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OMEGA-3 POLYUNSATURATED FATTY ACIDS AND CHICKEN IMMUNITY

BY

YANWEN WANG

A THESIS SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

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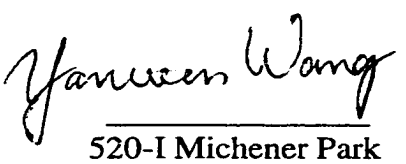

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The undersigned certify that they have read and recommend to the faculty of graduate studies and research for acceptance, a thesis entitled **OMEGA-3 POLYUNSATURATED FATTY ACIDS AND CHICKEN IMMUNITY** submitted by **YANWEN WANG** in partial fulfillment of the requirements of the degree of **DOCTOR OF PHILOSOPHY** in **NUTRITION AND METABOLISM.**

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ABSTRACT

Polyunsaturated fatty acids (PUFA) have received a great deal of attention for their effects on the immune system. The present study showed that feeding high levels of n-3 fatty acids (5% linseed oil or fish oil) to laying hens increased longer-chain n-3 PUFA, eicosapentaenoic, docosapentaenoic and docosahexaenoic acids in the immune cells, and consequently, decreased the estimated rate of lymphocyte proliferation and increased immunoglobulin G (IgG) concentrations in serum and egg yolk. The proportions of lymphocyte subsets were, however, unaffected. The effect of dietary n-3 PUFA on the fatty acid composition of immune tissues and the immune responses remained in the progeny if the same type of maternal and neonatal fat diets were provided. The maternal and neonatal n-3 PUFA elevated the proportion of $CD8^+$ T-cells and IgM^+ B-cells, which were, however, not associated with the alterations of immune responses. Higher levels of maternal and neonatal dietary PUFA promoted the growths of thymus, spleen and bursa of the progeny before 4 wk. This effect disappeared for thymus and spleen during the period from 5 to 8 wk, while the bursa weight was lower in the groups with higher levels of n-3 PUFA, especially the fish oil group. Solely maternal dietary n-3 PUFA increased the incorporation of these fatty acids into the immune tissues of the progeny and suppressed inflammatory response until the age of 4 wk. The ratio of n-6 to n-3 PUFA plays a major role of dietary fatty acids in regulating chicken immune responses. Decreasing the ratio of dietary n-6 to n-3 PUFA suppressed chicken inflammatory response and associated with the increase of total IgG and specific antibody IgG transfer from yolk to the embryo. A significant correlation was

found between the total IgG and antibody IgG in the sera of the hens, 11-d embryo, and hatching chicks. The modulating effects of dietary n-3 PUFA on chicken immune responses may be used to improve poultry health and productivity. For practical use, however, more experimentation is warranted.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF APPENDICES

LIST OF ABBREVIATIONS

AA	Arachidonic acid
AC	Adenylate cyclase
AO	Animal oil
BSA	Bovine serum albumin
CCM	Cell culture medium
Con A	Concanavalin A
CTL	Cytotoxic T-lymphocyte
d	Day(s)
DAG	Diacylglycerol
DHA	Docosahexaenoic acid
DPA	Docosapentaenoic acid
DTH	Delayed-type hypersensitivity
EDTA	Ethylenediaminetetraacetic acid
ELAM-1	Endothelial leukocyte adhesion molecule 1
EPA	Eicosapentaenoic acid
FBS	Fetal bovine serum
FO	Fish oil
h	Hour(s)
ICAM-1	Intercellular adhesion molecule 1
IFN	Interferon
Ig	Immunoglobulin

IL	Interleukin
IP_3	Inositoil-1,4,5-triphosphate
LA	Linoleic acid
LFA-1	Lymphocyte functional associated antigen 1
LNA	Linolenic acid
LO	Linseed oil
LPS	Lipopolisaccharide
LT	Leukotriene
MHC	Major histocompatibility
MHC-II	Major histocompatibility class II
MUFA	Monounsaturated fatty acids
NK	Natural killer
PBS	Phosphate buffered saline
PG	Prostaglandin
PHA	Phytohaemagglutinin
PK-C	Protein kinase C
PUFA	Polyunsaturated fatty acids
PWM	Pokeweed mitogen
SFA	Saturated fatty acids
SO	Sunflower oil
TGF-β	Transforming growth factor-β
Th	Helper T lymphocyte
TNF	Tumor necrosis factor

TX	Thromboxane
VCAM-1	Vascular cell adhesion molecule 1
wk	Week(s)

INTRODUCTION

In the past over 20 years, epidemiological and clinical investigations, and animal experiments have expanded our knowledge of the properties of dietary fatty acids in health and disease. The impetus on the research and expansion in our knowledge resulted from research advances in prostaglandin metabolism, membrane structure and function, the role of n-3 fatty acids in gene expression, and the epidemiological investigations that demonstrated a much lower rate of coronary heart disease in Eskimos (Simopoulos, 1994). Nutritional modification of cellular functions by dietary lipids offers a method to correct and to modify or to prevent many pathophysiological processes (Editorial, 1983; Simopoulos, 1994).

The effect of dietary fatty acids upon the immune system was suggested by early epidemiological studies of the incidence of multiple sclerosis and by the observations that the blood, cells, and tissues of patients with multiple sclerosis are deficient in polyunsaturated fatty acid (PUFA) (Mertin and Meade, 1977). Since then the research in the effects of dietary fatty acids on the immune functions has been initiated (Meade and Mertin, 1978) and intensified with the later elucidation of the roles of eicosanoids derived from n-6 PUFA, especially arachidonic acid (AA) in modulating inflammation and immunity (Goodwin and Ceuppens, 1983; Hwang, 1989; Kinsella et al., 1990; Roper and Phipps, 1994) and with the knowledge that the metabolism of AA to yield these mediators can be inhibited by the n-3 PUFA found in some marine fish oils (Hwang, 1989; Kinsella et al., 1990; Calder 1996b). Thereafter, accumulating evidence has demonstrated that both the amount and type of dietary lipids modulate the immune

responses in animals and humans (Gershwin et al., 1985; Johnston, 1985, 1988a,b; Harbige, 1998; Belch and Hill, 2000).

PUFA are involved in maintaining a variety of physiological processes and play key roles in nutrition both in normal health and disease states (Fernandes and Venkatraman, 1993). The n-3 and n-6 fatty acids are two series of PUFA that are not interconvertible and form a significant part of cell membranes (Clandinin et al., 1990). A number of studies conducted in mammals (rats, mice, rabbits) have shown that feeding animals with diets rich in n-3 PUFA resulted in a significant reduction of AA and a concomitant increase of n-3 PUFA in the immune tissues or cells. This change makes the tissue or cell have less AA release from phospholipids for the synthesis of eicosanoids, which are reported to regulate immune responses. In addition, n-3 PUFA, eicosapentaenoic acid (EPA) acts as substrate to produce less amount and less potency of eicosanoids, which are different series from that derived from AA. As a result, n-3 PUFA suppress specific cell-mediated immune response such as lymphocyte proliferation (Alexander and Smythe, 1988; Kelley et al., 1988; Yaqoob et al., 1994a) and non-specific cell-mediated immunity, e.g., inflammation (Prickett, 1984; James et al., 2000) and appear to enhance antibody production (Prickett, 1982; Fritsche et al., 1991a). The suppressive effects of n-3 PUFA on cell-mediated immunity and inflammation are beneficial to the patients with chronic inflammation and autoimmunity, and increases the survival of patients after tissue or organ transplantation (Calder, 1997b). The increased antibody production is thought to strengthen the protection of the host from infectious challenge by enhancing the fast line of immune defense against pathogens invading through openings, and facilitate clearance of pathogens.

In poultry, it has been shown that dietary fatty acids can also modulate immune responses, such as lymphocyte proliferation in response to mitogen stimulations and antibody production (Fritsche et al., 1991a; Friedman and Sklan, 1995), and inflammatory response (Korver and Klasing, 1997). Dietary modulation of immunity may be of critical importance (Klasing, 1998). For practical use of dietary fatty acid manipulation to maintain health and productivity in poultry, more investigations are warranted.

1. LITERATURE REVIEW

1.1. Structure of Immune System

Lymphoid and nonlymphoid systems constitute two broad structural categories of the chicken (and mammalian) immune system. Among lymphoid components, the bursa of Fabricius and the thymus are considered to be the primary lymphoid organs where lymphocytes develop and differentiate, whereas the spleen is usually termed a secondary lymphoid organ. In addition, lymphoid structures distributed throughout the intestinal tract represent the "intestinal arm" of the immune system (Qureshi et al., 1998). The nonlymphoid components of the immune system include cells that provide a nonspecific immunological defense to the host. Being the first line of immunological defense, the cells of the mononuclear phagocytic system are the primary players in this category. Blood monocytes and tissue macrophages are unique because of their wide distribution throughout the body fluids, organs, and cavities (Qureshi et al., 1998; Kelly and Coutts, 2000). Additional nonlymphoid cells with phagocytic potential in avian include heterophils (counterparts of the mammalian neutrophils) (Andreasen et al., 1993) and thrombocytes (Chang and Hamilton, 1979; Harmon et al., 1992).

The immune cells mainly include lymphocytes and phagocytic cells. The lymphocytes are present as circulating cells in blood and lymph, as anatomically defined collections of cells in lymphoid organs (thymus, spleen, bursa, lymph node) or as scattered cells in other tissues (Calder, 1998b). Lymphocytes exist as distinct subsets that have quite different functions and protein products, although they appear to be

morphologically similar. The principal types of lymphocytes are T- and B-lymphocytes and natural killer (NK) cells; along with monocytes, lymphocytes are termed mononuclear cells (Calder, 1998b). The lymphoid progenitor cell has the capacity to differentiate into T-cells or B-cells, and this is largely determined by the microenvironment to which they migrate (Kelly and Coutts, 2000). The thymus is a site of T-lymphocyte development and differentiation; bursa (bone marrow in mammals) is a site of B-lymphocyte development and differentiation. Both cells arise in the cells of the bone marrow and both have the ability to acquire a memory of encounters with specific antigens (Kelly and Coutts, 2000). The major function of T-cells is in antigen recognition and clearance, and in coordinating the response of B-cells (Kelly and Coutts, 2000). T-lymphocytes are further subdivided into functionally distinct populations, the best defined of which are helper (Th) lymphocytes and cytotoxic lymphocytes (CTL); these classes of T-lymphocytes are defined by the presence on their surfaces of CD4 and CD8 molecules, and respectively are termed $CD4^+$ and $CD8^+$ cells (Johnston, 1988b; Kelly and Coutts, 2000). They recognize peptide antigens attached to major histocompatibility complex (MHC) proteins on the surface of so-called antigen-presenting cells, which are mainly macrophages and dendritic cells. B-cells also have antigen-presenting function (Kuby, 1997). After an antigenic encounters there are metabolic changes in the cells; ultimately, cell division occurs leading to the formation of specific clones, which share a memory for a specific antigen. When reexposed to the same antigen, the effector T-cells respond by releasing cytokines (Johnston 1988b), which serve as the communication tools between immune cells to promote the proliferation and differentiation of T-lymphocytes as well as other cell types, including B cells, NK cells and macrophages.

The CTL lyse the cells that produce foreign antigens, such as cells infected by viruses or intracellular microbes (Calder, 1998b). The T-cell branch of the immune response is known as cell-mediated immunity (Johnston, 1988b).

The other branch of the immune system is the circulatory and secretary system of immunoglobulins (Ig) and antibodies, which is termed humoral immunity. This branch involves the bone marrow-dependent (bursa of Fabricius dependent in young chickens) B-cells. These cells are activated primarily by bacteria and differentiate into mature B-cells. Further stimulation causes them to proliferate and differentiate into memory B-cells, which retain their ability to respond to an antigen, and the effector B-cells or plasma cells which produce antibodies. The role of these antibodies is to neutralize and eliminate the antigen and also promote phagocytosis by macrophages (Johnston, 1988b). The antibodies produced belong to different Ig classes, depending on the type of stimulus and the anatomical site of the lymphocytes involved (Calder, 1998b). Cytokines determine the types of antibodies produced by stimulating B lymphocyte proliferation.

The Th lymphocytes are subdivided into Th1 and Th2 according to the pattern of cytokines they produce (Fig. 1.1). More recently a cytokine pattern to describe a Th0 and Th3 response by T cells has been described (Chen et al., 1994; Mackinnon, 1997). It is believed that naive Th cells produce mainly interleukin (IL)-2 on initial encounter with antigen (Calder, 1998b). These cells may differentiate into a population sometimes referred to as Th0 cells. Th0 cells differentiate further into Th1, Th2, and Th3 cells (MacDonald, 1998). This differentiation is regulated by cytokines; IL-12 and interferon-γ (IFN-γ) promote the development of Th1 cells, while IL-4 and IL-10 promote the development of Th2 cells (Calder, 1998b). Th1 cells produce IL-2 and IFN-γ, which

activate macrophages, NK cells and CTL and are the principal effector of cell-meditated inflammatory (Romagnani, 1995). Th1 cells induce responses to intracellular infections/pathogens. Th2 cells produce IL-4, IL-5, IL-10 and IL-13, which support a humoral antibody response (Romagnani, 1995). Th2 cells induce responses to extracellular pathogens. Th2 cells are responsible for defense against helminthic parasites and for allergic reactions, which are due to IgE-mediated activation of mast cells and basophils (Calder, 1998b). Th3 cells, mucosally derived $CD4^+$ cells producing mainly TGF-β, have both mucosal T helper function and down-regulatory properties for Th1 cells (Chen et al., 1994).

Phagocytes are derived from myeloid progenitor cells, and function to remove particulate antigen by phagocytosis (Kelly and Coutts, 2000). There are two major classes of phagocytes, the circulating phagocytes such as monocytes and granulocytes (neutrophils, eosinophils, and basophils) and the fixed phagocytes that are mainly macrophages. Neutrophils are major granulocytes in man, and they are also known as polymorphonuclear leukocytes (PMN) (Johnston, 1988b). Avian heterophils are functionally equivalent to mammalian neutrophils (Andeasen et al., 1993). Monocytes and macrophages are involved in the phagocytosis and killing of microorganisms, removal of debris, various regulatory actions, the synthesis of biologically active compounds such as prostaglandins and other eicosanoids, cytokines and in cooperative and effector functions in the immune responses (Johnston, 1988b). Macrophages also have an important role in antigen presentation to T-cells (Kelly and Coutts, 2000).

1.2. Mechanisms of Immune Responses

The primary function of the immune system is to eliminate infectious agents and to minimize the damage they cause. However, the immune system also processes harmless antigens, and has evolved intricate mechanisms to discriminate between antigens with the potential to cause damage and those without. Dietary antigens and bacterial antigens from the indigenous microflora trigger tolerance induction in healthy individuals, whereas pathogens induce strong activation of immune defense mechanisms required for pathogen clearance (Kelly and Coutts, 2000). Failure to regulate properly the immune responses often causes problems such as allergy, autoimmunity and inflammation (Kelly and Coutts, 2000).

The immune defense relies on the two 'arms' of immune system, the innate and the acquired. The innate immune system is evolved before the acquired or adaptive immune system (Medzhitov and Janeway, 1997; Dyrynda and Ratcliffe, 1998). The major function of the innate immune response is as a first line of defense to effectively limit the spread of an infectious challenge. The major immune cell types involved in mediating innate responses are macrophages, heterophils (counterparts of mammalian neutrophils) and natural killer cells. These cells are capable of discriminating self from non-self, and recognize molecular arrays or patterns that seem to be shared among groups of pathogens. The specific molecules expressed by pathogens are recognized by pattern-recognition receptors expressed on the activated macrophages, and induce mechanisms of kill including endocytosis, phagocytosis and opsinization (Kuby, 1997). For many years the innate immune system has been considered as quite distinct from the adaptive system.

However, recent studies have shown that function of the innate immune system is integrated with the acquired immune system such that the innate system operates to initiate and regulate the adaptive response (Jullien et al., 1997).

The adaptive immune responses are initiated following antigen uptake and presentation to T- and B-cells. The major antigen presenting cells are macrophages and dendritic cells. This process involves antigen uptake, processing, presentation, and recognition and thus has fine molecular specificity (Kelly and Coutts, 2000). Essential to the activation of T-cells is antigen presentation in combination with MHC, recognition involving the T-cell receptor, and a co-stimulation signal mediated through ligation of molecules such B7 expressed on antigen-presenting cells or CD28 on T-cells (June et al., 1994). Co-stimulation is very important in T-cell activation and absence of this signal leads to a partial T-cell response or tolerance induction. Following antigen priming, T- and B-lymphocytes migrate through the lymph and reach the peripheral blood for migration to the effector sites. Activated lymphocytes secrete cytokines that together with the cytokines secreted by macrophages regulate immune cell functions.

The function of the immune system is to defend and protect the host from various invaders and autoreactive transformed cells. This system has evolved to be complicated and finely regulated. The critical characteristic of the immune system is to be able to discriminate the self from nonself and to respond instantly quickly. Appropriate immunity is very important. Overreaction often causes disease such as autoimmunity and inflammation. Many factors may influence the immune responses, in which nutrition has been established to be one factor in recent years.

1.3. Nutrition and Immune Functions

Nutritional deficiencies alter immunocompetence and increase the risk of infection (Keusch, 1998; Woodward, 1998; Hulsewe, et al., 1999). However, excessive intake of some dietary components can also exert a deleterious effect on the immune responses (Corman, 1985; Birt 1990; Hunter and Willett, 1996). It is now generally accepted that nutrition is an important determinant of immune responses. Of the nutrients that may have immuno-modulating functions dietary fatty acids have been widely studied in mammals in the past over two decades. Research on poultry lags behind. An understanding of the mechanisms through which nutrition influences the immune system is necessary to appreciate the many complex interactions between diet and infectious diseases and maintain health and profitability (Klasing, 1998).

1.4. Biochemistry of Fatty Acids

1.4.1. Structure and Origin

Fatty acids, the basic components of fat, are made up of carbon, hydrogen and oxygen atoms. They have a carboxyl group at one end and methyl group at another with two groups connected by a carbon chain. Fatty acids are either saturated or unsaturated straight aliphatic chains, with even-numbered carbon atoms. A small proportion of non-aliphatic (branch, cyclic) and odd-numbered fatty acids are sometimes present in fats of either plant or animal origin. The stereochemistry of fatty acids influences the physical, chemical and biological properties of the fat.

Fatty acids have their origin in land and marine animal fats, terrestrial plants and organic synthesis (Pryde, 1979). Fatty acids that are present in animal tissues could be of dietary origin, *in vivo* synthesis or modification of dietary fatty acids, and those with 12 to 24 carbon atoms are the most common.

1.4.2. Fatty Acids Classification

Fatty acids are primarily classified on the basis of chain length and degree of unsaturation (Sanders and Roshanai, 1983). Based on the carbon-chain length, fatty acids are divided into short (4-6 carbons), medium (8-10 carbons), and long (12-18 carbons) chain fatty acids. This distinction is important primarily for absorption and oxidation. Fatty acids are also classified, according the degree of unsaturation, as saturated fatty acids (SFA, no double bond), monounsaturated fatty acids (MUFA, one double bond) and PUFA (two or more double bonds). Four classes of unsaturated fatty acids are commonly recognized, the parent molecules being palmitoleic acid (C16:1n-7), oleic acid (C18:1n-9), linoleic acid (C18:2n-6; LA), and α-linolenic acid (C18:3n-3; LNA) (Gurr, 1992). The 'n' symbol refers to the position of the first double bond relative to the methyl end of the fatty acid chain. This classification is important for biological functions of fatty acids. In recent years, the main focus of the research has been put on the effects of n-6 and n-3 PUFA on the immune functions in health and disease.

1.4.3. Existence of Fatty Acids

Fatty acids rarely exist in the form of free fatty acids, but constitute lipids. The major component of food lipids is triacylglycerols of which fatty acids are the main

components. The type of fatty acids and its position at which it is esterified to glycerol determine the characteristics of the triacylglycerols. There is some specificity in the position occupied by fatty acids. Animal depot fats tend to have a saturated fatty acid in position 1 and an unsaturated fatty acid in position 2; fatty acids in position 3 are more randomly distributed, with PUFA often accumulating there (Calder, 1996a).

Phospholipids are the major components of cell membranes which occur in very small amounts in most foods and extracted oils (Calder, 1996a). Other minor components that make up fat are mono and diacylglycerols (DAG), free fatty acids, sterols, fatty alcohols, fat soluble vitamins and other fat soluble substances. Non-glyceride lipid components of food are minor constituents of foods in terms of their concentrations relative to triacylglycerols. However, they can have potent biological properties. The level of each type of lipid consumed depends on the ingredients/components of the diet and the method of food (feed) preparation and storage (Calder, 1996a).

1.4.4. Essential Fatty Acids and Their Biosyntheses

The first step in saturated fatty acid synthesis *in vivo* is carboxylation of the activated form of acetate (acetyl-CoA) to form malonate prior to a series of elongation of the carbon chain from the carboxyl end. The elongation process occurs by the successive condensation of two carbon units (Bloch and Vance, 1977). The end product of the fatty acid synthetase enzyme is palmitic acid (16:0). The reactions are catalyzed by a group of enzymes, collectively known as fatty acid synthetase (Gurr and James, 1980). The major site of fatty acid synthesis is the cytosol of liver. Palmitic acid can be elongated to stearic acid (18:0). The carbon chain length of the fatty acids synthesized depends on the tissue

(Enser, 1984). Palmitic acid and stearic acid are the major SFA in foods (Simopoulos, 1994).

Animals have a strong ability to synthesize SFA. However, cell membranes require unsaturated fatty acids to maintain their structure, fluidity and function. Therefore, a mechanism for the introduction of double bounds (desaturation) exists (Calder, 1996b). The introduction of a single double bond between carbon atoms 9 and 10 is catalyzed by the enzyme Δ-9 desaturase, which is universally present in both plants and animals (Fig. 1.3). This enzyme results in the conversion of stearic acid into oleic acid (18:1n-9). Plants, unlike animals, can insert an additional double bond into oleic acid between existing double bond at the 9 position and the methyl terminus of the carbon chain; a Δ-12 desaturase converts oleic acid into LA, while a Δ-15 desaturase converts LA into LNA. Since mammalian tissues are unable to synthesize LA and LNA, these two fatty acids must be consumed from diet and so are termed essential fatty acids (Calder, 1996a).

LA is found in large amounts in corn oil, safflower oil, canola oil, and soybean oil and sunflower oil. LNA is found in the chloroplasts of green leafy vegetables, such as purslane and spinach, and in the seeds of flax, linseed, walnuts (Simopoulos, 1987).

1.4.5. Source of Longer-chain PUFA

Longer-chain (C20 and C22) PUFA are particularly considered for their important immunological functions. Starting from LA or LNA, animal cells can synthesize n-6 and n-3 series of longer-chain fatty acids (Fig. 1.3). The LA is converted via γ-linolenic acid (18:3n-6) and dihomo-γ-linolenic acid (20:3n-6) into AA; the LNA is converted into EPA

(20:5n-3), by a series of reactions. The EPA and AA can be further converted into longer-chain unsaturated fatty acids. The EPA is converted into docosapentaenoic acid (DPA, 22:5n-3) and docosahexaenoic acid (DHA, 22:6n-3). Many marine plants, especially single cellular algae can carry out chain elongation and desaturation of LNA to yield the longer-chain EPA, DPA and DHA. It is the formation of these n-3 PUFA by marine algae and their transfer through the food chain to fish that counts for the abundance of the n-3 PUFA in some marine fish oils (Calder, 1996a). The n-9, n-6, and n-3 families of fatty acids are not metabolically interconvertible in mammals, and there is competition between members of these families for the desaturase enzymes.

1.5. Effects of Dietary Fatty Acids on Immune Functions

1.5.1. Lymphocyte Proliferation

The function of lymphocyte can be assessed by lymphocyte proliferation, lymphocyte-derived cytokine production, lymphocyte-mediated cytotoxicity and cell-mediated immunity (Calder, 1998b). Experimentally, a widely used estimate of lymphocyte function is proliferation of the cells in response to mitogenic stimulation, measured by uptake of [^{3}H] thymidine (Calder, 1998b). Commonly used mitogens are concanavalin A (Con A) and phytohaemagglutinin (PHA) which primarily stimulate T-lymphocytes in the presence of other cells, bacterial lipopolisaccharide (LPS) which primarily stimulates B-lymphocytes, and pokeweed mitogen (PWM) which stimulates both T- and B-lymphocytes. An estimate of the rate of proliferation of lymphocytes is

conducted by measuring the rate of [^{3}H]thymidine incorporation into the DNA of the cells (Calder, 1998b).

1.5.1.1. Effects of Amount and Type of Dietary Fatty Acids

Many studies comparing the effect of dietary fat levels on the lymphocyte function have demonstrated that diets with high levels of fat (SFA, MUFA and PUFA) result in a suppression of lymphocyte proliferation *in vitro* (Erickson et al., 1980; Levy et al., 1982; Morrow, et al., 1985; Alexander and Smythe, 1988; Berger et al., 1993). The precise effect depends on the level of fat used in the high-fat diet and its source. Some studies have revealed that high saturated fat diets do not affect lymphocyte proliferation compared with low mixed fat diets (Kollmorgen et al., 1979; Locniskar et al., 1983; Alexander and Smythe, 1988) while others have shown that they are suppressive but less that PUFA-rich diets (Friend et al., 1980; Levy et al., 1982; Morrow et al., 1985; Sanderson et al., 1995b). It appears that lymphocyte proliferation is affected by the nature of the principal saturated fatty acid in the rat diet, with a high-fat diet rich in palmitic acid at the sn-2 position of dietary triacylglycerols leading to enhanced Con A-stimulated spleen lymphocyte proliferation compared with diets rich in medium chain, lauric, or stearic acids (Jeffery et al., 1997b).

Few studies have investigated the effects of consuming large amount of MUFA (such as oleic acid) upon lymphocyte functions (Calder, 1998a). A small number of studies have suggested that there may be beneficial effects of olive oil consumption on rheumatoid arthritis (Kremer et al., 1990), an autoimmune disease characterized by infiltration of synovial tissues and fluid by cells of the immune system and vigorous

overactivity and inflammation therein. Studies investigating the effects of MUFA-rich diets on immune functions have been often influenced by n-6 or n-3 PUFA. There is now growing evidence that MUFA-rich oils, which were previously thought to be neutral with respect to immune function, have effects which are similar to those of fish oils (Yaqoob, 1998).

There are a large number of studies that investigated the effects of n-6 PUFA on lymphocyte proliferation *in vitro*. Several studies have reported lower Con A- or PHA-stimulated T-lymphocyte proliferation rate following the feeding of diets rich in n-6 PUFA (maize or safflower oils) to laboratory rodents compared with feeding diets rich in SFA (Kollmorgen et al., 1979; Erickson et al., 1980; Friend et al., 1980; Levy et al., 1982; Locniskar et al., 1983; Yaqoob et al., 1994a,b; Sanderson et al., 1995b). In contrast, some other studies have reported no effect of feeding LA-rich diets upon rodent T-lymphocyte proliferation (Cathcart et al., 1987; Alexander and Smythe, 1988; Fritsche et al., 1991a; Berger et al., 1993). The outcome of such measures of lymphocyte function is strongly influenced by conditions used to culture the cells. It has been reported that feeding female rats throughout gestation and lactation a diet containing 10% (w/w) olive oil did not affect Con A-stimulated proliferation of spleen lymphocytes; in this study the cells were cultured in fetal calf serum (Berger et al., 1993). However, another study showed that feeding 20% (w/w) olive oil to rats diminished *in vitro* lymphocyte proliferation; in this study the cells were cultured in autologous serum (Yaqoob et al., 1994a). This finding has been confirmed by a later study (Jeffery et al., 1996c) using 20% LA-rich sunflower oil diet. A significant inverse relationship between rat spleen lymphocyte proliferation and the ratio of oleic acid to LA in the diet has been reported

(Jeffery et al., 1997a). This might account for the discrepancies in the literature (Calder, 1998a,b).

Recent studies have investigated the effect of AA on lymphocyte function, with no significant effect being found. For example, Jolly et al. (1997) reported that feeding mice on a diet containing 20 g safflower oil plus 10 g AA/kg did not affect Con A-stimulated spleen lymphocyte proliferation compared with feeding a diet containing 30 g/kg of safflower oil. Peterson et al. (1998) also observed that inclusion of 4.4 g AA/100 g fatty acids in the rat diet did not affect Con A-stimulated spleen lymphocyte proliferation. These observations agree with those of Kelley et al. (1997), who reported that feeding human volunteers with 1.5 g AA/d for 50 d did not affect the proliferative response of peripheral blood polymorphological nuclear cells to Con A, PHA or PWM. However, Field et al. (2000) reported that infants fed formula with addition of AA and DHA (0.49 and 0.35% of total fatty acids, respectively) increased the percentage of $CD3^+$ $CD4^+$ T cells and the percentage of $CD20^+$ cells compared with infants fed the formula without supplementation of these two fatty acids. The CD4+ cells from the infants supplemented with AA and DHA expressed more CD45R0 (antigen mature) and less CD45RA (antigen naive) (Field et al., 2000).

The studies that compared the effects of different types of fatty acids on lymphocyte proliferation have shown stronger suppressive effect of n-3 PUFA than n-6 PUFA and MUFA when they were given in high amounts. In contrast, lower amounts of n-3 PUFA (0-3.3%, w/w) increase cellular immunity such as natural killer cell cytotoxicity (Robinson and Field, 1998). Feeding rats on diets rich in LNA suppressed spleen T lymphocyte proliferation to PHA compared with feeding diets rich in SFA

(Marshall and Johnston, 1985) or n-6 PUFA (Jeffery et al., 1996b). And within n-3 PUFA, different individual fatty acids have different potencies in suppressing lymphocyte proliferation. Many studies have reported that feeding diets rich in n-3 PUFA from fish oil to rabbits, rats, or mice results in a stronger suppressed effect on the proliferation of lymphocytes compared with linseed oils (Alexander and Smythe, 1988; Kelley et al., 1988; Sanderson et al., 1995b; Yaqoob and Calder, 1995b).

1.5.1.2. Effects of n-3 PUFA

In recent years, there has been increased interest in the effects of n-3 PUFA containing oils (linseed oil or fish oil) on lymphocyte function. It has been reported that feeding these oils to laboratory animals (rats, mice, rabbits) suppresses the responses of spleen lymphocytes to mitogenic stimuli including Con A, PHA or PWM (Calder, 1997b). For example, feeding rats for 20 wk with fish oil (20%, w/w) diet resulted in suppressed proliferative response of lymphocytes from lymph node, blood and thymus in response to Con A or PHA (Yaqoob, et al., 1994a; 1995b). When rabbits were fed for 20 wk with a diet containing 7.6% (w/w) fish oil, spleen and blood lymphocyte proliferation to the stimulation of Con A, PHA, or LPS was significantly suppressed (Kelley et al., 1988). Wu et al. (1996) fed monkeys for 14 wk on diets containing 3.5 or 5.3% energy as LNA, 28% PUFA of the total fatty acids, and 1.0 or 0.5 of n-6 to n-3 PUFA ratio, proliferation of peripheral blood polymorphonuclear cells to Con A or PHA stimulation was not affected compared with the basal diet which had 36 of n-6 to n-3 PUFA ratio. In a recent study, rats were fed diets containing 18% fat but different in PUFA content (17.5% or 35% of the total fatty acids) and the ratio of n-6 to n-3 PUFA (100, 20, 5, 1). It

was found that lymphocyte proliferation decreased as the ratio of n-6 to n-3 PUFA in the low PUFA diets; whereas there was no significant effect of the n-6 to n-3 PUFA ratio in the high PUFA diets (Jeffery et al., 1997c). This study indicates that LNA reduces lymphocyte proliferation, but that its effect depends on the PUFA level and the ratio LA to LNA in the diets.

In human studies, Meydani et al. (1991) reported that supplementing the diets of healthy young (22-33 years old) or older (51-68 years old) women with encapsulated n-3 PUFA (approximately 2.4 g/d); the mitogenic response of peripheral blood lymphocytes to PHA was lowered after 12 wk of supplementation in the older women. Meydani et al. (1993) reported a decreased response of peripheral blood lymphocytes to Con A and PHA following supplementation of the diet of volunteers on a low-fat low-cholesterol diet with n-3 PUFA. Endres et al. (1993) found that 18 g/d for 6 wk resulted in lowered PHA-stimulated proliferation of peripheral blood lymphocytes 10 wk after the supplementation had ended (but not at the end of supplementation period).

The lymphocyte proliferation is not the only measure of T cell function. Other measures include cytokine production, lymphocyte mediated cytotoxicity, lymphocyte phenotyping and the expression of cell surface molecules, antibody production, cell-mediated immunity and cell metabolic activity (Calder, 1998b; Field et al., 2000). The effects of n-3 PUFA on most of these functions are briefly reviewed as follows.

1.5.2. Effect of n-3 PUFA on Antibody Production

Studies on the effects of dietary n-3 PUFA on antibody production produced contradictory results. Prickett et al. (1982) reported that feeding rats 25% (w/w) fish oil

enhanced production of antibody IgG and IgE to egg albumin as compared with feeding the same amount of tallow (rich in LA and oleic acid). Antifluorent antibody titers were higher in rats fed either soybean or canola oil (containing moderate levels of LNA) than those fed corn oil (containing high level of LA) (Fly and Johnston, 1990). The mouse serum haemagglutinin titer was slightly but significantly higher in the high LNA (LNA to LA, 3.91) diet group than in the dietary group with the low LNA diets (LNA to LA, 0.36 or < 0.01) (Yashino and Ellis, 1989). In patients undergoing surgery for upper gastrointestinal malignancies when supplemented n-3 fatty acids, arginine, and RNA with a nutritional complete nature diet, the mean IgM concentration was significantly higher on postoperative day 10 and mean IgG concentration was higher on postoperative day 16; B-lymphocyte indices were significantly higher in the supplemented group on postoperative day 10 (Kemem et al., 1995).

However, Kelley et al. (1988) found no effect of feeding rabbits linseed oil or fish oil on production of antibodies to bovine serum albumin. Similar result was reported by Kim and Lee (1992) that IgG and IgM responses to sheep red blood cells when estimated as plaque-forming cells present in spleen, were not affected significantly by the dietary fats fed to rats. In contrast, Prickett et al. (1984) reported that dietary fish oil decreased antibody production in rats. Virella et al. (1989) reported that supplementation of the diet of healthy human volunteers with fish oil (6 g/d) for 6 wk decreased the circulating levels of IgM and IgG following tetanus toxin challenge, but they used only a single subject and a later study with more subjects (Virella et al., 1991) found no significant effect of supplementing with fish oil. Feeding flaxseed oil diet decreased the concentration of Ig in serum and salivary IgA (Kelley et al., 1991). Recently, Atkinson and Maisey (1995)

reported that the production of anti-rat erythrocyte antibodies by mice injected with rat erythrocytes was significantly reduced if the mice were fed a 20% fish oil diet for 8 or 9 wk.

