# Effect of Dietary Fatty Acids, Time of Feeding and Immune Response in Poultry

by
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Abstract approved:

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Gita Cherian

In the presence of internal or external stressors, the body requires sources of energy that aide cells to combat inflammation. Fatty acids are an important source of energy and are vital components of cell membranes. Dietary fatty acids (n-6 and n-3) are of importance in immune function because they are precursors to metabolites that are potent mediators of inflammation. Poultry diets are high in n-6 fatty acids, which exert pro-inflammatory effects, and low in n-3 fatty acids, which tend to be less inflammatory. Delayed access to feed after hatching has been reported to impact the development of organs associated with immunity. Based on this information, two experiments were conducted in broiler chickens to determine the effect of dietary polyunsaturated fatty acid (PUFA) and the influence of time of feeding on bird performance, tissue lipid status, immune responses and expression of cyclooxygenase 2 (COX-2) upon challenge.

In experiment 1, birds were fed diets that contained 3.5% oxidized yellow grease (low n-3) or 3.5% canola oil (high n-3). Birds were fed early (<5 hrs post-hatch) or late (>24 hrs post-hatch). Intramuscular injection of lipopolysaccharide (LPS) or phosphate buffered saline (control) was used as the immune challenge. Feeding high n-3 diets

resulted in an increase in n-3 fatty acids in the liver in all treatment groups upon LPS challenge ($P<0.05$). LPS injection led to a decrease in total n-6 fatty acids in the liver when compared with control birds ($P<0.05$) fed early high n-3 and late low n-3. However, in spleen tissue, upon LPS challenge, increase in total n-3 fatty acids was observed only in birds fed early high n-3 and birds fed late high n-3. Plasma non-esterified fatty acids were lowest in high n-3 birds fed early ($P<0.05$). The spleen tissue total fat content was lowest in early high and late high n-3 birds ($P<0.05$). Breast muscle thiobarbituric acid reactive substances (TBARS) were higher in birds fed high n-3 compared to birds fed diets low in n-3 ($P< 0.05$). The delayed type hypersensitivity response was higher in birds fed high n-3 diet when compared to all other treatments ($P<0.05$). Thigh muscle of LPS challenged birds from early low n-3 and late high n-3 was significantly higher in TBARS when compared to control birds ($P<0.05$). There was no difference in final body weight, cut-up yield and organ weight of birds ($P>0.05$) except liver and thigh muscle weight percents were lowest in birds fed low n-3 ($P<0.05$).

In the second experiment, birds were fed diets containing 3.5% sunflower oil (low n-3) or 3.5% fish oil (high n-3). The birds were either fed early (<5 hrs post hatch) or late (>48 hrs post-hatch). No effect due to time of feeding was observed ($P>0.05$). Birds fed high n-3 diets had higher C20:5n-3, C22:5n-3, C22:6n-3 and total n-3 fatty acids and birds that were fed a low n-3 diet had higher levels of total n-6 PUFAs ($P<0.05$). LPS challenged led to a decrease in spleen C22:5n-3 of birds fed late high n-3 when compared to control birds within the same treatment group ($P<0.05$). LPS challenged birds showed an increase in C20:4n-6, total polyunsaturated fats and total n-6 fatty acids in birds that were late fed low n-3 compared to control birds within the same treatment group

($P<0.05$). LPS challenged birds from early and late high n-3 had higher liver total saturated fats when compared to control birds of the same diet ($P<0.05$). LPS challenge led to an increase in liver total n-6 fatty acids in birds fed late low n-3 when compared to control birds within the same treatment ($P<0.05$). LPS birds from early and late high n-3 diets were higher in total liver n-3 fatty acid content when compared to birds fed low n-3 diets ($P<0.05$).

Plasma isoprostanes showed no difference among treatment groups ($P>0.05$). Liver vitamin E was higher in control birds from early high n-3 groups when compared to the other treatments ($P<0.05$). Plasma vitamin E was highest in early low n-3 upon challenge when compared to the other treatments ($P<0.05$). LPS challenge resulted in an increase in vitamin E in the lung, small intestine and plasma of low n-3 birds. COX-2 expression in the spleen tissue increased due to LPS challenge. Time of feeding and diet had a significant effect on COX-2 protein expression ($P<0.05$).

These results indicate that type of dietary fat and time of feeding may alter the inflammatory response upon challenge in broiler birds. During inflammation, lipid substrates for the activated immune system are provided by fatty acids. Therefore, dietary management strategies directed at attenuating immune tissue lipid content may prove to be beneficial in enhancing bird health and in increasing production performance in broiler chickens.

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APPROVED:

Major Professor, representing Animal Science

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Dean of the Graduate School

I understand that my thesis will become part of the permanent collection of Oregon State University libraries. My signature below authorizes the release of my thesis to any reader upon request.

Dulmelis Gonzalez, Author

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# ABBREVIATIONS

| | |
|---|---|
| AA: | Arachidonic acid |
| Ab: | Antibody |
| ALA: | $\alpha$-linolenic acid |
| ANOVA: | Analysis of Variance |
| ATP: | Adenosine triphosphate |
| B-cell: | B lymphocyte (originates in bone marrow) |
| BSA: | Bovine serum albumin |
| BHT: | Butylated hydroxytoulene |
| CoA: | Coenzyme A |
| COX: | Cyclooxygenase |
| D: | Diversity (gene segments) |
| DHA: | Docosahexaenoic acid |
| DTH: | Delayed type hypersensitivity |
| EDTA: | Ethylenediaminetetraacetic acid |
| EFA: | Essential fatty acid |
| EPA: | Eicosapentaenoic acid |
| $FADH_2$: | Flavine-adenine dinucleotide (reduced) |
| FAME: | Fatty acid methyl ester |
| FID: | Flame inonization detector |
| FO: | Fish oil |
| GALT: | Gut associated lymphoid tissue |
| GLA: | Gamma linolenic acid |
| HETE: | Hydroperoxytetraenoic acid |
| HNF: | Hepatic nuclear factor |
| HPLC: | High pressure liquid chromatography |
| HPETE: | Hydroperoxyeicosatetraenoic acid |
| Ig: | Immunoglobulin |
| IL: | Interleukin |
| IFN-$\gamma$: | Interferon gamma |
| IS: | Internal standard |
| IU: | International unit |
| IUPAC: | International Union of Pure and Applied Chemistry |
| J: | Joining (gene segments) |
| LA: | Linoleic acid |
| LCFA: | Long-chain fatty acid |
| LCPUFA: | Long-chain polyunsaturated fatty acid |
| LOX: | Lipoxygenase |
| LPS: | Lipopolysaccharide |
| LT: | Leukotriene |
| LXR: | Liver X receptor |
| MUFA: | Monounsaturated fatty acid |
| n-3: | Omega 3 |
| n-6: | Omega 6 |

| | |
|---|---|
| NADH: | Nicotinamide adenine dinucleotide (reduced) |
| NEFAs: | Non-sterified fatty acids |
| NO: | Nitric oxide |
| NRC: | National research council |
| OD: | Optical density |
| PAF: | Platelet activator factor |
| PBS: | Phosphate buffered saline |
| PG: | Prostaglandin |
| $PGH_2$: | Prostaglandin endoperoxide $H_2$ |
| PGHS: | Prostaglandin endoperoxidase H synthase enzymes |
| PGI: | Prostaglandin I or Prostacyclin |
| PLA2: | Phospholipase-2 |
| PPAR: | Peroxisome proliferator activated factor |
| PUFA: | Polyunsaturated fatty acid |
| PVDF: | Polyvinylidene fluoride |
| RNS: | Reactive nitrogen species |
| ROS: | Reactive oxygen species |
| RXR: | Retinoid X receptor |
| SDS-PAGE: | Sodium dodecyl sulfate polyacrylamide gel electrophoresis |
| SEM: | Standard error of mean |
| SREBP: | Sterol regulatory element binding protein |
| TBA: | Thiobarbituric acid |
| TBARS: | Thiobarbituric acid reactive species |
| TBST: | Tris-buffered saline tween-20 |
| T-cell: | Thymus lymphocyte |
| TCR: | T-cell receptor |
| TMUFA: | Total monounsaturated fatty acid |
| Tn-3: | Total omega 3 |
| Tn-6: | Total omega 6 |
| TNF-α: | Tumor necrosis factor α |
| TPUFA: | Total polyunsaturated fatty acid |
| TSFA: | Total saturated fatty acid |
| TX: | Thromboxane |
| V: | Variable (gene segments) |
| VLCFAs: | Very long chain fatty acids |
| YG: | Yellow grease |

# 1. Introduction

In modern poultry industry practices, hatchlings spend a period of time without exposure to feed or water. The waiting period will depend on factors such as transport availability, distance to production facility and hatchery practice. During this time, the chicks are dependent on the nutrients remaining from the yolk sac to maintain metabolism, immune functions and skeletal muscle development. The developmental events important for immuno-competence in chickens are initiated in the pre and early post-hatch period (Kaspers *et al*., 1996; Chen *et al.*, 1996). The early post hatch is a period of rapid increase in leukocyte populations, seeding of lymphoid organs, and formation of unique clones of lymphocytes that will mediate immunity later in life (Klasing, 1998).

Among other nutrients, fats and oils are important sources of energy that influence metabolism and immune development. Fats are important for thermal insulation, absorption of fat soluble vitamins, nerve tissue constitution, and cell membrane composition. The development, function and maintenance of the immune system depend on adequate nutrition, as well as composition of dietary fatty acids. Identification of fatty acids is defined by the presence, position and number of the double bonds. Some of these fatty acids are essential in mammals and cannot be synthesized endogenously. Therefore, they need to be present in the diet. The two essential fatty acids are linoleic (C18:2n-6) and α-linolenic (C18:3n-3) acids. Polyunsaturated fatty acids of the n-6 series are derived from linoleic acid (LA), gamma linoleic acid (GLA) and arachidonic acid (AA). In contrast, polyunsaturated fatty acids (PUFAs) of the n-3 series derived from alpha linolenic acid (ALA) are

eicosapentaenoic acid (EPA), docosanpentaenoic (C22:5n-3) and docosahexaenoic acid (DHA).

Fatty acids are important constituents of immune cell function by contributing to changes in molecular composition of signaling platforms called lipid rafts. Immune cells are rich in PUFAs. Fatty acids also interfere with nuclear receptor activation (e.g. peroxisome proliferator-activated receptor) and eicosanoid formation (Stulnig, 2003). Eicosanoids are 20-carbon metabolites that are derived from n-6 and n-3 PUFA. AA and EPA, lead to formation of eicosanoids such as prostaglandins (PGs), prostacyclins (PGIs), thromboxanes (TXs). This is done via pathways involving cyclooxygenase (COX). Other metabolites that are synthesized from n-6 and n-3 PUFA are known as leukotrienes (LTs), which are produced via lipoxygenase (LOX). Synthesis of eicosanoids is mediated via enzymes phospholipase $A_2$ and cyclooxygenase-1 (COX-1) (housekeeping) or cyclooxygenase-2 (COX-2) (induced) due to activation by acetylcholine, stressors, or humoral responses. Due to competition for the enzyme's binding site, eicosanoid activity depends on the ratio and content of n-6 and n-3 fatty acids (Calder, 1998). n-6 PUFAs exert pro-inflammatory properties which lead to increased inflammatory eicosanoid, cytokine production and immuno-suppression. In contrast, n-3 PUFAs possess anti-inflammatory or less inflammatory properties by decreasing the release of pro-inflammatory eicosanoids and cytokines (Stulnig, 2003). Therefore, dietary supply of n-3 PUFA during early post hatch may impact the development of a strong immune system that can quickly and efficiently adapt to the different environments.

Metabolic and inflammatory problems are major causes of morbidity and mortality in broiler production. Understanding the role of dietary n-3 PUFA and early feeding in modulating the immune system in birds may increase our knowledge in increasing poultry productivity, reducing disease and thereby contributing to increased economic returns to the US poultry industry.

**Thesis Objective:**

To investigate the effects of dietary n-3 PUFA, time of feeding and inflammatory challenge on:

1) Bird performance
2) Tissue fatty acid composition and content of lipid oxidation products
3) Tissue vitamin E status
4) Expression of COX-2 protein in the immune tissue of broiler birds

**It is hypothesized that:**

1) Delayed feeding and immune challenge will affect:
    a) Bird performance
    b) n-6/n-3 PUFA and vitamin E content of tissues
    c) Induce expression of inflammatory proteins in organs of the immune system upon challenge

2) Substitution of n-3 PUFA in the late-fed birds may protect them from n-3 PUFA depletion upon challenge.

# 2. Literature Review

## 2.1 Fats in Poultry Diets

A standard broiler diet is composed of corn-soybean meal, oils (fats) and other essential nutrients such as vitamins and minerals. Fats in poultry diets are a good source of energy and are involved in several biological functions. Broiler diets contain mainly a mixture of fats and oils. Fats come from animal sources (e.g. tallow), and plant oil blends composed of hydrogenated vegetable cooking fats and oils (e.g. restaurant grease).

### 2.1.1 Definition of Fats

Compounds that are generally insoluble in water but soluble in organic solvents are categorized as fats. The chemical composition of fats includes tri-esters of glycerol and fatty acids. Fats may be either liquid or solid at room temperature.

### 2.1.2 Role of Fats

Fats (lipids) are important molecules that serve both structural and metabolic functions of animal systems (Leeson and Summers, 2001). Lipids play important roles as part of poultry diets both physically and physiologically. From a physical aspect, fats are associated with processes related improvement of feed quality, reduction of dust in feed, decreased feed particle separation, increased palatability, digestive lubrication (i.e. emulsification and rate of passage), and increased feed digestibility. From a physiological aspect, lipids play roles in processes associated with energy production, nutrient transport, and cellular structure (Leeson and Summers, 2001).

### 2.1.3 Composition of Fats

Triglycerides of different fatty acid profiles are usually a term in reference to fats and oils. Glycerol esters which are solid at room temperature are called fats and those which are liquid at room temperature are called oils. Lipids are classified into 3 different categories: simple lipids, compound lipids, and derived lipids (Leeson and Summers, 2001).

***Simple Lipids***: These are simple esters of fatty acids and some alcohols. Waxes are included in this group, which are fatty acid esters and have little nutritional value.

***Compound Lipids***: Compound lipids are esters of glycerol that have 2 fatty acid residues in addition to another chemical group (i.e. phospholipids).

***Derived Lipids***: This group includes substances that are derived from fatty acids, alcohols and sterols (Leeson and Summers, 2001).

### 2.1.4 Fatty Acid Classification

Fatty acids are classified based on the number of carbons (C) present in the chain. Short chain fatty acids are usually 2-8 C in length (important in ruminant nutrition, i.e. propionate, butyrate, and acetate). Medium chain fatty acids are usually 10 to 12 C in length, e.g. lauric acid. Long chain even number fatty acids are usually 14 to 20 C and include palmitic, stearic, EPA and AA. Very long chain fatty acids are 22 to 24 C in length and include docosapentaenoic, and DHA (Heird and Lapillonne, 2005). The trivial name, systemic name and designation of fatty acids are shown in table 2.1.

### 2.1.5 Saturated vs. Unsaturated Fatty Acids

Fatty acids are classified as saturated or unsaturated according to the absence or presence of double bonds. Fatty acids such as stearic acid, which possess no double bonds are classified as saturated. Monounsaturated fatty acids contain only one double bond, such as, oleic acid. PUFAs, such as EPA and AA, contain three or more double bonds (Heird and Lapillonne, 2005). PUFAs are divided into two different groups, omega 3 (n-3) and omega 6 (n-6), according to the positioning of the first double bond relative to the terminal methyl end of the molecule.

### 2.1.6 Functions of Polyunsaturated Fatty Acids

PUFAs affect physiological and pathophysiological processes leading to effects in health. Metabolic function, plasma lipid levels, neuronal development, regulation of the immune system, cardiovascular function, and insulin action have been closely related to PUFAs (Stulnig, 2003).

### 2.1.7 Fatty Acid Supplementation in Poultry Diets

Commercial diets vary in composition, but they possess the basic nutrients to provide chicks with the appropriate level of nutrients required for growth. Some common sources for LA in poultry diets are corn, safflower, soybean, and sunflower oils. ALA can be obtained from sources that come from flaxseed oil and canola oil (Shaikh and Edidin, 2006).

### 2.1.8 Essential Fatty Acids

Mammals are not able to endogenously synthesize either (LA, 18:2n-6) nor (ALA, 18:3n-3); thus they are considered essential fatty acids (EFAs). Diet is the source for intake of EFAs (Shaikh and Edidin, 2006). Some sources of LA are present in Table 2.1

LA, 18:2n-6 has two double bonds, the first between the sixth and seventh carbon from the methyl terminal. Conversion of LA to AA can be a precursor to other bioactive metabolites and molecules with great physiological effects.

ALA, 18:3n-3 has three double bonds. The first double bond is between the third and the fourth carbon from the methyl terminal. Further elongation and conversion of ALA can be a precursor to other bioactive metabolites with great physiological effects.

**Table 2.1 Common Fatty Acid Nomenclature and Sources (Adopted from Stulnig, 2003)**

| Trivial Name | Short Designation | Systemic name | Sources |
|---|---|---|---|
| **Saturated Fatty Acids** | | | |
| Palmitic | 16:0 | n-hexadecanoic | De novo synthesis, milk, eggs animal fats and meat, cocoa butter, palm oil and fish oils |
| Stearic | 18:0 | n-octadecanoic | De novo synthesis, milk, eggs animal fats and meat, cocoa butter |
| **Monounsaturated Fatty Acids** | | | |
| Palmitoleic | 16:1n-7 | Cis-9-hexadecenoic | Desaturation of palmitic acid, fish oil |
| Oleic | 18:1n-9 | Cis-9-octadecenoic | Desaturation of stearic acid, milk, eggs animal fats and meat, cocoa butter, most vegetable oils |
| **PUFAs n-6** | | | |
| Linoleic | 18:2n-6 | Cis,cis-9,12-octadecadienoic | Cannot be synthesized in mammals. Present in milk, eggs animal fats and meat, cocoa butter, leafy greens, corn oil, sunflower safflower, and soybean oils |
| γ-Linolenic | 18:3n-6 | 6,9,12-octadecatrienoic | Synthesized from linoleic acid, borage, and evening primrose oils |
| Dihomo-γ-Linolenic | 20:3n-6 | 8,11,14-eicosatrienoic | Synthesized from γ-linolenic |
| Arachidonic | 20:4n-6 | 8,11,14,17-eicosatetraeinoic | Synthesized from linoleic via γ-linolenic and dihomo-γ-linolenic acid |
| **PUFAs n-3** | | | |
| α-Linolenic | 18:3n-3 | All-cis-9,12,15-octadecatrienoic | Cannot be synthesized in mammals. Present in leafy greens, soybean oil, canola and linseed oils |
| Eicosapentaenoic | 20:5n-3 | 5,8,11,14,17- eicosapentaenoic | Synthesized from α-linolenic, present in marine fish oils |
| Docosahexaenoic | 22:6n-3 | 4,7,10,13,16,19-docosahexaenoic | Synthesized from α-linolenic, present in marine fish oils |

## 2.2 The Avian Digestive Tract

The avian digestive tract is composed of several organs which are involved in feed digestion and immune function (Figure 2.1). The digestive tract includes an expansion of the esophagus known as the crop which leads to the proventiculus and gizzard. The small intestine includes the duodenal loop, which is enclosed by the pancreas. It is also composed of jejunum and ileum. A remnant attachment of the yolk sac and Meckel's diverticulum is found at the end of the jejunum. Valves at the end of the ileum control passage to two ceca. After some fermentation digesta is released into a short large intestine that empties into the cloaca and is eventually excreted through the vent (Sklan, 2005).

### 2.2.1 Avian Fat Digestion and Absorption

Digestion occurs at physical and chemical levels. The physical aspect of it involves grinding of food, i.e. stomach, gizzard, as well as peristaltic movement which aides with passage through the intestinal tract. In the chicken, digestion of fats occurs mainly in the small intestine. The digestion process involves the breakdown of large and complex molecules into smaller ones that are eventually absorbed into the blood. Chemical digestion involves the secretion of enzymes which help degrade food particles even further for molecular level absorption and transport (Leeson and Summers, 2001).

### 2.2.2 Digestive Fat Formation and Emulsification

Most lipids are ingested in the form of triglycerides, but in order to be absorbed through the intestinal epithelium they are broken down into fatty acids

(Leeson and Summers, 2001). Emulsification of fats by bile salts enhances its digestion. Pancreatic lipases are able to break down the emulsified fats into fatty acids and monoglycerides. Also, fatty acids and cholesterol are products of fat digestion and absorption by action of cholesterol esterase through hydrolysis of cholesterol ester fatty acids. These digestion products are assembled into micelles, which allow fatty acids and other lipid-like compounds to be transported and absorbed trough the plasma membrane (Berg *et al*., 2002). Once inside the enterocyte, the chylomicrons are formed which have a TG-rich core. The chylomicrons are secreted into the lymph system.

***Monoglycerides*** and short-chain fatty acids can be taken into portal circulation. Figure 2.2 illustrates fat digestion and absorption mechanisms.

***Triglycerides*** are one of the major components of fats and are composed of 3 different fatty acids esterified to a molecule of glycerol. They are hydrolyzed in the intestinal lumen and then reassembled within the enterocyte along with phospholipids, monoglycerides, diglycerides, and sterol esters, which are absorbed and eventually reach the blood stream (Heird and Lapillonne, 2005).

### 2.2.3 Fat Transport into the Blood

Triglycerides are reassembled from fatty acids and monoglycerol in intestinal mucosal cells. They are packaged into chylomicrons which are composed of droplets of non-polar lipid, triglycerols, and cholesterol esters and the molecule is surrounded by a protein membrane-like structure, cholesterol and phospholipids. The main

component of chylomicrons is apolipoprotein B-48. Chylomicrons play important roles in function and transport of fat soluble vitamins and cholesterol (Kendrick *et al*., 2001).

After chylomicrons are released into the lymph system and subsequently into the circulation these particles bind to membrane bound lipoprotein lipases in muscle and adipose tissues where triglyceride molecules are degraded back into fatty acids and monoglycerides. The triglycerides are re-synthesized and stored in the adipose tissue, which can be used later for production of energy through oxidation.

***Transport of Fatty Acids*** is done through binding to serum albumin which acts as a carrier. Fatty acids are then carried on serum albumin and are able to enter the blood stream and provide energy to other tissues.

