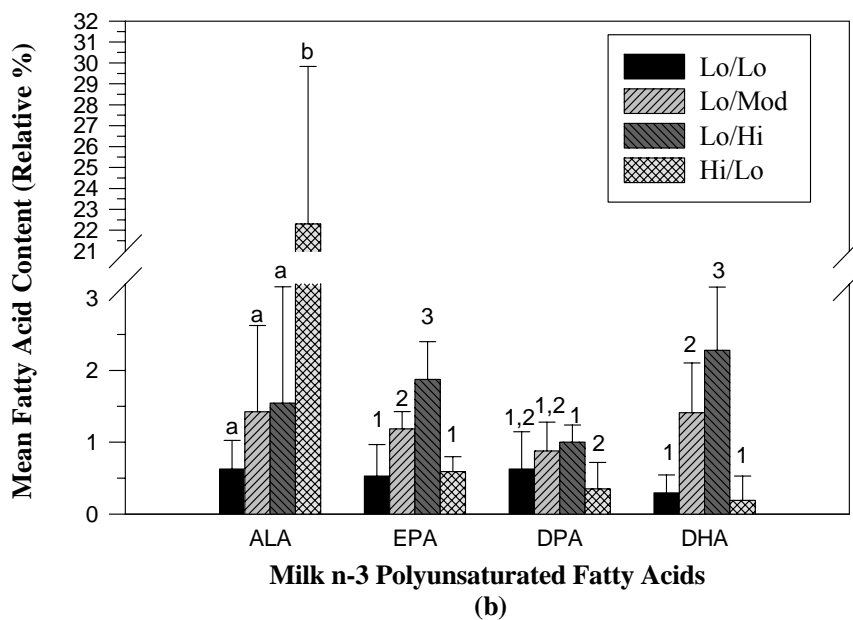
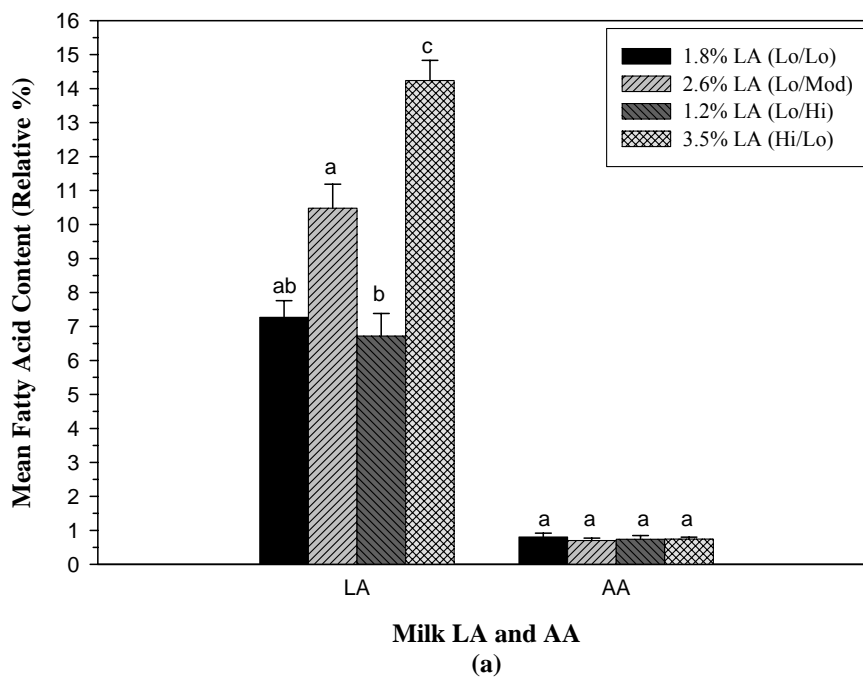
fatty acids were markedly lower in the Lo/Lo group than all other diet groups; however for DPA and DHA, these differences did not attain statistical significance.

### **Canine Milk**

Canine milk contained, on average,  $8.0 \pm 2.0\%$  S.D. total fat on an as-is basis, and no significant differences due to diet or day of lactation were found. Also, no main time effects were observed with respect to the individual fatty acids. However, significant diet effects were observed among milk fatty acid contents in the groups.

Among the n-6 fatty acids, a dose response was seen for LA as a function of its dietary concentration. Differences due to diet were statistically significant especially among the Lo/Lo, the Lo/Hi, and the Hi/Lo diet groups (Figure 4.2a). However, in spite of a doubling of dietary LA contents and a modest increase in dietary AA, milk AA remained unchanged in all groups. Dose responses of n-3 fatty acids in milk were also observed as a function of increasing dietary n-3 LCPUFA contents (Figure 4.2b). Thus, as dietary EPA and DHA increased, statistically significant elevations of these fatty acids in milk were noted. In the case of DPA, nominal increases were also seen, but the only statistically significant difference occurred between the Lo/Hi and Hi/Lo diets.

Finding no enrichment of milk DHA in dogs fed the Hi/Lo diet (i.e., the ALA enriched diet) was of particular interest in this study. This diet, which contained 6.8 % ALA DM basis, resulted in no significant differences in EPA, DPA, or DHA compared to the Lo/Lo diet that only contained 0.14 % ALA DM basis. This was the case even though the Hi/Lo diet contained nearly 50-fold more ALA precursor of the n-3 LCPUFA. Thus, no enrichment of canine milk n-3 LCPUFA occurs when ALA is fed during gestation and lactation.



**Figure 4.2** Relative concentrations (mean  $\pm$  S.D.) of LA and AA (a) and n-3 PUFA (b) in canine milk in dogs fed the experimental diets. Dietary concentrations of LA in the diets are indicated on a % DM basis in (a). The diets are designated as Lo/Lo, Lo/Mod, Lo/Hi, and Hi/Lo based on their ALA/n-3 LCPUFA contents. Letters not in common for each fatty acid are significantly different at  $p < 0.001$ . Numbers not in common are significantly different at  $p < 0.05$  ( $n = 3$  per group).

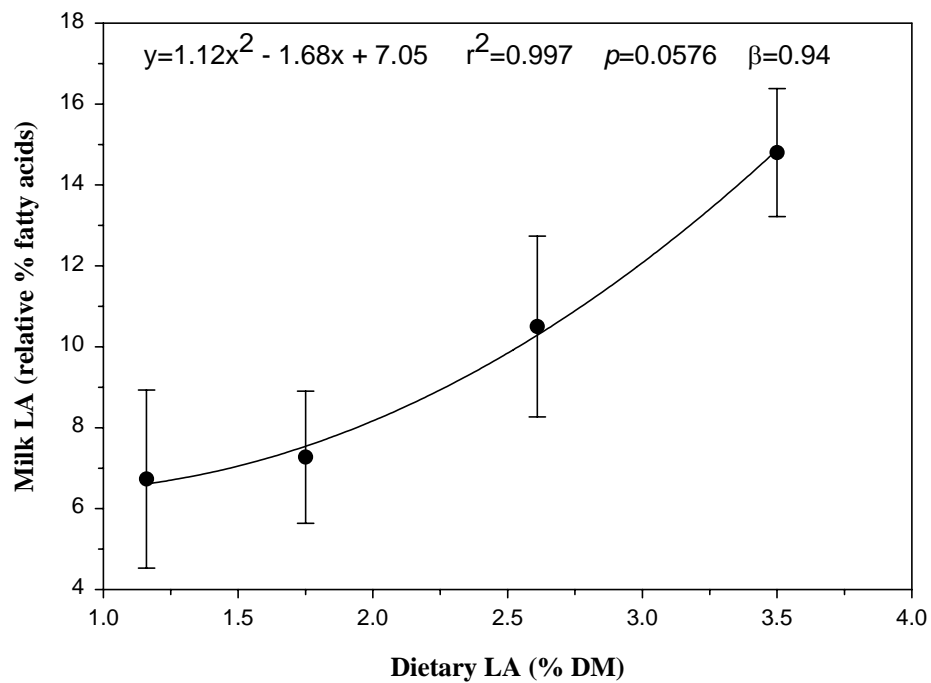
Regression analysis revealed useful relationships between milk PUFA and dietary PUFA content (Figures 4.3 and 4.4). Strong correlations existed for LA ( $r^2 = 0.997$ ), EPA ( $r^2 = 0.999$ ), and DHA ( $r^2 = 0.988$ ). The resultant quadratic equations may be of use in formulating future canine gestation/lactation diets such that optimum PUFA enrichment in milk is obtained. The predictive equations for each PUFA are listed below, where  $x$  = the dietary concentration of the respective fatty acid.

$$\text{Milk LA} = 1.12x^2 - 1.68x + 7.05 \quad (1)$$

$$\text{Milk EPA} = -2.96x^2 + 4.27x + 0.48 \quad (2)$$

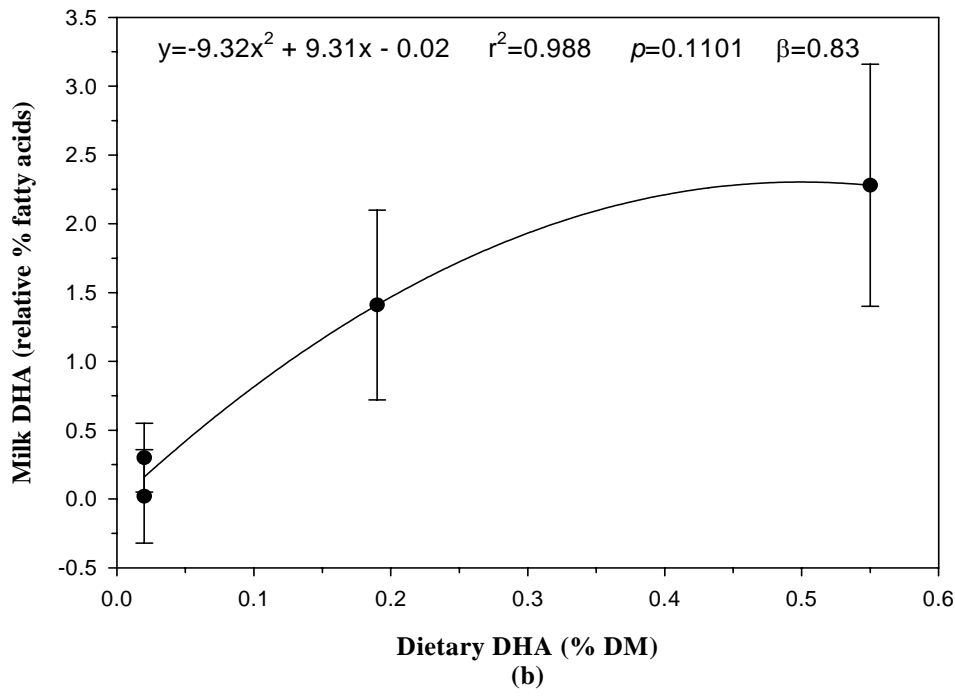
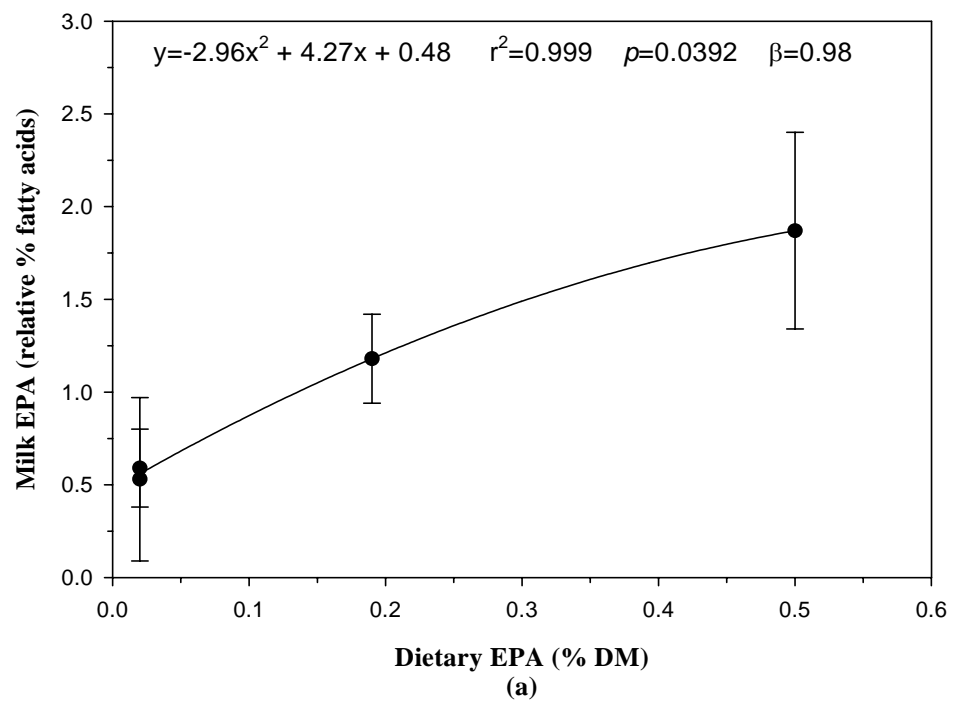
$$\text{Milk DHA} = -9.32x^2 + 9.31x - 0.02 \quad (3)$$