A recent study has examined in detail the effects of culturing rat mesenteric lymph node lymphocytes with different fatty acids on Ig production (Yamada et al., 1996). These workers measured total IgM, IgG, IgA, and IgE production by the cell, which are not stimulated in culture. Saturated fatty acids (lauric, myristic, palmitic, stearic) at a concentration of 10 μM did not alter IgM, IgE or IgG production, but they reduced IgA production by approximately 30% (Yamada et al., 1996). Unsaturated fatty acids (oleic, linoleic, and arachidonic) did not affect Ig production at concentrations below 100 μM. However, at a concentration of 1 mM these fatty acids strongly inhibited production of IgM, IgG and IgA and enhanced the production of IgE (Yamada et al., 1996).

1.5.3. Effect of n-3 PUFA on Inflammatory Response

It is evident that the outcome of *in vitro* studies is strongly affected by experimental conditions. *In vivo*, cells exist as a part of network being influenced by other cell types and the components in the environment fluids including chemicals or mediators secreted by other cells; such interactions are often disturbed by the purification of the particular cell types to be studied (Calder, 1998a). Therefore, the results of *in vitro* assays of immune cell do not always reflect the true situations *in vivo*. It is necessary to investigate the effects of dietary fatty acids on the intact, fully functioning system in which all normal cellular interactions are in place. The ability to make *in vivo* measures

of inflammatory response and of cell-mediated immunity offers the prospect of investigating the effects of dietary manipulations upon overall responses of these systems (Calder, 1998a).

Because n-3 PUFA diminish the production of mediators from AA, theoretically n-3 PUFA should exert an anti-inflammation effect. In fact, it was found that administrating healthy rats with EPA (500 mg/kg/d) and DHA (333 mg/kg/d) by a gastric tube for 50 days did not affect either antigen-induced inflammation of the air pouch or carrageenan-induced inflammation of the footpad (Yoshino and Ellis, 1987). This was despite a significant reduction in the production of pro-inflammatory eicosanoids (PGE_2 and LTB_4). Similar dietary n-3 PUFA-induced changes in the pattern of inflammatory eicosanoid production were also reported in a different model of acute inflammation (intraperennial injection of zymosan) in rodents (Lefkowith et al., 1990). The latter study also showed that dietary n-3 PUFA inhibited the influx of neutrophils into the peritoneal cavity, which accompanies such treatment. Feeding rats with 100g/kg cod liver oil for 10 wk significantly lowered (by 40%) the inflammatory response to carrageenan injection into the footpad compared with feeding coconut oil or groundnut oil (Reddy and Lokesh, 1994). In accordance with that observation, feeding rats high-fat diets containing 20g/kg of ethyl esters of EPA or DHA resulted in a 50% reduction in footpad swelling in response to carrageenan injection compared with feeding safflower oil; both n-3 PUFA were equally effective (Nakamura et al., 1990).

Delayed-type hypersensitivity (DTH) response, an important inflammatory response (repeatedly a Th1 response), results from non-specific cell-mediated immune response to an antigen challenge (Calder, 1997b). A significant reduction in DTH

response was observed following the feeding of diets rich in fish oil to rodents (Yoshino and Ellis, 1987; Kelley et al., 1989). Taki et al. (1992) reported suppression of the DTH response to sheep red blood cells in mice following tail-vein injections of emulsions of triacylglycerols rich in EPA or DHA. In human studies, a 40-day reduction in fat intake (from 40% to 35% to 30% of energy) by healthy volunteers did not later the DTH responses to seven recall antigens (Kelley et al., 1992a); these responses were also unaffected by differences in the PUFA level of the diet (3.2% or 9.1% of energy) (Kelley et al., 1992a) or by consuming a salmon-rich diet (500 g/d for 40 days) (Kelley et al., 1992b). However, feeding a linseed oil-rich diet to healthy human subjects for 8 wk lowered the DTH response to seven recall antigens (Kelley et al., 1991). Supplementation of the diet of human subjects consuming a low-fat, low-cholesterol diet with 1.25 g n-3 PUFA/day diminished the DTH responses to seven recall antigens (Meydani et al., 1993).

1.5.4. Effects of Dietary n-3 PUFA on Lymphocyte Phenotypes

Since different immune actions are performed by different phenotypes of immune cells, diet-induced changes of immune functions are influenced by distribution of various phenotypes of immune cells in immune organs or tissues (Robinson and Field 1998). Meydani et al. (1993) observed that consumption of a low fat-low cholesterol and n-3 PUFA-rich diet resulted in a lower proportion of $CD4^+$ and a higher proportion of $CD8^+$ peripheral blood T-lymphocytes, with the total proportion of $CD3^+$ cells (total T-cells) unaffected in humans. Kelley et al. (1991) observed that flaxseed oil did not affect the number of $CD4^+$ and $CD8^+$, nor total T- and B-cell concentrations in human peripheral blood leukocytes. Feeding fish oil to rats did not change the proportion of $CD4^+$ and

$CD8^+$ lymphocytes in spleen, thymus and lymph node, either in freshly prepared cells or in cells cultured in the presence of mitogen (Yaqoob et al., 1994a). Shapiro et al. (1994) reported that n-3 PUFA supplementation significantly decreased the percentages of T-cells, $CD4^+$, $CD8^+$ in spleen of mice, while Huang et al. (1992) observed that mice fed the n-3 PUFA had a greater percentage of T-cells but not the percentages of B-cells. The reason for such contradictory results is not clear. It is likely due to differences in the animal model, immune status, duration of fatty acid supplementation as well as fatty acid manipulation including the amount and types of fatty acids, especially PUFA employed by different researchers.

1.6. Mechanisms Involved in Fatty Acid Modulation of Immune Functions

1.6.1. Effects of n-3 PUFA on the Physical Properties of Cell Membrane

The lipid biolayer of a biological membrane is a mosaic of several molecular species. The basic brick of this edifice is the phospholipid (Cribier et al., 1993). A biological membrane also contains integral proteins and enzymes. A number of movements occur in the lipid biolayer of cell membrane which include intramolecular motions, rotational diffusion, transverse diffusion and lateral diffusion (Cribier et al., 1993). Lipid molecules collide with proteins, and interact with embedded proteins such as membrane enzymes, to regulate their functions. Thus fatty acids can potentially influence the functions of membrane proteins and intracellular proteins in several ways (Calder, 1996a). It is the physical properties, especially the structure of fatty acid chains of phospholipids, determining the cell membrane fluidity (Stubbs and Smith, 1984). The

fatty acid composition of phospholipids in cell membranes is usually characteristic for the cell type, but may change with progress through cell cycle, with age, in response to stimuli or to change in the environment or the diet; these changes may have functional consequences (Calder, 1996a).

It is now recognized that domains exit in cell membranes, where lipid-lipid and lipid-protein interactions occur. Often functional proteins require a specific lipid, which has led to the concept of an annulus of lipid providing a specific region of membrane proteins. A number of membrane-bound enzymes have been shown to be particularly sensitive to their fatty acid environments; these include adenylate cyclease, 5'-nucleotidase and the Na^+/K^+-ATPase (Brenner, 1984; Stubbs and Smith, 1984; Murphy, 1990). The same is true for a number of receptors, including adrenergic and insulin receptors (Calder, 1997b). The mechanisms by which membrane lipids modulate enzyme activity or receptor function are not fully understood, but suggestions include changes in membrane fluidity (Brenner, 1984; Murphy, 1990) and fatty acid-dependent effects on the conformation of the protein complex (Murphy, 1990).

The stimulation of immune cells such as lymphocytes is accompanied by *in vivo* synthesis and turnover of membrane phospholipids (Resch et al., 1971,1972; Resch and Ferber, 1972; Ferber et al., 1975). Within minutes of stimulation, remodeling of the membrane phospholipids begins with SFA in phospholipids being substituted by PUFA (Ferber et al., 1975). It has been reported that culture of mitogen-stimulated lymphocytes for several days results in a very significant increase in the content of unsaturated fatty acids in the membrane phospholipids (Anel et al., 1990; Calder et al., 1994). The culture of lymphocytes, macrophages or monocytes in the presence of n-3 PUFA results in

significant increase of these fatty acids in the membrane phospholipids. Such changes result in increased lymphocyte plasma membrane fluidity (Anel et al., 1990; Calder et al., 1994), although this has been a controversial subject for many years (Calder et al., 1994). The membrane fluidity changes are proposed to be an inherent component of lymphocyte activation and subsequent proliferation. This has been supported by Curtain et al. (1978), who found that a non-mitogenic lectin did not affect lymphocyte membrane fluidity, in contrast to the effect of the mitogenic lectins Con A and PHA. However, others reported that enrichment of n-3 PUFA in the membrane phospholipids did not significantly alter the fluidity of the plasma membranes of lymphocytes (Yaqoob, 1993; Yaqoob et al., 1995a), macrophages (Grimble and Tappia, 1995; Sherrington et al., 1995a) and monocytes (Lee et al., 1985). In contrast to the situation with lymphocytes, stimulation of macrophages with LPS appears to decrease plasma membrane fluidity (Grimble and Tappia, 1995).

It is well established that both qualitative and quantitative changes in dietary fat can induce significant alteration in the composition of membranes in immune cells, such as lymphocytes (Tiwari et al., 1987; Peterson et al., 1998), macrophages (Chapkin and Carmichael, 1990), and polymorphonuclear cells (Cleland et al., 1990). Since much of the function of the immune system depends on interactions involving the plasma membrane of both effector and target cells, incorporation of dietary lipids into plasma membrane of such cells could influence immune responses.

1.6.2. Effects of n-3 PUFA on Eicosanoid Production

1.6.2.1. Pathways of Eicosanoid Synthesis

Reactions of oxygen with polyunsaturated fatty acids produce several classes of biologically active compounds, including prostaglandins (PG), thromboxanes (TX), and leukotrienes (LT). These compounds are collectively known as eicosanoids as most of them are derived from 20-carbon fatty acids, dihomo-γ-linolenic acid, AA and EPA (Calder, 1997b). The pathways of eicosanoid synthesis begin with cyclooxygenase, which yields the PGs and TXs, or with the 5-, 12- or 15-lipoxygenases, which yield the LTs and related eicosanoids (Fig. 1.4). In most conditions, the principal precursor for these compounds is AA, and the eicosanoids produced from AA appear to have more potent biological functions that those released from dihomo-γ-linolenic acid and EPA (Miller et al., 1993; Spurney et al., 1994). The metabolism of AA yields 2-series PGs and 4-series of LTs and metabolism of EPA produces 3-series PGs and 5-series LTs, of which PGE and LTB are mainly considered for their important immunomodulating functions (Calder, 1997b).

The main source of AA is membrane phospholipids, although free fatty acids are also a significant source of eicosanoid precursors (Lands, 1989). Esterified AA is released by the action of phospholipase A_2, a membrane-bound enzyme activated by calcium that releases AA primarily from phosphatidylcholine, and by phospholipase C, which are specific for membrane phophatidylinositol-4,5-biphoaphate (IP_2) and diacylglycerol (DAG) lipase, which releases AA from DAG (Janniger and Racis, 1987).

Most mammalian cells, except erythrocytes, can metabolize AA, but different cells generate different profiles of AA end-products (Goodwin and Ceuppens, 1983; Janniger and Racis, 1987; Levine, 1988). Only a few cell lines, however, including leukocytes, hepatocytes, and gastric and adrenal cells, have specific prostaglandin

receptors (Parker, 1986). Furthermore, only minimal concentrations (10^{-9} g/g) are needed for physiologic effects in target cells (Peck, 1994). This necessity for small concentrations, together with the very short half-lives, suggest that eicosanoids have autocrine or paracrine effects on nearby cells, rather than on distant organ systems (Goodwin and Ceuppens, 1983; Parker, 1986; Levine, 1988). Eicosanoid release is stimulated by a variety of substances. Collagen, thrombin, bradykin, antigen-antibody complexes, oxygen free radicals, growth factors, and cytokines are all capable of stimulating release of esterified AA and further enzymatic modification to eicosanoid products (Levine, 1988).

1.6.2.2. Immunomodulatory Functions of Eicosanoids

Eicosanoids can be synthesized in varying amounts by all cells of the immune system (splenocytes, thymocytes, and leukocytes), especially by macrophages and monocytes which are apparently the principal sources of the eicosanoids affecting immune cells (Goodwin, 1985). Many of the eicosanoids are highly potent agents and they exert a wide variety of biologic effects, which are different in different organs or tissues (Needleman et al., 1986). Certain functions vary with species, concentration of PGs, length of PG-target cell interaction, and the state of differentiation of target cell, and some functions depend on the simultaneous presence of other mediators (Goodwin and Ceuppens, 1983; Parker, 1986; Robinson, 1987).

When the immune system is stimulated, PGs generally function to provide feedback inhibition (Peck, 1994). Of the PGs, PGE_2 has been shown to play important roles in regulating immune responses (Peck, 1994). Its proinflammatory effects include

fever erythema, increased vascular permeability, vasodilation, and enhancement of the pain and edema caused by bradykin and histamine (Parker, 1986).

Production of PGE_2 from AA by suppressor cells is enhanced in chronic inflammatory conditions such as multiple sclerosis, rheumatoid arthritis, fever, and infections. High levels of PGE_2 are present during acute and chronic inflammation and during infections (Hwang, 1989; Kinsella et al., 1990). PGE_2 at physiologically relevant concentrations generally suppresses T cell function, such as mitogen-induced blastogenesis, clonal expansion, antigenic stimulation, lymphokine production (Goodwin and Ceuppens, 1983; Ninnemann and Stockland, 1984; Parker, 1986; Janniger and Racis, 1987; Parker, 1987; Kunkel 1988). For example, T lymphocyte suppression in human diseases is associated with increased prostaglandin activity (Fisher and Bostick-Bruton, 1982; Holdstock et al., 1982). Moreover, monocytes, which are responsible for a large portion of PGE_2 production, also have increased activity in chronic inflammatory conditions (Goodwin and Ceuppens, 1983).

PGE_2 suppresses cytokine release (Parker 1987; Waymack et al., 1990) including IL-1 and tumor necrosis factor (TNF). Indeed, one of the primary roles of PGE_2 is to down-regulate the release of cytokines, which has been implicated in the pathogenesis of septic shock (Plescia and Racis, 1988; Waymack et al., 1990; Rock and Lowry, 1991). PGs depress chemotaxis, chemokinesis, aggregation, spreading, and oxidative metabolism of leukocytes (Parker, 1986; 1987). Biphasic responses of PGs are also noted in natural killer cells and macrophages such that low doses enhance and high doses suppress cellular function (Goodwin and Ceuppens, 1983). The effects of PGE_2 on B cell function and antibody production are dose dependent (Peck, 1994). At low doses, PGE_2

tends to enhance antibody production (Goodwin and Ceuppens, 1983; Staite and Panayi, 1984; Janniger and Racis, 1987; Plescia and Racis, 1988).

Lipoxygenase products (in particular, 5-lipoxygenase) play important roles in allergic and immune reactions and are produced by all leukocytes except lymphocytes (Lewis and Austen, 1984; Needleman et al., 1986; Packer, 1986,1987; Serafin and Austen, 1987). LTC_4, LTD_4, and LTE_4 are the slow-reacting substances released in response to IgE and are responsible for the smooth muscle restriction of anaphylactic reactions (Peck, 1994). They increase vascular permeability and cause arteriolar and bronchial constriction. Thus the LTs are prime candidates for mediators of bronchial asthma and immediate hypersensitivity reactions (Robinson, 1987).

The major LTs involved in modulation of the immune system, however, is LTB_4. Most leukocytes do not synthesize 5-lipoxygenase products on a constitutive basis, and the main source of LTB_4 is polymorphonuclear cells (Davies, 1987-1988). LTB_4 is a potent chemotactic agent that also increases leukocyte motility (chemokinetics). It stimulates aggregation lysosomal release and chemotaxis of polymorphonuclear cells (Peck, 1994). It acts on endothelial cells to cause adherence of neutrophils to endothelium, preceding their migration into extravascular tissues. LTB_4 promotes the secretion of inflammatory products by neutrophils, including active oxygen molecules, hydrogen peroxide, superoxide, and hydroxyl radical and degradative enzymes. LTB_4 acts synergistically with other mediators to increase vascular permeability (Robinson, 1987). LTB_4 also has a role in the regulation of cytokine synthesis (James et al., 2000).

1.6.2.3. Regulation of Eicosanoid Production by n-3 PUFA

The feasibility of modifying eicosanoid synthesis in immune and accessory cells by manipulation of dietary fatty acids is well documented. It has been generally accepted that the synthesis of eicosanoids is limited by the availability of AA in the membrane phospholipid pools in macrophages, lymphocytes, and polymorphonuclear cells (Marshall et al., 1987, Kinsella, et al., 1990). Although AA is abundant in phospholipids in all tissues and provides a readily available source for the synthesis of eicosanoids, modification of the tissue fatty acid composition has been shown to affect the synthesis of eicosanoids derived from AA (Calder, 1998a).

Factors which influence the metabolism of LA may be manipulated to alter the AA in immune and accessory cells and thus alter eicosanoid mediated responses. There are a number of studies, mostly with mammalian species indicating that dietary n-3 PUFA can alter the composition and functions of immune cells (Calder, 1998a,b). Dietary lipid modulation results in significant modification of the fatty acid composition of macrophages isolated from the peritoneal cavity of mice (Lokesh et al., 1986b; Chapkin et al., 1992; Hardardottir and Kinsella, 1992), rats (Brouard and Pascaud, 1990; Watanabe et al., 1991; Sherrington et al., 1995a), or hamsters (Surette et al., 1995), of macrophages isolated from the lungs of pigs (Fritsche et al., 1993), of lymphocytes isolated from rodent lymphoid tissues (Brouard and Pascaud, 1990; Robinson et al., 1993; Yaqoob et al., 1995a), and of monocytes isolated from human peritoneal blood (Lee et al., 1985; Endres et al., 1989). As a result, few AA-derived eicosanoids are produced by these cells when animals are fed n-3 PUFA-rich diets.

Feeding laboratory animals with n-3 PUFA-containing oils such as linseed oil (rich in LNA) or fish oil (rich in EPA, DPA, and DHA) results in decreased production of

AA-derived eicosanoids (Calder, 1997b). When LNA replaced LA in rat diet containing 10% (w/w) fat, PG synthesis in spleen and thymus following an immune challenge was reduced (Marshall and Johnston, 1985). Yoshino and Ellis (1987) showed that EPA and DHA fed to rats for 50 days reduced production of PGE_2, LTB_4, and inflammatory exudates following an antigenic challenge. N-3 PUFA of fish oil reduce inflammatory reactions that are relevant to immune diseases (Johnston, 1988a). The n-3 PUFA of fish oil reduce the synthesis of LTs (Calder, 1997b). This results in a significant reduction of chemotaxis by monocytes and polymorphonuclear cells, and also results in amelioration of rheumatoid arthritis (Harbige, 1998; Belch and Hill, 2000; Kremer, 2000). EPA and DHA competitively inhibit the oxygenation of AA by cyclooxygenase (Calder, 1997b). In addition, EPA is able to act as a substrate for both cyclooxygenase and 5-lipoxygenase (Calder, 1997b). Thus ingestion of fish oils that contain n-3 PUFA will result in a decrease in membrane AA levels and a concomitant decrease in the capability to synthesize eicosanoids from AA. EPA gives rise to the 3-series PGs and TXs and the 5-series LTs. But the eicosanoids produced from EPA have less biological potencies than those formed from AA (Calder, 1996a).

1.6.3. Effect of n-3 PUFA on Cytokine Production

1.6.3.1. Immune Functions of Cytokines

Cytokines are soluble protein mediators produced by cells following their activation with specific stimuli (Calder, 1997a) and serve as communication signals between cells. Cytokines act in an autocrine or paracrine manner to influence the activity of cell types which express specific receptors to which they can bind. The cells of the

immune system are the main source of the cytokines, although other cell types can also synthesize these proteins. The target cells for cytokines include both immune and non-immune cells. Cytokines comprise the families of IL, TNF, and IFN and the hematopoietic growth factors. IL-1, IL-2, IL-4, IL-6, TNF-α, and INF-γ have been increasingly studied because of their significant effects on immune functions. The information on chicken cytokine secretion and function is limited, we assume that chickens are similar with mammals in the synthesis and functions of cytokines.

The term TNF designates two different polypeptides, TNF-α and TNF-β. TNF-α is the first cytokine released in response to bacterial endotoxin (or LPS). It is produced mainly by activated monocytes and macrophages but can also be produced by lymphocytes, natural killer cells, neutrophils and mast cells (Calder, 1997a). TNF-β is derived from lymphocytes (Meydani and Dinarello, 1993). TNF activates neutrophils, monocytes and macrophages to initiate bacterial and tumor killing, increases adhesion molecule expression on the surface of neutrophils and endothelial cells, stimulates T- and B-cell function, upregulates major histocompatibility antigens and initiates the production of other proinflammatory cytokines such as IL-1 (Calder, 1997a). Thus TNF is a mediator of both natural and acquired immunity and an important link between specific immune responses and acute inflammation. In addition, TNF-α mediates the systemic effects of inflammation such fever and hepatic acute-phase protein synthesis (Meydani and Dinarello, 1993; Calder, 1997a). Production of small amount of TNF is clearly beneficial in response to infection, but overproduction can be dangerous and TNF is implicated in causing some of the pathological responses which occur in endotoxic shock and other inflammatory conditions (Calder, 1997a). TNF-α decreases phagocytic activity

of human neutrophils and is involved in activation of eosinophils (Meydani and Dinarello, 1993).

IL-1 appears to be the second cytokine released in response to inflammatory stimuli, including LPS and TNF, and it shares many of the proinflammatory effects of TNF (Calder, 1997a). IL-1 exists as two polypeptides, IL-1α and IL-1β, which have similar biological activities and share cell surface receptors. They are produced by a variety of cell types, with the major of macrophages (Dinarello, 1989). The biologic effects of IL-1 include regulation of T and B lymphocyte development and activation, induction of synthesis of other cytokines and cytokine receptors, microbial invasion, immunologic reactions, inflammatory responses, and tissue injury (Dinarello, 1989; Meydani and Dinarello, 1993). Overproduction of IL-1 contributes to the pathogenesis of several chronic and acute inflammatory diseases including rheumatoid arthritis, cancer cachexia, atherosclerosis (Meydani and Dinarello, 1993). IL-1 increases production of PGE_2, which suppress the production of IL-1 (Knusden et al., 1986).

IL-6 is produced by activated monocytes and macrophages (and also endothelial cells, Th $CD4^+$ cells and keratinocytes) in response to IL-1 and TNF (Calder, 1997a). IL-6 can influence growth and differentiation of both T and B cells. Biologic effects of IL-6, like IL-1 and TNF, extend beyond immune cells. IL-6 acts synergistically with IL-1 in T-cell proliferation; it includes generation of cytotoxic T cells and causes differentiation of B cells to antibody-secreting cells. IL-6 acts as a hepatocyte-stimulating factor and induces various acute-phase proteins in liver cells. In corporation with IL-1, IL-6 promotes the transformation of hematopoietic stem cells from G_0 to G_1 phase of the cell cycle, and it induces maturation of megakaryocytes resulting in an increase in the number

of platelets (Kishimoto et al., 1992). Elevated levels of IL-6 are detected in synovial fluids from affected joints and in sera of patients with active rheumatoid arthritis. A correlation between serum IL-6 levels and severity of the disease has been observed (Meydani and Dinarello, 1993).

IL-2 is produced by Th1 T-lymphocytes and involves in cell-mediated immunity, macrophages and natural killer cell activation, transplantation rejection, and inflammation (Wallace et al., 1999a,b). It also needed for antigen- and mitogen-stimulated T-cell proliferation, and contributes to antibody production by B cells and stimulates cytotoxic activity and proliferation of natural killer cells (Meydani and Dinarello, 1993). IL-2 production decreases with age and with several pathologic conditions such as cancer, autoimmune diseases, and congenital and acquired immunodeficiency states (Miller, 1989). IL-2 production is also under negative control by PGE_2 (Gordon et al., 1976; Rappaport and Dodge, 1982).

1.6.3.2. Regulation of Cytokine Production by n-3 PUFA

High concentrations of PGE_2 inhibit *in vitro* production of TNF by macrophages (Kunkel et al., 1987; Renz, et al., 1988) and human monocytes (Renz, et al., 1988; Endres, et al., 1991). In contrast, low concentrations of PGE_2 appear to enhance TNF-α production (Kovacs et al., 1988). Inhibition of PG production *in vivo* resulted in increased circulating concentrations of TNF (and IL-6) during endotoxaemia in humans (Spinas et al., 1991). In contrast, LTB_4 enhances TNF-α production by human monocytes (Gagnon et al., 1989) and *in vivo* treatment with lipoxygenase inhibitors reduced circulating TNF

concentrations after LPS injection as well as reduced *in vitro* TNF production by peritoneal or alveolar macrophages (Schade et al., 1989; Dubois et al., 1989).

Although it has been observed that IL-1 production is inhibited by PGE_2 (Knudsen et al., 1986; Kunkel et al., 1987), there are some contradictory reports (Scales et al., 1989; Endres et al., 1991). LTB_4 and LTC_4 enhance *in vitro* IL-1 production by macrophages (Kunkel et al., 1987) and monocytes (Rola-Pleszczynski and Lemaire, 1985). However, again there are contradictory reports: LT synthesis inhibitors did not reduce IL-1 production by mononuclear cells (Parkar et al., 1990; Hoffman et al., 1991). LTB4 enhances *in vitro* production of IL-6 by human monocytes (Poubelle et al., 1991).

T lymphocytes have receptors for PGE_1 and PGE_2 and these compounds suppress IL-2 production *in vitro* (Rappaport and Dodge, 1982; Walker et al., 1983; Chouaib et al., 1985; Paliogianni et al., 1993). LTB_4 enhances IFN-γ production by lymphocytes (Rola-Pleszczynski et al., 1983). Thus it appears that PGE_2 suppresses the production of TNF, IL-1, IL-2, and IL-6 while LTB_4-series enhances production of these cytokines (Fig. 1.5) (Calder, 1997a). Since AA derived eicosanoids modulate the production of inflammatory and immunoregulatory cytokines, feeding n-3 PUFA rich diets should influence the production of those cytokines. Furthermore, n-3 PUFA should affect the pathophysiological responses to stimuli which enhance cytokine production.

Biller et al. (1988) demonstrated that IL-1 production by Kupffer cells from rats fed 15% energy as fish oil (rich in n-3 PUFA) for 6 wk was significantly lower than from those fed 15% energy as corn oil (rich in n-6 PUFA). Human studies reported to date have consistently demonstrated a decrease in production of proinflammatory cytokines when moderate to high levels of marine oils are taken orally (Endres et al., 1989;

Meydani et al., 1991, 1992). Endres et al. (1989) reported that young male volunteers, given 18 g/d of fish oil containing 2.7 g of EPA and 1.9 g of DHA for 6 wk, lowered IL-1β production, decreased further 10 wk after ending n-3 PUFA supplementation, and returned to the presupplement level 20 wk after ending the supplementation. The supplementation of EPA and DHA in old and young women volunteers decreased IL-1β and IL-2 production and T-cell-mediated functions (Meydani et al. 1991). Fish oil feeding decreases *in vitro* production of TNF-α, IL-1 and IL-6 by murine thioglycollate-elicited peritoneal macrophages (Renier et al. 1993; Yaqoob and Calder, 1995a,b) and of TNF-α and IL-6 by rat peripheral blood mononuclear cells (Grimm et al. 1994). In humans, fish oil supplementation has been shown to decrease *in vitro* production of TNF-α (Endres et al., 1989; Meydani et al., 1991, 1993; Caughey et al., 1996), IL-1 (Endres et al., 1989; Meydani et al., 1991, 1993; Caughey et al., 1996; Cooper and Rothwell, 1993), and IL-6 (Meydani et al., 1991,1993; Meydani and Dinarello, 1993; Cooper and Rothwell, 1993) by peripheral blood mononuclear cells.

Wallace et al. (1999a) reported that inflammatory macrophages from mice fed low fat diet produced highest concentrations of TNF-α, IL-1β, IL-6, and IL-10 than did resident macrophages. Diets enriched in longer-chain n-3 PUFA decreased the production of IL-2 by murine and human lymphocytes (Calder and Newsholme, 1992a; Jolly et al., 1997). This observation has been supported by the finding that both EPA and DHA inhibit IL-2 production by cultured human peripheral blood polymorphonuclear cells (Calder and Newsholme, 1992b). IL-2 and IFN-γ are produced by the Th1 class of lymphocyte involved in cell-mediated immunity, macrophages and natural killer cell activation, transplant rejection, and inflammation. There is little information about the

effects of dietary fatty acids on the production of TFN-γ or IL-4 produced by Th2 lymphocytes involved in antibody-dependent responses and allergic reactions. Wallace et al. (1999b) reported that feeding mice, for 6 wk, diets containing 20% (w/w) olive oil, safflower oil or fish oil (rich in unsaturated fatty acids) decreased IL-2 production compared with feeding 20% (w/w) coconut oil (containing mainly mid-chain saturated fatty acids); IFN-γ production was reduced by feeding safflower oil or fish oil compared with feeding coconut oil or olive oil (rich in 18:1n-9); IL-4 production was not influenced by dietary fatty acids. Similar findings have been reported that feeding mice fish oil (10-20%, w/w), the production of IL-4, IL-10, and IFN-γ by spleen lymphocytes were not reduced compared with feeding the same levels of corn oil or safflower oil (Fernandes et al., 1994; Yaqoob and Calder, 1995b). One possible mechanism may be deceased 5-lipoxygenase metabolites such as LTB_4 (Lee et al., 1985; Strasser et al., 1985) as adding exogenous LTB_4 to human mononuclear cells enhances endotoxin stimulated production of IL-1 (Rola-Pleszczynski and Lemaire, 1985). Conversely, inhibitors of 5-lipoxygenase reduce the production of IL-1 (Dinarello et al., 1984; Kunkel and Chensue 1985).

The effects of n-3 PUFA upon the production cytokines are complicated, because PGE_2 inhibits and 4-series LTs enhance the production of TNF, IL- and IL-6 and fish oil feeding decreases both PGE_2 and 4-series LT production. Thus n-3 PUFA feeding decreases the production of both inhibitory and stimulatory factors. The precise effect of n-3 PUFA upon production of those cytokines might related to the changed balance in production of PGE_2 and 4-series LTs and also to the balance in production between these mediators and the analogues produced from EPA (Calder, 1997a). Field et al. (2000) reported that feeding preterm infants with formula without added longer-chain PUFA

lowered IL-10 production by peripheral blood lymphocytes compared with feeding human milk. However, the production of IL-10 by the cells of infants fed the formula with added DHA and AA was not different from that produced by the human milk group cells.

1.6.4. Effects of n-3 PUFA on Cell Surface Molecules

A requirement for antigen presenting function is the expression of MHC class II (MHC-II) and class I (MHC-I) on the surface of antigen-presenting cells. MHC-I is required for presenting the antigens of altered self-cells, whereas MHC-II is required for presenting antigens of foreign pathogens (Kuby, 1997). The T-cell proliferative response is related to the number of MHC-II molecules on the surfaces of antigen-presenting cells. The percentage of MHC-II positive cells and the density of these molecules on the cell surface can alter the degree of immune responsiveness of an individual (Janeway et al., 1984). Feeding mice for 6 wk a diet containing 200g/kg of menhaden fish oil decreased the expression of MHC-II on the peritoneal macrophages from *Listera*-infected mice (Huang et al., 1992). Supplementing rats or mice by esophageal gavage for 4 wk with 100 μL/day or 50 μL/day of fish oil, respectively, produced a similar result (Mosquera et al., 1990).

In addition to the MHC-II expression, cell-cell adhesion is critical for initiation of a primary immune response. The importance of adhesion molecules such as lymphocyte functional associated antigen-1 (LFA-1, CD11a) and intercellular adhesion molecule-1 (ICAM-1, CD54) in the process of T-cell activation is demonstrated by the ability of monoclonal antibodies against these molecules to inhibit the T-cell proliferative response

to mitogens and antigens *in vitro* (Dougherty et al., 1988). Feeding menhaden fish oil diet significantly decreased the levels of cell surface adhesion molecules, LFA-1, ICAM-1, and CD2 (Sanderson et al., 1995b). CD18 forms CD18/CD11 intergrin molecule complexes that include LFA-1, CD3 and Mac-1 molecules. All these molecules play important roles in a number of macrophage functions including adhesion and phagocytosis. Therefore, the decrease in CD18 expression on CD2 positive macrophages from menhaden fish oil fed animals may alter the adherent and phagocytic properties of these macrophages (Sherrington et al., 1995b). As IFN-γ stimulates the expression of MHC-II, that n-3 PUFA may affect MHC-II expression by decreasing macrophage sensitivity to IFN-γ or decreasing the production of IFN-γ by lymphocytes (Mosquera et al., 1990). Calder et al. (1990) reported that murine thioglycollate-elicited peritoneal macrophages cultured in the presence of EPA or DHA were less adherent to artificial surfaces (the adhesion to one of these surfaces is mediated by LFA-1) than those cultured with some other fatty acids; LNA was without effect. The primary function of dendritic cells is the capture and processing of antigen and its presentation of processed antigen to T lymphocytes (Austyn, 1987). Feeding fish oil diets significantly diminished the expression of CD18, CD11a, CD2, CD54, and MHC II on the surfaces of long dendritic cells, and their antigen presentation activity compared with feeding low fat or high n-6 PUFA diets (Sanderson et al., 1997). Dietary lipid manipulation also alters the expression of lymphocyte cell surface molecules (Yaqoob et al., 1994a).

Culture of human adult saphenous vein endothelial cells with DHA (but not EPA) significantly decreased the cytokine-induced expression of vascular cell adhesion molecule 1 (VCAM-1), endothelial leukocyte adhesion molecule 1 (ELAM-1) and

ICAM-1 in a dose-dependent manner (De Caterina et al., 1995). The effects of DHA were independent of eicosanoid production (De Caterina et al., 1995). The adhesion of human peripheral blood monocytes to endothelial cells diminished following incubation of the latter with DHA (De Caterina et al., 1995). The binding between monocytes and endothelial cells partially depends on VCAM-1 expression on the endothelial cells; thus the reduced expression of VCAM-1 caused by DHA appears to have a functional effect. The incubation of LPS-stimulated pig aortic endothelial cells with EPA resulted in diminished binding between these cells and monocytes (Kim et al., 1995).