**Figure 2.1 Avian Digestive Tract (Adopted from www.ca.uky.edu)**

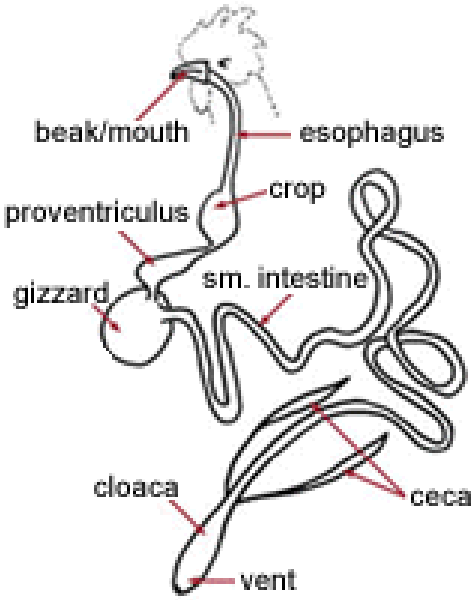



**Figure 2.2 Fat Digestion and Absorption (Adopted from Berg *et al*., 2002)**

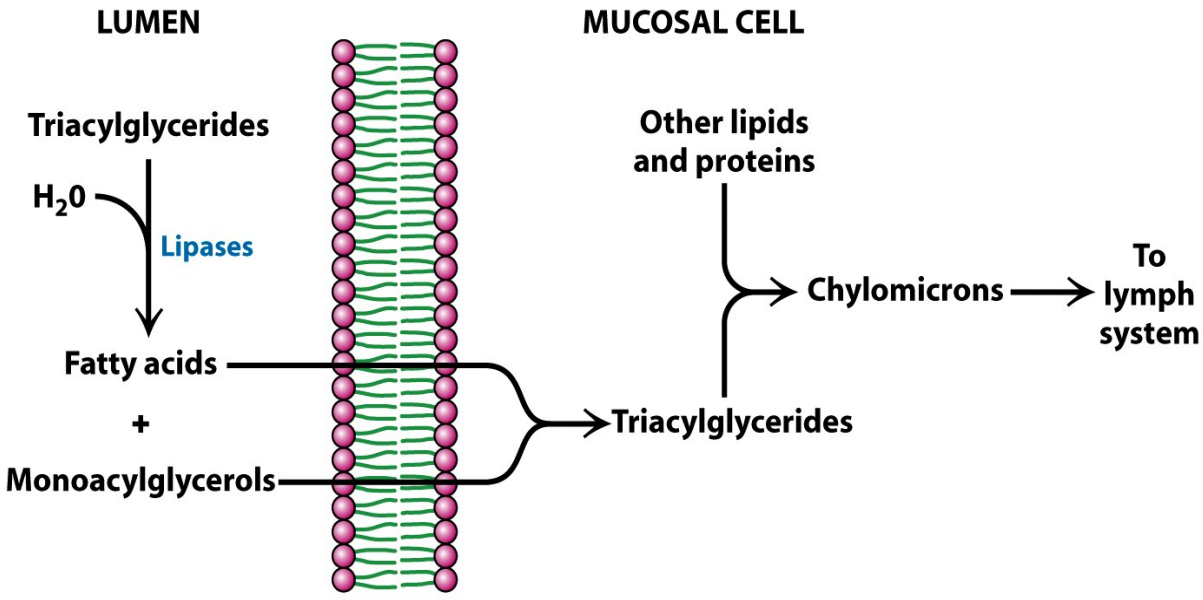



Figure 22-5
*Biochemistry, Sixth Edition*

## 2.3 Metabolic Functions of Fats

Fats are an important source of metabolic energy (triglycerides and cholesterol esters) (McArthur *et al*., 1999). PUFAs and monounsaturated fatty acids (MUFAs) can be stored as triglycerides in the body in the form of adipose tissues. The glycerol moiety of fats can be used as metabolic energy through its conversion to fructose and then glucose or converted to pyruvate through catabolic pathways in the body.

### 2.3.1 Role of Lipids in Membranes

Lipids have an important role in the structures of the cell membranes and organelles. Fats as part of cellular membrane composition play roles associated with integrity (rigidity and form) and functionality during many cellular processes (signaling, cellular development, gene expression) (McArthur *et al*., 1999).

***Phospholipids*** are a major component of cellular membranes. Phospholipids are comprised of fatty acids, a glycerol backbone, a phosphate and an alcohol attached to the phosphate. The phospholipid (polar head) portion provides a hydrophobic barrier while the rest of the molecule (tail) provides its hydrophilic properties allowing for external interaction (Berg *et al*., 2002). Phospholipid derivatives act as messenger molecules within cells during cell signaling process involved with cell proliferation, movement, differentiation and survival (Cooper and Hausman, 2004).

***Glycolipids*** are sugar-containing lipids. They are lipids consisting of two hydrocarbon chains linked to a polar head group containing carbohydrates (Berg *et al*., 2002). Glycolipids play important roles in cell signaling, and membrane transport.

***Cholesterol*** is a steroid built from four linked hydrocarbon rings (Berg *et al*., 2002). In membranes, cholesterol constitutes almost 25% of membrane lipids, which helps with membrane rigidity. Lipids such as cholesterol play important roles in hormone synthesis and sexual reproduction. Cholesterol is the precursor to steroid hormones such as testosterone and estradiol, which serve as chemical messengers involved in reproduction (Cooper and Hausman, 2004).

### 2.3.2 Other Roles of Lipids

Lipids also play important roles in the synthesis and metabolism of other important compounds in the body. Lipids synthesized in the intestine are involved in transport of fat and fat-soluble vitamins into the blood (Hussain, 2000). Fat soluble vitamins A, D, E and K have important roles in eye function, bone metabolism, lipid anti-oxidation, and blood clotting respectively.

Another important function of fats is the ability to become part of the structural composition of the body by providing protection of internal organs, maintenance of homeostasis, regulation, mediation, immune responses, insulation, and facilitation of nerve impulse conduction (Merrill *et al.,* 1996). Supplementation of fats, especially PUFAs, to poultry diets, has been closely associated with immune regulatory effects on both the innate and adaptive immunity through various mechanisms (dePablo *et al*., 2002).

### 2.3.3 Fatty Acid Metabolism

In order for peripheral tissues to utilize fatty acids as fuel, three stages of processing must take place. The first part of the process involves lipid mobilization to tissues that require the energy from fatty acids and glycerol that were released from adipose tissues. This process is initiated by a hormone-sensitive lipase. In these energy-requiring tissues, activation and transport of fatty acids into the mitochondria allows for further degradation into acetyl Coenzyme A (CoA), which is later used in the tricarboxylic acid cycle (Berg *et al*., 2002).

***Lipolysis*** is associated with the first step that is involved in fat utilization as an energy source. Lipase-sensitive hormones such as epinephrine, norepinephrine, glucagon, and adrenocorticotropic hormone trigger the activation of adenylate cyclase which initiates a cascade event that conclude in the making of free fatty acids and glycerol. Glycerol is absorbed by the liver, where it is made into glycerol-3-phosphate or dihydroacetone phosphate and can be used to make pyruvate or glucose (Berg *et al*., 2002).

### 2.3.4 Fatty Acid Oxidation

Due to the high content of stronger and more readily oxidizable bonds between carbon and hydrogen, fatty acids are able to provide a more efficient source of energy when compared to other nutrients (Jones and Kubow, 1999). Fatty acids can be oxidized in the mitochondrial matrix, in the peroxisome, and in the microsome, which is involved in oxidative metabolism (Cooper and Hausman, 2004).

#### 2.3.4.1 Mitochondrial Beta Oxidation

Beta (β)-oxidation is the process by which fatty acids, in the form of acyl-CoA molecules, are broken down in the mitochondria to generate acetyl-CoA (Berg *et al*., 2002). Fatty acids are activated outside the mitochondria, and then oxidized in the inner-mitochondrial matrix. Fatty acids must first be activated for degradation by being conjugated to acetyl-CoA. Long chain fatty acids and CoA derivatives are not able to enter the mitochondria, thus transport is made through conjugation with carnitine. Inside the mitochondria, β-oxidation occurs in four steps: dehydrogenation, hydration, dehydrogenation and thiolitic cleavage. Fatty acid oxidation yields one nicotinamide adenine dinucleotide, reduced (NADH) and one flavine-adenine dinucleotide, reduced ($FADH_2$) per acetyl CoA formed from acyl CoA. Additional metabolic reactions are required for fatty acids that have odd number of carbons or have unsaturated bonds (Berg *et al*., 2002).

#### 2.3.4.2 Peroxisomal β-Oxidation

Peroxisomal β-oxidation's primary function is to shorten very long chain fatty acids (VLCFAs) for degradation by the mitochondria. VLCFAs can be partly degraded and then attached to carnitine for transport to the mitochondria. One of the advantages in peroxisomal oxidation is that fatty acids can diffuse across the peroxisomal membrane thus not requiring a carrier molecule. One of the disadvantages is that peroxisomal oxidation is less efficient than mitochondrial oxidation because during oxidation, oxygen electrons are used to make hydrogen peroxide instead of $FADH_2$, creating less energy available for oxidation processes (Berg *et al*., 2002 and Jones and Kubow, 1999).

#### 2.3.4.3 Lipid Peroxidation

PUFAs are more susceptible to lipid peroxidation due to abundance of double bonds in fatty acid side chain, which allows for easier removal of hydrogen atoms. Cellular membranes are vulnerable to lipid peroxidation due to the high amount of fatty acids present. Lipid peroxidation is initiated by the attack on a fatty acid or a fatty acyl side chain by a reactive oxygen species and free radicals during regular metabolism becoming oxidized and turned into lipid peroxides (Halliwell and Chirico, 1993). Lipid hydroperoxide is one of the products from this reaction which can further decompose to an aldehyde, isoprostanes, and hydrocarbons (Kohen and Nyska, 2002). The highly unsaturated PUFAs in fish oils peroxidize at higher rates, which leads to shorter storage life. Reactive oxygen species (ROS) such as superoxide, hydroxyl radical, hydrogen peroxide, singlet oxygen and hypochlorous acid are generated during oxidation process in which a loss of electrons occurs. These oxygen derived pro-oxidants can cause damage to biological targets such as lipids, DNA, and proteins as well as the defending systems of the cell. Another example of a free radical involved in oxidative stress is nitric oxide ($NO^{\cdot}$), which is considered a reactive nitrogen species (RNS). Peroxynitrite, a derivative of $NO^{\cdot}$, has been associated with diseases such as atherosclerosis and ischemia as well as endothelial cell injury and hepatocellular necrosis. Inflammatory diseases and immune activation have also been associated with $NO^{\cdot}$ due to the capability of its enzyme, inducible nitric oxide synthase, to induce overstimulation of the expression of inflammatory enzyme proteins and pro-inflammatory agents (Halliwell and Chirico, 1993; Kohen and Nyska, 2002).

Oxidative stress due to endogenous and exogenous factors has been attributed to the increased formation of ROS. Another cause has been linked to the decreased intake of antioxidant agents such as vitamin E (α-tocopherol) which are able to terminate oxidation by donating a hydrogen atom to a lipid radical. The result of this is a chain braking effect on the propagation of lipid peroxidation.

### 2.3.5 Fatty Acid Synthesis

The synthesis of fatty acids is almost the same for all organisms. The first reaction in fatty acid synthesis involves the conversion of acetyl-CoA to malonyl-CoA and then the sequential combination to malonyl-CoA, which leads to palmitic acid (C16:0) as end product. In order to generate long chain fatty acids from palmitic acid, mammals require desaturases and elongases. Synthesis of fatty acids longer than 16 carbons occurs in the endoplasmic reticulum.

Desaturases are highly specific for the position of the double bond and they involve three components that are part of the microsomal membranes: desaturases, cytochrome $b_5$ NADH-cytochrome $b_5$ reductase, which provides electrons to desaturases. Desaturases $\Delta^9$, $\Delta^6$, $\Delta^5$, and $\Delta^4$ are involved in the desaturation reactions of unsaturated fatty acids derived from palmitate (C16:0), oleate (C18:1), linoleate (C18:2), and linolenate (C18:3). Different unsaturated fatty acids can be formed from combinations of elongation and desaturation reactions, i.e. oleate can be elongated to 20:1 cis-$\Delta^{11}$ fatty acid (Berg *et al*., 2002).

Plants are able to convert oleic acid into LA and to ALA through $\Delta^{12}$ and a $\Delta^{15}$ desaturases. Mammals are not able to synthesize LA (18:2 cis-$\Delta^9$, $\Delta^{12}$) and ALA (18:3

cis-$\Delta^9$, $\Delta^{12}$, $\Delta^{15}$) because they lack the enzymes that introduce double bonds beyond carbon 9 and the methyl end of fatty acids (Stulnig, 2003). Furthermore, desaturases and elongases in animal cells are able to convert ALA into EPA (20:5 n-3) and DHA (22:6 n-3) and LA to AA (Berg *et al*., 2002; Jones and Kubow, 1999). Table 2 shows a schematic representation of PUFA biosynthesis in the mammalian system.

**Table 2.2 Biosynthesis of Polyunsaturated Fatty Acids (Adopted from Kurlak & Stephenson, 1999)**

| n-6 acids | Enzymes | n-3 acids |
|---|---|---|
| 18:2 (9,12) Linoleic | | 18:3 (9,12,15) Alpha-linolenic |
| ↓ | | ↓ |
| 18:3 (6,9,12) Gamma-linolenic | Δ6-Desaturase | 18:4 (6,9,12,15) Stearidonic acid |
| ↓ | | ↓ |
| 20:3 (8,11,14) Dihomo-gamma-linolenic | Elongase | 20:4 (8,11,14,17) |
| ↓ | | ↓ |
| 20:4 (5,8,11,14) Arachidonic (AA) | Δ5-Desaturase | 20:5 (5,8,11,14,17) Eicosapentaenoic (EPA) |
| ↓ | | ↓ |
| 22:4 (7,10,13,16) | Elongase | 22:5 (7,10,13,16,19) Docosapentaenoic |
| ↓ | | ↓ |
| 22:5 (4,7,10,13,16) | | 22:6 (4,7,10,13,16,19) Docosahexaenoic (DHA) |

## 2.4 Fatty Acid Metabolite Formation

Metabolites that are derived from a large group of hormonally active, oxygenated fatty acids, $C_{18}$, $C_{20}$ are collectively known as eicosanoids, which includes prostanoids (PGs, TXs and PGIs), LTs, lipoxins, hepoxilins, monohydroxy fatty acids, isoprostanes, epoxy and monohydroxy fatty acids, isoleukotrienes, and other peroxidized fatty acid products (Funk, 2001; Smith *et al*., 2000).

Cellular modulation of cardiovascular, pulmonary, immune, reproductive, and secretory functions is mediated by eicosanoids. The effect of n-3 PUFAs on eicosanoid production has been associated with decreased inflammatory and anti-inflammatory actions. Thus, regulation of n-6 to n-3 ratios highly influences the production of different eicosanoids that will be involved in immune effects and inflammation. EPA both inhibits the production of AA-derived eicosanoids (E2) and acts as a precursor for less inflammatory eicosanoids (E3) (Yaqoob, 2003). The formation of different eicosanoids from n-6 and n-3 PUFA is shown in Figure 2.3.

Dietary modification of fatty acids has shown to play a key role in determining the action of immune cells by altering the different expression of eicosanoids, chemokines and gene expression factors. Fatty acid composition has been linked to the immune response due to its role as a component of the cellular membrane as well as being part of several mechanisms that lead to effective pathological and physiological effects in immunomodulation.

### 2.4.1 Prostaglandin Endoperoxide H Synthase Function

Biosynthesis of prostanoids involves three steps: First, a stimulus-initiated hydrolysis of a fatty acid from glycerophospholipids via phospholipase $A_2$, second, oxygenation to yield prostaglandin endoperoxide $H_2$ ($PGH_2$) via prostaglandin endoperoxidase H synthase enzymes 1 and 2 (PGHS-1 and 2) and last, conversion of $PGH_2$ to bioactive metabolites such as PGs, TXs and PGIs. The resulting products are used in activation of prostanoid G protein or nuclear receptor interaction (Murakami *et al*., 2002; Smith *et al*., 2000).

The committed step in prostanoid synthesis is catalyzed by prostaglandin endoperoxidase H synthase enzymes 1 and 2 (PGHS-1 and 2). It is also known as cyclooxygenase 1 and 2 (COX-1 and 2) isoforms. COX-1 is considered to be constitutive and COX-2 is considered to be inducible. Both enzymes catalyze a cyclooxygenase reaction in which a fatty acid, e.g. AA and 2 molecules of $O_2$, are converted to $PGG_2$ or reduced to PGHs via a peroxidase reaction by two electrons. The catalysis of cyclooxygenase reaction is peroxidase dependent, which requires the oxidation of its heme group by a hydroperoxide, (e.g. $PGG_2$). These enzymes are membrane bound and located inside the endoplasmic reticulum (Murakami *et al*., 2002; Smith *et al*., 2000).

AA is considered to be major prostanoid precursor, but EPA also produces prostanoid metabolites. PGs, PGIs and TXs are derived via COX pathway from AA ($PG_2$, $PGI_2$, and $TX_2$) or EPA ($PG_3$, $PGI_3$, and $TX_3$). Conversion of AA and EPA to chemically active molecules is shown in figure 2.3 Prostaglandins are classified from prostaglandin A (PGA) through prostacyclins (PGI) with different indications

denoting the number of double bonds (Berg *et al*., 2002; Stulnig, 2003; Smith *et al*., 2000).

### 2.4.2 Leukotriene Biosynthesis and Function

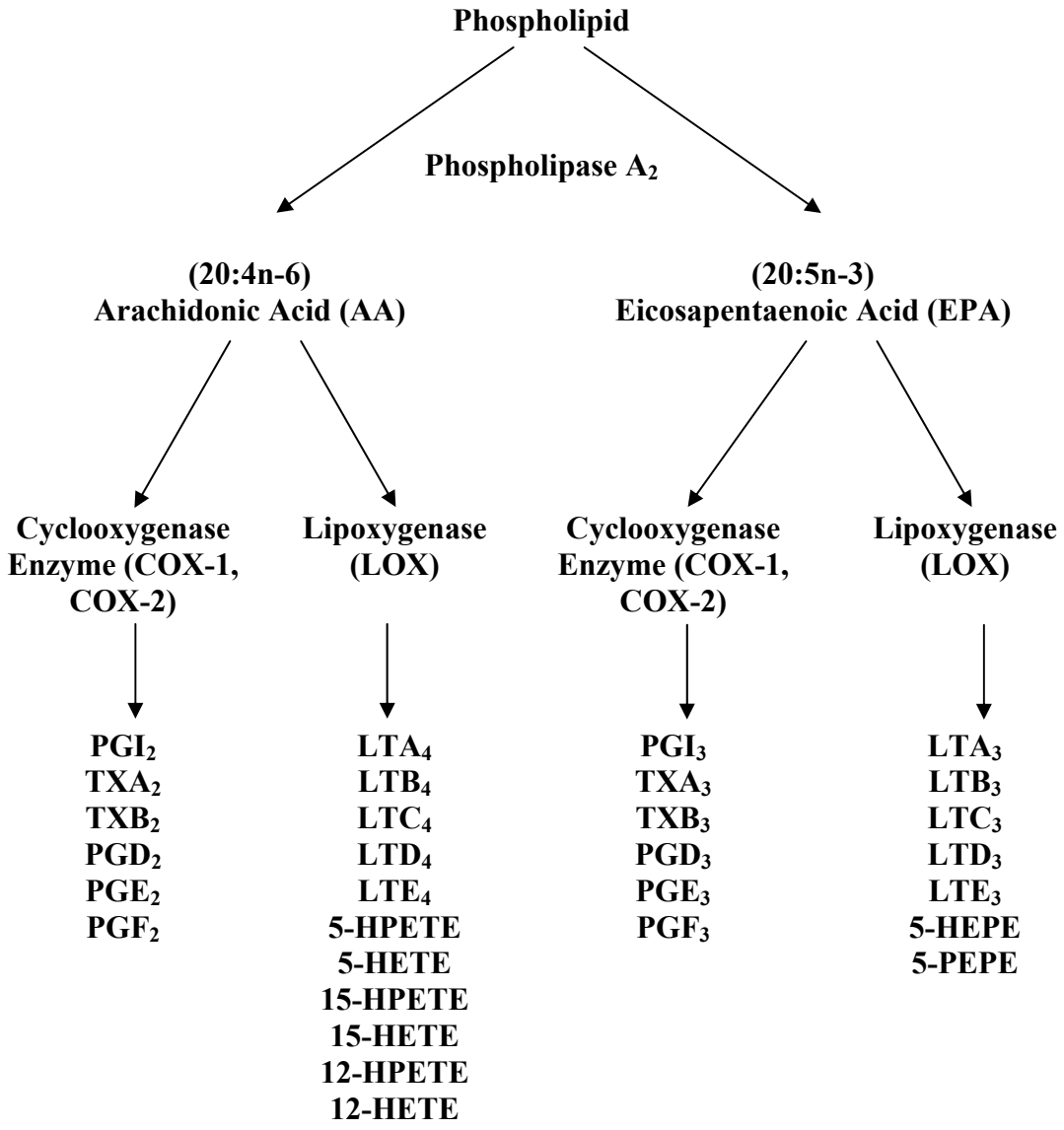
Another major pathway involved in production of AA metabolites is through 5-lipogenase (LOX) action. AA can be converted to leukotrienes, i.e., $LTB_4$, $LTC_4$, $LTD_4$, $LTE_4$, $HETE_5$, $HPETE_5$, $HETE1_2$, and $HETE_{15}$) and EPA can be converted into ($LTB_5$, $LTC_5$, $LTD_5$, and $LTE_5$). These metabolites are mainly found in leukocytes (Stulnig, 2003; Berg *et al*., 2002). Leukotrienes are considered to be responsible for the effects in inflammatory response and regulation of the body's response through different signaling processes. Leukotrienes ($LTD_4$ and $LTE_4$) have been considered to be potent activators of cystenyl leukotriene receptors which are involved in astrocyte proliferation and are predominant in brain tissue and linked to increased incidence of ischemia. $LTB_4$ has been associated with allergic responses and bronchio-constriction due to involvement in increased vascular permeability and neutrophil aggregation (Huang *et al*., 2008)

### 2.4.3 Prostaglandin and Leukotrienes: Effects on Membranes

Prostaglandins are related to mediation of inflammatory response and type I hypersensitivity reaction through inhibition of platelet aggregation, an increase in vascular permeability, and induction of smooth muscle contraction. Important inflammatory mediators (TXs, PGs, LTs, and platelet-activator factors) are produced by the breakdown of membrane phospholipids. Cells such as monocytes,

macrophages, and neutrophils generate different prostaglandins via COX enzymes (COX-1 and COX-2). COX-1 is synthesized at a constant rate, but expression of COX-2 is induced by inflammation via activation of inflammatory cells that produce PGs. COX-2 dependent biological functions have been found to be involved with ovulation, bone formation as well as various human diseases including inflammation, pain, fever, cancer, and Alzheimer's. Leukotrienes are produced by cells such as monocytes, macrophages and mast cells (Goldsby *et al*., 2000; Murakami *et al.,* 2002; Stulnig, 2003).

**Figure 2.3 Formation of Eicosanoids from Arachidonic Acid and Eicosapentaenoic Acid (Adopted from Calder, 1997)**




Abbreviations:

PG: Prostaglandin
TX: Thromboxane
LT: Leukotriene
HPETE: Hydoperoxyeicosatetraenoic acid
HETE: Hydroperoxytetraenoic acid

## 2.5 Fats and Inflammation

Inflammation is a physiological response to different stimuli that may be related to tissue damage, irritation, and infections. Inflammation can be characterized by pain, swelling, redness, heat, and in some cases, loss of function. A rapid increase in levels of a certain plasma protein, known as acute phase protein, is one of the first responses to acute inflammation (Goldsby *et al*., 2000). Dietary fatty acids have been associated with immuno-regulatory responses through chemical changes as well as structural modification of cell membranes and receptors. PUFAs are also important for modulation of immune responses and play essential roles in inflammation. PUFAs have been associated with alterations in T lymphocyte signaling through a change in the composition of lipid rafts. PUFAs alter eicosanoid synthesis of PGs and LTs, as well as affecting in activation of nuclear receptors such as peroxisomal proliferator-activated receptors (PPAR) and liver X-receptors (LXR) (Stulnig, 2003).