The best-fit curves in Figure 4.4 are close to 0% when dietary amounts of either EPA or DHA are also zero. By contrast, milk (triacylglycerols) LA content is approximately 6 relative% at a “theoretical” zero dietary LA. Milk LA likely also reflects tissue stores of this fatty acid while EPA and DHA are not stored to any appreciable extent in triacylglycerols.



**Figure 4.3** Regression analysis of milk LA vs. dietary LA.





**Figure 4.4** Regression analysis of milk EPA (a) and DHA (b) vs. dietary EPA and DHA.

## Discussion

### Maternal Plasma Phospholipids

In this study the dietary LA content varied up to two-fold while the AA supply was relatively constant. Consequently, the plasma PL content of LA varied in a dose-dependent manner in response to dietary LA (Figure 4.1a). However, although plasma AA among groups remained unchanged over time during lactation, there was a precipitous drop in plasma PL AA when lactation samples are compared with gestation. Although reasons for this finding are unclear, it appears that this decrease may be related to the need to provide AA in milk fat for neonates during suckling. Similar results among the n-3 fatty acids were not apparent in this regard, presumably because of the increased amounts of n-3 fatty acids concomitantly supplied in the diets.

Given the abundance of LA precursor, 3.5% DM, in the Hi/Lo group, it would not have been unreasonable to expect at least a modest increase in plasma AA in this group. Previous data, however, suggest that a competitive and “saturable” relationship exists between dietary PUFA and plasma PUFA in dogs (215). Similar results have been obtained in humans (219). The predictive equations for dog plasma devised by Bauer *et al.* (215) support the belief that adequate and saturable concentrations of dietary essential fatty acids in dogs may be as low as 0.5% energy. Thus, any dietary concentration of LA greater than 0.5% energy would result in tissue saturation of AA. Previous work determined the  $K_m$  and  $V_{max}$  of both LA and ALA for the  $\Delta^6$  desaturase enzyme (216). Although the  $K_m$  for LA is twice that of ALA, LA is present in greater amounts in canine diets and thus readily accumulates such that its  $K_m$  is easily met. In the present study, the lowest dietary LA content was 1.2% DM (0.8 en%), which was more than sufficient,

according to the derived prediction equations, to effect tissue saturation of AA. Therefore, even when dietary LA was doubled, further increases in plasma AA did not occur.

Also, substantial conversion of ALA to EPA and DPA occurred when large amounts of ALA were fed (Hi/Lo diet), yet the relative amounts of these LCPUFA remained significantly lower than those observed in the Lo/Hi group. The Hi/Lo diet contained 6.8% ALA and minimal amounts of preformed n-3 LCPUFA, yet plasma PL EPA and DHA in this group were not significantly different from the Lo/Mod group, whose diet contained approximately 10 times the preformed n-3 LCPUFA of the Hi/Lo diet. Unlike its n-6 counterpart, ALA is readily beta-oxidized and tends not to accumulate, in the absence of high dietary concentrations, to the extent required for conversion to occur. Furthermore, once the  $K_m$  for ALA is achieved, its rate of conversion ( $V_{max}$ ) is many times greater than the rate of conversion of LA (216). However, in order to reach the  $K_m$  for ALA, and for conversion to n-3 LCPUFA to proceed, the amount of dietary ALA must be exceedingly high (216). The data presented here indicate that when dietary ALA is high (6.8% DM), the amounts of n-3 LCPUFA produced by conversion are roughly equivalent to the enrichment attained by feeding moderate amounts of EPA.

The high ALA content of the Hi/Lo diet resulted in a product that had a strong varnish-like odor and at times was poorly received by many of the dogs in the study. Such side effects must be taken into consideration when formulating diets that are intended to maximize n-3 LCPUFA metabolism.

Although conversion of ALA undoubtedly occurs, it is many times less efficient than DHA at attaining a given degree of tissue enrichment. It has been demonstrated in various mammalian species that dietary DHA is a more effective source of tissue DHA

than dietary ALA (220). A labeling study in young rats revealed a 20-fold difference in the recovery of radiolabeled DHA from labeled DHA versus DHA from a labeled ALA precursor (221). Additional studies in baboons and guinea pigs suggest that 7 to 10 times more ALA than DHA is required to achieve equivalent tissue enrichment of DHA (48, 222-224).

Furthermore, ALA labeling studies in humans showed that the conversion of ALA to long-chain metabolites was 11-19% of the dose, and that conversion was decreased by as much as 54% when the diet was rich in LA (225). The presence of large amounts of LA in the diet has been shown to suppress the expression of  $\Delta^6$  desaturase, thus reducing the possibility for conversion of 18:3 n-3 (ALA) to 18:4 n-3 and 24:5 n-3 to 24:6 n-3 (226). Dietary LA in the Hi/Lo diet in this study was quite high, and thus could be a contributing factor to the poor conversion of ALA to long-chain metabolites beyond EPA.

A dose-effect of fish oil was observed among the Lo/Lo, Lo/Mod and Lo/Hi diet groups. As shown in Appendix Table B-I, the Lo/Hi diet contained roughly twice as much of each n-3 LCPUFA as did the Lo/Mod diet. With the exception of DPA, this two-fold difference in n-3 LCPUFA was also present in the plasma PL of the respective diet groups. The differences in plasma PL DPA values were less pronounced than the differences in the other n-3 LCPUFA, and any dose-response observed was not statistically significant. In the Lo/Hi group, plasma DPA was slightly, but not significantly, elevated with respect to the Lo/Lo, Lo/Mod and Hi/Lo diets. One possibility for the less predictable behavior of DPA is that it may primarily be an intermediate between EPA and DHA and may be rapidly shunted toward other n-3 LCPUFA synthesis, which subsequently modifies its tissue accumulation at any given time.

## Canine Milk

Neither n-6 nor n-3 LCPUFA (i.e., AA, EPA, DPA, or DHA) become enriched in milk when their respective 18-carbon precursors are fed throughout gestation and lactation in dogs. Similar small contributions of dietary LA to milk AA composition has been observed in humans using stable isotope techniques (227). With respect to the n-3 fatty acids, only ALA was significantly increased in milk in response to dietary sources and no changes were seen in any of the derived n-3 LCPUFA. This finding is also similar to that reported in humans by Francois *et al.* (157) in which seven lactating mothers ingested dietary flaxseed oil supplements at the rate of 20 g oil (10.7 g ALA) per day for 4 wk during lactation. In that study, milk sample analyses were reported at baseline, 2 and 4 wk of supplementation and at 4 wk post-supplementation. In the present canine study, dogs were fed larger amounts of ALA approximating 18-37 g of ALA per day depending on stage of gestation or lactation. Nonetheless, no significant enrichment of milk DHA occurred at any time. Francois *et al.* (157) did report that a modest yet statistically significant trend toward increased EPA and DPA was found over time. However, inspection of their data show that the mean values of both EPA and DPA appeared to decrease from their modest increases at 2 wk by the 4 wk time period. Thus it is possible that the milk samples of these subjects at 2 wk may not have been representative of a diet-supplement induced metabolic steady state. Instead, they may have been a reflection to varying extents of the mothers' previous diets, previous DHA status, or tissue stores immediately prior to beginning the dietary supplements (227). Indeed our previous studies show that it will generally require more than 3-4 wk of dietary lipid modification to result in steady state plasma fatty acid concentrations (187). Also, Francois *et al.* (157) supplemented human mothers only during lactation. In the present study dogs

had been fed the high linseed oil diet (Hi/Lo diet) from the onset of breeding and throughout gestation (ca. 63 d) and lactation periods (ca. 28 d). These differences may, in part, explain the modest elevations of EPA and DPA seen in human but not canine milk even though more ALA was fed to dogs in the present study.

The finding that plasma PL AA significantly declined between the onset of gestation and the lactation period is potentially important in terms of AA being used to enrich milk during lactation and perhaps during fetal development as well. The plasma PL fatty acid content at d 3 of gestation does not necessarily reflect only the experimental diets, but, to some extent, the pre-breeding diets as well. However, the LA and AA contents of the pre-breeding diet were not broadly different from those used in this study. Thus, this decrease likely is not attributable to dietary differences *per se*.

An additional consideration is the fact that the experimental diets contained n-3 fatty acids, which also may contribute to the lowering of AA in plasma PL. However, AA content decreased by 100% in each case, including the Lo/Lo diet, which was nearly devoid of n-3 PUFA. Thus, it appears that a marked decrease occurs in plasma PL AA in gestating (non-lactating) canines compared to lactation. Such an effect may reflect AA incorporation into the milk, which would assure delivery of this important fatty acid to the developing neonate.

The observations that dietary LA did not increase AA and that dietary ALA did not increase milk n-3 LCPUFA support the possibility that the biosynthetic pathways specifically relating to desaturation and chain elongation of milk lipids are poorly developed in canine mammary tissue. An alternate explanation is that these pathways are competitively inhibited in the presence of either small dietary amounts of the LCPUFA or their existing tissue stores. In either case, supplementation of gestation/lactation diets with LA or ALA

does not appear to be an effective method of increasing milk fat LCPUFA for developing canines.