1.6.5. Effects of n-3 PUFA on Signal Transduction

1.6.5.1. The Effect on the Enzyme Activity of Cell Membrane

Enzymes and proteins of the cAMP and the protein kinase C (PK-C) signaling pathways and those involving ion fluxes and mobilization are both activated and/or inhibited by fatty acids (Sumida et al., 1993). Adenylate cyclase (AC) is involved in the intracellular signaling of a number of hormones and neurotransmitters, through the synthesis of cAMP (Houslay, 1990). It has been reported that EPA inhibits the activity of adipocyte AC (Laustiola et al., 1986; Tisdale, 1993). Rat spleen PK-C is less active in the presence of n-3 PUFA-rich phosphatidylserine compared with a PUFA-poor phosphatidylserine (Bell and Sargent, 1987). Speizer et al. (1991) found that that in the presence of both DAG and phosphatidylserine EPA and DHA caused up to 60% inhibition of PK-C activity. Another study has shown that EPA and DHA inhibit rat lymphocyte PK-C activity in the presence of Ca^{2+}, phosphatidylserine and DAG (May et

al., 1993). EPA and DHA also inhibit PK-C activity of peritoneal macrophages in rats (Tappia et al., 1995).

1.6.5.2. Ion Channel Activity

Fatty acids themselves regulate ion fluxes and Ca^{2+} mobilization directly, much as they regulate the action of many enzymes. A change in the concentration of intracellular free Ca^{2+} is often a key component in the intracellular signaling pathway which follows the stimulation of lymphocytes, macrophages and other cells by growth factors, cytokines and antigens (Calder, 1997b). AA induces release of Ca^{2+} sequestered in endoplasmic reticulum of cultured isolated pancreatic islets (Wolf, et al., 1986) in a manner similar to but independent of inositol-1,4,5-trisphosphate (IP_3) (Chow and Jondal, 1990). N-3 PUFA (LNA, EPA, and DHA) inhibit the anti-CD3-induced increase in intracellular free Ca^{2+} in the JURKAT T-cell line (Chow et al., 1990; Breittmayer et al., 1993).

1.6.5.3. Second Messenger

Hydrolysis of membrane phospholipids such as PIP_2 and phosphatidylcholine by phospholipases generates second messengers such as AA and DAG. The activation of PK-C by AA is a good example of the role of fatty acids as second messenger in signal transduction (Naor, 1991). PK-C is more active in the presence of DAG containing AA than in the presence of DAG containing n-3 PUFA (Kishimoto et al., 1980). DAG also increases the affinity of PK-C for Ca^{2+} and permits the full activation of the enzyme (Naor, 1991). The generation of the intracellular lipid second messengers DAG and ceramide plays a vital role in regulating cellular function, especially the lymphocyte (Fernandes et al., 1998). The marked reduction in generation of the intracellular second

messengers DAG and ceramide in Con A-stimulated cells from EPA or DHA-fed mice (Jolly et al., 1997) is suggestive of effects of fish oil-derived n-3 PUFA on intracellular signaling pathways which control the functional activities of the cells (Calder, 1998b).

1.6.6. Effect of n-3 PUFA on Gene Expression

The effects of dietary fatty acids on signal transduction pathway ultimately leads to the alteration of gene expression. A recent study showed that feeding mice with n-3 PUFA increased the levels of mRNA for IL-2, IL-4, and TGF-β and reduced the levels of mRNA for oncogenes (*c-myc* and *c-ras*) in the spleen and delayed autoimmune diseases (Fernandes et al., 1994). Dietary fish oil completely abolished mRNA production for IL-1β, IL-6 and TNF-α in the murine kidneys (Renier et al., 1993; Chandrasekar and Fernandes, 1994), which might be due to impaired synthesis (Robinson et al., 1995). Recent studies conducted in healthy young female mice showed that feeding highly purified EPA or DHA influenced lymphocyte gene expression relative to AA-fed controls (Chapkin et al., 1998; Jolly et al., 1998). These studies suggest that n-3 PUFA might affect immune cell functions by control at the transcriptional level.

1.7. Dietary PUFA and Chicken Immunity

The fatty acid composition of chicken immune tissues or cells has been reported to be altered by dietary fat composition (Fritsche, et al., 1991b). The proliferation of spleen lymphocytes induced by Con A or PWM stimulation is suppressed in chickens fed n-3 PUFA rich oils (linseed oil or fish oil at 7%, w/w) compared with those fed n-6

PUFA rich oils (lard, corn oil) (Fritsche et al., 1991a). Inflammatory response as measured by the synthesis of acute phase protein in chicken liver is also decreased by feeding fish oil (≤2%) (Korver and Klasing 1997). The immunomodulatory effect of fish oil depends on the type of grain used in the diet, with fish oil/cereal diets resulting in greater cell-mediated immunity and lower indices of inflammation than fish/corn diets (Korver and Klasing 1997).

In contrast, feeding chickens with 7% (w/w) fish oil increased antibody production compared with feeding the same levels of lard (rich in SFA) and maize oil (rich in LA) (Fritsche et al., 1991a; Fritsche and Cassity, 1992). Antibody production developed more rapidly reached a higher level and was more persistent in the chicks fed higher levels of LNA Friedman and Sklan (1995). Whereras, others reported no effect of dietary n-3 PUFA on the antibody production to sheep red blood cells in chickens (Fritsche et al., 1992; Phetteplace and Watkins, 1992).

N-3 PUFA derived from fish oil has been reported to alter eicosanoid production by chicken leukocytes (Fritsche and Cassity, 1992) and cytokine production by macrophages (Korver and Klasing, 1997).

1.8. Conclusions

There is increasing evidence implicating both level and type of dietary fatty acids as important factors in modulating cell-mediated and humoral immune responses. The evidence from animal studies indicates that dietary fatty acid manipulation have strong potentials to be used to alter the immune response under different conditions towards health. Animal research also suggests that n-3/n-6 PUFA balance plays a major role in

regulating the immune functions. The complex relationships between dietary fatty acids and immune functions are not currently clear. However, dietary fatty acids have attracted recent attention for their potential immunoregulating properties.

Figure 1.1. Differentiation and activities of helper T (Th) lymphocytes. PC, precursor cell; CTL, cytotoxic T lymphocytes; IFN-γ, interferon-γ; IL, interleukin; NK, natural killer; TGF-β, transforming growth factor-β; +, promote; -, suppress.

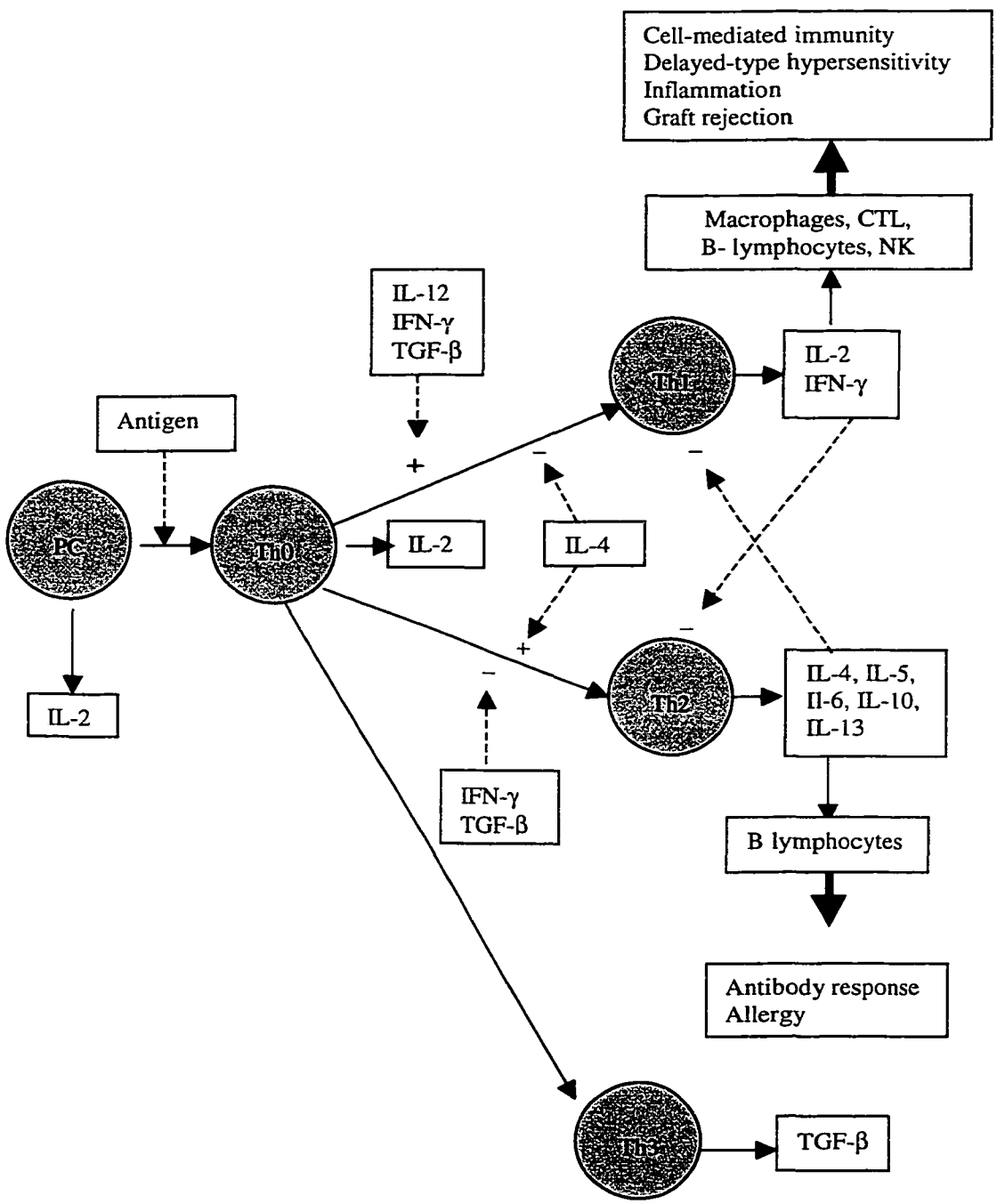


Figure 1.2. B-lymphocyte differentiation and class switching in the antibody production. IFN-γ, interferon -γ; Ig, immunoglobulin; IL-interleukin; TGF-β, transforming growth factor-β (Calder, 1998b).

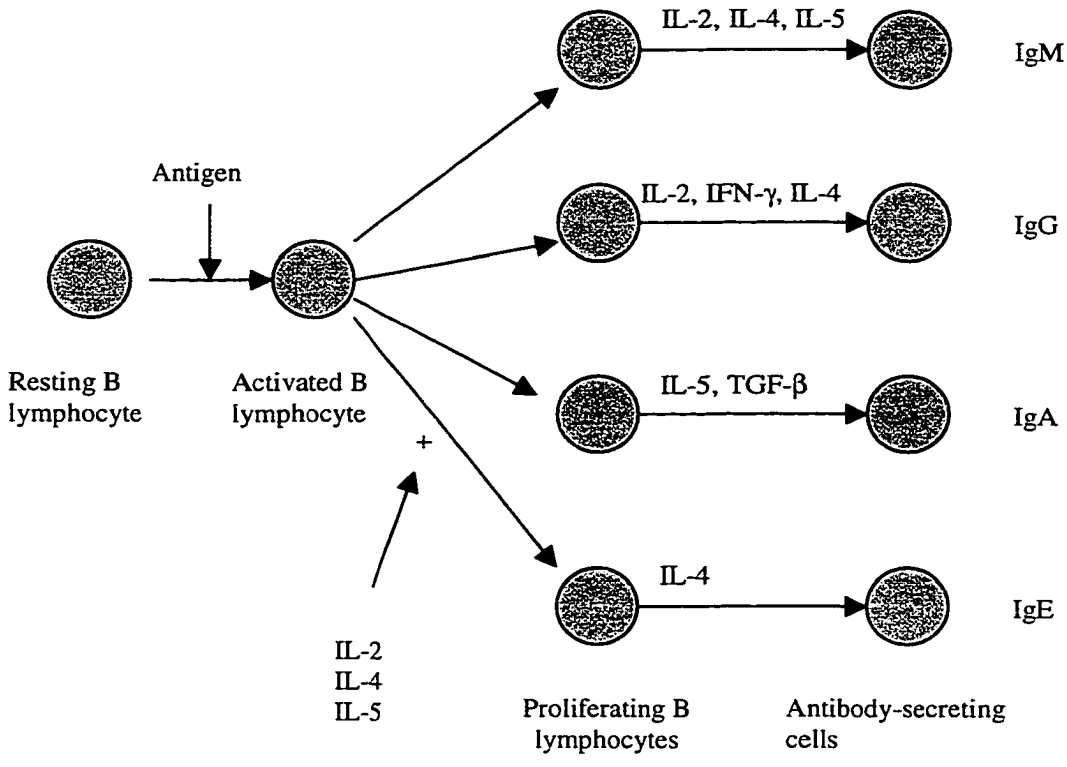


Figure 1.3. Pathways of synthesis of unsaturated fatty acids. Δ4, Δ5, Δ6, Δ9, Δ12, Δ15, respective desaturase enzymes (Calder, 1998a).

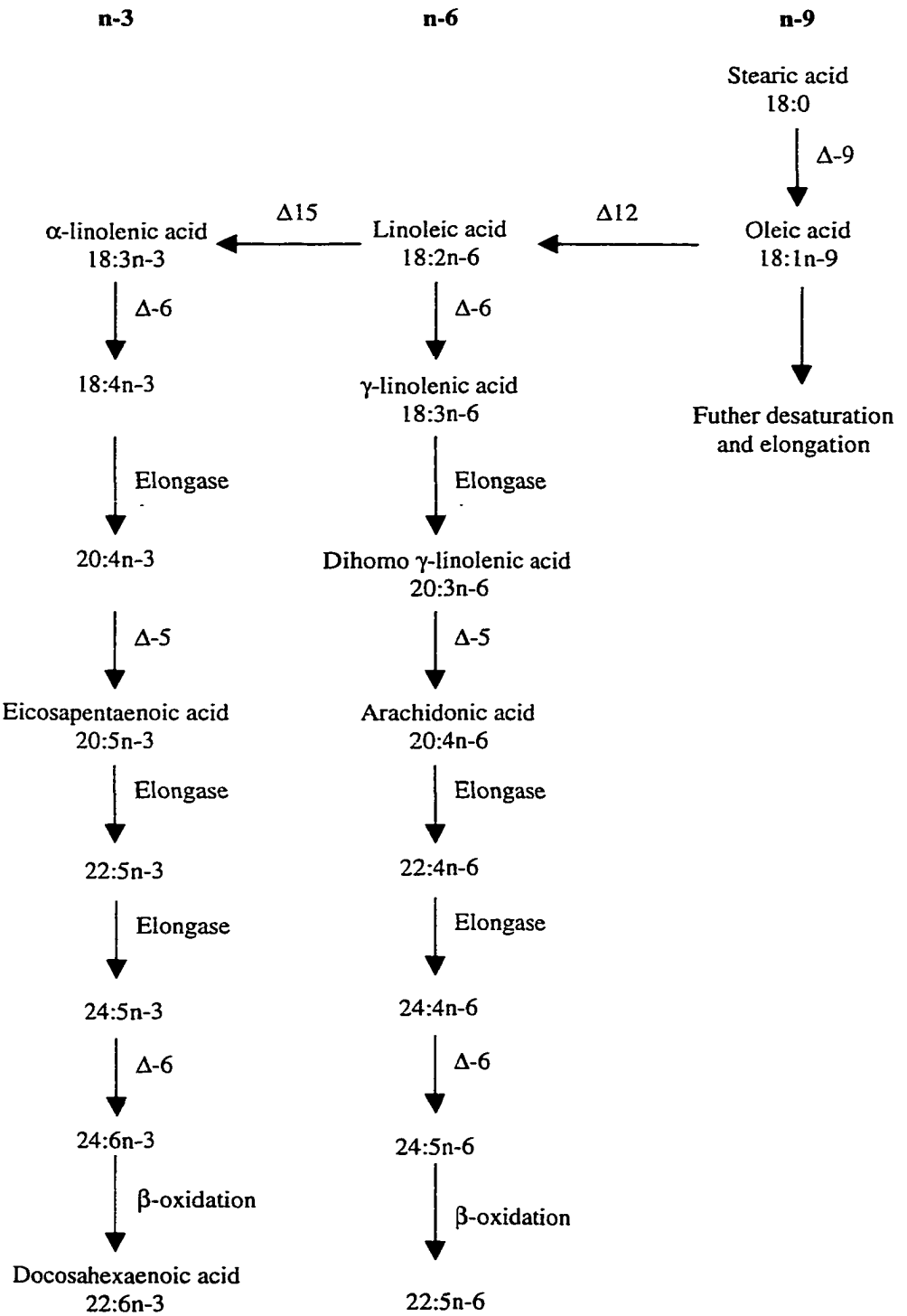


Figure 1.4. Pathways of eicosanoid synthesis. PG, prostaglandin; LT, leukotriene; HPETE, hydroperoxyeicosatetraenoic acid; HETE, hydroxyeicosatetraenoic acid (Calder, 1997b).

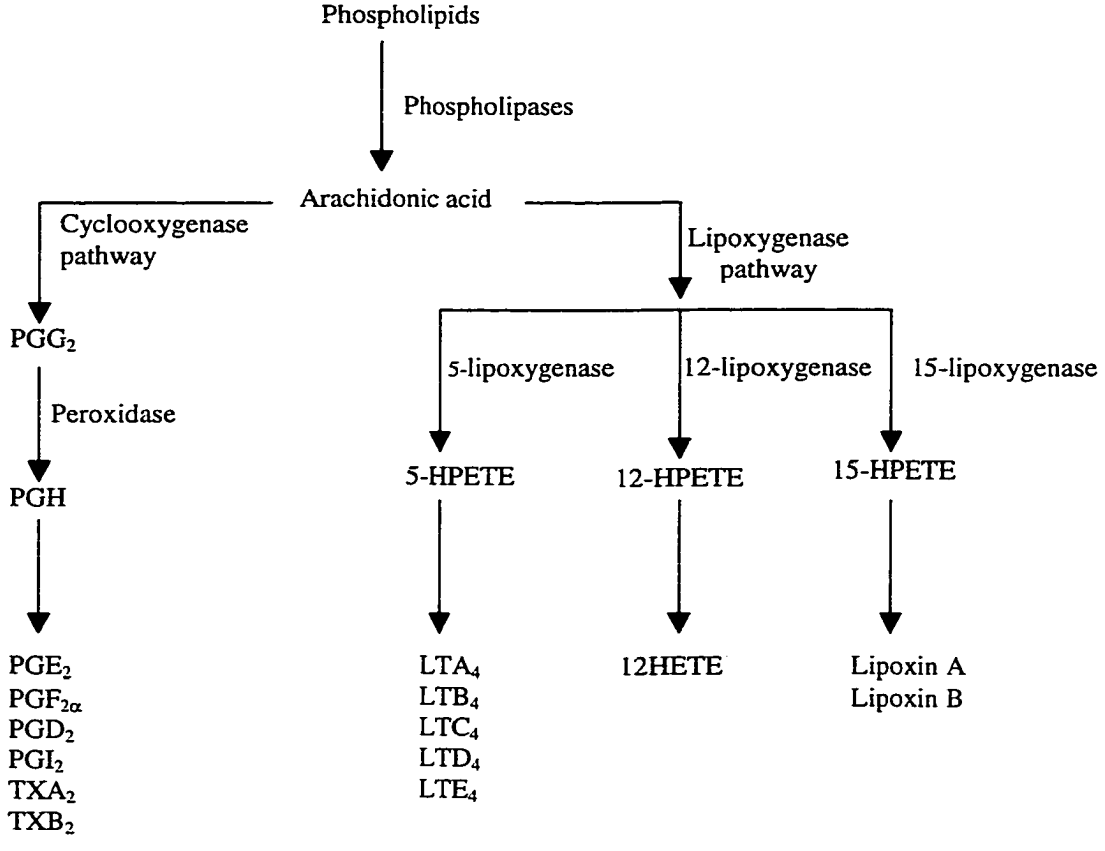
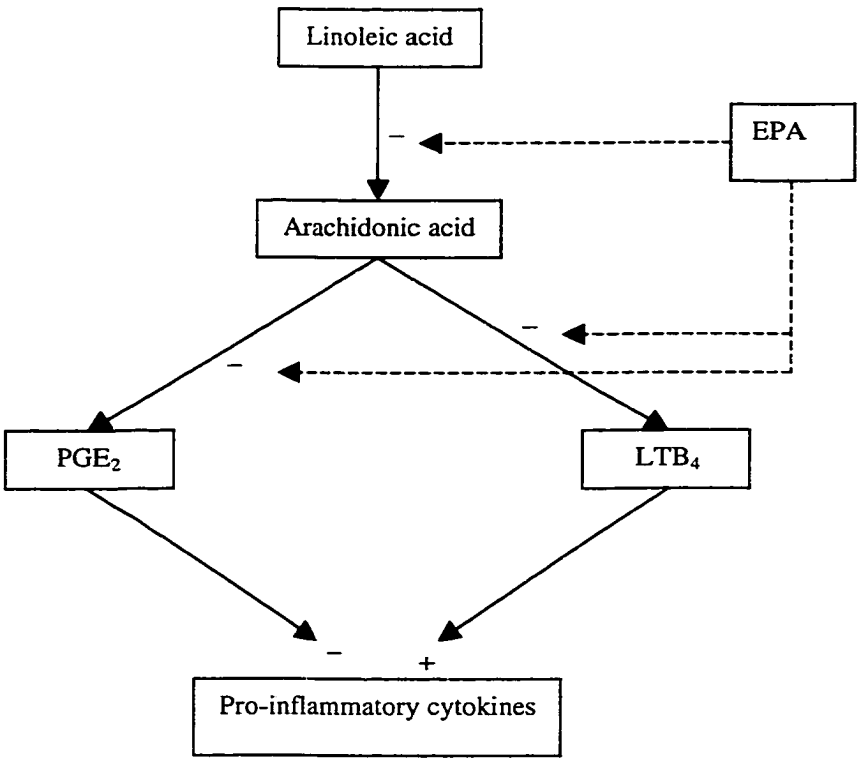


Figure 1.5. Summary of the general effects of PUFA on cytokine production. EPA, eicosapentaenoic acid; PGE_2, prostaglandin E_2; LTB_4, leukotriene B_4; -, suppress; +, enhance (Calder, 1997a).



2. STUDY RATIONALE

2.1. Introduction

Infectious disease causes serious economic losses to the poultry industry (Kover et al., 1997). Dietary characteristics can modulate a bird's susceptibility to infectious challenges and subtle influences due to the level of nutrients or the types of ingredients may at times be of critical importance (Klasing, 1998). From a practical viewpoint, a bird's susceptibility to an infectious challenge can be subdivided into two components, resistance and resilience. Resistance refers to the capacity of a variety of anatomical and physiological systems, including the immune system, to exclude pathogens. Resilience refers to the capacity of the bird to maintain productivity during an infectious challenge (Klasing, 1998). The appropriate balance between these two parameters is crucial to animals in protecting infection, maintaining health and profitability.

Inflammation is an important cell-mediated immune process to kill and clear pathogens. However, under certain circumstances, an inflammatory response can overreact or not appropriately down regulated, resulting in significant tissue damage or even death. In poultry, an inflammatory response can reduce feed consumption and muscle protein accretion, and increase metabolic rate, synthesis of acute phase protein, and organ mass relative to body mass (Klasing and Korver 1997; Roura et al. 1992). This change results in the partitioning of nutrients away from growth and therefore, decreases growth rate and feed efficiency. For example, coccidial infection results in gastrointestinal tract lesions, which in turn, result in decreased performance (Ruff and

Allen, 1990). The immune response to a coccidial infection is primarily mediated by pro-inflammatory phagocytic cells recruited to the site of infection (Tellez et al., 1994).

Antibody response provides protection to extracellular pathogens. Studies have shown significant up regulatory effect of dietary n-6 and n-3 PUFA on the antibody production in chickens (Fritsche et al., 1991a; Fritsche and Cassity, 1992; Friedman and Sklan, 1995), while some have shown no effect (Fritsche et al., 1992; Phetteplace and Watkins, 1992). The contradictory results might come from the differences in the chicken physiological status, fat source, other nutrients in the diets and the measuring method. The total immunoglobulins in serum might reflect the potential antibody response when chickens are exposed to an infectious challenge. IgG is the major immunoglobulin class produced in the secondary humoral response. The IgG concentration may thus be used to assess humoral immune response in combination with specific antibody production or as an independent index. In addition, maternal-fetal transfer of antibodies provides passive immunity that is essential to hatching chicks, in particular, in the early 2 wk following hatching (Smith et al., 1994). Whether maternal dietary fatty acids influence the antibody transfer from egg yolk to the embryo needs to be elucidated.

It is well established from mammalian studies that dietary PUFA have profound effects on the fatty acid composition of immune cell membrane and associated membrane functions. However, the effect of dietary PUFA on the compositions of n-3 and n-6 fatty acids in the immune tissues or cells has not been widely studied in chicken. In addition, in the study reported by Fritsche et al. (1991b), chickens were fed different fat diets for only a few weeks. For practical use, long-term (such as maternal and neonatal) effect of dietary fatty acids on the fatty acid profiles of immune tissues or cells and further on the

immune function of the progeny awaits to be examined. In addition, dietary n-3 and n-6 fatty acids may be differently incorporated into different immune tissues (Fritsche et al., 1991b) or the same class of immune cells (e.g., lympohcytes) from different origins, therefore, imposing different regulatory effects on the immune functions of different tissue sites.

In addition, egg yolk provides the solely nutrients for the embryo during incubation. The egg yolk fatty acid composition has been reported to alter the compositions of fatty acids in the serum and tissues of hatching chick (Cherian and Sim, 1991; 1992). It is not known how egg yolk fatty acids influence the fatty acid composition of immune tissues and the immune responses of chicks following hatching.

2.2. Objectives and Hypotheses

The objectives of this research were to test following general working hypotheses:

I. Dietary n-3 PUFA will decrease the ratio of n-6 to n-3 fatty acids in the immune tissues or cells and thus the immune functions in laying hens.

II. Maternal and neonatal dietary n-3 PUFA will decrease the composition of n-6 PUFA and increase n-3 fatty acids in the immune tissues or cells and affect the immune tissue growth of chicks.

III. Maternal and neonatal dietary n-3 fatty acids will suppress cell-mediated and enhance humoral immune responses of the progeny as in the hens.

IV. Dietary source of n-3 PUFA will increase the components of n-3 fatty acids incorporated into the immune tissues or cells, thus affect the immune functions in chicken.

V. Maternal dietary n-3 and n-6 PUFA will affect the ratio of n-6 to n-3 PUFA in the immune tissues and alter the inflammatory response of chicks following hatching.

VI. Low ratios of dietary n-6 to n-3 PUFA will suppress cell-mediated immune responses and increase antibody response of chickens.

2.3. Chapter Format

The hypotheses posed were tested in a sequence of experiments. These experiments are organized as thesis chapters and have been prepared for submission for scientific publication as individual papers.

2.3.1. Study one

It is hypothesized that:

1. Dietary supplementation of n-3 fatty acids will increase the compositions of these fatty acids in laying hen immune cells.
2. High levels of dietary n-3 fatty acids will suppress mitogenic responses of lymphocytes in chicken.
3. The dietary ratio of n-3 to n-6 fatty acids will change the proportions of T and B cell populations.

4. Low ratio of n-6 to n-3 PUFA will increase total IgG concentrations in laying hen serum and egg yolk.
5. Incorporation of longer-chain n-3 PUFA into chicken immune cells will depend on the chain-length of dietary n-3 PUFA.
6. N-3 PUFA derived from linseed oil will have less potency than fish oil in affecting cell-mediated and antibody-mediated immune responses in chicken.

Chapter 3 examines the effects of laying hen dietary fat composition on the fatty acid composition of spleen lymphocytes (*hypotheses 1 and 5)*, spleen and blood lymphocyte proliferative responses to Con A (*hypotheses 2 and 6*), proportions of different lymphocyte classes (*hypotheses 3 and 6)* and total IgG concentrations in the serum and egg yolk (*hypotheses 4 and 6*).

2.3.2. Study Two and Three

It is hypothesized that:

7. The effect of dietary PUFA on the composition of n-3 fatty acids in the immune tissues or cells will be remained in the next generation if the progeny were fed the same types of fat diets as the maternal.
8. Dietary n-3/n-6 PUFA will differently affect the incorporation of these fatty acids into the immune tissues (spleen, thymus, bursa of Fabricius) and lymphocytes from different tissue sites.
9. Amount and type of maternal and neonatal dietary PUFA will alter the immune tissue development of chicks.

10. Maternal and neonatal dietary n-3 fatty acids will suppress the lymphocyte proliferation to the stimulation of mitogens Con A, PHA, or PWM.
11. Maternal and neonatal dietary n-3 fatty acids will increase $CD8^+$ T-cells and B lymphocyte populations of chick immune tissues.
12. Increasing n-3 PUFA in maternal and neonatal diet will enhance chick IgG production.

Chapter 4 details the findings from study two. The experiment was designed to investigate the long-term (maternal and neonatal, two generations) effect of dietary fat composition on the fatty acid incorporation into the three major immune tissues and two origins of lymphocytes (*hypotheses 7*). The different effects of n-3 PUFA derived from linseed oil and fish oil on the n-3 fatty acid composition of immune tissue or cells were repeatedly investigated (*hypotheses 5*). This experiment was also aimed to examine if there is heterogeneity effect of dietary fat on the fatty acid composition among the immune tissues and between cell origins of lymphocytes (*hypotheses 8).* Since immune tissue development is fundamental to the function of immune system, the growths of three immune tissues were also investigated in this study (*hypothesis 9*).

Chapter 5 examines the effect of maternal and neonatal dietary n-3 PUFA on the mitogenic responses of the lympohcytes from the thymus and spleen to Con A, PHA, and PWM (*hypothesis 10*), lymphocyte subset populations (*hypothesis 11*), and total immunoglobulin G production (*hypothesis 12*). The different immunomodulating effects of n-3 fatty acids derived from linseed oil and fish oil were repeatedly examined (*hypothesis 6).*

2.3.3. Study Four

It is hypothesized that:

13. Dietary n-3 fatty acids will suppress inflammatory response of the hens.
14. Dietary n-3/n-6 fatty acids will change the n-3 PUFA incorporation into the egg yolk.
15. Maternal dietary n-3 fatty acids will, via egg yolk, alter the incorporation of these fatty acids into the embryo immune tissues during incubation. This effect will last for a certain time after hatching.
16. Maternal dietary n-3 fatty acids will suppress the inflammatory response of the offspring for a certain time following hatching.

Chapter 6 examines the effect of dietary fat source on the fatty acid composition of egg yolk of broiler breeders and the effect of egg yolk fatty acids on the fatty acid composition of embryo immune tissues upon hatching (*hypotheses 14-15).* This study was also expanded to investigate the duration of the effect of solely maternal fatty acids on the fatty acid composition of the offspring immune tissues (*hypothesis 15).* In addition, the effects of maternal dietary n-3 fatty acids on the inflammatory response in the breeders (*hypothesis 13*) and their progeny (*hypothesis 16)* were studied.

2.3.4. Study Five

It is hypothesized that:

17. Decreasing dietary n-6 to n-3 fatty acid ratio will suppress the chicken inflammatory response.
18. Altering the dietary ratio of n-6 to n-3 fatty acids will change the total IgG and antibody IgG productions in chicken.
19. Total serum IgG concentration will reflect the capability of chickens for producing antibody IgG.
20. Maternal-fetal transfer of specific antibody IgG will be affected by maternal dietary n-6 and n-3 fatty acids via egg yolk.
21. The net transfer of specific antibody IgG to the circulating system of the embryo will be associated with the total IgG transportation via yolk sac.

Chapter 7 presents the observations obtained from study five. The objectives of this study were to examine the effect of changing the dietary ratio of n-6 to n-3 fatty acids on the inflammatory response in chickens (*hypothesis 17*). The ratio of n-6 to n-3 fatty acids in the diet on the humoral immune response was assessed by measuring the total IgG and antibody IgG production (*hypothesis 18*). Through yolk fatty acids the maternal dietary fatty acids will affect the total IgG and antibody IgG transfer from egg yolk to the embryo during incubation (*hypothesis 20*). Total IgG in serum is thought to reflect the potential of antibody production when the chicken is exposed a pathogenic challenge (*hypothesis 19).* This study was also designed to examine if specific antibody transfer from egg yolk to the circulating system of embryo is paralleled with the amount of total IgG transferred (*hypothesis 21*).

3. EFFECT OF DIETARY N-3 POLYUNSATURATED FATTY ACIDS ON THE LYMPHOCYTE PROPORTION AND PROLIFERATION AND SERUM IGG CONCENTRATION IN LAYING HENS[1]

3.1. Introduction

As in other animals, immune responses are important to poultry for protection from various infections and maintaining health. It has been reported that dietary fatty acids can modify the fatty acid profiles of immune tissues or cells and thus alter immune responses (Fritsche et al., 1991a; Watkins, 1995). Such alterations may be used to benefit poultry and other animals under certain conditions. In mammals, evidence has shown that both type and amount of fatty acids in a diet can alter immune functions (Gershwin et al., 1985; Johnston, 1985, 1988a,b; Fritsche et al., 1991a). The n-3 PUFA, LNA and its longer-chain EPA and DHA suppress lymphocyte proliferation to polyclonal mitogen stimulations (Kelley et al., 1988; Meydani et al., 1991, 1993; Sanderson et al., 1995a,b).

The influences of dietary fatty acids on humoral immunity are quite contradictory in mammals (Calder, 1997b). In poultry, limited literature reports indicate that n-3 PUFA suppress spleen lymphocyte proliferation and increased antibody titer against sheep red blood cells or bovine serum albumin (Fritsche et al., 1991a; Friedman and Sklan, 1995). We assume that the concentration of total Ig in serum is related to the potential antibody production and may reflect the capacity of humoral immunity in chickens. In addition,

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[1]A version of this chapter is in press. Wang, Y. W., Sunwoo, H. H., Sim, J. S., and Cherian, G., 2000. Canadian Journal of Animal Science.

the IgG transferred from the egg yolk (IgG in egg yolk is also termed IgY) via yolk sac to the embryo provides immuno-protection to the embryo and newly hatched chick (Brambell, 1969). The amount of IgY transferred from egg yolk to the embryo during incubation may be influenced by the total amount of IgY in egg yolk and thus impact the early resistance of chicks to various infections.

Since different immune actions are performed by different phenotypes of immune cells, diet-induced changes of immune functions are likely associated with distribution of various phenotypes of immune cells in immune organs or tissues (Robinson and Field, 1998). Meydani et al. (1993) observed that consumption of a low fat, low cholesterol, n-3 PUFA-rich diet resulted in a lower proportion of $CD4^+$ and a higher proportion of $CD8^+$ peripheral blood T-lymphocytes, with the proportion of $CD3^+$ cells (total T-cells) being unaffected in humans. It has also been reported that feeding a fish oil diet did not affect the proportions of $CD4^+$, $CD8^+$, total T-cells, total B-cells, and macrophages in the spleen, thymus, lymph nodes or blood of rats (Yaqoob et al., 1994a; Sanderson et al., 1995b). The present experiment was designed to determine the effect of amount and type of n-3 PUFA in diets with a constant level of total fat on the indices of chicken immune responses including lymphocyte subset population, an estimation of proliferation and blood and egg yolk IgG concentration.