### 2.5.1 Role of Essential Fatty Acids Metabolites on Gene Expression

Lipid metabolism has also been associated with diverse actions seen by peroxisome proliferator-activated receptors (PPARs), which are ligand activated transcription factors in different cell types. Metabolites from n-3 PUFAs, eicosanoids, have been known to suppress hepatic lipogenesis. These eicosanoids are molecules that have been termed lipid mediators because they have many roles in intracellular regulation involving activation of enzymes at specific sites. Eicosanoids are also known to induce peroxisomal and microsomal oxidization by regulating transcription factors that include PPARs, LXRs, sterol regulatory element binding protein (SREBP

1 or 2) (Jump *et al.*, 2005; Bandeira-Melo *et al.*, 2002). Long chain fatty acids such as EPA and DHA demonstrate regulatory properties of the ligands that bind to PPARs. PPARs influence cellular proliferation, differentiation and apoptosis through the regulation of lipid and lipoprotein metabolism as well as glucose homeostasis. It has been suggested that PPARα and PPARγ interact with fatty acid-binding proteins to direct ligands to their responsive genes which would represent a mechanism for how cellular fatty acids could be directed to interact with target genes (Jump *et al*., 2005; Yaqoob, 2003). PPARα is known to regulate genes that regulate lipid metabolism including fatty acid transport, fatty acid-binding proteins, fatty acid acyl-CoA synthesis, microsomal, peroxisomal and mitochondrial oxidation, ketogenesis, and $\Delta^9$, $\Delta^6$, $\Delta^5$ desaturation. PPARγ is also expressed in tissues such as adipose, muscle and vascular cells. It induces lipoprotein lipase, fatty acid transporters and enhances adipocyte differentiation and inhibits nuclear factor kappa-light-chain-enhancer of activated B cells ($NF_kB$) function and expression of COX-2 and other cytokines (Jump, 2001).

### 2.5.2 Essential Fatty Acid Effects on Cytokines and Inflammatory Mediators

Cytokines play significant roles throughout the development of acute or chronic inflammation by exertion of different effects on lymphocytes and other immune cells. Cytokines produced by white blood cells serve as regulators to the whole body in response to infection and injury. Eicosanoids play an essential role in modulating inflammatory response intensity and duration. They are involved in the increase in vascular permeability and vasodilation which enhances the production of

inflammatory cytokines: tumor necrosis factor (TNF-α), interleukins-1β, 6 and 8 (IL-1β, IL-6, and IL-8). Eicosanoids are potent chemotactic agents for leukocytes. n-3 fatty acids, especially DHA and EPA, have demonstrated anti-inflammatory properties by inhibiting the production of IL-1β and TNF-α as well as IL-6 and IL-8. DHA and EPA also decrease cytokine-mediated induction of the expression of COX-2 gene. n-3 PUFAs can act directly by either replacing or inhibiting AA eicosanoid production and metabolism, as well as indirectly by altering the expression of inflammatory genes (Calder, 2002).

### 2.5.3 Non-Esterified Fatty Acids (NEFA) and Inflammation

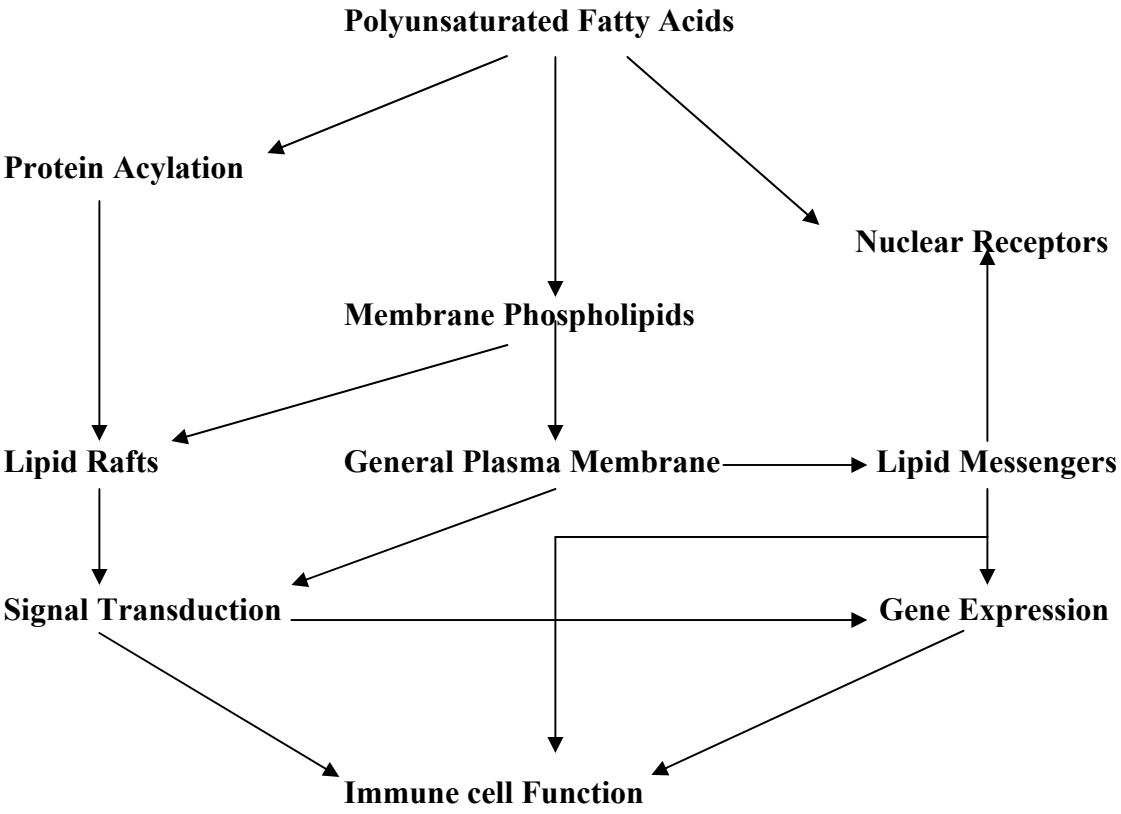
When fatty acids are not sterified to molecules such as glycerol or cholesterol they are called "free" fatty acids also known as non-esterified fatty acids (NEFAs) (Stulnig *et al*., 2000). It has been suggested that the predominant type of PUFA "free" fatty levels play essential roles in cellular functions such as activation, degranulation and cytolytic function of lymphocytes; as well as stimulation of macrophages, cytokines, growth factors and antigens (Calder, 1998; Stulnig *et al*., 2000; Brassard *et al*., 2007). NEFAs are essential for glucose and fatty acid metabolism as well as to provide cells with energy (e.g. ATP). Previous studies have shown that an increase in NEFA concentration due to increased lipolysis can induce oxidative stress and have pro-inflammatory effects (Stulnig *et al*., 2000). NEFAs likely play important roles in inflammation due to their influence on T-cell functions via modulation of eicosanoid metabolism by producing PG, TX and LT intermediates that act directly on T-cells. Other possible mechanisms are based on NEFA modulation of gene expression by

interacting with PPARγ. NEFA interferes with intracellular signaling pathways after activation of T-cell receptor and CD28; as well as NEFA modification of lipid membrane composition and raft organization. This interference can affect protein scaffolding and function of the transmembrane receptor (Brassard *et al*., 2007). Assessing NEFAs, although complex, can give an interpretation of *in vivo* modulation, which can be linked to immune function and responses in the presence of different PUFA (Brassard *et al*., 2007)

### 2.5.4 Fatty Acid Effects on Delayed Type Hypersensitivity (DTH) Response

Fatty acids, especially PUFAs, have been associated with different effects on T-cell responses *in vivo* (Stulnig *et al*., 2000; DeWille *et al*., 1981). Changes in DTH response provide indirect evidence that in vivo T-lymphocyte function is altered due to the fact that DTH responses involve a complex assortment of inflammatory mediators, chemokines, and cells (including monocytes, macrophages and antigen-specific T-cells). DTH tests are considered a recall response that is intrinsically different from the initial response to antigen by naïve T-cells (Anderson and Fritsche, 2004)**.** Some studies have reported a decrease in DTH response in humans, mice, and chickens in the presence of n-3 fatty acids (Meydani *et al*., 1993; Fowler *et al*., 1993; DeWille *et al*., 1981, Wang *et al*., 2002). A schematic representation on the effects of PUFAs in relation to mechanisms associated with immune cell modulation is shown in Figure 2.4.

**Figure 2.4 Possible Mechanisms of Immune Cell Functional Modulation by Polyunsaturated Fatty Acids (Adopted from Stulnig, 2003)**



## 2.6 Immune System of the Chicken

The immune system's primary purpose in the body is to protect it from abnormal cells of self or foreign origin that may compromise the organism's health. Different tissues carry out functions that relate to immune protection of the body. Some of the primary tissues that are in charge of providing immunity are: bone marrow, lymph nodes, spleen, thymus, bursa of Fabricius, lymphatic and intestinal tissues. There are also cells that deal with immune response within the organism such as lymphocytes, macrophages, plasma cells, neutrophils, and dendritic cells (Selvaraj, 2002).

One of the principal sources of immunological active cells is the bone marrow, which contains a large numbers of lymphocytes, macrophages, and plasma cells. Lymph nodes are small chambers that act as filters and ingest foreign materials. Nevertheless, the chicken's immunological capacity depends on antigens and other immunological cells (Selvaraj, 2002). The bursa of Fabricius is very important in the development of B lymphocytes and production of antibodies in avian species. (Aliahmad *et al*., 2005). The intestinal tissues in addition to their role in nutrient absorption play important roles in immunological activity and antigen development of the body (Bar-Shira and Friedman, 2005).

***The small intestine's*** ability to create and maintain its differences within intestinal mucosa structure, such as villus height makes it a very important organ in regards to nutrient absorption. Nutrient absorption by the small intestine occurs after hydrolysis of macromolecules by gastric, pancreatic and finally brush-border enzymes. Brush border enzymes appear to have different temporal patterns of development

immediately post-hatch; making lipid absorption higher when close to hatching time and amino acids and carbohydrate absorption rates higher during feed intake. The functional development of the intestine plays an important factor as a lymphoid organ which creates immune responses and antigen development of the body (Bar-Shira and Friedman, 2005).

***The hindgut*** is one of the primary generators of antigen against the different responses, which would require the generation of immune responses through sampling exogenous antigen material. Through oral induction, the gut associated lymphoid tissue (GALT) can be associated with development antigen induced tolerance which can be programmed towards the development of a stronger immune system. The maturation and development of GALT can be influenced by exposures to microflora, which allows the maturation of hindgut GALT to precede that of the foregut. Thus, early feeding of birds may promote immunity through early exposure of microflora for faster development of hindgut GALT (Bar-Shira and Friedman, 2005; Uni and Ferket, 2004; Geyra *et al*., 2001).

***The bursal canal*** contains effector plasma cells in the connective tissue proximal to the follicular associated epithelium and the lamina propria. It is associated with immune function and antigen transport into the bursal lumen. The antigen is bound by follicular associated epithelium and is known to induce immune responses in both the canal and the bursa.

***The bursa of Fabricius*** is considered a primary and a secondary lymphoid immune organ in birds. It is known to undergo changes involving immune function and immune cell differentiation during incubation and later life. The bursa of Fabricius is the site where T-cell receptor diversification and B-cell receptor rearrangement take place. During incubation, bursal follicles are formed due to migration of lymphocytes to secondary lymphoid organs and spleenic pre-bursal stem cell migration via bloodstream to the bursa. During incubation, gene conversion of the different immunoglobulin (Ig) repertoire of B-cells is also formed. Ig diversified B-cells migrate from the bursa to the periphery before hatch. Also, a bursal size increase from 2% to 3% of body weight in broiler chickens before hatch; then the bursa regresses. At hatch, the proliferative activity of bursal cells is very high, but this activity is reduced when chicks do not have access to feed post-hatch. It is during this time that antigens can be sampled from the cloaca and transferred via the bursal canal into the bursal lumen (Sklan, 2005). This type of antigen sampling process is done via retrograde contractions of the intestinal duct, which improves resorption of water from urine. This pathway allows for the chicks to rectally obtain bursa-derived antibodies right up to the small intestine (Bar-Shira and Friedman, 2005; Sklan, 2005).

***The thymus*** is considered a primary immune organ. It serves in the development of lymphocytes. T-cell differentiation occurs in the avian thymus which involves complex developmental changes in gene rearrangement and acquisition of cell surface differentiation. In the thymus, through recombination of the variable (V), diversity (D), and joining (J) gene segments, genes that encode the variable regions of the T-cell receptor (TCR)-α, -β, -γ, and -δ chains are assembled (Kong *et al*., 1999).

The thymus is also important for DTH and graft versus host reactions. The bursa of Fabricius and the thymus work together to execute complex processes that characterize the immune response of the chicken. Removal of the thymus or bursa shows significant decrease in lymphocyte development and function as well as inability of the chicken to fight off allergic response and rejection of skin homografts (Cooper *et al*., 1980).

***The Spleen*** of the chicken is considered a secondary immune organ. The spleen is composed of a reticulum meshwork supplied by arteries and veins. The mesh (pulp tissue) includes red pulp, composed of red blood cells, and white pulp, composed of lymphocytes. The white pulp is considered the lymphoid part of the spleen and it is intimately involved with the immune system by aiding in disease resistance (John, 1994; Janeway *et al.,* 2005). The chicken spleen is located near the gizzard and the proventiculus. Spleen weight is closely associated with bird body weight, thus size varies considerably. The spleen of the bird is also closely associated with the liver because the blood supply is served by the celiac artery which drains into the larger of two hepatic portal veins leading into the liver (John, 1994). In chickens, the development of the spleen increases rapidly after hatching when it is exposed to antigens, but decreases closer to sexual maturity stage. The major spleenic functions in the bird are oxygen supply to tissues and immunity. Avian spleenic function within oxygen transmission to tissues is only linked with involvement in removal of aberrant or parasitized red blood cells from the circulation. In regards to immune function, the spleen is a major site for lymphocyte recirculation and final stages of lymphocyte differentiation, which involves the production of memory cells necessary for

secondary immune responses. The immune functions of the spleen also include antibody biosynthesis, formation of the complement system, and development of macrophages. It also includes phagocytosis, destruction of antigens, immune complexes and parasitized cells and granulocyte phagocytosis and immunogenesis (John, 1994).

## 2.7 Importance of Early Feeding in Poultry

One problem that commercial hatcheries face is the lack of access to feed and water at early stages in the chicken's life. Different hatching times of birds have pushed the industry to look for efficient ways to collect the greatest numbers of birds hatched for shipping to growers. As a result, many chicks may have already waited up to 2 days (30 to 48 hours) in the hatchery and transportation to the farms before any feed and water is provided (Moran and Reinhart, 1980). Timing and form of nutrients supplied during early hatching is critical for the development of chicks (Noy and Sklan, 1998). Previous experiments show how important early access to nutrients is for chicks and how delayed feeding has been associated with chick loss in body weight, decreased development of immune organs, and poor meat yield as well as irregular morphology in jejunal crypts and clumping of intestinal microvilli (Uni and Ferket, 2004). With access to early feed, birds have a rapid development of cell activity that increases skeletal growth, therefore impacting muscle development (Halevy *et al*., 2000). Post-hatch, early fed birds have been observed to have rapidly developed their intestinal system and have a greater numbers of cells per crypt and number of crypts per villi (Uni *et al*., 1998).

Previous research in turkey poults and chicks has reported that a 54 hour delay in access to feed and water led to a reduction in growth and development of the small intestine, reduced organ weights (crop, liver, proventiculus) affecting digestive capacity, final body weight and meat yield (Corless and Sell, 1999; Uni *et al*., 1998). In addition, feed deprivation during the first 48 hours has reported increases in plasma NEFAs indicating fatty acid breakdown for energy purposes (Noy *et al*., 2001). The results from these reports clearly demonstrate that withholding feed from post hatch chicks and poults affected intestinal growth and productivity.

### 2.7.1 Early Feeding and Immunity

Initial immunity of the chick is closely related to the development of its immune organs. One of the main organs associated with immunity is the small intestine. Post-hatch, the small intestine of the chick undergoes a rapid developmental change that allows the immediate transition from the endogenous nutrient supply in yolk to exogenous feed dependence (Geyra *et al*., 2001). Studies have shown that the transition begins during the first 48 hours post-hatch, which makes feed availability during this time a key element in the future development of the intestinal tract. Chicks feed deprived post-hatch undergo decreases in the development of the intestinal segments of the jejunum and duodenum (Geyra *et al*., 2001; Palo *et al*., 1995). During the time (up to 48 hours) that feed intake is restricted, morphological changes that occur during the transition of yolk nutrient absorption to feed nutrient absorption are impaired due to the lack of fast development and differentiation of enterocytes,

intestinal crypts, and decreased absorptive surface area within the small intestine (Uni and Ferket, 2004).

## 2.8 Why Feed n-6 and n-3 Fatty Acids to Poultry?

During the first hours post-hatch, chicks are exposed to different stressors, including environmental and immune challenges. Yolk-sac nutrients are an important source of energy for the development or immune cells. Supplementation of feed with n-6 and n-3 EFA during this period would allow for better development of the immune system of the chick as well as proper nutrient balance for growth. Dietary fats are part of an integral and functional broiler-diet composition necessary for growth in chickens. The presence of essential n-6 and n-3 fatty acids in poultry diets is necessary since chickens are not able to synthesize these fatty acids. Commercial diets are usually higher in n-6 fatty acids and low in n-3 fatty acids. PUFAs of the n-6 and n-3 series are particularly important due to the fact that they modulate immune response by altering eicosanoid production, cell membrane composition, gene expression and immune cell function. PUFAs of the n-3 series are considered to be beneficial because EPA and DHA are major precursors of eicosanoids that are less inflammatory than those of the n-6 series. Provision of a diet high in n-3 fatty acids after hatch may be beneficial to broiler chickens by allowing them to develop an efficient immune system throughout growth by decreasing synthesis of inflammatory eicosanoids, thus decreasing incidences of inflammatory disorders.

Considering the importance of early access to feed and dietary content of n-6 and n-3 fatty acids in regards to avian immune system, the following chapters

investigate the role of time of feeding and diet in tissue lipid profile of broiler chickens during lipopolysaccharide (LPS) challenge.

# 3. Effect of Dietary Lipids and Time of Feeding on Immune Tissue n-6 and n-3 Fatty Acid Distribution During Lipopolysaccharide Challenge in Broiler Chickens

## 3.1 Introduction

Due to shipment delays chicks are subject to late access to feed. The time period between chick processing and placement varies and depends on transport availability, placement facility and hatchery practice. The developmental events important for immuno-competence in chickens are initiated in the pre and early post hatch period (Uni, 1996; Dibner *et al*., 1998). During hatching time, changes in the chick's gastrointestinal tract show rapid development of GALT, which impacts adaptive immunity and provides protection against microorganism colonization throughout growth (Bar-Shira and Friedman, 2005). The early post hatch is a period of rapid increase in leukocyte populations, seeding of lymphoid organs, and formation of unique clones of lymphocytes that will mediate immunity later in life. Therefore, in order to support potential growth and development of internal organs, chicks need to acquire ability to assimilate external nutrients (Dibner *et al*., 1998).

Fats are included in poultry diets to meet the necessary nutrients and energy needs required for growth. Dietary fatty acids also play an essential role in the immunity of the chicks because type of PUFA in the diet will determine type of eicosanoids formed. PUFAs are important in immunity as they are components of membranes. They can act as signaling molecules by changing the composition of lipid rafts as well as controlling the expression of receptors involved with fatty acid synthesis. Commercial diets contain an animal-vegetable blend, high in n-6 and very low in n-3 PUFAs (Hall *et al*., 2007). High bioavailability of n-6 PUFAs and AA,

leads to production of pro-inflammatory eicosanoids increasing the incidence of inflammatory related disorders in poultry (Calder, 1998).

Dietary supply of n-3 PUFAs during early post hatch may impact the development of a strong immune system that quickly and efficiently adapts to the different immune challenges. n-3 PUFAs, such as EPA and DHA, have been associated with immuno-modulatory and anti-inflammatory effects by interfering with eicosanoid production derived from AA (Calder, 1998). They help by generating less effective messenger molecules from AA via competitive inhibition (Stulnig, 2003). Understanding the role of dietary n-3 PUFA and early feeding, along with modulation of the immune system in birds, may enhance poultry productivity and health.

The present study aimed at determining whether there is a difference and effect by PUFAs and time of feeding in tissues that regulate fat metabolism and immunity after a given inflammatory challenge. It is hypothesized that early feeding and availability of n-3 PUFA in the immune tissues will modulate fatty acid metabolism and immune response of broiler birds upon challenge.

## 3.2 Experiment 1

### 3.2.1 Composition of Diets

Experimental rations were formulated with 3.5% oxidized yellow grease (Diet 1: low n-3) or 3.5% canola oil (Diet 2: high n-3). Diets were corn-soybean based with 22% crude protein and 3200 Kcal/kg. NRC guidelines were followed to meet minimum nutrient requirement as well as calcium, phosphorus, lysine, methionine, and other nutrient requirements for broiler from 0-42 days of age. Oxidation process

of yellow grease was performed by heating approximately 3000 ml (~2.5 kg) yellow grease to 60-65 $^{o}$C for 8 hours daily for 4 days.

### 3.2.2 Design

One-day old Cobb broilers (mixed sexes, n=60) were weighed and randomly assigned to four treatments (n=15, 3 replicates of 5 birds). Chicks were placed on deep wood shaving litter at temperatures and lighting based on Cobb broiler guidelines. Water was allowed *ad libitum*. Feeding times were divided into two groups 0-5 hours post-hatch (early fed) or given more than 24 hours post-hatch (late fed). Dietary treatments were either high n-3 (canola) or low n-3 (yellow grease). There were a total of four dietary treatment groups: Diet 1 (early fed, high n-3), Diet 2 (early fed, low n-3), Diet 3 (late fed, high n-3) and Diet 4 (late fed, low n-3). Standard management practices were applied to all treatments.

### 3.2.3 Inflammatory Challenge

Delayed type hypersensitivity (DTH) response was performed at 3 weeks of age on the bird's foot pad (1 bird/pen). DTH response was measured by administering bovine serum albumen (BSA) as described by Wang *et al.* (2002). The right foot pad was injected with 1 ml of 1 mg BSA /ml phosphate buffered saline (PBS) and the left foot pad was injected with 1 ml PBS as control. Swelling index measurement of foot pad was performed at 24, 48 and 72 hrs post-injection with caliper three times and averaged to decrease experimental error.

Inflammatory responses were triggered by breast muscle intramuscular lipopolysaccharide (LPS) injection twice; first at 3rd week, then the second injection at 72 hrs later. Two birds per pen were injected with 1 ml of LPS/PBS solution (1mg/kg body weight) and two birds per pen were injected with PBS (1ml/kg body weight) as control.

### 3.2.4 Sample Collection

At day 42 of age, 48 birds (12 birds per treatments of 4 birds per replicate) were randomly chosen and sacrificed by decapitation. Approximately, 15-20 ml of blood was collected in 50 ml screw-capped tubes containing 2% EDTA. Final body weight, cut-up yield, and organ weights were documented. Organs (spleen, liver, heart and whole gut: esophagus to cloaca) were washed in PBS, separated into different labeled bags and immediately frozen at -80 $^{o}$C until used for analytical assays. Cut-up yield of breast, thigh/leg muscle, wing, and whole gut was documented.

## 3.3 Analytic Assays

### 3.3.1 Total Lipids

Lipid content of liver and spleen were analyzed according to Folch *et al*. (1957). In a 50 ml screw-cap test tube, approximately 2 grams of minced liver or spleen were homogenized using a polytron with 18 ml of chloroform:methanol (2:1 vol/vol). Samples were stored overnight at 4 $^{o}$C to allow settling and separation. To each sample, 0.88% sodium chloride (NaCl) solution (4 ml) was added and mixed. Samples were centrifuged at 2000 rpm for 10 minutes. Bottom lipid layer was

removed and stored in glass vials at -20 $^{o}$C. Total lipids were determined gravimetrically. Total lipids were also assessed for the experimental diets using the same procedure for tissues.