In summary, dietary ALA supplementation during gestation/lactation is an ineffective means of increasing milk DHA content to supply dietary amounts of this LCPUFA for neonatal nutritional modification. Whether sources of pre-formed dietary n-3 LCPUFA are necessary to support puppy development during suckling or whether puppies are themselves capable of synthesizing sufficient n-3 and n-6 LCPUFA from 18-carbon precursors is a question that was also addressed in the present study and which will be reported in Chapter V of this dissertation. Finally, the consistency of the milk AA concentration independent of dietary LA content and the dose responses seen with the n-3 LCPUFA will assist in future efforts to approximate dietary PUFA amounts needed to support specific milk PUFA concentrations for puppies during suckling. However, it should be noted that exact amounts that are most beneficial for puppies themselves remains undetermined.

**CHAPTER V**  
**EFFECT OF DIETARY ALA AND N-3 LCPUFA ON PLASMA**  
**PHOSPHOLIPIDS AND ON ELECTRORETINOGRAM (ERG)**  
**PARAMETERS IN YOUNG CANINES**

**Introduction**

In the last 20 years, the importance of n-3 fatty acids in brain and retinal development has become increasingly evident. One question that remains, however, is whether ALA provided as a precursor is sufficient for optimal development or if preformed DHA is required. The greatest concern is for premature infants, whose intrauterine development is shortened, often by several weeks (26).

Neural development in primates begins in the third trimester of gestation, peaks about the time of birth, and continues for about 18-24 months after parturition (49, 50). During this developmental period, AA and DHA are rapidly incorporated into the neural tissues (47, 48). The high amounts of DHA in the brain and especially in the retina suggest a functional role in these tissues (108). Deficiency of n-3 PUFA during the developmental phase in neural tissues can result in irreversible functional abnormalities. Electroretinogram (ERG) data from humans and monkeys indicate decreased amplitudes and increased implicit times of both the a- and b-waves in response to n-3 PUFA insufficiency (126, 136). Reduced a- and b-wave amplitudes have also been reported in n-3 deficient rats; however, retinal function was restored when the rats were fed n-3 replete diets (54).



### **Canine Neurologic Development**

DHA is highly conserved in the retina, and it has a role in neurologic function in this tissue (83). Canine retina is capable of synthesizing DHA from its 22-carbon precursor, docosapentaenoic acid (22:5 n-3, DPA) (58). Bauer *et al.* (187) reported the accumulation of DPA, but not DHA, in canine plasma phospholipids when the precursor ALA is fed. It, therefore, is likely that canine retina, and presumably other nervous tissues, synthesize and utilize DHA in a manner similar to other mammalian species and that plasma DPA provides a likely substrate for such synthesis. Thus, a dietary source of pre-formed DHA or one of its precursors may be necessary during gestation and suckling for normal neural development in dogs.

It is possible that ALA may be sufficient as a dietary precursor for the synthesis of requisite amounts of DHA during pre- and postnatal development. However, what quantity of ALA may be needed to optimize neural development in companion animals currently is not known. Additionally, because both n-6 and n-3 fatty acids metabolically compete for the same enzyme systems, it also is unclear what relative amounts may be needed.

### **The Electroretinogram**

Electroretinography is a sensitive and quantitative measure of retinal function in humans and animals (228, 229). The ERG recording represents the response of the photoreceptors, and their subsequent post-synaptic signals, to a series of varying-intensity flash stimuli. It is the sum of responses across the retina and includes the responses of many retinal cell types. Major ERG components have been studied in dogs (230), and

many studies suggest the use of the dog as a suitable model for the study of human retinal physiology and pathology (231).

One goal of this study was to assess the effect of dietary n-3 fatty acid supply on retinal function in canine neonates. To our knowledge, such a study has not been reported.

### **Materials and Methods**

An existing breeding colony of dogs provided bred hound/Labrador retriever dogs and their puppies for this study. Twelve dogs (3 each per diet group) were fed one of four complete and balanced, extruded-type diets from the time of insemination and throughout gestation, parturition, and lactation. These diets have been described in detail in Chapter II. Fatty acid compositions and nutrient contents of the diets can be found in Appendix B, Tables B-I and B-II. After parturition, puppies were allowed to suckle *ad libitum*. At 21 d postpartum, a gruel consisting of the mothers' respective diets and water was offered to the puppies three times a day, in addition to suckling. Gradually, the time the puppies spent suckling was decreased until they were completely weaned by d 28. Upon weaning, puppies were continued on the same diets as their mothers until 12 wk of age. The puppies were weighed daily until 6 wk of age and every other day thereafter to ensure proper growth and development. If any failure-to-thrive issues arose, the puppy was removed from the study and supplemented to ensure proper nutrition. Generally this occurred only when litter size was large; small puppies were the most likely to be removed.

EDTA blood samples were taken from the puppies on d 4, 10, 16, 28, 70 and 84 of age. The puppies were removed from their mothers three hours prior to blood collection on d 4, 10, 16 and 28, and food was withheld overnight on d 70 and 84. Two milliliters of blood was taken on each sample day through d 16. On d 28, 4 mL were collected, and on

days 70 and 84, 7 mL were obtained. Plasma total lipids and erythrocyte total PL were extracted using chloroform:methanol (2:1, v/v) and subsequently separated into subclasses via thin-layer chromatography. Fatty acid methyl esters were prepared, and fatty acid profiles were determined via gas chromatography (187). Statistical analyses were performed by repeated measures ANOVA with Bonferroni comparisons performed at  $p < 0.05$  for plasma PL fatty acids.

At 12 wk of age, retinal development of the puppies was assessed via electroretinography (ERG). The ERG procedure is outlined in detail in Chapter II. The completion of the ERGs marked the end of the study, at which time suitable homes were found for the puppies. Statistical analyses were performed on ERG parameters using data obtained from the 8<sup>th</sup> flash intensity via one-way ANOVA with Bonferroni comparisons performed at  $p < 0.05$ .

An additional parameter,  $\ddot{a}$ , was derived from the slope of the a-wave (140). The ERG software used in the study calculated the slopes of the descending limb of the a-wave for the three highest intensity responses; the increase in magnitude of these slopes as a function of intensity is reported as the parameter  $\ddot{a}$ . The slope for each of the three intensities was then plotted against flash intensity. The resultant data were modeled with linear regression to yield  $\ddot{a}$  as the calculated slope parameter (140). The slope  $\ddot{a}$  represents the increase in initial response with increasing light intensity (232).

## **Results**

Because no time effects were observed for any fatty acid, numerical values reported in the text and presented in figures are mean values for all four sample days during

suckling and both sample days during the postweaning period. The experimental unit was “litter,” and  $n = 3$  for each diet group.

### Suckling Period

A dose response of dietary LA (from milk) was observed in neonatal plasma (Figure 5.1a). Milk from the Hi/Lo group contained the highest concentration of LA (Table 5.1); consequently so did plasma PL from neonates in this group. Plasma PL arachidonate in neonates from the Lo/Lo group, was significantly higher than all other groups (Figure 5.1a). A summary of the mean plasma PL content of major fatty acids during suckling is presented in Table 5.2. Values presented are mean values for each sample day during the suckling period.

**Table 5.1** Mean  $\pm$  S.D. dietary content (in milk) of major fatty acids during suckling

DIET	FATTY ACID					
	18:2 n-6	20:4 n-6	18:3 n-3	20:5 n-3	22:5 n-3	22:6 n-3
Lo/Lo	7.27 $\pm$ 1.63 <sup>a</sup>	0.81 $\pm$ 0.35	0.63 $\pm$ 0.40 <sup>a</sup>	0.53 $\pm$ 0.44 <sup>a</sup>	0.63 $\pm$ 0.52 <sup>ab</sup>	0.30 $\pm$ 0.25 <sup>a</sup>
Lo/Mod	10.5 $\pm$ 2.23 <sup>b</sup>	0.70 $\pm$ 0.22	1.42 $\pm$ 1.20 <sup>a</sup>	1.18 $\pm$ 0.24 <sup>b</sup>	0.88 $\pm$ 0.40 <sup>ab</sup>	1.41 $\pm$ 0.69 <sup>b</sup>
Lo/Hi	6.73 $\pm$ 2.20 <sup>a</sup>	0.74 $\pm$ 0.34	1.54 $\pm$ 1.62 <sup>a</sup>	1.87 $\pm$ 0.53 <sup>c</sup>	1.00 $\pm$ 0.24 <sup>a</sup>	2.28 $\pm$ 0.88 <sup>c</sup>
Hi/Lo	14.8 $\pm$ 1.58 <sup>c</sup>	0.73 $\pm$ 0.17	22.3 $\pm$ 7.53 <sup>b</sup>	0.59 $\pm$ 0.21 <sup>a</sup>	0.35 $\pm$ 0.37 <sup>b</sup>	0.19 $\pm$ 0.34 <sup>a</sup>