3.2. Materials and Methods

3.2.1. Animals and Diets

The experiment was reviewed and approved by the Faculty of Agriculture and Forestry Animal Policy and Welfare Committee, University of Alberta and was conducted in accordance with the Canadian Council on Animal Care guidelines. Forty-eight Single Comb White Leghorn laying hens (at the age of 24 wk) were randomly assigned to one of four dietary treatments with 12 birds each. All birds were housed in cages with 2 birds per cage and had free access to feed and water. The experimental diets (Table 3.1) were formulated to meet the nutrient requirements of laying hens (National Research Council, 1994). To a wheat-soybean diet, sunflower oil (SO), animal oil (AO), linseed oil (LO), and menhaden fish oil (FO) were added at 5% (w/w), respectively. The diets were isocaloric and isonitrogenic. Fatty acid composition of the diets was analyzed using the method described by Cherian and Sim (1992) and presented in Table 3.2. The SO, AO and LO provided different ratios of PUFA to SFA and different ratios of n-6 to n-3 PUFA. The LO and FO had different PUFA to SFA ratios, similar n-6 to n-3 PUFA ratios but different n-3 PUFA components. Fresh feed was prepared weekly and stored in a cool dark room.

3.2.2. Sample Collection

After 5 wk feeding the experimental diets, 12 eggs were collected from each group for analysis of egg yolk IgY. Six healthy birds were randomly chosen from each group. Five (5) mL of blood were taken from wing vein into a 15 mL tube without anti-

clotting reagent [such as ethylenediaminetetraacetic acid (EDTA)] and allowed to clot for 4 h at room temperature; another 8 mL of blood were collected into a 15 mL tube with EDTA and placed on ice. Immediately, the birds were killed by cervical dislocation. The spleen was removed, weighed, and put into a 50 mL sterile centrifuge tube containing 20 mL of complete culture medium (CCM) and placed on ice. The CCM was composed of RPMI-1640 (Sigma Chemical Co. St. Louis, MO 63178-7794; Sigma is used in the following) with the addition of 1% (v/v) antibiotic and antimycotic mix (GibcoBRL, Grand Island, N.Y., 14072).

Sera were separated from the clot by spinning at 250 x *g* for 10 min, sterile filtered and placed on ice until use in the lymphocyte proliferation assays. The blood, collected for isolation of blood lymphocytes, was diluted (1:2, v/v) with CCM, mixed gently and laid on the lymphocyte separation medium (density, 1.07-1.08; Sigma) and then centrifuged at 1,100 x *g* for 15 min at 20 °C. Cells at the interface were collected, washed twice with CCM. The cells were resuspended in CCM with 5% (v/v) fetal bovine serum (5% FBS-CCM), then were counted and adjusted to $1x10^7$ cells/mL in 5% FBS-CCM. The cell viability was determined by trypan blue exclusion (Robinson and Field 1998).

Suspensions of spleen lymphocytes were made as the method described by Field (1995). Briefly, each tissue was cut into small pieces using sterile scissors, and pushed through a tissue sieve equipped with 80-mesh stainless steel screen (Sigma). Cell clumps were dispersed by several gentle washings with CCM through the sieve. The red blood cells and dead cells were removed by laying the cells and CCM mixture on the lymphocyte separation medium (density 1.07-1.08) and centrifuging at 1,100 x *g* for 15

min. The cells were washed 2-3 times with CCM and resuspended in 5 mL of 5% FBS-CCM. The cells were counted using a hemacytometer and cell viability was determined by trypan blue exclusion. The cell suspension was diluted to a final concentration of 1 x 10^7 cells/mL in 5% FBS-CCM.

3.2.3. Lymphocyte Proliferation Assay

An estimate of lymphocyte proliferation was assayed according to the method reported by Field (1995) with some modifications. Spleen lymphocytes ($1x10^6$) and peripheral blood lymphocytes ($5x10^5$) were cultured in triplicate using 96-well microtiter plates (Corning Inc., Corning, NY 14830). The cells were incubated with Con A (10 μg/mL) in humidified atmosphere containing 5% CO_2 at 40 °C for 72 h and 48 h, respectively. The assays were conducted in 5% FBS-CCM and 5% (v/v) autologous chicken serum (Fritsche et al., 1991a). Eight hours before harvesting cells, 1 μCi of [^{3}H]thymidine was added to each well. The cells were harvested with the aid of an automatic cell-harvesting device (Skatron Instruments AS, N-3401 Lier, Norway) and incorporation of the radioactivity was determined by liquid scintillation counting. Data were presented by stimulation index, which was calculated as stimulation index = (amount of [^{3}H]thymidine incorporated by the stimulated cells – the amount of [^{3}H]thymidine incorporated by the unstimulated cells)/amount of [^{3}H]thymidine incorporated by unstimulated cells.

The remaining spleen lymphocytes of each sample were centrifuged again. The cell pellets were individually kept at –20 °C for fatty acid analyses. The remaining sera were also kept at –20 C for fatty acid analyses and IgG measurements.

3.2.4. Lymphocyte Phenotyping

Lymphocyte subsets from freshly isolated splenocytes and blood mononuclear cells were identified by direct single label (one color) immunofluorescence assay (Robinson and Field 1998) using purified monoclonal antibodies (Southern Biotechnology Associates, Inc. Birmingham, AL 35226) produced by mouse specific for the different chicken lymphocyte subsets. CT-4 recognizes T helper lymphocytes ($CD4^+$), CT-8 recognizes cytotoxic/suppressor T lymphocytes ($CD8^+$), M-1 recognizes IgM^+ cells and L-1 recognizes Ig^+ cells. For each sample, 5 $x10^5$ lymphocytes were added to each well, then 200 µL of phosphate buffered saline (PBS) with 4% (v/v) FBS (4% FBS-PBS) were added to each well and centrifuged at 900 x *g* for 2 min at 4 °C. The supernatants were aspirated and the pellets were broken up by gentle vortexing. Each monoclonal antibody conjugated with fluorescein isothiocyanate (0.4 µg) in 50 µL of 4% FBS-PBS was added. Fifty µL of 4% FBS-PBS was added into the blank wells. The plates were covered and incubated for 30 min at 4 °C. Then the cells were washed three times with 4% FBS-PBS. The pellets were vortexed gently and 200 µL of PBS containing 1% (v/v) paraformaldehyde was added. The cells were identified using FACScan (Becton Dikinson, Sunnyvale, CA) set at a wavelength of 488 nm. Resulting percentages were corrected for background fluorescence determined by incubating cells with 4% FBS-PBS only. Unwanted events (dead cells and debris) were detected on the basis of forward scatter and side scatter and were excluded from subsequent phenotype analyses by electronic gating of the viable spleen and blood lymphocyte population.

3.2.5. Content of IgG in Serum and IgY in Egg Yolk

Egg yolk IgY is contained in the water-soluble fraction. Egg yolk was separated from the white albumin using a yolk separator, gently rolled on paper towel to remove the attached white, and then was transferred into a graduated cylinder. The yolk volume was recorded and diluted (1:6, v/v) with acidified deionized water (pH 2.5), adjusted with 0.1N HCl, mixed well and kept at 4 °C for 6 h or overnight. Then the solution was centrifuged at 12,100 x *g* for 15 min at 4 °C and the supernatant was collected. The contents of IgG in serum and egg yolk were determined by radial immunodiffusion technique as described by Sunwoo et al. (1996).

3.2.6. Fatty Acid Analyses

Fatty acid extraction and methylation were done as described by Folch et al. (1957) and Metcalfe, et al. (1961), respectively. Fatty acid methyl esters of spleen lymphocyte and feed samples were separated and quantified using automated gas chromatograph equipped with autosampler and ionization detector (Model 3600, Varian Associates Inc., Sunnyvale, CA). A 30 m x 0.25mm i.d. fused silica capillary (Supelco Canada, Ltd., Oakville, ON) was used as described by Cherian and Sim (1992). A Shimadzu EZChrom laboratory data integration system (Shimadzu Scientific Instruments, Inc., Columbia, MD) was used to integrate peak areas.

3.2.7. Statistical Analysis

Data were analyzed by one-way ANOVA using general linear model procedure of SAS software (SAS Institute Inc. 1990). Significant differences among dietary treatments

were analyzed by the method of least squares means test after a significant main effect by ANOVA. Significance level was set at $P < 0.05$ (Myers and Well, 1995).

3.3. Results and Discussion

3.3.1. Fatty Acid Composition of Splenocytes

The detailed fatty acid profiles of splenocytes were presented in Table 3.3. There was no effect of fat source on the level of C18:0 and total SFA in the isolated splenocytes as reported by Friedman and Sklan (1995) and Fritsche et al. (1991b). The relative content of MUFA and PUFA were also fairly resistant to dietary modifications under this experimental dietary condition. The ratios of PUFA to SFA in the splenocytes from the hens fed SO, AO, LO and FO were 0.7, 0.6, 0.6 and 0.6, respectively, with the ratio in splenocytes from the hens fed SO being higher than the other diets ($P < 0.05$). The ratios of n-6 to n-3 PUFA changed dramatically ($P < 0.0001$), which were 21.0, 8.8, 3.7 and 1.3, respectively for hens fed SO, AO, LO and FO. The pattern of n-6 to n-3 PUFA ratio in the splenocytes reflected that in the diets.

The relative amount of C18:1 in splenocytes changed reciprocally with the amount of total PUFA incorporated. The impact of dietary fat source on chicken splenocyte fatty acid profile is significant for the individual fatty acids of PUFA. Similar results were obtained by Fritsche et al. (1991b) in the fatty acid composition of the isolated splenocytes by feeding chicks diets containing 7% (w/w) of lard, corn oil, canola oil, linseed oil, or fish oil. The amount of LA was affected ($P < 0.0001$) by its dietary level. The AA in the splenocytes accounted for up to 17% of the total fatty acids, and

feeding n-3 PUFA-rich diets significantly lowered ($P < 0.0001$) the level of AA in splenocytes with a concomitant increase ($P < 0.0001$) of n-3 PUFA level. Similar observations have been reported for liver, heart, brain, muscle and embryo lipids in chickens (Anderson et al. 1989; Huang et al. 1990; Cherian et al. 1995).

The LNA was poorly incorporated into splenocytes even though high amount was supplied in diet (32% in diet LO). The incorporation of longer-chain n-3 PUFA (EPA and DHA) in splenocytes depended on the source of the n-3 PUFA in the diets (Fritsche et al., 1991b). Feeding the preformed longer-chain n-3 PUFA (fish oil) resulted in a greater elevation of these fatty acids compared with feeding the precursor, LNA (linseed oil). Greater enrichment of EPA and DHA has also been observed in the immune tissues of rats or mice fed a fish oil diet compared with those fed linseed oil diet, in spite of higher overall n-3 PUFA content in the linseed oil diet (Croft et al., 1984; Fritsche and Johnston, 1990).

3.3.2. Lymphocyte Proliferation

Spleen lymphocyte proliferation induced by mitogen Con A was significantly affected by dietary fat source ($P < 0.05$; Fig. 3.1). There was no difference ($P > 0.05$) among the hens fed SO, AO and LO, and among the hens fed AO, LO and FO. The hens fed FO showed lower ($P < 0.05$) spleen lymphocyte proliferative response to Con A stimulation compared with those fed SO. Feeding FO to hens suppressed the spleen lymphocyte proliferation around 30% relative to feeding SO. This was in agreement with the suppression of 30-50% by feeding chicks with diets containing 7% (w/w) of n-3 PUFA rich oils (linseed oil or fish oil) compared with 7% of n-6 PUFA rich oil (maize

oil), or SFA rich oil (lard) (Fritsche et al., 1991a). The blood lymphocyte proliferation in response to Con A stimulation was suppressed ($P < 0.001$) with the lower n-6 to n-3 PUFA ratios (Fig. 3.2). The proliferative responses of blood lymphocytes from the chicks fed LO or FO were lower ($P < 0.05$) than that from the chicks fed SO or AO. The chicks fed AO had lower ($P < 0.05$) response than the chicks fed SO. No difference was observed between the two groups fed LO and FO ($P > 0.05$). The results of the current study are in accordance with previous studies conducted in mammals. For example, Meydani et al. (1993) reported that the proliferative response of peripheral blood lymphocytes to Con A was suppressed when volunteers on a low-fat, low-cholesterol diet were supplemented with n-3 PUFA. A reduction of blood lymphocyte proliferation in response to Con A was also observed in rats by feeding, for 10 wk, a diet containing 20% fish oil (Yaqoob et al., 1995b).

The results of our present study support the observations in mammals that dietary fat source can affect lymphocyte proliferative response to polyclonal mitogen stimulation (Marshall and Johnston, 1985; Kelley et al., 1988,1991; Yaqoob et al., 1994a,1995b). The ratios of PUFA to SFA were similar among the four groups of hens, with a little higher level observed in the hens fed diet SO. The greatest changes that occurred in the immune cells of the laying hens are the n-6 to n-3 PUFA ratios, which affected the lymphocyte proliferation. With the increase of n-3 PUFA, i.e., decrease of n-6 to n-3 PUFA ratio, the lymphocyte proliferation was significantly diminished. This suggests that the ratio of n-6 to n-3 PUFA play a major role in regulating chicken spleen and blood lymphocyte proliferation. The stronger suppressive effect of n-3 PUFA derived from fish oil than the n-3 PFUA from linseed oil indicates that different n-3 fatty acids possess different

potencies in regulating chicken immune cell proliferation. Similarly it has been reported that EPA from fish oil appears to be the most inhibitory among the n-3 PUFA on lymphocyte proliferation (Calder and Newsholme 1992a, b; Devi and Das, 1994; Das, 1994).

3.3.3. Content of Serum IgG and Yolk IgY

The concentration of IgG in serum and in egg yolk was impacted by dietary level of n-3 PUFA ($P < 0.05$; Fig. 3.3). The IgG concentration in serum from the laying hens fed the LO diet was significantly higher ($P < 0.05$) than that from the laying hens fed AO, SO or FO with no significant differences ($P > 0.05$) detected among the latter three groups. The hens fed SO had the lowest IgG concentration in the serum. Egg yolk IgY was lower ($P < 0.05$) by feeding the SO diet to laying hens compared with feeding the other three diets. No differences were observed in the concentration of yolk IgY among the hens fed AO, LO, and FO ($P > 0.05$).

To our knowledge, this is the first study on the effect of dietary fat source on total IgG in serum and IgY in egg yolk. We assumed that total IgG production would reflect the capacity that a chicken produces specific antibodies when exposed to pathogenic challenges. In accordance with our observation, enhanced serum antibody production were observed by Friedman and Sklan (1995) in broiler chicks fed relatively higher levels of n-3 PUFA (LNA) compared with feeding higher levels of n-6 PUFA (LA) when the chicks were challenged with bovine serum albumin. However, the results of present study is inconsistent with that reported by Fritsche et al. (1991a) that feeding chicks 7% (w/w) fish oil diet rather than 7% (w/w) linseed oil diet increased serum antibody titer against

sheep red blood cells. An increase in antibody production was also observed in rats when they were fed a 25% fish oil diet compared with those fed a 25% tallow diet (Pricket et al., 1982).

3.3.4. Lymphocyte Subset

N-3 fatty acids did not significantly alter the distribution of lymphocyte subset populations present in spleen and blood ($P > 0.05$; Tables 3.4 and 3.5). Similar results were obtained from other studies. Kelley et al. (1991) observed that flaxseed oil did not affect the number of $CD4^+$ and $CD8^+$, and also total T- and B-cells in human peripheral blood leukocytes. Feeding fish oil to rats did not change the proportion of $CD4^+$ and $CD8^+$ lymphocytes in spleen, thymus and lymph node, either in freshly prepared cells or in cells cultured in the presence of mitogen (Yaqoob et al., 1994a). Thus the alterations in lymphocyte proliferation and Ig formation in the current study could not be attributed to the modification of lymphocyte subset proportions. Different results have been reported by others. Shapiro et al. (1994) reported that n-3 PUFA supplementation significantly decreased the percentages of T-cells, $CD4^+$, $CD8^+$ in spleen of mice, while Huang et al. (1992) observed that mice fed the n-3 PUFA had a greater percentage of T-cells but not the percentage of B-cells. The reason causing such contradictory results is not clear. From the authors' viewpoint, it is likely due to differences in the animal model, immune status, duration of fatty acid supplementation as well as fatty acid manipulation including the amount and individual fatty acids of n-3 PUFA employed by different researchers.

3.4. Implication

The results of current study imply that the immune functions of laying hens could be modulated by dietary fatty acid manipulation towards healthier and higher production and economy. It has been reported that inflammatory response causes nutrient partitioning away from chicken growth and thus reduces growth and feed efficiency (Korver and Klasing 1997). Inflammatory response is a non-specific cell-mediated response. *In vitro* cell proliferation in response to polyclonal mitogens is one of the most commonly used procedures in assessing cell-mediated immune responses. Our current study showed that feeding hens with n-3 PUFA rich diets significantly reduced cell proliferation compared with feeding n-6 PUFA rich diet, suggesting that cell-mediated immune responses could be reduced by supplementing hens with n-3 PUFA. Indeed, indices of inflammatory response were lower in fish oil-fed birds, and the growth and feed efficiency in the chicks with inflammatory response were improved when they were fed a diet containing 2% fish oil (Korver and Klasing 1997).

Chickens produce immunoglobulins against antigens including bacteria, virus and foreign substances in host defense. Antibodies interfere with the adhesion of pathogens to the intestinal wall and neutralize partially, or completely, their colonization potential (Rutter and Jones 1973; Moon 1981). Once pathogens enter into blood, antibodies induce hypersensitivity, activation of complement system and antibody-dependent cell cytotoxicity to facilitate the clearance of the pathogens. The total IgG in serum is thought to be related to the potential of specific humoral immune responses. Immunoglobulins in avian blood are transferred to the yolk of eggs to give acquired immunity to the offspring (Rose and Orlans 1981). As such, the antibodies in eggs originated from the mother hen

are used to protect the newly hatched chick from a variety of infectious diseases. The increased serum IgG concentration (LO diet) and IgY in egg yolk (AO, LO, FO) by feeding laying hens a n-3 PUFA diet may provide a novel strategy to improve the health of hens and chicks, and thus poultry production. However, further experiments are required to make better understanding of the effects of amount and ratio of dietary n-6 to n-3 PUFA, as well as of individual n-6 and n-3 fatty acids on the immune responses and the performance of chickens.

Table 3.1. The composition of basic laying hen diet

Gradients	g/kg
Wheat	669
Soybean meal	162
Limestone ground	77
Dicalcium-phosphate	28
Sodium chloride	3.3
DL-Methionine	0.6
Layer premix[1]	5
Choline chloride premix[2]	5
Calculated composition	
ME (kcal/kg)	2800
Crude protein (%)	16.3

[1]Layer premix provides per kg diet: vitamin A, 12,000 IU; vitamin D3, 3,000 IU; vitamin E, 40 IU; vitamin K, 2.0 mg; pantothenic acid, 14.0 mg; riboflavin, 6.5 mg; folacin, 1.0 mg; niacin, 40.0 mg; thiamine, 3.3 mg; pyridoxine, 6.0 mg; vitamin B12, 0.02 mg; biotin, 0.2 mg; iodine, 0.5 mg; manganese, 75.0 mg; copper, 15.0 mg; zinc, 80.0 mg; selenium, 0.1 mg; iron, 100.0 mg.

[2]50 kg choline chloride premix contains choline chloride (60%) 1.7 kg, wheat shorts 48.3 kg.

Table 3.2. The fatty acid composition of the experimental diets

	Diet[1]			
	SO	AO	LO	FO
Fatty acid[2]	*% of total fatty acids*			
C16:0	9.8	21.1	16.3	18.7
C18:0	3.2	9.5	5.7	3.1
C16:1	0.3	2.3	0.3	7.5
C18:1	17.8	37.9	19.0	14.2
C18:2n-6	64.5	21.6	26.2	18.0
C20:4n-6	Nd[3]	0.14	nd	0.57
C18:3n-3	2.07	2.57	32.16	3.04
C20:5n-3	nd	0.17	nd	7.14
C22:5n-3	nd	0.06	nd	1.56
C22:6n-3	nd	0.24	nd	8.77
SFA	14.6	33.0	22.4	29.8
MUFA	18.8	41.2	19.2	26.0
PUFA	66.67	25.41	58.36	40.51
n-6 PUFA	64.60	22.34	26.20	19.75
n-3 PUFA	2.07	3.07	32.16	20.76
P/S	4.58	0.77	2.60	1.35
n-6/n-3	31.19	7.27	0.82	0.95

[1]SO, AO, LO and FO represent diets containing 5% (w/w) sunflower oil, animal oil, linseed oil, or menhaden fish oil.

[2]SFA, saturated fatty acids; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids; P/S, ratio of PUFA to SFA, n-6/n-3; ratio of n-6 to n-3 PUFA.

[3]Not detectable.

Table 3.3. Effect of dietary fat source on the fatty acid composition of laying hen splenocytes

	Diet[1]				
	SO	AO	LO	FO	SEM[3]
Fatty acids[2]	*% of total fatty acids*				
C16:0	21.43[b]	22.20[ab]	21.83[b]	23.68[a]	0.39
C16:1	0.71[b]	0.92[b]	0.88[b]	1.33[a]	0.08
C18:0	19.54	18.76	19.35	18.84	0.37
C18:1	14.08[b]	17.97[a]	17.01[ab]	17.95[a]	0.75
C18:2n-6	12.41[a]	7.20[c]	10.32[b]	7.51[c]	0.42
C18:3n-3	0.05[b]	0.11[b]	1.16[a]	0.18[b]	0.86
C20:4n-6	17.21[a]	17.73[a]	12.41[b]	7.72[c]	0.54
C20:5n-3	0.10[c]	0.19[c]	1.17[b]	3.39[a]	0.08
C22:4n-6	4.13[a]	3.78[a]	2.10[b]	1.05[c]	0.10
C22:5n-3	0.36[d]	0.70[c]	1.94[b]	2.76[a]	0.05
C22:6n-3	1.19[c]	2.42[b]	3.02[b]	7.57[a]	0.21
SFA	47.58	47.35	47.73	48.83	0.78
MUFA	15.21[b]	19.40[a]	18.48[ab]	19.88[a]	0.80
PUFA	37.21[a]	33.25[b]	33.79[b]	31.30[b]	0.63
n-6PUFA	35.51[a]	29.83[b]	26.49[c]	17.41[d]	0.59
n-3PUFA	1.70[d]	3.42[c]	7.30[b]	13.89[a]	0.25
P/S	0.71[a]	0.64[b]	0.64[b]	0.59[b]	0.02
n-6/n-3	20.99[a]	8.80[b]	3.65[c]	1.26[d]	0.40

[a-d]For each fatty acid, values that have common superscripts are not significantly different ($P \geq 0.05$). Values are means (n = 6).

[1]SO, AO, LO, and FO mean diets containing sunflower oil, animal oil, linseed oil or menhaden fish oil at 5% (w/w), respectively.

[2]SFA, saturated fatty acids; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids; P/S, ratio of PUFA to SFA; n-6/n-3, ratio of n-6 to n-3 PUFA.

[3]pooled SEM.

Table 3. 4. Effect of dietary fat source on single-label immune phenotypes in the splenocytes of laying hens

	Diet[1]			
	SO	AO	LO	FO
Lymphocytes[2]	*% of total spleen lymphocytes*			
$CD4^+$	22 ± 2	20 ± 2	23 ± 3	21 ± 1
$CD8^+$	49 ± 3	48 ± 3	47 ± 4	48 ± 4
Ig^+	25 ± 0.2	22 ± 2	26 ± 0.5	24 ± 2

Values are means ± SEM (n = 6). No significant differences were found for each phenotype among the four dietary treatments ($P \geq 0.05$).

[1]SO, AO, LO, and FO mean diets containing sunflower oil, animal oil, linseed oil or fish oil at 5% (w/w), respectively.

[2]$CD4^+$ and $CD8^+$ T-lymphocytes are recognized by CT-4 and CT-8 monoclonal antibodies, respectively, and Ig^+ cells are recognized by L-1 monoclonal antibodies.

Table 3. 5. Effect of dietary fat source on single-label immune phenotypes in the blood lymphocytes of laying hens

	Diet[1]			
	SO	AO	LO	FO
Lymphocytes[2]	*% of total spleen lymphocytes*			
$CD4^+$	16 ± 1	15 ± 1	16 ± 1	14 ± 0.4
$CD8^+$	45 ± 2	41 ± 1	44 ± 1	44 ± 4
IgM^+	13 ± 0.4	13 ± 1	14 ± 1	13 ± 1

Values are means ± SEM (n = 6). No significant differences were found in each phenotype among the four dietary treatments ($P \geq 0.05$).

[1]SO, AO, LO, and FO mean diets containing sunflower oil, animal oil, linseed oil or fish oil at 5% (w/w), respectively.

[2]$CD4^+$ and $CD8^+$ T-lymphocytes are recognized by CT-4 and CT-8 monoclonal antibodies, respectively, and IgM^+ cells are recognized by M-1monoclonal antibodies.

FIGURE 3. 1. Effects of dietary fat source on spleen lymphocyte proliferative response to polyclonal mitogen Con A in laying hens after 5 wk on experimental diets. The SO, AO, LO and FO represent diets containing sunflower oil, animal oil, linseed oil and fish oil at 5% (w/w), respectively. Stimulation index was calculated as (amount of [^{3}H]thymidine incorporated by stimulated cells – the amount of [^{3}H]thymidine incorporated by unstimulated cells)/amount of [^{3}H]thymidine incorporated by unstimulated cells. Bars are means ± SEM (n = 6). Bars that do not have common superscripts are significantly different ($P < 0.05$).

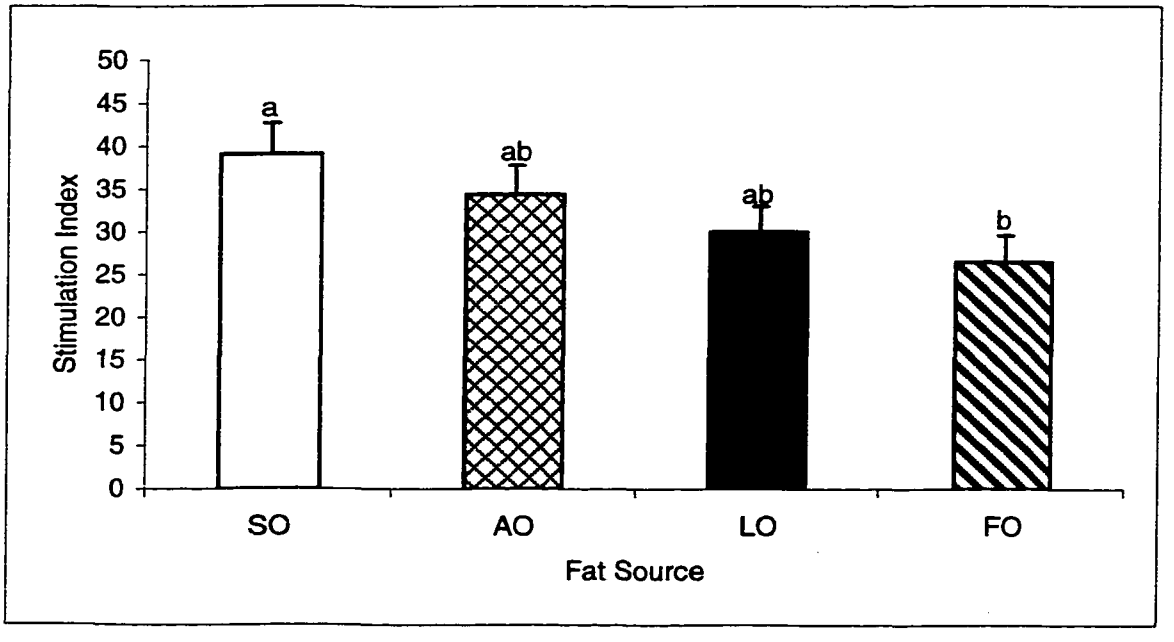


FIGURE 3. 2. Effects of dietary fat source on blood lymphocyte proliferative response to polyclonal mitogen Con A in the laying hens after 5 wk of feeding the experimental diets. The SO, AO, LO and FO mean diets containing sunflower oil, animal oil, linseed oil or fish oil at 5% (w/w). Stimulation index was calculated as (amount of [^{3}H]thymidine incorporated by stimulated cells – the amount of [^{3}H]thymidine incorporated by unstimulated cells)/amount of [^{3}H]thymidine incorporated by unstimulated cells. Bars are means ± SEM (n = 6). Bars that do not have common superscripts are significantly different ($P < 0.05$).

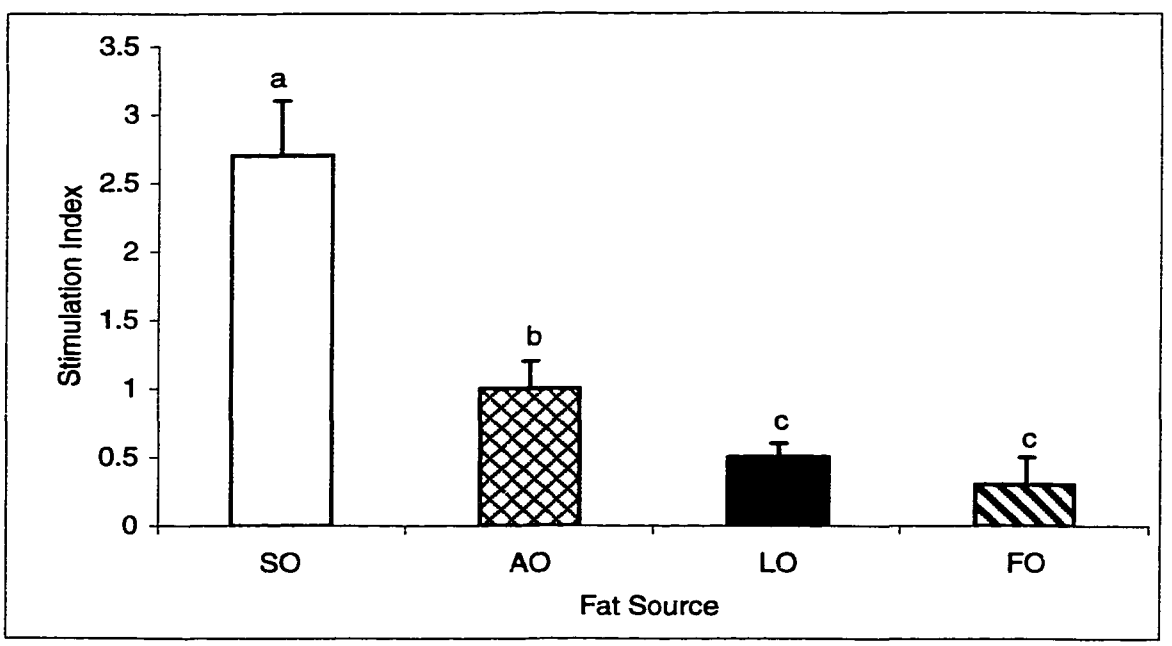
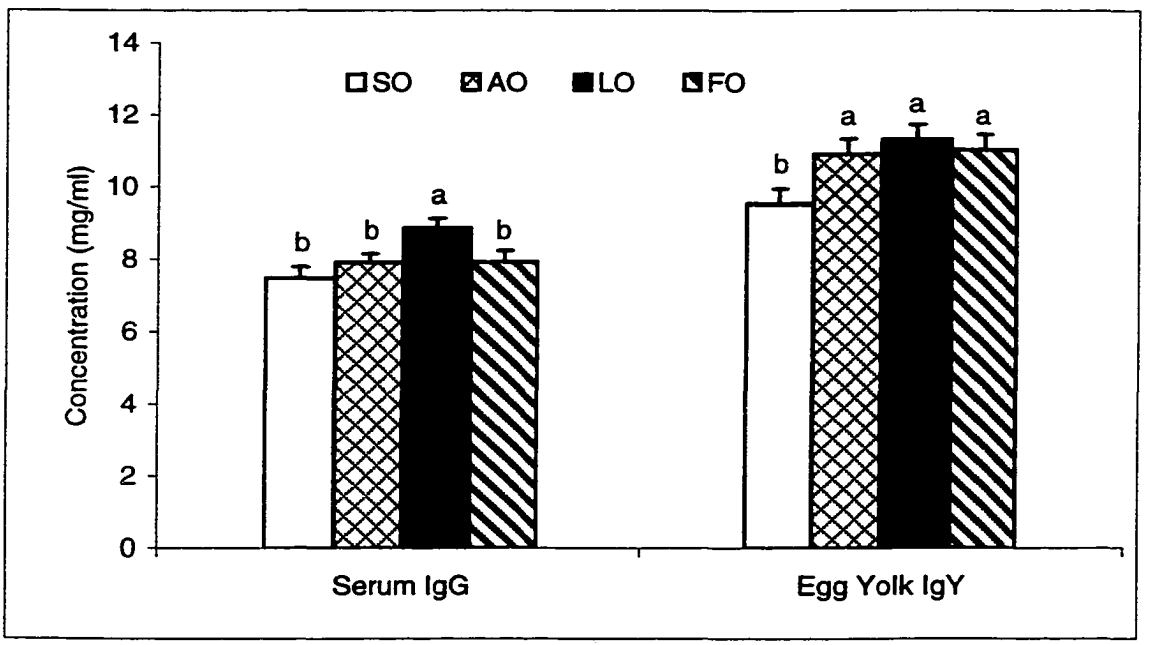


FIGURE 3. 3. Effects of dietary fat source on the concentration of serum IgG and egg yolk IgY of the laying hens after 5 wk of feeding different oil diets. The SO, AO, LO and FO mean diets containing sunflower oil, animal oil, linseed oil or fish oil at 5% (w/w). The IgG and IgY were measured by the method of radial immunodiffusion (RID). Bars are means ± SEM (n = 6). For each antibody, bars that do not have common superscripts are significantly different ($P < 0.05$).



4. EFFECT OF MATERNAL AND NEONATAL DIETARY FAT COMPOSITION ON THE FATTY ACID COMPOSITION OF IMMUNE TISSUES/CELLS AND IMMUNE TISSUE DEVELOPMENT IN CHICKS[2]

4.1. Introduction

The type and level of dietary lipids impact immune responses in humans and animals (Barone et al. 1989; Kinsella et al. 1990; Jeffery et al., 1996a,b,c,1997c; Calder, 1998a). Mainly concerned are polyunsaturated fatty acids (PUFA) such as LA and its longer-chain product, AA, and LNA and its longer-chain products, EPA, DPA and DHA acids. AA and EPA serve as the precursors of eicosanoids, such as prostaglandins and leukotrienes, which are recognized as important modulators of cellular and humoral immunities (Goldyne and Stobo, 1981; Stenson and Parker, 1982). However, the eicosanoids derived from EPA have different functions or similar functions but less potency from that formed from AA. In addition, n-6 and n-3 PUFA differently affect cell membrane functions, such as membrane fluidity, receptor expression and activity, signal transduction, ion channel activity, expression of surface adhesive molecules, etc., so as to affect immune responses (Calder, 1997b).