### 3.3.2 Fatty Acid Determination

In a 15 ml screw-capped test tube, 2 ml of lipid extract were dried under nitrogen, resolubilized in 2 ml of boron-trifluoride-methanol (10% wt/wt) and esterified by heating to 90 $^{o}$C for 1 hour. Fatty acid methyl esters (FAME) of feed, liver and spleen were analyzed using the modified methods described by Cherian *et al*. (2002). Fatty acid composition was determined using an HP 6890 Gas Chromatograph equipped with an autosampler, flame ionization detector (FID), and fused-silica capillary column (SP-2330, 30m x 0.25mm x 0.2 µl film thickness). From samples, 1 µl was injected into the column with helium as a gas carrier (flow rate of 1 ml/min). Oven temperature was set at an initial temperature of 110 $^{o}$C for 1 min, progressively increased at 15 $^{o}$C/min to 190 $^{o}$C, held for 5 min, then increased at 5 $^{o}$C/min to 230 $^{o}$C and held for 5 min. Inlet and detector temperatures were 250 $^{o}$C. Calculation of peak areas and percentages of fatty acids was performed by Hewlett-Packard ChemStation software. FAME retention times were compared to standards. Fatty acid content was reported as percentages of total fatty acids.

### 3.3.3 Non-Esterified Fatty Acids (NEFA)

Plasma NEFA concentrations were determined using an in vitro enzymatic colorimetric method using the NEFA C kit (Wako Chemical USA, Richmond, VA)

according to the manufacturer's instructions. NEFAs were determined in plasma from all birds injected with LPS.

### 3.3.4 Thiobarbituric Acid Reactive Substances (TBARS)

Lipid oxidation products were measured in breast and thigh muscle tissues. Approximately 2 grams of minced tissue samples were weighed into 50 mL test tubes, and 18 mL of 3.86% perchloric acid were added. Samples were homogenized with a polytron for 15 seconds and butylated hydroxytoulene (BHT) (50 µl in 4.5% ethanol (EtOH)) was added to each sample during homogenization to control lipid oxidation. The homogenate was filtered through Whatman #1 filter paper. Filtrate (2ml) was mixed with 2 ml of 20 mM thiobarbituric acid (TBA) in distilled water and incubated in the dark for 15-17 hr. Absorbance was determined at 531 nm. TBARS values were expressed as mg of malondialdehyde per kg of tissue (Cherian *et al*., 1996).

## 3.4 Statistical Analysis

The effects of dietary fat source and time of feeding on organ weights was determined by two-way ANOVA using SAS (version 9.2). The effects of dietary fat source, time of feeding and LPS challenge on organ total lipids, fatty acid profile, DTH, NEFA, and TBARS were determined by three-way ANOVA using SAS (version 9.2). Differences among groups were compared by using Student-Newmann-Keuls multiple range tests. Statistical significance was set at $P<0.05$. Mean values ± SEM are reported.

## 3.5 Results

### 3.5.1 Feed Composition

The composition of the basal diet is shown in table 3.1. The source of dietary fat influenced the fatty acid composition of the diets (table 3.2). Higher n-3 fatty acid concentrations were present in Diet 1 and Diet 3 due to inclusion of canola oil. Higher n-6 fatty acid concentrations were present in Diet 2 and Diet 4 due to inclusion of oxidized yellow grease. Diet 1 and Diet 3 were lower in total saturated fatty acids (TSFA) and PUFA when compared to Diet 2 and 4 (table 3.2).

**Table 3.1 Ingredient List of Broiler Diet: Experiment 1**

| Ingredients | Percent |
|---|---|
| Corn Grain | 47.0 |
| Soybean Meal, 44% | 37.0 |
| Oil[1] | 3.5 |
| Limestone Ground | 1.5 |
| Salt | 0.3 |
| Dicalcium Phosphate | 0.2 |
| Broiler Premix[2] | 0.5 |
| Wheat Middling | 10.0 |

[1] Oil source includes yellow grease or canola oil

[2] Broiler premix supplied per kg/feed:

Vit. A: 12500 IU
Riboflavin: 8 mg
Choline: 500 mg
d-Biotin: 0.15 mg
Vit. D3: 4000 IU
Panthotenic Acid: 12 mg
Thiamine: 1.75 mg
Vit. E: 25 IU
Niacin: 40mg
Folic Acid: 0.75 mg
Vit. $B_{12}$: 0.014 mg
Menadione: 2.5 mg
Pyridoxine: 2 mg
Ethoxyquin: 2.5 μg

**Table 3.2 Fatty Acid Composition of Diets: Experiment 1**

| | Diets | |
|---|---|---|
| **Fatty Acid** | **High n-3** | **Low n-3** |
| | **----------(%)----------** | |
| TSFA: | 18.14 | 16.24 |
| TMUFA: | 36.83 | 36.87 |
| PUFA: | 45.03 | 46.89 |
| T n-6 PUFA: | 33.51 | 38.26 |
| T n-3 PUFA: | 11.52 | 5.80 |

A 3.5% canola oil (source of n-3 fatty acid) was present in high n-3 fatty acid diet. A 3.5% oxidized yellow grease (high source of n-6 fatty acid) was present in low n-3 fatty acid diet.

TSFA: Total saturated fatty acids- includes 16:0, 18:0, 20:0, 22:0, 24:0; TMUFA: total monounsaturated fatty acids- includes 16:1,18:1, 20:1,24:1; PUFA: Polyunsaturated fatty acids- includes 18:2n-6, 18:3n-6 and 18:3n-3, 20:2n6, 20:3n-6, 20:4n-6, 22:4n-6. T n-6 PUFA-includes 18:2n-6, 18:3n-6, 20:2n6, 20:3n-6, 20:4n-6, and 22:4n-6. T n-3 PUFA- includes 18:3n-3.

### 3.5.2 Growth Parameters

No significant differences were observed in final body, spleen, heart, whole gut, wing, and breast weights among treatment groups (Table 3.3).

Liver weight as percent of body weight showed to be different among treatment groups. Diet 1 (early fed, high n-3) showed the highest percent liver weight and Diet 4 had the lowest ($P<0.05$) (Table 3.3).

Thigh muscle weight as percent of body weight was different among treatment groups ($P<0.05$). Diet 1 (early fed, high n-3) showed the highest percent thigh muscle weight and Diet 2 had the lowest ($P<0.05$) (Table 3.3).

**Table 3.3 Body and Organ Weights of Birds at Day 42 of Growth: Experiment 1**

| Treatments | Body Weight | Breast | Wing | Thigh | Whole Gut | Liver | Heart | Spleen |
|---|---|---|---|---|---|---|---|---|
| | (g) | ----------(%)---------- | | | | | | |
| Diet 1 | 1648 | 20.3 | 1.71 | 9.77[a] | 6.38 | 2.04[a] | 0.55 | 0.13 |
| Diet 2 | 1782 | 21.1 | 1.75 | 8.90[b] | 5.74 | 1.60[ab] | 0.53 | 1.11 |
| Diet 3 | 1648 | 19.8 | 1.86 | 8.63[ab] | 5.44 | 1.87[ab] | 0.52 | 0.10 |
| Diet 4 | 1639 | 22.1 | 1.72 | 9.00[ab] | 5.95 | 1.73[b] | 0.51 | 0.10 |
| P-value | ns | ns | ns | < 0.05 | ns | < 0.05 | ns | ns |
| *SEM* | 99.4 | 1.49 | 0.16 | 0.45 | 0.58 | 0.12 | 0.031 | 0.015 |

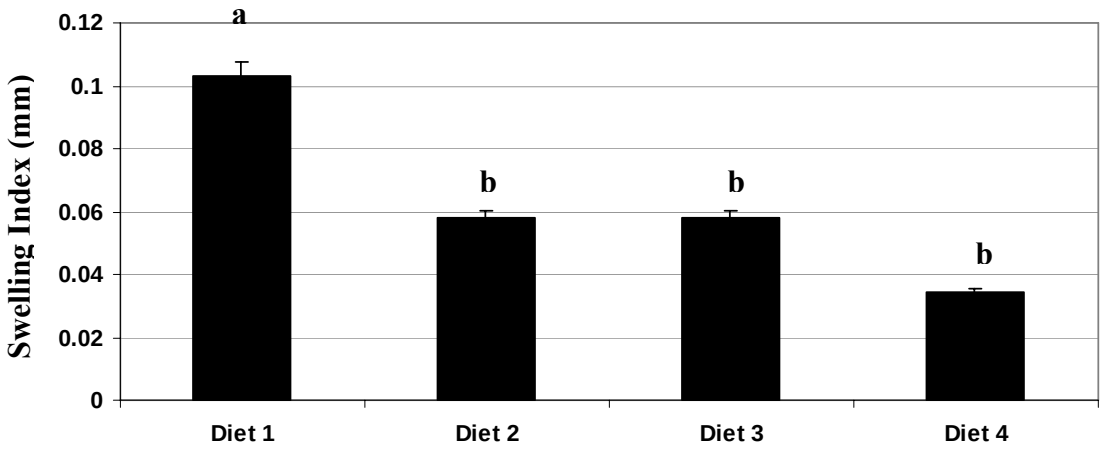
Diets 1-4 represent a basal corn-soybean broiler chicken diet (Table 1). A 3.5% canola oil (source of n-3 fatty acid) was present in Diet 1 & 3. A 3.5% oxidized yellow grease (high source of n-6 fatty acid) was present in Diet 2 & 4.

Each number represents a mean of 6 (n=6). No significant difference was observed among treatments (P>0.05) in body weight, breast, wing, whole gut, heart and spleen percentage. Bars with different superscripts among treatments in thigh and liver are significantly different (P<0.05). Pooled SEM= pooled standard error of mean. ns= not significant.

### 3.5.3 Delayed Type Hypersensitivity Response Reaction

Initial foot pad measurement (0 hrs) showed no significant differences among dietary groups ($P>0.05$) (data not shown). At 24 hrs post injection, broiler birds from Diet 1 (Early fed, high n-3) showed more swelling when compared to broilers from Diets 2, 3 and 4 ($P<0.05$). At 48 and 72 hrs post BSA injection there was no significant difference among the different diet groups ($P>0.05$) (data not shown). DTH response among treatments at 24 hours post-injection is shown in Figure 3.1

**Figure 3.1 DTH Response Measured as Difference between the Footpad Thickness of Birds at 24 Hours Post-Injection: Experiment 1**




Diets 1-4 represent a basal corn-soybean broiler chicken diet (Table 1). A 3.5% canola oil (source of n-3 fatty acid) was present in Diet 1 & 3. A 3.5% oxidized yellow grease (high source of n-6 fatty acid) was present in Diet 2 & 4.

Sensitization with *BSA* (0.01 g) in the foot pad was given at week three of age. Second injection was given 2 weeks later. DTH response was induced on left foot pad by injection with 1.0 mL (1mg/mL) BSA/PBS solution. 1.0 mL of PBS was injected on right foot pad as control. Swelling index measurements were taken at 0, 24, 48 and 72 hrs post injection.

Swelling index formula= [(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 represents mean ± SEM (n=3). SEM= standard error of mean. $^{a-b}$ represents significant difference ($P<0.05$).

### 3.5.4 Total Lipids and Fatty Acid Composition of Liver and Spleen Tissue After LPS Challenge

Tissue (liver and spleen) total lipid content in PBS injected (control) birds was not significantly different ($P>0.05$) (data not shown). Liver total lipid content of LPS injected birds was higher ($P<0.05$) in Diet 4 (late fed, low n-3) when compared to Diet 1 (early fed, high n-3) or Diet 2 (early fed, low n-3) treatment groups, but not different from Diet 3 (late fed, high n-3) ($P>0.05$). Time of feeding among high n-3 groups was not significant ($P>0.05$).

Spleen total lipid content of LPS injected birds fed Diet 2 (early fed, low n-3) was significantly higher when compared to Diet 1(early fed, high n-3), Diet 3 (late fed, high n-3) and Diet 4 (late fed, low n-3) ($P<0.05$). Time of feeding did not affect tissue total lipids ($P>0.05$) [Figures 3.2 (a) and (b)].

Effect of diet composition on liver fatty acid composition was minimal. However, n-3 fatty acids such as ALA, EPA and 22:5n-3 were significantly higher in the liver tissue of control birds from Diet 1 and Diet 3 (high n-3) when compared to control birds from Diet 2 and Diet 4 (low n-3) ($P<0.05$). LPS injection led to significant differences in liver and spleen fatty acid composition among groups. After LPS injection, liver 18:2n-6 and total n-6 were lowest in LPS challenged birds from Diet 1 (early fed, high n-3) when compared to control birds within the same treatment group ($P<0.05$). Similarly, DHA and total n-3 fatty acid were significantly higher in LPS injected birds from Diet 1 (early fed, high n-3) when compared to the control birds from the same treatment group ($P<0.05$). In Diet 2 (early fed, low n-3), LPS injection led to significant increases in EPA, 22:5n-3 and DHA when compared to

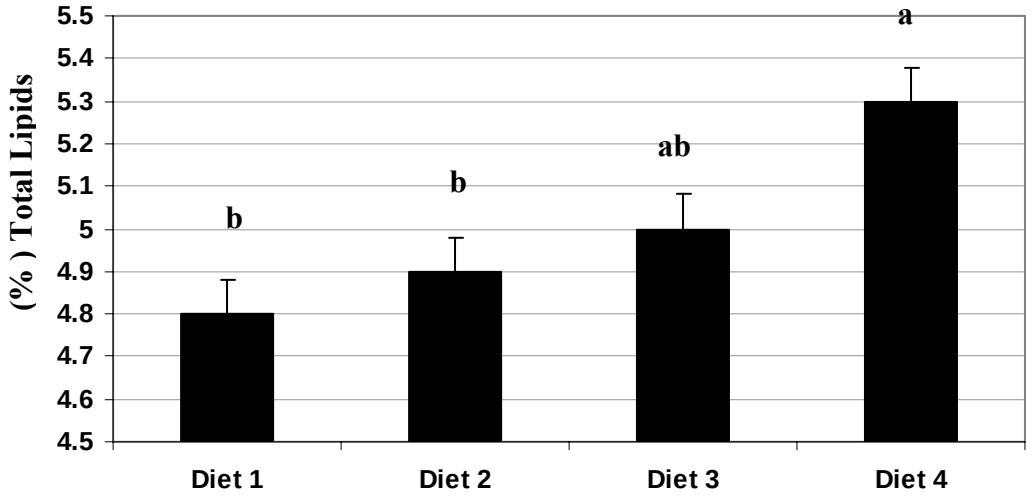
control within the same treatment group ($P<0.05$). AA content in the liver was significantly higher in control birds from Diet 3 and Diet 4 when compared to LPS challenged within the same treatment group ($P<0.05$). No significant differences were found in liver 18:3n-3, TMUFA, and PUFA fatty acid contents after LPS challenge within treatment groups. However, LPS injection led to a decrease in total n-6 fatty acid in Diet 1 (early fed, high n-3), Diet 3 (late fed, high n-3) and Diet 4 (late fed, low n-3) ($P<0.05$). Interestingly, an increase in total n-3 fatty acid content was seen in liver of LPS challenged birds of Diets 1-4 when compared to control birds within the same treatment group ($P<0.05$). The content of DHA and EPA was higher in LPS injected birds from Diet 4 (late fed, low n-3) when compared to control birds within the same treatment group ($P<0.05$) (Table 3.4).

Spleen fatty acid content (Table 3.5) showed no significant difference in regard to diet type or time of feeding among treatment groups ($P>0.05$). However, the content of LA was significantly higher in control birds from Diet 4 (late fed, low n-3) when compared to control birds from Diet 3 (late fed, high n-3). Other fatty acids in the spleen were not affected by diet. Upon LPS challenge, a significant decrease was shown in spleen total n-6 in birds from Diet 1 (early fed, high n-3) when compared to control birds within the same treatment group ($P<0.05$). Total n-3 was significantly higher in LPS birds from Diet 1 (early fed, high n-3) and Diet 3 (late fed, high n-3) when compared to control birds within the same treatment group ($P<0.05$). Similarly, LPS challenge led to an increase in 18:1 content in birds from Diet 1 (early fed, high n-3), Diet 2 (early fed, low n-3) and Diet 4 (late fed, low n-3) when compared to control birds from the same treatment ($P<0.05$). Saturated fatty acid (16:0) decreased

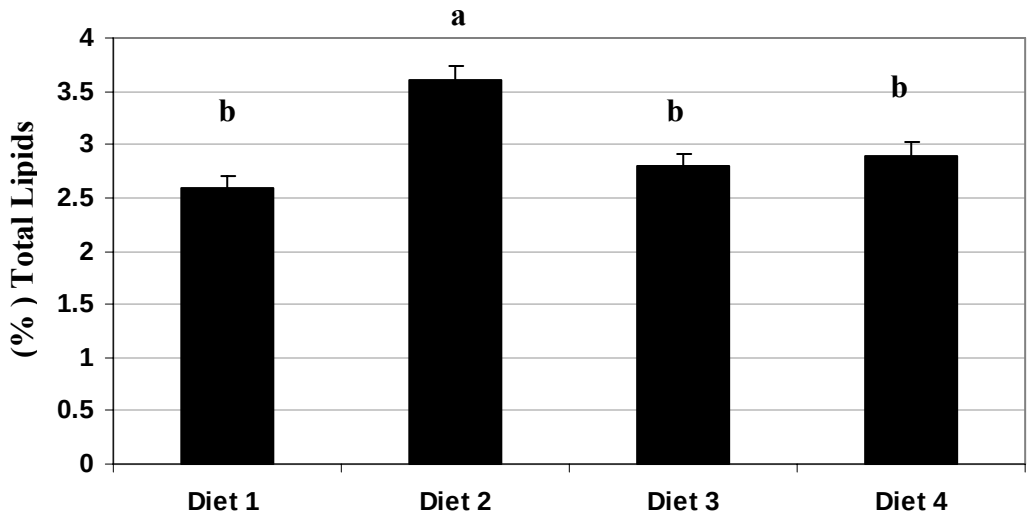
in Diet 1 (early fed, high n-3), Diet 3 (late fed, high n-3) and Diet 4 (late fed, low n-3) upon LPS challenge when compared to control birds from the same treatment group ($P<0.05$).

**Figure 3.2 Effects of Early vs. Late Feeding and LPS Challenge on Liver (a) and Spleen (b) Total Lipids: Experiment 1**

**(a)**




**(b)**




Diets 1-4 represent a corn-soybean broiler chicken diet (Table 1). A 3.5% canola oil (source of n-3 fatty acid) was present in Diet 1 & 3. A 3.5% oxidized yellow grease (high source of n-6 fatty acid) was present in Diet 2 & 4.

Each bar represents mean of six observations (n=6). $^{a-b}$ represents significant difference among dietary groups ($P<0.05$).

**Table 3.4 Effect of Diet and Time of Feeding on Liver Fatty Acid after LPS Challenge: Experiment 1**

| | Treatments | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | Diet 1 | | Diet 2 | | Diet 3 | | Diet 4 | | *Pooled* |
| Fatty Acid (%) | (+) | (-) | (+) | (-) | (+) | (-) | (+) | (-) | *SEM* |
| C16:0 | $17.88^{b}$ | $18.77^{ab}$ | $18.11^{b}$ | $19.06^{ab}$ | $17.80^{b}$ | $19.54^{a}$ | $18.60^{ab}$ | $19.83^{a}$ | 0.42 |
| C18:0 | $23.10^{a}$ | $22.51^{ab}$ | $22.93^{a}$ | $20.91^{abc}$ | $19.04^{c}$ | $21.07^{abc}$ | $19.65^{bc}$ | $21.94^{ab}$ | 0.86 |
| C18:1 | $12.24^{c}$ | $12.12^{c}$ | $12.50^{c}$ | $14.56^{bc}$ | $21.04^{a}$ | $17.06^{abc}$ | $18.94^{ab}$ | $13.55^{bc}$ | 1.74 |
| C18:2n-6 | $15.95^{c}$ | $18.15^{ab}$ | $17.25^{bc}$ | $18.24^{ab}$ | $18.08^{ab}$ | $19.24^{a}$ | $17.17^{bc}$ | $18.74^{ab}$ | 0.57 |
| C18:3n-3 | $0.94^{a}$ | $0.76^{ab}$ | $0.75^{ab}$ | $0.59^{bc}$ | $0.44^{c}$ | $0.76^{abc}$ | $0.01^{d}$ | $0.14^{d}$ | 0.09 |
| C20:4n-6 | $16.69^{ab}$ | $17.66^{ab}$ | $15.42^{abc}$ | $16.15^{ab}$ | $11.52^{d}$ | $14.45^{bc}$ | $12.90^{dc}$ | $17.04^{ab}$ | 0.92 |
| C20:5n-3 | $1.12^{ab}$ | $0.87^{bc}$ | $1.54^{a}$ | $0.45^{c}$ | $0.96^{b}$ | $0.67^{bc}$ | $0.94^{b}$ | $0.01^{d}$ | 0.16 |
| C22:4n-6 | $1.01^{ab}$ | $1.14^{a}$ | $0.77^{ab}$ | $1.02^{ab}$ | $0.78^{ab}$ | $1.00^{ab}$ | $0.68^{b}$ | $1.16^{a}$ | 0.14 |
| C22:5n-3 | $1.73^{a}$ | $1.36^{a}$ | $1.36^{a}$ | $0.73^{b}$ | $1.28^{a}$ | $1.18^{ab}$ | $1.35^{a}$ | $1.53^{a}$ | 0.17 |
| C22:6n-3 | $8.45^{a}$ | $5.58^{bc}$ | $8.65^{a}$ | $5.28^{c}$ | $7.06^{ab}$ | $4.24^{c}$ | $8.31^{a}$ | $5.72^{bc}$ | 0.51 |
| TSFA | $41.09^{ab}$ | $41.36^{ab}$ | $41.03^{ab}$ | $40.79^{ab}$ | $36.84^{c}$ | $40.61^{ab}$ | $38.25^{bc}$ | $41.76^{a}$ | 1.01 |
| TMUFA | $12.51^{c}$ | $13.98^{c}$ | $12.71^{c}$ | $16.22^{abc}$ | $21.77^{a}$ | $17.82^{abc}$ | $19.28^{ab}$ | $13.91^{bc}$ | 1.89 |
| PUFA | $46.41^{a}$ | $45.65^{ab}$ | $46.26^{a}$ | $42.99^{abc}$ | $41.39^{c}$ | $41.57^{c}$ | $42.99^{bc}$ | $44.30^{abc}$ | 1.10 |
| Tn-6 | $34.77^{c}$ | $37.89^{a}$ | $34.19^{c}$ | $36.39^{abc}$ | $30.94^{d}$ | $35.48^{bc}$ | $30.86^{d}$ | $37.08^{ab}$ | 0.73 |
| Tn-3 | $11.62^{a}$ | $7.80^{bc}$ | $12.06^{a}$ | $6.60^{b}$ | $10.53^{a}$ | $6.09^{b}$ | $11.54^{a}$ | $7.24^{b}$ | 0.56 |

Mean of 6 observations. Means with different letters within a row significantly differ from each other ($P<0.05$). Pooled SEM = pooled standard error of mean. Diet 1: (early fed, high n-3), Diet 2: (early fed, low n-3), Diet 3: (late fed, high n-3), Diet 4: (late fed, low n-3) (+): LPS Injected, (-): PBS

TSFA: Total saturated fatty acids- includes 16:0, 18:0, 20:0, 22:0, 24:0; TMUFA: total monounsaturated fatty acids- includes 16:1,18:1, 20:1,24:1; PUFA: Polyunsaturated fatty acids- includes 18:2n-6, 18:3n-6 and 18:3n-3, 20:2n6, 20:3n-6, 20:4n-6, 20:5n-3, 22:4n-6, 22:5n-3, 22:6n-3. Tn-6 PUFA-includes 18:2n-6, 18:3n-6, 20:2n-6, 20:3n-6, 20:4n-6, and 22:4n-6. Tn-3 PUFA- includes 18:3n-3, 20:5n-3, 22:5n-3, and 22:6n-3.