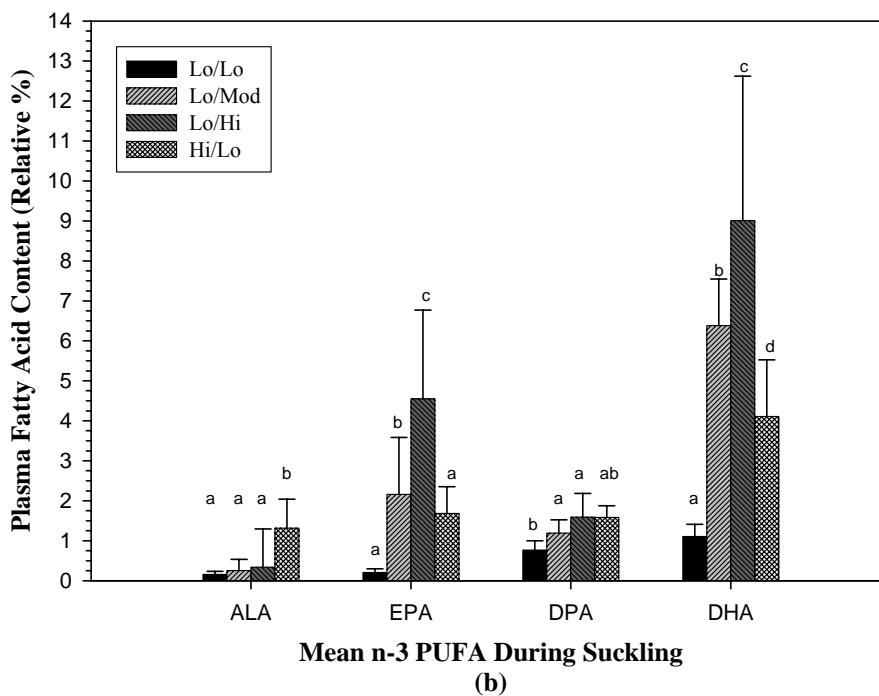
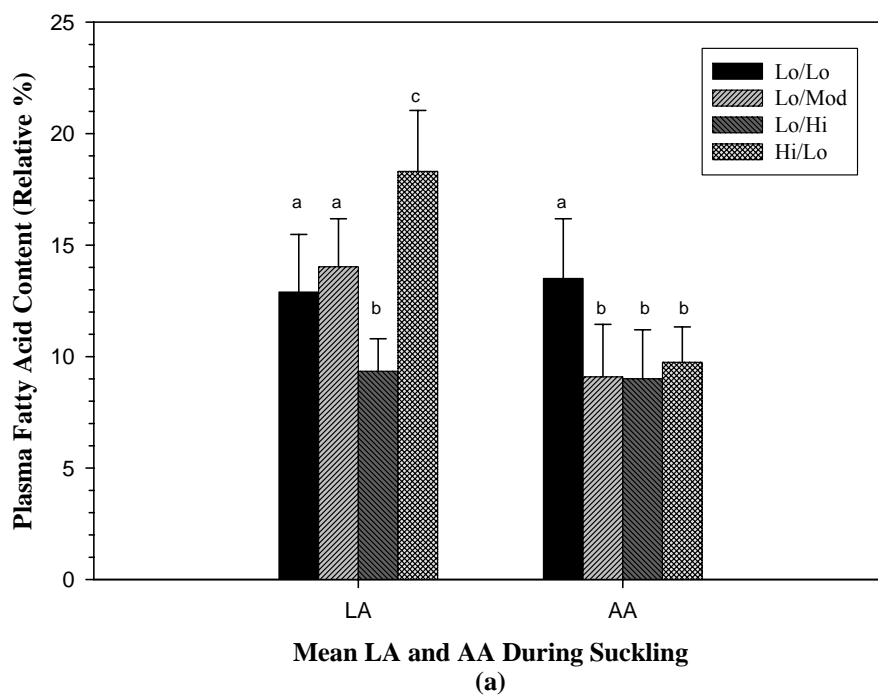
Letters not in common for individual fatty acids are significantly different at  $p < 0.05$  ( $n = 3$  dogs per group). Mean milk fat concentration was  $8.0 \pm 2.0$  % on an as-is basis.

With respect to the n-3 fatty acids, dose responses were observed for ALA, EPA and DHA. Puppies in the Hi/Lo group received the largest relative amount of ALA in their diets ( $22.3 \pm 7.5\%$  S.D.), and their plasma PL also contained the highest concentration of ALA,  $1.3 \pm 0.7\%$  S.D. These values were significantly different from all other diets at  $p < 0.05$ . Neither dietary nor neonatal plasma ALA were different among the remaining dietary groups (Figure 5.1b).

**Table 5.2** Relative percent (mean  $\pm$  S.D.) of major n-6 and n-3 fatty acids in neonatal plasma PL during suckling

		FATTY ACID					
DIET	DAY	18:2 n-6	20:4 n-6	18:3 n-3	20:5 n-3	22:5 n-3	22:6 n-3
Lo/Lo	4	13.2 $\pm$ 0.5	15.0 $\pm$ 2.7	0.2 $\pm$ 0.1	0.1 $\pm$ 0.1	0.6 $\pm$ 0.1	1.0 $\pm$ 0.4
	10	14.1 $\pm$ 2.7	13.9 $\pm$ 2.4	0.2 $\pm$ 0.1	0.2 $\pm$ 0.0	0.8 $\pm$ 0.0	1.1 $\pm$ 0.3
	16	15.2 $\pm$ 0.9	13.6 $\pm$ 1.5	0.2 $\pm$ 0.1	0.2 $\pm$ 0.0	0.9 $\pm$ 0.4	1.2 $\pm$ 0.1
	28	9.4 $\pm$ 1.0	11.7 $\pm$ 3.9	0.1 $\pm$ 0.0	0.3 $\pm$ 0.1	0.7 $\pm$ 0.2	1.2 $\pm$ 0.4
Hi/Lo	4	18.2 $\pm$ 0.9	11.2 $\pm$ 1.9	0.87 $\pm$ 0.4	1.1 $\pm$ 0.6	1.4 $\pm$ 0.4	2.7 $\pm$ 0.2
	10	20.0 $\pm$ 2.4	9.1 $\pm$ 1.2	1.4 $\pm$ 0.1	1.6 $\pm$ 0.7	1.5 $\pm$ 0.1	3.8 $\pm$ 0.1
	16	18.1 $\pm$ 4.0	8.9 $\pm$ 1.4	1.1 $\pm$ 0.6	1.8 $\pm$ 0.8	1.8 $\pm$ 0.3	4.3 $\pm$ 0.7
	28	16.5 $\pm$ 3.1	9.8 $\pm$ 1.4	1.9 $\pm$ 1.2	2.2 $\pm$ 0.3	1.7 $\pm$ 0.3	5.6 $\pm$ 2.0
Lo/Mod	4	13.3 $\pm$ 2.5	10.8 $\pm$ 1.0	0.3 $\pm$ 0.1	1.3 $\pm$ 0.5	1.3 $\pm$ 0.3	7.2 $\pm$ 0.6
	10	12.4 $\pm$ 2.7	8.1 $\pm$ 1.7	0.6 $\pm$ 0.4	1.5 $\pm$ 0.3	1.0 $\pm$ 0.3	5.9 $\pm$ 1.1
	16	15.5 $\pm$ 0.6	8.7 $\pm$ 4.1	0.04 $\pm$ 0.08	3.3 $\pm$ 2.4	1.3 $\pm$ 0.5	6.7 $\pm$ 1.4
	28	14.8 $\pm$ 1.6	8.8 $\pm$ 1.8	0.09 $\pm$ 0.08	2.5 $\pm$ 1.1	1.2 $\pm$ 0.5	5.7 $\pm$ 1.2
Lo/Hi	4	9.2 $\pm$ 2.1	8.5 $\pm$ 0.8	0.03 $\pm$ 0.05	2.0 $\pm$ 1.2	1.2 $\pm$ 0.2	5.2 $\pm$ 1.9
	10	10.3 $\pm$ 0.05	9.6 $\pm$ 1.4	0.1 $\pm$ 0.2	5.4 $\pm$ 1.8	1.5 $\pm$ 0.5	9.3 $\pm$ 2.9
	16	9.3 $\pm$ 2.2	10.1 $\pm$ 4.4	1.2 $\pm$ 2.0	4.1 $\pm$ 0.7	2.2 $\pm$ 1.3	10.3 $\pm$ 3.5
	28	8.6 $\pm$ 0.2	7.8 $\pm$ 0.2	0.1 $\pm$ 0.2	6.7 $\pm$ 1.9	1.7 $\pm$ 0.3	11.2 $\pm$ 3.9
ANOVA ( <i>p</i> )	time	0.0597	0.2850	0.7250	0.0024	0.0537	0.0663
	diet	0.0000	0.0001	0.0002	0.0000	0.0000	0.0000

No time effects within diet groups were observed at  $p < 0.05$  ( $n = 3$  litters per diet group). Dietary differences for individual fatty acids are indicated in Figure 5.1.



**Figure 5.1** Mean plasma PL content of LA and AA (a) and major n-3 fatty acids (b) during suckling. Because no time effects were observed for any fatty acid, data from all four sample days during suckling were pooled. Letters not in common for individual fatty acids are significantly different with respect to diet at  $p < 0.05$  ( $n = 3$  litters per diet group). Error bars indicate S.D. values.

Milk EPA and DHA were highest in the two fish-oil diet groups. Likewise neonatal plasma from these groups contained the largest amounts of EPA and DHA in the PL fraction (Figure 5.1b). Neonatal plasma PL from the Lo/Hi group contained  $4.6 \pm 2.2\%$  S.D. EPA compared to  $2.2 \pm 1.4\%$  S.D. in the Lo/Mod group. These values were significantly different from each other as were the concentrations of dietary (milk) EPA in these two groups.

Despite a two-fold, and statistically significant, difference between milk EPA in the Lo/Mod and Hi/Lo groups, the EPA in plasma PL from neonates in these groups were not significantly different. However, milk ALA differed considerably between these two diet groups. Mean plasma PL EPA from puppies in the Hi/Lo group was  $1.7 \pm 0.7\%$  S.D. (Figure 5.1b).

Dietary (milk) DPA was highest in the two fish-oil groups and lowest in the Hi/Lo group (Table 5.1). However, DPA concentrations in plasma PL were similar between the Hi/Lo and Lo/Mod groups, with values of  $1.6 \pm 0.3\%$  S.D. and  $1.2 \pm 0.3\%$  S.D., respectively. Puppies in the Lo/Hi group had the highest plasma PL DPA content,  $1.59 \pm 0.59\%$ , while the Lo/Lo group had the lowest,  $0.77 \pm 0.23\%$ . None of these differences attained statistical significance (Figure 5.1b).

A dose response of DHA was also observed in the plasma of puppies fed diets containing n-3 LCPUFA. DHA concentrations in plasma PL were  $9.0 \pm 3.6\%$  S.D.,  $6.4 \pm 1.2\%$  S.D. and  $1.1 \pm 0.3\%$  S.D, respectively, in the Lo/Hi, Lo/Mod and Lo/Lo groups. Puppies in the Hi/Lo group received the lowest relative amount of DHA in milk, yet their plasma PL contained fairly high amounts,  $4.10 \pm 1.44\%$ , of this fatty acid. Plasma DHA concentrations in all diet groups were significantly different from each other at  $p < 0.05$ .

### Post-weaning Period

Plasma PL fatty acid profiles of the puppies consuming the dry diets were similar to those obtained during suckling. Fatty acid compositions of the dry diets can be found in Appendix B, Table B-I. A dose response was again observed for LA (Figure 5.2a). An interesting occurrence was the significantly higher arachidonate concentration in the plasma PL of Lo/Lo puppies. This finding is peculiar because all groups received similar (not significantly different) amounts of AA in both the milk and dry diets. Moreover, the Hi/Lo diet contained the greatest concentration of LA, which was up to two times higher than the LA content of the remaining three diets. This apparent “increase” in plasma AA in the Lo/Lo group is most likely the result of a decrease in plasma AA due to competition from n-3 PUFA in the other three groups such that the plasma AA content in Lo/Lo puppies appears elevated relative to the others. A summary of the mean plasma PL content of major fatty acids after weaning is presented in Table 5.3. Values presented are mean values for each sample day during the post-weaning period.

The Hi/Lo group again had significantly higher ALA,  $2.2 \pm 0.4\%$  S.D., in plasma PL (Figure 5.2b). The other three groups, whose plasma PL ALA was on the order of 0.15% of total fatty acids, were not significantly different from each other.

