AN ABSTRACT OF THE THESIS OF

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Title: Dietary Polyunsaturated Fatty Acids and Leukotriene Production in Poultry

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Inflammation is the body's response to injury and is characterized by pain, swelling, redness, and heat. Eicosanoids are lipid mediators of inflammation. Leukotrienes (LT) are 20-carbon eicosanoids produced from arachidonic acid (AA), an n-6 fatty acid (FA), and eicosapentaenoic acid (EPA), an n-3 FA. LT produced from AA are proinflammatory (LTB_4) and those produced from EPA (LTB_5) are less inflammatory. Two experiments were conducted. The objective of the first experiment was to optimize the assay conditions for LT production by platelets from chickens, and neutrophils from horses and dogs. Optimal production of LT from equine and canine neutrophils and chicken platelets was characterized in terms of incubation time (2.5, 5, 10, 15 or 20 minutes), temperature (25 or 37°C), and calcium ionophore A23187 concentration (0.1, 1, 10 or 20 μM). In all species, incubation at 37°C resulted in optimal LTB_4 production compared to 25°C ($p \leq 0.05$). Production of LTB_4 was maximum when neutrophils were stimulated with 20 μM calcium ionophore A23187 in all species ($p \leq 0.05$). Incubation times greater than 2.5 minutes did not further increase LTB_4 production in chickens and horses; in dogs, incubation for 2.5 and 10 minutes resulted in the highest concentrations

of LTB_4 ($p \leq 0.05$). These results indicate that platelets from chickens, and neutrophils from horses and dogs, are capable of producing LTB_4; optimum conditions for LTB_4 production are similar in all three species.

In the second study, the effect of feeding diets that differed in n-6 and n-3 FA ratios to breeder hens was investigated with regard to changes in composition of immune tissue, alteration of delayed-type-hypersensitivity (DTH) response, and LT production by platelets. Chicks hatched to hens fed these diets were also studied with regard to fatty acid composition of immune tissues and LT production by platelets at various stages of growth (7, 14, 21 days). A total of 72 breeder hens were randomly divided into three groups (n=24) and fed diets supplemented with either 3.0 % (by weight) sunflower oil (SFO; rich in n-6 FA; Diet I), a mixture of 1.5 % SFO and 1.5 % fish oil (Diet II), or 3.0 % fish oil (FO; rich in n-3 FA; Diet III). Production of LTB_4 and LTB_5 by platelets stimulated with calcium ionophore A23187 were assessed by RP-HPLC. The hens fed Diet I synthesized 43.9 ± 2.5 ng of LTB_4 per 5×10^6 cells compared to 13.3 ± 0.9 ng of LTB_4 from hens fed Diet II ($p \leq 0.05$). However, no LTB_4 was produced by hens fed Diet III. Production of LTB_5 by platelets of hens fed Diet III was 36.7 ± 4.9 ng compared to 47.4 ± 5.7 ng of LTB_5 from hens fed Diet II. No LTB_5 was produced by hens fed Diet I. The DTH reaction was smaller at 48 hrs post injection of bovine serum albumin in hens fed the 3% FO Diet III ($p \leq 0.05$). Fatty acid composition spleen and platelets in hens reflected the fatty acid composition of diets consumed by them ($p \leq 0.05$).

Hatched chicks from hens fed Diet I produced significantly less LTB_4 at 14 days ($p \leq 0.05$) compared to 7- and 21-day-old chicks, which were not different from each other. Chicks from hens fed Diet II produced more LTB_4 at 21 days ($p \leq 0.05$) compared

Chicks from hens fed Diet III produced more LTB_4 at 7 and 21 days ($p \leq 0.05$) compared to 14-day-old chicks. There were no significant differences in LTB_5 production from chicks hatched to hens fed Diet III at 7 and 14 days of growth. By 21 days of growth, chicks hatched to hens fed Diet III showed decreased production of LTB_5 compared to 7- and 14-day-old chicks. The spleen and bursa tissue fatty acid composition in chicks at 7 and 14 days of age were similar to the maternal diet fatty acid composition, however, there were no significant differences in platelet fatty acid composition between the groups at different stages of growth. These results indicate that the type of fat in diets fed to breeder hens may alter the inflammatory response in hatched chicks, which could lead to less mortality and increased production performance in poultry.

Dietary Polyunsaturated Fatty Acids and Leukotriene Production in Poultry

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CHAPETR 1. INTRODUCTION

The term "lipid" encompasses a large number of hydrophobic non-polar natural compounds with various chemical structures, for examples fats, cholesterol, fatty acids, lipid soluble vitamins, waxes, soaps, phospholipids, sphingolipids, oxylipins, sterols, and others. Lipids are an important component in poultry rations because of their high-energy value and because they provide vitamins and essential fatty acids that are associated with them.

1.1 ROLE OF LIPIDS IN POULTRY DIET

The role of lipids in poultry diets can be divided in two major subdivisions.

1.1.1 Physical Role

- They improve the physical quality of feeds.
- They act as lubricants.
- They reduce dust and particle separation.
- They increase the palatability of feeds.
- The digestibility of fat generally is higher than protein or carbohydrate.

1.1.2 Physiological Role

The major physiological functions of lipids are stated below

1.1.2.1 Lipids as an energy source for poultry

Fatty acids (FA) in the form of triglycerides constitute an important source of energy for mammals. Polyunsaturated fatty acids (PUFA), monounsaturated fatty acids, and saturated fatty acids can be stored in the body as triglycerides. They give rise to free fatty acids after the action of lipases. Triglycerides are the principal storage form of fatty acids in adipose tissues. While proteins and carbohydrates yield around 4 kilocalories of energy per gram, fats yield 2 ¼ times as much or 9 kilocalories per gram. They are the most important for providing energy. The use of high energy rations is a popular trend and in poultry rations it is now common to add 10% fat to the feed to get maximum productivity in a shorter length of time.

1.1.2.2 Structural role of fats

Lipids are an integral part of cell membranes and organelles. Lipids involved in membrane structure are mainly phospholipids and cholesterols. An important FA in cell membranes is linoleic acid, which serves to maintain the integrity and functionality of membranes. The concentration of PUFA in membrane lipids can affect cell receptor binding, activity of membrane-bound enzymes, and permeability properties of the membrane.

1.1.2.3 Fats serve as transporters

Another very important function of fats is the transportation of fat soluble vitamins A, D, E, K, and the transportation of other substances that are fat soluble. Without fat in the diet, these vitamins would not be present. This would result in severe problems with eyesight, skin, blood clotting, kidney function, bone growth and repair, reproductive functions, and cellular energy metabolism.

1.1.2.4 Metabolic functions of fats and fatty acids

Lipids take part in diverse aspects of metabolism. Fatty acids are not only responsible for providing energy and contribute to the structure of cell, but they act as intermediates in the synthesis and metabolism of other important compounds in the body. The various metabolic functions of fat can be outlined under the subheadings listed below:

1.1.2.4.1 Phospholipids: The primary lipids of biological membranes are phospholipids, a group of phosphate-containing molecules with structures similar to triglycerides. They have a hydrophilic head and hydrophobic tail. The main function of phospholipids is to act as emulsifying agents to maintain the proper colloidal state of protoplasm.

1.1.2.4.2 Glycolipids: The term glycolipid designates any compound containing one or more monosaccharide residues bound by a glycosidic linkage to a hydrophobic moiety such as an acylglycerol, a sphingoid, a ceramide (*N*-acylsphingoid) or a prenyl phosphate. Glycolipids are present ubiquitously in cell membranes. Glycolipids can play important roles in biological functions, such as cellular transport processes, communication, and defense mechanisms.

1.1.2.4.3 Steroid hormones: These can be classified as sex hormones (testosterone, androsterone, estrone, estradiol, progesterone), and adrenal cortical hormones (aldosterone, hydrocortisone, cortisone). The various functions of these hormones in the body are well known.

1.1.2.4.4 Sterols: Cholesterol is the most abundant animal sterol. Cholesterol is primarily found in membranes and it serves to increase membrane fluidity. Cholesterol is used in the biosynthesis of other cell materials, including bile acids (which are involved in the digestion and emulsification of lipids) and steroid hormones.

1.1.2.4.5 Eicosanoids: The eicosanoids consist of the prostaglandins (PG), thromboxanes (TX), and leukotrienes (LT). Eicosanoids play a regulatory role in physiological processes. They are produced from 20-carbon polyunsaturated fatty acids, such as arachidonic acid (AA) and eicosapentaenoic acid (EPA). The eicosanoids have a wide range of biological effects, e.g., on inflammatory responses, on the intensity and duration of pain and fever, and on reproductive function.

1.1.3 Additional Functions of Fat

Fats, in the form of structural body fat, provide important protection for the vital internal organs. Fat is a fairly poor conductor of heat; body fat in the subcutaneous tissues (under the skin) acts as insulation, and tends to prevent loss of body heat. Fats are also required for brain structural lipids. Long chain PUFA is required in nervous tissue and the retina. Substances made from fats also provide the covering for nerves (myelin), and thereby allows nerves to carry impulses.

As early as the 1930s, lipids were known to provide energy for the body. Later in the 1960s, it was discovered that lipids had other major roles, e.g., in maintaining homeostasis. During the two last decades, and especially in the 1990s, many studies have shown that lipids also act as important biological effectors, regulators, and mediators. They participate in virtually all biological processes (such as immune responses, transmission of neuronal information, regulation of vessel and muscle tone, homeostasis, inflammation, etc.) and in cellular biochemical reactions. As second messengers they transmit external signals into the cell, and then they serve as intracellular messengers (Merrill et al., 1996).

CHAPTER 2. REVIEW OF LITERATURE: FATTY ACIDS

Fatty acids are the basic structural components of triglycerides and are also found in phospholipids and cholesterol esters. Fatty acids are simple in structure, with a carboxyl group at one end and a methyl group at the other end of the carbon backbone. In poultry rations, commonly added fatty acids contain C14:0 to C22:0 fatty acids. Commonly fed lipids and their fatty acid composition are listed in Table 1.

2.1 CLASSIFICATION OF FATTY ACIDS

2.1.1 Classification on the Basis of Number of Double Bonds

- No double bonds: saturated fatty acids, e.g., stearic acid
- Single double bond: monounsaturated fatty acids, e.g., oleic acid
- Two or more double bonds: polyunsaturated fatty acids (PUFA), e.g., arachidonic acid

2.1.2 Classification on the Basis of Chain Length

- Short chain fatty acids: These are 2- to 4-carbon molecules of great importance in intermediary metabolism. They are the mainstay of ruminant nutrition. They are represented by acetic, butyric and propionic acids.
- Medium chain fatty acids: 4 to 12 carbon molecules, e.g., palmitic acid
- Long chain fatty acids: 12 to 20 carbon molecules, e.g., arachidonic acid (AA)
- Very long chain fatty acids: 22 carbon molecules, e.g., docasahexaenoic acid (DHA)

Table 1. Dietary fatty acids in poultry rations.*

Source	Saturated fatty acids	Polyunsaturated fatty acids		Mono-unsaturated fatty acids
		(n-6)	(n-3)	
Canola Oil	7	22	10	61
Hempseed	8	55	25	12
Flaxseed Oil	10	17	55	18
Safflower Oil	10	76	Trace	14
Sunflower Oil	12	71	1	16
Corn Oil	13	57	1	29
Olive Oil	15	9	1	75
Soybean Oil	15	54	8	23
Peanut Oil	19	33	Trace	48
Cottonseed Oil	27	54	Trace	19
Lard	43	9	1	47
Beef Tallow	48	2	1	49
Palm Oil	51	10	Trace	39
Butterfat	68	3	1	28
Coconut Oil	91	2	0	7
Fish oil	16.8	10.9	26.4	41.5
Menhaden fish oil	26.9	2.2	29.5	25

* Values reported as percentages

2.1.3 Classification on the Basis of Essentiality

- Non-essential fatty acids: These can be synthesized endogenously in the body.
- Essential fatty acids: They are called essential because they are required for normal development and maintenance and cannot be synthesized endogenously in the body (e.g., linolenic acid).

2.1.4 Classification on the Basis of Position of Omega Carbon

Fatty acids are named based on the position of the endmost double bond, counting from the methyl (CH_3) carbon, called the omega end (also known as 'n' classification). The two most important omega fatty acids are listed.

Omega-6 fatty acids: (e.g., linoleic acid and arachidonic acid). Linoleic acid is designated 18:2n-6. It has an 18-carbon backbone, two double bonds, with the first double bond at the sixth carbon from the methyl end. Arachidonic acid is designated 20:4n-6. It has a 20-carbon backbone, four double bonds, with the first double bond at the sixth carbon from the methyl end.

Omega-3 fatty acids: (e.g., α-linolenic acid, eicosapentaenoic acid (EPA), and docosahexaenoic acid (DHA). α-linolenic acid is designated 18:3n-3. It has an 18-carbon backbone, three double bonds, with the first double bond at the third carbon from the methyl end. Eicosapentaenoic acid is designated 20:5n-3. It has a 20-carbon backbone, five double bonds, with the first double bond at the third carbon from the methyl end. Similarly, docosahexaenoic acid is designated 22:6n-3. It has a 22-carbon backbone, six double bonds, with first the double bond at the third carbon from the methyl end (Calder, 1997).

2.2 DIGESTION AND ABSORPTION OF LIPIDS IN POULTRY

Digestion is the process of breaking down large, complex molecules into smaller components that can be absorbed into the blood stream. The process involves changes in both physical and chemical properties of dietary components.

The digestibility of fats by poultry has been shown to vary widely depending upon many factors, including type of fat, degree of saturation, age of bird, amount of fat included in the diet, and presence of other dietary components (Krogdahl, 1985). The bulk of dietary lipid is in the form of triglycerides, which consist of a glycerol backbone with each carbon linked to a fatty acid. Additionally, feedstuffs may contain phospholipids, sterols like cholesterol, and many minor lipids, including fat-soluble vitamins.

In order for the body to make use of dietary lipids, they must first be absorbed from the small intestine and for this, two processes must occur.

- Large aggregates of dietary triglyceride, which are virtually insoluble in an aqueous environment, must be broken down physically and held in suspension, a process called emulsification.

- Triglyceride molecules must be enzymatically digested to yield monoglycerides and fatty acids, both of which can efficiently diffuse into the enterocyte.

Dietary lipids do not undergo any significant digestion in the proventriculus of chickens in spite of the presence of a gastric lipase. They are, however, partially emulsified by the high temperature in the stomach as well as by peristaltic movements. Because the feed is retained in the proventriculus for some time, up to 30% digestion of triglycerides

may occur in the stomach. Because the short-chain fatty acids released are moderately hydrophilic, they enter into the portal vein. The bulk of the dietary lipids are, however, passed into the duodenum along with the acid chyme. Most of the digestion of dietary lipids occurs in the intestine by enzymes present in pancreatic juice. The middle section of the small intestine is the most active site of fat absorption in chickens. The pancreatic enzymes are lipase, cholesterol esterase and phospholipase A_2.

2.2.1 Emulsification, Hydrolysis, and Micelle Formation

Fat digestion is enhanced by emulsification with bile salts. The emulsification of dietary fats renders them accessible to pancreatic lipases, which generate free fatty acids and a mixture of mono- and diacyl-glycerols from dietary triacylglycerols. Free fatty acids and monoglycerides are not water-soluble. A further action of bile is the formation of micelles. Micelles are essentially small aggregates of mixed lipids and bile salts suspended within the ingesta. As the ingesta mixes, micelles goes into the brush border of enterocytes and lipids, including monoglycerides and fatty acids, are absorbed. Once inside the intestinal mucosal cell, fatty acids are reesterified to form triglycerides. The re-esterification of fatty acids within the mucosal cell occurs by addition of two fatty acids to the mono-acylglycerol molecules absorbed. Fatty acids are first activated to their coenzyme A derivatives before they can be esterified to the monoacylglycerol (Burley et al., 1984).

2.2.2 Transport into Blood

Out of the total plasma lipids, roughly 40% are triglycerides, 20% are cholesterol, 2% are free fatty acids, and the rest are phospholipids. Because lipids are insoluble in water, they cannot be transported as such in plasma. Most lipids are transported in the

blood as triglycerides within lipoproteins, which are a core of hydrophobic lipids surrounded by a shell of proteins, phospholipids and cholesterol. There are four major groups of lipoproteins: 1. Chylomicrons, 2. Very low-density lipoproteins (VLDL), 3. Low-density lipoproteins (LDL), and 4. High-density lipoproteins (HDL). In poultry, unlike mammals, lipoprotein rich triglycerides are absorbed directly into the portal blood system and transported to the liver. These types of lipoproteins have been referred to as portomicrons as opposed to chylomicrons in mammals. Their size (mean diameter of about 150 nm) and composition (about 90% triglycerides) are very similar to those of mammalian chylomicrons. Apolipoprotein B-100 and apo A-I are the major apolipoproteins of chicken VLDL and HDL, respectively (Burley et al., 1984; Hermier et al., 1984; Perry et al., 1984; Whitehead et al., 1984).

Chylomicrons are the major exogenous lipoproteins synthesized by intestinal mucosal cells and contain apo-A, apo-B-48, apo-C and apo-E. Major sites of metabolism of chylomicrons are adipose tissue and skeletal muscle. The enzyme lipoprotein lipase is located in the endothelial layer of capillaries. Apo-C present in the chylomicrons activates lipoprotein lipase and this enzyme hydrolyzes triglycerides and liberated fatty acids are taken up by muscle or adipose tissue. As the triglyceride content is progressively decreased, some apo-A, apo-C and phospholipids from the surface are released, and the chylomicrons shrink in size. The small chylomicron remnants containing apo-E and apo-B-48 are taken up by hepatic cells, by receptor mediated endocytosis. Thus, chylomicrons are the transport form of dietary triglycerides enabling them to be delivered to adipose tissue for storage (Perry et al., 1984). VLDL is the major carrier of endogenous triglycerides. Triglycerides are synthesized in the liver from fatty acids and glycerol, and get

incorporated into VLDL along with hepatic cholesterol, apo-E, apo-B-100, and apo-C-II. When they reach the peripheral tissues, apo-C-II activates lipoprotein lipase, which liberates fatty acids that are taken up by adipose tissue and muscle. In mammals, lipoprotein lipase (LPL) must be activated by apo-C-II. The equivalent of apo-C-II remains to be identified in birds, although it is known that HDL constitutes the major reservoir of LPL activator in chicken plasma (Whitehead et al., 1984). The remnant is designated as intermediate density lipoprotein (IDL) and contains less triglyceride and more cholesterol. A small part of IDL is taken up in the liver by receptor-mediated endocytosis, which is aided by apo-B-100 and apo-E. Further loses of triglyceride and apo-E from IDL enables it to become low density lipoprotein (LDL). Thus, VLDL carries triglycerides from liver to peripheral tissues for energy needs.

LDL molecules are cholesterol rich lipoprotein molecules containing only apo-B-100. They transport cholesterol from liver to the peripheral tissues. LDL receptors are present on all cells, but most abundant in hepatic cells and adrenal cortex. There is a specific LDL receptor on plasma membranes of extra hepatic cells, located in specialized regions called coated pits, which contain the protein called clathrin. These receptors recognize the apo-B-100 and, therefore, can take up LDL particles. When the apo-B-100 binds the receptor, the receptor-LDL complex is internalized by endocytosis. These vesicles fuse with lysosomes, and lysosomal enzymes degrade apoproteins and also hydrolyze cholesterol ester to free cholesterol. The free cholesterol can be incorporated into cell membranes, metabolized to steroid hormones, re-esterified and stored, or can be finally excreted through the liver (Krogdahl, 1985).

2.2.3 Transport of Fatty Acids

When infused intravenously, free fatty acids are carried physiologically largely on albumin. Free fatty acids are delivered within seconds directly to the cardiac phospholipid membranes, where they are in place to exert their antiarrhythmic potential. When they are ingested in the diet, however, the situation is different and circuitous. They are ingested largely as triglycerides and absorbed as free fatty acids and monoglycerides, but are rapidly and efficiently resynthesized in gut wall and liver back to triglycerides. They appear in the circulation largely in chylomicrons and LDL's, from which they are liberated in the periphery and liver by lipoprotein and hepatic lipases, and then picked up on the fatty acid binding sites of serum albumin. They are carried on the albumin to membrane phospholipids of heart, brain, and other tissues into which they very rapidly partition. DHA is the preferred storage form of n-3 fatty acids in heart and brain, where it accounts for a large percentage of fatty acids incorporated into the sn-2 position of membrane phospholipids. A summary of digestion in poultry is presented in Table 2.

Table 2. Fat digestion in poultry.

G I Tract region	Enzyme or secretion	Substrate/Function	End product
Mouth	Saliva	Lubricates and softens food	_
Crop	Mucus	Lubricates and softens food	_
Gizzard and Proventriculus	HCl	Lowers stomach pH	_
	Lipase	Fats	Fattty acids, mono-glycerides and glycerol
Duodenum and Jejunum	Bile	Fats	Emulsification
	Lipase	Fats	Fattty acids, mono-glycerides and glycerol
	Cholesterol estrase	Fatty acid-cholesterol esters	Fatty acids, cholesterol

2.3 METABOLISM OF FATTY ACIDS

Fatty acids are stored in the adipose tissue as triacylglycerols, and when needed, they are mobilized from adipose tissue by lipolysis. Released fatty acids undergo oxidation and this process is known as ß-oxidation because the splitting of carbon units occurs at the ß-carbon atom. Before fatty acids undergo oxidation, they have to be activated and converted to their coenzyme derivatives (Bierbach et al., 1979).

Beta-oxidation of the even-numbered, carbon-chain, fatty acids involves mainly 4 reactions, which are sequentially repeated until there is complete oxidation of a fatty acid. The activated fatty-acyl CoA is dehydrogenated to a trans-enoyl CoA, which is further hydrated to from ß-hydroxyl fatty acyl CoA. This intermediate product is again oxidized to from ß-keto fatty-acyl CoA. The ß-keto fatty-acyl CoA undergoes thiolytic cleavage leaving behind a fatty acid with 2 less carbon atoms. The newly formed fatty-acyl CoA will undergo further cycles of ß-oxidation until it is completely converted to acetyl CoA.