**Table 3.5 Effect of Diet and Time of Feeding on Spleen Fatty Acid after LPS Challenge: Experiment 1**

| | Treatments | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | Diet 1 | | Diet 2 | | Diet 3 | | Diet 4 | | *Pooled* |
| Fatty Acid (%) | (+) | (-) | (+) | (-) | (+) | (-) | (+) | (-) | *SEM* |
| C16:0 | $20.96^{d}$ | $23.59^{ab}$ | $22.04^{bcd}$ | $23.36^{abc}$ | $21.64^{cd}$ | $23.59^{ab}$ | $21.91^{bcd}$ | $23.92^{a}$ | 0.56 |
| C16:1 | $0.29^{bc}$ | $0.90^{bc}$ | $0.01^{c}$ | $1.15^{ab}$ | $0.24^{bc}$ | $1.93^{ab}$ | $0.01^{c}$ | $1.02^{abc}$ | 0.32 |
| C18:0 | $14.73^{ab}$ | $15.61^{a}$ | $14.79^{ab}$ | $15.12^{ab}$ | $13.62^{b}$ | $14.37^{ab}$ | $13.53^{b}$ | $15.01^{ab}$ | 0.57 |
| C18:1 | $28.18^{abc}$ | $23.34^{d}$ | $30.57^{ab}$ | $25.81^{cd}$ | $29.24^{abc}$ | $25.76^{cd}$ | $31.93^{a}$ | $26.76^{bcd}$ | 1.32 |
| C18:2n-6 | $14.29^{b}$ | $16.11^{ab}$ | $16.11^{ab}$ | $15.43^{ab}$ | $15.36^{ab}$ | $14.59^{b}$ | $16.18^{ab}$ | $16.95^{a}$ | 0.67 |
| C20:4n-6 | $11.45^{ab}$ | $13.21^{a}$ | $11.19^{ab}$ | $12.70^{ab}$ | $10.61^{b}$ | $11.34^{ab}$ | $10.61^{b}$ | $11.70^{ab}$ | 0.67 |
| C22:4n-6 | 3.42 | 3.94 | 2.78 | 3.69 | 3.44 | 3.77 | 3.15 | 3.25 | 0.34 |
| TSFA | $38.14^{ab}$ | $41.63^{a}$ | $38.69^{ab}$ | $40.01^{ab}$ | $37.34^{b}$ | $40.23^{ab}$ | $36.69^{b}$ | $39.97^{ab}$ | 1.11 |
| TMUFA | $28.48^{ab}$ | $24.62^{b}$ | $30.57^{a}$ | $26.96^{ab}$ | $29.63^{ab}$ | $28.70^{ab}$ | $31.93^{a}$ | $27.78^{ab}$ | 1.56 |
| PUFA | 33.39 | 33.75 | 30.74 | 33.03 | 33.03 | 31.07 | 31.37 | 32.25 | 1.04 |
| Tn-6 | $29.40^{b}$ | $33.26^{a}$ | $30.08^{b}$ | $32.04^{ab}$ | $29.63^{b}$ | $30.36^{b}$ | $29.94^{b}$ | $32.06^{ab}$ | 0.83 |
| Tn-3 | $3.99^{a}$ | $0.49^{b}$ | $0.67^{b}$ | $1.00^{b}$ | $3.40^{a}$ | $0.71^{b}$ | $1.43^{b}$ | $0.20^{b}$ | 0.53 |

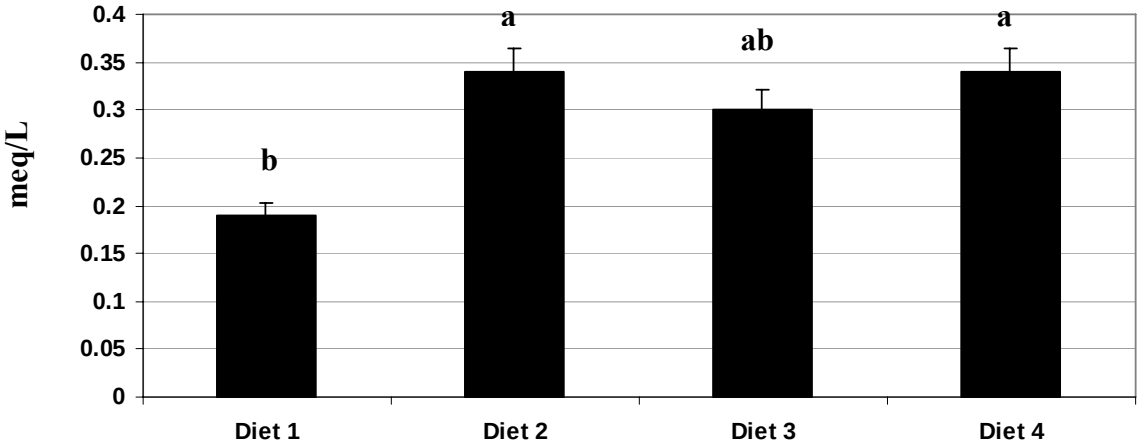
Mean of 6 observations. Means with different letters within a row significantly differ from each other ($P<0.05$). Pooled SEM = pooled standard error of mean. Diet 1: (early fed, high n-3), Diet 2: (early fed, low n-3), Diet 3: (late fed, high n-3), Diet 4: (late fed, low n-3) (+): LPS Injected, (-): PBS

TSFA: Total saturated fatty acids- includes 16:0, 18:0, 20:0, 22:0, 24:0; TMUFA: total monounsaturated fatty acids- includes 16:1,18:1, 20:1,24:1; PUFA: Polyunsaturated fatty acids- includes 18:2n-6, 18:3n-6 and 18:3n-3, 20:2n-6, 20:3n-6, 20:4n-6, 20:5n-3, 22:4n-6, 22:5n-3, 22:6n-3. Tn-6 PUFA-includes 18:2n-6, 18:3n-6, 20:2n-6, 20:3n-6, 20:4n-6, and 22:4n-6. Tn-3 PUFA- includes 18:3n-3, 20:5n-3, 22:5n-3, and 22:6n-3.

### 3.5.5 Plasma Non-Esterified Fatty Acids

Plasma NEFA showed significant differences between dietary groups upon LPS challenge (Figure 3.3). Early fed high n-3 birds (Diet 1) showed the lowest concentrations of plasma NEFA upon challenge ($P<0.05$), while the highest concentrations were seen in birds fed Diet 2 (early fed, low n-3) and Diet 4 (late fed, low n-3). No differences were observed in the NEFA of Diet 3 (late fed, high n-3) when compared to Diet 2 and Diet 4 ($P>0.05$).

**Figure 3.3 Effects of Early vs. Late Feeding and LPS Challenge on Plasma Non-Esterified Fatty Acids: Experiment 1**




Diet 1: (early fed, high n-3), Diet 2: (early fed, low n-3), Diet 3: (late fed, high n-3), Diet 4: (late fed, low n-3).

Each bar represents mean of six observations (n=6). $^{a-b}$ represents significant difference among dietary groups ($P<0.05$).

### 3.5.6 Tissue Thiobarbituric Acid Reactive Substances (TBARS)

Effect of feeding and diet on TBARS content of thigh muscle following LPS challenge is shown in Figure 3.4. Among the control groups, Diet 1 (early fed, high n-3) birds had a higher content of TBARS when compared to Diet 4 (late fed, low n-3). No significant differences were present in Diet 2 (early fed, low n-3) and Diet 3 (late fed, high n-3) ($P>0.05$). In the presence of LPS challenge, TBARS content in thigh muscle from Diet 2 (early fed, low n-3) and Diet 3 (late fed, high n-3) were significantly higher when compared to Control birds within the same treatment group ($P<0.05$). No difference were detected in the TBARS of LPS and control birds of Diet 1 and Diet 4 ($P>0.05$).

Breast tissue TBARS content in regards to diet and time of feeding showed no significant difference among treatment groups ($P>0.05$) (data not shown). However, upon LPS challenge, birds from Diet 1 (early fed, high n-3) had significantly higher TBARS content when compared to Diet 2 (early fed, low n-3) and Diet 4 (late fed, low n-3) ($P<0.05$).

**Figure 3.4 Effect of Diet and Time of Feeding on Thigh Muscle Thiobarbituric Acid Reactive Substances (TBARS) upon LPS Challenge: Experiment 1***

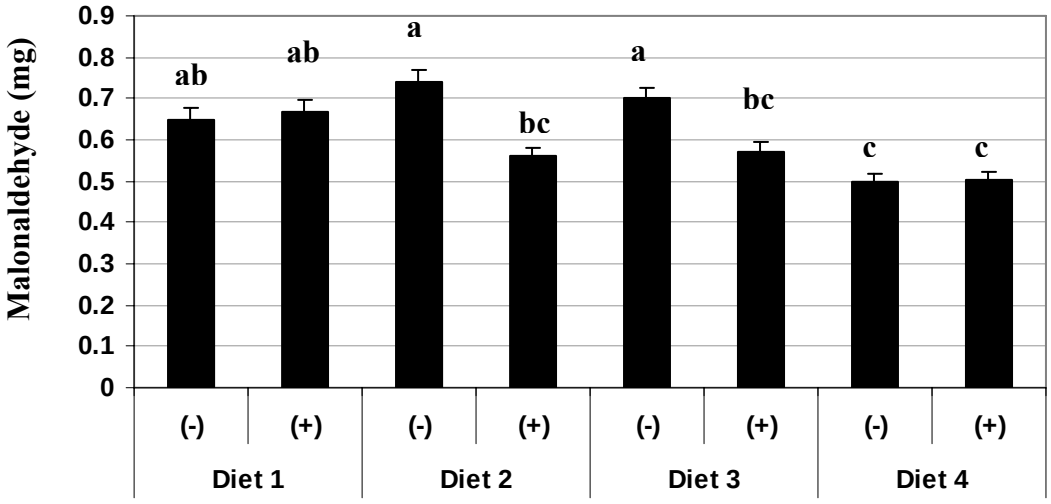
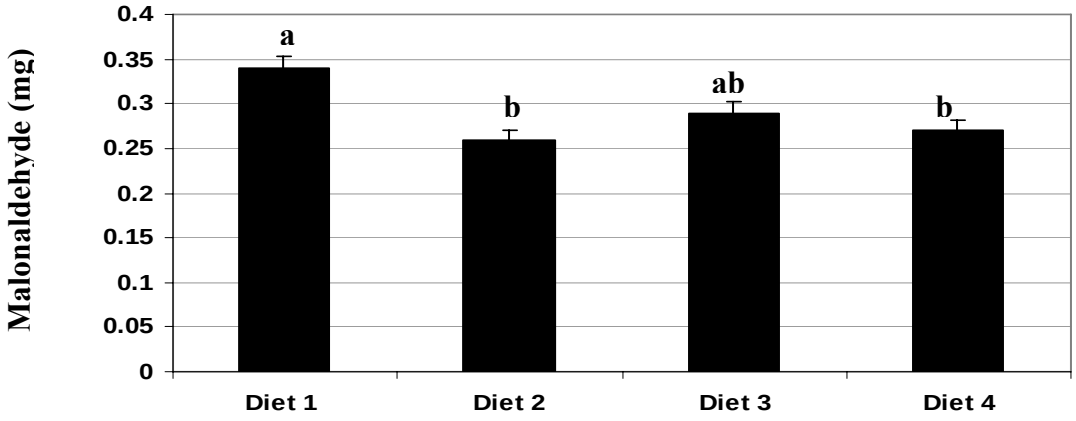



**Figure 3.5 Effect of Diet and Time of Feeding on Breast Muscle TBARS upon LPS Challenge: Experiment 1****




Mean of 6 observations (n=6). Diet 1: (early fed, high n-3), Diet 2: (early fed, low n-3), Diet 3: (late fed, high n-3), Diet 4: (late fed, low n-3). (+): LPS Injected, (-): PBS Each bar represents mean (n=6). $^{a-b}$ represents significant difference among dietary groups ($P<0.05$).

* Figure 3.4 (-) represents control birds and (+) represents LPS challenged birds.
** Figure 3.5 represents only LPS challenged birds.

## 3.6 Discussion

The effect of early and late feeding of high and low n-3 diets in the presence of LPS challenge in broiler birds was investigated. The main focus was to measure the differences among treatments in tissue lipid and fatty acid profile, degree of lipid peroxidation in muscle tissues, production of plasma NEFA, and DTH response measurement. The rationale behind this experimental setting is that early access to a diet that is high in n-3 PUFAs will lead to an increase in availability of n-3 PUFAs upon LPS challenge. Lipid substrate mobilization in the immune system during challenge is dependent on fatty acid availability which will allow fatty acids to be used as an energy source by immune tissue cells.

### *Body and Organ Weights*

Previous studies have reported that in order to satisfy the rapid transition to external nutrients, the chick's gastrointestinal tract undergoes dramatic changes during the first days of life (Bar-Shira and Friedman, 2005; Sklan, 2005). During this time, the intestinal epithelium is exposed to foreign materials which lead to oral tolerance, which is a selective and natural immunosuppressive mechanism (Worbs *et al.,* 2006). Usual hatchery practices result in a 24-48 hr transitional delay between hatching, transport, and placement of the chicks on the farm. This type of practice has shown that dietary restriction leads to regression of mucosal development, lower digestive and immune organ weights, and higher vulnerability to diseases (Dibner *et al*., 1998; Uni *et al.,* 1996; Bar-Shira and Friedman, 2005; Geyra *et al.,* 2001). In the current study, time of feeding did not seem to have an effect on final body weight, spleen, heart, whole gut, wing, and breast tissue with the exception of liver and thigh muscle.

At 42 days of age, birds fed high n-3 and placed on feed 0-5 hrs post hatch had significantly higher liver and thigh weights. These results were in agreement with Palo *et al*. (1995) and Fassbinder-Orth and Karasov (2006) who reported that final body weights were not significantly different because initial feed restriction accompanied with later stages adlib feed supplementation lead to compensatory growth in adult chickens. The liver plays an important role in fatty acid assembly and distribution. The increase in liver weight in early high n-3 birds suggests that extra lipid in this tissue may provide added fuel source to the birds. In addition, during the early stages of life, incorporation of DHA plays an essential role in brain and retina function and development. DHA is also involved in production of eicosanoids involved in inflammation. Thus inclusion of an early fed high n-3 diet may ensure higher liver weights which may help with future energy supply to cells in the presence of inflammation or other growth related stressors.

***Delayed Type Hypersensitivity (DTH) Response***

DTH response is used as a reflection of development of immunity that is antigen specific. DTH response is also used to correlate T-cell response by lymphocyte proliferation which causes inflammation. Diets higher in n-3 PUFA have been shown to diminish DTH response when compared to diets high in saturated fatty acids or diets rich in n-6 PUFA (Hall *et al*., 2007, Sijben *et al*., 2000; Calder, 1996; and Meydani *et al*., 1993). Previous studies in poultry reported by Hall *et al*. (2007) and Selvaraj and Cherian (2004), showed that inclusion of n-3 PUFAs significantly decreased DTH response when compared to diets high in n-6 PUFAs. Fatty acid composition of immune cell membranes dictates the production of cytokines and

eicosanoids in the presence of inflammatory agents. At 24 hrs post LPS injection, signs of inflammation were observed in birds from Diet 1, but no significant differences were observed during the following measurements which could be due to the low number of birds that were used for this test. Studies in humans, mice, rats, dogs and chickens have shown significant differences in DTH response in the presence of different dietary fatty acids. These results indicate that type of fatty acid present in the lipid membrane of immune cells lead to the modification of eicosanoids produced (Hall *et al*, 2007, Sijben *et al*., 2000; Wander *et al*., 1997; and Meydani *et al*., 1993). n-3 PUFAs are considered less potent mediators in the presence of antigens due to production of eicosanoids that are less inflammatory; thus causing less T-cell proliferation and overall inflammation.

***Total Lipids and Fatty Acid Composition after LPS Challenge***

The liver accounts for 95% of the de novo fatty acid synthesis in broiler chickens (O'Hea and Leveille, 1969). High intakes of dietary fats strongly inhibit dietary lipogenesis in chickens. It has been previously reported that dietary fat type on fat metabolism and deposition in broiler chickens was influenced by diets that contained saturated or unsaturated fats (Sanz *et al*., 2000). Lower fat deposition in broilers fed unsaturated fat-enriched diets had an increased rate of lipid catabolism and lower rate of fatty acid synthesis despite higher dietary fat absorption (Sanz, *et al*., 2000). The liver also plays an essential role in modification of fatty acids derived from diet as well as distribution to tissue membranes for maintenance, storage and generation of signaling molecules. The liver and spleen of birds are closely related because spleenic blood supply is served by the celiac artery which drains into the

larger of two hepatic portal veins leading into the liver (John, 1994). The spleen is considered a secondary immune organ in the chicken, but it plays key role in immunity by allowing recirculation of immune cells in the presence of inflammation. The increase in total lipid in spleen tissue of Diet 2 (early fed, low n-3) indicates that after LPS challenge, birds from this dietary group were able to synthesize or uptake more lipids to be used during inflammatory challenge

In this study, a high n-3 diet elevated the content of n-3 fatty acid in the liver and spleen, but distribution of n-3 PUFAs was altered in the presence of inflammatory challenge. LPS endotoxin, a component of the cell wall of gram-negative bacteria, is frequently administered to animals in order to invoke acute phase response changes. It has been previously determined that a dose of 1 mg LPS/kg body weight is sufficient to create inflammation and expression of inflammatory cytokines in liver and spleen of avians without resulting in mortality (Koutsos and Klasing, 2001).

LPS challenged birds fed early high n-3 and low n-3 diets retained a higher percentage of liver n-6 fatty acids when compared to the other LPS challenged groups, which indicates that birds having late access to feed would have greater liver n-6 depletion in the presence of inflammatory challenge. Total liver n-6 fatty acid was highest in control birds; thus LPS challenge injection led to a decrease in liver n-6 fatty acids. The same result was observed in spleen total n-6 fatty acid. Spleen total n-3 fatty acid showed to be highest in LPS challenged birds that were fed early and late high n-3 fatty acid diets when compared to the LPS challenged birds fed low n-3 fatty acid diets. This could indicate that in the presence of inflammation, feeding a diet that is high in n-3 fatty acids may increase the presence of n-3 fatty acids in spleen tissue,

thus supplying the spleen with a beneficial source of energy and less pro-inflammatory fatty acids.

***Plasma NEFA***

NEFA concentrations can induce oxidative stress to the vascular endothelium and can have pro-inflammatory effects (Sainsbury *et al.,* 2004; & Stulnig, 2000). NEFA measurement in the presence of different PUFA, although complex, has been linked to immune function and hepatic oxidation and response (Brassard *et al.,* 2007; Hamano, 2007). In regards to inflammation, the type of NEFA has been known to influence T-cell functions via modulation of eicosanoid metabolism by producing PGs, TXs and LT intermediates, as well as interfering with intracellular signaling pathways.

Upon LPS challenge, NEFA release from adipose tissue is initiated by action of hormone sensitive lipase. NEFA bound to serum albumen for transport is taken to other tissues such as liver and muscle where they can be activated and function in fatty acid and glucose metabolism. NEFA have influence over immune function via modification of the membrane lipid composition and lipid raft modulation (Brassard *et al*, 2007). NEFA effects on the immune system shows that PUFA of the n-3 series tend to have immunosuppressive effects, while other NEFA, including n-6 and linoleic acid, tend to be immuno-stimulatory in chronic diseases such as atherosclerosis and diabetes type II. In this study, plasma NEFA was lowest in LPS challenged birds fed a high n-3 diet when compared to the other LPS challenged groups. This could indicate that in the presence of inflammation, a diet that is high in n-3 fatty acids may decrease the NEFA concentration, thus modulating immune functions and decreasing stress

related oxidation. A previous study by Hall *et al*. (2007) showed that birds fed diets containing high n-3 PUFAs helped to minimize the NEFA content as well as pro-inflammatory eicosanoid production. Thus, dietary modification in broilers may be beneficial by modulation of immune response.

***Lipid Peroxidation Products: TBARS***

The increase in thigh muscle TBARS content in the current study could be because Diet 2 was highly oxidized due to the heating process. However, Diet 4 had no increase in TBARS. In Diets 1 and 3 the larger number of double bonds may promote greater lipid peroxidation product formation. Increased TBARS content in breast muscle of early high n-3 after LPS challenged birds could be due to the presence of long chain fatty acids with higher number of double bonds that are highly peroxidized as well as the presence of inflammatory response against LPS challenge. Therefore, it seems that lipid oxidation product formation can be different in muscle tissues (i.e. breast vs. thigh). This seems to be dependent on type of fatty acid present in the diet and level of peroxidation that occurs in the muscle. Thigh muscle is rich in red fibers full of fast muscle twitch, oxygen, and metabolic key enzymes that play significant roles in aerobic and anaerobic activities (Kiessling, 1977). Thigh muscle of chickens also has higher fat content and higher number of mitochondria when compared to breast muscle, a slow twitch muscle. Thus, the effect of LPS challenge in combination with peroxidation would have an effect on overall TBARS content depending on muscle functionality.

Oxidative stress, especially lipid peroxidation of fatty acids, is known to increase the number of products involved in inflammatory pathways. Studies have

shown that *in vivo* modification of lipids by lipid peroxidation (i.e. malonaldehyde) products can lead to chronic inflammation such as increased atherosclerotic lesions and foam cell formation (Esterbauer, 1993). Increased fatty acid concentration is also known to increase lipid peroxidation by increasing ROS generation, $NF_kB$ plasma concentration, and endothelial $PGE_2$ synthesis in the presence of inflammation and stressors (Tripathy *et al*., 2003; & Wander *et al*., 1997). In addition to increased lipid peroxidation from PUFAs, plasma NEFA has also been associated to induce oxidative stress. Studies using dogs have shown an increased consumption of n-3 PUFAs from fish oil leads to an increase in lipid peroxidation products in plasma and urine (Wander *et al*., 1997). Formation of lipid peroxidation products has been associated with increased incidences of vascular permeability, cancer cell proliferation, damage to DNA and overall expression of cytokines involved in inflammatory pathways.

# 4. Tissue n-3 and n-6 Fatty Acid Contents, Vitamin E Status and Cyclooxygenase-2 (COX-2) Protein Expression During Lipopolysaccharide Challenge in Broiler Chickens: Effects of Dietary Lipids and Time of Feeding

## 4.1 Introduction

Current practices in the poultry industry prevent chicks from accessing feed after hatch. Once they arrive to the farm (up to 48 hrs post hatch) the chicks are placed on a standard starter diet that is high in n-6 fatty acids and low in both n-3 fatty acids and antioxidants. This dietary imbalance may contribute to the acute inflammatory response and the prevalence of inflammatory-related disorders in broiler chickens. Based on the results from the previous chapter, in support with other studies, there is a strong correlation with fatty acid distribution in the presence of inflammation. The results from the previous study showed that dietary n-3 PUFA and prompt feeding following hatching, when evaluated following LPS immune challenge had significant effects on tissue fatty acid distribution, plasma NEFA content, DTH response, and muscle TBARS content.