A number of investigations on the effect of dietary fat on the fatty acid composition of immune tissues or cells have been accomplished in mammals (Caler, 1997b). The chicken immune system is, to a certain extent, different from mammals. For

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example, the bursa of Fabricius is unique in avian. Fritsche et al. (1991b) reported that the dietary fat source significantly modifies the fatty acid composition of chicken thymus, bursa and splenocytes. However, different immune tissues contain different immune cell types and may perform, to certain extent, different immune functions. It is not clear whether dietary fatty acids modify the fatty acid composition of different immune tissues or cells from different origins in the same pattern and potency. In addition, for a long term (two generations), dietary fatty acids may influence the immune tissue development and immune cell function in life. To manipulate dietary fatty acid composition for improving the immune capacity, it is necessary to know the effect of dietary fatty acids on the immune tissue fatty acid profile and immune tissue development with different durations of feeding of the experimental diets. The objective of the present study was to investigate the effect of dietary lipids on the fatty acid composition of the immune tissues (thymus, spleen, and bursa) and cells (blood and spleen lymphocytes), and on the immune tissue development of chicks from the hens fed different dietary oils and continued to be fed the same type of diets following hatching.

4.2. Materials and Methods

4.2.1. Animals and Diets

Single Comb White Leghorn laying hens were fed a wheat-soybean meal basal diet with addition of 5 % (w/w) of SO, AO, LO, or FO (Tables 2.1 and 2.2; Chapter 3). After 6 wk of feeding, fertilized eggs were collected and incubated. Upon hatching, thirty healthy chicks in each group were selected and housed in separate floor pens with

free access to feed and water. The chicks in each group were given the same type of diet as the maternal. The fatty acid composition of the diet analyzed by gas chromatography (Cherian and Sim, 1992) was summarized in Table 4.1. The experiment was approved by the Faculty of Agriculture and Forestry Animal Policy and Welfare Committee, University of Alberta and was conducted in accordance with the Canadian Council on Animal Care guidelines.

4.2.2 Sample Preparation

At the age of 4 wk, six chicks from each group were selected and anesthetized by CO_2 inhalation. Blood (10 mL) was collected by cardiac puncture using a syringe into a 15-mL tube with EDTA and placed on ice. Immediately, the chicks were killed by cervical dislocation, the spleens were removed into 15-mL tubes containing 10 mL CCM per tube, and left on ice. The blood was diluted by 1:2 with CCM (Sigma), mixed gently, laid carefully on the lymphocyte separation medium, density 1.07-1.08 (Sigma), and centrifuged at 2,200 rpm, at 20 °C for 25 min. The cells at the interface were collected and washed twice with CCM. Spleen lymphocytes were obtained as described by Field (1995). Next day, another six chicks from each group were killed by cervical dislocation, and the thymus, spleen and bursa of Fabricius (bursa) were removed and weighed. The isolated splenocytes and blood lymphocytes and the immune tissues were kept at –20 °C before fatty acid analysis. At the age of 8 wk, another six chicks from each group were selected and killed by cervical dislocation and the three immune tissues were removed, weighed, and frozen at –20 °C before fatty acid analysis.

4.2.3. Fatty Acid Analyses of Immune Tissues and Cells

For the immune tissues, about 0.2 g of each immune tissue was homogenized in 15 mL of Folch-solution (chloroform: methanol, 2:1, v/v) using a polytron homogenizer (Virtis Company, Inc., Gardiner, NY 12525). Another 5 mL of Folch-solution was used to rinse the polytron head and was added to the homogenate. The homogenate was kept overnight, then 4 mL of 0.88% NaCl solution was added, mixed, and left to separate into phases. For lymphocytes, 9 mL of the Folch-solution was added and shaken well. After 1 h, 2 mL of 0.88% NaCl solution was added, mixed, and left for separation of the phases. The bottom layer containing the lipid extract was collected into a 15 mL tube and dried under a nitrogen atmosphere. Approximately 4 mL of methylating reagents (boron trifluoride: methanol: hexane, 35:45:20, v/v/v) was added and refluxed in a water bath for 1 h at 95 °C. After cooling, 5 mL of water and 3 mL of hexane were added and shaken well to extract the fatty acid methyl esters. The fatty acid methyl esters were separated and quantified using an automated gas chromatograph equipped with an autosampler and flame ionization detector (Model 3600, Varian Associates, Inc., Palo Alto, CA 94304), using a 30 m x 0.25mm i.d. fused silica capillary (Supelco Canada, Ltd., Oakville, ON, L6H 6J8) as described by Cherian and Sim (1992). A Shimadzu EZChrom (Shimadzu Scientific Instruments, Inc., 7120 Riverwood Drive, Columbia, MD 21406) laboratory data integration system was used to integrate peak areas.

4.2.4. Statistical Analysis

ANOVA was used to analyze the main effects of dietary lipids on the fatty acid composition of immune tissues or cells. Significant differences among the four treatments were tested using Tukey's studentized range test. The significance level was set at $P < 0.05$. Computations were done using general linear model procedure of SAS software (SAS Institute Inc., 1990).

4.3. Results and Discussion

In mammals, it has been well documented that n-3 PUFA at high levels suppress lymphocyte functions such as proliferation (Wu et al., 1996; Jeffery et al., 1997c), cytokine production (Jolly et al., 1997) and natural killer cell activity (Yaqoob et al., 1994b; Sanderson et al., 1995b). However, lower, more physiologic, levels of n-3 PUFA have been found to be stimulatory (Robinson and Field, 1998). Many factors have been found to be involved in the regulation of immune functions such as eicosanoid production, cytokine formation, expression of cell surface molecules and cell membrane enzyme activity (Calder, 1998a, b; Jolly et al., 1997; Wallace et al., 1999a). These factor can be influenced fatty acid composition of cell membrane (Calder 1997b; 1998a,b). Because of the differences existed between chickens and mammals in the immune system, it is important to know the effect of dietary fat on the fatty acid composition of chicken immune tissues and cells in order to manipulate the immune function in chicken.

The effect of dietary fat source on the fatty acid composition of thymus, spleen and bursa is shown in Tables 4.2 to 4.4. The levels of each fatty acid in the SFA were similar among the chicks fed SO, AO and LO regardless of two-fold differences among the diets. Feeding of fish oil resulted in a higher ($P < 0.05$) incorporation of total SFA in the spleen, bursa, and thymus. This is different from that reported by Fritsche et al. (1991b) who found that feeding chicks of 7% (w/w) fish oil only elevated total SFA in thymus rather than in bursa compared with feeding 7% (w/w) of linseed oil, corn oil, canola oil, or lard. The levels of individual fatty acids of MUFA and PUFA were dramatically modified by dietary fatty acids, with the total MUFA and PUFA being changed reciprocally. Oleic acid (C18:1) was the predominant MUFA in the three immune tissues, and its relative content paralleled that in the diet. Large differences existed among the three tissues as an evidence of over two-fold as much MUFA in bursa (29 - 40%) as that in spleen (10 - 18%).

The relative content of LA and LNA in the three immune tissues also paralleled those in the diet, but were greatly reduced compared with that in the diets. A very little LNA was incorporated despite 37% dietary level of this fatty acid (LO). The fats fed contained little or no preformed AA, yet levels of the fatty acid in the immune tissues constituted up to 15% of total lipids. Second, EPA and DPA were seen in the immune tissues of chicks fed SO and AO, and significant elevations of these fatty acids were shown in the chicks fed LO. This suggests that chickens possess certain capacity to synthesize the longer-chain PUFA from their precursors, which may require desaturase activity (Fritsche et al., 1991b). Although there was three-fold as much LA in the SO as in the AO, the levels of its product AA in the immune tissues were similar between the

two groups of chicks fed these diets, indicating that chick has regulated ability to convert LA to AA or the incorporation of AA into the immune tissues is a saturation process. Similar phenomenon was observed in other tissues of the chicken (Cherian and Sim, 1991, 1992, 1995). This result may also implicate that chick has a certain demand for AA, and that mechanisms exist to control AA metabolism and incorporation.

The incorporation of longer-chain n-3 PUFA into the immune tissues has been studied (Kinsella et al., 1990, Calder, 1997b, 1998a,b). Our data showed that the percent composition of n-3 PUFA was dependent on the source of n-3 fatty acids which is similar with that reported by Fritsche et al. (1991b). Feeding the preformed n-3 fatty acids (FO) resulted in a higher ($P < 0.05$) elevation of these fatty acids in the immune tissues than feeding the parent LNA (LO). Similar results have been reported in the immune tissues of rat or mice (Croft et al., 1984; Fritsche and Johnston, 1990) and in other chicken tissues (Cherian and Sim, 1992). The capacity of the chick to convert the parent LNA to its longer-chain products, EPA, DPA, and DHA is limited. For maximized incorporation of longer-chain n-3 products, the supplementation of the preformed longer-chain PUFA is required. The elevation of n-3 PUFA by feeding high levels of n-3 PUFA resulted in a significant decrease ($P < 0.05$) of n-6 PUFA, mainly AA and C22:4n-6. As with other chicken tissues, such as liver, grain, and egg yolk (Cherian and Sim, 1991, 1992), a strong competition exists between n-3 and n-6 PUFA in their metabolism and incorporation into the immune tissues of chicks. Similar results have been reported in mammals (Carbonell et al., 1997; Babu et al., 1997; Kuratko and Becker, 1998). LNA competes with LA for the delta-6 desaturase enzyme, thus limits the synthesis of AA (Brenner et al., 1969).

On the other hand, significant tissue-specific differences were observed in the composition of each PUFA. For example, the thymus had a higher proportion of LA, but lower level of AA, and similar level of each longer-chain n-3 PUFA with spleen. Bursa had the lowest proportion of each n-3 and n-6 fatty acid. Immune organ contains different types of tissues such as connective tissue, blood cells and immune cells. The immune cells are grouped into several classes and subclasses, which are not evenly distributed across various immune tissues. Typically, thymus contains mainly T-lymphocytes, and bursa contains mainly B-lymphocytes. The differences of immune cell types in different immune organs could be one of factors contributing to the differences in the fatty acid composition among the immune tissues. Different types of immune cells undergo differences in the diet-induced modification of the fatty acid composition (Jeffery, 1997a), which might result from different patterns of phospholipids present in different types of immune cells. It has been reported that dietary fatty acid manipulation has different effects on the fatty acid composition of different phospholipid classes and these effects were unique for each phospholipid class (Anderson, 1970; Poovaiah et al., 1976; Conroy et al., 1986; Lokesh et al., 1986a; Aukema and Holub, 1989; Berger and German, 1990; Chapkin and Carmichael, 1990; Huang and Fritsche, 1992). The tissue-specific differences in PUFA content may also due to the differences in the desaturase activity or acyl-coenzyme A in different tissues (Willis, 1981).

As with the immune tissues, the lymphocyte fatty acid composition was significantly modified by feeding different oil diets (Tables 4.5 and 4.6). It is worth noting that large differences were observed in the relative contents of lymphocyte fatty acids between the two origins. The spleen lymphocytes incorporated significantly higher

LA and n-3 PUFA than blood lymphocytes. The amount of AA was higher in spleen lymphocytes than blood lymphocytes when the relative content was over 8% (w/w) (i.e., the chicks fed SO and AO). Thus, the total PUFA concentration was lower in blood lymphocytes (25 to 31%) than in spleen lymphocytes (34 to 41%), although the pattern reflected that in the diets. LNA was a minor component in both blood and spleen lymphocytes. EPA, DPA, and DHA were the major n-3 PUFA, suggesting a strong demand for the longer-chain n-3 fatty acids rather than the parent LNA in chicken lymphocytes. The ratio of n-6 to n-3 PUFA was doubled ($P < 0.05$) in blood lymphocytes compared with spleen lymphocytes. This effect might be due to the difference of the proportions of different immune cells between these two origins of lymphocytes.

This is the first report to provide a detailed comparison of the differences in the fatty acid composition of the same class of immune cells (lymphocytes) between different origins in chickens. To date, the mechanisms to explain this have not been clearly elucidated. The marked heterogeneity of responses to dietary fatty acids in different origins of lymphocytes may be due to the differences in tissue affinity of the fatty acid (Fritsche et al., 1991b) or differences of desaturase activity in different tissues (Willis, 1981).

Whether the fatty acid composition of an immune tissue reflects that in the immune cells of the same tissue is not often reported. Comparing the fatty acid composition of spleen with the spleen lymphocytes (Tables 4.2 and 4.5), we found that spleen lymphocytes had higher C18:0, lower C16:1 and C18:1 than the whole spleen tissue, with the relative contents of other fatty acids were similar. When a high level of n-6 PUFA was supplied in the diet, spleen lymphocytes incorporated a higher amount of

AA but a lower amount of its precursor, LA than the spleen. The total MUFA was higher, but total SFA was lower in the spleen than the spleen lymphocytes. These results suggest that when spleen is used to monitor the diet-induced alterations of fatty acid composition in the spleen lymphocytes, biases exist in some fatty acids.

Tissue specific differences in n-6 and n-3 fatty acids may have important immunological consequences with regard to the role that fatty acids play through the production of eicosanoids and cytokines, or direct involvement in the regulation of the immune cell activity. Immune tissue development is the base of immune system functionality. The increase of PUFA (n-6 or n-3) in chick diets consistently and significantly ($P < 0.05$) promoted the growth of thymus, spleen and bursa before 4 wk compared to animal oil diet. Interestingly, the growth promoting effect of high PUFA diets on immune tissues stopped ($P > 0.05$) during the period from 4 to 8 wk. At the age of 8 wk, the bursa weight of chicks was decreased with the increase of dietary PUFA and significantly decreased ($P < 0.05$) with the increase of n-3 PUFA, especially EPA and DHA from fish oil compared with the moderate level of PUFA (AO). Anatomically, the bursa in the chicks fed 5% (w/w) fish oil diet became flat and almost withered at the age of 8 wk. It is not known whether n-3 PUFA, in particular, n-3 PUFA from fish oil promoted chick bursa growth and maturity, and then started withering early or inhibited growth or even damaged the bursa in the course of 4 to 8 wk of age.

TABLE 4. 1. Fatty acid composition of chick diets

	Diet [2]			
	AO	SO	LO	FO
Fatty acid[1]	*% of total fatty acids*			
C16:0	22.16	10.10	9.73	20.78
C18:0	10.26	3.60	3.76	3.62
C16:1	2.34	0.14	0.15	7.51
C18:1	36.86	17.92	17.03	16.56
C18:2n-6	22.5	64.60	30.76	21.02
C20:4n-6	0.11			0.51
C18:3n-3	2.42	2.21	37.51	3.39
C20:5n-3				7.27
C22:5n-3				1.57
C22:6n-3				7.41
SFA	34.95	15.06	14.24	32.43
MUFA	40.11	18.33	17.49	26.01
PUFA	24.94	66.01	68.27	41.56
n-6 PUFA	22.52	64.40	30.76	21.95
n-3 PUFA	2.42	2.21	37.51	19.61
PUFA:SFA	0.71	4.42	4.79	1.28
n-6:n-3 PUFA	9.32	29.09	0.82	1.12

[1]SFA, saturated fatty acids; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids.

[2]AO, SO, LO, and FO are diets with 5% (w/w) of animal tallow, sunflower oil, flaxseed oil and menhaden fish oil, respectively.

TABLE 4. 2. Fatty acid composition of chick spleen at the age of 8 wk

	Diet [2]			
	AO	SO	LO	FO
Fatty acid[1]	*% of total fatty acids*			
C16:0	23.65[a]	20.53[b]	21.60[b]	24.55[a]
C18:0	16.43[b]	18.02[a]	18.66[a]	17.53[ab]
SFA	46.54[b]	45.58[b]	47.02[b]	49.71[a]
C16:1	1.20[a]	0.38[b]	0.45[b]	1.55[a]
C18:1	17.98[a]	10.60[c]	12.41[bc]	13.09[b]
MUFA	20.59[a]	11.85[c]	13.88[bc]	15.71[b]
C18:2n-6	8.78[c]	19.52[a]	14.47[b]	8.69[c]
C20:4n-6	14.13[a]	13.77[a]	8.28[b]	5.13[c]
C22:4n-6	3.88[b]	4.90[a]	1.55[c]	0.86[d]
C18:3n-3	0.18[b]	0.14[b]	3.09[a]	0.27[b]
C20:5n-3	0.31[c]	0.00[c]	2.90[b]	6.69[a]
C22:5n-3	1.13[c]	0.48[d]	3.64[b]	4.32[a]
C22:6n-3	1.64[c]	0.61[d]	2.24[b]	7.08[a]
PUFA	31.89[d]	41.89[a]	38.16[b]	34.36[c]
n-3PUFA	3.26[c]	1.24[d]	11.87[b]	18.36[a]
n-6PUFA	28.40[b]	40.66[a]	26.29[c]	16.00[d]
PUFA/SFA	0.69[c]	0.92[a]	0.81[b]	0.69[c]
n-6/n-3PUFA	8.75[b]	33.02[a]	2.22[c]	0.87[c]

[a-d] For each fatty acid, the values with different superscripts are significantly different ($P < 0.05$). Data are means (n = 6).

[1]SFA, saturated fatty acids; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids.

[2] AO, SO, LO, and FO are diets with 5% (w/w) of animal tallow, sunflower oil, flaxseed oil and menhaden fish oil, respectively.

TABLE 4. 3. Fatty acid composition of chick thymus at the age of 8 wk

	Diet[2]			
	AO	SO	LO	FO
Fatty Acid[1]	*% of total fatty acids*			
C16:0	24.88[b]	20.98[d]	22.99[c]	27.15[a]
C18:0	13.59	15.13	14.58	14.45
SFA	41.34[b]	39.04[b]	40.20[b]	45.64[a]
C16:1	1.63[b]	0.84[d]	1.22[c]	3.62[a]
C18:1	27.84[a]	16.17[c]	18.08[bc]	19.72[b]
MUFA	32.29[a]	18.20[c]	19.98[c]	24.43[b]
C18:2n-6	9.05[c]	24.60[a]	15.16[b]	8.87[c]
C20:4n-6	8.19[a]	8.79[a]	3.52[b]	2.30[c]
C22:4n-6	2.29[b]	3.29[a]	0.79[c]	0.45[c]
C18:3n-3	0.29[b]	0.32[b]	8.32[a]	0.64[b]
C20:5n-3	0.51[c]	0.10[c]	3.05[b]	6.22[a]
C22:5n-3	1.21[c]	0.59[d]	3.85[b]	4.51[a]
C22:6n-3	1.66[b]	0.60[c]	1.81[b]	6.29[a]
PUFA	26.37[b]	42.38[a]	39.84[a]	29.95[b]
n-6PUFA	22.18[b]	40.66[a]	22.10[b]	13.06[c]
n-3PUFA	3.78[b]	1.72[c]	17.83[a]	16.75[a]
PUFA/SFA	0.64[b]	1.10[a]	0.99[a]	0.66[b]
n-6/n-3PUFA	5.89[b]	23.75[a]	1.24[c]	0.78[c]

[a-d]For each fatty acid, the values with different superscripts are significantly different ($P < 0.05$). Data are means (n = 6).

[1]SFA, saturated fatty acids; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids.

[2] AO, SO, LO, and FO are diets with 5% (w/w) of animal tallow, sunflower oil, flaxseed oil and menhaden fish oil, respectively.

TABLE 4. 4. Fatty acid composition of chick bursa at the age of 8 wk

	Diet [2]			
	AO	SO	LO	FO
Fatty Acid[1]	*% of total fatty acids*			
C16:0	22.10	22.19	21.81	22.77
C18:0	10.77	11.22	11.02	11.05
SFA	35.96^{b}	36.19^{ab}	35.69^{b}	38.54^{a}
C16:1	4.78^{ab}	3.68^{c}	4.17^{bc}	5.16^{a}
C18:1	39.99^{a}	32.46^{bc}	34.58^{b}	29.03^{c}
MUFA	46.73^{a}	37.36^{b}	39.94^{b}	35.43^{b}
C18:2n-6	7.62^{b}	15.65^{a}	9.40^{b}	8.75^{b}
C20:4n-6	4.60^{b}	6.18^{a}	2.16^{c}	2.74^{c}
C22:4n-6	0.62^{b}	1.27^{a}	0.50^{b}	0.60^{b}
C18:3n-3	0.33^{b}	0.28^{b}	4.64^{a}	0.78^{b}
C20:5n-3	0.35^{c}	0.16^{c}	2.28^{b}	5.07^{a}
C22:5n-3	0.96^{b}	0.44^{c}	3.19^{a}	2.88^{a}
C22:6n-3	1.23^{b}	0.45^{c}	1.29^{b}	4.28^{a}
PUFA	17.32^{b}	26.46^{a}	24.37^{a}	26.04^{a}
n-6PUFA	14.15^{b}	25.13^{a}	12.96^{b}	13.02^{b}
n-3PUFA	2.88^{c}	1.32^{c}	11.40^{b}	13.02^{a}
PUFA/SFA	0.48^{b}	0.73^{a}	0.68^{a}	0.68^{a}
n-6/n-3PUFA	4.95^{b}	20.36^{a}	1.14^{c}	1.00^{c}

[a-c]For each fatty acid, the values with different superscripts are significantly different ($P < 0.05$). Data are means (n = 6).

[1]SFA, saturated fatty acids; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids.

[2] AO, SO, LO, and FO are diets with 5% (w/w) of animal tallow, sunflower oil, flaxseed oil and menhaden fish oil, respectively.

TABLE 4. 5. Fatty acid composition of chick spleen lymphocytes at the age of 4 wk

	Diet [2]			
	AO	SO	LO	FO
Fatty Acid[1]	*% of total fatty acids*			
C16:0	22.98^{b}	21.83^{bc}	20.79^{c}	25.08^{a}
C18:0	21.11	19.94	20.56	21.37
SFA	50.30^{b}	48.79^{b}	48.09^{b}	53.77^{a}
C16:1	0.59^{b}	0.24^{c}	0.32^{c}	1.00^{a}
C18:1	13.94^{a}	8.91^{c}	10.83^{b}	9.68^{bc}
MUFA	15.51^{a}	9.80^{c}	11.81^{b}	11.54^{b}
C18:2n-6	8.06^{b}	16.07^{a}	15.58^{a}	9.12^{b}
C20:4n-6	17.30^{a}	15.78^{a}	8.78^{b}	5.29^{c}
C22:4n-6	3.76^{b}	5.02^{a}	1.44^{c}	0.75^{d}
C18:3n-3	0.00^{b}	0.04^{b}	2.88^{a}	0.20^{b}
C20:5n-3	0.30^{c}	0.05^{c}	3.32^{b}	7.11^{a}
C22:5n-3	1.23^{b}	0.58^{c}	3.85^{a}	4.07^{a}
C22:6n-3	1.83^{b}	0.61^{c}	1.89^{b}	7.10^{a}
PUFA	34.22^{b}	40.88^{a}	39.76^{a}	34.91^{b}
n-6PUFA	30.70^{b}	39.60^{a}	27.83^{c}	16.43^{d}
n-3PUFA	3.36^{c}	1.28^{d}	11.93^{b}	18.48^{a}
PUFA/SFA	0.68^{b}	0.84^{a}	0.83^{a}	0.65^{b}
n-6/n-3PUFA	9.18^{b}	31.10^{a}	2.35^{c}	0.89^{c}

[a-d]For each fatty acid, the values with different superscripts are significantly different ($P < 0.05$). Data are means (n = 6).

[1]SFA, saturated fatty acids; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids.

[2]AO, SO, LO, and FO are diets with 5% (w/w) of animal tallow, sunflower oil, flaxseed oil and menhaden fish oil, respectively.

TABLE 4. 6. Fatty acid composition of chick blood lymphocytes at the age of 4 wk

	Diet [2]			
	AO	SO	LO	FO
Fatty Acid[1]	*% of total fatty acids*			
C16:0	22.03[a]	20.54[ab]	20.05[b]	22.14[a]
C18:0	27.50	28.18	30.13	28.64
SFA	58.91	58.65	58.94	61.33
C16:1	0.49[b]	0.26[c]	0.30[c]	0.90[a]
C18:1	11.89[a]	8.10[b]	10.85[a]	10.71[a]
MUFA	14.14[a]	9.37[c]	12.02[b]	13.12[ab]
C18:2n-6	5.46[d]	9.93[b]	12.23[a]	7.72[c]
C20:4n-6	14.28[a]	13.32[a]	8.58[b]	5.52[c]
C22:4n-6	3.95[b]	5.13[a]	1.05[c]	0.77[c]
C18:3n-3	<0.01[b]	<0.01[b]	1.29[a]	<0.01[b]
C20:5n-3	0.12[c]	0.00[c]	1.28[b]	2.64[a]
C22:5n-3	0.45[c]	0.21[c]	1.79[b]	2.97[a]
C22:6n-3	0.57[b]	0.26[b]	0.76[b]	4.51[a]
PUFA	26.36[b]	30.90[a]	28.71[ab]	25.43[b]
n-6PUFA	25.09[b]	30.43[a]	23.59[b]	15.31[c]
n-3PUFA	1.15[c]	0.47[c]	5.12[b]	10.13[a]
PUFA/SFA	0.45	0.53	0.49	0.42
n-6/n-3PUFA	21.86[b]	66.25[a]	4.65[c]	1.52[c]

[a-c] For each fatty acid, the values with different superscripts are significantly different ($P < 0.05$). Data represent the means (n = 6).

[1]SFA, saturated fatty acids; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids.

[2]AO, SO, LO, and FO are diets with 5% (w/w) of animal tallow, sunflower oil, flaxseed oil and menhaden fish oil, respectively.

TABLE 4. 7. Effect of dietary fat source on the relative percentage of immune tissue weight to the body weight in chicks

	4 wk				8 wk			
Diet[1]	BW[2]	Thymus	Spleen	Bursa	BW	Thymus	Spleen	Bursa
		% of body weight				*% of body weight*		
SO	256 ± 14	7.8 ± 0.2[a]	2.9 ± 0.3[a]	5.6 ± 0.4[a]	820 ± 38	8.8 ± 0.6	4.1 ± 0.3	4.9 ± 0.2[ab]
AO	256 ± 14	4.8 ± 0.6[b]	1.7 ± 0.1[b]	3.8 ± 0.3[b]	831 ± 47	7.6 ± 0.7	4.3 ± 0.4	5.7 ± 0.5[a]
LO	238 ± 19	6.4 ± 0.5[a]	2.4 ± 0.1[a]	5.5 ± 0.2[a]	831 ± 28	7.6 ± 0.7	3.5 ± 0.3	4.3 ± 0.3[b]
FO	262 ± 18	6.8 ± 0.7[a]	2.9 ± 0.2[a]	5.2 ± 0.3[a]	786 ± 36	7.8 ± 0.3	4.2 ± 0.1	2.0 ± 0.2[c]

[a-c] For each tissue, values that do not have common superscripts are significantly different ($P<0.05$). Each value represents mean ± SEM (n = 6).

[1]SO, AO, LO and FO represent diets containing 5% (w/w) of sunflower oil, animal oil mixture, linseed oil and fish oil, respectively.

[2]Body weight (g).

5. EFFECT OF MATERNAL AND NEONATAL DIETARY PUFA ON THE LYMPHOCYTE SUBSET PROPORTION AND PROLIFERATION AND SERUM IGG CONCENTRATION OF CHICKS[3]

5.1. Introduction

In recent years there has been increased interest in the effects of PUFA on the immune responses of animals and humans. It has been reported that feeding rats or mice high levels of n-3 PUFA, EPA and DHA resulted in marked suppression of *in vitro* spleen, thymus, lymph node and peripheral blood lymphocyte proliferation (Alexander and Smythe, 1988; Yaqoob et al., 1994a; Sanderson et al., 1995b; Yaqoob and Calder, 1995b). Feeding rats or mice high levels of n-3 PUFA, LNA also suppressed *in vitro* lymphocyte proliferation (Marshall and Johnston, 1985; Jeffery et al., 1996b). Fritsche et al. (1991a) reported that n-3 PUFA, LNA or EPA and DHA significantly suppressed *in vitro* spleen lymphocyte proliferation in chicks.

Humoral immune response is another important aspect of the immunity and can be assessed by measuring antibody production and activity. Dietary supplementation of n-3 PUFA derived from fish oil decreased antibody production in rats (Prickett et al., 1984), mice (Atkinson and Maisey, 1995), and humans (Virella et al., 1991). It has also been reported that n-3 PUFA from linseed oil or fish oil had no effect on antibody production in rabbits (Kelley et al., 1988) and rats (Kim and Lee, 1992). However,

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[3]A version of this chapter has been published. Wang, Y. W., Field, C. J., and Sim, J. S., 2000. Poultry Science 79: 1741-1748.

Prickett et al. (1982) reported the production of IgG and IgE to alvobumin was enhanced when rats were fed high levels of n-3 PUFA (EPA and DHA) compared with those fed high levels of saturated fatty acids. Yoshino and Ellis (1989) reported that the mouse serum hemaegglutinin titer was significantly higher in the group fed a diet with the n-6 (LA) to n-3 PUFA (LNA) ratio of 0.25 than in the group fed a diet with 2.78 or 100 of n-6 to n-3 PUFA ratio. In chickens, feeding n-3 PUFA from fish oil produced more antibodies to sheep red blood cells than feeding saturated fat (lard) and n-6 PUFA rich oil (maize oil; Fritsche et al., 1991a). Antibody production developed more rapidly, reached a higher level and was more persistent in the chicks fed lower levels of n-6 to n-3 PUFA (LA to LNA) ratio (Friedman and Sklan, 1995).

Lymphocytes, with several phenotypes, play vital roles in determining an immune response. Alterations of lymphocyte subset proportion could change the functions of the immune system (Robinson and Field, 1998). Flaxseed oil (rich in LNA) supplementation did not affect the proportion of $CD4^+$, $CD8^+$, $CD3^+$ and B-cells in human peritoneal blood (Kelley et al., 1991). Fish oil feeding did not change the proportion of $CD4^+$ or $CD8^+$ lymphocytes in spleen, thymus and lymph nodes in rats (Yaqoob et al., 1994a). However, consumption of a low fat, low cholesterol, n-3 PUFA rich diet resulted in a lower proportion of $CD4^+$ and a higher proportion of $CD8^+$ peripheral blood lymphocytes in humans, with the proportion of $CD3^+$ cells (total T-cells) unaffected (Meydani et al., 1993). Huang et al. (1992) observed that dietary fat did not affect total yield or percentage of B-cells or macrophages, but n-3 PUFA feeding increased the percentage of T-cells in noninfected mice. In *Listeria* monocytogen- infected mice, the n-3 PUFA

feeding produced the highest percentage of B-cells and the lowest percentage of T-cells and macrophages in the peritoneum (Huang et al., 1992).

The chicken immune system is somewhat different from mammals such as rats and mice. The bursa is unique in birds and has been used to study B-lymphocyte development and functional maturation. Both the thymus and bursa wither when a chicken matures. Thereafter, the immune responses of the birds rely on the immune cells in spleen and peripheral lymph nodes, mucousal associated lymphoid tissue and gut associated lymphoid tissue. The antibodies transferred from egg yolk provide the only immune protection of the embryo and newly hatched chicks against various pathogens (passive immunity). The contradictory results obtained from mammalian studies on the effects of dietary n-6 and n-3 PUFA on antibody production and immune cell subset populations might be due to different species, basal diet, fat amount, ratio of n-6 to n-3 PUFA and different fatty acids of n-3 PUFA involved in various studies. Research on the effects of dietary fatty acids on chicken immunity is relatively low. Little is known about the effects of dietary n-3 PUFA on total IgG production and immune cell subset proportions. However, immune responses have been found to impact chicken nutrient utilization and performance (Klasing and Korver, 1997). Modulating the immune status of chickens may produce beneficial effects and provide a new avenue in improving poultry production. For example, the reduction of the suppressive effect of inflammation by adding fish oil to chicken diets improved the growth and feed efficiency (Korver and Klasing, 1997). Therefore, it is important to know the effects and mechanisms of dietary fatty acids on chicken immune responses. Our previous study showed that the fatty acid composition of chick immune tissues or cells could be modified by feeding hens and the

progeny with different oil diets. As a result, the immune tissue development was significantly affected. Continuing from the previous study, this experiment was designed to examine the effects of different dietary ratios of n-6 to n-3 PUFA and different n-3 fatty acids in maternal and neonatal diets on chick immune responses including *in vitro* lymphocyte proliferation, serum IgG concentration, and lymphocyte subset proportion.

5.2. Materials and Methods

5.2.1. Animals and Diets

The experiment was reviewed and approved by the Faculty of Agriculture and Forestry Animal Policy and Welfare Committee, University of Alberta, and was conducted in accordance with the Canadian Council on Animal Care guidelines. One-day-old chick pullets were used in this study with four dietary treatments of 30 chicks each. The chicks of each group were the progeny of Single Comb White Leghorn laying hens fed, for 6 wk, a wheat-soybean basal diet with addition of 5% (w/w) of SO, AO, LO or FO (Tables 2.1 and 2.2). The chicks were given the same kind of diets as the maternal upon hatching until 8 wk of age. The chick diets were formulated to meet the nutrient requirements for early growing chicks (Table.4.1; National Research Council, 1994). The fatty acid compositions of diets were analyzed using gas chromatography (Cherian and Sim, 1992) and are summarized in Table 4.2. The diets SO, AO, and LO provided n-6 to n-3 PUFA ratios of 29:1, 9:1 and 0.8:1, respectively. The FO gave a n-6 to n-3 PUFA ratio of 1.1:1, close to that of LO, but the main n-3 PUFA in FO is EPA and DHA instead

of LNA, the only n-3 PUFA in LO. The chicks were housed in separate floor pens with free access to feed and water.

5.2.2. Sample Collection

At 4 wk of age, six chicks in each group were selected and anaesthetized by CO_2 inhalation. Blood (5 mL) was collected by cardiac puncture and allowed to clot. The sera were separated from the clot by centrifuging at 250 x *g* for 10 min (Fritsche et al., 1991). An aliquot (1 mL) of each serum sample was sterile-filtered and kept on ice for lymphocyte proliferation assays, the remainder was frozen at –20 C for later IgG analysis. Immediately, the birds were killed by cervical dislocation. The thymus and spleen were removed, weighed, and put in CCM (Sigma) and placed on ice. Suspensions of spleen and thymus lymphocytes were made as described by Field (1995). Briefly, each tissue was cut into small pieces using sterile scissors, and pushed through a tissue sieve equipped with 80-mesh stainless steel. Cell clumps were dispersed by several gentle washings with CCM through the sieve. The red blood cells and dead cells were removed by placing the CCM and cell mixture over lymphocyte separation medium (density, 1.08; Sigma) and centrifuging at 1,100 x *g* for 15 min. The cells were washed two to three times with CCM and resuspended in 5 mL of CCM with 5% (v/v) fetal bovine serum (5% FBS-CCM). The cells were counted with a hemacytometer, and cell viability was determined by trypan blue exclusion (Field, 1995). The cell suspension was diluted to a final concentration of 1 x 10^7 cells/mL in 5% FBS-CCM. The same procedure was repeated with chicks (n = 6) at 8 wk.