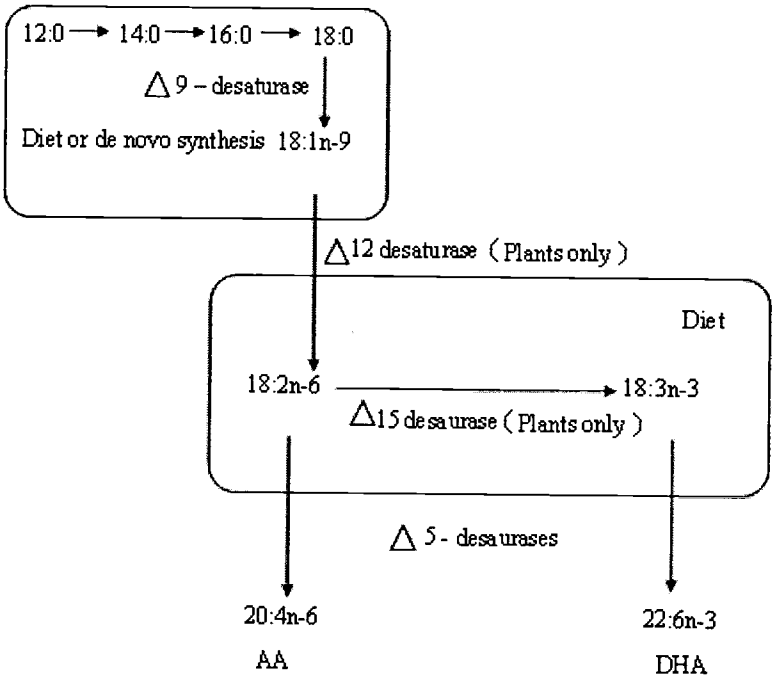
The odd-numbered, carbon-chain, fatty acids are also oxidized in the same manner as even-numbered, carbon-chain, fatty acids, however, in the terminal stage one 3-carbon unit, propionyl CoA, is produced. This is further metabolized by conversion to the TCA cycle intermediate, succinyl CoA.

All mammals can synthesize fatty acids *de novo* from acetyl CoA. It's not a reversal of oxidation. The end product of the fatty acid synthetase enzyme is palmitic acid (16:0). There are mitochondrial elongation system as well as a microsomal elongation and desaturation system by which other fatty acids can be formed from palmitate in the body.

The process of elongation and desaturation of fatty acids occurs mainly in liver, adipose tissue, kidney, brain, and mammary glands. Because cell membranes need

unsaturated fatty acids to maintain their functions, a mechanism for introducing double bonds into saturated fatty acids is needed. The introduction of a single double bond between C-9 and C-10 is catalyzed by the enzyme Δ9-desaturase, which is present in both plants and animals. This enzyme results in the conversion of stearic acid to oleic acid (18:1n-9). Plants, unlike animals, can insert additional double bonds into oleic acid between the existing double bond at the C-9 position and the methyl terminus of the C chain. A Δ12-desaturase converts oleic acid into linoleic acid (18:2 n-6), while a Δ15-desaturase converts linoleic acid into α-linolenic acid (18:3 n-3). Animal cells then convert α-linolenic acid into EPA (20:5 n-3) and DHA (22:6 n-3). As a result of the actions of the Δ9, Δ12, and Δ15 desaturase enzymes, four unsaturated fatty acid families exist: n-7, n-9, n-6 and n-3 families. These fatty acids families are not metabolically inter-convertible in mammals (Calder and Grimble, 2002c). Biosynthesis of PUFA is outlined in Figure 1.

Figure 1. Biosynthesis of polyunsaturated fatty acids. Elongation and desaturation of monounsaturated fatty acids by elongases and desaturases yields polyunsaturated fatty acids (adopted form Calder, 2001).



2.4 FATTY ACID COMPOSITION OF CELL MEMBRANES

The lipid requirement of all mammals and birds is met by their dietary intake or by *de novo* synthesis of fatty acids. The n-3 and n-6 FA (linolenate and linoleate, respectively) are essential fatty acids that can not be synthesized by mammals or birds and, therefore, must be obtained from dietary sources. Essential fatty acids are components of cell membranes. Cell membranes are partly made up of phospholipids, which contain fatty acids. The type of fatty acids in the diet will determine what type of fatty acids gets incorporated into cell membrane phospholipids. The relative amounts of n-3 and n-6 fatty acids in cell membranes also affect their function. Unlike cellular proteins, which are predominantly genetically determined, cell membrane PUFA composition is largely influenced by dietary intake. Thus, manipulation of dietary PUFA intake serves as a means of altering tissue lipid composition. Tissue arachidonic acid (AA), a desaturation-elongation product of dietary linoleic acid (LA), serves as a precursor for eicosanoids, i.e., the prostaglandins (PG) and leukotrienes (LT) collectively. Alterations in dietary lipids provide a nonpharmacologic means of altering eicosanoid synthesis. For example, ingestion of fish oil can modify tissue lipid PUFA composition by increasing tissue EPA and DHA and reducing AA content. This tissue alteration exerts a significant effect on eicosanoid synthesis because EPA competes with AA as a substrate for eicosanoid formation (Broughton and Wade, 2002).

2.5 PUFA IN CELL MEMBRANES

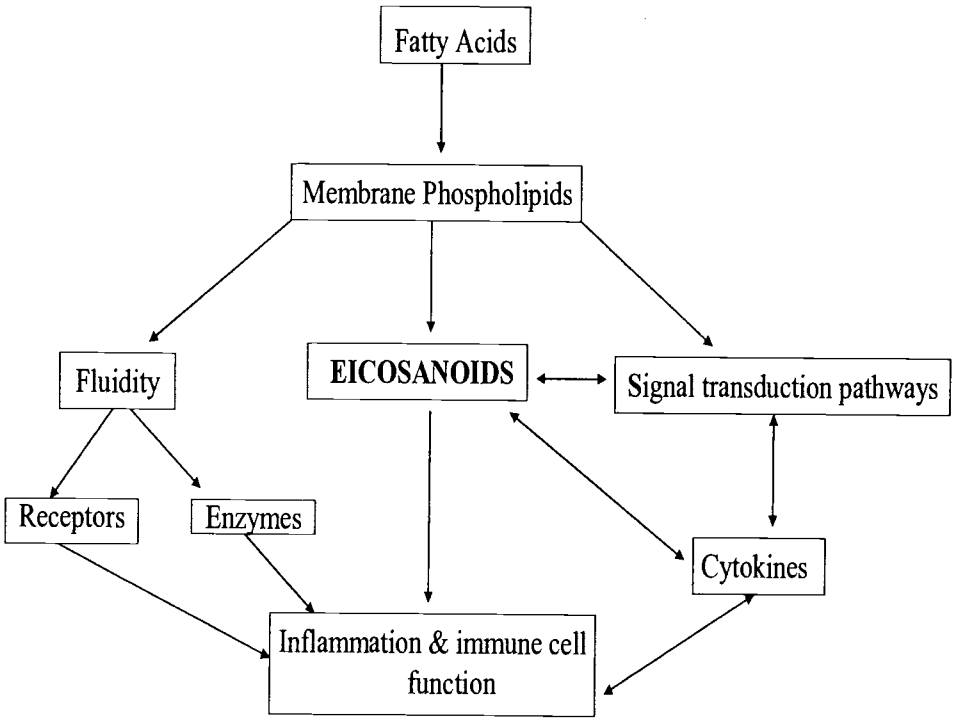
Laboratory animals and human subjects fed a normal fat diet had a high proportion of n-6 FA, e.g., AA, and a low proportion of n-3 FA, e.g., EPA, in inflammatory and

immune cells (Calder, 1998; Calder, 2001; Calder, 2003). Furthermore, it has been reported that the AA fatty acid composition of inflammatory cells varies according to cell type and lipid fraction examined (Sperling and Bluestone, 1993). The phospholipids of human mononuclear cells (approximately 70:20:10 mixture of T cells, B cells, and monocytes) contain 6 to 10 % linoleic acid (LA), 1 to 2% di-homo-γ-linolenic acid (DGLA) and 15 to 25% AA. In contrast, the percentages of n-3 fatty acids are much lower; α-linolenic acid (ALA) is rare, and EPA and DHA comprise only 0.1 to 0.8% and 2 to 4 % of the total fatty acids, respectively (Yaqoob and Calder, 1993).

2.6 FATTY ACIDS AND IMMUNITY

Dietary fatty acids can influence host immunity in two ways, i.e., structural alterations and chemical signaling modifications. Structural alterations are caused by changes in membrane phospholipid components, which ultimately affect membrane fluidity. Modifications of membrane phospholipid composition, e.g., substitution of n-3 for n-6 PUFA, may alter the structure and function of receptor binding sites involved in initiation of cell responses. This could affect cell division and hormonal signal transduction mechanisms. Chemical signaling modifications may affect synthesis, release, and binding of chemokines or other chemotactic compounds. Such changes may also lead to overproduction of eicosanoids (Wan et al., 1988). Figure 2 depicts the overall effects of fatty acids on immune function and immunity. (Re-drawn from Calder, 2003, Nutrition and Immune Function.)

Figure 2. Relationship between fatty acids and immune function (adopted from Calder, 2003).



2.7 EICOSANOIDS: A LINK BETWEEN FATTY ACIDS AND CELL FUNCTION

Eicosanoids are a group of chemical messengers synthesized from 20 carbon PUFA. They include prostaglandins, prostacyclins, leukotrienes, and thromboxanes. They are responsible for many of the beneficial effects of the "good fats." However, some eicosanoids, such as those derived from arachidonic acid, are potentially harmful if excessive amounts are produced in the body. Of the different eicosanoids, prostaglandins (PG) and leukotrienes (LT) are the most important.

2.7.1 Metabolism of Arachidonic Acid and Pathways for Eicosanoid Biosynthesis

Arachidonic acid (AA) is the most common precursor for eicosanoids. A signal activates phospholipase in cell membranes of immune cells, which cleaves the 20-carbon PUFA, AA, from cell membrane phospholipids, usually at the central carbon. Thus, membrane-bound phospholipase-A_2 frees AA. After AA is released, it is converted to eicosanoids by one of two enzyme systems (Figure 3).

2.7.1.1 Cyclooxygenase (COX) pathway

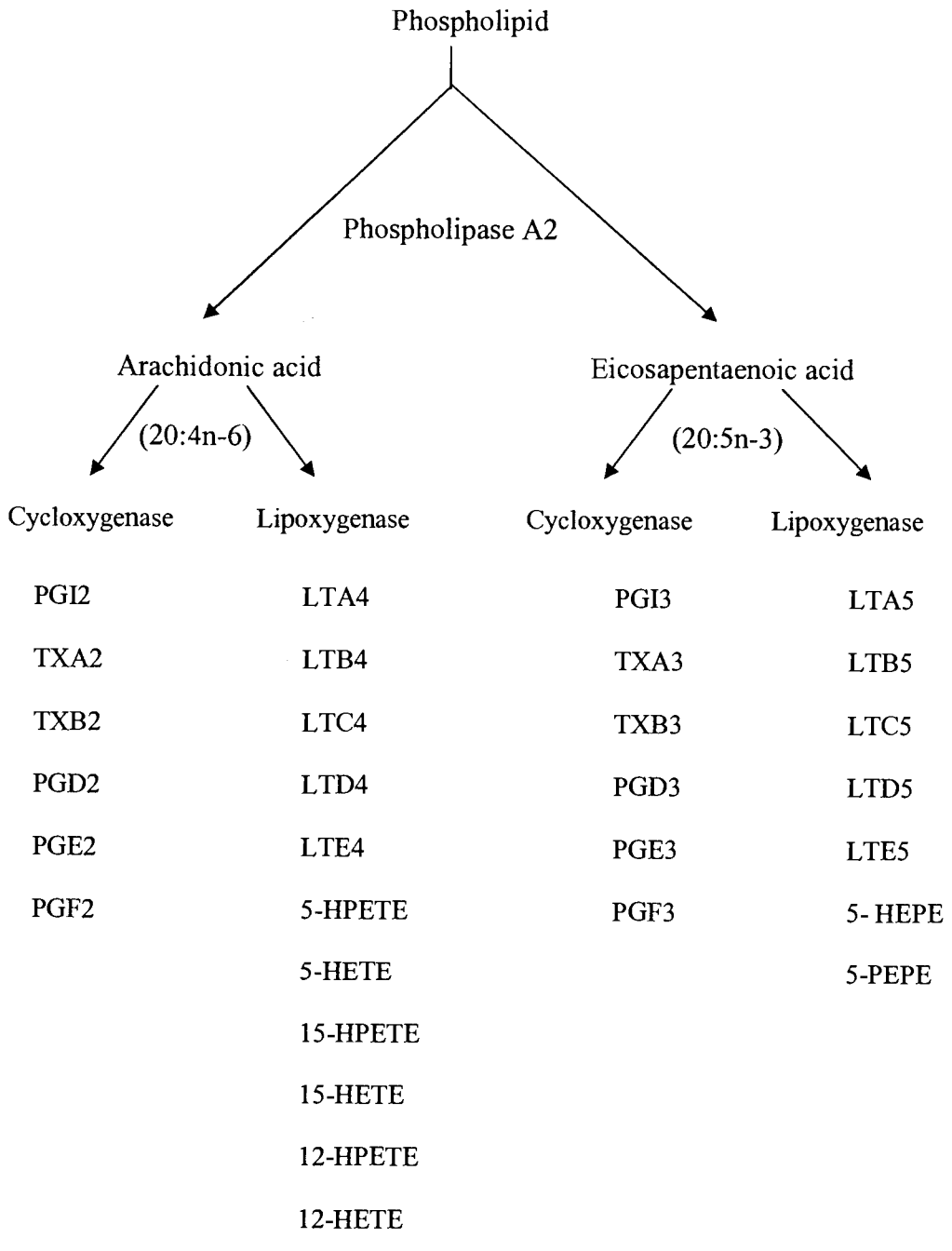
This pathway is initiated through the action of prostaglandin GH synthase, (PGS; also called prostaglandin endoperoxide synthetase). This enzyme possesses two activities. It acts as a cyclooxygenase (COX) and a peroxidase. COX-1 (PGS-1) is expressed constitutively in gastric mucosa, kidney, platelets, and vascular endothelial cells. COX-2 (PGS-2) is inducible, and is expressed in macrophages and monocytes in response to inflammation. The primary triggers for COX-2 induction in monocytes and macrophages are platelet-activating factor (PAF) and interleukin-1 (IL-1). Both COX-1 and COX-2

catalyze the 2-step conversion of AA to prostaglandin G_2 (PGG_2) and then to prostaglandin H_2 (PGH_2).

2.7.1.2 Lipoxygenase pathway

This pathway is initiated through the action of lipoxygenases. It is the enzyme 5-lipoxygenase (5-LOX) that gives rise to leukotrienes of the 4- and 5-series.

Figure 3. Release of arachidonic acid and eicosapentaenoic acid from membrane phospholipids and their metabolism to eicosanoids (adopted from Calder, 1997).



2.7.2 Eicosanoids Derived from Gamma Linolenic Acid (GLA)

The body processes GLA (18:3 n-6) through several steps, eventually leading to beneficial eicosanoids, the most important of which is prostaglandin E1 (PGE_1). PGE_1 has three basic functions: it reduces inflammation, dilates blood vessels, and inhibits blood clotting. Its strong anti-inflammatory properties help the body recover from injury by reducing pain, swelling and redness. The other two functions keep blood vessels open and blood flowing freely.

2.7.3 Eicosanoids Derived from Arachidonic Acid (AA)

The body processes AA (20:4 n-6) into potentially harmful eicosanoids, the most potent of which is prostaglandin E2 (PGE_2). PGE_2 increases inflammation, constricts blood vessels, and encourages blood clotting. These properties come into play when the body suffers a wound or injury. Without this eicosanoid, an animal could bleed to death from the wound. However, in excess, this eicosanoid may be harmful. Many diseases are directly linked to excessive inflammation and blood clotting. Examples include rheumatoid arthritis and some forms of stroke and heart attack in humans. High levels of PGE_2 derived from AA in the body have also been linked to other diseases including diabetic nerve damage, high blood pressure, allergies, skin inflammation, and cancer in both humans and animals (Pace et al., 2004).

2.7.4 Eicosanoids Derived from Alpha-Linolenic Acid and Eicosapentaenoic Acid

The body processes α-linolenic acid (18:3 n-3) and eicosapentaenoic acid (20:5 n-3) into eicosanoids with various functions; some of them dilate blood vessels, while others constrict blood vessels. This group of eicosanoids includes prostaglandin E3 (PGE_3), which is a weak pro-inflammatory agent that inhibits blood clotting. In general, PGE_3 is a useful defense mechanism against trauma and infection. The body must maintain a delicate balance between the eicosanoids. Of particular concern is the balance between PGE_1, PGE_2, and PGE_3. The body must produce enough beneficial eicosanoids such as PGE_1 and PGE_3 or otherwise the pro-inflammatory, blood-vessel-constricting, and blood-clotting effects of PGE_2 will overwhelm the system. Researchers have reported that dietary modulation with n-3 FA could be helpful in maintaining this required balance.

2.8 INFLUENCE OF THE TYPE OF FATTY ACIDS IN THE DIET ON WHITE BLOOD CELL FUNCTION AND EICOSANOID PRODUCTION

2.8.1 Saturated Fatty Acids

In vitro and animal feeding studies suggest that saturated fatty acids have a limited impact on lymphocyte proliferation, Th-1 and Th-2 type cytokine production, or NK cell activity (Calder, 2002; Calder, 2003; Calder, 2003; Calder, 2004). The lack of any effect of varying the intake of saturated fatty acids on NK cell activity is supported by human studies (Hebert et al., 1990).

2.8.2 Oleic Acid

In vitro and animal feeding studies have shown that oleic acid in adequate amounts can partially inhibit lymphocyte proliferation, IL-2 production, IL-2 receptor and adhesion molecule expression, and NK cell activity (Calder, 2002; Calder, 2003; Calder, 2003;

Calder, 2004). These studies show that oleic acid has the potential to modulate lymphocyte function, although the amount of oleic acid added to animal diets in these studies was greatly in excess of that in diets of free living humans. Yaqoob et al. (2000) supplemented the diet of a group of subjects and with an extra 9 gram of olive oil per day for 12 weeks. This had no effect on lymphocyte proliferation, NK cell activity, or the production of tumor necrosis factor (TNF-alpha). Neither were interleukins such as IL-alpha, IL-beta, IL-2 or IFN-gamma produced by mononuclear cells affected. In another study conducted by the same researchers, they reported that an increase in olive oil consumption from 11 to 18% decreased the function of NK cells, caused a non-significant reduction in lymphocyte proliferation, and significantly decreased the number of mononuclear cells expressing CD54.

2.8.3 Linoleic Acid (LA)

In vitro and animal feeding studies have shown that LA in sufficient amounts can partially inhibit lymphocyte proliferation, production of IL-2, cytotoxic lymphocyte activity, NK cell activity, and the production of IgG and IgM (Calder, 1996; Calder, 1998; Calder, 1999). The DTH response was suppressed after feeding LA enriched rich diets. These observations suggest that high levels of LA in the diet impair cell mediated immunity (CMI) and antibody responses. Jeffery et al. (1997) reported that more modest changes in the rat diet did not markedly affect lymphocyte proliferation, production of IL-2, CTL activity, and NK cell activity. Kelley et al. (2001, 1991) performed the most detailed human study, which involved providing volunteers with low fat diets (25% energy as fat) that were rich in LA (12% energy as fat) or poor in linoleic acid (3.5% energy as fat). Surprisingly, no differences were observed in the response of lymphocytes to various

T cell mitogens, in circulating IgM, IgG, IgE or IgA levels, or in the DTH response to seven recall antigens. Yaqoob et al. (2000) supplemented the habitual diet of a group of subjects with 9 g sunflower oil per day for 12 weeks. This had no effect on lymphocyte proliferation, NK cell activity, or the production of TNF-alpha, IL-alpha, IL-beta, IL-2 or IFN-gamma by mononuclear cells. These studies suggest a limited ability for LA at levels of 3.5% to 12.9% dietary energy to affect human immune function.

2.8.4 Conjugated Linoleic Acid (CLA)

Cook et al. (1993) reported that CLA in the diet of chickens (0.5g/100g diet) increased lymphocyte proliferation in response to T cell mitogens. More recent studies have reported some immunological effects of feeding a mixture of isomers of CLA to laboratory rodents. In these studies, diets were fairly low in fat (5% fat by weight) and CLA comprised 20% of FA present. Feeding this diet to mice increased lymphocyte proliferation and IL-2 production, but did not affect NK cell activity. Feeding the same diet to rats decreased TNF and IL-6 production by macrophages (Hayek et al., 1999).

2.8.5 Gamma Linoleic Acid (GLA) and Di-homo Gamma Linolenic Acid (DGLA)

Santoli et al. (1990) and Purasiri et al. (1997) have reported that both GLA and DGLA inhibit the proliferation of cultured human lymphocytes. Furthermore, Santoli et al. (1990) in the same year reported that DGLA decreases the production of IL-2 by human lymphocytes. DeLuca et al. (1999) reported that GLA and DGLA inhibit production of IL-beta and TNF-alpha by human monocytes. NK cell activity was reduced when it was cultured with GLA (Purasiri et al., 1997). Feeding rats a diet rich in GLA (evening prime rose oil) decreased their lymphocyte proliferation and NK cell activity, and decreased the expression of the IL-2 receptor as well as some of adhesion molecules on the surface of

lymphocytes (Peterson et al., 1999; Sanderson et al., 2000; Sanderson et al., 1995; Yaqoob, 2004). These observations suggest that GLA in sufficient quantities will diminish the Th-1 type response. GLA has been shown to increase total and ovalbumin-specific IgE production by spleen lymphocytes and to increase circulating total and ovalbumin-specific IgE levels following ovalbumin challenge in sensitive Brown Norway rats. In contrast, production of IgG was decreased by lymphocytes in the same study suggesting that GLA acts to diminish the Th-1 type lymphocyte response (Matsuo et al., 1996). In human supplementation studies, GLA rich oils were given to healthy human volunteers. A GLA intake of somewhere between 1 to 2.4 g per day was required to exert immunological effects in these healthy humans (DeLuca et al., 1999; Thies et al., 2001; Yaqoob and Calder, 1993).

2.8.6 Arachidonic Acid (AA)

AA serves as a substrate for synthesis of eicosanoids (PG, LT, TX, and HETE). In fact, several 20 carbon PUFA are able to serve as precursors for eicosanoid biosynthesis. However, because membranes of most cells contain large amounts of AA, compared to EPA, another precursor of eicosanoids, AA is the usual precursor in eicosanoid biosynthesis. The metabolism of AA to eicosanoids has been explained in Figure 3.

It was reported that AA increased IL-beta production by cultured human monocytes (Baldie et al., 1993). AA also inhibits the proliferation of cultured lymphocytes, decreases the production of IL-2, and inhibits cytotoxic lymphocytes (CTL) degranulation (Calder, 1996; Calder, 1998; Calder, 1999). It was reported that feeding rats or mice diets containing significant amounts of AA did not affect spleen lymphocyte proliferation, IL-2 production, NK cell activity, or the graft vs. host response, despite the increased capacity of

cells to produce PGE_2. Thus, these studies suggest that even significant amounts of AA in a rodent's diet does not influence cell-mediated immunity (Jolly et al., 1997; Peterson et al., 1998).

Including arachidonic acid (1.5g/day) in a low fat diet (27% energy as fat) for 8 weeks did not alter lymphocyte proliferation, NK cell activity, DTH response, TNF-alpha, IL-1beta, IL-6 or IL-2 production by monocytes (Kelley, 2001). Arachidonic acid did, however, increase production of PGE_2 and LTB_4 by lipopolysaccharide stimulated mononuclear cells. Although these studies suggest that increasing arachidonic acid consumption in healthy adults does not have any adverse immunological effects, the duration of arachidonic acid administration was only 12 weeks.