EFAs are important constituents of cell membranes which can determine the behavior of membrane-bound enzymes and receptors (Das, 2006). Fatty acid composition of immune cells can also modulate the type and intensity of inflammatory response in the presence of immunological challenge. LA and AA are the major precursors for the synthesis of inflammatory bioactive mediators such as PGs, TXs and LTs. The presence of these bioactive mediators derived from AA is found to be higher in blood and tissues when exposed to inflammatory conditions.

Long chain n-3 fatty acids, EPA and DHA, which are predominant in fish and marine sources, are known to be precursors of metabolites that alter the production of pro-inflammatory eicosanoids, chemokines, and gene expression (Calder, 1998). Dietary n-3 PUFAs contribute to two main effects in decreasing synthesis of inflammatory eicosanoids by immune cells. First, EPA is an inhibitor of AA release and secondly, EPA inhibits oxygenation of AA by COX because there is an enzymatic binding site competition between n-6 and n-3 fatty acids because EPA inhibits oxygenation of AA by COX (Calder, 2002; Obata *et al.*, 1999). At the level of gene expression, n-3 PUFAs, EPA and DHA, exert anti-inflammatory effects by decreasing the expression of COX-2, TNF-α, and IL-1α. Metabolism of AA by COX enzymes is known to give rise to the 2-series PGs and TXs, which have pro-inflammatory effects (Babcock *et al*., 2002). It has been suggested that n-3 PUFA (EPA) may have an effect in lowering AA eicosanoid mediators by decreasing the expression of COX-2 because it is able to bind to COX enzymes (COX -1 and 2) giving rise to 3-series eicosanoids which are known to exert anti-inflammatory properties (Calder, 2002; Bagga *et al.,* 2003).

PUFAs easily undergo auto-oxidation because auto-oxidation is dependant on the number of double bonds present. Some of the primary products of lipid peroxidation are lipid hydroperoxides and radicals. Lipid peroxidation of EPA and DHA hypothetically yields 8-10 different monohydroperoxides. Endogenous lipid peroxidation products can exert biological properties and are expressed in high quantities in the presence of stressors such as chronic and acute diseases, inflammation, injury, cancer and atherosclerosis (Esterbauer, 1993). Products of in

vivo free radical lipid peroxidation from AA and EPA such as prostaglandin-like compounds called $F_2$-isoprostanes and $F_3$-isoprostanes, respectively, are used to provide a measure of in vivo oxidative stress in plasma and urine. Although isoprostanes behave like prostanoids, they differ in their stereochemistry and do not require the presence of the COX enzyme for their formation (Singh *et al*., 2005; Mori *et al*., 2003). Therefore, a high level of dietary PUFAs may cause vulnerability to lipid peroxidation products; thus increasing the level of production of lipid radical peroxidation products. Interestingly, other results show that oxidized PUFAs present in fish oil may prevent inflammation by inhibition of NF-κ B and modulation of endothelial cell adhesion molecule expression, which would suggest a natural mechanism for decreasing inflammation caused by lipid peroxidation *in vivo* (Mishra *et al*., 2004; Mori *et al*., 2003).

In mice, humans, and dogs the presence of PUFAs (EPA and DHA) in combination with antioxidants such as vitamin E (alpha-tocopherol) in the diet may contribute to a decrease in the expression of COX activity as well as a decrease in $F_2$-isoprostanes (Wu *et al*., 1998; Dommels *et al*., 2003; Venkatraman & Chu, 1999).

It is hypothesized that feeding diets high in n-3 PUFAs after hatch to broiler chickens, will lead to accumulation of n-3 PUFAs in tissues and immune organs. This will be beneficial to chicks in the presence of inflammation by reducing the expression of inflammatory enzymes and other inflammatory markers. The current study investigated the effects of time of feeding and type of diet in comparison to commercial practices and measured the effects and retention of PUFAs and vitamin E

in immune organs, effects on COX-2 enzyme expression and isoprostanes production in broiler chickens.

## 4.2 Experiment 2

### 4.2.1 Diet Composition

All experimental diets were corn-soybean based with added 3.5 % sunflower oil (low n-3) or 3.5% menhaden fish oil (high n-3). The diet had 22% crude protein and 3200 Kcal/kg as depicted in table 4.1. NRC guidelines were followed in order to meet minimum nutrient requirement as well as calcium, phosphorus, lysine, methionine, and other nutrient requirements for broilers from 0-42 days of age. Vitamin E in broiler diet consisted of a mixture of tocopherols. The vitamin E concentration in the high n-3 diet was 21 IU/ kg and in the low n-3 diet was 19 IU/kg of feed vitamin E. All diets were stored in the cold room (4 $^{o}$C) to decrease the possibility of rancidity.

**Table 4.1 Feed Composition: Experiment 2**

| Ingredients | Percent (%) |
|---|---|
| Corn Grain | 55.03 |
| Soybean Meal, 44% | 30.02 |
| Oil[1] | 3.50 |
| Limestone Ground | 1.60 |
| Salt | 0.25 |
| Dicalcium Phosphate | 0.10 |
| Broiler Premix[2] | 0.50 |
| Wheat Middling | 9.00 |

[1] Oil source includes sunflower or menhaden fish oil

[2] Broiler premix supplied per kg/feed:

| | |
|---|---|
| Vit. A: 12500 IU | Vit. E: 25 IU |
| Riboflavin: 8 mg | Niacin: 40mg |
| Choline: 500 mg | Folic Acid: 0.75 mg |
| d-Biotin: 0.15 mg | Vit. $B_{12}$: 0.014 mg |
| Vit. D3: 4000 IU | Menadione: 2.5 mg |
| Panthotenic Acid: 12 mg | Pyridoxine: 2 mg |
| Thiamine: 1.75 mg | Ethoxyquin: 2.5 μg |

### 4.2.2 Design

A total of 144 Cobb broiler eggs were obtained from a commercial hatchery. Eggs were set and incubated according to standard protocol. Hatchability and mortality was recorded for comparing to commercial records. Hatchability was 85 % with 123 hatched, 15 infertile, 3 early dead and 3 late dead. On the day of hatching, a total of 64 chicks were randomly selected and assigned to 4 different treatments with 16 birds per treatment in replicates of 4. Time of feeding consisted of early feeding (<5 hrs post-hatch) or late feeding (>48 hrs post-hatch). There were a total of 4 treatments: Diet 1(early fed, high n-3), Diet 2 (late fed, high n-3), Diet 3 (early fed, low n-3) and Diet 4 (late fed, low n-3). Water was provided *ad libitum*. Standard management practices were applied to all treatments from incubation to the end of the trial.

### 4.2.3 Inflammatory Challenge

At day 36 of age, a total of 6 birds per treatment were sensitized by intramuscular injection in the breast muscle with 1.0 ml of sterile PBS (control) or LPS (from lyophilized powder) dissolved in saline at 1mg/kg body weight. At day 39 of age, the birds were injected a second dose of 1.0 ml of sterile PBS or LPS (1 mg/kg body weight). Tissues were collected 72 hrs (day 42 of age) after 2$^{nd}$ injection.

### 4.2.4 Sample Collection

At day 42 of age, a total of 64 birds (32 PBS and 32 LPS challenged) were chosen at random and sacrificed by decapitation. Approximately 15-20 ml of blood

was collected in 50 ml screw-capped tubes containing 2% EDTA. Plasma was collected by centrifugation at 1500 x g for 15 minutes. Organs (liver, lung, heart, spleen and small intestine) were collected, washed in PBS, flash frozen in acetone/dry ice and stored in -80 $^{o}$C until used for analytical assays. Plasma and a smaller cut portion of the organs were separately stored for COX-2 protein assays at -80 $^{o}$C.

## 4.3 Analytic Assays

### 4.3.1 Total Lipid Extraction and Fatty Acid Analysis

Lipid and fatty acid contents of the feed, liver, spleen, lung, and plasma were determined using methods described by Folch *et al*. (1957) as described in Chapter 3.

### 4.3.2 Plasma 8-Isoprostane Analysis

Frozen plasma samples were sent out in dry ice overnight to Assay Services, Cayman Chemical Company, Ann Arbor, MI for plasma isoprostanes assay.

### 4.3.3 Cyclooxygenase-2 Assay of Spleen Tissue

#### 4.3.3.1 Spleen Tissue Sample Preparation

Approximately 0.1-0.3 gm of spleen tissue was weighed and recorded then placed in a homogenizer tube. All samples were diluted 4 times the volume per weight in lysis buffer [(50 mM Tris-HCL-pH 7.4, 150 mM NaCl, 2 mM EDTA, 0.1% SDS, 1% Triton X) plus 1 Complete-mini protease inhibitor (Roche Diagnostics) tablet per 10 ml lysis buffer and used immediately]. Spleen tissue was homogenized for about 5 seconds at a time until tissue was blended. Homogenate was transferred to 1.7 ml

eppendorf tubes, taken to the cold room (4 $^{o}$C) and spun at 12,000 x g for 15 minutes. The supernatant was collected and transferred in aliquots to clean 1.7 ml eppendorf tube and frozen at -80$^{o}$C until used for protein determination assay.

#### 4.3.3.2 Bradford Protein Assay

In a culture tube, 5 ml of ultrapure MilliQ water was added. Standards were made by removing 0, 2.5, 5, 10, 20, 25, and 30 µl of MilliQ water from culture tubes and replaced with BSA. To determine amount of protein in unknown samples, 5 µl of ultrapure MilliQ water was removed and same amount was replaced with of unknown spleen homogenate. All tubes were vortexed to allow mixing. On a 96 well plate 150 µl of standards and unknown spleen homogenates were added in replicates of 3. To each well, 150 µl of Bradford Coomasie Plus$^{TM}$ (Pierce Biotechnology, Catalog # 23238) was added. The plates were shaken and placed in a dark drawer for 10 minutes. Absorbance value, mean OD value, standard deviation, %CV, and $R^2$ using quadratic fit were obtained from the plate reader using SoftMaxPro software. Formula for calculating protein concentration was using quadratic fit: $Y = A + Bx + Cx^2$ where values for A, B and C were calculated using concentration vs. mean OD values.

#### 4.3.3.3 Loading Sample Preparation

Based on protein concentration obtained, 75 µg of total protein was pipetted from sample aliquots into clean eppendorf tubes containing 2 µl of NuPAGE® sample reducing buffer 10x and NuPAGE® LDS sample buffer 4x (Invitrogen, catalog #: NP0009 and NP007) respectively. All samples were made up to 20 µl by adding

sample buffer with protease inhibitor. Samples were denatured at 70 $^{o}$C for 10 minutes and placed in ice.

#### 4.3.3.4 SDS-PAGE of Spleen Proteins and Identification

SDS-PAGE was performed according to manufacturer recommendations using NuPAGE® 4-12% Bis-Tris gel, 1.0 mm x12 well (Invitrogen, catalog #: NP0322BOX) and transferred into Invitrolon$^{TM}$ PDVF, 0.45 0.45-µm pore size, 8.5 cm x 13.5 cm (Invitrogen, catalog #: LC2007). Samples were loaded into individual wells. Twelve µl of protein ladder were added to 1 well/gel, 1:1-MagicMark$^{TM}$ XP: SeeBlue Plus 2 (Invitrogen, catalog #: LC5602 and LC5925 respectively). Whole cell lysates from mouse macrophages (RAW 264.7 + LPS/IFN-γ) were purchased (Santa Cruz Biotechnologies, catalog #: sc24767) and placed in one well/gel as COX-2 positive control.

After transfer, the PVDF membrane was rinsed in tris-buffered saline tween-20 (TBST) and blocked on shaker for 1-2 hrs at room temperature in 3% milk/TBST. The primary Ab used was a rabbit polyclonal IgG for COX-2 (Santa Cruz, catalog #: sc7951), used at 1:500 dilution in 3% milk/TBST and was left blocking overnight at 4 $^{o}$C on shaker. Application of secondary antibody was done using compatible goat anti-rabbit IgG-HRP in TBST at 1:50,000 dilution for 1 hr at room temperature on shaker. Mouse β-actin (Chemicon, catalog # MAB1501R) was used as housekeeping protein with a 1: 2000 dilution of primary in 3% milk/TBST and a 1:20,000 dilution for secondary using sheep anti-mouse. Quantification of protein was done using Li-Cor

Biosciences Odyssey® infrared detection. Calculations to quantify COX-2 protein was done using the COX-2 intensity/Beta-actin intensity.

### 4.3.4 Vitamin E Assay

Vitamin E assay was performed on plasma, feed, lung, liver and small intestine using methods previously described by McMurray *et al*. (1980) and Surai *et al*. (1996) with some modifications. About 0.8 ml of plasma or 0.2-0.3 g of sample was weighed and homogenized using 2 ml of PBS. Standards were prepared by taking the same weight or volume from one of samples (i.e. plasma, tissue, feed) and adding 0.025 ml of the working vitamin E standard to one tube, 0.05 ml to the next and 0.1 ml in addition to 2 ml of 1% ascorbic acid in ethanol. Saturated KOH (0.3 ml) was added to all tubes, capped, vortexed and placed in the 70 ºC water bath. After 30 minutes, samples were cooled to room temperature. 0.1 ml of 0.25 mg BHT/ml ethanol and 1.0 ml 1% ascorbic acid in $H_2O$ was added to all tubes. Hexane (2.5 ml) was added to all tubes, capped and vortexed for 1 min. Samples were centrifuged for 5 min at 1500 g. Upper hexane layer was extracted and evaporated under nitrogen and the residue was dissolved in 0.3 ml ethanol. In eppendorf tubes, 0.5 ml of sample was microcentrifuged at 7000 rpm for 5 min and upper layer was collected and transferred into HPLC vials. Sample (0.025 ml) was injected in the HPLC. The mobile phase consisted of 97.5 % methanol, 2.5% ultra pure water (v/v). The internal temperature was set at 40 $^{o}$C with a flow of 1min/min and a wavelength of 295 nm. Total running time per sample was 16 minutes.

Vitamin E content was calculated as μg of vitamin E per gram of feed or tissue by using the slope and intercept of standards and peak area of the unknowns.

$$\textit{Vitamin E Content } (\mu g) = \frac{\textit{Y-intercept} - (\textit{ vitamin E peak area} \div \textit{IS peak area})}{(\textit{ Slope} \div \textit{grams of sample})}$$

## 4.4 Statistical Analysis

The effects of dietary fat source, time of feeding and inflammatory challenge on fatty acid profile, isoprostanes, COX-2 expression and vitamin E were determined by three-way ANOVA using SAS (version 9.2). Differences among groups were compared by using Student-Newmann-Keuls multiple range tests. Statistical significance was set at $P<0.05$. Mean values ±SEM were reported.

## 4.5 Results

### 4.5.1 Feed Fatty Acid Composition

As shown in table 4.2, the high n-3 diets were significantly higher in total saturated fatty acid and total n-3 content when compared to the low n-3 diet. In contrast, the low n-3 diet contained significantly greater amounts of total monounsaturated fatty acids, PUFAs and n-6 fatty acids content when compared to the high n-3 diet.

**Table 4.2 Fatty Acid Composition of the Diets: Experiment 2**

| | Diets | |
|---|---|---|
| **Fatty Acid** | **High n-3** | **Low n-3** |
| | ----------(%)---------- | |
| C14:0 | 6.74 | 0.09 |
| C16:0 | 23.4 | 13.2 |
| C16:1 | 7.35 | 1.56 |
| C18:0 | 5.99 | 4.87 |
| C18:1 | 19.3 | 27.9 |
| C18:2n-6 | 26.3 | 48.9 |
| C18:3n-3 | 2.97 | 2.85 |
| C20:4n-6 | 0.01 | 0.53 |
| C20:5n-3 | 2.50 | 0.01 |
| C22:6n-3 | 2.39 | 0.01 |
| TSFA: | 39.1 | 18.2 |
| TMUFA: | 26.7 | 29.5 |
| PUFA: | 34.2 | 52.3 |
| Tn-6 PUFA: | 26.3 | 49.4 |
| Tn-3 PUFA: | 7.86 | 2.85 |

Mean of 4 observations. High n-3 diet contained 3.5 % fish oil. Low n-3 diet contained 3.5 % sunflower oil.

Total saturated fatty acids (TSFA) includes-14:0, 16:0, and 18:0. Total monounsaturated fatty acids (MUFA) includes-16:1 and 18:1. Total polyunsaturated fatty acids (PUFA) includes- 18:2n-6, 18:3n-3, 20:4n-6, 20:5n-3, 22:5n-3 and 22:6n-3. Tn-6 PUFA-includes 18:2n-6, 18:3n-6. Tn-3 PUFA- includes 18:3n-3, 20:5n-3, 22:5n-3, and 22:6n-3.

### 4.5.2 Tissue Fatty Acid Composition

Fatty acid composition of liver, spleen, lung and plasma showed significant difference between treatment groups based on diet, time of feeding and LPS injection (Tables 4.3-4.6).

#### *Liver*

Time of feeding (early vs. low) showed no significant effect on liver fatty acid content (Table 4.3) of broiler birds (Diets 1, 2 and 3) ($P>0.05$), except in birds fed late (Diet 4) showed the highest C18:2n-6 when compared to early fed birds when challenged ($P<0.05$). Diet composition showed significant effect on liver fatty acid content. Birds fed high n-3 had significantly higher levels of C20:5n-3, C22:5n-3, C22:6n-3 and total n-3 when compared to birds that were fed low n-3 ($P<0.05$). Birds fed low n-3 (Diet 3 and Diet 4) showed significant difference in liver C18:2n-6 when compared to birds fed high n-3 diets. Diet did not affect C16:1, C16:0 and C18:0 among treatment groups.

LPS challenge showed significant effect within groups. LPS challenged birds from early and late fed high n-3 (Diet 1, Diet 2) had higher content of liver TSFA when compared to PBS (control) birds of the same diet ($P<0.05$). LPS challenge led to a decrease in liver total MUFA content of late fed low n-3 (Diet 4) birds when compared to control ($P<0.05$). LPS challenge led to an increase in total n-6 fatty acids in birds fed late low n-3 (Diet 4) when compared to control birds within the same treatment ($P<0.05$). LPS challenge also showed significant effect among different treatment groups. LPS injected birds from early high n-3 (Diet 1) had significantly higher liver TSFA content when compared to LPS challenged birds from late low n-3

(Diet 4) ($P<0.05$). Early fed low n-3 (Diet 3) LPS challenged birds showed high TMUFA, but low TPUFA liver content when compared to all other LPS challenged birds from all treatment groups ($P<0.05$). Total n-6 was highest in late fed low n-3 (Diet 4) LPS challenged birds when compared to all other LPS birds ($P<0.05$). LPS birds from early and late high n-3 (Diet 1, Diet 2) were significantly higher in total n-3 liver content when compared to low n-3 (Diet 2, Diet 4) ($P<0.05$).

***Spleen***

No significant effects in spleen fatty acid content in regards to time of feeding among all treatment groups were observed ($P>0.05$) (Table 4.4). Diet had significant effects among different dietary treatments. Birds that were fed high n-3 had significantly higher C20:5n-3, C22:5n-3, C22:6n-3 and total n-3 fatty acids when compared to birds fed low n-3 diets ($P<0.05$). However, birds that were fed a low n-3 diet had significantly higher levels of total n-6 PUFAs when compared to birds fed a high n-3 fatty acid diet ($P<0.05$).

In regards to LPS challenge within same treatment groups, LPS challenge led to a decrease in spleen C22:5n-3 of birds fed late high n-3 (Diet 2) when compared to control birds within the same treatment group ($P<0.05$). LPS challenge showed a decreased content of TMUFA, but an increase in C20:4n-6, TPUFA and total n-6 in birds that were late fed low n-3 (Diet 4) compared to control birds within the same treatment group ($P<0.05$).

***Plasma***

Time of feeding did not show any significant difference in plasma fatty acid content (Table 4.5) among early and late fed treatment groups ($P>0.05$). However, dietary treatment showed that birds fed high n-3 had significantly higher plasma C20:5n-3, C22:5n-3, C22:6n-3 and total n-3 fatty acid content compared to low n-3 fed dietary treatment groups ($P<0.05$). Birds fed low n-3 had significantly higher plasma LA and 20:4n6 when compared to high n-3 dietary treatment groups ($P<0.05$). In regards to LPS challenge within treatment groups, a decrease in plasma 16:1 was shown in late low n-3 (Diet 4) birds compared to control birds within the same treatment group ($P<0.05$). No difference in plasma TSFA and TPUFA was observed among LPS challenged birds from all the treatment groups ($P>0.05$). However, TMUFA was highest in late low n-3 (Diet 4) LPS challenged birds when compared to early and late fed high n-3 (Diet 1, Diet 2) LPS challenged birds ($P<0.05$).

***Lung***

Time of feeding did not have any significant effect on lung fatty acid content (Table 4.6) among early and late fed treatment groups ($P>0.05$). However, dietary treatment showed that groups fed high n-3 had significantly higher levels of lung C20:5n-3, C22:5n-3, C22:6n-3 and total n-3 fatty acid content when compared to low n-3 dietary treatment groups ($P<0.05$). Lung C18:2n-6, C20:4n-6, C22:4n-6 and total n-6 fatty acid content was significantly higher in birds fed low n-3 when compared to high n-3 dietary treatment groups ($P<0.05$). LPS challenge showed no significant difference in lung TSFA, TMUFA and TPUFA among all treatment groups ($P>0.05$). Lung total n-6 fatty acid content in low n-3 (Diet 3 and Diet 4) was highest in LPS

challenged birds when compared to early high n-3 (Diet 1 and Diet 2) ($P<0.05$). Total n-3 fatty acid content in the lung was highest in LPS injected birds from high n-3 treatment groups when compared to low n-3 diets ($P<0.05$).