5.2.3. Mitogenic Responses of Lymphocytes

Lymphocyte proliferation was assayed according to the method reported by Field (1995) with some modifications. Spleen or thymus lymphocytes were cultured ($1x10^6$ cells/well) in triplicate using 96-well microtiter plates (Corning Inc., Corning, NY 14830). The plates were incubated in a humidified atmosphere with 5% (v/v) CO_2 at 40 C for 72 h. The concentration of Con A, PHA and PWM were 10, 50 and 50 μg/mL for spleen lymphocytes, and 5, 50 and 5 μg/mL for thymus lymphocytes, respectively. All mitogens were bought from Sigma Science, Inc. The optimum concentration of each mitogen was determined in preliminary studies (data not shown). Assays were conducted in CCM containing 5% (v/v) FBS and 5% (v/v) chicken serum from the same bird from which lymphocytes were derived (Fritsche et al., 1991a). Eight hours before harvesting cells, 1 μCi of [^{3}H]thymidine was added to each well. The cells were harvested on glass fiber filter using a multiwell harvester (Skatron Instruments AS, N-3401 Lier, Norway) and counted using Ecolite in the Betacounter (Skatron Instruments AS, N-3401 Lier, Norway). Data were presented by stimulation index, calculated as (amount of [^{3}H]thymidine incorporated by the stimulated cells – the amount of [^{3}H]thymidine incorporated by the unstimulated cells)/amount of [^{3}H]thymidine incorporated by unstimulated cells.

5.2.4. Splenocyte Phenotyping

Lymphocyte subsets from freshly isolated splenocytes and blood mononuclear cells were identified by direct single label (one color) immunofluorescence assay (Robinson and Field, 1998) by using purified mouse monoclonal antibodies (South Biotechnology

Associates Inc. Birmingham, AL 35226) specific for the different chicken lymphocyte subsets. CT-4 recognizes T-helper lymphocytes ($CD4^+$), CT-8 recognizes cytotoxic/suppressor T-lymphocytes ($CD8^+$), M-1 recognizes IgM^+ cells, and L-1 recognizes Ig^+ cells. For each sample, 5×10^5 lymphocytes were added in each of the four wells, and then 200 µl of phosphate buffered saline (PBS) with 4% (v/v) FBS (the mixture was named as 4% FBS-PBS) were added to each well and spun at 900 x *g* at 4 C for 2 min. The supernatants were aspirated, and the pellets were broken up by gentle vortexing. Each monoclonal antibody, conjugated with fluorescein isothiocyanate (0.4 µg) in 50 µL of 4% FBS-PBS, was added. An equal amount of 4% FBS-PBS was added into the blank wells. The plates were covered and incubated at 4 C for 30 min. Next, the cells were washed three times with 4% FBS-PBS. The pellets were vortexed gently and fixed in paraformaldehyde (1% in PBS, v/v). All samples were analyzed, within 5 d, by flowcytometry on the same FACScan (South Biotechnology Associates Inc. Birmingham, AL 35226). The incident/excitation light from the laser light source was set at a wavelength of 488 nm. The fluorescence from the excitation of FIT-C was detected at a wavelength of 530 nm using the Lysis II program. The resulting percentages were corrected for background fluorescence determined by incubating cells with 4% FBS-PBS. Unwanted events (dead cells and debris) were (dead cells and debris) detected on the basis of forward scatter and side scatter and were excluded from subsequent phenotype analyses by electronic gating of the viable lymphocyte populations.

5.2.5. Measurement of Serum IgG Concentration

The serum IgG content was determined by radial immunodiffusion as described by Sunwoo et al. (1996).

5.2.6. Statistical Analysis

One-way ANOVA was used to analyze the overall difference of the main effects among the four dietary treatments by using general linear model procedure of SAS software (SAS Institute Inc., 1990). Significant differences among the treatment means were analyzed by the method of least squares means test after a significant ANOVA. Significance level was set at 0.05.

5.3. Results

5.3.1. Mitogenic Response of Splenocytes

Dietary fat source, providing different ratios of n-6 to n-3 PUFA, significantly affected the spleen lymphocyte proliferation in response to Con A (T-cell mitogen) at 4 wk ($P < 0.0003$, Table 5.3). The chicks fed SO produced a higher response than the chicks fed LO ($P < 0.005$) or FO ($P < 0.0001$). A significantly higher response was also achieved by the chicks fed AO relative to the chicks fed LO ($P < 0.03$) or FO ($P < 0.02$). The response of chicks fed LO was 42.5% lower ($P < 0.05$) than the chicks fed FO. At 8 wk, different fat sources continued to produce significant effects on spleen lymphocyte proliferation to Con A stimulation ($P < 0.0001$). The chicks fed SO had significantly higher responses than the chicks fed LO ($P < 0.0003$) or FO ($P < 0.0003$). The response

of the chicks fed AO was also significantly higher than the chicks fed LO ($P < 0.0001$) or FO ($P < 0.0001$).

PWM (T- and B-cell mitogen)-induced spleen lymphocyte proliferative response did not differ among the four dietary treatments at the age of 4 wk ($P = 0.10$, Table 5.3). At 8 wk, the response from the chicks fed SO was higher than the chicks fed AO ($P < 0.003$), LO ($P < 0.003$) and FO ($P < 0.0001$). No significant differences were found among the chicks fed AO, LO and FO ($P > 0.05$). The splenocyte proliferative response to T-cell mitogen PHA stimulation was not significant different among the four dietary treatments at 4 wk or 8 wk ($P > 0.05$).

5.3.2. Mitogenic Response of Thymocytes

Dietary fat source also significantly influenced the mitogenic response of thymus lymphocytes to Con A at 4 wk ($P < 0.0001$, Table 5.3). This effect was evident as a 61% decrease in the chicks fed AO ($P < 0.0002$), 86% decrease in the chicks fed LO ($P < 0.0001$), and 79% decrease in the chicks fed FO ($P < 0.0001$) relative to the chicks fed SO. There were no significant differences among the four dietary treatments in the thymus lymphocyte proliferation in response to stimulation with PHA ($P = 0.11$) or PWM ($P = 0.44$).

5.3.3. IgG Concentration in Serum

There were no significant differences in serum IgG concentration among the four dietary treatments ($P > 0.05$) at 4 wk (Fig. 5.1). However, the serum IgG concentration in the chicks fed FO was 73% higher than in the chicks fed SO ($P < 0.0002$), 37% higher

than in the chicks fed AO ($P < 0.005$), and 40% higher than in the chicks fed LO ($P < 0.005$) at 8 wk, with no significant differences observed among the groups fed SO, AO, and LO ($P > 0.05$).

5.3.4. Phenotypes in Spleen

The phenotypes of freshly isolated splenocytes were determined at 4 wk only. Dietary fat source did not affect ($P > 0.05$) the proportion of $CD4^+$ T-lymphocytes and Ig^+ B-lymphocytes in spleen (Table 5.4). The proportion of $CD8^+$ T-lymphocytes in the spleen of chicks fed LO was higher ($P < 0.05$) than the chicks fed SO or AO. There were no significant differences among the chicks fed SO, AO, and FO ($P > 0.05$), or between the chicks fed LO and FO ($P > 0.05$). The ratio of $CD4^+$ to $CD8^+$ T- cells did not differ ($P > 0.05$) among the four treatments. The chicks fed LO and FO, respectively, had 58% more ($P < 0.05$) and 46% more ($P < 0.05$) spleen IgM^+ B-lymphocyte than the chicks fed SO. No significant differences ($P > 0.05$) were detected in the proportion of IgM^+ B-lymphocytes among the chicks fed AO, LO, and FO or between the chicks fed SO and AO.

5.4. Discussion

We found that increases of dietary n-3 PUFA (LNA) in chick diets resulted in marked suppression of the spleen and thymus lymphocyte mitogenic responses to Con A and PWM with n-6 to n-3 PUFA (LA to LNA) ratios ranging from 0.8 to 29. Similar results were reported in chicks in an even larger range of dietary n-6 to n-3 PUFA (LA to

LNA) ratios from 0.3 to 59 (Fritsche et al., 1991a). Although Con A and PHA are both T-cell mitogens, they bind to different parts or determinants on the T-cell (Leca et al., 1986; Benichou et al., 1989; Chilson and Kelly-Chilson, 1989). The amount of PHA used resulted in a significantly lower response in both the spleen and thymus lymphocytes, which might explain why no significant diet-induced effects were observed for T-cell response to PHA. Many studies in mammalian species also demonstrated that n-3 PUFA from both linseed oil (rich in LNA) and fish oil (rich in EPA and DHA) can suppress lymphocyte proliferation in response to mitogen stimulation (Marshall and Johnston, 1985; Endres et al., 1993; Meydani et al., 1993; Jeffery et al., 1996a). Nevertheless, due to different n-3 fatty acid components provided by linseed oil and fish oil, the intensities of suppressive effects of 5% linseed oil diet (LO) and 5% fish oil diet (FO) on splenocyte proliferation were different. The LNA had a stronger suppressive effect on splenocyte proliferation in response to Con A at 4 wk than EPA and DHA. This result is opposite that obtained from most studies in mammals (Calder and Newsholme, 1992a, b; Das, 1994; Devi and Das, 1994) that EPA from fish oil appears to be the most inhibitory, but similar with the result obtained in chickens by Fritsche et al. (1991a).

Antibodies block the antigenic sites of an antigen and mucosal adhesive molecules to protect the host from infections and to facilitate the clearance of blood-borne antigens. Antibodies also induce hypersensitivity, activation of complement system, and antibody dependent-cell cytotoxicity to facilitate clearance of pathogens from infectious sites. We assume that total IgG might represent the potential humoral immune response of chickens exposed to antigenic challenges. Based on this assumption we made the following comparisons. In this study, the chick serum IgG concentration did

not differ among the three groups of chicks fed the diets with the ratio of LA to LNA as 29:1, 9:1 and 0.8:1. The production of antibodies to albumin was also not affected when feeding rabbits linseed oil (Kelley et al., 1988), whereas Friedman and Sklan (1995) reported in broilers that antibody (IgG) developed more quickly and reached a higher level and was more persistent when dietary LA to LNA ratio was reduced to 12:1 from 31:1, 28:1 or 24:1. Our data showed that feeding a diet containing 1.1:1 of n-6 to n-3 PUFA (5% fish oil) significantly increased IgG concentration in chick serum compared with the chicks fed the other three diets. The enhanced IgG activity was also reported in rats fed 25% (wt/wt) fish oil diet compared with those fed 25% tallow diet (Pricket et al., 1982) and in chickens fed 7% (wt/wt) fish oil diet relative to those fed a diet with 7% lard, corn oil, or canola oil (Fritsche et al., 1991a). The data we compared here indicated that alterations of total IgG production induced by dietary fatty acids in chicks without an antigenic challenge might reflect the potential of specific antibody IgG production when chicks were challenged with an antigen. However, further investigations are required.

Diet-induced alterations in immune responses might be determined by assessing changes in the proportions of different lymphocyte subsets (Hoffman-Goetz and Pedersen, 1994). To our knowledge, this is the first study to examine the effect of dietary LA to LNA ratio on the proportions of lymphocyte subsets in chick spleen. In comparison to chicks fed diets with 29:1 or 9:1 of LA to LNA ratio, the chicks fed the diet with 0.8 of n-6 to n-3 PUFA ratio did not significantly change the ratio of $CD4^+$ to $CD8^+$ T-cells although the proportions of $CD8^+$ lymphocytes were significantly increased. The diet containing linseed oil or fish oil significantly increased the proportion of IgM^+ B-cells in spleen, but only the fish oil diet increased serum IgG concentration. The

proportions of $CD4^+$ and $CD8^+$ T-cells and the ratio of $CD4^+$ to $CD8^+$ T-lymphocytes in the chicks fed fish oil diet did not differ from any others. These results suggested that the differences in lymphocyte proliferation and IgG formation by feeding different ratios of n-6 to n-3 PUFA or n-3 fatty acids may be attributable to the functional alterations of immune cells rather than the changes of immune cell subset proportions.

It is worth noting that effect of n-3 PUFA on immune responses appears to be dose dependent. In our study, relatively higher level of oils (5%, wt/wt) in the diet was utilized. An even higher level (7%, wt/wt) was employed by Fritsche et al. (1991a) in chicks. Both studies showed that the increase of dietary n-3 PUFA diminished chick lymphocyte proliferative responses to Con A and PWM, and LNA (linseed oil) showed stronger potency than EPA and DHA (fish oil). However, Korver and Klasing (1997) reported that when moderate levels of n-3 PUFA ($\leq$ 2% fish oil, wt/wt) were applied, increased dietary n-3 PUFA resulted in greater cell-mediated immunity in chickens as determined by the wattle delayed-type hypersensitivity. In humans, inclusion of fish oil at 0.54% of total energy in a low fat diet decreases T-cell proliferation in response to Con A and PHA, whereas inclusion of only 0.13% of calories as fish oil in a similar diet results in an increase in the same indices. Delayed-type hypersensitivity is decreased vs. baseline at the higher level of fish oil, but there was no change at the low level of fish oil (Meydani et al., 1993). Fish oil is immunosuppressive in the host vs. graft model in mice only at high concentrations ($\geq$ 10 g/100g diet; Hinds and Sanders, 1993). It has also been reported that the immunomodulating effect of dietary n-3 PUFA is related to physiological status. For sedentary rats, long-chain n-3 PUFA augmented cell-mediated

immune function and NK cell cytotoxicity. For exercise-trained rats, there was no effect (Robinson and Field, 1998).

The results of this experiment give insights into a potential dietary method to modulate chicken immune responses toward improving chicken performance under a given condition. For example, inflammatory response is the first line of defense against novel pathogens, but cells and mediators of the inflammatory responses have been implicated in the pathology of many poultry diseases, including coccidiosis (Trout and Lillehoj, 1993) and *S. enteritidis* (Tellez et al., 1994; Kogut et al., 1995). It has been reported that the indices of an inflammatory (non-specific cell-mediated immunity) response was lower in fish oil-fed (≤ 2% fish oil, wt/wt) birds, whereas indices of specific immunity were either unchanged or greater in the chicks fed fish oil diet (Korver and Klasing, 1997). The growth and feed efficiency in the chicks with an inflammatory response were improved when the chicks were given fish oil diet (Korver and Klasing, 1997). Modification of antibody production and activity by dietary fatty acid manipulation may provide an avenue to strengthen chick humoral immunity and protection against various pathogens. However, long-term effects of immunomodulation induced by dietary n-3 PUFA on the resistance of chickens to commercially relevant infectious challenges and chicken performances remain to be investigated.

TABLE 5. 1. Composition of chick basal diets

Ingredients	g/kg
Wheat	567
Barley	100
Soybean meal	230
Limestone	14
Dicalcium phosphate	25
DL-methionine	1.8
L-Lysine-HCl	0.2
Layer premix[1]	5
Choline chloride premix[2]	5
Sodium chloride	2.3
Amproline	0.5
ME (kJ/g)	12.3
Crude protein (g/kg)	195

[1]Layer premix provides per kg diet: vitamin A, 12,000 IU; vitamin D3, 3,000 IU; vitamin E, 40 IU; vitamin K, 2.0 mg; pantothenic acid, 14.0 mg; riboflavin, 6.5 mg; folacin, 1.0 mg; niacin, 40.0 mg; thiamine, 3.3 mg; pyridoxine, 6.0 mg; vitamin B12, 0.02 mg; biotin, 0.2 mg; iodine, 0.5 mg; manganese, 75.0 mg; copper, 15.0 mg; zinc, 80.0 mg; selenium, 0.1 mg; iron, 100.0 mg.

[2]50 kg choline chloride premix contains choline chloride (60%) 1.7 kg, wheat shorts 48.3 kg.

TABLE 5. 2. Fatty acid composition of chick diets

	Diet[1]			
	SO	AO	LO	FO
Fatty acid[2]	*% of total fatty acids*			
16:0	10.1	22.2	9.7	20.8
18:0	3.6	10.3	3.8	3.6
16:1	0.1	2.3	0.2	7.5
18:1	17.9	36.9	17.0	16.6
18:2n-6	64.4	22.2	30.8	21.0
20:4n-6		0.1		0.5
18:3n-3	2.2	2.4	37.5	3.4
20:5n-3				7.3
22:5n-3				1.6
22:6n-3				7.4
SFA	15.1	35.0	14.2	32.4
MUFA	18.3	40.1	17.5	26.0
PUFA	66.6	24.9	68.3	41.6
n-6 PUFA	64.4	22.5	30.8	22.0
n-3 PUFA	2.2	2.4	37.5	19.6
P/S	4.4	0.7	4.8	1.3
n-6:n-3 PUFA	29.1	9.3	0.8	1.1

[1]SO, AO, LO, and FO represent diets containing 5% (w/w) of sunflower oil, animal tallow, linseed oil, or menhaden fish oil, respectively.

[2]SFA, saturated fatty acids; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids; P/S, ratio of PUFA to SFA.

TABLE 5. 3. Effect of dietary fat source on spleen and thymus lymphocytes proliferative response[1] to the stimulation of three mitogens

	Spleen lymphocytes						Thymus lymphocytes		
	4 wk			8 wk			4 wk		
Diet[2]	CON A[3]	PHA-P[4]	PWM[5]	CON A	PHA-P	PWM	CON A	PHA-P	PWM
SO	26.4±2.5[a]	5.6±0.7	3.1±0.6	25.1±3.4[a]	9.2±1.9	13.2±0.7[a]	31.1±11.3[a]	13.3±4.6	7.9±2.7
AO	33.8±3.4[a]	5.8±1.1	3.6±1.1	27.5±3.0[a]	6.6±0.6	7.0±1.0[b]	12.0±1.0[b]	7.1±2.2	3.7±1.2
LO	10.6±0.6[c]	5.4±0.9	1.6±0.1	7.3±1.6[b]	7.3±1.3	6.9±1.0[b]	4.3±0.7[b]	3.6±1.3	4.6±1.3
FO	18.5±2.7[b]	6.0±1.2	2.0±0.3	11.4±0.9[b]	8.4±1.7	3.9±1.3[b]	6.5±2.4[b]	4.0±1.5	3.9±1.8

[a-c]For each mitogen, values that do not have common superscripts are significantly different ($P < 0.05$).

[1] Lymphocyte proliferative response to each mitogen is expressed as stimulation index, which is equal to (the amount of [^{3}H] thymidine incorporated by stimulated lymphocytes – the amount of [^{3}H] thymidine incorporated by unstimulated lymphocytes) / the amount of [^{3}H] thymidine incorporated by unstimulated lymphocytes. Each value represents mean ± SEM (n = 6).

[2] SO, AO, LO and FO represent diets containing 5% (w/w) of sunflower oil, animal oil mixture, linseed oil and menhaden fish oil, respectively.

[3-5] Con A, concanavalin A; PHA-P, phytohaemagglutinin-P; PWM, pokeweed mitogen.

TABLE 5. 4. Effect of dietary fatty acids on single-labeled immune phenotypes in chick splenocytes

Antibody	Diet [1]				P <
	SO	AO	LO	FO	
	% of the total spleen lymphocytes				
CT-4 ($CD4^+$ cells)	23.2±1.7	22.6±1.1	24.4±0.8	23.6±0.4	NS[2]
CT-8 ($CD8^+$ + NK^3 cells)	40.4±2.4^b	40.7±2.7^b	49.6±1.5^a	44.4±0.7^{ab}	0.01
CT-4/CT-8	0.6±0.1	0.6±0	0.5±0	0.5±0	NS
L-1^+ (Ig^+ cells)	21.8±1.8	20.5±2.7	22.5±1.1	24.3±0.4	NS
M-1^+ (IgM^+ cells)	14.0±1.4^b	19.0±2.8^{ab}	22.2±0.8^a	20.5±1.0^a	0.01

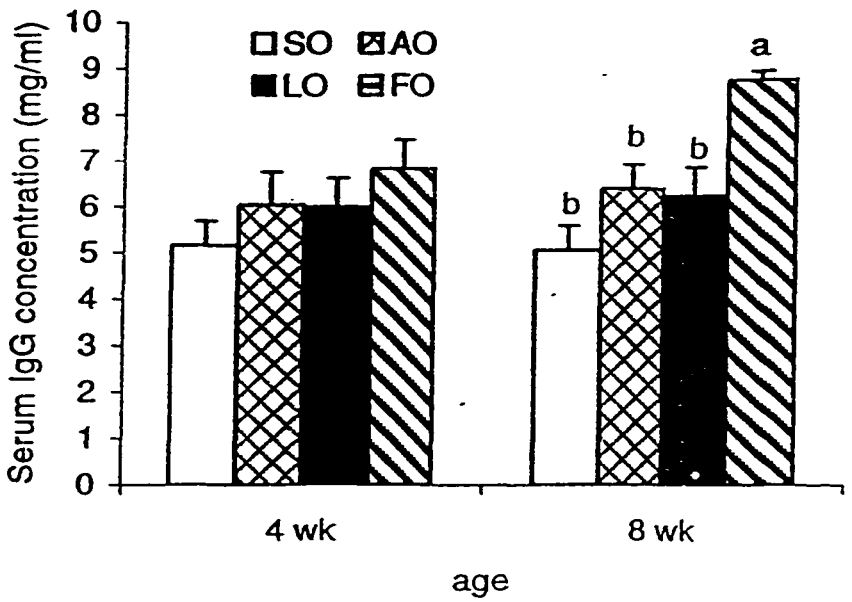
[a,b]For each antibody, values that do not have common superscripts are significantly different ($P < 0.05$). Values are means ± SEM (n = 6) for freshly isolated splenocytes.

[1]SO, AO, LO and FO represent diets containing 5% (w/w) of sunflower oil, animal tallow, linseed oil or fish oil, respectively.

[2]Not significant ($P \geq 0.05$).

[3]Natural killer.

FIGURE 5. 1. Effect of dietary fat source on IgG concentration in chick serum. SO, AO, LO, and FO represent diets containing 5% (w/w) of sunflower oil, animal oil mixture, linseed oil and menhaden fish oil, respectively. Each bar represents mean ± SEM (n = 6). For each age, bars that do not have common letters are significantly different ($P < 0.05$).



6. EFFECT OF MATERNAL DIETARY FATTY ACID PROFILE ON THE SPLEEN FATTY ACID COMPOSITION AND DELAYED-TYPE HYPERSENSITIVITY OF BROILERS

6.1. Introduction

The modulation of immune functions may be produced as a consequence of several factors, but one event involved in this process is associated with changes in the cell membrane due to dietary fatty acid manipulation (Fritsche, et al., 1991a; Kinsella et al., 1990; Clamp et al., 1997). Fatty acids can be incorporated into the plasma membrane after dietary lipid manipulation, so that the composition of lipids in the cell membrane will reflect to some extent the composition of dietary lipids (Clamp et al., 1997; Wang et al., 2000a,b). Fatty acids, especially n-6 and n-3 PUFA regulate the productions of amount and type of eicosanoids and cytokines, the signal transduction, gene expression, and cell membrane fluidity (Calder, 1997a,b; De Pablo et al., 2000). As a result, dietary fatty acid manipulation can dramatically change the immune cell functions (Wu and Meydani, 1998; Calder, 1997b, 1998a,b). It has been reported that feeding high levels of n-3 PUFA to chickens suppressed mitogen-induced lymphocyte proliferation (Fritsche et al., 1991a, Wang et al., 2000a,b), inflammatory response as measured by acute phase protein production in liver (Korver and Klasing, 1997), or by DTH response (Korver and Klasing 1997; Wang et al., Chapter 7), and also affect the total IgG and antibody production (Fritsche et al, 1991a, Friedman and Sklan, 1995, Wang et al., 2000a, b).

The beneficial effects of unsaturated fatty acids on the immune system of both humans and animals have been widely studied, and n-3 PUFA, especially EPA and DHA has been used in clinical patients who suffer from chronic inflammation or autoimmunity (Calder, 1997b). In these cases, the benefits of n-3 PFUA are generated by suppressing cell-mediated immunity thus ameliorating the symptoms resulting from the overreaction of inflammatory responses (Grimble, 1998; James et al., 2000). In poultry, it has also been reported that feeding chickens with n-3 PUFA from fish oil (2%) reduces inflammatory response and improves the growth rate and feed efficiency of those chickens incurring coccidial infection compared with feeding the same level of n-6 PUFA.

The developmental events important for immunocompetence begin in the embryo and continue during the first week following hatching (Gobel, 1996; Ratcliffe et al., 1996). The first week of life is a period of rapid expansion of leukocyte populations, seeding of lymphoid organs, and educational events that produce the unique clones of lymphocytes that will mediate immunity later in life (Klasing, 1998). So it is not surprising that this is a critical period during which nutritional status may impact the immune system (Klasing, 1998). In our previous study (Wang et al., 2000b), we found that the immunomodulatory effects of dietary PUFA in the offspring were similar with that in the hens when the offspring were continued to be fed the same type of fat diets as their corresponding maternal diets following hatching (Wang et al., 2000a, b). It has been well documented that maternal dietary fatty acids have a profound influence on the fatty acid composition of non-immune organs (liver, heart, brain and muscle) in developing embryo until hatching, but it is not known how long this maternal effect can be sustained

in these tissues following hatching (Cherian and Sim, 1991,1992). The question was raised that whether and how long maternal dietary lipids affect the immune tissue fatty acid composition and functions of the offspring following hatching.

6.2. Materials and Methods

One hundred twenty broiler breeders (26 wk old) were randomly assigned to one of the three nutritionally complete diets (40/diet). The diets were wheat-soybean meal based diet with addition of 5% (w/w) of SO, the mix of SO and FO (1:1), and FO, respectively (Table 6.1). The fatty acid composition of the diets was analyzed by gas chromatography (Cherian and Sim, 1992) and is presented in Table 6.2. All the birds were housed in cages with free access to feed and water. The experiment was approved by the University Animal Policy and Welfare Committee and was conducted in accordance with the Canadian Council on Animal Care guidelines.

The egg samples were collected and analyzed weekly. After 2 wk of feeding experimental diets, the breeder hens were artificially inseminated. Two batches of fertilized eggs were collected and incubated. The first batch was collected for 4 wk, and the second batch was collected for 2 wk. Upon hatching, 500 hatching chicks (300 from the first batch, and 200 from the second batch) were selected from each treatment and randomly assigned to five floor pens with 100 each. All chicks were fed the identical diets (broiler starter, 1-3 wk and grower, 4-6 wk; Appendixes 1 and 2). The feed intake, body weight, and daily mortality were recorded. Five spleen samples from each treatment were collected on day 0, 14, 28, and 42 following hatching for fatty acid analyses. DTH

responses were measured in breeders at the end of collection of the fertilized eggs and in the offspring (broiler chicks) at the ages of 2 and 4 wk. An aliquot 0.4 mL of BSA solution (1 mg/mL in PBS) was injected subcutaneously into the flat area of both wing webs (two sites at left wing and two sites at right wing). The thicknesses of the injection sites in both wing webs were measured using a caliper before injection and 24 h post-injection, respectively. The DTH response was presented as wing web swelling following the injection of BSA, which was calculated as the increase of wing web thickness = the thickness of wing webs following BSA injection - the initial thickness of the wing webs. At the end of experiment, any birds that had died were dissected to determine reasons for death.

6.3 Results and Discussion

The fatty acid composition of egg yolk after 2 wk of feeding experimental diets was summarized in Table 6.3. The fatty acid composition of egg yolk reflected was consisted with what would be predicted by the fat composition of the diets fed to the hens. The SFA were fairly constant as documented by Cherian and Sim (1991, 1992). The percentages of total SFA and MUFA in the egg yolk were much higher than that in the diets, indicating a vigorous synthesizing activity and certain demands of those two types of fatty acids for chickens. The most significant changes happened in the percent composition of each fatty acid in MUFA and PUFA families, which were changed inversely (Cherian and Sim, 1991, 1991, 1995).

Two-fold more C18:1 was incorporated into the egg yolk compared with that in the diets (Table 6.3). The amounts of both C16:1 and C18:1 were mainly influenced by the total PUFA incorporated. The amounts of LA and LNA in the egg yolk were affected by the dietary supplementation ($P < 0.001$) and greatly reduced compared with their amounts in the diets. The relative amount of AA in the egg yolk was very low (≤2.5%), and was significantly decreased with the increase of n-3 PUFA ($P < 0.001$). This suggests that breeders have limited capability in synthesizing AA from LA (Cherian and Sim, 1991) or egg yolk limits the deposition of AA; N-3 PUFA inhibit the conversion of LA to AA or inhibit AA incorporation into the egg yolk (Cherian and Sim, 1992). The amount of each longer-chain n-3 PUFA was increased with its increase in the diets ($P < 0.01$), especially DHA. Although a large amount of EPA (8.6% in 5% fish oil diet) was supplied by the diets, the amount of this fatty acid in the egg yolk was very low (≤ 1%). The preferential accumulation of n-3 PUFA, DHA, into the egg yolk may reflect a strong demand of embryo for this fatty acid during the incubation.

The fatty acid profile in the chick spleen of day-old chick reflected that in the egg yolk (Table 6.4). Similar results were reported in other tissues such as brain and muscle of chickens (Cherian and Sim, 1991,1992,1993,1995). Except for C16:0 and total SFA, all other fatty acids in the spleen were significantly different among the treatments ($P < 0.05$). In comparison to the fatty acids in the yolk of eggs incubated, the amount of C18:0 in the spleen was almost doubled; C18:1 was greatly reduced and more strongly influenced by the amount of total PUFA incorporated ($P < 0.0001$). The percentage of AA was dramatically increased in the newly hatched chick, which was about eight-fold as much as that in the egg yolk. The each longer-chain n-3 PUFA was also increased. These

suggest that chick spleen demands high levels of both n-6 and n-3 PUFA, which are achieved by mainly reducing MUFA incorporation, which may also suggest that embryo has strong ability to synthesize AA and longer-chain n-3 PUFA or preferentially incorporate these n-6 and n-3 PUFA from the egg yolk (Table 6.4).

After 2 wk of feeding the identical diet (broiler starter), the amount of EPA ($P < 0.03$), DHA ($P < 0.0003$), total N-3 PUFA ($P < 0.0003$) or the ratio of n-6 to n-3 PUFA was still different among the three groups ($P < 0.001$) (Table 6.5). The broilers from the eggs laid by the hens fed higher levels of n-3 PUFA maintained more these fatty acids in the spleen, with no differences were observed between the broilers from the eggs laid by the hens fed the moderate (2.5% of SO and 2.5% FO, w/w) and high (5% FO) levels of n-3 PUFA ($P > 0.05$). There were no differences among the treatments for each of n-6 fatty acids ($P > 0.05$). This is not surprising as considerable amount of n-6 (LA; 28% of total fatty acids) was supplied by the broiler diet (Appendix 3). It is interesting that DHA differed ($P < 0.006$) among the groups after 4 wk of feeding the same broiler diet (after 3 wk, the chicks transited to the grower diet). The chicks from the eggs laid by the hens fed n-3 rich diet (5% FO) maintained higher level ($P < 0.05$) of DHA than the chicks from the eggs laid by the hens fed n-6 rich diet (5% SO). These results might indicate a strong demand of broiler immune organ (spleen) for n-3 PUFA and reserving response to the n-3 PUFA depletion. It may also indicated that the amount of n-3 PUFA in the broiler diet fed in this study does not meet the requirements of the immune tissues, which may affect the immune tissue development and later functions in life. This is the first report on the effect of maternal dietary lipids on the fatty acid composition of the offspring immune tissue upon and following hatching.

As discussed in Chapter 5, inflammatory responses are the first line of immune defenses that are required for protecting from infectious challenges. However, too high response can result in negative effects on the health (tissue damage, fever, protein wasting, disease and even death) and production. It has been reported that dietary fatty acids modulate the inflammatory responses (Harbige, 1998; James et al., 2000; Kremer, 2000). The suppressive effect of n-3 PUFA, especial from marine oil on the inflammation has been used in the patients with chronic inflammation or autoimmune diseases (Calder, 1997b). In poultry, although the information is limited, feeding n-3 PUFA from fish oil ($\leq$ 2%) has been reported to benefit the chickens incurred with moderate coccidial infection by suppressing inflammatory response (Korver and Klasing, 1997; Korver et al., 1997). It has not been reported whether chicken becomes more sensitive to infectious challenges when inflammatory response is suppressed by feeding n-3 PUFA.

The results of this study showed that DTH response of the breeder hens as measured by wing-web-swelling was significantly affected by dietary fatty acid composition (Fig. 6.1). The hens fed the high level of n-3 PUFA had significantly lower ($P < 0.0001$) DTH response than the hens fed moderate level of n-3 PUFA and high level of n-6 PUFA. The breeders fed the moderate level of n-3 PUFA also had lower DTH response ($P < 0.0001$) than the hens fed the high level of n-6 PUFA. The suppressed DTH response by feeding the broiler breeder hens with n-3 PUFA derived from fish oil (2.5-5%, w/w) was in agreement with our previous study that the DTH response of laying hens was reduced by feeding n-3 PUFA derived from linseed oil (5%, w/w) (Wang et al., submitted).

The development of immune system begins in the embryo and continues following hatching (Gobel, 1996; Ratcliffe et al., 1996; Klasing, 1998). So it is not surprising that during this period nutritional status may impact the immune system (Klasing, 1998). The maternal dietary fatty acids significantly affected the fatty acid profile of an immune tissue (spleen) of the offspring (broiler) up to 4 wk after hatching. This modification of fatty acid composition in the offspring immune tissue had significant effect on the cell-mediated immune response as measured by wing-web-swelling 24 h post-injection of BSA. Two weeks following hatching, the broilers from the eggs laid by hens fed the high and moderate levels of n-3 PUFA had significantly lower ($P < 0.05$) DTH responses than the chicks from the eggs laid by the hens fed the high level of n-6 PUFA diet (Fig. 6.2). Four weeks after hatching, the DTH response was still significantly different ($P < 0.001$; Fig. 6.3). There was no difference between the chicks from the hens fed the moderate and high levels of n-3 PUFA, but the both were lower ($P < 0.001$) than the chicks from the hens fed the high level of n-6 PUFA.