2.9 INFLUENCE OF N-3 POLYUNSATURATED FATTY ACIDS ON PLATELETS FUNCTION AND LEUKOTRIENE PRODUCTION

Platelets are also known as thrombocytes. It is well known that platelets participate in blood coagulation and thrombus formation. They are small fragments of cells that clump together and stick to the inner surface of blood vessels to plug leaks. Platelets are formed in the bone marrow. The number of circulating platelets is normally between 250,000 and 350,000 per dL of blood. Platelets can be activated in inflammation, which results in the release of lipid mediators and various granules into the extracellular space. Platelets are capable of attaching themselves to injured vessel walls (adhesion) and of sticking to each other (aggregation).

Neutrophils and blood platelets co-operate in several pathophysiological processes, and together they produce eicosanoids in response to activation (Chabannes et al., 2003). Polymorphonuclear neutrophils and platelets interact to produce both inflammatory and

anti-inflammatory lipid mediators during human disease (Stahl et al., 1997). Experiments in rabbits provide *in vivo* evidence that platelets are involved in an acute inflammatory event contributing to the transcellular production of LTs (Evangelista et al., 1999).

It has been shown that endotoxin directly affects platelets and other cells, and stimulates them to produce thromboxane and prostacyclin. Very high concentrations of endotoxin are required to stimulate neutrophils to produce rather small increases in LTC_4. Platelets are easily activated by minute concentrations of endotoxins and calcium ionophore A23187 (Bolton-Smith et al., 1988). The effect of dietary fish oil, corn oil and coconut oil on the induction of atherosclerosis secondary to serum sickness in rabbits was investigated by Chabannes et al. (2003). It was concluded that platelets may inhibit the neutrophil 5-lipoxygenase activity at the integrin level, and, in turn may play a role in slowing down the production of LTB_4 during the course of inflammation. There is evidence that platelets are also capable of producing LT alone as well as in conjunction with neutrophils (Chabannes et al., 2003, Stahl et al., 1997).

2.10 IMMUNOREGULATORY AND ANTI-INFLAMMATORY EFFECTS OF N-3 POLYUNSATURATED FATTY ACIDS

Fish oil is rich in long-chain n-3 FA like EPA and DHA. Linseed oil and green plants are rich in the precursor fatty acid, alpha-linolenic acid. Most vegetable oils are rich in the n-6 FA linoleic acid, the precursor of arachidonic acid. Arachidonic acid-derived eicosanoids such as PGE_2 are pro-inflammatory and regulate the functions of cells of the immune system. Consumption of fish oil leads to replacement of AA in cell membranes by EPA. This changes the amount and alters the balance of eicosanoids produced.

Consumption of fishoil diminishes lymphocyte proliferation, T-cell-mediated cytotoxicity, natural killer cell activity, macrophage-mediated cytotoxicity, monocyte and neutrophil chemotaxis, major histocompatibility class II expression and antigen presentation, production of pro-inflammatory cytokines (interleukins 1 and 6, tumor necrosis factor) and adhesion molecule expression. Feeding laboratory animals fish oil reduces acute and chronic inflammatory responses, improves survival to endotoxin and prolongs the survival of grafted organs. Feeding fish oil reduces cell-mediated immune responses. Fish oil supplementation may be clinically useful in acute and chronic inflammatory conditions and following transplantation (Sanderson et al., 1995).

N-3 FA may exert their effects by modulating signal transduction and/or gene expression within inflammatory and immune cells (Calder, 2003; Calder, 2004). Dietary fish oil supplementation suppresses the T-lymphocyte proliferative response *in vitro* of cells isolated from healthy humans, and is associated with decreased production of IL-2, a potent autocrine and paracrine T-lymphocyte growth factor (Endres et al., 1993). Most of the supplementation studies have used large amounts of fish oil taken daily in fish oil capsules. However, in another human study it has been shown that incorporation of fish in the diet suppresses the delayed-type hypersensitivity (DTH) response, an *in vitro* T-lymphocyte dependent phenomenon, and an *in vitro* T-lymphocyte proliferative response (Meydani and Dinarello, 1993).

2.10.1 Alpha Linolenic Acid

In vitro and animal feeding studies indicate that α-linolenic acid inhibits lymphocyte proliferation, the production of IL-2, cytotoxic T-lymphocyte activity, NK cell activity and the graft vs. host response (Calder et al., 2002; Peterson et al., 1998; Sanderson

et al., 1995). But, it has been reported that the precise effect of α-linolenic acid on lymphocyte functions appears to depend on the level of linolenic acid and the total PUFA content of the diet (Jeffery et al., 1997). A human study in which subjects were fed a high dose of α-linolenic acid, i.e., 13 grams per day, to healthy volunteers found that it decreased IL-1 and TNF production by lipopolysaccharide-stimulated monocytes (Caughey et al., 1996). After adding 15 g per day linseed oil to a low fat diet containing 29% energy from fat, researchers found significant decreases in the DTH response to seven recall antigens after 6 weeks of diet consumption, but circulating antibody levels were unaffected (Burri et al., 1991). Thies et al. (2001) showed that there was no effect of α-linolenic acid diet modification on the immune response in elderly subjects. Therefore, these studies suggest that marked increases in α-linolenic acid intake can induce immunomodulatory effects. It's not clear whether these are exerted by α-linolenic acid itself or EPA, a product of α-linolenic acid metabolism.

2.10.2 Eicosapentaenoic Acid and Docosahexaenoic Acid

In-vitro studies have revealed a marked influence of long chain n-3 FA (EPA and DHA) on inflammatory and immune cell functions. Long chain PUFA inhibit cytokine – induced cell surface expression of MHC II on macrophages and monocytes (Huges et al., 1996). In culture, EPA and DHA inhibited the production of IL-beta, TNF-alpha, IL-6 and IL-2, lymphocyte proliferation, and NK cell activity (Calder, 1996, 1998). Dietary fishoil decreased expression of the IFN-gamma receptor on murine macrophages and splenocytes (Feng et al., 1999). Feeding fish oil to rodents decreased TNF-gamma, IL-1B, and IL-6 production by inflammatory macrophages and monocytes *ex-vivo* (Calder, 1998). These studies suggest that dietary fish oil might diminish cell-mediated immunity by decreasing

the activity of antigen presenting cells and by decreasing the sensitivity of macrophages to T-lymphocyte derived cytokines. High level of fish oil decreased NK cell activity, CTL activity, lymphocyte proliferation, expression of IL-2 receptor and adhesion molecules on lymphocytes (Calder, 1998). Calder et al. (1998) also reported that fish oil diminishes the DTH and graft vs. host responses, compared to a low fat or low n-6 FA diets.

2.11 INFLUENCE OF FATS ON INFLAMMATORY DISEASES IN ANIMAL MODELS AND HUMAN SUBJECTS

Clinical studies have concentrated on the effects of fish oil, while the influence of a range of fats has been studied in animal models of inflammation. Inflammatory symptoms in psoriasis, asthma, Crohn's disease and ulcerative colitis are ameliorated by fish oil (Forbes, 2003; Graham and Kandil, 2002; Simopoulos, 2002). The substantial weight loss that occurs in pancreatic cancer is prevented by a daily supplement of fish oil (Barber et al., 1998). There are many models of animal inflammation, both acute and chronic. Fats have been shown to modulate these processes. Fish oil protected pigs, rats and guinea-pigs from the lethal effect of endotoxins (Murray et al., 1993). Fish oil exerted protective effects in experimental colitis in rats. Diets rich in medium chain triglyceride produced similar ameliorative effects for colon adenocarcinoma in mice (Bartoli et al., 2000; Hong et al., 2003; Karmali, 1987; Kim and Park, 2003).

2.12 DIETARY LEVELS OF N-6 VS. N-3 FATTY ACIDS

N-3 fatty acids offer considerable potential for disease prevention and for therapeutic management of a wide variety of clinical disorders. Consequently, n-3 fatty acid supplements have been used in veterinary trials, predominantly in animals with

inflammatory and immune-based conditions, with some favorable clinical responses. Many lipid experts recommend a ratio of 3 to 1 (n-6 to n-3 FA) in the total diet to achieve optimal inflammatory responses in canids (Boudreaux et al., 1997; Mooney et al., 1998). There is little scientific evidence that dietary fatty acid composition influences the profile of eicosanoids produced by cell types which are important in inflammatory conditions, i.e., platelets, leukocytes and endothelial cells. Also, it must be remembered that eicosanoid production does not equate to inflammatory potential, because inflammation is a complex phenomenon, involving a number of other factors in addition to eicosanoids.

Feeding vaccinated dogs diets with a low (1.4 to 1) n-6 to n-3 FA ratios was associated with an increase in total lymphocyte count and a decrease in CD4 to CD8 ratio (Hall et al., 1999). It is likely that there is a complex relationship between the dietary n-6 to n-3 fatty acid ratio and the phospholipid fatty acid composition of cell membranes of the key cell types responsible for eicosanoid production. It is possible that the dietary n-6 to n-3 fatty acid ratio that has favorable effects on inflammatory potential might not be optimal, and may indeed be detrimental, for immune and aggregatory functions (Calder, 2004). Thus, a single ratio of fatty acids might not be beneficial for all animals under all conditions. Research indicates that the ideal ratio of n-6 to n-3 FA in the diet is between 5 to 1 and 10 to 1 (Calder, 2004, Yaqoob et al., 2001, Mooney et al., 1998).

2.13 LEUKOTRIENES

Leukotrienes (LT) are a subgroup of eicosanoids derived from the 5–lipoxygenase (5-LOX) pathway in myeloid cells. 5-LOX enzyme is present in polymorphonuclear neutrophils, basophils, eosinophils, mast cells, macrophages and platelets.

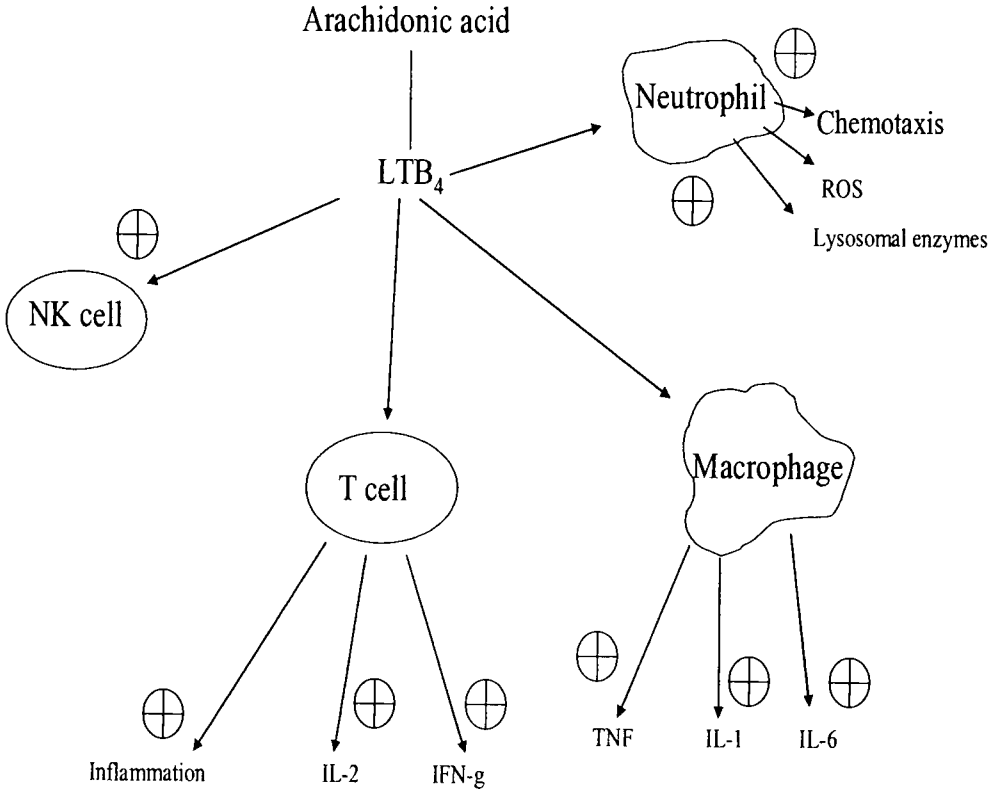
2.13.1 Functions and role of leukotrienes

1. LT's are involved in many immune-mediated inflammatory processes. They are also regarded as slow reacting substances of anaphylaxis.
2. LT's are potent mediators of inflammation, causing increased vascular permeability, and smooth muscle contraction.
3. LT's play a role in the immune response by mediating leukocyte activation. They are one of the most potent chemotactic factors for neutrophils to the site of infection.
4. LT's increase the activity of NK cells and cytotoxic T cells by enhancing binding to target cells. They are involved in proliferation of T cells, modulate production of TNF, and may play a role in mediating the response of these cells to antigen.
5. LT's have been reported to be important in the pathophysiology of asthma and allergic rhinitis in humans.

2.13.2 Control and regulation of leukotriene production

The type and availability of fatty acids in plasma membranes of leukocytes, such as linoleic acid and α-linolenic acid, which are precursors of AA and EPA, respectively, regulate leukotriene production. The metabolism of leukotrienes is extremely rapid and the body can increase or decrease the amount produced very quickly. LT half-life is less than 5 minutes. The significance is that leukotrienes have profound effects even at low concentrations. LT production in tissue is also regulated by the presence of enzymes. Immunoregulatory functions of LTB_4 are summarized in Figure 4.

Figure 4. Immunoregulatory functions of leukotriene B_4 (LTB_4).



⊕ = increased action or increased proliferation of affected cells

2.13.1 Effects of N-3 and N-6 Polyunsaturated Fatty Acids on Leukotriene Production

As discussed earlier, the type and amount of PUFA in cell membranes is determined and influenced by dietary intake of fatty acids, unlike protein deposition in cell membranes, which is determined by genes (Calder, 2004). Thus, cell PUFA composition can be easily manipulated by feeding different fatty acids. AA, which is a desaturation elongation product of linoleic acid, is the main precursor of eicosanoids, i.e., prostaglandins and leukotrienes.

The type of eicosanoid synthesized depends upon the type of PUFA in the diet, e.g., n-3 or n-6 FA (Broughton et al., 1991; Hwang et al., 1988). For example, dietary uptake of fish oil, which is a rich source of n-3 FA, will lead to incorporation of EPA and DHA into membrane phospholipids of tissues and circulating cells (Broughton et al., 1991; Whelan et al., 1991). Diets enriched in n-3 fatty acids result in the incorporation of n-3 fatty acids into biological membranes, with a corresponding decrease in concentration of n-6 fatty acids such as AA. This has been shown in many tissues including skin, heart, aorta, platelets, erythrocytes, testis and adipose tissue in dogs, pigs, rabbits, rats and humans. Arachidonic acid metabolism leads to the pro-inflammatory four series of leukotrienes (4-LT), and EPA and DHA metabolism leads to the less anti-inflammatory five series of leukotrienes (5-LT) (Ziboh, 1996). It has been has shown that production of four-series-leukotrienes decreases progressively with increased dietary n-3 FA content and five series leukotriene production generally increases with increased dietary intake of n-3 FA (Broughton and Wade, 2002). Activation of phospholipase-A_2 in plasma membranes of immune cells and other circulating cells initiates a cascade of reactions in which n-3 and n-

6 FA competitively react with enzymes and are converted into different isomers of leukotrienes and prostaglandins. See Figure 5. It has also been shown that dietary intake of n-3 FA inhibits Δ6-desaturase activity. This is probably one of the mechanisms by which n-3 FA decrease n-6 eicosanoid synthesis (Garg et al., 1988). Broughton et al. (1991) and Whelan et al. (1991) have suggested that while n-3 FA ingestion decreases AA based eicosanoid synthesis and increases EPA based leukotriene synthesis, differences in total dietary fat levels may also have the potential to alter total eicosanoid production.

It is now believed that diets enriched with a combination of n-6 and n-3 FA and in the appropriate ratio will be beneficial. It has been suggested that the absolute amount of n-3 FA in the diet suppresses AA metabolism, and therefore, the ratio of n-6 to n-3 FA in the diet should be balanced (Boudreau et al., 1991). LTB_5 has been reported to be 30 to 100 times less active at stimulating leukotriene receptors on immune cells than the corresponding n-6 FA derived LTB_4 (Kragballe et al., 1987; Lee et al., 1984; Shimizu et al., 1988). Stimulation of LTB_4 receptors on neutrophils is one of the primary steps involved in chemotaxis. It has been shown that LTB_5 will inhibit LTB_4 induced inflammatory responses and can diminish allergy symptoms and other inflammatory conditions (Croft et al., 1988).

N-3 fatty acids not only modulate prostaglandin metabolism, but also decrease triglyceride levels and, in high doses, lower cholesterol. In addition, they have anti-thrombotic and anti-inflammatory properties. EPA competes with AA for prostaglandin and leukotriene synthesis at the enzymatic level. (See Figure 6). It has been shown through various research studies on humans, animals, and laboratory animals and in *in-vitro* studies that dietary fish oil, containing EPA and DHA, leads to (1) decreased production of PGE_2

Figure 5. Oxidative metabolism of arachidonic acid and eicosapentaenoic acid by the cyclooxygenase and 5-lipoxygenase pathways (adopted from Calder, 1997).

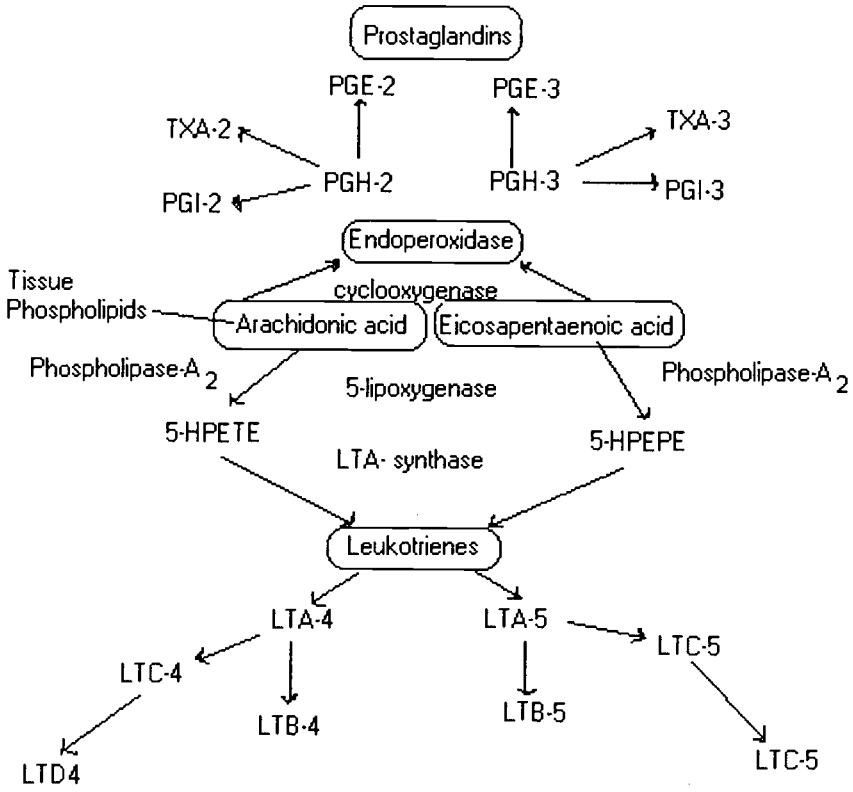
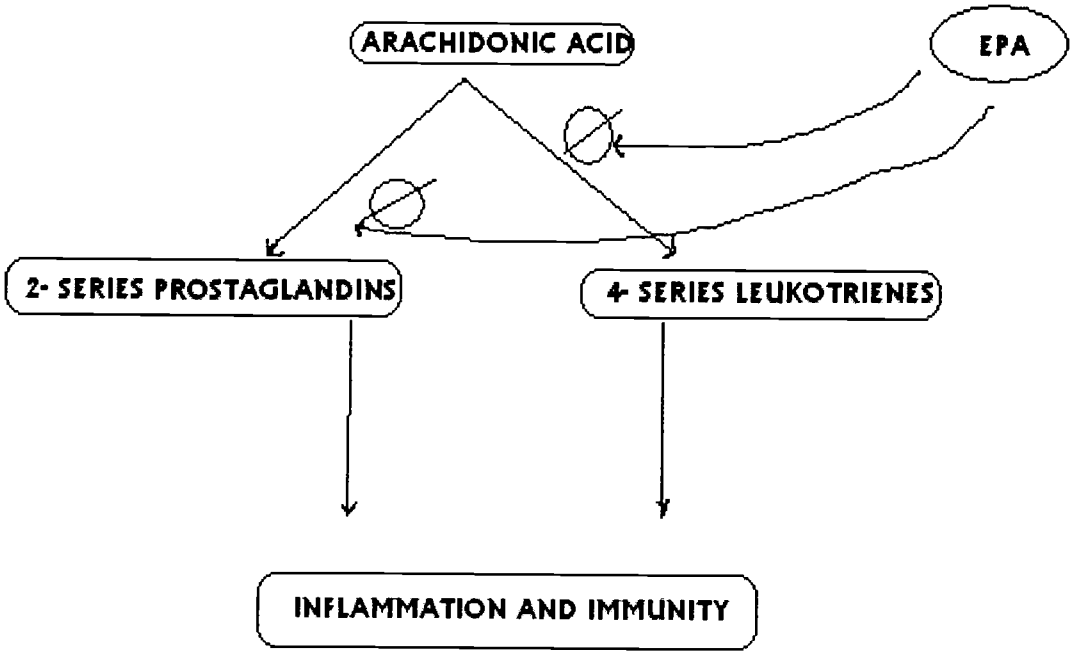


Figure 6. Mechanism by which eicosapentaenoic acid affects the immunoregulatory activities of arachidonic acid (adopted from Calder, 2001).



metabolites, (2) decreased thromboxane A2 production, which is a potent aggregator and vasoconstrictor, (3) decreased LTB_4 formation, which is an inducer of inflammation and a powerful inducer of leukocyte chemotaxis and adherence, (4) increased thromboxane A_3 production, which is a weak platelet aggregator and a weak vasoconstrictor, (5) increased prostacyclin PGI_3 production, which leads to an overall increase in total prostacyclin PGI_3 without a decrease in PGI_2 (both PGI_2 and PGI_3 are active vasodilators and inhibitors of platelet aggregation), and (6) increased LTB_5 production, which is a weak inducer of inflammation and a weak chemotactic agent.

2.13.2 Effect of Dietary Fatty Acids on Leukotriene Production: Animal Studies

Stenson et al. (1984) studied the production of LTB_4 by mouse neutrophils *in vitro* in the presence and absence of exogenous EPA. They recorded that the in the presence of exogenous EPA, the production of LTB_4 declined by 88%, and as the concentration of exogenous EPA was increased, the production of LTB_4 declined. Whelan et al. (1997) reported that inflammatory peritoneal macrophages from chickens lacked the capacity to produced leukotrienes *in vivo*, following opsonised zymosan stimulation, or *in vitro*, in response to calcium ionophore A23187 stimulation. Fritsche and Cassity (1992) found that after feeding n-3 FA to broilers there was no significant difference in the amount of leukotrienes produced by chicken immune cells. The effects of dietary n-6 to n-3 fatty acid ratio on leukotriene synthesis by dog skin and neutrophils was reported; neutrophils from dogs fed a 5:1 or 10:1 n-6 to n-3 FA ratio synthesized 30 to 33% less LTB_4 and 370 to 500 % greater LTB_5 after 6 to 12 weeks of experimental diet consumption (Mooney et al., 1998).