**Table 4.3 Effect of Dietary Fatty Acids and Time of Feeding on Liver Fatty Acid Profile of Broiler Birds upon LPS Challenge: Experiment 2**

| | Treatment | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | Diet 1 | | Diet 2 | | Diet 3 | | Diet 4 | | *Pooled* |
| Fatty Acid (%) | (+) | (-) | (+) | (-) | (+) | (-) | (+) | (-) | *SEM* |
| 14:0 | $0.47^{ab}$ | $0.56^{a}$ | $0.13^{bc}$ | $0.47^{ab}$ | $0.09^{c}$ | $0.11^{bc}$ | $0.01^{c}$ | $0.07^{c}$ | 0.12 |
| 16:0 | $26.00^{a}$ | $23.17^{abc}$ | $23.75^{abc}$ | $22.52^{abc}$ | $24.55^{ab}$ | $22.77^{abc}$ | $20.74^{c}$ | $22.25^{bc}$ | 1.13 |
| 16:1 | 2.25 | 2.71 | 1.97 | 2.81 | 3.72 | 3.29 | 2.15 | 3.67 | 0.53 |
| 18:0 | $19.64^{a}$ | $17.13^{ab}$ | $20.50^{a}$ | $15.27^{b}$ | $18.45^{ab}$ | $19.47^{a}$ | $20.48^{a}$ | $17.52^{ab}$ | 1.24 |
| 18:1 | $18.93^{c}$ | $25.74^{abc}$ | $18.84^{c}$ | $22.16^{bc}$ | $32.86^{a}$ | $28.07^{abc}$ | $20.34^{c}$ | $31.23^{ab}$ | 3.16 |
| 18:2n-6 | $12.45^{b}$ | $11.51^{b}$ | $12.26^{b}$ | $12.53^{b}$ | $13.06^{b}$ | $16.02^{b}$ | $24.19^{a}$ | $14.89^{b}$ | 1.73 |
| 20:4n-6 | $3.36^{b}$ | $3.03^{b}$ | $3.98^{b}$ | $8.57^{ab}$ | $7.27^{ab}$ | $10.27^{a}$ | $11.51^{a}$ | $10.37^{a}$ | 1.92 |
| 20:5n-3 | $3.34^{a}$ | $2.98^{a}$ | $3.56^{a}$ | $2.99^{a}$ | $0.01^{b}$ | $0.01^{b}$ | $0.01^{b}$ | $0.01^{b}$ | 0.34 |
| 22:5n-3 | $2.49^{a}$ | $2.36^{a}$ | $2.59^{a}$ | $2.20^{a}$ | $0.01^{b}$ | $0.01^{b}$ | $0.24^{b}$ | $0.01^{b}$ | 0.20 |
| 22:6n-3 | $11.07^{a}$ | $10.62^{a}$ | $12.34^{a}$ | $10.15^{a}$ | $0.01^{b}$ | $0.01^{b}$ | $0.35^{b}$ | $0.01^{b}$ | 1.11 |
| TSFA | $46.11^{a}$ | $40.87^{cd}$ | $44.47^{ab}$ | $38.26^{d}$ | $43.08^{abc}$ | $42.35^{bc}$ | $41.22^{cd}$ | $39.84^{cd}$ | 1.02 |
| TMUFA | $21.17^{b}$ | $28.44^{ab}$ | $20.81^{b}$ | $24.97^{ab}$ | $36.58^{a}$ | $31.35^{ab}$ | $22.49^{b}$ | $34.90^{a}$ | 3.62 |
| TPUFA | $32.72^{a}$ | $30.69^{ab}$ | $34.72^{a}$ | $36.77^{a}$ | $20.33^{b}$ | $26.29^{ab}$ | $36.29^{a}$ | $25.26^{ab}$ | 3.57 |
| Tn-6 | $15.81^{d}$ | $14.54^{d}$ | $16.24^{cd}$ | $21.09^{bcd}$ | $20.33^{bcd}$ | $26.29^{b}$ | $35.70^{a}$ | $25.26^{bc}$ | 2.92 |
| Tn-3 | $16.90^{a}$ | $16.15^{a}$ | $18.48^{a}$ | $15.67^{a}$ | $0.01^{b}$ | $0.01^{b}$ | $0.59^{b}$ | $0.01^{b}$ | 1.54 |

Mean of 6 observations. Means with different letters within a row significantly differ from each other ($P<0.05$). Pooled SEM = pooled standard error of mean. Diet 1: (early fed, high n-3), Diet 2: (late fed, high n-3), Diet 3: (early fed, low n-3), Diet 4: (late fed, low n-3) (+): LPS Injected, (-): PBS.

TSFA: Total saturated fatty acids- includes 14:0, 16:0, 18:0; TMUFA: total monounsaturated fatty acids- includes 16:1, 18:1; TPUFA: Polyunsaturated fatty acids- includes 18:2n-6, 18:3n-6 and n-3, 20:4n-6, 20:5n-3, 22:5n-3, 22:6n-3. Tn-6 PUFA-includes 18:2n-6, 18:3n-6, 20:2n-6, 20:3n-6, 20:4n-6, and 22:4n-6. Tn-3 PUFA- includes 18:3n-3, 20:5n-3, 22:5n-3, and 22:6n-3.

**Table 4.4 Effect of Dietary Fatty Acids and Time of Feeding on Spleen Fatty Acid Profile of Broiler Birds upon LPS Challenge: Experiment 2**

| | Treatment | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | Diet 1 | | Diet 2 | | Diet 3 | | Diet 4 | | *Pooled* |
| **Fatty Acid (%)** | **(+)** | **(-)** | **(+)** | **(-)** | **(+)** | **(-)** | **(+)** | **(-)** | ***SEM*** |
| 14:0 | $2.05^{a}$ | $1.90^{a}$ | $1.63^{a}$ | $1.78^{a}$ | $0.44^{b}$ | $0.14^{b}$ | $0.29^{b}$ | $0.83^{b}$ | 0.28 |
| 15:0 | $1.47^{ab}$ | $1.88^{ab}$ | $2.44^{a}$ | $1.83^{ab}$ | $0.54^{b}$ | $1.75^{ab}$ | $1.67^{ab}$ | $1.42^{ab}$ | 0.50 |
| 16:0 | $25.28^{ab}$ | $24.55^{ab}$ | $25.68^{a}$ | $24.32^{ab}$ | $22.39^{b}$ | $23.67^{ab}$ | $23.56^{ab}$ | $23.29^{ab}$ | 1.06 |
| 16:1 | $4.16^{a}$ | $3.78^{ab}$ | $2.27^{abc}$ | $3.94^{ab}$ | $2.24^{abc}$ | $2.17^{bc}$ | $1.66^{c}$ | $3.45^{abc}$ | 0.68 |
| 18:0 | $11.98^{ab}$ | $11.71^{ab}$ | $15.63^{a}$ | $11.80^{ab}$ | $13.77^{ab}$ | $12.92^{ab}$ | $15.21^{ab}$ | $10.99^{b}$ | 1.44 |
| 18:1 | $24.91^{bc}$ | $25.71^{bc}$ | $21.46^{c}$ | $27.29^{abc}$ | $29.64^{ab}$ | $29.50^{ab}$ | $24.37^{bc}$ | $32.97^{a}$ | 2.49 |
| 18:2n-6 | $16.53^{bc}$ | $15.80^{bc}$ | $15.65^{bc}$ | $14.83^{c}$ | $21.76^{a}$ | $22.33^{a}$ | $21.52^{a}$ | $19.99^{ab}$ | 1.62 |
| 18:3n-6 | $0.88^{a}$ | $1.11^{a}$ | $0.63^{ab}$ | $0.99^{a}$ | $0.01^{b}$ | $0.52^{ab}$ | $0.01^{b}$ | $0.69^{ab}$ | 0.28 |
| 20:4n-6 | $2.87^{c}$ | $2.85^{c}$ | $3.94^{bc}$ | $3.36^{c}$ | $7.92^{a}$ | $5.93^{b}$ | $9.20^{a}$ | $4.77^{bc}$ | 0.76 |
| 20:5n-3 | $3.60^{b}$ | $4.06^{ab}$ | $4.68^{a}$ | $3.79^{ab}$ | $0.01^{c}$ | $0.01^{c}$ | $0.01^{c}$ | $0.01^{c}$ | 0.33 |
| 22:4n-6 | $0.01^{c}$ | $0.01^{c}$ | $0.01^{c}$ | $0.01^{c}$ | $1.28^{abc}$ | $1.10^{bc}$ | $2.53^{a}$ | $1.62^{ab}$ | 0.48 |
| 22:5n-3 | $2.35^{a}$ | $2.50^{a}$ | $1.25^{b}$ | $2.38^{a}$ | $0.01^{c}$ | $0.01^{c}$ | $0.01^{c}$ | $0.01^{c}$ | 0.39 |
| 22:6n-3 | $3.92^{a}$ | $4.14^{a}$ | $4.74^{a}$ | $3.69^{a}$ | $0.01^{b}$ | $0.01^{b}$ | $0.01^{b}$ | $0.01^{b}$ | 0.71 |
| TSFA | $40.78^{ab}$ | $40.04^{ab}$ | $45.38^{a}$ | $39.73^{ab}$ | $37.15^{b}$ | $38.48^{ab}$ | $40.72^{ab}$ | $36.52^{b}$ | 2.79 |
| TMUFA | $29.09^{ab}$ | $29.50^{ab}$ | $23.73^{b}$ | $31.23^{ab}$ | $31.88^{ab}$ | $31.67^{ab}$ | $26.03^{b}$ | $36.42^{a}$ | 3.05 |
| TPUFA | $30.13^{ab}$ | $30.47^{ab}$ | $30.89^{ab}$ | $29.04^{ab}$ | $30.96^{ab}$ | $29.85^{ab}$ | $33.25^{a}$ | $27.06^{b}$ | 1.76 |
| Tn-6 | $19.40^{c}$ | $18.65^{c}$ | $19.59^{c}$ | $18.19^{c}$ | $30.96^{a}$ | $29.33^{ab}$ | $33.25^{a}$ | $26.37^{b}$ | 1.64 |
| Tn-3 | $10.73^{a}$ | $11.82^{a}$ | $11.30^{a}$ | $10.85^{a}$ | $0.01^{b}$ | $0.52^{b}$ | $0.01^{b}$ | $0.69^{b}$ | 1.13 |

Mean of 4 observations. Means with different letters within a row significantly differ from each other ($P<0.05$). Pooled SEM = pooled standard error of mean. Diet 1: (early fed, high n-3), Diet 2: (late fed, high n-3), Diet 3: (early fed, low n-3), Diet 4: (late fed, low n-3) (+): LPS Injected, (-): PBS.

TSFA: Total saturated fatty acids- includes 14:0, 16:0, 18:0; TMUFA: total monounsaturated fatty acids- includes 16:1,18:1; TPUFA: Polyunsaturated fatty acids- includes 18:2n-6, 18:3n-6 and n-3, 20:4n-6, 20:5n-3, 22:4n-6, 22:5n-3, 22:6n-3; Tn-6 and Tn-3 fatty acids are components of PUFA. Tn-6 PUFA-includes 18:2n-6, 18:3n-6, 20:2n-6, 20:3n-6, 20:4n-6, and 22:4n-6. Tn-3 PUFA- includes 18:3n-3, 20:5n-3, 22:5n-3, and 22:6n-3.

**Table 4.5 Effect of Dietary Fatty Acids and Time of Feeding on Plasma Fatty Acid Profile of Broiler Birds upon LPS Challenge: Experiment 2**

| | Treatment | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | Diet 1 | | Diet 2 | | Diet 3 | | Diet 4 | | *Pooled* |
| **Fatty Acid (%)** | **(+)** | **(-)** | **(+)** | **(-)** | **(+)** | **(-)** | **(+)** | **(-)** | ***SEM*** |
| 14:0 | 1.07 | 0.22 | 0.91 | 0.78 | 0.48 | 0.29 | 0.15 | 0.41 | ± 0.30 |
| 16:0 | $25.82^{a}$ | $25.70^{a}$ | $25.03^{ab}$ | $26.32^{a}$ | $21.44^{b}$ | $21.30^{b}$ | $23.41^{ab}$ | $23.41^{ab}$ | ± 1.20 |
| 16:1 | $1.62^{b}$ | $1.96^{ab}$ | $1.60^{b}$ | $1.63^{b}$ | $1.53^{b}$ | $1.66^{b}$ | $1.75^{b}$ | $2.46^{a}$ | ± 0.21 |
| 18:0 | $14.87^{bc}$ | $13.53^{c}$ | $14.12^{bc}$ | $13.65^{c}$ | $17.78^{a}$ | $16.34^{ab}$ | $15.18^{bc}$ | $13.00^{c}$ | ± 0.80 |
| 18:1 | $14.14^{c}$ | $17.94^{bc}$ | $15.77^{c}$ | $14.88^{c}$ | $17.98^{bc}$ | $19.56^{bc}$ | $23.73^{ab}$ | $28.77^{a}$ | ± 2.27 |
| 18:2n-6 | $21.80^{c}$ | $21.42^{c}$ | $20.86^{c}$ | $21.03^{c}$ | $29.98^{a}$ | $30.58^{a}$ | $28.47^{ab}$ | $23.75^{bc}$ | ± 1.91 |
| 18:3n-6 | $1.43^{a}$ | $0.94^{ab}$ | $1.21^{ab}$ | $0.73^{ab}$ | $0.20^{b}$ | $0.58^{ab}$ | $0.83^{ab}$ | $0.74^{ab}$ | ± 0.31 |
| 20:4n-6 | $2.63^{c}$ | $2.86^{c}$ | $2.70^{c}$ | $2.59^{c}$ | $9.59^{a}$ | $8.78^{ab}$ | $6.48^{b}$ | $6.75^{ab}$ | ± 0.94 |
| 20:5n-3 | $5.88^{a}$ | $5.63^{a}$ | $5.57^{a}$ | $5.57^{a}$ | $0.01^{b}$ | $0.01^{b}$ | $0.01^{b}$ | $0.01^{b}$ | ± 0.37 |
| 22:5n-3 | $2.67^{a}$ | $2.12^{a}$ | $2.54^{a}$ | $2.35^{a}$ | $0.01^{b}$ | $0.01^{b}$ | $0.01^{b}$ | $0.01^{b}$ | ± 0.23 |
| 22:6n-3 | $8.08^{a}$ | $7.68^{a}$ | $9.69^{a}$ | $9.81^{a}$ | $0.47^{b}$ | $0.01^{b}$ | $0.01^{b}$ | $0.01^{b}$ | ± 0.84 |
| TSFA | 41.76 | 39.45 | 40.06 | 40.75 | 39.71 | 37.93 | 38.74 | 36.82 | ± 1.80 |
| TMUFA | $15.76^{c}$ | $19.90^{bc}$ | $17.37^{c}$ | $16.51^{c}$ | $19.51^{bc}$ | $21.22^{bc}$ | $25.49^{ab}$ | $31.23^{a}$ | ± 2.45 |
| TPUFA | $42.48^{a}$ | $40.64^{ab}$ | $42.57^{a}$ | $42.74^{a}$ | $40.79^{ab}$ | $40.85^{ab}$ | $35.77^{ab}$ | $31.94^{b}$ | ± 2.78 |
| Tn-6 | $24.42^{dc}$ | $24.28^{dc}$ | $26.56^{d}$ | $24.27^{dc}$ | $40.12^{a}$ | $40.27^{a}$ | $34.94^{ab}$ | $31.20^{bc}$ | ± 2.33 |
| Tn-3 | $18.05^{a}$ | $16.36^{a}$ | $19.01^{a}$ | $18.47^{a}$ | $0.66^{b}$ | $0.58^{b}$ | $0.83^{b}$ | $0.74^{b}$ | ± 1.04 |

Mean of 4 observations. Means with different letters within a row significantly differ from each other ($P<0.05$). Pooled SEM = pooled standard error of mean. Diet 1: (early fed, high n-3), Diet 2: (late fed, high n-3), Diet 3: (early fed, low n-3), Diet 4: (late fed, low n-3) (+): LPS Injected, (-): PBS.

TSFA: Total saturated fatty acids- includes 14:0, 16:0, 18:0; TMUFA: total monounsaturated fatty acids- includes 16:1,18:1; TPUFA: Polyunsaturated fatty acids- includes 18:2n-6, 18:3n-6 and n-3, 20:4n-6, 20:5n-3, 22:4n-6, 22:5n-3, 22:6n-3; Tn-6 and Tn-3 fatty acids are components of PUFA. Tn-6 PUFA-includes 18:2n-6, 18:3n-6, 20:2n-6, 20:3n-6, 20:4n-6, and 22:4n-6. Tn-3 PUFA- includes 18:3n-3, 20:5n-3, 22:5n-3, and 22:6n-3.

**Table 4.6 Effect of Dietary Fatty Acids and Time of Feeding on Lung Fatty Acid Profile of Broiler Birds upon LPS Challenge: Experiment 2**

| | Treatment | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | Diet 1 | | Diet 2 | | Diet 3 | | Diet 4 | | *Pooled* |
| **Fatty Acid (%)** | **(+)** | **(-)** | **(+)** | **(-)** | **(+)** | **(-)** | **(+)** | **(-)** | ***SEM*** |
| 14:0 | $2.11^{b}$ | $3.46^{a}$ | $1.97^{b}$ | $3.63^{a}$ | $0.68^{b}$ | $1.70^{b}$ | $0.85^{c}$ | $2.40^{b}$ | ± 0.25 |
| 16:0 | 29.90 | 28.88 | 30.35 | 29.14 | 28.67 | 26.47 | 28.06 | 26.72 | ± 1.26 |
| 16:1 | 4.27 | 4.05 | 4.26 | 4.24 | 2.97 | 3.73 | 3.23 | 3.13 | ± 0.51 |
| 18:0 | 12.01 | 13.18 | 11.80 | 12.17 | 11.74 | 9.07 | 11.16 | 12.01 | ± 1.45 |
| 18:1 | 24.52 | 25.93 | 25.01 | 27.28 | 28.33 | 31.14 | 27.71 | 27.65 | ± 2.67 |
| 18:2n-6 | $14.71^{bc}$ | $13.44^{c}$ | $13.44^{c}$ | $12.76^{c}$ | $19.11^{ab}$ | $21.51^{a}$ | $21.42^{a}$ | $20.72^{a}$ | ± 1.52 |
| 18:3n-6 | $1.41^{a}$ | $1.21^{ab}$ | $1.27^{a}$ | $1.07^{ab}$ | $0.67^{b}$ | $0.84^{ab}$ | $0.98^{ab}$ | $0.91^{ab}$ | ± 0.18 |
| 20:4n-6 | $2.13^{b}$ | $1.99^{b}$ | $2.65^{b}$ | $1.98^{b}$ | $6.49^{a}$ | $4.43^{ab}$ | $5.44^{a}$ | $5.20^{a}$ | ± 0.78 |
| 20:5n-3 | $3.12^{a}$ | $2.95^{a}$ | $3.28^{a}$ | $2.94^{a}$ | $0.01^{b}$ | $0.01^{b}$ | $0.01^{b}$ | $0.01^{b}$ | ± 0.17 |
| 22:4n-6 | $0.01^{b}$ | $0.01^{b}$ | $0.01^{b}$ | $0.01^{b}$ | $1.37^{a}$ | $1.19^{a}$ | $1.37^{a}$ | $1.31^{a}$ | ± 0.23 |
| 22:5n-3 | $1.90^{a}$ | $1.74^{a}$ | $1.97^{a}$ | $1.66^{a}$ | $0.01^{b}$ | $0.01^{b}$ | $0.01^{b}$ | $0.01^{b}$ | ± 0.16 |
| 22:6n-3 | $3.91^{a}$ | $3.34^{a}$ | $4.11^{a}$ | $3.34^{a}$ | $0.01^{b}$ | $0.01^{b}$ | $0.01^{b}$ | $0.01^{b}$ | ± 0.31 |
| TSFA | 44.00 | 45.40 | 44.00 | 44.91 | 41.00 | 37.22 | 39.96 | 41.11 | ± 2.64 |
| TMUFA | 28.72 | 30.01 | 29.24 | 31.45 | 31.31 | 34.90 | 30.90 | 30.76 | ± 3.13 |
| TPUFA | $27.23^{ab}$ | $24.62^{ab}$ | $26.74^{ab}$ | $23.77^{b}$ | $27.64^{ab}$ | $27.95^{ab}$ | $29.27^{a}$ | $28.23^{ab}$ | ± 1.43 |
| Tn-6 | $16.98^{b}$ | $15.30^{b}$ | $16.11^{b}$ | $14.77^{b}$ | $26.91^{a}$ | $27.17^{a}$ | $28.23^{a}$ | $27.33^{a}$ | ± 1.20 |
| Tn-3 | $10.33^{a}$ | $9.24^{a}$ | $10.61^{a}$ | $9.02^{a}$ | $0.67^{b}$ | $0.84^{b}$ | $0.98^{b}$ | $0.91^{b}$ | ± 0.59 |

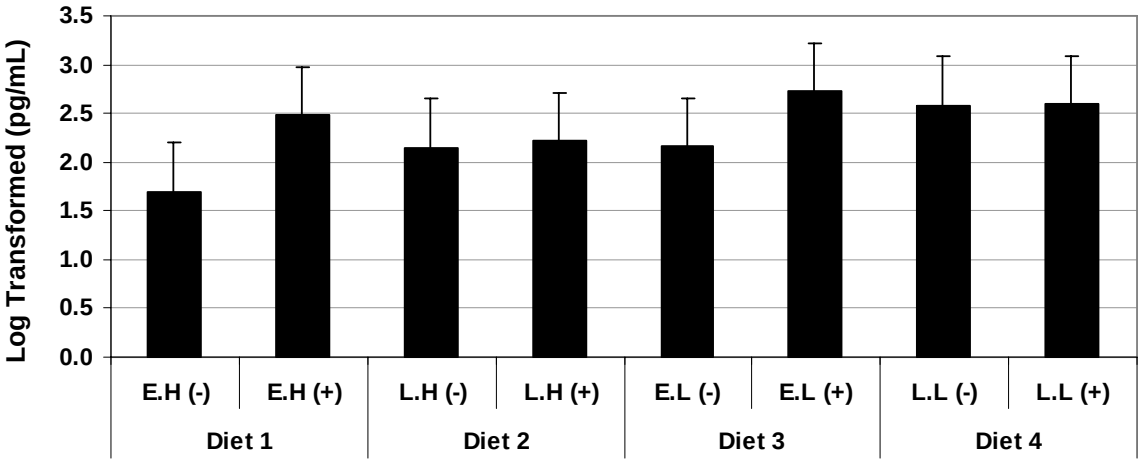
Mean of 4 observations. Means with different letters within a row significantly differ from each other ($P<0.05$). Pooled SEM = pooled standard error of mean. Diet 1: (early fed, high n-3), Diet 2: (late fed, high n-3), Diet 3: (early fed, low n-3), Diet 4: (late fed, low n-3) (+): LPS Injected, (-): PBS.

TSFA: Total saturated fatty acids- includes 14:0, 16:0, 18:0; TMUFA: total monounsaturated fatty acids- includes 16:1,18:1; TPUFA: Polyunsaturated fatty acids- includes 18:2n-6, 18:3n-6 and n-3, 20:4n-6, 20:5n-3, 22:4n-6, 22:5n-3, 22:6n-3; Tn-6 and Tn-3 fatty acids are components of PUFA. Tn-6 PUFA-includes 18:2n-6, 18:3n-6, 20:2n-6, 20:3n-6, 20:4n-6, and 22:4n-6. Tn-3 PUFA- includes 18:3n-3, 20:5n-3, 22:5n-3, and 22:6n-3.

### 4.5.3 Plasma Isoprostanes

Plasma isoprostanes shown in Figure 4.1 shows that there is no significance among treatment groups ($P>0.05$). Diet 3 showed that LPS injected chickens may have slightly elevated amount of isoprostanes when compared to control, but it was not statistically different. A similar trend was observed in early fed high n-3 birds, but also there was no significant difference due to the high variability of the data obtained.

**Figure 4.1 Effects of Dietary Treatment and LPS Challenge on Plasma Isoprostanes: Experiment 2**




Diets 1-4 represent a corn-soybean based broiler chicken diet. 3.5% fish oil (source of n-3 PUFAs) was present in Diet 1 and Diet 2. 3.5% sunflower oil (source of n-6 fatty acids) was present in Diet 3 and Diet 4. Time of feeding is also divided among Diets 1-4. Early fed (<5 hrs, Diets 1 and 3) and late fed (>48 hrs, Diets 2 and 4).

E.H= Early fed, High n-3; L.H= Late fed, High n-3; E.L= Early fed, Low n-3; L.L= late fed, Low n-3. (-): Control (PBS injected); (+): LPS injected

Each bar represents mean (n=4). No significant difference among treatments ($P>0.05$).

### 4.5.4 Tissue and Plasma Vitamin E Content

Liver vitamin E content (Figure 4.2) was significantly higher in control birds from early high n-3 groups (Diet 1) compared to all treatment groups except late low n-3 control birds (Diet 4) ($P<0.05$). LPS challenge led to a decrease in liver vitamin E content of early fed high n-3 (Diet 1) birds when compared to control birds within the same treatment group ($P<0.05$).

Lung vitamin E content (Figure 4.3) was highest in LPS challenged late fed low n-3 (Diet 4) compared to early fed high n-3 group (Diet 1) ($P<0.05$). An increase in lung vitamin E content was noted after LPS challenge in low n-3 birds (Diet 4) ($P<0.05$). However, the opposite was observed in LPS challenged birds from early fed low n-3 (Diet 3), where there was a decrease in lung vitamin E content after LPS challenge ($P<0.05$). No significant difference was observed among LPS challenged and control birds fed high and low n-3 (Diet 1, Diet 3) ($P>0.05$).