The suppressive effect of n-3 PUFA on the inflammatory response has been reported in other mammalian animals (Yoshino and Ellis, 1987; Kelley, et al., 1989; Taki, et al., 1992) and chickens (Benson et al., 1993; Klasing et al., 1987; Roura, et al., 1992; Takahashi et al., 1995). As such, the n-3 PUFA from fish oil has been found to be beneficial to the growth and feed efficiency of chicks infected with moderate levels of coccidiosis (Korver et al., 1997). The mechanism for the suppressive effect of n-3 fatty acids on inflammatory response has not been well established. The decreased production of pro-inflammatory eicosanoids and cytokines by n-3 PUFA might be involved. It has been shown in previous studies that decreasing the n-6 to n-3 PUFA ratio increases the n-

3 content in the lipids of immune cells (Wang et al. 2000a, b). This in turn results in a decreased production of pro-inflammatory eicosanoids (Boudreau et al. 1991, Broughton et al. 1991) and cytokines (Endres et al. 1989), and also decreased responsiveness of target cells to pro-inflammatory signals (Cooper and Rothwell 1993, Mulrooney and Grimble 1993).

This was the first study that investigated the effect of maternal dietary fatty acids on the immune tissue fatty acid composition and on the cell-mediated immune response of offspring following hatching. Under the dietary conditions in the present study, the effect of maternal dietary fatty acids on the composition of n-3 PUFA of the hatching chicks can be maintained up to 4 wk; this long-lasting effect of n-3 PFUA has significant influence on the immune responses of the broilers. The suppressed effect of maternal dietary n-3 PUFA on the inflammatory response of the offspring might be a result of the alteration of the immune tissue fatty acid composition that affect immune tissue development (Wang et al., 2000b) and functions (Fritsche et al., 1991a, Wang et al., 2000a). As broilers are raised commercially for only 5-6 wk, this long-lasting immunoregulatory effect of maternal fatty acids on the offspring immunity may be useful in the poultry industry to improve broiler productivity. Although benefit of n-3 PUFA (2% fish oil) in minimizing the catabolic effect of inflammation induced by pathogenic challenge has been reported (Korver and Klasing, 1997), the disease resistance has not been studied. Deleterious effects may be generated if high or very high levels of n-3 fatty acids applied in chicken diet, which suppress cell-mediated immune responses as indicated from this study and others in mammals (Chang et al., 1992; Mayatepek et al.

1994). This could place chickens at risk of infections. Thus for practical use, more investigations are required.

TABLE 6.1. Composition of broiler breeder diet

Ingredients	g/kg
Barley	150.0
Oats	100.0
Corn	143.1
Wheat short	12.9
Wheat	337.6
Soy meal	134.2
Limestone	76.8
Dicalcium phosphate	10.6
Tallow	20.0
Methionine	1.7
Lysine	0.3
Iodine fortified salt	2.8
Layer premix[1]	5.0
Choline premix[2]	5.0
Protein (%)	15.6
ME (kcal/kg)	2619

[1]Layer premix provides per kg diet: vitamin A, 12,000 IU; vitamin D3, 3,000 IU; vitamin E, 40 IU; vitamin K, 2.0 mg; pantothenic acid, 14.0 mg; riboflavin, 6.5 mg; folacin, 1.0 mg; niacin, 40.0 mg; thiamine, 3.3 mg; pyridoxine, 6.0 mg; vitamin B12, 0.02 mg; biotin, 0.2 mg; iodine, 0.5 mg; manganese, 75.0 mg; copper, 15.0 mg; zinc, 80.0 mg; selenium, 0.1 mg; iron, 100.0 mg.

[2]50 kg choline chloride premix contains choline chloride (60%) 1.7 kg, wheat shorts 48.3 kg.

TABLE 6.2. Composition of fatty acids in the breeder diets

	Diet [1]		
	5% SO	2.5% SO + 2.5% FO	5% FO
Fatty acids[2]	*% of total fatty acids*		
C16:0	10.11	15.68	20.05
C18:0	3.65	3.26	2.88
C16:1	0.18	4.21	8.92
C18:1	16.89	15.59	14.39
C18:2n-6	64.99	41.48	17.79
C20:4n-6		0.31	0.59
C18:3n-3	1.73	2.25	2.52
C20:5n-3		3.92	8.59
C22:5n-3		0.68	1.48
C22:6n-3		3.06	6.55
SFA	14.81	23.07	30.80
MUFA	17.35	20.91	25.54
PUFA	66.77	52.01	38.23
n-6 PUFA	65.02	42.03	18.91
n-3 PUFA	1.75	9.98	19.31
n-6/n-3	37.12	4.21	0.98

[1]SO, sunflower oil; FO, fish oil.

[2]SFA, saturated fatty acids; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids; n-6/n-3, the ratio of n-6 PUFA to n-3 PUFA.

TABLE 6.3. Fatty acid composition of egg yolk after 2 wk of feeding experimental diet

	Diet[1]			
	5% SO	2.5% SO + 2.5% FO	5% FO	
Fatty acids[2]	*% of total fatty acids*			*P* <
C16:0	26.88 ± 0.05[b]	26.88 ± 0.32[b]	29.32 ± 0.02[a]	0.0428
C18:0	9.34 ± 0.03[a]	8.28 ± 0.05[b]	8.28 ± 0.01[b]	0.0056
C16:1	2.85 ± 0.10[c]	3.84 ± 0.05[b]	5.44 ± 0.08[a]	0.0010
C18:1	34.45 ± 0.09[b]	36.73 ± 0.32[a]	37.85 ± 0.16[a]	0.0240
C18:2n-6	21.28 ± 0.05[a]	15.02 ± 0.02[b]	6.71 ± 0.02[c]	0.0001
C18:3n-3	0.24 ± 0.00[c]	0.41 ± 0.01[b]	0.52 ± 0.00[a]	0.0014
C20:4n-6	2.51 ± 0.04[a]	1.01 ± 0.01[b]	0.61 ± 0.02[c]	0.0011
C20:5n-3	0 ± 0.00[c]	0.38 ± 0.01[b]	1.01 ± 0.01[a]	0.0001
C22:5n-3	0.09 ± 0.00[c]	0.84 ± 0.01[b]	1.50 ± 0.00[a]	0.0002
C22:6n-6	0.52 ± 0.01[c]	4.14 ± 0.01[b]	5.48 ± 0.03[a]	0.0060
SFA	37.02 ± 0.01[b]	36.29 ± 0.29[b]	39.14 ± 0.02[a]	0.0301
MUFA	37.48 ± 0.10[c]	41.16 ± 0.31[b]	44.08 ± 0.11[a]	0.0069
PUFA	25.23 ± 0.10[a]	21.75 ± 0.01[b]	15.03 ± 0.07[c]	0.0003
n-6 PUFA	24.37 ± 0.09[a]	16.36 ± 0.02[b]	7.54 ± 0.04[c]	0.0001
n-3 PUFA	0.86 ± 0.01[c]	5.77 ± 0.01[b]	8.50 ± 0.04[a]	0.0001
n-6/n-3	28.37 ± 0.19[a]	2.84 ± 0.01[b]	0.89 ± 0.00[c]	0.0001

[1]SO, sunflower oil; FO, fish oil.

[2]SFA, saturated fatty acids; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids; n-6/n-3, the ratio of n-6 PUFA to n-3 PUFA.

TABLE 6.4. Fatty acid composition of day-old chick spleen

	Diet[1]			
	5% SO	2.5% SO + 2.5% FO	5% FO	
Fatty acids[2]	*% of total fatty acids*			P <
C16:0	24.59 ± 0.59	26.33 ± 0.74	26.04 ± 0.50	NS
C18:0	16.01 ± 0.32[a]	15.88 ± 0.27[a]	14.20 ± 0.33[b]	0.021
C18:1	16.48 ± 1.16[b]	19.32 ± 1.01[b]	27.07 ± 0.91[a]	0.0001
18:2n-6	14.55 ± 0.37[a]	13.63 ± 0.25[a]	7.40 ± 0.53[b]	0.0001
C20:3n-6	0.62 ± 0.04[b]	0.93 ± 0.05[a]	0.50 ± 0.05[b]	0.0001
C20:4n-6	18.30 ± 0.58[a]	10.90 ± 0.28[b]	5.07 ± 0.20[c]	0.0001
C20:5n-3	0.00 ± 0.00[c]	2.65 ± 0.14[b]	6.28 ± 0.46[a]	0.0001
C22:4n-6	3.49 ± 0.18[a]	1.25 ± 0.04[b]	0.39 ± 0.04[c]	0.0001
C22:5n-3	0.30 ± 0.03[c]	1.73 ± 0.13[b]	2.83 ± 0.21[a]	0.0001
C22:6n-6	1.59 ± 0.18[c]	5.90 ± 0.30[b]	8.33 ± 0.27[a]	0.0001
SFA	41.28 ± 0.74	43.55 ± 0.74	41.89 ± 0.82	NS
MUFA	16.92 ± 1.11[b]	19.32 ± 1.01[b]	27.07 ± 0.91[a]	0.0001
PUFA	39.35 ± 0.20[a]	36.98 ± 0.62[b]	30.80 ± 0.36[c]	0.0001
n-6	37.46 ± 0.32[a]	26.71 ± 0.40[b]	13.36 ± 0.65[c]	0.0001
n-3	1.89 ± 0.20[c]	10.27 ± 0.32[b]	17.44 ± 0.42[a]	0.0001
n-6/n-3	20.44 ± 2.02[a]	2.61 ± 0.07[b]	0.77 ± 0.06[b]	0.0001

[1]SO, sunflower oil; FO, fish oil.

[2]SFA, saturated fatty acids; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids; n-6/n-3, the ratio of n-6 PUFA to n-3 PUFA.

TABLE 6.5. Fatty acids composition of 2-wk-old chick spleen

	Diet[1]			
	5% SO	2.5% SO + 2.5% FO	5% FO	
Fatty acids[2]	*% of total fatty acids*			P <
C16:0	24.35 ± 0.33	23.74 ± 0.28	23.42 ± 0.41	NS
C18:0	13.22 ± 0.57	13.97 ± 0.11	13.68 ± 0.19	NS
C16:1	2.31 ± 0.31	1.71 ± 0.07	1.65 ± 0.13	NS
C18:1	27.96 ± 1.45	26.86 ± 0.48	28.1 ± 0.53	NS
C18:2n-6	12.54 ± 0.18	12.62 ± 0.48	13.3 ± 0.21	NS
C18:3n-3	0.81 ± 0.18	0.64 ± 0.03	0.78 ± 0.08	NS
C20:3n-6	1.34 ± 0.11	1.437 ± 0.04	1.454 ± 0.09	NS
C20:4n-6	6.66 ± 0.52	6.51 ± 0.39	6.11 ± 0.38	NS
C20:5n-3	0.98 ± 0.10	1.269 ± 0.07	1.2 ± 0.05	NS
C22:4n-6	1.56 ± 0.15	1.32 ± 0.14	1.14 ± 0.08	NS
C22:5n-3	1.46 ± 0.12[b]	1.75 ± 0.04[a]	1.72 ± 0.03[a]	0.03
C22:6n-6	0.56 ± 0.10[b]	1.2 ± 0.05[a]	1.25 ± 0.10[a]	0.0003
SFA	39.36 ± 0.85	39.68 ± 0.23	38.94 ± 0.52	NS
MUFA	32.71 ± 1.66	31.27 ± 0.58	32.24 ± 0.69	NS
PUFA	27.27 ± 0.84	28.16 ± 0.58	28.13 ± 0.62	NS
n-6	23.3 ± 0.76	23.14 ± 0.56	23.04 ± 0.63	NS
n-3	3.97 ± 0.18[b]	5.02 ± 0.05[a]	5.09 ± 0.16[a]	0.0003
n-6/n-3	5.9 ± 0.28[b]	4.61 ± 0.11[a]	4.54 ± 0.21[a]	0.0012

[1]SO, sunflower oil; FO, fish oil.

[2]SFA, saturated fatty acids; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids; n-6/n-3, the ratio of n-6 PUFA to n-3 PUFA.

TABLE 6.6. Fatty acid composition of 4-wk-old chick spleen

	Diet[1]			
	5% SO	2.5% SO + 2.5% FO	5% FO	
Fatty acids[2]	*% of total fatty acids*			*P* <
C16:0	25.10 ± 0.49	24.31 ± 0.29	23.97 ± 0.36	NS
C18:0	14.74 ± 0.13	14.88 ± 0.15	14.36 ± 0.16	NS
C16:1	1.86 ± 0.19	1.53 ± 0.09	1.53 ± 0.09	NS
C18:1	25.21 ± 0.37	24.98 ± 0.30	25.59 ± 0.44	NS
C18:2n-6	12.75 ± 0.59	12.39 ± 0.31	12.58 ± 0.28	NS
C18:3n-3	0.45 ± 0.03	0.55 ± 0.03	0.52 ± 0.02	NS
C20:3n-6	1.59 ± 0.10	0.60 ± 0.06	1.48 ± 0.04	NS
C20:4n-6	8.61 ± 0.33	8.47 ± 0.26	8.79 ± 0.35	NS
C20:5n-3	0.91 ± 0.07	1.04 ± 0.06	0.98 ± 0.04	NS
C22:4n-6	2.04 ± 0.10	2.15 ± 0.12	2.20 ± 0.09	NS
C22:5n-3	1.85 ± 0.28	2.18 ± 0.18	1.80 ± 0.10	NS
C22:6n-6	0.72 ± 0.03[b]	0.88 ± 0.04[ab]	1.05 ± 0.09[a]	0.006
SFA	41.47 ± 0.49	40.81 ± 0.36	40.05 ± 0.32	NS
MUFA	28.56 ± 0.36	28.40 ± 0.29	28.81 ± 0.44	NS
PUFA	29.95 ± 0.53	30.35 ± 0.36	30.50 ± 0.47	NS
n-6	26.02 ± 0.69	25.70 ± 0.36	26.14 ± 0.41	NS
n-3	3.93 ± 0.36	4.65 ± 0.26	4.36 ± 0.11	NS
n-6/n-3	6.82 ± 0.60	5.61 ± 0.35	6.00 ± 0.14	NS

[1]SO, sunflower oil; FO, fish oil.

[2]SFA, saturated fatty acids; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids; n-6/n-3, the ratio of n-6 PUFA to n-3 PUFA.

FIGURE 6.1. Effect of the dietary fatty acids on the DTH response of broiler breeders. DTH index is presented as the wing web swelling 24 h post-injection of BSA, which was calculated as the increase of the wing web thickness = the thickness of wing web 24 h following the injection - the thickness of wing web before the injection. Bars represent means ± SEM (n = 8). The bars with different letters are significantly different ($P < 0.0002$). SO, sunflower oil; FO, fish oil.

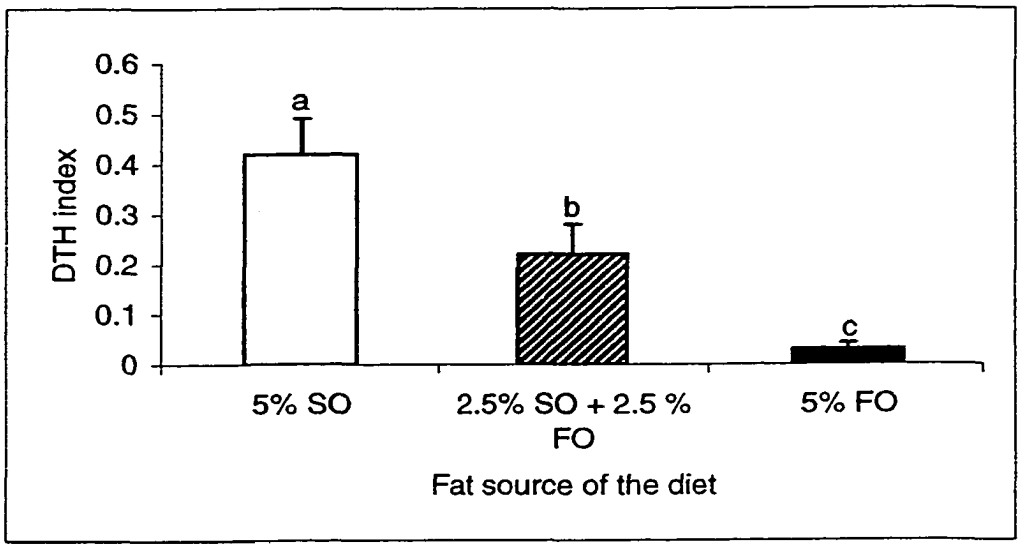


FIGURE 6. 2. Effect of the maternal fatty acids on the DTH response of offspring (broiler) 2 wk following hatching. DTH index is presented as the wing web swelling 24 h post-injection of BSA, which was calculated as the increase of the wing web thickness = the thickness of wing web 24 h post the injection - the thickness of wing web before the injection. Bars represent means ± SEM (n = 8). The bars with different letters are significantly different ($P < 0.0002$). SO, sunflower oil; FO, fish oil.

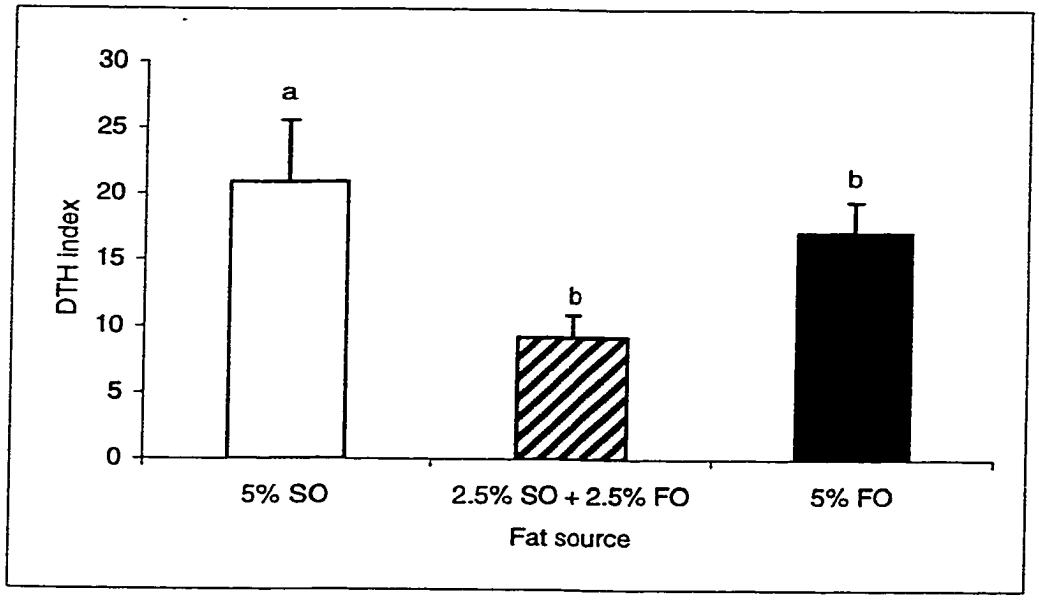
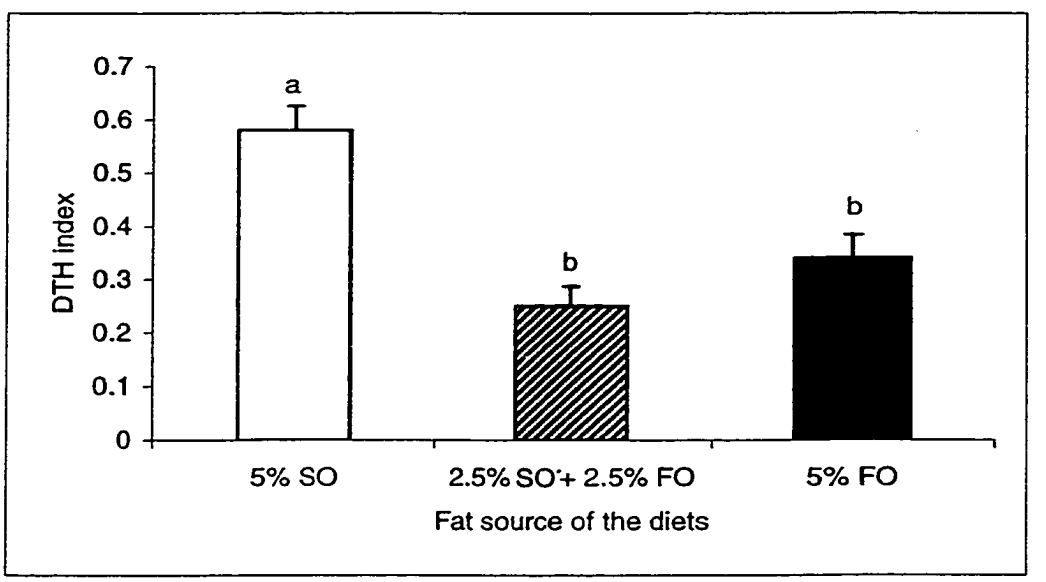


FIGURE 6. 3. Effect of the maternal fatty acids on the DTH response of offspring (broiler) 4 wk following hatching. DTH index is presented as the wing web-swelling 24 h post-injection of BSA, which was calculated as the increase of the wing web thickness = the thickness of wing web 24 h post the injection - the thickness of wing web before the injection. Bars represent means ± SEM (n = 8). The bars with different letters are significantly different ($P < 0.0002$). SO, sunflower oil; FO, fish oil.



7. EFFECT OF DIFFERENT RATIOS OF N-6 TO N-3 PUFA ON DELAYED-TYPE HYPERSENSITIVITY OF LAYING HENS AND PASSIVE IMMUNITY OF HATCHING CHICKS[4]

7.1. Introduction

Immune response evokes a battery of effector molecules and immune cells that act to remove antigen by various mechanisms. Those effector molecules and immune cells involved in a subclinical, localized inflammatory response generally eliminate antigen without extensive damage to the host. Under certain circumstances, however, this inflammatory response can have deleterious effects, resulting in significant tissue damage or even death. In poultry, an inflammatory response can reduce feed consumption and muscle protein accretion, and increase metabolic rate, synthesis of acute phase protein, and organ mass relative to body mass (Klasing and Korver 1997; Roura et al. 1992). This change results in the partitioning of nutrients away from growth and therefore, decreases growth rate and feed efficiency. Thus any strategies that can minimize the nutrient partitioning away from growth and muscle protein deposition are beneficial to poultry production.

DTH, one type of the inflammatory responses is characterized by large influxes of nonspecific inflammatory cells such as macrophages and neutrophils, with a major component of macrophages. The avian heterophil is functionally equivalent to the

[4]A version of this chapter has been submitted for publication. Wang, Y. W., Field, C. J., and Sim, J. S. 2000. Poultry Science (submitted).

mammalian neutrophil (Andreasen et al., 1993). It has been found that eicosanoids derived from AA (PGE_2 and LTB_4) are involved in inflammatory responses, and n-3 PUFA can modify both the quantity and diversity of eicosanoids produced. A decreased production of inflammatory LTB_4 has been reported in mammals fed n-3 PUFA from fish oil (Spurney et al., 1994), with the release of lower amount as well as less potent inflammatory mediator LTB_5 derived from n-3 PUFA (Miller et al., 1993). Dietary supplementation of n-3 PUFA also decreases the production of PGs from AA (Broughton et al., 1991; Haynes et al., 1992), of which PGE_2 mediates the production and function of pro-inflammatory cytokines (Kunkel et al., 1987; Davidson et al., 1990; Cooper and Rothwell, 1993). Although far less work has been done in avian, dietary fish oil has been shown to alter eicosanoid production by chicken leukocytes (Fritsche and Cassity, 1992) and cytokine production by macrophages (Korver and Klasing, 1997). In deed, fish oil has been shown to be suppressive on inflammatory response as measured by the synthesis of acute phase protein in chicken liver (Korver and Klasing, 1997).

Antibody-mediated humoral immunity plays important roles in protecting the host against infectious pathogens and facilitating the clearance of the pathogens by cell-mediated immune responses. IgG constitutes the majority of the antibodies produced in the second humoral response, and is the only class of immunoglobulins capable of crossing the placental barrier (Nysather et al., 1976). IgG neutralizes antigens and also activates the complement system and is frequently involved in opsinization, aiding in the phagocytosis of antigens by macrophages (Nysather et al., 1976). Increasing evidence has shown that n-3 PUFA in flaxseed oil or fish oil affects antibody production in mammals such as rats (Prickett et al., 1982,1984), mice (Atkinson and Maisey, 1995), and humans

(Virella et al., 1989). In chickens, feeding n-3 PUFA has also been reported to enhance antibody production (Fritsche et al., 1991a, Friedman and Sklan, 1995). An increased production of total IgG has been observed in the serum and egg yolk of hens fed n-3 PUFA from flaxseed oil (Wang et al., 2000a) and in the serum of young chicks (4 – 8 wk of age) fed n-3 PUFA from fish oil (Wang et al., 2000b). In avian (except pigeon), all the maternal immunoglobulins needed to protect hatching chick (hatchling) must be present in the egg laid, and transported from the yolk across the yolk sac to the circulation of the developing chick (Brambell, 1969). The question of whether dietary n-6 to n-3 PUFA ratio affects maternal-fetal transfer of total IgG and specific antibody IgG was raised.

Apart from PUFA, fatty acids in SFA and MUFA also differently influence indices of immunocompetence (Jeffery et al. 1997a,b; Yaqoob, 1998). The objective of the present study was to determine the precise effect of dietary n-6 to n-3 PUFA ratio on chicken immune response. The experiment was designed to given laying hens one of four diets that contained similar amounts of each fatty acid of SFA and MUFA, differing only in the compositions of LA and LNA, i.e., the ratio n-6 to n-3 PUFA.

7.2. Material and Methods

7.2.1. Animals and Diets

Thirty-two Single Comb White Leghorn laying hens (at the age of 24 wk) were randomly assigned to one of four dietary treatments with 8 birds per treatment and housed in cages (2 hens/cage). They were fed wheat and soybean meal-basal diet (Table

7.1), with added at 5% (w/w) of the mix of sunflower oil and linseed oil in varied proportions. The experimental diets provided 0.8, 5.4, 12.5, and 27.7 of LA to LNA ratio, respectively as determined by gas chromatography (Cherian and Sim, 1992; Table 7.2), and respectively referred to as diet I, II, III, and IV. All laying hens had free access to diet and water. The experiment was reviewed and approved by the University Animal Policy and Welfare Committee and was conducted in accordance with the Canadian Council on Animal Care guidelines.

7.2.2. Immunization of Hens with Bovine Serum Albumin (BSA)

After 5 wk of feeding experimental diets, the hens were injected intramuscularly with 1 mL BSA solution (1 mg/mL) in PBS and incomplete Freud's adjuvant emulsion (1:1, v/v) at four sites (two at left side and two at right side) in breast muscle. Identical booster vaccinations were given 2 wk after the first injection.

7.2.3. Sample Collection and Preparation

Three mL of blood (n = 8) was taken from wing vein weekly from before injection to 6 wk after the first injection. Blood samples were kept for 4 h at room temperature. After clotting the sera were collected by centrifuging at 250 x *g* for 10 min (Fritsche, et al., 1991a) and frozen at –20 C. Eggs were collected and counted daily, and weighed weekly. The water-soluble fraction of egg yolk was obtained using the method described by Wang et al. (2000a). After 23 wk of feeding the experimental diets, the hens were given the third booster immunization. One week later, the hens were inseminated. The fertilized eggs were collected and incubated. Sera from 11-day-old embryos and day-

old chicks (n = 8) were collected and stored at –20 °C before the analyses of IgG concentration and BSA-specific antibody IgG activity.

7.2.4. Antibody IgG Activity by ELISA

The ELISA method described by Li et al. (1998) was used to measure the specific antibody activity in serum and egg yolk. Briefly, 96-well polystyrene plates (Corning Inc., Corning, NY 14831) were coated by adding 150 μL of BSA solution (30 μg/mL) in coating buffer (1.59 g Na_2CO_3 and 2.93 g $NaHCO_3$ per liter distillated water, pH 9.6) to each well and incubating for 1.5 h at 37°C. The plates were washed with PBS containing 0.05% (v/v) Tween-20 (PBST) for three times. Samples (150 μL) of serum (1:2000 for hen serum, 1:1000 for hatchling serum and 1:100 for 11-d embryo in PBS) or WSF of egg yolk (1:3000 in PBS) were added in triplicate, and PBS was used as control. The plates were then incubated for 1.5 h at 37°C, washed with PBST, and incubated for 1 h at 37°C with 150 μL of a 1:1000 dilution of peroxidase conjugated goat anti-chicken IgG (Bethyl Laboratories, Inc., Montgomery, TX 77356). Plates were then washed with PBST, and added with 150 μL of substrate solution, 2, 2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (Sigma Chemical Co., St. Louis, MO 63178) in phosphate-citrate buffer (9.6 g $C_6H_8O_7$ and 14.1 g Na_2HPO_4 per liter, pH 5.0) containing 0.03% (w/w) sodium perborate. After 30 min reaction, the absorbence of mixture was read at a wavelength of 405 nm using an ELISA reader against the control.

7.2.5. IgG Concentration by Radial Immunodiffusion

Radial immunodiffusion as described by Wang et al. (2000a) was employed to measure the IgG concentration in the serum and egg yolk.

7.2.6. DTH Response

Two weeks after the second booster immunization, 0.2 mL of BSA solution (1 mg/mL in PBS) was injected subcutaneously into the flat area of both wing webs (two sites at left wing and two sites at right wing). The thicknesses of the injection sites in both wing webs were measured using a caliper (Beta Technology Inc., Cambridge, MD) before injection, 4 and 24 h post-injection, respectively. The DTH response was presented as wing web swelling following BSA injection, which was calculated as the increase of wing web thickness = the thickness of wing webs following BSA injection - the initial thickness of the wing webs.

7.2.7. Statistical Analysis

The results are presented as means ± SEM. All statistical analyses were conducted using the SAS statistical package (Version 7.0, SAS Institute, Cary, NC). One-way ANOVA was used to analyze the main effect of dietary n-6 to n-3 PUFA ratio on DTH response, IgG concentration and BSA-specific antibody IgG activity. The method of least squares means was employed to identify significant differences ($P < 0.05$) among the treatment means.

7.3. Results

The layer's body weight, egg production and egg weight were not affected ($P > 0.05$) by dietary ratio of n-6 to n-3 PUFA (LA to LNA) (Appendixes 1-4). After 5 wk of feeding experimental diets, the IgG content in hen serum (Table 7.3) was not different ($P > 0.05$) among the treatments, which were 5.6, 5.7, 7.5, and 6.4 mg/mL for the hens fed the diets with 0.8, 5.4, 12.4, and 27.7 of LA to LNA ratio, respectively. Two weeks after the primary immunization with BSA, a marked increase of IgG concentration in hen serum was observed as an evidence of 9.8, 11.2, 9.6, and 9.7 mg/mL, respectively for the hens fed diets with 0.8, 5.4, 12.4, and 27.7 of LA to LNA ratio. After the secondary immunization (booster), the serum IgG concentration did not show further elevation. There were no differences among the treatments ($P > 0.05$) after the primary and the secondary immunizations. The egg yolk IgG concentration was also not altered by feeding hens with the four diets ($P > 0.05$, Table 7.4). The immunization did not induce a significant elevation ($P > 0.05$) of yolk IgG concentration. The eggs from hens fed diets containing 0.8, 5.4, 12.4, and 27.7 of LA to LNA ratio had 14.3, 16.1, 14.3, and 16.3 mg/mL before the immunization, and 15.1, 16.6, 14.4, and 15.6 mg/mL after 6 wk of the primary immunization, respectively. The antibody IgG activity in both serum and egg yolk did not differ ($P > 0.05$) among the treatments at 2, 4, and 6 wk after the injection of BSA (Tables 7.3 and 7.4).

The total IgG (Fig. 7.1) and BSA-specific antibody IgG (Fig. 7.2) transferred from the egg yolk to the 11-day-old embryo showed a trend of decreasing with the increase of hen's dietary LA to LNA ratio, but these differences were not significant ($P >$

0.05). Upon hatching, however, the antibody IgG activity in the serum of hatching chicks differed significantly ($P < 0.05$) among the four maternal dietary treatments (Fig. 7.2). The chicks from hens fed the diet containing 0.8 of LA to LNA ratio had higher antibody IgG titer than those from hens fed the diet with 5.4 of LA to LNA ratio, and had significantly higher ($P < 0.05$) antibody IgG titer than those from the hens fed the diet containing 12.4 of LA to LNA ratio. When the maternal dietary ratio of LA to LNA was further increased from 12.4 to 27.7, the chick serum antibody IgG activity became not different form the others ($P > 0.05$). The IgG concentration in the sera of hatching chicks changed in a similar pattern with antibody IgG titer. The hatchlings from the hens fed the diet containing 12.4 of LA to LNA ratio had a lower ($P < 0.05$) serum IgG content than those from the hens fed diets containing 0.8, 5.4, and 27.7 of LA to LNA (Fig. 7.1). Similarly, when LA to LNA ratio was increased from 0.8 to 12.4, the serum IgG concentration in hatchling serum was decreased. When the ratio was increased from 12.4 to 27.7, the IgG concentration in the serum of hatching became not different ($P > 0.05$) from those chicks from hens fed diets containing 0.8 or 5.4 of LA to LNA ratio and higher than those from hens fed a diet with 12.4 of LA to LNA ratio.

As shown in Fig. 7.3, the DTH response measured after 4 h of the BSA injection was significantly influenced ($P = 0.005$) by dietary ratio of LA to LNA. The hens fed the diet with the lowest ratio of LA to LNA (0.8) got the smallest swelling at the injecting sites of wing webs, which did not differ from the hens fed diets with 5.4 and 12.4 of LA to LNA ratio, respectively. The largest swelling was observed in hens fed the diet containing the highest LA to LNA ratio (27.7), which was significantly larger that those fed the other three diets ($P < 0.05$). The swelling became smaller 24 h post-injection, but

significant differences ($P < 0.002$) still existed among the four treatments. The hens fed the diet containing the lowest LA to LNA ratio had the smallest swelling, which was significantly lower than the hens fed diets with 5.4 and 12.4 ($P < 0.01$), and 27.7 ($P < 0.0001$) of LA to LNA ratio, respectively. The hens fed the diet containing 5.4 of LA to LNA ratio had lower ($P < 0.05$) DTH response than those fed the diet with 27.7 of LA to LNA ratio.

7.4. Discussion

We found that different dietary ratios of n-6 to n-3 PUFA (LA to LNA) did not significantly affect the performance of laying hens, in terms of egg production, egg weight and body weight. The immunization of hens with BSA also did not evoke significant change of the performance in each one of groups fed the varied LA to LNA ratios.