Broughton and Wade (2002) reported that total fat and the n-3 to n-6 FA ratio influence eicosanoid production in mice. Feeding n-6 FA decreased four series leukotrienes synthesis by 37%, as the fat intake increased from 5 to 20g/100g diet. At a ratio of 0.1:1, production of LT-4 series decreased, and at a ratio of 1:1 there was an increase in 4-series leukotriene synthesis with increasing dietary fat up to 15%, after which 4-series leukotriene synthesis plateaued. Furthermore, increased n-3 FA ingestion led to an increase in 5-series leukotrienes production. PGF_1-α levels were decreased by increasing both dietary fat and by increasing the n-3 to n-6 FA ratio. PGE_2 levels in mice fed a 5 or 10% fat diet decreased, as the n-3 to n-6 FA ratio increased from 0.1:1 to 1:1. Overall production of prostaglandins was lowest in mice consuming a high total fat and a high n-3 FA diet. Production of PGE_2 by unstimulated and LPS-stimulated mononuclear cells decreased significantly from baseline values for all doses of n-3 FA. The production of prostaglandin was negatively correlated with the proportion of EPA in plasma phosphatidylcholine, and it was positively correlated with the proportion of AA in plasma phosphatidylcholine (Trebble et al., 2003a).

How the ratio of n-6 to n-3 FA influenced immune system function and eicosanoid production was studied in aged dogs. It was reported that after consuming a diet with 1.4:1 n-6 to n-3 FA ratio, dogs produced 52% less PGE_2 from stimulated mononuclear cells compared to dogs fed a diet n-6 to n-3 FA ratio of 31:1 (Wander et al., 1997). Amalsadvala and Vaughn (1992) characterized the synthesis and release of LTB_4 from canine polymorphonuclear cells in terms of incubation time, temperature and effects of calcium ionophore concentration. Their results indicated that canine neutrophils have the capacity to synthesize large quantities of LTB_4 when stimulated with calcium ionophore A23187

and that 5-LOX inhibitors will inhibit LTB_4 synthesis in these cells. It has also been shown that exogenous LTB_4 inhibits human neutrophil production of LTB_4 from endogenous AA during opsonized zymosan phagocytosis (Fiedler et al., 1998). In a study where a normal rat diet was supplemented with EPA for 4 weeks, a significant increase in LTB_5 synthesis and decrease in LTB_4 by leukocytes was noted (Terano et al., 1984).

2.14 TRANSFER OF FATTY ACIDS FROM HEN TO EGGS TO CHICKS

The major depot for fats and fatty acids in chicken eggs is egg yolk. During incubation of fertile eggs, egg yolk fatty acids serve as a major energy source and provide fatty acids for the synthesis of structural lipid (Noble and Shand, 1985; Wang et al., 2000). The bulk of egg yolk lipid is taken up by engulfment by cells of the yolk sac membrane; lipids are stored together with proteins to form lipoproteins. These are secreted into blood vessels and delivered to the circulation of the embryo (Powell et al., 2004). Upon entering blood capillaries of adipose tissue, heart, and skeletal muscle of the embryo, the lipoproteins encounter an enzyme, lipoprotein lipase, which hydrolyses the lipid component, releasing fatty acids, which are then taken up by adjacent tissues. They are oxidized for energy, incorporated into cell membrane phospholipids, or used to form fat stores. Ajuyah et al. (2003) studied the transfer of maternal n-3 FA to egg yolk and subsequent deposition in cardiac tissue of chicks. They documented that the cardiac tissues of 0-day-old chicks reflected the fatty acid composition of maternal diet. Progressively, the fatty acid content in tissues declined as the age of chicks increased. Noble (1985) has described the transfer and deposition of yolk lipids, including fatty acids, during incubation of avian eggs. Based on his work, carbohydrate and protein metabolism predominate during the first two weeks of egg incubation.

Evidence shows that there is an intense period of lipid metabolism and rapid embryo growth during the last seven days of incubation. It is during this time that 85% of the entire lipid content of the yolk is being absorbed into embryonic tissue (Noble, 1985). The type of fat transferred from the yolk to the embryo can be manipulated by dietary alteration of maternal diet. Cherian et al. (1992) has documented the net transfer of long chain n-3 FA during the incubation of eggs. They observed a significant increase in the incorporation of EPA and DHA into hepatic and brain tissue of hatched chicks when hens were fed an n-3 FA enriched diet. Their study also showed that at 0- and 14-day-old chicks from hens fed the high n-3 FA diet had high levels of n-3 FA in their cardiac tissue. Ajuyah et al. (2003) has noted that maternal diets with diverse n-6 to n-3 FA ratios affect the brain DHA content of growing chickens. They found that brain DHA content in 0- and 14-day-old chicks were highest in chicks hatched from hens fed a high n-3 FA diet.

2.15 STUDY OBJECTIVES

Modern commercial broilers are marketed at 42 to 48 days of growth. Metabolic disorders like sudden death syndrome (SDS) and ascites are major causes of increased mortality in broilers birds. The losses caused by high mortality account for a big economic loss in the poultry industry in the USA. Current corn–soybean broiler diets are rich in n-6 FA and low in n-3 FA. This dietary imbalance may contribute to increased inflammatory response leading to an increase in metabolic diseases. Because eicosanoids are important for bird health and productivity, developing dietary strategies to regulate LTB_4 production may modulate inflammatory processes, which could lead to increased productivity.

Inflammation plays a key role in the pathogenesis of many diseases of companion as well as production animals. An increased understanding of the role that mediators of

inflammation, such as LTB_4, play in disease processes has led to an emphasis on the role of diet in conjunction with anti-inflammatory therapy to limit progression of the inflammatory process. Improved methods for assaying leukotrienes will help animal scientists to understand the role that nutrients play in disease prevention.

The objectives of the following studies were:

1. To optimize the assay conditions for LT production by platelets from chickens, and neutrophils from horses and dogs.
2. To determine the effect of feeding diets that differed in n-6 and n-3 FA ratios to breeder hens with regard to changes in composition of immune tissue, alteration of delayed-type-hypersensitivity (DTH) response, and LT production by platelets.
3. Chicks hatched to hens fed these diets were also studied with regard to fatty acid composition of immune tissues and LT production by platelets at various stages of growth (7, 14, 21 days).

CHAPTER 3. OPTIMIZATION OF ASSAY CONDITIONS FOR LEUKOTRIENE B_4 SYNTHESIS BY NEUTROPHILS OR PLATELETS ISOLATED FROM PERIPHERAL BLOOD OF MONOGASTRIC ANIMALS

3.1 ABSTRACT

Eicosanoids are lipid mediators of inflammation. Neutrophils are involved in inflammation through leukotriene (LT) production. The predominant proinflammatory leukotriene released from neutrophils is LTB_4, which serves as a biological marker of inflammation. The purpose of this study was to optimize the conditions for LTB_4 production by neutrophils from horses and dogs and heterophils from chickens, although unintentionally platelets were isolated from chickens. Optimal production of LTB_4 from neutrophils or platelets was characterized in terms incubation time (2.5, 5, 10, 15 or 20 minutes), temperature (25 or 37°C), and calcium ionophore A23187 concentration (0.1, 1, 10 or 20 μM). In all species, incubation at 37°C resulted in optimal LTB_4 production compared to 25°C ($p \leq 0.05$). Production of LTB_4 was highest when neutrophils or platelets were stimulated with 20 μM calcium ionophore in all species ($p \leq 0.05$). Incubation times greater than 2.5 minutes did not further increase production of LTB_4 in chickens or horses; in dogs incubation for 2.5 and 10 minutes resulted in the highest concentrations of LTB_4 ($p \leq 0.05$). These results indicate that neutrophils isolated from horses and dogs, and platelets isolated from chickens, are capable of producing LTB_4. Optimum conditions for LTB_4 production are similar in all three species.

Keywords: Leukotrienes; LTB_4; Neutrophils; Platelets

3.2 INTRODUCTION

Inflammation is characterized by redness, fever, pain, swelling and loss of function. Neutrophils are important cells involved in the inflammatory process. Neutrophils are also known as heterophils in poultry. Major functions of neutrophils are to release chemical mediators of inflammation such as eicosanoids and oxygen radicals, and enzymes such as proteases, phospholipases, collagenases, and lysozyme. All these are involved in killing of pathogens inside phagolysosomes, but can also be used in extra cellular clearing of pathogens (Bellingan, 1999).

Neutrophil migration is an important step in inflammatory processes. Diapedesis of leukocytes, also known as transendothelial migration, results in recruitment of neutrophils to an area of inflammation (Nohgawa et al., 1997). In the process of transendothelial migration, there is margination and rolling of neutrophils along the endothelium and, finally, adhesion and transmigration (Azzali et al., 1990). These sequential events happen under the influence of complement components (e.g., C5a), cytokines (e.g., IL-8) (Dobrina et al., 2002) and leukotrienes (e.g., LTB_4) (Nohgawa et al., 1997). Some *in vivo* data in mice indicates that neutrophil platelet-endothelial-cell-adhesion-molecule-1 (PECAM-1), as well as endothelial PECAM-1, are involved in the recruitment of neutrophils into inflammatory sites (Christofidou-Solomidou et al., 1997). Studies have been done to define the role of LTB_4 in the recruitment of neutrophils. For example, LTB_4 present in exudative pleural effusions contributes to recruitment of neutrophils to the inflamed pleura (Pace et al., 2004).

LTB_4 is a potent chemotactic agent produced by almost all types of immune cells, but especially by neutrophils. Arachidonic acid (AA) in cell membrane phospholipids is

cleaved by phospholipase A_2 and acted on by 5-lipoxygenase (5-LOX) to form LTA_4. Neutrophils contain LTA_4-hydrolases, which convert the 5-LOX product LTA_4 into LTB_4 through addition of a triene epoxide. LTA_4 can also be converted to LTB_4 outside the cell by adjacent cells (Haeggstrom and Wetterholm, 2002). The major action of LTB_4 in the body is on leukocytes, where in nanomolar concentrations it elicits chemotaxis, adherence and aggregation. As one of the most potent chemoattractants discovered to date, LTB_4 modulates immune responses and participates in host defenses against infection and injury (Pace et al., 2004). LTB_4 can be thought of as an eicosanoid involved in developing and maintaining inflammatory reactions.

LTB_4 is also involved in certain pathological conditions because of overproduction. For example, overproduction of LTB_4 is associated with lung edema (Pace et al., 2004), inflammatory bowel disease (Singh et al., 2003; Stenson, 1990), eye diseases (Smith et al., 2004), diseases of skin such as atopic dermatitis (Chari et al., 2001), and other pathological conditions. LTB_4 has also been shown to be an important mediator of pulmonary dysfunction, causing transendothelial migration of neutrophils in lipopolysaccharide-induced acute lung injury in endotoxemic pigs (VanderMeer et al., 1995).

Many studies have been conducted to measure LTB_4 production and to study its synthesis by leukocytes and inflammatory cells. For example, studies in rats have shown that LTB_4 is produced by peritoneal inflammatory cells after calcium ionophore A23187 stimulation (Mansour and Al-Shabanah, 2003). The effect of LTB_4 on isolated canine neutrophils also has been studied. It was shown that LTB_4 affected isolated canine and human neutrophils similarly, e.g., chemotactic activity was increased in both (Gruber et al., 1989). LTB_4 produced by neutrophils in horses with recurrent airway obstruction (RAO)

may play a role in the inflammatory events of RAO (Lindberg et al., 2002). Recently it was shown that increased concentrations of LTB_4 in airways of RAO-affected horses contributes to infiltration of neutrophils into lungs and the ongoing inflammation associated with RAO (Lindberg et al., 2004). In another study, co-culturing equine platelets and granulocytes resulted in potentiated LT production by granulocytes when stimulated with calcium ionophore A23187 (Lindberg et al., 1998). In poultry there have been no reports on LTB_4 production by stimulated platelets.

Calcium ionophore A23187 is used to asses LT production by isolated cells *in vitro.* Calcium ionophore A23187 transports extracellular calcium by an electroneutral mechanism across cell membranes into the cell cytosol (Prabhananda and Kombrabail, 1998). Measurement of LT production *in vitro* can be studied very effectively as calcium ionophore A23187 is not cell-type specific in its action. Regardless of cell type, calcium ionophore A23187 stimulates all cells, enabling them to synthesize and release eicosanoids in amounts that can be quantitated by analytical methods, e.g., RP-HPLC (Maclouf and Murphy, 1988). In order for neutrophils to make LTB_4, enzymes such as phospholipase A_2, 5-LOX and LTB_4 hydrolases must be expressed (Haeggstrom and Wetterholm, 2002).

Because LTB_4 is involved in many pathophysiological processes, not only in humans but also in animals, there is a need to determine optimum conditions for assaying and measuring LTB_4 production by neutrophils. The purpose of this study was to determine the optimum assay conditions for LTB_4 production by neutrophils from horses and dogs and by platelets from chickens under a variety of experimental conditions. LTB_4 was quantitated using reverse phase-high pressure chromatography (RP-HPLC).

3.3 MATERIALS AND METHODS

3.3.1 Animals

A healthy horse from the College of Veterinary Medicine, Oregon State University, Corvallis, Oregon, was chosen for blood collection. Two healthy, adult, mixed-breed dogs and six 56-day-old broiler birds were also used for collection of blood. Whole blood was collected from the jugular vein into plastic tubes containing 2% EDTA (Sigma). The experimental protocol was reviewed and approved by the Oregon State University Animal Care and Use Committee in accordance with principles outline by the National Institutes of Health (National Research Council, 1985).

3.3.2 Reagents

Calcium ionophore A23187, $CaCl_2$, NH_4Cl, sodium diatrizoate, sodium citrate tribasic dehydrate, EDTA and histopaque 1119 were obtained from Sigma. Citric acid anhydrous and dimethylsulphoxide (DMSO) were obtained from Fisher; HBSS was obtained from Gibco; and, Zapoglobin II lytic reagent was obtained from Beckman Coulter. HPLC grade methanol and hexane were obtained from EM Science and JT Baker, respectively. Purified Leukotriene B_4 and ProstaglandinB_3 for making standard curves were obtained from Cayman.

3.3.3 Isolation of Neutrophils

Neutrophils were isolated by a modified version of the method used by Amalsadvala and Vaughn (1992). Briefly, a stock solution of histopaque 1119 was prepared by adding 1.5 grams of sodium diatriazoate to 100 mL of histopaque 1119. Whole blood was carefully layered over histopaque 1119 in a 1:1 ratio (20 mL of each) and then centrifuged for 30 min at 700 x g using a Beckman TJ-6 centrifuge with brake off. After

removing the neutrophil band, any RBCs that remained were lysed with buffered 0.83% NH_4Cl until contaminating RBCs were no longer visible. After this, cells were washed twice with HBSS without $CaCl_2$. Cells were then resuspended in HBSS with 0.8 mM $CaCl_2$. An aliquot of cell suspension was used for counting cells with a Coulter counter, and neutrophil concentration was determined using an automated cell counter.

3.3.4 Neutrophil Viability and Purity

Cell viability was assessed by trypan blue exclusion. Neutrophil purity was assessed by making a thin smear of cells on a slide, staining it with Wright Stain, and counting isolated cells under oil immersion using a light microscope.

3.3.5 Stimulation of Neutrophils for LTB_4 Production

Aliquots of $5x10^6$ cells were transferred to 8 mL plastic tubes and HBSS containing 0.8 mM $CaCl_2$ was added to achieve a final volume of 475 μL. Before stimulation, aliquots of cells were pre-incubated at 37°C for 10 minutes. To initiate leukotriene production, 25 μL of calcium ionophore A23187 was added to each tube. Control tubes received 25 μL of 0.2% DMSO in HBSS without $CaCl_2$. Control and calcium ionophore stimulated cells were incubated for 10 min at 37°C in a shaking water bath. After incubation, 2 mL of ice-cold methanol was added to each tube to terminate the reaction. Cells were chilled for 20 minutes on ice. Finally, tubes were centrifuged for 5 minutes at 1000 x g at 4°C to pellet cellular debris. Supernatants were transferred to another tube and were stored in a –70°C freezer until subsequent LTB_4 analysis. Experimental conditions, such as concentration of calcium ionophore, time of incubation, and temperature of incubation varied in the different experiments outlined below.

3.3.6 Extraction and Separation of LTB_4

LTB_4 was extracted and separated using a modified version of the method described by Terano et al. (1984). Briefly, stored supernatants were centrifuged for 15 min at 400 x g. Supernatants were transferred to 15 mL graded conical tubes containing 100 ng of PGB_3 as an internal standard. Freshly prepared citrate buffer was added to bring the volume up to approximately 14.5 mL.

A 12-mL syringe was attached to a C-18 solid phase extraction cartridge and 5 mL of HPLC-grade methanol was passed through the cartridge for pre wetting the cartridge. Samples were then loaded onto the cartridge by gravity flow and leukotrienes were absorbed onto the extraction column. The cartridges were rinsed with 5 mL of double deionised H_2O followed by 5 mL of HPLC-grade hexane. Leukotrienes were then eluted from the column, using gravity flow, with 5 mL of a 90:10 mixture of methanol and double distilled H_2O into 5 mL plastic test tubes. The eluted samples were placed in a 30°C water bath and solvent was evaporated under a stream of N_2. After evaporation, the residues were reconstituted in 125 µL of mobile phase made up of methanol: water: acetic acid (75:25:01) and pH was adjusted to 5.7 with NH_4OH. Samples were capped with N_2 and stored at -70°C until LTB_4 were quantitated by RP-HPLC.

3.3.7 Quantitation of Leukotrienes by RP-HPLC

LTB_4 was separated by HPLC using a C-18 reverse-phase column fitted with a C-18 guard column. The mobile phase was methanol: water: glacial acetic acid (75:25:01), with pH adjusted to 5.7 with NH_4OH. The flow rate of the pump was set at 0.7 mL/min and the variable wavelength UV detector was set at 270 nm.

A standard calibration curve for LTB_4 was made by adding 100 ng of PGB_3 as an

internal standard to samples containing 6.25, 12.5, 25, 50, and 100 ng of LTB_4. PGB_3 was chosen as the internal standard because it was widely separated from LTB_4 present in actual samples during HPLC separation, whereas PGB_2 coeluted with LTB_5 and PGB_1 coeluted with LTB_4 (Terano et al., 1984). The standard solutions were extracted as above and LTB_4 was detected by HPLC. The peak area ratio for LTB_4/PGB_3 was calculated and plotted against the concentration of LTB_4. (See Figure 7 and Figure 8). The concentrations of leukotrienes in test samples were calculated with reference to the standard curve. Final LTB_4 concentration in horse, dog and chicken samples were reported as nanograms of LTB_4 per 5 million cells.

Figure 7. Standard curve for LTB_4, using PGB_3 as an internal standard, after extraction and RP-HPLC. Each point is the mean ± SEM (n=3).

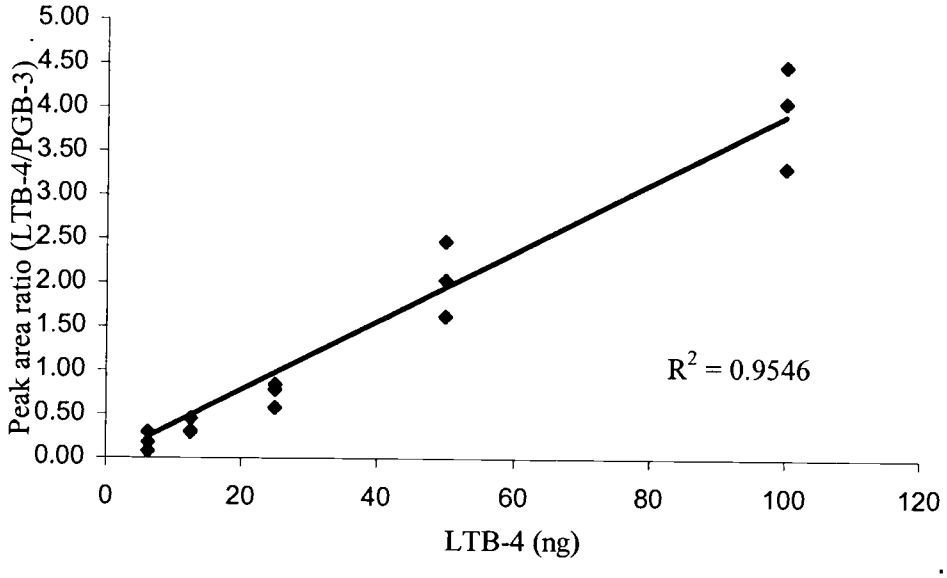
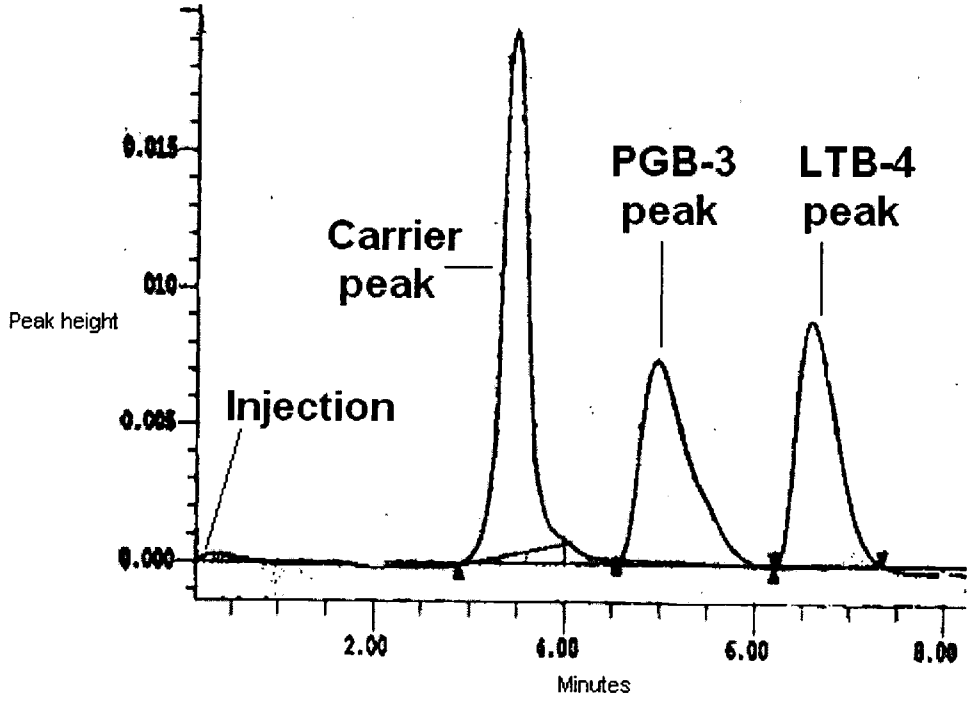


Figure 8. Reverse-phase HPLC separation of a mixture containing 100 ng of PGB_3 and 50ng LTB_4. The HPLC conditions are described in the text.