Vitamin E content in the small intestine (Figure 4.4) was highest in LPS challenged birds from late low n-3 (Diet 4) compared to control birds within the same group ($P<0.05$). Among birds that were LPS challenged, highest vitamin E content was shown in late low n-3 challenged birds (Diet 4) compared to early low n-3 (Diet 3) and early high n-3 LPS challenged birds (Diet 1) ($P<0.05$), but not late high n-3 LPS challenged birds (Diet 2) ($P>0.05$).

Plasma vitamin E (Figure 4.5) was highest in early fed low n-3 (Diet 3) birds compared to all other treatment groups ($P<0.05$). It also showed that after LPS challenge, there is an increase in vitamin E content compared to control birds within the same treatment group in Diet 3 ($P<0.05$).

**Figure 4.2 Effect of Dietary Treatment and LPS Challenge on Liver Vitamin E Content: Experiment 2**

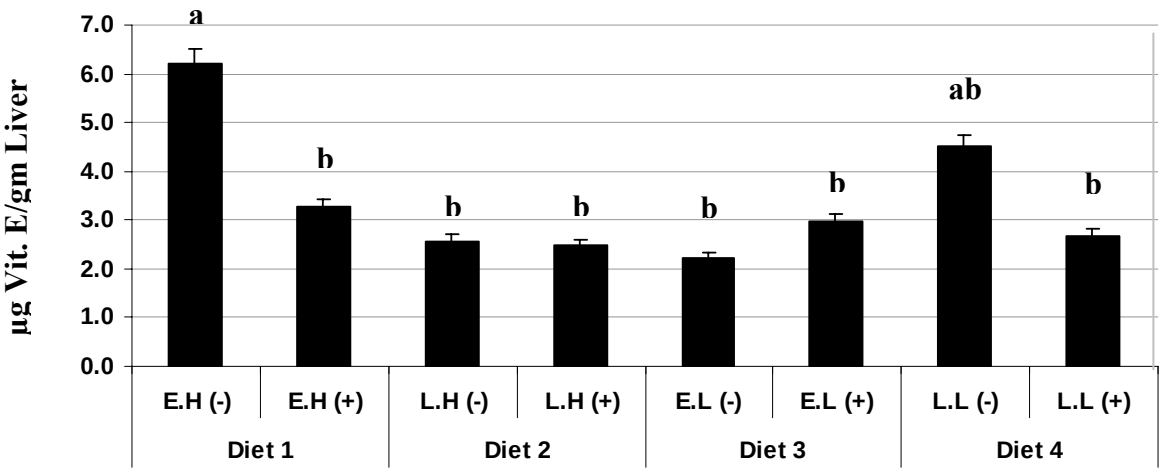
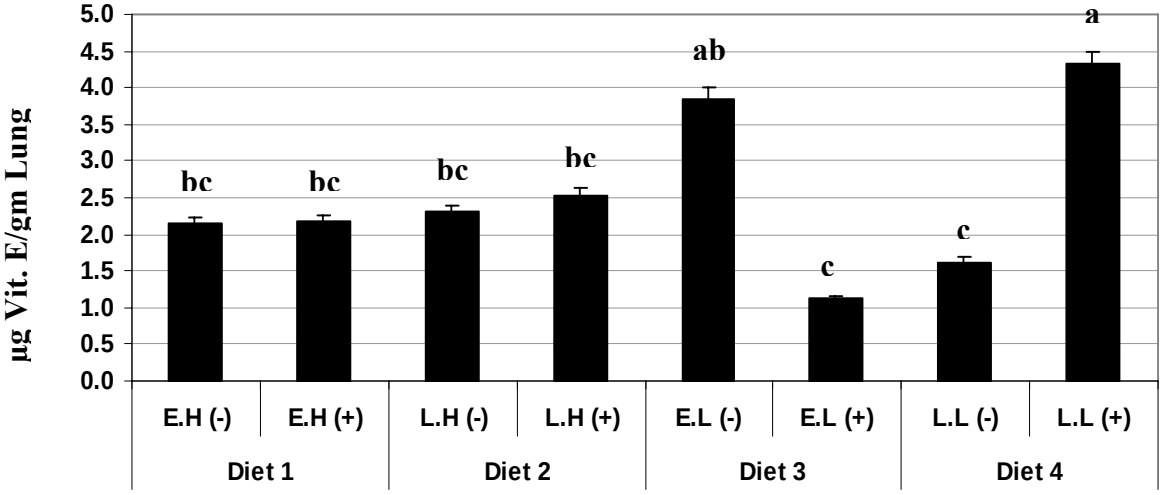



**Figure 4.3 Effect of Dietary Treatment and LPS Challenge on Lung Vitamin E Content: Experiment 2**




Diet 1: (early fed, high n-3), Diet 2: (late fed, high n-3), Diet 3: (early fed, low n-3), Diet 4: (late fed, low n-3). E.H= Early fed, High n-3; L.H= Late fed, High n-3; E.L= Early fed, Low n-3; L.L= late fed, Low n-3. (-): Control (PBS injected); (+): LPS injected

Each bar represents mean (n=4). $^{a\text{-}c}$ represents significant difference among dietary groups ($P<0.05$)

**Figure 4.4 Effect of Dietary Treatment and LPS Challenge on Small Intestine Vitamin E Content: Experiment 2**

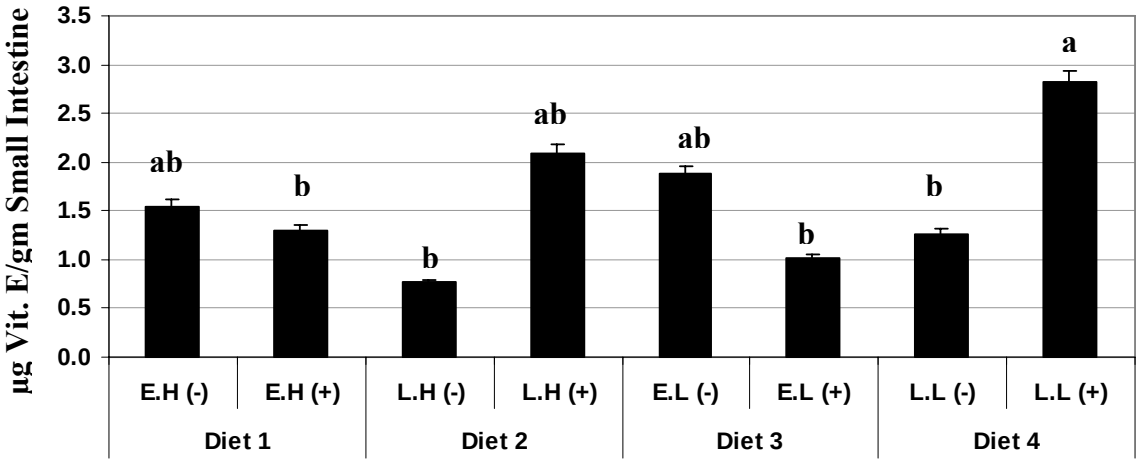
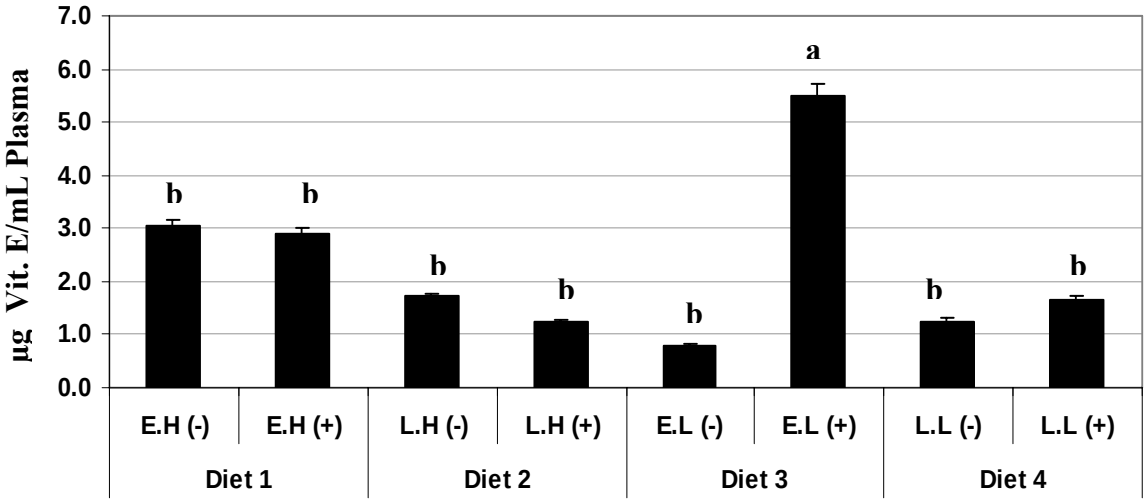



**Figure 4.5 Effect of Dietary Treatment and LPS Challenge on Plasma Vitamin E Content: Experiment 2**



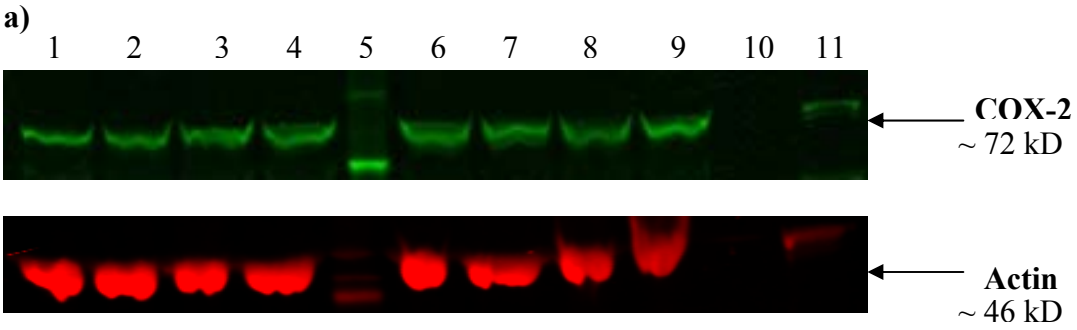

Diet 1: (early fed, high n-3), Diet 2: (late fed, high n-3), Diet 3: (early fed, low n-3), Diet 4: (late fed, low n-3). E.H= Early fed, High n-3; L.H= Late fed, High n-3; E.L= Early fed, Low n-3; L.L= late fed, Low n-3. (-): Control (PBS injected); (+): LPS injected

Each bar represents mean (n=4). $^{a-b}$ represents significant difference among dietary groups ($P<0.05$)

### 4.5.5 Cyclooxygenase-2 Protein Expression

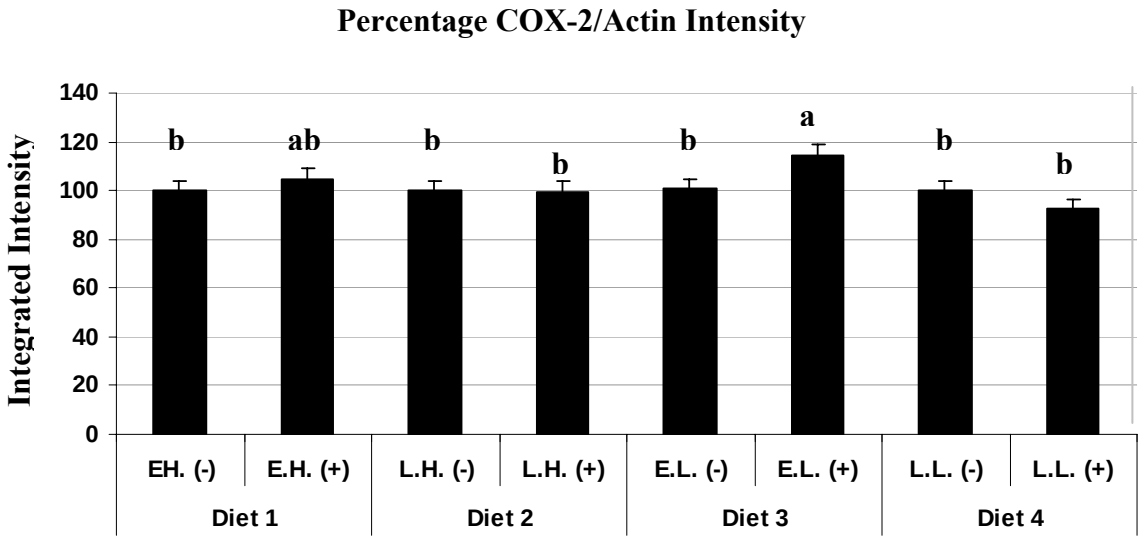
Injection of LPS led to an increase in spleen COX-2 expression in early high n-3 birds (Diet 1). Figure 4.6 shows both the bands for COX-2 and β-actin from control and LPS injected early high n-3 (Diet 1). LPS injection led to an increase in COX-2 protein expression in birds from early low n-3 (Diet 3) compared to control birds within the same treatment group ($P<0.05$). LPS birds from early low n-3 (Diet 3) had significantly higher COX-2 protein expression compared to LPS challenged birds from late high (Diet 2) and late low (Diet 4) treatment groups ($P<0.05$), but not to LPS challenged birds from early high n-3 (Diet 1) treatment group ($P>0.05$) (Figure 4.7).

**Figure 4.6 COX-2/Beta Actin Expression in the Spleen of Birds Fed an Early High n-3 Diet upon LPS Challenge: Experiment 2**




1-4: Early high n-3 Control, 5: Marker ladder, 6-9: Early high n-3 LPS, 10: Blank, 11: Santa Cruz Mouse COX-2 Positive Control, 12: Blank (not shown)

**Figure 4.7 COX-2/Beta Actin Expression of all Dietary Treatment Groups: Experiment 2**




Diet 1: (early fed, high n-3), Diet 2: (late fed, high n-3), Diet 3: (early fed, low n-3), Diet 4: (late fed, low n-3). E.H= Early fed, High n-3; L.H= Late fed, High n-3; E.L= Early fed, Low n-3; L.L= late fed, Low n-3. (-): Control (PBS injected); (+): LPS injected

Each bar represents mean (n=4). Pooled SEM (standard error of mean) value is ± 3.79. $^{a-b}$ represents significant difference among dietary groups ($P < 0.05$).

## 4.6 Discussion

The purpose of this study was to investigate the effect of diet, time of feeding and LPS challenge on the content of fatty acids (n-6 and n-3), isoprostanes and vitamin E in tissues and COX-2 protein expression of the spleen.

Early access to nutrients has been associated with greater organ weights and lymphocyte proliferation, and early maturation of the immune system. This allows hatchlings to utilize yolk components that are meant for antibody synthesis instead of utilizing it for muscle deposition (Dibner *et al*., 1998 and Sklan, 2005). Fatty acids are known to have different effects on immune and inflammatory responses. Broiler diets are high in saturated fats and n-6 fatty acids. PUFAs of the n-6 family (i.e. LA and AA) are known to have effects that stimulate inflammation by production of pro-inflammatory eicosanoids. PUFAs derived from n-3 sources are known for their beneficial suppressive effects on antibody, cytokine production and expression of adhesion molecules (Calder, 2002).

### *Fatty Acid Composition after LPS Challenge*

This study showed that inclusion of dietary n-6 and n-3 PUFA changed the composition of fatty acids in different tissues. Feeding preformed n-6 or n-3 PUFA increased the content of these fatty acids in liver, spleen, lung and plasma. This agrees with previous studies done by Selvaraj and Cherian (2004) and Hall *et al*. (2007), where inclusion of n-3 and n-6 PUFA led to higher deposition of these fatty acids in spleen, thrombocytes and plasma in chickens. In this study, LPS challenge among different treatment groups showed significant incorporation of either n-6 or n-3 fatty acid

depending on dietary fatty acid intake which is of importance for further eicosanoid synthesis. Incorporation of fatty acids into the membrane of spleen, liver and lung cells can influence the type of eicosanoids produced; thus addition of n-3 fatty acids in the diets might protect the birds in the presence of inflammation during growth.

### *Plasma Isoprostanes after LPS Challenge*

Plasma isoprostanes showed variations upon LPS injection but it did not show significant differences among treatments. In the presence of a free radical attack on the membrane of cell membranes, isoprostanes are released into the bloodstream and are excreted in the urine. Altered generation of isoprostanes in association with oxidative stress of fats (i.e. AA) has been liked to pulmonary distress, chronic inflammation, aging, Alzheimer's disease. Isoprostanes also act as modulators of platelet aggregation and activation of smooth cell constriction. Human studies have shown that there is a link in the increase level of expression of isoprostanes due to peroxidation of AA which leads to pulmonary inflammation (Halliwell and Chirico, 1993; Morrow 2005), but studies in avian models showing isoprostane production in the presence of inflammation have not been reported.

### *Vitamin E Content after LPS Challenge*

Broiler diets are usually low in antioxidants which leave broiler chickens more susceptible to oxidative stress due to fast growth and metabolism (Nain *et al*., 2008; Julian, 2005). Lipid peroxidation of cell membranes occurs due to the presence of PUFAs which under oxidative stress tends to increase (Lykkesfeldt and Svendsen, 2007;

Chow, 1991). Adequate antioxidant protection in the diet may prevent the peroxidation of PUFAs. Marine source derived PUFAs, which tend to be more oxidizable require intake of proper levels of antioxidants such as vitamin E (alpha-tocopherol). Cherian *et al*. (2007) reported that inclusion of vitamin E enhanced the oxidative stability and decreased the accumulation of TBARS in chicken eggs. Vitamin E is considered the major fat soluble chain-breaking antioxidant. Studies performed in dogs and rats, show that inclusion of menhaden fish oil increased lipid peroxidation based on plasma and urinary TBARS expression (Wander *et al*., 1997; Meydani *et al*., 1993). Previously, Chapter 3 described an increase in TBARS content in thigh and breast muscle of broiler birds that were fed diet that contained high n-3 when compared to low n-3 fatty acids. The current study showed that vitamin E content was lower in lung and plasma of birds fed high n-3 PUFA diets. Interestingly, liver vitamin E content of early high n-3 birds was significantly higher when compared to late high n-3 birds, but it decreased after LPS challenge. It was also interesting that LPS injection led to an increase in lung vitamin E content of birds from low n-3 diets when compared to control groups. Perhaps, in the presence of inflammation, tissues that have high oxidative stress (i.e. lung) tend to accumulate vitamin E to prevent increased production of pro-inflammatory cytokines and oxidative stress by lipid radicals by monocytes, macrophages and leukocytes.

### *Cyclooxygenase-2 (COX-2) Expression after LPS Challenge*

Expression of COX-2 in the presence of inflammation (i.e. LPS endotoxin induced inflammation) is considered the rate limiting step in the formation of PGs, and

TXs from n-6 (AA) and n-3 (EPA/DHA) fatty acids. Elevated levels of COX-2 have been found in the presence of acute/chronic inflammation, atherosclerosis, and cancer. Scott and Owens (2008) showed that nucleated chicken thrombocytes were able to increase the expression of mRNA COX-2 after LPS exposure, which led to increased $PGE_2$ production. Mitogen, viral and LPS stimulation is known to induce the expression of COX-2 in chicken cells (Khatri and Sharma, 2006; Xie *et al*., 1991; Scott and Owens, 2008).

In this study, COX-2 expression in chicken spleen tissue was highest in early low n-3 after LPS injection when compared to control within the same treatment group. Increased expression of COX-2 by supplementation of n-6 PUFAs causes an increase in the production of $PGE_2$ and $TX_2$ which leads to increased vascular permeability, smooth muscle contraction or dilation, pain, and fever. Spleen tissue of birds fed low n-3 (Diet 3 and Diet 4) had higher total n-6 fatty acids upon challenge. This increase in n-6 fatty acids may increase the COX-2 protein expression in these treatments. The effects of n-3 PUFAs on COX-2 occur via incorporation of n-3 PUFAs into cellular membranes, competition for the COX-2 binding site and eventual substrate produced ($PG_3$, $TX_3$, $Prosctacyclin_3$). Inclusion of n-3 PUFAs (DHA/EPA) in the broiler diets may have decreased the expression of COX-2 protein because metabolites of n-3 PUFAs (i.e. PGE3) are weaker inducers of COX-2 when compared to metabolites from n-6 PUFAs (i.e. PGE2). Therefore, n-3 PUFAs are able of creating a negative feedback mechanism of COX-2 and decrease PGE2 synthesis.

# 5. Conclusion

The experimental work that is presented in this thesis showed that dietary manipulation of the feed by altering the fatty acid source had a significant impact on the deposition of total lipids, n-6 and n-3 PUFA in the tissue and plasma lipids, TBARS, NEFA, vitamin E and COX-2 protein expression. Tissues that are high in n-6 PUFAs may exert more pro-inflammatory characteristics and lead to the increased expression of inflammatory proteins such as COX-2. On the other hand, feeding dietary fat sources high in n-3 PUFAs reduces the production of pro-inflammatory eicosanoids ($PGE_2$ and $TX_2$) via down-regulation of COX-2 protein expression.

This study showed that there was no significant difference in regards to time of feeding on bird performance and tissue weights, except thigh muscle and the liver. Breast and thigh muscle are of economic importance for the poultry industry; thus an increase in meat yield is of beneficial importance. The liver is the primary organ for fat metabolism and distribution to other organs. In the presence of immune challenge, the energy demand for cell formation increases dramatically. Thus, the ability of the chicks to fight off environmental stressors may depend on tissue weight. Although time of feeding has been reported to increase the organ weights and performance of broiler chicks, it is not conclusive that it affects their immune performance at later stages of their life. However, our study was conducted in a university facility with clean, hygienic and optimal dietary conditions. The results may have more application in suboptimal facilities with less hygienic conditions.

As observed in chapter 3, LPS challenge led to an increase in n-6 fatty acids (Diet 4) and TPUFA. This study showed that there are alterations in fatty acid profile in

tissues due to LPS. An increase in PUFA content could be due to the increase demand in tissue cells for energy and production of metabolites to fight off immune challenge.

In regards to lipid peroxidation products, this study showed that diets high in n-3 PUFA tended to have increased TBARS when compared to low n-3 upon LPS challenge. In the presence of immune challenge lipids are being oxidized by the increase influx of white blood cells (i.e. macrophages, lymphocytes, heterophils) to the site of inflammation. In the second study, there was no difference in isoprostane production due to the data variability, but studies in human, mouse and rat models have shown that in the presence of n-6 PUFAs (i.e. AA) and LPS injection, there is an increase in isoprostane production.

The presence of antioxidants is important to prevent lipid peroxidation products in diets high in PUFAs. In this study, vitamin E status in the tissues showed that in the presence of inflammation and high PUFAs, a higher demand for antioxidants is required. Previous studies have shown that inclusion of vitamin E in the diet protects from cells from lipid peroxidation as well as decreasing the expression of COX-2. Perhaps inclusion of vitamin E along with n-3 PUFAs, may dramatically decrease the expression of pro-inflammatory eicosanoids as well as down-regulate expression of COX-2 in immune tissues and cells.

Fatty acids are very important in poultry diets because they are fuels for the generation of energy. In addition, they are an important physical and functional component of cell membranes. Fatty acids are important key components that influence cellular function, expression of proteins, and regulators of gene expression, signaling processes and transcription factors. Fatty acids are also important because they are

precursors to bioactive phospholipids and eicosanoids. In addition, muscle fatty acids contribute to human diet which would modulate human health. Thus; it is essential to continue studies that show the importance of diet in modulation of the immune system in chickens. Dietary supplementation of n-3 PUFAs and their direct and indirect role in immune regulation and inflammatory responses should be further investigated.

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