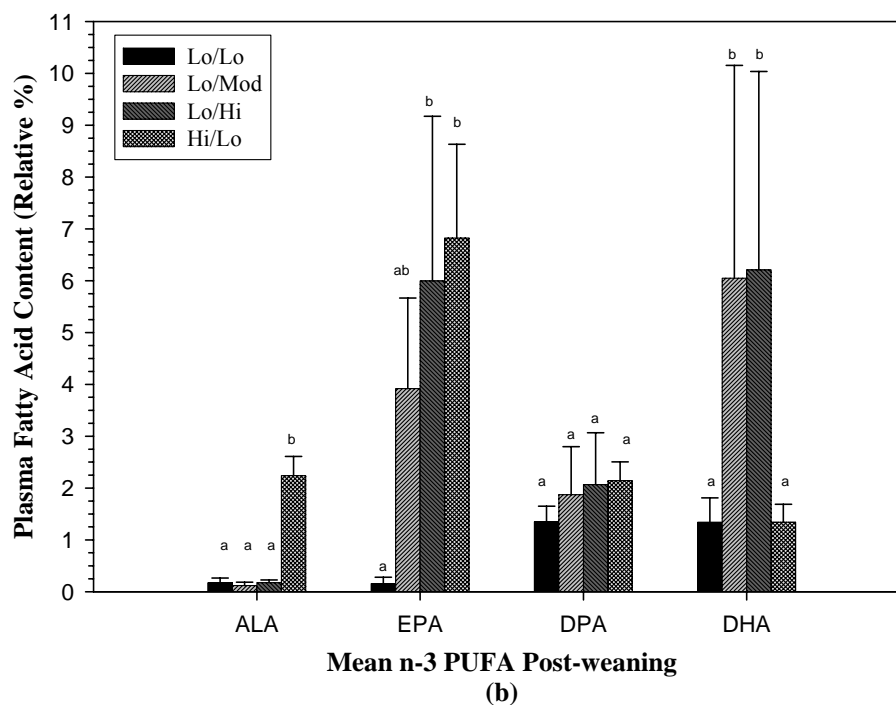
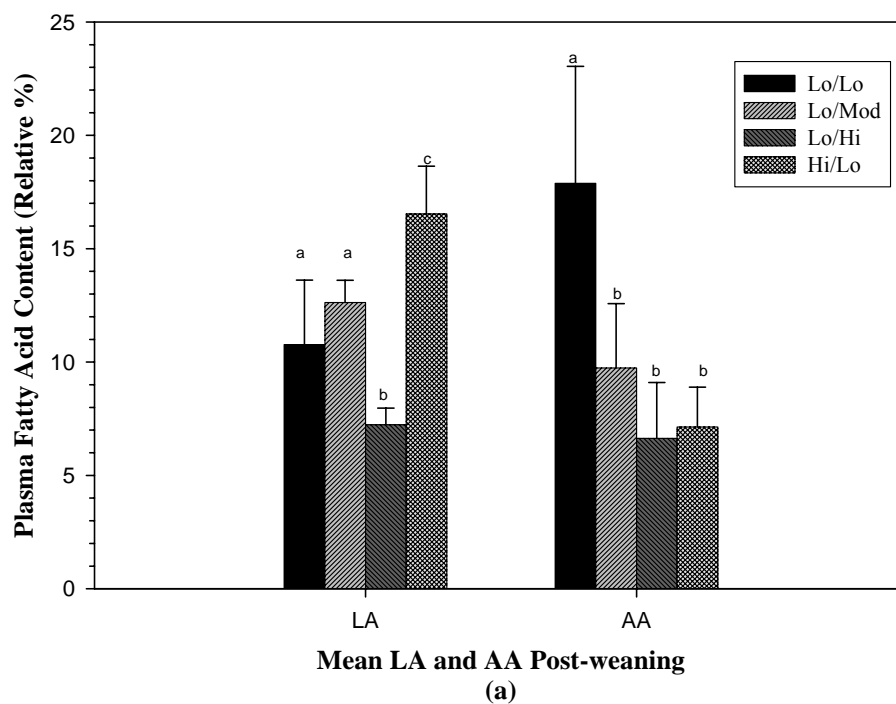
The Hi/Lo group also had the highest concentrations of EPA and DPA in the plasma PL fraction (Figure 5.2b). Mean EPA during the post-weaning period was  $6.8 \pm 1.8\%$  S.D. in the Hi/Lo group, compared to  $3.9 \pm 1.8\%$  S.D. and  $6.0 \pm 3.2\%$  S.D., respectively, in the Lo/Mod and Lo/Hi diet groups. These values, however, were not statistically different from each other. Plasma PL fatty acids from puppies in the Lo/Lo diet group contained considerably less EPA,  $0.2 \pm 0.1\%$  S.D., yet due to considerable



**Table 5.3** Relative percent (mean  $\pm$  S.D.) of major n-6 and n-3 fatty acids in neonatal plasma PL after weaning

		FATTY ACID					
DIET	DAY	18:2 n-6	20:4 n-6	18:3 n-3	20:5 n-3	22:5 n-3	22:6 n-3
Lo/Lo	70	11.0 $\pm$ 2.3	18.9 $\pm$ 4.0	0.2 $\pm$ 0.1	0.3 $\pm$ 0.0	1.3 $\pm$ 0.4	1.5 $\pm$ 0.4
	84	10.5 $\pm$ 3.8	16.9 $\pm$ 6.9	0.2 $\pm$ 0.1	0.6 $\pm$ 0.1	1.4 $\pm$ 0.2	1.2 $\pm$ 0.6
Hi/Lo	70	17.0 $\pm$ 2.3	7.2 $\pm$ 1.9	2.3 $\pm$ 0.6	6.5 $\pm$ 1.7	2.1 $\pm$ 0.1	1.5 $\pm$ 0.4
	84	16.1 $\pm$ 2.3	7.1 $\pm$ 2.1	2.2 $\pm$ 0.2	7.1 $\pm$ 2.3	2.2 $\pm$ 0.5	1.2 $\pm$ 0.3
Lo/Mod	70	11.8 $\pm$ 0.6	8.3 $\pm$ 2.7	0.2 $\pm$ 0.02	3.5 $\pm$ 1.7	1.6 $\pm$ 0.7	4.7 $\pm$ 2.5
	84	13.4 $\pm$ 0.2	11.1 $\pm$ 2.7	0.1 $\pm$ 0.1	4.4 $\pm$ 2.1	2.1 $\pm$ 1.2	7.4 $\pm$ 5.5
Lo/Hi	70	7.9 $\pm$ 0.2	6.9 $\pm$ 2.8	0.2 $\pm$ 0.05	5.7 $\pm$ 3.1	2.1 $\pm$ 1.1	5.0 $\pm$ 2.5
	84	6.6 $\pm$ 0.3	6.4 $\pm$ 2.6	0.1 $\pm$ 0.02	6.3 $\pm$ 4.0	2.0 $\pm$ 1.1	7.4 $\pm$ 5.1
ANOVA ( <i>p</i> )	time	0.7391	0.9623	0.5636	0.6279	0.6021	0.3007
	diet	0.0000	0.0000	0.0000	0.0004	0.3245	0.0032

No time effects within diet groups were detected at  $p < 0.05$  ( $n = 3$  litters per diet group). Dietary differences for individual fatty acids are indicated in Figure 5.2.



**Figure 5.2** Mean plasma PL content of LA and AA (a) and n-3 fatty acids (b) after weaning. Because no time effects were observed for any fatty acid, data from both sample days were pooled. Letters not in common for individual fatty acids are significantly different with respect to diet at  $p < 0.05$  ( $n = 3$  litters per diet group). Error bars indicate S.D. values.

variability in individual responses, this difference was not significantly different from puppies in the Lo/Mod group. In addition, statistical differences in plasma PL DPA concentrations were not observed amongst diets at  $p < 0.05$ .

During the post-weaning period, a dose response of fish oil was not as apparent in the plasma of the Lo/Lo, Lo/Mod and Lo/Hi puppies. This observation is in contrast to data from other parts of this study in which a dose effect of DHA was clearly observed in the plasma of both mothers and neonates from these diet groups. Plasma PL DHA content in the fish-oil groups were significantly higher than the remaining two groups, but were not significantly different from each other because of the large sample variability. Mean plasma PL DHA concentrations during the post-weaning period were  $6.2 \pm 3.8\%$  S.D. and  $6.1 \pm 4.1\%$  S.D., respectively, in the Lo/Hi and Lo/Mod groups (Figure 5.2b). These values compared with  $1.3 \pm 0.4\%$  and  $1.3 \pm 0.5\%$  S.D., respectively in the Hi/Lo and Lo/Lo diet groups. The failure of the Lo/Mod and Lo/Hi groups to attain statistical significance can be attributed to the large variability in individual responses.

**Red blood cell phospholipids.** Although RBC data were incomplete, total PL fatty acid profiles from at least one litter per diet were available for the d 28 and wk 12 sample days. Difficulties with implementing reliable separation of phospholipid subfractions for subsequent analyses resulted in the loss of a majority of the RBC samples. Small sample volumes did not allow further sample analysis of total RBC PL.

Mean RBC DHA content on d 28 was highest in the Lo/Hi group (5.8%, n=1), followed by the Hi/Lo (n = 2), Lo/Mod (n = 1), and Lo/Lo (n = 2) groups whose mean RBC DHA content were  $2.6 \pm 1.0\%$  S.D., 2.1%, and  $1.4 \pm 1.3\%$  S.D., respectively. At wk 12 the Lo/Hi group (n = 2) again had the highest DHA content,  $4.3 \pm 0.8\%$  S.D. Among

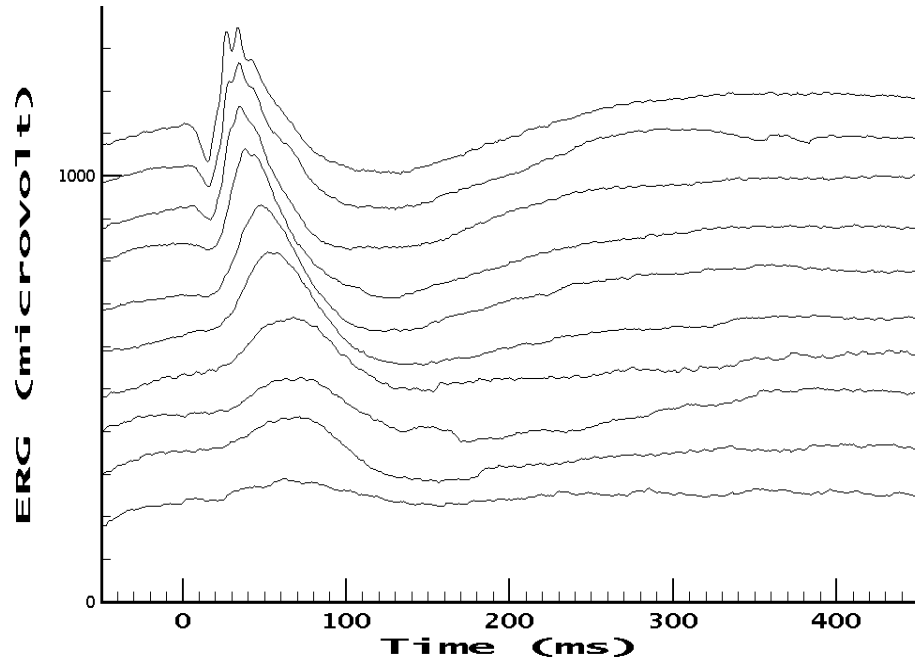
the remaining groups, erythrocyte DHA content was 3.1% in the Lo/Mod group (n = 1), 2.8% in the Hi/Lo group (n = 1). Puppies in the Lo/Lo group (n = 1) had only trace amounts of DHA in the RBC fraction.