3.3.8 Calcium Ionophore A23187 Concentration - Dependent Experiments

Calcium ionophore A23187 concentration was varied to determine the concentration that would lead to optimum LTB_4 production by horse and dog neutrophils, and chicken platelets. A cell suspension of $5x10^6$ neutrophils or platelets was incubated with 0.1, 1, 10 or 20 μM calcium ionophore A23187 at 37°C for 10 minutes.

3.3.9 Time - Dependent Experiments

Time of incubation was varied to characterize the time pattern for LTB_4 production. Cell suspensions of $5x10^6$ neutrophils or platelets were incubated with 20 μM calcium ionophore A23187 at 37°C for 0, 2.5, 5, 10 or 20 minutes. Cells from all three species were also incubated without calcium ionophore A23187 for the same time periods.

3.3.10 Temperature - Dependent Experiments

Temperature was varied to determine the effect of temperature on production of leukotrienes from horse and dog neutrophils, and chicken platelets. Cell suspensions of $5x10^6$ cells were incubated for 10 minutes with 10 μM calcium ionophore A23187 at 25 or 37°C.

3.3.11 Statistical Analysis

The effect of these experimental conditions on LTB_4 production was analyzed by ANOVA using SAS (version 8.2 SAS Institute). The Student-Newmann-Keuls multiple range tests were used to compare differences among treatment means (Steel and Torrie, 1980). Mean values ± SEM are reported. Values were considered significant at $p \leq 0.05$.

3.4 RESULTS

3.4.1 Neutrophil Viability and Purity

In horse, dog and chicken preparations, more than 90% of cells were alive after isolation from whole blood based on trypan blue exclusion. The neutrophil fraction in horse and dog samples was > 90% of total cells isolated. However, the predominant cell type in chicken preparations was platelets, and to a lesser extent other white blood cells, including heterophils and lymphocytes.

3.4.2 Effect of Calcium Ionophore A23187 Concentration on LTB_4 Production

Equine neutrophils stimulated with calcium ionophore A23187 showed maximal production of LTB_4 (95.4 ± 0.42 ng per $5x10^6$ cells) after incubation for 10 minutes. This represented a 100-fold increase over unstimulated cells ($p \leq 0.05$) and is shown in Figure 9a. A similar trend in production of LTB_4 from canine neutrophils was observed. When 5 x 10^6 cells were stimulated with varying concentrations of calcium ionophore A23187, from 0 to 20 μM, at 37°C for 10 minutes, there was a progressive increase in LTB_4 production as concentration of calcium ionophore A23187 increased. Maximal production (108 ± 19.5 ng LTB_4 per $5x10^6$ cells) occurred with 20 μM calcium ionophore A23187 concentration, which was 100 fold more than that produced by unstimulated cells ($p \leq 0.05$). See Figure 9b. The same effect of calcium ionophore A23187 concentration on LTB_4 production was noted for platelets from chickens. LTB_4 production increased linearly as calcium ionophore A23187 concentration increased, from 0.58 ± 1.17 ng LTB_4 per $5x10^6$ cells at 0 μM calcium ionophore A23187 concentration to 111.8 ± 0.42 ng LTB_4 per $5x10^6$ cells at 20 μM calcium ionophore A23187 concentration ($p \leq 0.05$). See Figure 9c.

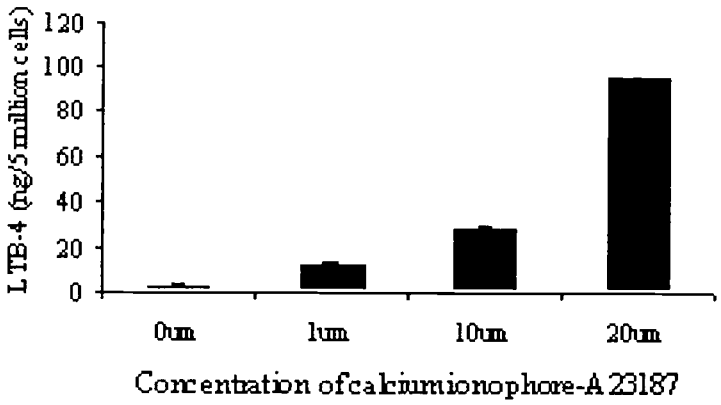


Figure 9a

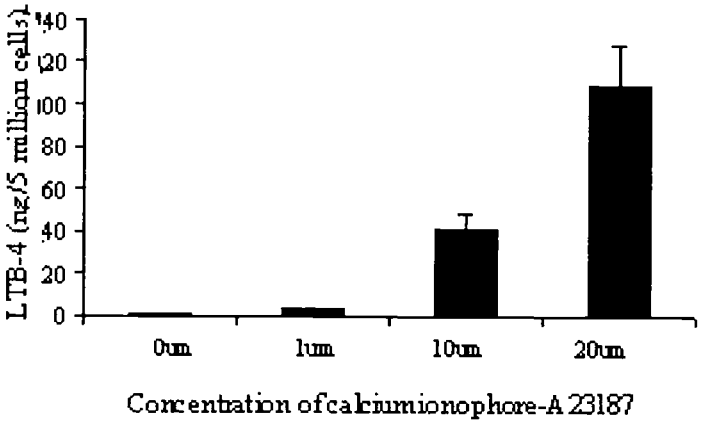


Figure 9b

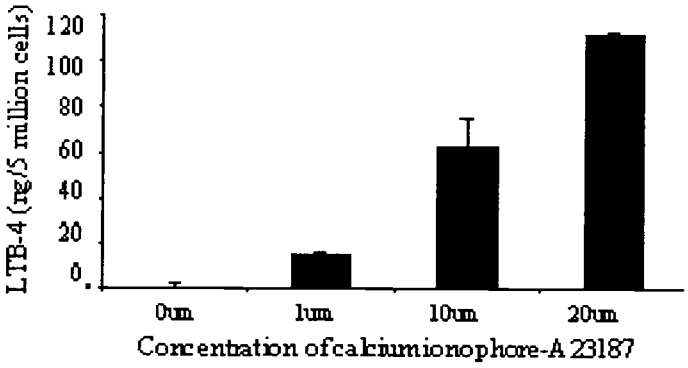


Figure 9c

Figure 9. Concentration-dependant effects of calcium ionophore A23187 on LTB_4 production by horse (9a) and dog (9b) neutrophils, and chicken (9c) platelets. Cells (5 x 10^6) were incubated with calcium ionophore A23187 at concentrations of 0, 1, 10 and 20 uM. The values for LTB_4 concentrations are mean ± SEM of three replicates.

3.4.3 Effect of Incubation Time on LTB_4 Production

Interestingly, LTB_4 production by horse neutrophils did not increase further after 2.5 minutes of incubation at 37°C and 10 μM calcium ionophore A23187 concentration ($p \leq 0.05$). However, in dogs, the greatest production of LTB_4 was seen at 2.5 and 10 minutes ($p \leq 0.05$). Chicken platelets produced maximum amount of LTB_4 at 10 minutes of incubation at 37°C and 10 μM calcium ionophore A23187 concentration ($p \leq 0.05$). See Figure 10.

3.4.4 Effect of Incubation Temperature on LTB_4 Production

In all three species, 10 minutes incubation time, 37°C, and 10 μM calcium ionophore A23187 concentration resulted in optimum production of LTB_4 ($p \leq 0.05$). The results for temperature-dependent experiments in all three species are shown in Figure 11.

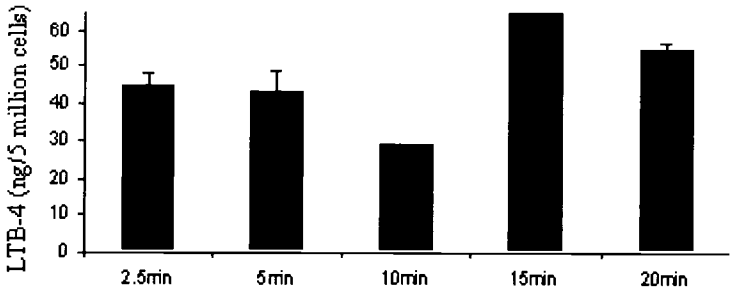


Figure 10a

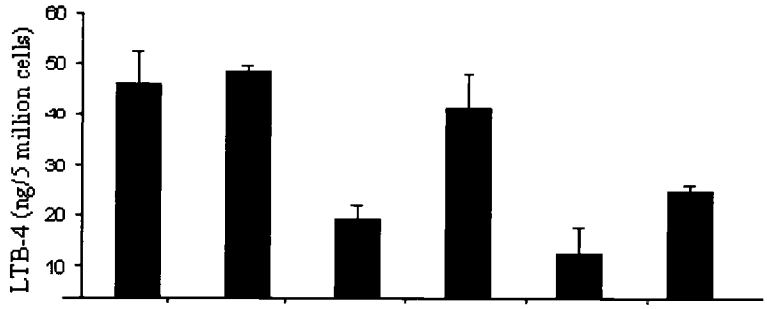


Figure 10b

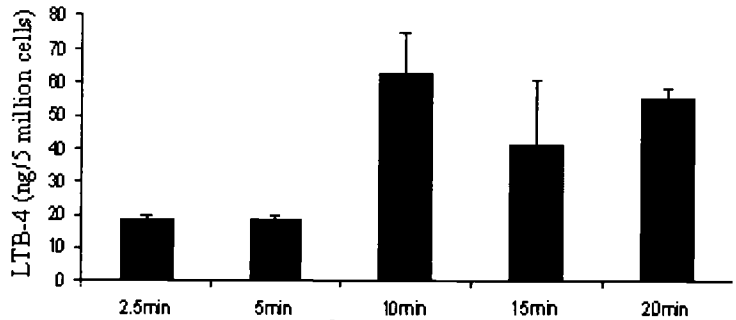


Figure 10c

Figure 10. Time-dependant effects of calcium ionophore A23187 on LTB_4 production by horse (10a) and dog (10b) neutrophils, and chicken (10c) platelets. Cells ($5x10^6$) were incubated with 10 μM concentration of calcium ionophore A23187 at 37°C for 0, 2.5, 5, 10, 15 and 20 min. The values for LTB_4 concentrations are mean ± SEM of three replicates.

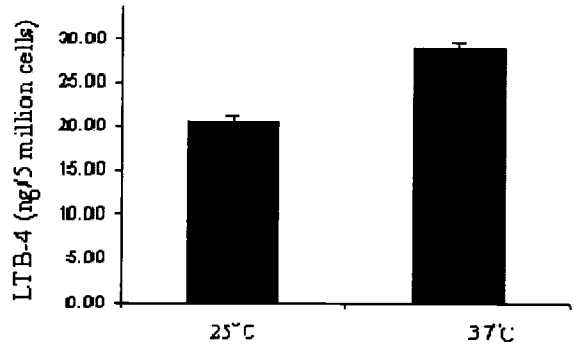


Figure 11a

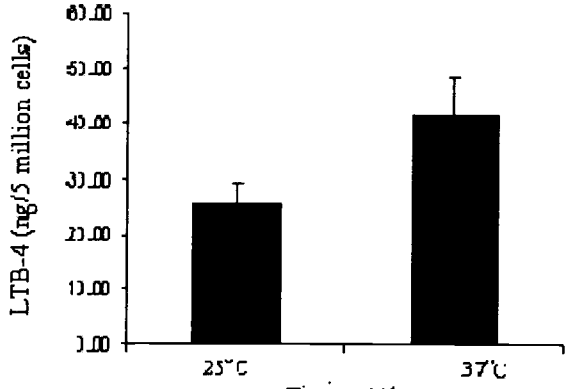


Figure 11b

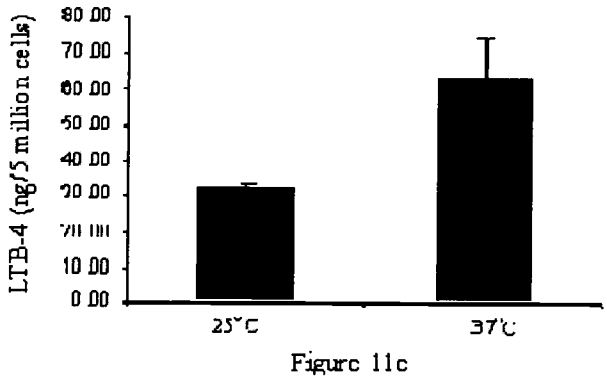


Figure 11c

Figure 11. Temperature-response effect of calcium ionophore A23187 on LTB_4 production by horse (11a) and dog (11b) neutrophils, and chicken (11c) platelets. Cells (5×10^6) were incubated with 10 μM concentration of calcium ionophore A23187 at indicated temperatures (25 and 37°C) as described in the methods. The values are the means ± SEM of the three replicates.

3.5 DISCUSSION AND CONCLUSIONS

Our goal was to isolate neutrophils from whole blood of horses, dogs and chickens using the same methodology as outlined by Amalsadvala and Vaughn (1992). We had previously shown that this method worked well for isolating relatively pure populations of neutrophils (>95%) from horse and dog whole blood. Following the same methodology, we found that white blood cells isolated from chicken whole blood were predominantly platelets, with <10% heterophils and lymphocytes. Because heterophils are nucleated cells, other methods using Ficoll-Hypaque discontinuous gradients have been shown to give better separation of avian heterophils from anticoagulated whole blood (Andreasen and Latimer, 1989). Thus, in this study, we compared LT production by neutrophils from horse and dog blood to LT production from cells that were predominantly platelets from chicken blood.

The results of this study indicate that, horse and dog neutrophils and chicken platelets readily synthesize and release large quantities of LTB_4 following stimulation with low micromolar concentrations of calcium ionophore A23187. These results also show that regardless of species, neutrophils and platelets from whole blood were able to produce a similar amount of LTB_4 from $5x10^6$ cells under similar conditions. These results are comparable to what has previously been reported in dogs (Amalsadvala and Vaughn, 1992). A dose-response increase in calcium ionophore A23187 concentration resulted in a dose-response increase in 5-LOX activity as reflected by increased LTB_4 concentrations, which were 100-fold higher than in unstimulated neutrophils. The data obtained from the time-response experiments indicate that the time for activation of phospholipase A_2 with subsequent liberation of AA and further metabolism by 5-LOX and LTA_4-hydrolase to

produce LTB_4 occurs very quickly (within 10 minutes). Activation of 5-LOX activity was also maximal at 37°C.

In this study, platelets from chickens were able to produce LTB_4 in quantities similar to that produced by horse and dog neutrophils. To our knowledge, nobody has investigated production of LTB_4 from stimulated chicken platelets under a variety of experimental conditions, such as incubation time (2.5, 5, 10, 15 or 20 minutes), temperature (25 or 37°C), and calcium ionophore A23187 concentration (0.1, 1, 10 or 20 μM). This study demonstrates the likely presence of LTA_4 hydrolase in chicken platelets, and that it is responsible for the production of LTB_4 upon stimulation of platelets with calcium ionophore A23187.

Platelets are generally considered to have LTC_4-synthase activity, which yields LTC_4, the main LT released by platelets. It has been difficult to document release of LTB_4 from isolated and stimulated platelets in animals. In one study, platelets were isolated from several species and the production of LT after stimulation and incubation with different agents was studied. Equine, human, ovine, rabbit, and rat platelets all produced LTC_4 after incubation with LTA_4. Thus, it can be inferred that LTC_4 synthase activity is a common feature among platelets from various species. In the same study, it was reported that pig platelets failed to transform LTA_4 to LTC_4, but rather LTA_4 was transformed to LTB_4, which points to the presence of LTA_4 hydrolase in porcine platelets, and probably the absence of LTA_4 hydrolase in platelets of the above mentioned group of animals (Tornhamre et al., 1998).

LTB_4 synthesis by chicken peritoneal macrophages stimulated with calcium ionophore A23187 was not reported by Whelan et al. (1997). In the Fritsche and Cassity

(1992) study, total leukotriene B release was not significantly altered by dietary fat source when chicken immune cells were stimulated with calcium ionophore A23187. Chicken myelomonocytic cells treated with arachidonic acid and calcium ionophore A23187 produced leukotrienes in the Habenicht et al. (1989) study.

LTB_4 is a local hormone derived from the oxidative metabolism of arachidonic acid (AA). This lipid mediator is a key signaling molecule in an array of inflammatory and allergic conditions, such as atherosclerosis in the cardiovascular system, atopy in skin disorders, arthritis in the musculoskeletal system, diseases like inflammatory bowel disease and ulcerative colitis in the gastrointestinal system, and asthma in the respiratory system. LTB_4 is a dihydroxylated derivative of AA that is generated through the 5-lipoxygenase (5-LOX) and LTA_4-hydrolase pathways. Neutrophils that are activated by external agents, e.g., bacterial polypeptides, or internal stimuli, e.g., immune complexes, or other stimuli such as calcium ionophore A23187, undergo a sequence of cellular events beginning with the translocation of phospholipase-A_2 and 5-LOX to the nuclear envelope and ending with release of AA from cell membranes, and subsequent production of LTA_4, which is further metabolized to LTA_4 and, subsequently, LTB_4. LTB_4 is one of the most powerful chemotactic agents known to date and participates in the recruitment of additional leukocytes (Haeggstrom and Wetterholm, 2002).

The contribution of platelets to arachidonic acid transcellular metabolism may represent an important pathway for modifying LT production. Neutrophils and platelets have been shown to co-operate in several pathophysiological processes, and together they produce eicosanoids in response to activation (Chabannes et al., 2003). In experiments with rabbit whole blood stimulated *in vitro* with calcium ionophore A23187, whole blood

produced more LTB_4, whereas the same blood enriched with platelets showed reduced LTB_4 production (Evangelista et al., 1999).

Inflammation plays a key role in the pathogenesis of many diseases of companion as well as production animals. An increased understanding of the role that mediators of inflammation, such as LTB_4, play in disease has led to an emphasis on therapy with anti-inflammatory agents. Thus, early treatment of inflammatory conditions with an effective anti-inflammatory agent inhibits excessive production of LTB_4 and may limit progression of the inflammatory process. Improved methods for assaying leukotrienes will help animal scientists in understanding the role of nutrients in disease prevention.

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CHAPTER 4. TISSUE FATTY ACID COMPOSITION AND LEUKOTRIENE PRODUCTION BY PLATELETS IN BREEDER HENS AND HATCHED CHICKS: EFFECT OF DIET

4.1 ABSTRACT

Inflammation is the body's response to injury. It is characterized by pain, swelling, redness, and heat. Eicosanoids are lipid mediators of inflammation. Leukotrienes (LT) are 20-carbon eicosanoids produced from arachidonic acid (AA), an n-6 fatty acid (FA), and eicosapentaenoic acid (EPA), an n-3 FA. LT produced from AA are proinflammatory (LTB_4) and those produced from EPA (LTB_5) are less inflammatory. Platelets and white blood cells (WBCs) are involved in inflammation through LT production. The objectives of this study were 1) To determine the effect of altering the ratio of dietary n-6 to n-3 polyunsaturated fatty acids (PUFA) fed to breeder hens on a) fatty acid composition in immune tissues, b) delayed-type-hypersensitivity (DTH) reaction, and c) leukotriene production by stimulated platelets; and 2) To determine the changes in a) immune tissue fatty acid composition, and b) leukotriene production in chicks at various stages of growth (7, 14 and 21 days). Chicks were hatched to breeder hens fed diets with different n-3 to n-6 FA ratio. A total of 72 breeder hens were randomly divided into three groups (n=24) and fed diets supplemented with either sunflower oil (SFO; rich in n-6 FA; Diet I), a mixture of 1.5 % SFO and 1.5 % Fish oil (Diet II) or 3.0 % Fish oil (FO; rich in n-3 FA; Diet III).

Production of LTB_4 and LTB_5 by platelets stimulated with calcium ionophore A23187 were assessed by reverse phase-high pressure liquid chromatography (RP-HPLC).

The hens fed Diet I synthesized 43.9 ± 2.5 ng of LTB_4 from $5x10^6$ cells compared to 13.3 ± 0.9 ng of LTB_4 from hens fed Diet II ($p \leq 0.05$). However, no LTB_4 was produced by hens fed Diet III. Production of LTB_5 from hens fed Diet III was 36.7 ± 4.9 ng from $5x10^6$ cells compared to 47.4 ± 5.7 ng of LTB_5 from hens fed Diet II. No LTB_5 was produced by hens fed Diet I. The DTH reaction in hens was significantly lower at 48 hrs post injection of bovine serum albumin in birds fed 3% fish oil (Diet III; $p \leq 0.05$). Fatty acid composition of spleen and platelets reflected the dietary fatty acid composition of ration fed to breeder hens ($p \leq 0.05$).

Hatched chicks from hens fed Diet I produced significantly less LTB_4 at 14 days ($p \leq 0.05$) compared to 7- and 21-day-old chicks, which were not different from each other. Chicks from hens fed Diet II produced significantly higher LTB_4 at 21 days ($p \leq 0.05$) compared to 7-day-old chicks; the latter also produced more than 14-day-old chicks ($p \leq 0.05$). Chicks from hens fed Diet III produced significantly more LTB_4 at 7 and 21 days ($p \leq 0.05$) compared to 14-day-old chicks. However, there were no significant differences in LTB_5 production by chicks from hens fed Diet III at 7 and 14 days of growth. The spleen and bursa tissue fatty acid composition in chicks at 7 and 14 days reflected the maternal diet fatty acid composition. There were no significant differences in platelet fatty acid composition between the groups at different stages of growth. These results indicate that the type of fat in maternal diet fed to breeder hens may alter the inflammatory response in hatched chicks, which could lead to less mortality and increased production performance in poultry.

Keywords: n-6 and n-3 fatty acids, Leukotrienes, Poultry.

4.2 INTRODUCTION

Inflammation is the body's response to external or internal injury and is characterized by redness, fever, pain, swelling, and loss of function. The important cells involved in the inflammatory process are platelets and WBCs. Major functions of these cells are to release mediators of inflammation, such as oxygen radicals and eicosanoids, and different enzymes such as proteases, phospholipases and collagenases (Stahl et al., 1997). Eicosanoids are 20-carbon compounds that are chemical mediators of inflammation and play a vital role in immune and inflammatory responses (Calder, 2002b). Dietary fatty acids influence the type of eicosanoids released from inflammatory cells by changing the fatty acid composition of immune tissue and cells (Calder, 2001b). The amount and ratio of n-6 to n-3 PUFA in cell membranes can affect the production of eicosanoids, and ultimately modulate immune and inflammatory responses (Broughton and Wade, 2002).

Among the different PUFA, AA, an n-6 FA, and EPA, an n-3 FA, are the major precursors for eicosanoids. The major eicosanoids produced from n-6 and n-3 fatty acids are leukotrienes (LT) and prostaglandins. Leukotrienes are a subgroup of eicosanoids derived from the action of 5-lipoxygenase (5-LOX) on fatty acids (Haeggstrom and Wetterholm, 2002). Eicosanoids derived from n-6 FA are pro-inflammatory and eicosanoids derived from n-3 FA are less inflammatory (Calder, 2002b). LTB_4 is derived from AA and is a potent mediator of inflammation. It causes increased vascular permeability, smooth muscle contraction, and increases the activity of NK cells and cytotoxic T cells (Calder, 2003; Pace et al., 2004). LTB_5 is less inflammatory. The potency of LTB_5 relative to LTB_4 is approximately 1:8 as a chemotactic agent and approximately 1:20 as an aggregating agent (Lee et al., 1984).

Inflammatory responses in poultry can decrease feed consumption and reduce muscle protein accretion, leading to reduction in bird performance and reduced productivity (Korver and Klasing, 1997). Inclusion of fish oil in poultry diets was reported to increase cell mediated immunity and lower indices of inflammation (Klasing, 1994; Korver and Klasing, 1997; Korver et al., 1997). Similarly, feeding n-3 PUFA to chicks reduced the febrile response against S. *typhimurium* and S. *aureus* lipopolysaccharide (LPS). It also decreased production of IL-1 from peritoneal macrophages. N-3 PUFA decreased footpad delayed-type-hypersensitivity (DTH) response against *Mycobacterium butyricum* and increased antibody production in white leghorn laying hens (Selvaraj and Cherian, 2004).

LTB_4 acts on leukocytes in nanomolar concentrations to elicit chemotaxis, adherence and aggregation. As one of the most potent chemoattractants discovered to date, LTB_4 modulates immune responses and participates in host defenses against infection and injury (Pace et al., 2004). LTB_4 can be thought of as an eicosanoid involved in developing and maintaining inflammatory reactions. LTB_4 is also involved in certain pathological conditions because of overproduction. For example, overproduction of LTB_4 is associated with lung edema (Pace et al., 2004), inflammatory bowel disease (Singh et al., 2003; Stenson, 1990), eye diseases (Smith et al., 2004), diseases of skin such as atopic dermatitis (Chari et al., 2001), and other pathological conditions. One study suggested that LTB_4 is an important mediator of pulmonary dysfunction and the transendothelial migration of neutrophils in LPS-induced acute lung injury in endotoxemic pigs (VanderMeer et al., 1995).

Metabolic disorders like sudden death syndrome (SDS) and ascites are major causes of increased mortality in broilers birds in recent years. The losses caused by these disorders account for a big economic loss in the USA poultry industry. Current corn–soybean broiler diets are rich in n-6 FA and low in n-3 FA. This dietary FA imbalance may contribute to increased inflammatory responses, and increased metabolic diseases. Because eicosanoids are important for bird health and productivity, developing dietary methods to control and regulate LTB_4 release to modulate inflammatory processes may lead to increased productivity.

It has been shown that maternal dietary supplementation of n-3 and n-6 FA affects the n-3 to n-6 ratio in spleen and cardiac tissue of hatched chicks (Ajuyah et al., 2003). The cardiac tissue of chicks hatched from hens fed diets high in n-3 FA showed deposition of higher amounts of EPA and DHA and simultaneous reduction in AA at 0 and 14 days of age (Ajuyah et al., 2003).

It is hypothesized that feeding diets enriched in n-3 PUFA to breeder hens will increase n-3 FA accumulation in tissue of chicks. This will increase LTB_5 and decrease LTB_4 production by platelets from hens and their progeny. The study was proposed with the following objectives: 1) To study the influence of dietary fatty acids on immune tissue fatty acid composition, DTH response, and LT production by platelets in breeder hens. 2) To investigate the influence of maternal diet on immune tissue retention of PUFA, and LT production by platelets from chicks hatched to hens fed diets enriched in n-3 PUFA.

4.3 MATERIALS AND METHODS

These experiments were reviewed by Oregon State University's Animal Care Committee to ensure adherence to Animal Care Guidelines.

4.3.1 Birds and Diet

4.3.1.1 Breeder hens

A total of 72 (36 to 42-week-old) New Hampshire breeder hens were weighed and randomly assigned to 3 groups. Group 1 hens were fed a diet containing sunflower oil (SFO) 3% by weight (SFO; rich in n-6 FA). Group 2 hens were fed a diet containing a mixture of 1.5% SFO and 1.5% fish oil. Group 3 hens were fed a diet supplemented with fish oil 3.0% by weight (FO; rich in n-3 FA). The birds were kept in individual cages. Standard management practices were followed uniformly for all treatment groups. Water and feed was provided ad libitum to the birds and feed consumption was measured biweekly. The following conventional zoo technical data were recorded: daily feed intake (g/laying hen), laying rate (%; eggs/100 laying hens), and feed efficiency (daily feed intake/daily egg mass; g/g). The experimental diets were fed for 46 days.

4.3.1.2 Chicks

All breeder hens at 36-42 weeks of age were artificially inseminated at the same time. Eggs were collected 3 to 10 days post insemination and stored in a cooler at 65°F. A total of 100 eggs from each treatment were incubated for 21 days. A total of 73 chicks were hatched from each treatment, which were then culled, weighed, and then placed in deep litter. Forty eight chicks, (1-day-old), were randomly selected from each diet group and divided into four replicates of 12. Chicks were provided starter feed (Purina) and water ad libitum.

4.3.2 Measurement of delayed-type-hypersensitivity reaction

Eight birds from each breeder hen group at 6 weeks of treatment were randomly selected for measuring DTH response. DTH response was measured following administration of bovine serum albumin (BSA) as described by Wang et al. (2002). Briefly, intramuscular injections in the thigh were made to deposit 200 μL of solution, which had a concentration of 1 mg of BSA per ml of phosphate buffered saline (PBS). Fourteen days after the first injection, birds were injected with 200 μL of BSA in the left footpad. As a control, 200 μL of PBS were injected subcutaneous into the right footpad. The thickness of the injection site was measured with pressure sensitive caliper before injection, and at 24, 48, and 72 hrs post injection. The footpad swelling reaction was recorded as swelling index, and calculated as follows:

Swelling index = [(thickness of left footpad following BSA injection-initial thickness of left footpad)-(thickness of right footpad following PBS injection-initial thickness of right footpad)]

4.3.3 Sample collection

4.3.3.1 Breeder Hens

After 8 weeks of treatment, a total of 3 birds from each replicate were chosen randomly and sacrificed by decapitation. Blood was collected in 50 mL plastic tubes containing 2% EDTA. Spleen was also collected for fatty acid analysis.

4.3.3.2 Hatched Chicks

On days 7, 14 and 21 of age, 4 chicks from each replicate were selected randomly and sacrificed by decapitation. Blood was collected in 50 mL plastic tubes containing 2% EDTA. Spleen and bursa were collected for fatty acid analysis.

4.3.4 Sample preparation

4.3.4.1 Isolation of Platelets and WBCs

Platelets and WBCs were isolated by a modified version of the method used by Amalsadvala and Vaughn (1992). Briefly, a stock solution of histopaque 1119 was prepared by adding 1.5 grams of sodium diatriazoate to 100 mL of histopaque 1119. Whole blood was carefully layered over histopaque 1119 in a 1:1 ratio (20 mL of each) and then centrifuged for 30 min at 700 x g using a Beckman TJ-6 centrifuge with brake off. After removing the WBC band, any RBCs that remained were lysed with buffered 0.83% NH_4Cl until contaminating RBCs were no longer visible. After this, cells were washed twice with HBSS without $CaCl_2$. Cells were then resuspended in HBSS with 0.8 mM $CaCl_2$. An aliquot of cell suspension was used for counting cells with a Coulter counter.

4.3.4.2 Cell Viability and Purity

Cell viability was assessed by trypan blue exclusion. Cell differential count was assessed by making a thin smear of cells on a slide, staining it with Wright Stain, and counting isolated cell types under oil emulsion using a light microscope.

4.3.4.3 Stimulation of Platelets and WBCs for LTB_4 and LTB_5 Production

Aliquots of $5x10^6$ cells were transferred to 8 mL plastic tubes and HBSS containing 0.8 mM $CaCl_2$ was added to achieve a final volume of 475 µL. Before stimulation, aliquots of cells were pre-incubated at 37°C for 10 minutes. To initiate leukotriene production, 25 µL of calcium ionophore A23187 was added to each tube. Control tubes received 25 µL of 0.2% DMSO in HBSS without $CaCl_2$. Control and 20 µM calcium ionophore A23187 stimulated cells were incubated for 10 minutes at 37°C in a shaking water bath. After incubation, 2 mL of ice-cold methanol was added to each tube to terminate the reaction.

for 20 minutes on ice. Finally, tubes were centrifuged for 5 minutes at 1000 x g at 4°C to pellet cellular debris. Supernatants were transferred to another tube and stored in a –70°C freezer until subsequent LTB_4 and LTB_5 analysis.

4.3.4.4 Extraction and Separation of LTB_4 and LTB_5

LTB_4 and LTB_5 were extracted and separated using a modified version of the methods described by Terano et al. (1984). Briefly, stored supernatants were centrifuged for 15 min at 400 x g. Supernatants were transferred to 15 mL graded conical tubes containing 100 ng of PGB_3 as an internal standard. Freshly prepared citrate buffer was added to bring the volume up to approximately 14.5 mL.

A 12-mL syringe was attached to a C-18 solid phase extraction cartridge and 5 mL of HPLC-grade methanol was passed through the cartridge for pre-wetting the cartridge. Samples were then loaded onto the cartridge by gravity flow and leukotrienes were absorbed onto the extraction column. The cartridges were rinsed with 5 mL of double distilled H_2O followed by 5 mL of HPLC-grade hexane. Leukotrienes were then eluted from the column, using gravity flow, with 5 mL of a 90:10 mixture of methanol and double distilled H_2O into 5 mL plastic test tubes. The eluted samples were placed in a 30°C water bath and solvent was evaporated under a stream of N_2. After evaporation, the residues were reconstituted in 125 µL of mobile phase made up of methanol: water: acetic acid (75:25:01) and pH was adjusted to 5.7 with NH_4OH. Samples were capped with N_2 and stored at -70°C until LT were quantitated by RP-HPLC.

4.3.4.5 Quantitation of Leukotrienes by RP-HPLC

LTB_4 and LTB_5 were separated by HPLC using a C-18 reverse-phase column fitted with a C-18 guard column. The mobile phase was methanol: water: glacial acetic acid

(75:25:01), with pH adjusted to 5.7 with NH_4OH. The flow rate of the pump was set at 0.7 ml/min and the variable wavelength UV detector was set at 270 nm.

Standard calibration curves for LTB_4 and LTB_5 were made by adding 100 ng of PGB_3 as an internal standard to samples containing 6.25, 12.5, 25, 50, and 100 ng of LTB_4 and LTB_5. PGB_3 was chosen as the internal standard because in studies by Terano et al. (1984) it was widely separated from LTB_4 present in actual samples during HPLC separation, whereas PGB_2 coeluted with LTB_5 and PGB_1 coeluted with LTB_4. The standard solutions were extracted as above and LTB_4 and LTB_5 were detected by HPLC. The peak area ratio for LT/PGB_3 was calculated and plotted against the concentration of LT. (See Figure 7 and Figure 8 in Chapter 3). Concentrations of leukotrienes in test samples were calculated with reference to the standard curve. Final LTB_4 and LTB_5 concentrations in samples were reported as nanograms of LT per 5 million cells.

4.3.4.6 Total Lipid Extraction

The lipid content of spleen and bursa were extracted as per methods described by Folch et al. (1957). Briefly, approximately 2 g of minced spleen and bursa were homogenized in 30 mL of Folch–I solution (CH_3: MeOH, 2:1) using a polytron. The sample was filtered, and 0.88% NaCl was added and allowed to phase separate. The bottom layer was stored in a glass vial at -20°C for later use. Total lipid content was determined gravimetrically. The total lipid of the experimental diet and peripheral blood cells were also extracted in this same way, but their total lipid content was not quantified.

4.3.4.7 Fatty Acid Analysis

Two mL of lipid extract was dried under nitrogen, resolubilised in 3 mL boron trifluoride-methanol (10%wt/wt), and esterified by heating to 95°C. The fatty acid methyl

esters of feed, spleen, bursa and platelets were determined by a modification of the methods described by Cherian and Sim (1992). Fatty acid analysis was performed with an HP 6890 gas chromatograph equipped with an autosampler, flame ionization detector, and SP-2330 fused silica capillary column (30 m × 0.25 mm i.d). The initial oven temperature was set at 110°C, held for 1.5 minutes, and then increased by 20°C/minute to 200°C, and held for 50 minutes. The temperature was then increased by 10°C/minute to 230°C and held for 5.0 minutes (Cherain et al., 2002). Inlet and detector temperatures are 250°C. Helium was used as the carrier gas at a flow rate of 1.0 mL/minute. Fatty acid methyl esters were identified by comparison with retention times of authentic standards.[1] Peak areas and percentages were calculated using Hewlett Packard ChemStation software.[2] Fatty acid values are reported as percentages.

4.3.4.8 Statistical Analysis

To determine the effect of dietary fat on LT production by platelets in breeder hens and chicks, data were analyzed by ANOVA using SAS (version 8.2 SAS Institutes). The Student-Newmann-Keuls multiple range tests were used to compare differences among treatment means (Steel and Torrie, 1980). Mean values ± SEM are reported. Values were considered significant at $p \leq 0.05$.

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[1] Nuchek, Elysian, MN 56028

[2] Agilent Technologies, Inc. Wilmington, DE 16808-1610

RESULTS

4.4.1 Breeder Hens

4.4.1.1 Feed intake, growth and feed conversion ratio

There were no significant differences in final body weight, growth, and feed conversion ratio between breeder hens fed Diets I, II and III. (See Table 3.)

4.4.1.2 Dietary fatty acid composition

The composition of the basal diet is shown in Table 4. Fatty acid composition of diets was influenced by the source of fat included in the diet. Total n-3 fatty acids were higher in Diet III compared to Diet I and II because of added fish oil. Diet III was higher in total saturated fatty acids (SFA), total monounsaturated fatty acids (MUFA) and total n-3 fatty acids when compared with Diet I and Diet II ($p \leq 0.05$). Diet I was higher in total n-6 fatty acids when compared with Diet II and Diet III. The ratio of n-6 to n-3 FA was highest in Diet I and lowest in Diet III ($p \leq 0.05$). Fatty acid compositions of the three experimental Diets are summarized in Table 5.

Table 3. Feed intake, growth, and feed conversion ratio of breeder hens fed the experimental diets.*

Treatment	Body weight (g)	HDEP** (%)	Feed Consumption/bird/day (g)	Feed Efficiency /dozen eggs
Diet I	1705	75	110	1.27
Diet II	1709	70.8	109	1.2
Diet III	1700	75	110	1.3
SEM***	26	1.4	1.2	0.09

*Diets I, II and III represent a basal corn-soybean breeder hen diet supplemented with 3% sunflower oil (rich in n-6 FA), a mixture of 1.5 % sunflower oil and 1.5 % fish oil, or 3% fish oil (rich in n-3 FA), respectively. Values represent the mean of three observations. None of the means differ significantly ($p \leq 0.05$).

** HDEP: Hen day egg production

***SEM: Standard error of mean.

Table 4. Composition of breeder hen diets.

Ingredients	Percent
Corn	38.5
Wheat middling	29.5
Wheat grain	10
Soybean meal	9
Limestone	6.5
Dicalcium phosphate	2.5
Salt	0.5
Oil source*	3
Broiler premix**	0.5
Calculated composition:	
ME (kcal/kg)	2900
Crude protein (%)	16

Oil source includes Sunflower oil, fish oil or a mix of sunflower oil and fish oil at 1.5% each

**Supplied per kilogram of feed: vitamin A, 12500 IU; vitamin D_3, 4000 IU; vitamin E, 25 IU; vitamin B_{12}, 0.014 mg; riboflavin, 8 mg; pantothenic acid, 12 mg; niacin, 40 mg; menadione, 2.5 mg; choline, 500 mg; thiamine, 1.75 mg; folic acid, 0.75 mg; pyridoxine, 2 mg; d-biotin, 0.15 mg; ethoxyquin, 2.5 µg.

Table 5. Fatty acid composition of breeder hen diets.

	Dietary treatments*		
Fatty acids (%)	Diet I	Diet II	Diet III
C16:0	12.7	15.9	23.9
C18:0	2.6	3.17	4.28
C18:1	21.5	21.5	21.3
C18:2	57	44.7	30.1
C18:3 n-3	2.4	2.8	2.5
C20:5 n-3	0	2.0	3.14
C22:5 n-3	0	0	0.59
C22:6 n-3	0	1.99	3.2
Total SFA	19	23.8	31.8
Total MUFA	21.5	23.9	28.0
Total PUFA	59.5	52.3	40.1
Total n-6	57	45.4	31.3
Total n-3	2.4	6.8	9.4
n-6:n-3	23.7	6.8	3.3

*Diet I, II and III represent a basal corn-soybean breeder hen diet supplemented with 3% sunflower oil (rich in n-6 FA; Diet I), a mixture of 1.5 % sunflower oil and 1.5 % fish oil (Diet II), or 3% fish oil (rich in n-3 FA; Diet III). Values are means ± SEM (n=6).

Total SFA=Total saturated fatty acids and includes C16:0, C18:0, C20:0, C22:0, and C24:0.

Total MUFA=Total monounsaturated fatty acids and includes C16:1, C18:1, C20:1, C22:1, and C24:1.

Total PUFA=Total polyunsaturated fatty acids and includes C18:2 n-6, C18:3 n-6, C18:3 n-3, C20:2 n-6, C20:3 n-6, C20:4 n-6, C20:5 n-3, C22:4, C22:5 n-3, and C22:5 n-3.

Total n-6 and Total n-3=Total n-6 and n-3 fatty acids.

4.4.1.3 Fatty acid composition of spleen tissue from breeder hens

The fatty acid profile of spleen tissue from breeder hens is shown in Table 6. Deposition of AA in spleen tissue was similar for hens fed the three diets, which varied in n-6 to n-3 FA ratio. EPA was increased in spleen tissue of hens fed Diet III and Diet II when compared to Diet I ($p \leq 0.05$). Breeder hen spleen tissue showed no differences in DHA content between Diet III and Diet II, but DHA was significantly less in the spleen tissue of hens fed Diet I. Fatty acid composition of spleen from breeder hens fed Diet I had higher amounts of total n-6 fatty acids compared to spleen from birds fed Diet II and Diet III ($p \leq 0.05$). The ratio of n-6 FA to n-3 FA was higher in birds fed Diet I, but was similar in the other two groups ($p \leq 0.05$).

4.4.1.4 Fatty acid composition of breeder hen platelets

The fatty acid composition of platelets resembled the FA composition of the respective diets fed to breeder hens. AA content was not different amongst birds fed the three diets. However, EPA content of platelets was affected. Hens fed Diet III had the most EPA in their cells followed by hens fed Diet II. No EPA was detected in cells from hens fed Diet I ($p \leq 0.05$). DHA content of cells was similar for birds fed Diet III and Diet II, but DHA was significantly less in cells from breeder hens fed Diet I ($p \leq 0.05$). There were also significant differences in total n-3 FA composition. Cells obtained from breeder hens fed Diet III had significantly higher total n-3 FA content, but the lowest n-6 to n-3 FA ratio ($p \leq 0.05$). The FA composition of platelets of breeder hens is summarized in Table 7.

4.4.1.5 Delayed-type-hypersensitivity reaction in breeder hens

At 24 hrs post footpad injection with BSA, breeder hens fed Diet I and Diet II had more inflammed footpads than breeder hens fed Diet III. This finding persisted at 48 hours, at which time maximum swelling was noted. (See Figure 12.) Birds fed Diet I had a greater swelling index compared to birds fed Diet III ($p \leq 0.05$). By 72 hrs, the overall swelling index had decreased in all three groups. The DTH reaction in breeder hens was significantly suppressed by feeding a diet enriched in n-3 FA.

Table 6. Spleen tissue fatty acid composition in breeder hens fed experimental diets.

	Dietary treatments*			
Fatty acids (%)	Diet I	Diet II	Diet III	SEM
C16:0	21.57[b]	22.82[ab]	22.84[a]	0.44
C16:1	3.82[c]	4.51[b]	5.58[a]	0.14
C18:0	9.34	9.24	9	0.92
C18:1	32	33.73	34.39	2.65
C18:2	21.63[a]	16.8[b]	15.0[b]	1.08
C20:4 n-6	5.56	4.85	3.11	0.89
C20:5 n-3	0.10[b]	0.57[a]	0.65[a]	0.12
C22:6 n-3	0.61[b]	2.23[a]	2.49[a]	0.55
Total SFA	33.3	34.8	36	1.42
Total MUFA	36.17	38.4	40.2	2.57
Total PUFA	30.18[a]	26.68[ab]	23.65[b]	1.34
Total n-6	7.52	6	4	1.2
Total n-3	1.03[b]	3.6[a]	4.6[a]	0.72
n-6:n-3	7.35[a]	1.63[b]	0.88[b]	0.92

*Diet I, II and III represent a basal corn-soybean breeder hen diet supplemented with 3% sunflower oil (rich in n-6 FA; Diet I), a mixture of 1.5 % sunflower oil and 1.5 % fish oil (Diet II), or 3% fish oil (rich in n-3 FA; Diet III). Values are means ± SEM (n=6).

Total SFA=Total saturated fatty acids and includes C16:0, C18:0, C20:0, C22:0, and C24:0.

Total MUFA=Total monounsaturated fatty acids and includes C16:1, C18:1, C20:1, C22:1, and C24:1.

Total PUFA=Total polyunsaturated fatty acids and includes C18:2 n-6, C18:3 n-6, C18:3 n-3, C20:2 n-6, C20:3 n-6, C20:4 n-6, C20:5 n-3, C22:4, C20:5 n-3, and C22:5 n-3.

Total n-6 and Total n-3=Total n-6 and n-3 fatty acids respectively.

a-c For each fatty acid, values with different superscripts are significantly different ($p \leq 0.05$).

Table 7. Fatty acid composition of platelets from breeder hens.

	Dietary treatments*			
Fatty acids (%)	Diet I	Diet II	Diet III	SEM
C16:0	18.25	18.94	19.14	0.8
C16:1	3.02	3.37	2.74	0.63
C18:0	15.68	15.18	16.4	0.48
C18:1	21.48	23.42	21.53	3.21
C18:2	11.75	11.14	12.72	0.88
C20:4 n-6	15.1	13.19	12.67	1.69
C20:5 n-3	0^{c}	0.54^{b}	0.98^{a}	0.57
C22:6 n-3	0.66^{b}	2.01^{a}	2.8^{a}	0.28
Total SFA	41.54	41.35	42.29	1.12
Total MUFA	24.74	27.04	24.59	3.02
Total PUFA	22.4	22.4	22.3	0.8
Total n-6	33.05	28.74	28.66	2.52
Total n-3	0.66^{c}	2.84^{b}	4.45^{a}	0.06
n-6:n-3	5.24^{a}	3.75^{b}	2.5^{c}	0.8

*Diet I, II and III represent a basal corn-soybean breeder hen diet supplemented with 3% sunflower oil (rich in n-6 FA; Diet I), a mixture of 1.5 % sunflower oil and 1.5 % fish oil (Diet II), or 3% fish oil (rich in n-3 FA; Diet III). Values are means ± SEM (n=6).