Because of the limited data available for the RBC PL fatty acid profiles, valid estimates of retinal DHA content at 12 wk of age cannot be made. However, from the available data, it appears that RBC DHA content was greatest in the Lo/Hi puppies and lowest in the Lo/Lo puppies, while the Lo/Mod and Hi/Lo groups were intermediate. Due to the small sample size, there were not enough degrees of freedom to declare statistical significance.

### **Electroretinography**

Retinal function was assessed at 12 wk of age via flash electroretinography. A representative ERG is shown in Figure 5.3. Parameters of interest were the a- and b-wave amplitudes (a-amp and b-amp, respectively), the implicit times of the a- and b-waves ( $a_i$  and  $b_i$ , respectively), and the derived parameter  $\bar{a}$ . Statistical analyses were performed on data obtained at the eighth light intensity because the highest intensity is known to saturate the rod response in canines (213). A summary of these parameters, including sample sizes, is presented in Table 5.4.

The puppies in the Lo/Hi group demonstrated the greatest response in their a-waves. Mean a-amp in this group was  $49.5 \pm 16.3\mu\text{V}$  S.D. This value was not significantly different from the mean a-amp of the Hi/Lo group, which was  $43.5 \pm 18.4\mu\text{V}$  S.D. Both groups, however, were significantly different from the Lo/Mod and Lo/Lo groups, which had mean a-amp of  $24.6 \pm 8.8\mu\text{V}$  S.D. and  $31.6 \pm 19.6\mu\text{V}$  S.D., respectively.



**Figure 5.3** An ERG series from a puppy in the Lo/Hi group. The intensity of the stimulus increased in half log-unit steps, eliciting ERGs starting from the bottom. For all animals, the amplitudes and implicit times of both the a- and b-waves were determined at the 8<sup>th</sup> (third most intense) flash intensity. Mean values are reported in Table 5.4. The  $\bar{a}$  is calculated from the top three a-wave slopes only.

**Table 5.4** Mean  $\pm$  S.D. values for ERG parameters obtained at 8<sup>th</sup> light intensity

<b>DIET</b>	<b>a-amp (<math>\mu</math>V)</b>	<b>b-amp (<math>\mu</math>V)</b>	<b>a<sub>i</sub> (ms)</b>	<b>b<sub>i</sub> (ms)</b>	<b>ä</b>	<b>Threshold Intensity</b>
Lo/Lo n=36	31.6 $\pm$ 19.6 <sup>a</sup>	172.3 $\pm$ 60.0 <sup>ab</sup>	6.1 $\pm$ 2.3 <sup>a</sup>	35.5 $\pm$ 4.1 <sup>a</sup>	1.8 $\pm$ 1.0 <sup>a</sup>	6.2 $\pm$ 1.0 <sup>a</sup>
Lo/Mod n=25	24.6 $\pm$ 8.8 <sup>a</sup>	153.2 $\pm$ 49.5 <sup>b</sup>	5.6 $\pm$ 1.5 <sup>ab</sup>	34.9 $\pm$ 2.9 <sup>a</sup>	1.6 $\pm$ 0.5 <sup>a</sup>	5.8 $\pm$ 0.8 <sup>ab</sup>
Lo/Hi n=20	49.5 $\pm$ 16.3 <sup>b</sup>	197.8 $\pm$ 47.5 <sup>a</sup>	4.4 $\pm$ 1.2 <sup>b</sup>	33.0 $\pm$ 4.3 <sup>ab</sup>	2.5 $\pm$ 1.0 <sup>b</sup>	5.3 $\pm$ 0.6 <sup>b</sup>
Hi/Lo n=30	43.5 $\pm$ 18.4 <sup>b</sup>	169.7 $\pm$ 42.6 <sup>ab</sup>	5.0 $\pm$ 1.4 <sup>ab</sup>	32.1 $\pm$ 3.2 <sup>b</sup>	1.9 $\pm$ 0.7 <sup>ab</sup>	5.9 $\pm$ 0.8 <sup>a</sup>
<i>p</i> -value (ANOVA)	0.0000	0.0350	0.0026	0.0013	0.0066	0.0017

Letters not in common for a given parameter are significantly different at  $p < 0.05$ .

Mean  $a_i$  was lowest in the Lo/Hi group and highest in the Lo/Lo group. Implicit times were not significantly different among the Lo/Mod, Lo/Hi and Hi/Lo diet groups, whose mean  $a_i$  were  $5.6 \pm 1.5$  ms S.D.,  $4.4 \pm 1.2$  ms S.D., and  $5.0 \pm 1.4$  ms S.D., respectively. The  $a_i$  of the Lo/Lo group was  $6.1 \pm 2.3$  ms S.D., which was not significantly different ( $p < 0.05$ ) from values obtained in the Lo/Mod or Hi/Lo groups.

The  $\ddot{a}$  parameter, as described by Breton *et al.* (140, 232), is a measure of the time-course of activation of the phosphodiesterase cascade in the rod outer segments (ROS). Because the calculation of  $\ddot{a}$  is based on the a-amp, the results obtained follow a similar pattern as those obtained for the a-amp. Again, visual performance in the Lo/Hi group was significantly greater than the Lo/Lo and Lo/Mod groups. Values of  $\ddot{a}$  for these groups were  $2.5 \pm 1.0$ ,  $1.8 \pm 1.0$ , and  $1.6 \pm 1.5$ , respectively. The value of  $\ddot{a}$  in the Hi/Lo group was  $1.9 \pm 0.7$ , which was not significantly different from any other dietary group.

Dietary content of LCPUFA did not appear to have a direct effect on either of the b-wave parameters. The greatest b-amp,  $197.8 \pm 47.5\mu$ V, was observed in the Lo/Hi group, followed by the Lo/Lo, Hi/Lo and Lo/Mod groups, respectively. On average,

puppies in the Hi/Lo group elicited the quickest b-wave response (lowest mean  $b_i$ ), at  $32.1 \pm 3.2$  ms, which was significantly different from the Lo/Lo and Lo/Mod groups. The mean  $b_i$  of puppies in the Lo/Hi group was  $33.0 \pm 4.3$  ms, while the mean  $b_i$  of the Lo/Lo and Lo/Mod groups was  $35.5 \pm 4.1$  ms and  $34.9 \pm 2.9$  ms, respectively. No significant differences were detected among these latter three diet groups at  $p < 0.05$ .

Not all animals responded equally to all intensities of light. Some puppies did not elicit an a-wave response until the 7<sup>th</sup> or 8<sup>th</sup> flash intensity, whereas others responded as early as the 4<sup>th</sup> flash intensity. Based on this observation, mean values were obtained for the threshold intensity ( $I_t$ ), which was the intensity at which the a-wave was first detected. To our knowledge, such a parameter has not been reported before. On average, the Lo/Hi group responded to flashes at lower intensities than did the other three groups. The Lo/Mod and Lo/Hi diet groups were not significantly different from each other, but the Lo/Hi group was significantly different from the Lo/Lo and Hi/Lo groups (Table 5.4).

## **Discussion**

### **Plasma Phospholipids**

For the first 21 d of the suckling period, maternal milk served as the sole source of nutrition for the neonates. By 28 d of age, the puppies had been weaned onto the same experimental diets as their mothers. With few exceptions, the plasma PL fatty acid profiles from both the suckling and post-weaning periods were not different.

The response of neonatal plasma PL composition to dietary fatty acids is similar to that observed in adult dogs during the gestation and lactation portions of this study. Dose responses were observed for LA and ALA in all neonates and for DHA in the Lo/Lo, Lo/Mod and Lo/Hi groups.

The puppies in the Hi/Lo and Lo/Lo groups received nearly identical amounts of EPA from mothers' milk, yet the concentration of EPA in the plasma PL of the Hi/Lo puppies was many times higher than that of the Lo/Lo puppies. Although the differences between the Hi/Lo and Lo/Mod puppies in plasma PL concentrations of EPA were statistically significant, EPA in the Hi/Lo puppies compared more closely with that of the Lo/Mod puppies, whose diet contained twice the EPA, than with the Lo/Lo puppies. Furthermore, milk DHA in the Hi/Lo group was the lowest among all four groups, yet the plasma PL from these puppies contained markedly elevated concentrations of DHA. From these data, it is apparent that neonatal canines are indeed capable of synthesizing EPA and DHA from ALA. This is consistent with results from studies in human infants, which have reported the ability of both fetuses and neonates to synthesize LCPUFA from their 18-C precursors (17-21). Additionally, when adult dogs are fed high dietary amounts of ALA, there is little additional accumulation of PL DHA in plasma (187, 233), yet neonates appear to have considerable PL DHA in their plasma under similar dietary conditions. Although reasons for this are unknown, it appears that neonatal canines may preferentially synthesize at a time of life in which demand is especially high. Peroxisomal metabolism in young dogs may be increased compared to adults, whereby DPA is more readily converted to DHA.

Mean plasma PL AA in the Lo/Lo group was significantly higher than all other groups during both the suckling and post-weaning periods. This occurred despite the facts that no significant dietary differences existed in milk AA and that all experimental diets contained nearly identical amounts of AA. This perceived "enrichment" was the result of a blunting of AA incorporation into plasma PL by increased concentrations of n-3 LCPUFA



in the Lo/Mod, Lo/Hi and Hi/Lo diets. When n-3 PUFA content is high, EPA will be substituted for AA in PL. Thus, the Lo/Lo diet group was not necessarily enriched in AA; instead, the other diet groups had decreased AA and increased n-3 PUFA. Another possible explanation for the increased plasma PL arachidonate is that the low concentration of dietary ALA facilitated increased rates of elongation and desaturation of LA by means of reduced competition for  $\Delta^6$  desaturase. A study of  $\Delta^6$  desaturase enzyme kinetics in adult canine liver microsomes reported that, despite its twofold larger  $K_m$ , LA is readily converted to long-chain metabolites in the absence of high dietary amounts of ALA (215). Since most canine diets are replete in LA, and physiological concentrations of ALA are typically much less than its  $K_m$ , elongation of LA usually proceeds unhindered. This competition for  $\Delta^6$  desaturase is of particular concern in developing neonates. Therefore, careful consideration must be taken when formulating infant formula, either for dogs or humans, in order to assure adequate tissue enrichment of both n-6 and n-3 LCPUFA, which may be conditionally essential during the perinatal period.

Noted differences in the plasma distribution of EPA were observed between the suckling and post-weaning periods. During suckling, the Lo/Hi and Lo/Mod diet groups were significantly different from each other, and both were significantly higher than the Hi/Lo group. However, after weaning, the Hi/Lo group contained the highest concentration of EPA in plasma PL. Although this value was markedly increased from suckling values, the difference was not significant due to large variability of the response. The lower plasma EPA during suckling may indicate a more efficient conversion of ALA to DPA and DHA in younger puppies, when the demand for DHA is greatest. After weaning, demand for DHA by neural tissues is decreased and EPA accumulates. This is

consistent with studies of human infants that suggest LCPUFA formation is a function of gestational age, and that the elongation/desaturation machinery is more active at earlier gestational ages (29).