Total SFA=Total saturated fatty acids and includes C16:0, C18:0, C20:0, C22:0, and C24:0.

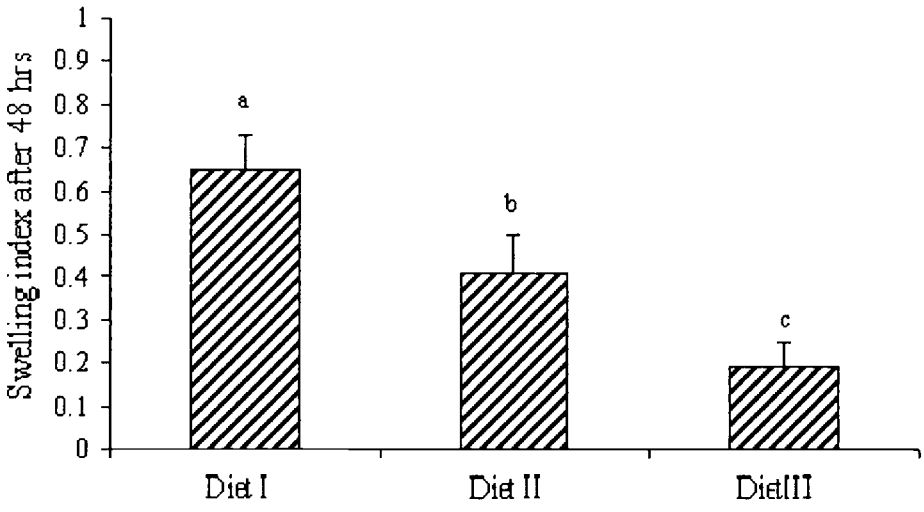
Total MUFA=Total monounsaturated fatty acids and includes C16:1, C18:1, C20:1, C22:1, and C24:1.

Total PUFA=Total polyunsaturated fatty acids and includes C18:2 n-6, C18:3 n-6, C18:3 n-3, C20:2 n-6, C20:3 n-6, C20:4 n-6, C20:5 n-3, C22:4, C20:5 n-3, and C22:5 n-3.

Total n-6 and Total n-3=Total n-6 and n-3 fatty acids respectively.

a-c For each fatty acid, values with different superscripts are significantly different ($p \leq 0.05$).

Figure 12. Delayed-type-hypersensitivity reaction in breeder hens.



Diets I, II and III represent a basal corn-soybean breeder hen diet supplemented with 3% sunflower oil (rich in n-6 FA; Diet I), a mixture of 1.5 % sunflower oil and 1.5 % fish oil (Diet II), or 3% fish oil (rich in n-3 FA; Diet III).

The DTH foot pad swelling response was induced by immunizing the birds by intramuscular injection with 200 µL of solution having 1mg of BSA per mL of PBS. After 14 days, a second subcutaneous injection was made in the left footpad. As a control vehicle, 200 µL of PBS was injected subcutaneously into the right footpad.

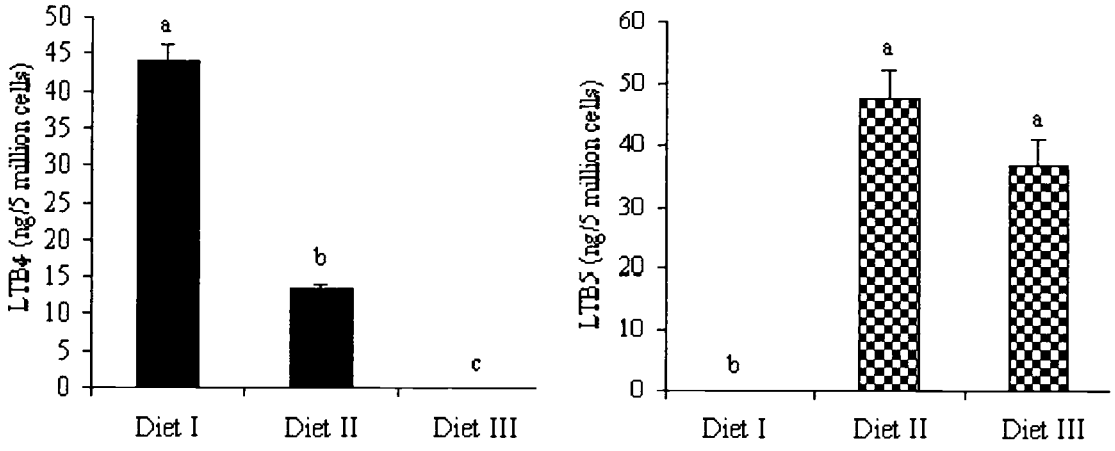
Swelling index was calculated = [(thickness of left footpad following BSA injection-initial thickness of left footpad)-(thickness of right footpad following PBS injection-initial thickness of right footpad)].

Each bar represent mean ± SEM (n=8). Bars with different letters are significantly different ($p \leq 0.05$).

4.4.1.6 Leukotriene production in breeder hens

In breeder hens, the type of diet fed influenced the type of the leukotrienes produced by isolated platelets. (See Figure 13.) Hens fed Diet I, which was rich in n-6 FA, produced 43.9 ± 2.5 ng of LTB_4 from $5x10^6$ cells compared with 13.3 ± 0.9 ng of LTB_4 from hens fed Diet II ($p \leq 0.05$). However, hens fed Diet III, which was rich in n-3 FA produced no LTB_4. Production of LTB_5 by platelets of hens fed Diet III was 36.7 ± 4.9 ng from $5x10^6$ cells compared to 47.4 ± 5.7 ng of LTB_5 from hens fed Diet II. Hens fed Diet I produced no LTB_5.

Figure 13. Leukotriene production by platelets from breeder hens.



13a 13b

Diets I, II and III represent a basal corn-soybean breeder hen diet supplemented with 3% sunflower oil (rich in n-6 FA; Diet I), a mixture of 1.5 % sunflower oil and 1.5 % fish oil (Diet II), or 3% fish oil (rich in n-3 FA; Diet III).

Shown are LTB_4 (13a) and LTB_5 (13b) production (*ex vivo*) by 5 x 10^6 platelets following stimulation with 20 uM calcium ionophore A23187 at 37°C for 10 minutes.

Each bar represent mean ± SEM (n=4). Bars with different letters are significantly different ($p \leq 0.05$).

4.4.2 Chick Performance

Commercial diets for chicks contained grain products and had a total crude protein of 18%, crude fiber of 3%, lysine of 0.88% methionine of 0.32%, calcium of 1.25%, phosphorus of 0.60%, salt of 0.85%, Vit A of 5000 IU/lb, and Vit E of 14 IU/lb.

4.4.2.1 Feed intake, growth and feed conversion ratio in hatched chicks

There were no significant differences in final body weight, growth, and feed conversion ratio among chicks hatched to breeder hens fed Diets I, II, and III.

4.4.2.2 Fatty acids composition of chick diets

The fatty acid composition of chick starter diet is summarized in Table 8. Starter Diet was subjected to fatty acid analysis to determine the relative percentages of different fatty acids. The n-6 FA were the major PUFA (44.3%). The diet was a poor source of n-3 FA (3.4%). The n-6 to n-3 FA ratio was high at 13 to 1.

4.4.2.3 Fatty acid composition of spleen tissue from chicks

Fatty acid compositions of spleen from chicks at 7, 14 and 21 days of age are shown in Table 9, Table 10, and Table 11, respectively. No significant differences were found in spleen content of AA and EPA in hatched chicks among the three groups. Groups represent chicks hatched from hens fed different diets. Spleen DHA content was highest in chicks from hens fed Diet III, followed by chicks from hens fed Diet II, and least in chicks from hens fed Diet I at 7, 14 and 21 days of age ($p \leq 0.05$). In 7-day-old chicks, there were no differences in total PUFA and total n-6 FA in the spleen tissue. However, total n-3 FA content of spleen was higher in chicks from hens fed Diet III compared to chicks from hens fed Diets II and I ($p \leq 0.05$). Spleen from 14-day-old chicks showed no differences in total PUFA. Interestingly, when the FA composition of spleen from 21-day-old chicks was

compared, it was found that total PUFA were higher in Diet II and Diet III chicks, and lower in Diet I chicks. Spleen total n-6 FA content was not different among the dietary groups, but spleen total n-3 FA was highest in chicks from hens fed Diet III, followed by chicks from hens fed Diet II and Diet I, respectively ($p \leq 0.05$).

Table 8. Fatty acid composition of commercial starter diet fed to chicks.

	Starter feed for chicks
Fatty acids	%
C16:1	6.4
C18:0	2.6
C18:1	21
C18:2	44.3
C18:3 n-3	3.4
Total SFA	31.2
Total MUFA	21
Total PUFA	47.7
Total n-6	44.3
Total n-3	3.4
n-6: n-3	13.1

The diet contained grain products, plant-protein products, processed-grain by-products, calcium carbonate, monocalcium phosphate, molasses products, salt, L-lysine, choline chloride, DL-methionine, riboflavin supplement, vitamin E supplement, calcium pantothenate, vitamin B-12 supplement, biotin, vitamin A supplement, menadione dimethylpyrimidinol bisulfite (source of vitamin K), folic acid, pyridoxine hydrochloride, niacin supplement, vitamin D-3 supplement, manganous oxide, zinc oxide, copper sulfate, calcium iodate, and sodium selenite.

Total SFA=Total saturated fatty acids and includes C16:0, C18:0, C20:0, C22:0, and C24:0.

Total MUFA=Total monounsaturated fatty acids and includes C16:1, C18:1, C20:1, C22:1, and C24:1.

Total PUFA=Total polyunsaturated fatty acids and includes C18:2 n-6, C18:3 n-6, C18:3 n-3, C20:2 n-6, C20:3 n-6, C20:4 n-6, C20:5 n-3, C22:4, C20:5 n-3, and C22:5 n-3.

Total n-6 and Total n-3=Total n-6 and n-3 fatty acids, respectively.

Table 9. Spleen tissue fatty acid compositions of 7-day-old chicks hatched from hens fed experimental diets.

	Dietary treatments*			
Fatty acids (%)	Diet I	Diet II	Diet III	SEM
C16:0	26.4	26.6	26.5	0.4
C16:1	4.5	4.7	5.8	0.65
C18:0	12.8	11.3	11.4	0.85
C18:1	23.3	26.8	25.3	1.52
C20:4 n-6	9.5	8.1	8.1	1.35
C20:5 n-3	0.48	0.69	0.91	0.13
C22:6 n-3	0.84^{b}	1.28^{ab}	1.76^{a}	0.25
Total SFA	42.33^{a}	39.7^{b}	41.3^{ab}	0.67
Total MUFA	28.3	31.9	31.6	2.03
Total PUFA	28.9	28.1	27.1	1.64
Total n-6	14.6	11.2	10.9	1.8
Total n-3	1.9^{b}	2.8^{ab}	3.7^{a}	0.48
n-6: n-3	8.1^{a}	3.8^{b}	3.5^{b}	1.02

*Diet I, II and III represent a basal corn-soybean breeder hen diet supplemented with 3% sunflower oil (rich in n-6 FA; Diet I), a mixture of 1.5 % sunflower oil and 1.5 % fish oil (Diet II), or 3% fish oil (rich in n-3 FA; Diet III). Values are means ± SEM (n=6). Chicks were fed identical diets.

Total SFA=Total saturated fatty acids and includes C16:0, C18:0, C20:0, C22:0, and C24:0.

Total MUFA=Total monounsaturated fatty acids and includes C16:1, C18:1, C20:1, C22:1, and C24:1.

Total PUFA=Total polyunsaturated fatty acids and includes C18:2 n-6, C18:3 n-6, C18:3 n-3, C20:2 n-6, C20:3 n-6, C20:4 n-6, C20:5 n-3, C22:4, C20:5 n-3, and C22:5 n-3.

Total n-6 and Total n-3=Total n-6 and n-3 fatty acids, respectively.

a-c For each fatty acid, values with different superscripts are significantly different ($p \leq 0.05$).

Table 10. Spleen tissue fatty acid compositions of 14-day-old chicks hatched from hens fed experimental diets.

	Dietary treatments*			
Fatty acids (%)	Diet I	Diet II	Diet III	SEM
C16:0	26.5	26	27.5	1.12
C16:1	4	5	3.6	0.57
C18:0	12.7	11	14.2	1.06
C18:1	23.5^{ab}	26.9^{a}	21.7^{b}	1.55
C18:2	13.9	14.7	11.9	1.49
C20:4 n-6	8.7	7.8	7.8	0.96
C20:5 n-3	0.45	0.49	0.69	0.14
C22:6 n-3	0.48^{b}	0.60^{ab}	1.0^{a}	0.15
Total SFA	39.9	37.8	42.9	2.24
Total MUFA	28.3	32.6	25.6	2.12
Total PUFA	29.2	27.4	29	0.85
Total n-6	13.7	10.8	14.6	1.82
Total n-3	1.6	1.8	2.4	0.32
n-6: n-3	8.6	5.8	9.5	3.2

*Diet I, II and III represent a basal corn-soybean breeder hen diet supplemented with 3% sunflower oil (rich in n-6 FA; Diet I), a mixture of 1.5 % sunflower oil and 1.5 % fish oil (Diet II), or 3% fish oil (rich in n-3 FA; Diet III). Values are means ± SEM (n=6). Chicks were fed identical diets.

Total SFA=Total saturated fatty acids and includes C16:0, C18:0, C20:0, C22:0, and C24:0.

Total MUFA=Total monounsaturated fatty acids and includes C16:1, C18:1, C20:1, C22:1, and C24:1.

Total PUFA=Total polyunsaturated fatty acids and includes C18:2 n-6, C18:3 n-6, C18:3 n-3, C20:2 n-6, C20:3 n-6, C20:4 n-6, C20:5 n-3, C22:4, C20:5 n-3, and C22:5 n-3.

Total n-6 and Total n-3=Total n-6 and n-3 fatty acids, respectively.

a-c For each fatty acid, values with different superscripts are significantly different ($p \leq 0.05$).

Table 11. Spleen tissue fatty acid compositions of 21-day-old chicks hatched from hens fed experimental diets.

	Dietary treatments*			
Fatty acids (%)	Diet I	Diet II	Diet III	SEM
C16:0	25.3	24.6	25.6	0.28
C16:1	4.6[a]	3.8[b]	3.5[b]	0.22
C18:0	12.3	13.6	12.9	0.65
C18:1	24.3	21.2	23.1	1.68
C18:2	14.4	14.8	15.4	0.77
C20:4 n-6	8.9	10.5	10	0.94
C20:5 n-3	0.51	0.61	0.65	0.05
C22:6 n-3	0.46[b]	0.75[ab]	1.02[a]	0.1
Total SFA	38.5	39	39	0.55
Total MUFA	29.6	25.8	27.3	1.77
Total PUFA	28.8[b]	31.8[a]	31.5[ab]	0.85
Total n-6	12.8	15	13.9	1.33
Total n-3	1.5[b]	1.9[ab]	2.2[a]	0.19
n-6: n-3	9	7.7	6.6	1.05

*Diet I, II and III represent a basal corn-soybean breeder hen diet supplemented with 3% sunflower oil (rich in n-6 FA; Diet I), a mixture of 1.5 % sunflower oil and 1.5 % fish oil (Diet II), or 3% fish oil (rich in n-3 FA; Diet III). Values are means ± SEM (n=6). Chicks were fed identical diets.

Total SFA=Total saturated fatty acids and includes C16:0, C18:0, C20:0, C22:0, and C24:0.

Total MUFA=Total monounsaturated fatty acids and includes C16:1, C18:1, C20:1, C22:1, and C24:1.

Total PUFA=Total polyunsaturated fatty acids and includes C18:2 n-6, C18:3 n-6, C18:3 n-3, C20:2 n-6, C20:3 n-6, C20:4 n-6, C20:5 n-3, C22:4, C20:5 n-3, and C22:5 n-3.

Total n-6 and Total n-3=Total n-6 and n-3 fatty acids, respectively.

a-c For each fatty acid, values with different superscripts are significantly different ($p \leq 0.05$).

4.4.2.4 Fatty acid composition of bursa from chicks

Fatty acid profiles of bursa from chicks at 7, 14 and 21 days of age are shown in Table 12, Table 13, and Table 14, respectively. Bursa from 7-day-old chicks had the highest content of LA in chicks from hens fed Diet I ($p \leq 0.05$). The bursa content of AA, EPA and DHA were highest in the chicks from hens fed Diet II; chicks from hens fed Diet I and III were not different. Total PUFA and n-6 FA, and the ratio of n-6 to n-3 FA were similar among the groups, but chicks from hens fed Diet II showed higher content of total n-3 FA, followed by chicks from hens fed Diet III and Diet I ($p \leq 0.05$). See Table 12.

Fatty acid profiles of bursa from 14-day-old chicks showed no difference in LA content, although AA was significantly higher in chicks from hens fed Diet I ($p \leq 0.05$). In contrast to the findings in 7-day-old chicks, EPA and DHA were higher in chicks from hens fed Diet III, followed by chicks from hens fed Diet II and Diet I, respectively, in 14-day-old chicks ($p \leq 0.05$). Total n-6 FA content was higher in chicks from hens fed Diet I, and total n-3 FA content was higher in chicks from hens fed Diet III ($p \leq 0.05$). Ratios of n-6 to n-3 FA were significantly higher in bursa obtained from 14-day-old chicks hatched to hens fed Diet I ($p \leq .05$). See Table 13.

Chicks at 21 days of age showed no differences in LA and AA content of bursa. Bursa tissue FA composition showed a similar trend in EPA, DHA, total n-6 and total n-3 content as that seen in 14-day-old chicks. EPA and DHA contents were highest in chicks from hens fed Diet III; n-6 FA content was higher in chicks from hens fed Diet I; and, n-3 FA content was higher in chicks from hens fed Diet III ($p \leq 0.05$). See Table 14.

Table 12. Bursa tissue fatty acid compositions of 7-day-old chicks hatched from hens fed experimental diets.

	Dietary treatments*			
Fatty acids (%)	Diet I	Diet II	Diet III	SEM
C16:0	26.3^{ab}	25.3^{b}	28.0^{a}	0.53
C16:1	7.9	8.1	7.6	0.45
C18:0	8.1	10.1	9	0.63
C18:1	34.33	34.1	33.5	1.13
C18:2	14.9^{a}	11.5^{b}	12.1^{b}	0.81
C20:4 n-6	2.8^{ab}	3.3^{a}	1.9^{b}	0.37
C20:5 n-3	0.12^{b}	0.41^{a}	0.18^{b}	0.1
C22:6 n-3	0.45^{b}	1.71^{a}	0.92^{b}	0.16
Total SFA	37.3	38.9	39.7	0.99
Total MUFA	42.7	42.6	41.5	1.5
Total PUFA	19.9	18.4	18.7	0.87
Total n-6	3.9	4.2	4.8	0.98
Total n-3	1.0^{c}	2.7^{a}	1.8^{b}	0.21
n-6: n-3	3.9	1.6	3.1	0.76

*Diet I, II and III represent a basal corn-soybean breeder hen diet supplemented with 3% sunflower oil (rich in n-6 FA; Diet I), a mixture of 1.5 % sunflower oil and 1.5 % fish oil (Diet II), or 3% fish oil (rich in n-3 FA; Diet III). Values are means ± SEM (n=6). Chicks were fed identical diets.

Total SFA=Total saturated fatty acids and includes C16:0, C18:0, C20:0, C22:0, and C24:0.

Total MUFA=Total monounsaturated fatty acids and includes C16:1, C18:1, C20:1, C22:1, and C24:1.

Total PUFA=Total polyunsaturated fatty acids and includes C18:2 n-6, C18:3 n-6, C18:3 n-3, C20:2 n-6, C20:3 n-6, C20:4 n-6, C20:5 n-3, C22:4, C20:5 n-3, and C22:5 n-3.

Total n-6 and Total n-3=Total n-6 and n-3 fatty acids, respectively.

a-c For each fatty acid, values with different superscripts are significantly different ($p \leq 0.05$).

Table 13. Bursa tissue fatty acid compositions of 14-day-old chicks hatched from hens fed experimental diets.

	Dietary treatments*			
Fatty acids (%)	Diet I	Diet II	Diet III	SEM
C16:0	23.3	23.3	25.3	0.2
C16:1	7.2	7.2	7.1	0.17
C18:0	10.3	10.1	10.2	0.2
C18:1	30	30.5	30.2	0.72
C18:2	11.7	12.1	11.8	0.38
C20:4 n-6	5.6^{a}	4.9^{ab}	4.8^{b}	0.24
C20:5 n-3	0.64^{b}	0.71^{b}	0.88^{a}	0.05
C22:6 n-3	0.63^{c}	0.84^{b}	1.3^{a}	0.04
Total SFA	39.1	38.7	39.2	0.32
Total MUFA	38.2	38.8	38.4	0.89
Total PUFA	22.4	22.4	22.3	0.69
Total n-6	9.0^{a}	8.0^{ab}	7.5^{b}	0.35
Total n-3	1.7^{c}	2.1^{b}	7.5^{a}	0.09
n-6: n-3	5.24^{a}	3.75^{b}	2.5^{c}	0.22

*Diet I, II and III represent a basal corn-soybean breeder hen diet supplemented with 3% sunflower oil (rich in n-6 FA; Diet I), a mixture of 1.5 % sunflower oil and 1.5 % fish oil (Diet II), or 3% fish oil (rich in n-3 FA; Diet III). Values are means ± SEM (n=6). Chicks were fed identical diets.

Total SFA=Total saturated fatty acids and includes C16:0, C18:0, C20:0, C22:0, and C24:0.

Total MUFA=Total monounsaturated fatty acids and includes C16:1, C18:1, C20:1, C22:1, and C24:1.

Total PUFA=Total polyunsaturated fatty acids and includes C18:2 n-6, C18:3 n-6, C18:3 n-3, C20:2 n-6, C20:3 n-6, C20:4 n-6, C20:5 n-3, C22:4, C20:5 n-3, and C22:5 n-3.

Total n-6 and Total n-3=Total n-6 and n-3 fatty acids, respectively.

a-c For each fatty acid, values with different superscripts are significantly different ($p \leq 0.05$).

Table 14. Bursa tissue fatty acid compositions of 21-day-old chicks hatched from hens fed experimental diets.

	Dietary treatments*			
Fatty acids (%)	Diet I	Diet II	Diet III	SEM
C16:0	24.3	23.9	24	0.21
C16:1	7	7.3	7.2	0.11
C18:0	10	9.9	10.3	0.23
C18:1	29.54	30.9	30.1	0.52
C18:2	12.7	12.4	11.7	0.32
C20:4 n-6	5.4	4.5	4	0.24
C20:5 n-3	0.52^{c}	0.72^{b}	1.2^{a}	0.03
C22:6 n-3	0.64^{c}	1.0^{b}	1.7^{a}	0.06
Total SFA	39.6	38.9	39.7	0.32
Total MUFA	37	39.1	38.3	0.61
Total PUFA	22.9	21.9	21.9	0.42
Total n-6	8.5^{a}	7.0^{b}	6.2^{b}	0.35
Total n-3	1.6^{c}	2.4^{b}	3.9^{a}	0.1
n-6: n-3	5.1^{a}	2.9^{b}	1.6^{c}	0.23

*Diet I, II and III represent a basal corn-soybean breeder hen diet supplemented with 3% sunflower oil (rich in n-6 FA; Diet I), a mixture of 1.5 % sunflower oil and 1.5 % fish oil (Diet II), or 3% fish oil (rich in n-3 FA; Diet III). Values are means ± SEM (n=6). Chicks were fed identical diets.

Total SFA=Total saturated fatty acids and includes C16:0, C18:0, C20:0, C22:0, and C24:0.

Total MUFA=Total monounsaturated fatty acids and includes C16:1, C18:1, C20:1, C22:1, and C24:1.

Total PUFA=Total polyunsaturated fatty acids and includes C18:2 n-6, C18:3 n-6, C18:3 n-3, C20:2 n-6, C20:3 n-6, C20:4 n-6, C20:5 n-3, C22:4, C20:5 n-3, and C22:5 n-3.

Total n-6 and Total n-3=Total n-6 and n-3 fatty acids, respectively.

a-c For each fatty acid, values with different superscripts are significantly different ($p \leq 0.05$).

4.4.2.5 Fatty acid composition of chick platelets

There were no significant differences in the FA profiles of peripheral blood platelets isolated from 14 or 21-day-old chicks hatched to hens fed experimental diets. (See Table 15 and Table 16.)

Table 15. Platelet fatty acid compositions of 14-day-old chicks hatched from hens fed experimental diets.

	Dietary treatments*			
Fatty acids (%)	Diet I	Diet II	Diet III	SEM
C16:0	21.00	20.04	19.40	0.33
C16:1	2.11	0.00	1.80	0.47
C18:0	17.70	18.45	18.00	0.15
C18:1	17.90	18.43	17.80	0.11
C18:2	10.40	10.13	11.70	0.34
C20:4 n-6	17.70	17.37	17.50	0.07
C20:5 n-3	0.00	0.00	1.03	0.24
C22:6 n-3	0	2.17	0	0.51
Total SFA	38.9	41.51	37.4	0.85
Total MUFA	20.05	18.43	19.60	0.34
Total PUFA	36.10	38.17	37.30	0.43
Total n-6	25.76	25.87	24.5	0.36
Total n-3	0	2.17	1.03	0.44
n-6: n-3	25.7	11.9	23.9	3.06

*Diet I, II and III represent a basal corn-soybean breeder hen diet supplemented with 3% sunflower oil (rich in n-6 FA; Diet I), a mixture of 1.5 % sunflower oil and 1.5 % fish oil (Diet II), or 3% fish oil (rich in n-3 FA; Diet III). Values are means ± SEM (n=6). Chicks were fed identical diets.

Total SFA=Total saturated fatty acids and includes C16:0, C18:0, C20:0, C22:0, and C24:0.

Total MUFA=Total monounsaturated fatty acids and includes C16:1, C18:1, C20:1, C22:1, and C24:1.

Total PUFA=Total polyunsaturated fatty acids and includes C18:2 n-6, C18:3 n-6, C18:3 n-3, C20:2 n-6, C20:3 n-6, C20:4 n-6, C20:5 n-3, C22:4, C20:5 n-3, and C22:5 n-3.

Total n-6 and Total n-3=Total n-6 and n-3 fatty acids, respectively.

Table 16. Platelet fatty acid compositions of 21-day-old chicks hatched from hens fed experimental diets.

	Dietary treatments*			
Fatty acids (%)	Diet I	Diet II	Diet III	SEM
C16:0	14.10	16.94	15.60	0.31
C16:1	3.70	4.93	4.30	1.32
C18:0	22.40	19.03	21.50	0.79
C18:1	19.90	18.60	23.70	2.04
C18:2	9.65	10.62	9.94	0.22
C20:4 n-6	12.50	14.54	12.53	0.87
C20:5 n-3	0.19	0.41	0.13	0.10
C22:6 n-3	0.22	0.42	0.19	0.13
Total SFA	37.9	36.9	38.4	1.10
Total MUFA	24.50	24.50	28.60	2.64
Total PUFA	32.20	34.40	31.80	1.26
Total n-6	21.5	22.5	20.9	0.88
Total n-3	1.03	1.32	0.88	0.21
Ratio n-6: n-3	21.9	17	28.6	4.28

*Diet I, II and III represent a basal corn-soybean breeder hen diet supplemented with 3% sunflower oil (rich in n-6 FA; Diet I), a mixture of 1.5 % sunflower oil and 1.5 % fish oil (Diet II), or 3% fish oil (rich in n-3 FA; Diet III). Values are means ± SEM (n=6). Chicks were fed identical diets.

Total SFA=Total saturated fatty acids and includes C16:0, C18:0, C20:0, C22:0, and C24:0.

Total MUFA=Total monounsaturated fatty acids and includes C16:1, C18:1, C20:1, C22:1, and C24:1.

Total PUFA=Total polyunsaturated fatty acids and includes C18:2 n-6, C18:3 n-6, C18:3 n-3, C20:2 n-6, C20:3 n-6, C20:4 n-6, C20:5 n-3, C22:4, C20:5 n-3, and C22:5 n-3.

Total n-6 and Total n-3=Total n-6 and n-3 fatty acids, respectively.

4.4.2.6 Leukotriene production in chicks

Platelets isolated from 7-day-old chicks and stimulated with calcium ionophore A23187 produced LTB_4 regardless of the diet fed to hens from which chicks hatched. Those chicks hatched to hens fed 3% SFO supplemented diet (Diet I) produced significantly more LTB_4 compared to chicks from hens fed Diets II and III ($p \leq 0.05$). (See Figure 14.) Platelets isolated from 14-day-old chicks and stimulated with calcium ionophore A23187 produced LTB_4 regardless of the diet fed to hens from which chicks hatched. There were no significant differences among groups. (See Figure 15.) Platelets isolated from 21-day-old chicks and stimulated with calcium ionophore A23187 produced LTB_4 regardless of the diet fed to hens from which chicks hatched. (See Figure 16.) Those chicks hatched to hens fed the 3% fish oil supplemented diet (Diet III) produced significantly less LTB_4 compared to chicks hatched to hens fed Diets I and III ($p \leq 0.05$). The 7-day-old and 14-day-old chicks from hens fed fish oil (Diet III) produced similar amounts of LTB_5. By 21 days, however, stimulated platelets were no longer able to produce measurable quantities of LTB_5.

All the chicks were fed a commercial starter diet low in n-3 fatty acids. Diets I, II and III refer to diets fed to breeder hens. Chicks from hens fed Diet I produced significantly less LTB_4 at 14 days ($p \leq 0.05$) compared to 7- and 21-days-old chicks from hens fed Diet I; the latter were not different from each other. Chicks from hens fed Diet II produced significantly more LTB_4 at 21 days ($p \leq 0.05$) compared to 7-day-old chicks from hens fed Diet II; the latter also produced significantly more than 14-day-old chicks ($p \leq 0.05$). Chicks from hens fed Diet III produced significantly more LTB_4 at 7 and 21days ($p \leq 0.05$) compared to 14-day-old chicks from hens fed Diet III. However, there were no significant

differences in LTB_5 production from chicks hatched to hens fed Diet III at 7 or 14 days of growth.

Figure 14. Leukotriene production by platelets from 7-day-old chicks.

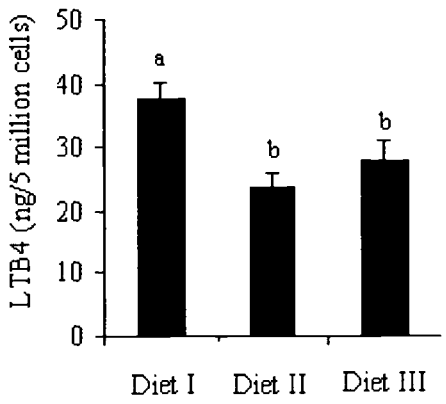


Figure 14a

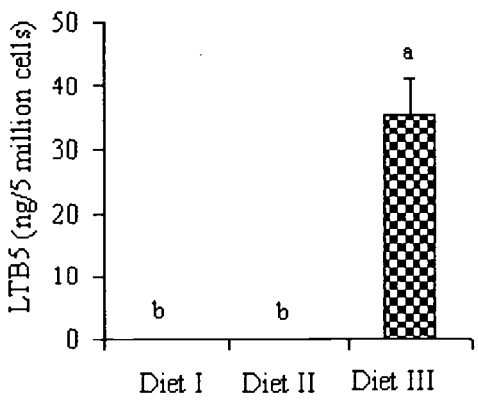


Figure 14b

Diets I, II and III represent a basal corn-soybean breeder hen diet supplemented with 3% sunflower oil (rich in n-6 FA; Diet I), a mixture of 1.5 % sunflower oil and 1.5 % fish oil (Diet II), or 3% fish oil (rich in n-3 FA; Diet III). Chicks were fed identical diets.

Shown are LTB_4 and LTB_5 production (*ex vivo*) by 5 x 10^6 platelets from 7-day-old chicks following stimulation with 20 μM calcium ionophore A23187 at 37°C for 10 minutes.

Each bar represents the mean ± SEM (n=4). Bars with different letters are significantly different ($p \leq 0.05$).

Figure 15. Leukotriene production by platelets from 14-day-old chicks.

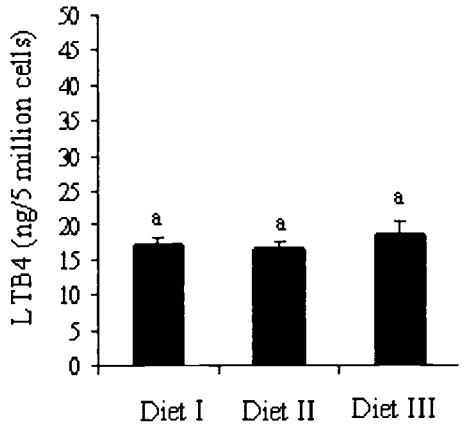


Figure 15a

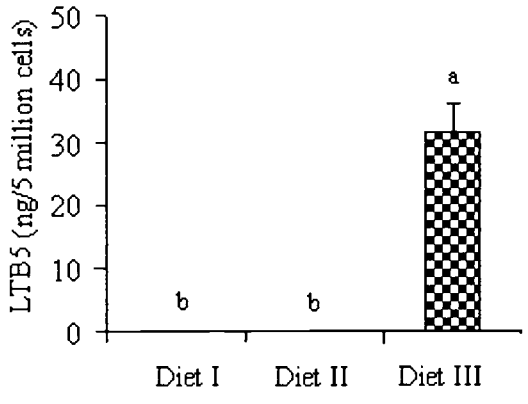


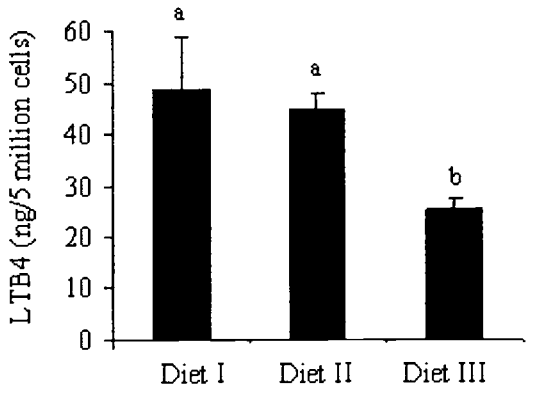
Figure 15b

Diets I, II and III represent a basal corn-soybean breeder hen diet supplemented with 3% sunflower oil (rich in n-6 FA; Diet I), a mixture of 1.5 % sunflower oil and 1.5 % fish oil (Diet II), or 3% fish oil (rich in n-3 FA; Diet III). Chicks were fed identical diets.

Shown are LTB_4 and LTB_5 production (*ex vivo*) by 5 x 10^6 platelets from 14-day-old chicks following stimulation with 20 μM calcium ionophore A23187 at 37°C for 10 minutes.

Each bar represents the mean ± SEM (n=4). Bars with different letters are significantly different ($p \leq 0.05$).

Figure 16. Leukotriene production by platelets from 21-day-old chicks.



Diets I, II and III represent a basal corn-soybean breeder hen diet supplemented with 3% sunflower oil (rich in n-6 FA; Diet I), a mixture of 1.5 % sunflower oil and 1.5 % fish oil (Diet II), or 3% fish oil (rich in n-3 FA; Diet III). Chicks were fed identical diets.

Shown is LTB_4 production (*ex vivo*) by 5 x 10^6 platelets from 21-day-old chicks following stimulation with 20 μM calcium ionophore A23187 at 37°C for 10 minutes.

Each bar represents the mean ± SEM (n=4). Bars with different letters are significantly different ($p \leq 0.05$).

4.4 DISCUSSION AND CONCLUSIONS

The purpose of the current study was to investigate deposition of n-3 and n-6 FA in breeder hen tissues and cells after feeding diets that contained varied ratios of n-6 to n-3 FA. After consuming diets for 6 weeks, a DTH test was performed, and LT production by isolated platelets was assessed in breeder hens. We also examined the effect of feeding diets that contained varied ratios of n-6 to n-3 FA to breeder hens on hatched chick tissue FA composition. All chicks were fed a common ration. The goal of the study was to determine how long changes in tissue FA composition persisted based on the maternal diets. FA composition of chick tissues, and LT production by platelets, were assessed in chicks at 7, 14, and 21 days of age.

Tissue and cell membrane PUFA composition is largely influenced by dietary FA intake. Thus, these results are in agreement with previous reported research in that alterations of dietary PUFA intake serve as a means of altering tissue lipid composition in birds and hatched chicks (Cherian et al., 1997). Tissue AA, a desaturation-elongation product of dietary LA, serves as a precursor for eicosanoids, i.e., the prostaglandins (PG) and leukotrienes (LT) collectively. Alterations in dietary lipids provide a non-pharmacologic means of altering eicosanoid synthesis. Ingestion of fish oil can modify tissue PUFA content by increasing tissue EPA and DHA and reducing AA content. This tissue alteration changes eicosanoid synthesis because EPA competes with AA as a substrate for eicosanoid formation (Calder, 2001a).

Fatty acid composition of platelets and bursa of breeder hens reflected the dietary FA composition of their respective diets. Diet III-fed birds had higher amounts of n-3 PUFA in their bursa as well as in their platelets when compared to Diet I fed birds.

The DTH responses in laboratory animals and dogs have been reported to be suppressed after consuming a diet enriched in n-3 FA (Hayashi et al., 1998; Wander et al., 1997). Kelley et al. (1991) reported a decreased DTH response after fish oil feeding in rats. Meydani and Dinarello (1993) reported a diminished DTH response in human subjects fed fish oil. The suppressed DTH response in this study in birds fed Diet III may be attributed to the less inflammatory activities of n-3 PUFA. N-3 PUFA influence MHC class I antigen expression, reduce biosynthesis of AA from LA, and decrease production of pro-inflammatory AA metabolites, such as LTB_4. Consumption of the n-3 PUFA enriched diet might also have led to reduced production of inflammatory cytokines, such as IL1, IL2 and TNF-α, which are important in cell-mediated immunity (Santoli et al., 1990).

It is known from various laboratory animal studies and from human trials that leukotriene production by immune cells can be altered by modulating the diet fatty acid composition. Stenson et al. (1984) reported on the production of LTB_4 from mouse neutrophils in the presence and absence of exogenous EPA. In the presence of exogenous EPA, the production of LTB_4 declined by 88%. Whelan et al. (1997) reported that inflammatory peritoneal macrophages from chickens lacked the capacity to produce leukotrienes either *in vivo*, following opsonized zymosan stimulation, or *in vitro*, in response to calcium ionophore A23187 stimulation. Fritsche et al. (1992) fed n-3 FA enriched diets to broilers and found that there was no significant difference in the amount of leukotrienes produced by chicken immune cells. Mooney et al. (1998) evaluated the effects of dietary n-6 to n-3 FA ratio on LTB_4 synthesis in dog skin. Neutrophils from dogs fed diets with an n-6 to n-3 FA ratio of 5:1 and 10:1 synthesized 30 to 33% less LTB_4 and 370 to 500 % more LTB_5 after 6 to 12 weeks of feeding. In another study, it was shown

that alterations in total fat, as well as n-3 to n-6 FA ratios, influenced eicosanoid production in mice. Broughton and Wade (2002) reported that n-6 PUFA decreased series-four leukotriene production by 37% as the fat intake increased from 5 to 20g per 100g diet. Increased n-3 PUFA ingestion led to increased five-series leukotriene production.

Activation of phospholipase A_2 in plasma membranes of immune cells would initiate a cascade of reactions in which n-3 and n-6 PUFA competitively react with enzymes for conversion into different isomers of leukotrienes. EPA competes with AA for enzymes needed to be converted into prostaglandins and leukotrienes (Calder and Grimble, 2002). Eicosanoid biosynthesis depends upon the type of PUFA present in phospholipids of cell membranes, i.e., n-3 or n-6 PUFA (Broughton et al., 1991; Hwang et al., 1988). In this study, ingestion of diets enriched in n-3 PUFA decreased LTB_4 production and increased LTB_5 production in breeder hens, which can be explained on the basis of the above phenomenon.

Chicks at 7 and 14 days of age showed a similar trend, whereby fatty acids in their tissues reflected the diets fed to breeder hens. At 7 days there was more n-6 PUFA in tissues of chicks hatched from hens fed Diet I, which was rich in AA, and more n-3 PUFA in tissues of chicks hatched from hens fed Diet III, which was rich in EPA. Similar findings were present in 14-day-old chicks. Chicks at 21 days of age did not show any significant differences in the FA content of their tissues or cells. These results suggest that the potential for differences in eicosanoid production in 7- and 14-day-old chicks exists, because their fatty acid composition can be modified according to the FA composition of the maternal diet.

LTB_4 production was noted in platelets from all chicks at 7, 14 and 21 days of age, with 7- and 21-day-old chicks producing significantly more LTB_4 compared to 14-day-old chicks. Lesser quantities of LTB_4 were produced by 14-day-old chicks. Seven and 14-day-old chicks hatched to hens fed Diet III were able to produce a significant amount of LTB_5 compared to chicks hatched from hens fed Diets I and II. Chicks at 21 days of age were unable to produce LTB_5 because platelets lacked significant quantities of EPA, which is the precursor for LTB_5, at 21 days.

Overall, this data indicates that there is an integral relationship between maternal diet and FA content of chicks' tissue (spleen and bursa). Retention of n-3 PUFA in platelets was possible up to 21 days of age in chicks, and was able to affect leukotriene production in chicks. These findings show that modulating the maternal dietary PUFA composition, and, thereby yolk fatty acid composition, can change the type of LT produced in the progeny for the first few weeks of life. Chicks from hens fed a diet rich in n-3 FA at 7 and 14 days of age were able to produce increased amounts of LTB_5. Given the importance of leukotrienes in human and bird health, the role of maternal diet in manipulating biosynthetic capabilities needs further attention.

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CHAPTER 5. CONCLUDING THOUGHTS

In poultry, the fatty acid composition of tissue changes in response to altering the fatty acid composition of the diet. Furthermore, it is possible to alter the chick's tissue fatty acid composition through maternal dietary modulation. The fatty acids are deposited in all tissues including platelets. The n-6 PUFA arachidonic acid (AA) is the precursor of proinflammatory prostaglandins and leukotrienes, which have important roles in inflammation and in the regulation of immunity. Fish oil contains the n-3 PUFA eicosapentaenoic acid (EPA). Feeding fish oil results in partial replacement of AA in cell membranes by EPA. This leads to decreased production of AA-derived mediators and, thus, EPA can serve as a precursor of less inflammatory prostaglandins and leukotrienes.

Eicosanoids such as LTB_4 and LTB_5 affect the function of inflammatory cells, including white blood cells, neutrophils and thrombocytes (platelets). These cells interact to produce both inflammatory and anti-inflammatory lipid mediators during disease. The contribution of platelets to arachidonic acid transcellular metabolism may represent an important pathway of modifying leukotriene production.

The results of this study indicate that horse and dog neutrophils and chicken platelets readily synthesize and release large quantities of LTB_4 following stimulation with low micromolar concentrations of calcium ionophore A23187. These results also suggest that regardless of species, cells were able to produce a similar amount of LTB_4 from $5x10^6$ cells under similar conditions. Because inflammation plays a key role in the pathogenesis of many diseases of companion as well as production animals, increased understanding of the role that dietary modification plays in modifying the type of mediators produced, such

as LTB_4, may lead to an emphasis on nutritional therapy along with anti-inflammatory agents.

In this study it was shown that dietary fish-oil supplementation in breeder hens results in alterations in tissue deposition of EPA and DHA, and in a suppressed DTH response. Furthermore, (and similar to what is reported in the literature) it was shown that dietary supplementation of n-3 fatty acids to hens can modulate the fat depot in yolk and can affect the deposition of EPA and DHA in tissues of hatching chicks. Chick tissue fatty-acid composition reflected the fatty acid profile of maternal diets. Because of the deposition of specific fatty acids in spleen and bursal tissue (important immune organs), chicks were able to synthesize different amounts of LTB_4 and LTB_5 at later stages of their life. In 7-day-old chicks, LTB_4 as well as LTB_5 production was observed. In 21-day-old chicks, there was no production of LTB_5 by isolated platelets, likely because the n-3 fatty acid depot in tissues was depleted. This study shows that we can have an impact on hatched chicks by modulating the maternal diet, as evidenced by differences in production of LTB_4 and LTB_5. This is important because clinical studies have reported that fish oil supplementation has beneficial effects for a range of inflammatory conditions in animals as well as in humans, supporting the idea that the n-3 FA in fish oil are anti-inflammatory and immunomodulatory in action.

The goal of this project was to isolate heterophils from chicken whole blood, but unintentionally platelets were isolated. The major LT produced from platelets is LTC_4 and to our knowledge, nobody has investigated production of LTB_4 from stimulated chicken platelets under a variety of experimental conditions, such as incubation time (2.5, 5, 10, 15 or 20 minutes), temperature (25 or 37°C), and calcium ionophore A23187 concentration

(0.1, 1, 10 or 20 μM). This study demonstrates the likely presence of LTA_4 hydrolase in chicken platelets, and that it is responsible for the production of LTB_4 upon stimulation of platelets with calcium ionophore A23187.

It would be interesting to isolate heterophils from chicken whole blood in future studies to see if heterophils perform similar to platelets in leukotriene production under different experimental conditions e.g., time of incubation, temperature of incubation and concentration of calcium ionophore A23187. Furthermore, it is advised that the RP-HPLC fractions of leukotriene should be examined using mass spectrometry to confirm the isomers LTB_4 and LTB_5 by mass to charge ratio comparison. Given the significance of dietary n-3 FA in altering human and animal inflammatory disorders, the role of maternal dietary manipulation with n-3 FA in hens, on progeny's productivity and health, needs further attention.

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