A dose response of DHA was observed among the Lo/Lo, Lo/Mod and Lo/Hi groups during suckling; however, this effect was not evident in post-weaning plasma PL. Concentrations of DHA in plasma PL were nearly identical between the Lo/Mod and Lo/Hi groups 6 to 8 wk after weaning, despite the two-fold difference in total dietary n-3 LCPUFA. However, marked variability was again observed in post-weaning plasma samples from these two groups.

Additionally, the enrichment of DHA in plasma PL of the Hi/Lo group did not parallel the increase in plasma EPA in this group during the post-weaning period. This finding emphasizes the fact that feeding diets rich in ALA results in the enrichment of DPA in plasma PL, especially in older dogs. Bauer *et al.* (187) also reported the accumulation of plasma DPA, but not DHA, in adult dogs fed flaxseed oil (10 wt% ALA) diets. Although DHA is not enriched in the plasma, tissue needs may still be met by further conversion of DPA. This conversion, however, is far less efficient at enriching tissue DHA than when preformed DHA is supplied in the diet. Fu and Sinclair (234) reported that guinea pigs fed a high-ALA (17% total FA) diet had significantly more whole body ALA and EPA, but not DPA or DHA, compared to those fed a low-ALA (0.3% total FA) diet. Similarly, humans fed 15 g ALA/day for 4 wk experienced small, but significant increases in the PL content of ALA, EPA, and DPA, but little to no increase in DHA in plasma, platelets, white blood cells and erythrocytes (235-237). However, in these studies, accumulation of DHA in neural tissues was not measured. Moreover, studies in guinea

pigs and baboons have shown that dietary DHA was ten and seven times, respectively, as effective as a substrate for neural DHA than dietary ALA (48, 222-224). These findings, in combination with the data presented here, underscore the concept that dietary ALA, unless markedly increased, is not an adequate substitute for dietary DHA.

### **Electroretinography**

Numerous studies have evaluated the effects of breast milk or n-3 LCPUFA-supplemented formula on retinal development in neonatal humans and primates (5, 51, 112-127, 135-143). Although the debate regarding benefits to term infants is ongoing, the evidence for preterm infants seems to be more conclusive. Studies in humans and primates indicate that LCPUFA synthesis may be more active in late-term and premature fetuses than in older neonates (29, 238). All puppies in this study were born at full-term, and all received milk, rather than formula, during suckling. Nonetheless, beneficial effects of dietary n-3 PUFA on ERG response were seen in the puppies in the present study.

ERG data obtained at the 8<sup>th</sup> light intensity was used for analysis of all ERG parameters because the highest (10<sup>th</sup>) intensity is known to saturate the b-wave response in canines. Such a saturation effect could possibly mask measurable differences in retinal function amongst diet groups.

The descending segment of the a-wave represents photoreceptor activity, whereas the b-wave is the composite post-synaptic response of the bipolar and Müller cells (111, 232, 239). The  $\bar{a}$  parameter is related to the time-course of activation for the phosphodiesterase cascade in the ROS (232).

Diau *et al.* reported in 4-wk old baboons that improvement of the b-wave response occurs independently of retinal DHA content and that “DHA per se is not the limiting

factor in the development of the b-amplitude in formula-fed neonates (140).” Although RBC DHA data in this study were not reliable, differences due to diet appeared to exist between the Lo/Hi and Lo/Lo groups, while the differences between the Lo/Hi and Lo/Mod groups were less pronounced. However, the lowest b-amp was observed in the Lo/Mod puppies rather than the Lo/Lo puppies. Thus, the data suggest that magnitude of the b-wave response in breast-fed canines may also be independent of retinal DHA content and that other factors may be involved. Further studies, however, should be conducted, and reliable biomarkers of retinal DHA status are needed.

Although DHA did not appear to affect b-wave amplitude in this study, modest differences were observed in the implicit times of the b-wave. Puppies in the Hi/Lo group had significantly shorter  $b_i$  when compared to the Lo/Lo and Lo/Mod groups. The  $b_i$  of the Lo/Hi group was not significantly different from any group, but was most similar to that of the Hi/Lo puppies. A reduction in  $b_i$  may indicate increased efficiency of post-synaptic signal transduction of the visual response in the presence of increased retinal DHA.

Puppies in the Lo/Lo group had significantly poorer retinal function as measured by the a-amp and  $a_i$  when compared to puppies in the Lo/Hi group. Mean a-amp in the Hi/Lo puppies was not significantly different from the Lo/Hi group, but was superior to both the Lo/Mod and Lo/Lo groups. Although the best rod response (highest a-amp, lowest  $a_i$ ) occurred in those animals that received the highest amounts of dietary DHA during suckling, it was not significantly better than the rod response of animals fed a diet markedly rich in ALA. Similarly, no difference in rod photoreceptor function was found in monkeys fed either 8% ALA or 0.6% DHA (136) or between monkeys fed either ALA as the sole n-3 PUFA or a combination of AA and DHA (139). Furthermore, a study in

guinea pigs reported the highest ERG amplitudes in the group fed ALA only rather than in the group fed ALA in combination with n-3 LCPUFA (240). Thus, it can be concluded from the data that gestation diets containing 0.55% DM DHA and milk containing 2.5 relative% DHA are sufficient to effect increased photoreceptor activity in young canines. Additionally, it seems that gestation diets and milk high in ALA are able to achieve adequate DHA synthesis and neural enrichment such that photoreceptor activity is at least moderately improved. However, the formulation of high-ALA diets, such as the one in this study, may be of little practical use due to the undesirable odor and lowered palatability associated with it.

A decrease in the  $\bar{a}$  parameter is indicative of a reduction in the initial amplification cascade, which is induced when a photon is absorbed by rhodopsin (140). Therefore, a higher  $\bar{a}$  represents an increase in the initial amplification. Although a distinction cannot be made as to whether such an increase means an increase in rhodopsin content or an increased efficiency of photon absorption, greater amplification demonstrates a favorable response when the overall value is elevated. In light of the significantly higher value of  $\bar{a}$  from the Lo/Hi puppies, it can be concluded that DHA conveys a beneficial effect (i.e., an increase) on the initial amplification of the photoreceptor response.

A novel parameter devised in this study is the threshold intensity, i.e. the light intensity at which the initial a-wave was observed. Puppies that consumed the highest amount of n-3 LCPUFA (Lo/Hi) elicited the earliest photoreceptor response, which was significantly lower than those of the Lo/Lo and Hi/Lo groups, but not different from the Lo/Mod group. These data indicate that puppies whose diets contained more n-3 LCPUFA had lower rod thresholds, i.e. greater rod sensitivity, than puppies in the other groups. This

is a possible consequence of the higher dietary concentrations of n-3 LCPUFA in these two groups. Studies in both term and preterm human infants have reported an association between DHA status and retinal sensitivity (241, 242).

Taken together, the data from this study indicate an advantage of dietary DHA on retinal function in young canines. Puppies consuming the highest concentrations of DHA in both milk and dry diet consistently demonstrated the greatest rod sensitivity (as measured by a-amp,  $a_i$ , and  $I_i$ ) and elicited the greatest increase in the amplification of the phosphodiesterase cascade. Although visual performance in puppies fed the high ALA diet was not significantly lower than those fed DHA, it did not match the level of retinal function observed in the DHA-fed puppies.

Erythrocyte DHA has been used as a marker for retinal and cortical DHA status (140), although the usefulness of this marker has been challenged (243). Gibson, *et al.* reported poor correlation ( $r^2=0.16$ ) between erythrocyte and brain DHA in piglets and in post-mortem human infants (243). They also declared the relationship between plasma and brain DHA to be non-existent. In the present study, RBC data was insufficient to make either reliable estimates of retinal DHA or correlations of RBC DHA with retinal function. However, in four-week old baboon neonates, retinal tissue DHA status was most closely associated with a-wave parameters (140). Additionally, retinal response was optimized in breast-fed or DHA-supplemented formula-fed compared to non-supplemented infants (140). Thus, when data from these and the present studies are collectively considered, the likelihood of dietary DHA in canines resulting in retinal enrichment and its associated improvement in ERG-related measures helps to confirm and extend the importance of DHA in fetal and neonatal development among mammalian species. In addition, new data

on the relationship of dietary PUFA and milk fatty acid composition will aid in the development of the most appropriate diets for gestation and lactation.

## CHAPTER VI

### SUMMARY AND CONCLUSIONS

Long-chain polyunsaturated fatty acids are essential for proper growth and neural development in mammalian neonates. Although evidence exists which supports the concept that neonates can synthesize n-3 LCPUFA, namely DHA, from ALA, it remains uncertain whether or not such conversion is sufficient to meet the demands of rapidly developing neural tissues. Thus, because placental transfer is the only means of n-3 LCPUFA delivery to the fetus, and because maternal milk is the sole exogenous source of n-3 LCPUFA for the newborn, maternal dietary n-3 LCPUFA intake during gestation and lactation could profoundly impact the n-3 LCPUFA status, and subsequent development, of both the fetus and the neonate.

The objectives of this research were to investigate the effects of dietary ALA and n-3 LCPUFA on the following: 1) plasma PL fatty acid composition of adult canines during the gestation and lactation periods; 2) fatty acid composition of canine milk; 3) plasma PL fatty acid composition of canine neonates during the suckling and post-weaning periods; 4) retinal development and function in 12-wk-old canine neonates.

In this study adult dogs were fed one of four experimental diets, which contained varying ratios of ALA to n-3 LCPUFA. The combinations of ALA and n-3 LCPUFA content were: low ALA/low n-3 LCPUFA (Lo/Lo); low ALA/moderate n-3 LCPUFA (Lo/Mod); low ALA/high n-3 LCPUFA (Lo/Hi); high ALA/low n-3 LCPUFA (Hi/Lo). Adult dogs were maintained on their randomly assigned diets for the entirety of gestation and lactation. Following parturition, neonates were reared on mother's milk until the time of weaning, at which point they were transitioned to the same dry experimental diet



as their mother had consumed. The major findings of this study can be summarized as follows:

- 1) The modification of dietary n-3 and n-6 fatty acid contents in canine diets resulted in altered lipid metabolism during gestation and lactation. The plasma phospholipid (PL) contents of nearly all PUFA varied in a dose-dependent manner as a function of their dietary concentrations. Despite large dietary differences in LA, no enrichment of AA occurred in the plasma PL fraction. Moreover, when compared with early gestation, plasma AA during lactation was significantly reduced in all diet groups. Such an effect may reflect a channeling of AA to the mammary tissue for incorporation into the milk, which would assure delivery of this fatty acid to the growing newborn. Additionally, the plasma PL fatty acid data from this study confirmed the ability of adult canines to convert ALA to both EPA and DPA; however, only minimal synthesis of DHA occurred in response to high dietary ALA. These results suggest a reduced capacity for DHA synthesis in older dogs.
  
- 2) Enriching canine gestation/lactation diets with LA and ALA does not result in the enrichment of either AA or DHA in the milk. This observation suggests that the biosynthetic pathways specifically relating to desaturation and chain elongation of milk lipids are poorly developed in canine mammary tissue or that these pathways are competitively inhibited in the presence of either small dietary amounts of LCPUFA or their existing tissue stores. In either case, dietary

supplementation of canine gestation/lactation diets with LA or ALA is an ineffective means of increasing milk AA and DHA content.

3) Not only are canine neonates capable of converting ALA to DHA, but their synthetic capacity is greater during the suckling period when compared with the post-weaning period. These results, in combination with the observed lack of DHA synthesis in adult dogs, suggest that neonatal canines may preferentially synthesize DHA at a time of life in which demand is especially high. It is possible that peroxisomal metabolism in young dogs is increased compared to adults, whereby DPA is more readily converted to DHA.

4) Canine neonates reared on a diet of milk containing 2.5 relative % DHA in combination with an experimental diet containing 0.55% DM DHA displayed superior photoreceptor function, that is, greater rod sensitivity, when compared to those on a low ALA/low n-3 LCPUFA diet. Additionally, it seems that gestation diets and milk high in ALA are able to achieve adequate DHA synthesis and neural enrichment such that photoreceptor activity is at least moderately improved. However, the plasma enrichment and photoreceptor function of dogs reared on this diet did not match that of the dogs fed the low ALA/high n-3 LCPUFA diet.

The findings of this research underscore the importance of providing n-3 LCPUFA, rather than ALA, in the canine gestation/lactation diet as a means of enriching

milk with DHA. Furthermore, dietary n-3 LCPUFA was more effective than ALA for the enrichment of plasma PL DHA during the developmental period in neonatal canines. In addition, dietary DHA appears to be related to improved visual performance in developing canids. Although a diet high in ALA was able to effect moderate improvements in ERG parameters when compared to a low ALA/low n-3 LCPUFA diet, visual function did not equal that of dogs fed higher amounts of dietary DHA. This reinforces the notion that dietary ALA, unless exceedingly high, is not an adequate substitute for DHA. Moreover, the formulation of high-ALA diets, such as the one in this study, may be of little practical use due to the undesirable odor and lowered palatability associated with it.

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## **APPENDIX A**



**TABLE A-I**  
**BODY WEIGHTS\* OF ADULT DOGS**

<b>DIETS</b>	<b>DOG</b>	<b>DAY</b>								
		<b>G0</b>	<b>G7</b>	<b>G14</b>	<b>G28</b>	<b>G42</b>	<b>G56</b>	<b>L7</b>	<b>L14</b>	<b>L28</b>
<b>Lo/Lo</b>	Anne	23.5	23.8	23.8	23.0	23.7	28.9	22.2	23.1	22.8
	Doris	18.2	17.9	17.8	17.4	18.8	22.6	19.1	19.6	19.2
	Alexa	24.0	23.8	23.4	22.0	25.6	29.7	21.8	22.4	22.2
<b>Hi/Lo</b>	Bambi	22.5	23.0	23.5	22.2	23.3	26.2	18.1	18.2	15.1
	Belle	22.3	21.3	23.6	22.1	25.5	29.9	24.2	25.5	22.1
	Dolly	22.5	22.8	25.1	23.2	26.8	29.9	24.2	24.2	24.3
<b>Lo/Mod</b>	Brandi	21.4	21.2	21.2	20.7	23.3	26.9	21.3	24.1	25.0
	Bonnie	21.0	22.9	22.7	20.3	23.7	26.7	20.7	21.8	20.9
	Bethany	22.9	22.5	24.2	23.2	25.3	27.3	23.1	23.0	24.7
<b>Lo/Hi</b>	Bettina	24.6	24.5	24.5	24.2	26.5	31.4	23.6	25.1	24.3
	Dana	20.9	20.5	20.0	19.4	23.9	26.1	21.9	23.2	23.2
	Agnes	19.6	19.2	19.8	18.0	17.6	20.1	15.8	16.4	16.7

\*Body weights are in kilograms.

**TABLE A-II**  
**AVERAGE LITTER BODY WEIGHTS\* OF PUPPIES**

DIETS	LITTER	DAY								
		0	7	14	21	28	42	56	70	84
<b>Lo/Lo</b>	N	0.43 (8)	0.49 (8)	0.73 (8)	0.98 (8)	1.60 (8)	3.56 (8)	5.54 (8)	7.20 (8)	9.20(8)
	O	0.41 (6)	0.70 (6)	1.08 (6)	1.56 (6)	2.26 (6)	3.89 (6)	5.74 (6)	7.85 (4)	10.00 (4)
	V	0.40 (10)	0.61 (10)	0.85(10)	1.16 (10)	1.79 (10)	3.38(10)	5.13 (10)	6.56 (7)	9.29 (7)
<b>Hi/Lo</b>	S	0.36 (10)	0.47 (10)	0.61(10)	0.92 (10)	2.10 (10)	3.71 (10)	5.25 (10)	7.20 (7)	9.12 (7)
	U	0.48 (7)	0.62 (7)	0.91 (7)	1.02 (7)	1.25 (7)	2.58 (7)	3.59 (7)	5.50 (3)	7.00 (3)
	W	0.39 (8)	0.51 (8)	0.88 (8)	1.24 (8)	1.56 (8)	2.98 (8)	4.33 (8)	6.53 (8)	8.51 (8)
<b>Lo/Mod</b>	M	0.51 (7)	0.82 (7)	1.26 (7)	1.83 (7)	2.67 (7)	4.20 (7)	6.55 (7)	7.42 (5)	10.40 (5)
	P	0.35 (8)	0.51 (8)	0.79 (8)	1.10 (8)	1.66 (8)	3.18 (8)	5.18 (8)	6.32 (6)	7.25 (6)
	Q	0.48 (7)	0.76 (7)	1.12 (7)	1.59 (7)	2.32 (7)	3.59 (7)	5.61 (7)	6.79 (2)	8.66 (2)
<b>Lo/Hi</b>	K	0.49 (8)	0.71 (8)	1.02 (8)	1.37 (8)	1.93 (8)	3.30 (8)	4.64 (8)	7.22 (5)	8.02 (5)
	R	0.45 (4)	0.70 (4)	1.14 (4)	1.61 (4)	2.49 (4)	4.24 (4)	6.04 (4)	7.06 (2)	10.20 (2)
	T	0.39 (6)	0.52 (6)	0.74 (6)	1.02 (6)	1.71 (6)	3.13 (6)	4.80 (6)	6.55 (3)	8.70 (3)

\*Body weights are in kilograms. Values in parentheses indicate litter size.

**APPENDIX B**

**TABLE B-I**  
**MATERNAL DIET FATTY ACID CONCENTRATION**  
**(% dry matter basis)\***

Fatty Acid	Diet			
	Lo/Lo	Lo/Mod	Lo/Hi	Hi/Lo
Saturated	6.02	5.46	6.95	2.55
Monounsaturated	6.55	6.14	6.17	4.22
18:2n-6	1.75	2.61	1.16	3.5
20:4n-6	0.02	0.03	0.05	0.02
18:3n-3	0.14	0.29	0.2	6.82
20:5n-3	0.02	0.19	0.5	0.02
22:5n-3	0.02	0.05	0.11	0.02
22:6n-3	0.02	0.19	0.55	0.02
%total fatty acids	14.54	14.96	15.69	17.17
%total dietary fat	15.33	15.90	16.10	17.47

\*The diets are designated based on their ALA/n-3 LCPUFA contents. For details see text.

**TABLE B-II**  
**MATERNAL DIET NUTRIENT PROFILES**  
**(% dry matter basis)\***

Fatty Acid	Diet			
	Lo/Lo	Lo/Mod	Lo/Hi	Hi/Lo
Crude protein	30.7	30.4	29.9	31.4
Fat	15.3	15.8	16.1	17.5
Nitrogen-free extract	47.8	47.5	47.9	44.6
Ash	6.2	6.2	6.1	6.5
Energy (kcal/g)	5.1	5.1	5.2	5.0

\* Ingredients of each diet included Supro 620<sup>®</sup>, Brewers milled rice, taurine, potassium chloride, vitamin Super Premix, dehulled soybean meal, poultry by-product meal, pea fiber, dry calcium chloride, dicalcium phosphate, NaCl, mineral premix, liquid choline chloride, vitamin E 50%, safflower oil, and linseed oil, beef tallow, or Menhaden fish oil.

TABLE B-III

**COMPLETE FATTY ACID PROFILE OF EXPERIMENTAL DIETS**  
(expressed as % fatty acids)

<b>Fatty Acid</b>	<b>Lo/Lo</b>	<b>Hi/Lo</b>	<b>Lo/Mod</b>	<b>Lo/Hi</b>
13:0	<0.10	<0.10	<0.10	<0.10
14:0	2.32	0.34	2.94	4.45
14:1	0.47	<0.10	0.32	0.39
15:0	0.37	<0.10	0.39	0.46
15:1	<0.10	<0.10	<0.10	<0.10
16:0	22.70	9.45	21.00	23.70
16:1	2.97	0.57	4.02	5.54
17:0	1.11	0.18	0.84	0.90
17:1	<0.10	<0.10	<0.10	<0.10
18:0	14.2	5.18	10.60	10.80
18:1	40.31	23.95	34.42	32.55
18:2	11.67	20.00	16.69	6.83
18:3	0.89	39.00	1.80	1.05
19:0	0.18	<0.10	0.12	0.12
20:0	0.16	0.18	0.21	0.14
20:1	0.23	0.14	0.38	0.61
20:2	<0.10	<0.10	<0.10	0.18
20:3	<0.10	<0.10	<0.10	0.21
20:4	0.12	<0.10	0.20	0.31
20:5	<0.10	<0.10	1.22	2.83
21:0	<0.10	<0.10	<0.10	<0.10
22:0	<0.10	0.16	0.13	<0.10
22:1	<0.10	<0.10	<0.10	0.10
22:2	<0.10	<0.10	<0.10	<0.10
22:5	<0.10	<0.10	0.26	0.59
22:6	<0.10	<0.10	1.19	3.07
23:0	<0.10	<0.10	<0.10	<0.10
24:0	<0.10	0.13	0.10	<0.10

## VITA

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Texas A & M University, Nutrition, Ph.D.	2004
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Magna Cum Laude graduate, Texas A & M University  
 Who's Who Among American College Students  
 Dean's List, College of Agriculture & Life Sciences, Texas A & M University  
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2000-2004	Graduate Student, Comparative Nutrition Laboratory, Texas A & M University
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#### Publications

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 Maternal diet alpha-linolenic acid during gestation and lactation does not increase canine milk docosahexaenoic acid, *J Nutr*, accepted.

#### Scholarships

Graduate Assistantship, Nutrition, Texas A & M University, 2001-2004.  
 Graduate Fellowship in Nutrition, Texas A & M University, 2000.  
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