After 5 wk of feeding hens with the diets that LA to LNA ratio ranged from 0.8 to 27.7 did not result in alteration of total IgG in serum or egg yolk. This result is similar with our previous study (Wang et al., 2000a). The primary immunization of the hens with BSA caused a marked increase of total IgG in the serum. Because of difficulty in obtaining a reference of antibody IgG against BSA, we did not measure absolute amount of antibody IgG. However, the antibody activity (titer) is often used as a convenient quantification of the relative antibody concentration (Carlsson and Lindberg, 1978). The immunization of laying hens with BSA resulted in a significant elevation of specific antibody IgG in serum and egg yolk, and peaked at 2 wk after the primary challenge and

kept slow increase after the booster until 6 wk, the last measurement performed. Different dietary LA to LNA ratios had no effect on the antibody production or activity in the serum and egg yolk of the hens. This result is in agreement with that reported by Fritsche et al. (1991a) that feeding chicks a diet containing 7% (w/w) fish oil (high in n-3 PUFA, LNA, EPA, and DHA) resulted in higher antibody production; but feeding the same amount of flaxseed oil (only rich in LNA) failed to alter antibody production. In contrast, Friedman and Sklan (1995) observed increased antibody titer to BSA in broiler chicks fed relatively high levels of n-3 PUFA (LNA). The discrepancy may come from the differences in the strain, sex, age, environment, immune status, and the composition of diet. It is evidenced that immuno-modulating effect of n-3 PUFA from fish oil is dependent on the type of grain used in the diet (Korver and Klasing, 1997). Jeffery et al. (1997b) reported that the nature of principal saturated fatty acids in the diet influences the immune responses. The level and source of dietary fat determine the precise modulating effect of dietary fatty acids on the indices of immunocompetence (Erichson et al., 1980; Friend et al., 1980; Berger et al., 1993). In the present study, different from most others, we kept the composition of other fatty acids in the maternal diets relatively constant, differing only in the ratio of LA to LNA.

A significant correlation between serum IgG concentration and BSA-specific antibody activity was found with 0.44 of the correlation coefficient (Fig. 7.4; $P = 0.01$). This suggests that total IgG may be used to assess the potential antibody production. It is very interesting that although the total IgG content and antibody IgG activity were not affected by the ratio of LA to LNA in the hen diets, the amounts of IgG and BSA-specific antibody IgG transferred from the yolk to embryo during the incubation were

significantly affected by the maternal dietary treatments. After 11 days of incubation, the amount of antibody IgG transferred from egg yolk to the embryo appeared to be decreased with the increase of the ratio of LA to LNA from 0.8 to 12.4 in the maternal diets. However, it was backed up when this ratio was further increased from 12.4 to 27.7, and became not different from the others. The total IgG transferred from egg yolk to embryo followed the same pattern with the antibody IgG. A significant correlation was found between the IgG concentration and specific antibody IgG in the serum of 11-day-old embryo (Fig. 7.5) and day-old chick (Fig. 7.6), with 0.49 ($P < 0.01$) and 0.44 ($P < 0.02$) of correlation coefficient, respectively. These results indicate that the alterations of BSA-specific antibody IgG activity in the serum of embryo or hatching chick were not a result of the modification of antibody affinity or binding activity. It is due to the alterations in the amount of antibody IgG transferred. The transportation of both total IgG and antibody IgG were affected by maternal dietary ratio of LA to LNA, and the amount of antibody IgG altered in parallel with the total IgG. The maternal antibodies are known to across the yolk sac intact (Rose et al., 1974; Kramer and Chow, 1970). Brambell (1969) proposed that the transfer of IgG in chicken, as well as in other species, occurs via a selective, receptor-mediated mechanism. This hypothesis has been supported by Linden and Roth (1978) who have characterized specific receptors for IgG. The results of the present study indicate that ratio of n-6 to n-3 fatty acids (LA to LNA) in yolk phospholipids may alter the activity of IgG-receptor or the permeability of yolk sac, and therefore, alter the IgG and antibody IgG transfer via yolk sac. It is well established that passive immunity plays critical roles in the immune defense of early chicks against various bacterial and viral infections while the chick's immune system is developing

(Rose, 1972; Smith et al., 1994). The increased total IgG and antibody IgG concentration in the circulation system of embryo or hatchling is, therefore, predicted to be beneficial to poultry health and production.

DTH response is an important inflammatory response, resulting from non-specific cell-mediated immune response to an antigen challenge (Calder, 1997b). Our study demonstrated that a low n-6 to n-3 PUFA (LA to LNA) ratio in the diet is associated with lower DTH response in laying hens. Similar results have been reported in mammals. For instance, a significant reduction in DTH response was observed following feeding diets rich in fish oil to rodents (Yoshino and Ellis, 1987; Kelley et al., 1989). Taki et al. (1992) reported suppression of the DTH response to sheep red blood cells in mice following tail-vein injections of emulsions of triacylglycerols rich in EPA or DHA. Both Kelley et al. (1991) and Meydani et al. (1993) reported that supplementation of the human diet with n-3 PUFA diminished the DTH responses to seven recall antigens. In avian, Korver and Klasing (1997) reported that incorporation of less than 2% (w/w) fish oil in chick diet resulted in a suppressed synthesis of acute phase protein in liver. This suppressed inflammatory response improved the growth and feed efficiency in chicks incurred moderate levels of coccidiosis (Korver et al., 1997). Similar effects have been observed in several other studies in chickens (Benson et al. 1993; Klasing et al., 1987; Roura et al., 1992; Takahashi et al., 1995), pigs (McCrachen et al., 1995), and rats (Peisen et al., 1995). The mechanism for the suppressive effect of n-3 fatty acids on inflammatory response has not been well established. The decreased production of pro-inflammatory eicosanoids and cytokines by n-3 PUFA might be the factor involved. It has been shown in previous studies that the decrease of dietary ratio of n-6 to n-3 PUFA increases the n-3

content in the phospholipids of immune cells (Fritsche, et al., 1991a; Wang et al., 2000a,b). This in turn results in a decreased production of pro-inflammatory eicosanoids (Boudreau et al., 1991; Broughton et al., 1991) and cytokines (Endres et al., 1989), and also decreased responsiveness of target cells to pro-inflammatory signals (Cooper and Rothwell, 1993; Mulrooney and Grimble, 1993).

Thus in practical poultry husbandry, n-3 PUFA may benefit growth when the birds are challenged with pathogens. Additionally, when birds are reared in commercial-type environments with the built-up of dust, dander, and feces, the inflammatory responses are constantly stimulated (Korver and Klasing, 1997). N-3 PUFA might be useful to minimize the catabolic effect of inflammation induced by environmental challenges, permitting a higher productivity. However, deleterious effects may also be generated from feeding an inappropriate low ratio of n-6 to n-3 PUFA to chickens by suppressing cell-mediated immune response as indicated from mammal studies (Chang et al., 1992b; Mayatepek et al., 1994). However, there might be a range of dietary n-6 to n-3 PUFA ratio in a certain range of total fat within which animal can maintain appropriate cell-mediated immunity, and meanwhile reduce inflammatory responses, thus maintain health and high productivity.

TABLE 7.1. Composition of laying hen basal diet

Gradients	g/kg
Wheat	669
Soybean meal	162
Limestone ground	77
Dicalcium-phosphate	28
Sodium chloride	3.3
DL-Methionine	0.6
Layer Premix[1]	5
Choline chloride Premix[2]	5
Calculated composition	
ME (kcal/kg)	2800
Crude protein (%)	16.3

[1]Layer premix provides per kg diet: vitamin A, 12,000 IU; vitamin D3, 3,000 IU; vitamin E, 40 IU; vitamin K, 2.0 mg; pantothenic acid, 14.0 mg; riboflavin, 6.5 mg; folacin, 1.0 mg; niacin, 40.0 mg; thiamine, 3.3 mg; pyridoxine, 6.0 mg; vitamin B12, 0.02 mg; biotin, 0.2 mg; iodine, 0.5 mg; manganese, 75.0 mg; copper, 15.0 mg; zinc, 80.0 mg; selenium, 0.1 mg; iron, 100.0 mg.

[2]Choline chloride premix contains (per kg) 34 g choline chloride (60%) and 966 g wheat shorts.

TABLE 7.2. Fatty acid composition of the diets

	Diet [1]			
	I	II	III	IV
Fatty acid[2]	*% of total fatty acids*			
16:0	10.3	10.7	10.7	10.7
18:0	3.3	3.6	3.7	3.7
16:1	0.2	0.2	0.2	0.2
18:1	19.8	20	20.9	21.1
18:2(n-6)	26.2	53.8	58.6	60.9
18:3(n-3)	39.6	10.0	4.7	2.2
SFA	10.4	11.3	11.4	11.4
MUFA	20.5	21.3	21.5	21.7
PUFA	65.8	63.8	63.4	63.1
P/S ratio	6.3	5.7	5.6	5.5
n-6/n-3 ratio	0.7	5.4	12.4	27.7

[1]I, II, III, and IV mean diets containing 5% (w/w) linseed oil, mix of 1% linseed oil and 4% sunflower oil, mix of 0.35% linseed oil and 4.65% sunflower oil, and 5% sunflower oil, respectively to provide 0.7, 5.4, 12.4, and 27.7 of n-6 to n-3 PUFA ratio

[2]SFA, sum of saturated fatty acids; MUFA, sum of monounsaturated fatty acids; PUFA, sum of polyunsaturated fatty acids; P/S ratio, ratio of polyunsaturated to saturated fatty acids; n-6/n-3 ratio, ratio of n-6 to n-3 polyunsaturated fatty acids.

TABLE 7.3. Effect of dietary linoleic acid to linolenic acid ratio on serum IgG concentration and BSA-specific antibody IgG activity in laying hens[1]

	Post-injection of BSA in weeks							
	0	2	4	6	0	2	4	6
Diet[2]	IgG concentration (mg/mL)				IgG activity			
I	5.6 ± 0.9	9.8 ± 1.4	9.1 ± 1.3	9.4 ± 1.6	0.05 ± 0	0.50± 0.05	0.63± 0.12	0.97 ± 0.11
II	5.7 ± 0.7	11.0 ± 1.5	11.8 ± 1.2	11.5 ± 1.4	0.05 ± 0	0.74± 0.16	0.83± 0.09	1.09 ± 0.10
III	7.5 ± 1.3	9.6 ±. 2.2	8.7 ± 1.1	8.8 ± 2.1	0.06± 0.01	0.53± 0.09	0.66± 0.19	1.23 ± 0.04
IV	6.4 ± 1.3	9.7 ± 2.3	11.0 ± 1.4	9.6 ± 2.0	0.05± 0.01	0.69± 0.05	0.78± 0.09	1.20 ± 0.04

[1]BSA, bovine serum albumin. Values are mean ± SEM (n = 8). For IgG concentration and antibody IgG activity at each week, there was no significant effect of dietary linoleic acid to linolenic acid ratio by one-way ANOVA ($P > 0.05$)

[2]I, II, III, and IV mean diets containing 0.7, 5.4, 12.4, and 27.7 of linoleic acid to linolenic acid ratio, respectively.

TABLE 7.4. Effect of dietary linoleic acid to linolenic acid ratio on egg yolk IgG concentration and BSA-specific antibody IgG activity in laying hens[1]

	Post-injection of BSA in weeks							
	0	2	4	6	0	2	4	6
Diet[2]	IgG concentration (mg/mL)				IgG activity (titer)			
I	14.3 ± 1.6	15.7 ± 1.2	14.4 ± 1.1	15.1 ± 0.9	0.05 ± 0.01	0.75 ± 0.04	0.88 ± 0.07	1.07 ± 0.02
II	16.1 ± 1.0	16.7 ± 0.6	15.5 ± 0.7	16.6 ± 0.4	0.06 ± 0.01	0.82 ± 0.03	0.96 ± 0.04	1.06 ± 0.02
III	14.3 ± 1.0	15.6 ± 0.9	14.4 ± 0.6	14.1 ± 0.8	0.05 ± 0	0.80 ± 0.05	0.92 ± 0.07	1.15 ± 0.09
IV	16.3 ± 0.8	15.9 ± 0.7	15.6 ± 0.8	15.9 ± 0.7	0.05 ± 0.01	0.93 ± 0.01	1.02 ± 0.02	1.08 ± 0.01

[1]BSA, bovine serum albumin. Values are mean ± SEM (n = 8). For IgG concentration and antibody IgG activity at each week, there was no significant effect of dietary linoleic acid to linolenic acid ratio by one-way ANOVA ($P > 0.05$)

[2]I, II, III, and IV mean diets containing 0.7, 5.4, 12.4, and 27.7 of linoleic acid to linolenic acid ratio, respectively.

FIGURE 7.1. Effect of maternal dietary linoleic acid to linolenic acid ratio on the total IgG transferred from egg yolk to the circulation of 11-day-old embryo and day-old chick. Values are mean ± SEM (n = 8). For each total IgG, the main effects were analyzed by a one-way ANOVA and the method of least squares means was used to determine significant ($P < 0.05$) differences among groups. For each total IgG, values that do not have a common superscript are significant different ($P < 0.05$). I, II, III, and IV mean the diets containing 0.8, 5.4, 12.4, and 27.7 of linoleic acid to linolenic acid ratio, respectively.

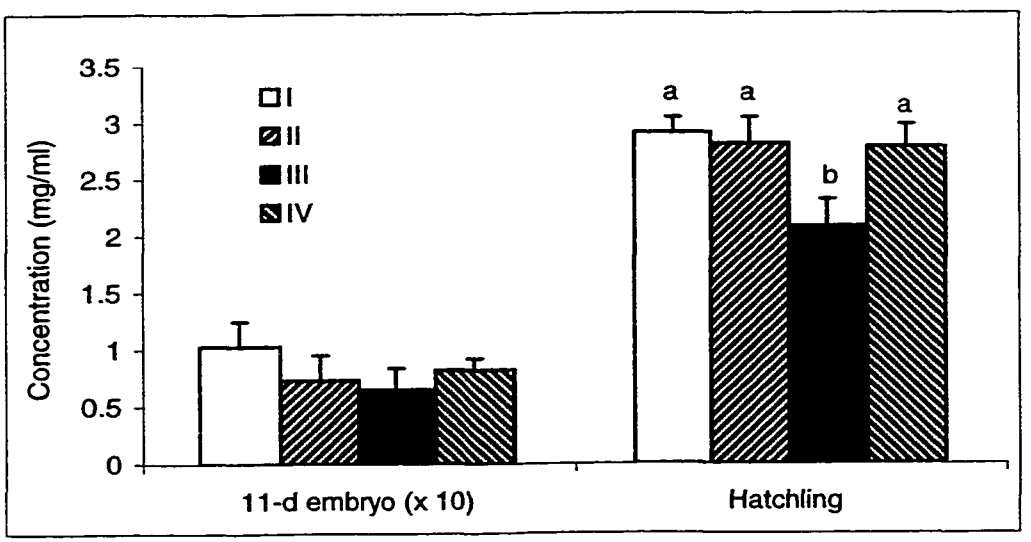


FIGURE 7.2. Effect of maternal dietary linoleic acid to linolenic acid ratio on the bovine serum albumin-specific antibody IgG transferred from egg yolk to the circulation of 11-day-old embryo and day-old chick. Values are mean ± SEM (n = 8). For each antibody IgG, the main effects were analyzed by a one-way ANOVA and the method of least squares means was used to determine significant ($P < 0.05$) differences among groups. For each antibody IgG, values that do not have a common superscript are significant different ($P < 0.05$). I, II, III, and IV mean the diets containing 0.8, 5.4, 12.4, and 27.7 of linoleic acid to linolenic acid ratio, respectively.

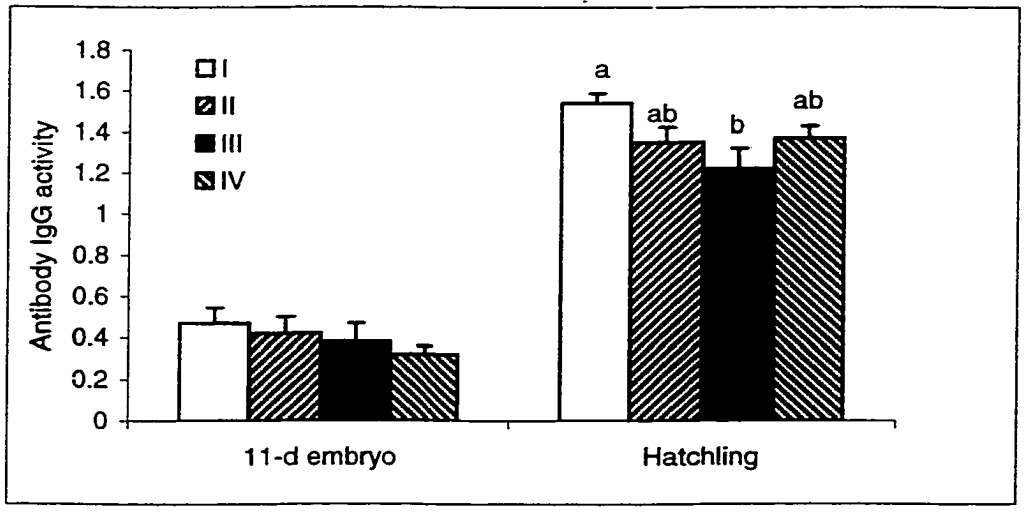


FIGURE 7.3. Effect of dietary linoleic acid to linolenic acid ratio on hen's delayed-type hypersensitivity (DTH) response. The DTH index was the average swelling thickness at the sites injected with bovine serum albumin in both wing webs, which was calculated as the increase of wing web thickness following the injection of bovine serum albumin = the thickness of wing web following the injection of bovine serum albumin – the initial thickness of the wing webs. The main effects were analyzed by a one-way ANOVA and the method of least squares means was used to determine significant ($P < 0.05$) differences among groups. Values that do not have a common superscript are significant different ($P < 0.05$). I, II, III, and IV mean the diets containing 0.8, 5.4, 12.4, and 27.7 of linoleic acid to linolenic acid ratio, respectively.

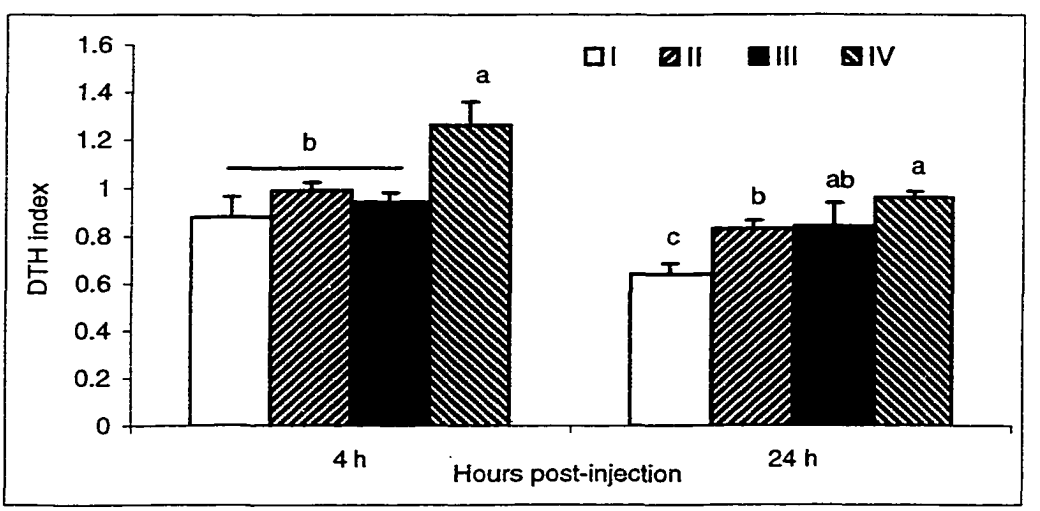


FIGURE 7.4. Relationship between the total IgG concentration and the bovine serum albumin-specific antibody IgG activity in the serum of the laying hens. The total IgG and bovine serum albumin-specific antibody IgG activity from the hens fed diets containing different ratios of LA to LNA were paired for each hen and pooled together to determine correlation using the CORR procedure in SAS package ($P = 0.01$).

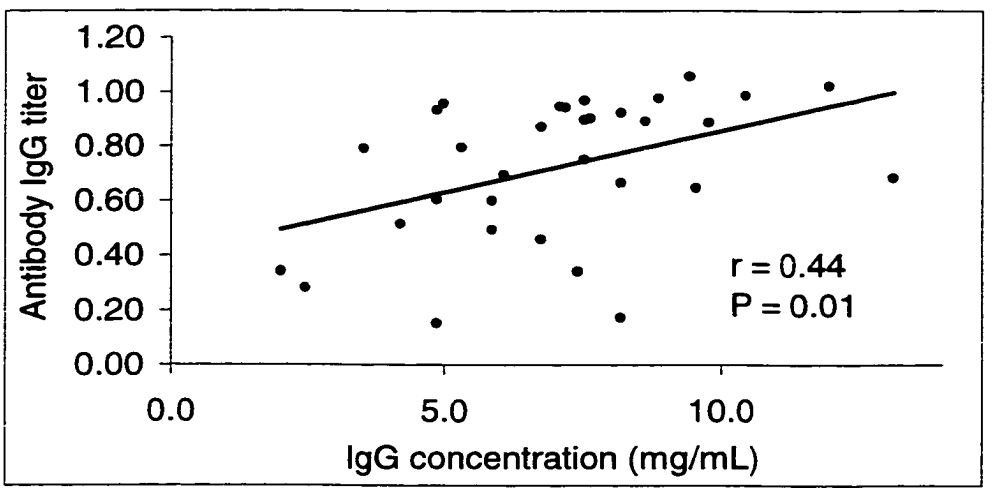


FIGURE 7. 5. Relationship between the total IgG concentration and the bovine serum albumin-specific antibody IgG activity in the serum of 11-day-old embryo. The pairs of total IgG and bovine serum albumin-specific antibody IgG activity for hens from the four groups were pooled together and used to determine correlation using the CORR procedure in SAS package ($P < 0.01$).

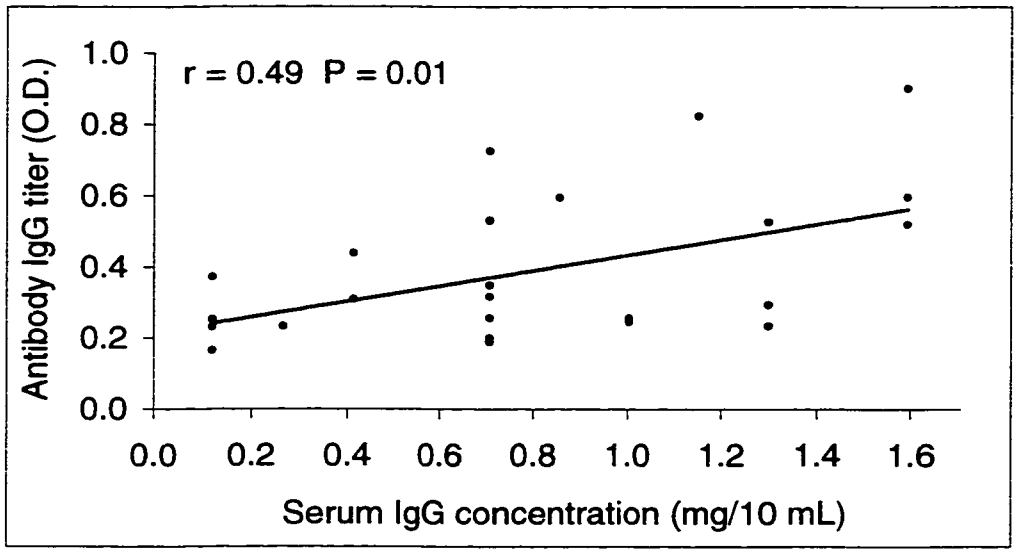
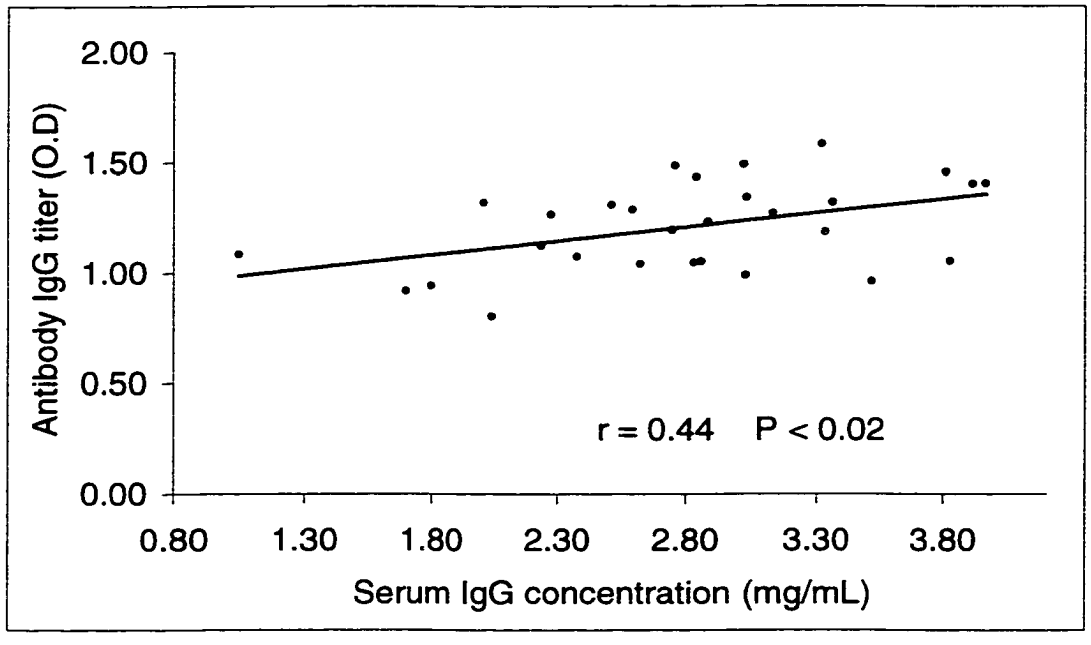


FIGURE 7.6. Relationship between the total IgG concentration and the bovine serum albumin-specific antibody IgG activity in the serum of day-old chick. The pairs of total IgG and bovine serum albumin-specific antibody IgG activity for hens from the four groups were pooled together and used to determine correlation using the CORR procedure in SAS package ($P < 0.02$).



8. GENERAL SUMMARY AND DISCUSSION

8.1. Summary of Results

The objectives of this research were to test the following general working hypotheses:

I. DIETARY N-3 PUFA WILL DECREASE THE RATIO OF N-6 TO N-3 FATTY ACIDS IN THE IMMUNE TISSUES OR CELLS AND THUS THE IMMUNE FUNCTIONS IN LAYING HENS

This hypothesis was supported by the results reported in Chapter 3, 4, and 5. Specifically, it was hypothesized that:

A. Dietary supplementation of n-3 fatty acids will increase the composition of these fatty acids in laying hen immune tissues or cells with concomitant decrease of n-6 fatty acids.

The results reported in Chapter 3 and 4 supported this hypothesis. The hens fed higher levels of n-3 PUFA derived from linseed oil or fish oil has significantly higher n-3 fatty acid content and a significantly lower ratio of n-6 to n-3 PUFA ratio in the spleen lymphocytes (Chapter 3). Higher levels of dietary n-3 fatty acids resulted in higher proportional amount of n-3 PUFA in the thymus, spleen, and bursa and the lymphocytes isolated from both blood and spleen of chicks (Chapter 5).

B. High levels of dietary n-3 fatty acids will suppress mitogenic responses of immune cells in chicken.

Our findings in Chapter 3 and 5 supported this hypothesis. Feeding high levels of n-3 PUFA significantly suppressed spleen and blood lymphocyte proliferation to Con A stimulation in laying hens (Chapter 3). The thymus and spleen lymphocyte proliferation to Con A or PWM was also significantly suppressed in the chicks fed higher levels of n-3 PUFA (Chapter 5).

C. Dietary ratio of n-3 to n-6 fatty acids will change the proportions of T and B cell populations.

This hypothesis was not supported by the results in Chapter 3 and partially supported by the results in Chapter 5. Dietary fat composition had no significant effect on the proportions of T- and B-lymphocytes in both spleen and blood of laying hens (Chapter 3). However, the chicks from the hens fed high levels of n-3 PUFA derived from linseed oil and continued, following hatching, to be fed the same type of fat diet had a higher proportion of $CD8^+$ T cells and IgM^+ B cells (Chapter 5).

D. The ratio of dietary n-6 to n-3 PUFA will alter the total IgG concentrations in chicken serum and egg yolk.

The results in Chapter 3 and 5 supported this hypothesis. In laying hen, higher levels of dietary n-3 PUFA produced higher serum and egg yolk IgG concentrations. In contrast, higher levels of n-6 PUFA were associated with lower IgG concentration in serum and egg yolk (Chapter 3). The chicks from the hens fed higher levels of n-3 PUFA derived from fish oil and continued to be fed the same type of fat diet following hatching had a higher total IgG concentration in the serum (Chapter 5).

II. MATERNAL AND NEONATAL DIETARY FAT TYPE WILL CHANGE THE COMPOSITIONS OF N-6 AND N-3 FATTY ACIDS IN THE IMMUNE TISSUES OR CELLS AND THE IMMUNE TISSUE GROWTH OF CHICKS

This hypothesis was supported in Chapter 4. Specially, it was hypothesized that:

E. Dietary n-3 and n-6 PUFA will differently affect the incorporation of these fatty acids into the immune tissues (spleen, thymus, and bursa of Fabricius) and lymphocytes from different tissue sites.

The results in Chapter 4 supported this hypothesis. The thymus had a higher proportion of LA, but lower level of AA, and similar level of each longer-chain n-3 PUFA with spleen. Bursa had the lowest proportion of each n-3 and n-6 fatty acid. The lymphocytes from spleen had significantly higher LA, AA, and each n-3 PUFA than the lymphocytes from blood (Chapter 4).

F. Effects of dietary PUFA on the compositions of n-3 fatty acids in the immune tissues or cells will be remained in the next generation if the progeny were fed the same types of diets as the maternal.

This hypothesis was supported by the results in Chapter 3 and 4. The fatty acid composition in laying hen splenocytes was significantly increased by feeding higher levels of n-3 fatty acids, with concomitant decrease of n-6 PUFA (Chapter 3). The expanded study from this experiment showed that this effect could be remained in the next generation if the progeny were fed diets containing the same amount and type of fat as in the maternal (Chapter 4).

G. Amount and type of maternal and neonatal dietary PUFA will alter immune tissue development of chicks.

This hypothesis was supported by the findings in Chapter 4. Significantly higher weights of the thymus, spleen and bursa were observed in the chicks with higher levels of maternal and neonatal dietary PUFA in spite of n-3 or n-6 at the age of 4 wk. This effect disappeared in the period of 5-8 wk for thymus and spleen and the weights of these two tissues became not different among the four treatments. Whereas, the bursa weight was significantly lower at 8 wk for the chicks with higher levels of n-3 PUFA, especially n-3 PUFA from fish oil.

III. MATERNAL AND NEONATAL DIETARY N-6/N-3 FATTY ACIDS WILL AFFECT THE IMMUNE RESPONSES OF THE PROGENY

The immunomodulatory effects of maternal and neonatal (long-term) dietary fatty acids on the immune functions of the chicks were reported in Chapter 5. Specifically, it was hypothesized that:

H. Maternal and neonatal dietary n-3 fatty acids will suppress the thymus and spleen lymphocyte proliferation to Con A, PHP, and PWM stimulation.

The results in Chapter 5 partially supported this hypothesis. Higher levels of maternal and neonatal dietary n-3 PUFA significantly suppressed spleen and thymus lymphocyte proliferation to Con A stimulation and spleen lymphocyte proliferation to PWM stimulation. The PHA-induced proliferation of the thymus and spleen lymphocytes was not affected by dietary n-3/n-6 fatty acids.

I. Maternal and neonatal dietary n-3/n-6 fatty acids will change T and B lymphocyte populations in chick spleen.

The results in Chapter 5 supported this hypothesis. Maternal and neonatal dietary n-3 PUFA derived linseed oil significantly increased chick spleen $CD8^+$ T lymphocytes. An increased proportion of the spleen IgM^+ B-lymphocytes was observed in the chicks from

hens fed higher levels of n-3 PUFA derived from linseed or fish oil and continued, following hatching, to be fed the same types of diets as the maternal.

J. Increase of n-3 PUFA in maternal and neonatal diets will elevate total IgG concentration in chick serum.

This hypothesis was supported by the results in Chapter 5. The chicks from the hens fed higher level of n-3 PUFA derived from fish oil and given the same type of fat diet following hatching had significantly higher serum IgG concentration than the chicks fed diets containing higher levels of n-6 PUFA.

IV. DIETARY SOURCE OF N-3 PUFA WILL AFFECT THE COMPONENTS OF N-3 FATTY ACIDS INCORPORATED INTO THE IMMUNE TISSUES OR CELLS, THUS DIFFERENTLY AFFECT THE IMMUNE FUNCTIONS IN CHICKEN

This hypothesis was supported by the results in chapter 3, 4 and 5. Specifically it was hypothesized that:

K. The enrichments of longer-chain n-3 PUFA, EPA, DPA and DHA, in chicken immune tissues or cells will depend on the forms of dietary n-3 fatty acids.

Feeding preformed longer-chain n-3 PUFA derived from fish oil resulted in higher levels of these fatty acids in the immune tissues or cells than feeding the same amount of parent, linolenic acid (Chapter 3 and 4).

L. N-3 fatty acids derived from linseed oil and fish oil will differently affect cell-mediated and humoral immune responses of chickens.

Our findings in Chapter 3 and 5 partially supported this hypothesis. N-3 PUFA derived from fish oil and linseed oil had no significantly different effect on spleen and blood lymphocyte proliferation to Con A and lymphocyte subset populations in laying hens (Chapter 3). The hens fed a diet containing higher level of n-3 PUFA derived from linseed oil had significantly higher serum IgG than the hens fed n-3 fatty acids derived from fish oil (Chapter 3). Higher level of n-3 PUFA derived from fish oil in maternal and neonatal diets significantly increased the IgG concentration in chick serum (Chapter 5). Compared with the same level of n-3 fatty acids derived from fish oil, high level of n-3 PUFA derived from linseed oil in maternal and neonatal diets had stronger suppressive effect on the spleen lymphocyte proliferation to Con A stimulation in chicks (Chapter 5).

V. MATERNAL DIETARY N-3/N-6 PUFA WILL AFFECT THE RATIO OF N-6 TO N-3 PUFA IN THE IMMUNE TISSUES AND ALTER THE INFLAMMATORY RESPONSE OF CHICKS FOLLOWING HATCHING

The results in Chapter 6 supported this hypothesis. Specifically, it was hypothesized that:

M. Dietary n-3/n-6 fatty acids will change the n-3 PUFA incorporation into the egg yolk.

N. Maternal dietary n-3 fatty acids will via egg yolk alter the incorporation of these fatty acids into the embryo immune tissues during incubation. This effect will last for certain time after hatching.

Our findings reported in Chapter 7 supported these hypotheses. The hens fed higher levels of n-3 PUFA derived from fish oil significantly increased the incorporation of longer-chain n-3 fatty acids into egg yolk. As a result, a significant increase of n-3 PUFA in the immune tissues (such as spleen) of hatching chicks was observed. Under the experimental dietary conditions (18% n-6 and 7% n-3 fatty acids, w/w), two and four weeks following hatching, the amount of n-3 PUFA in chick spleen was still significantly higher in the chicks from the eggs laid by the hens fed higher levels of n-3 PUFA.

O. Dietary n-3 fatty acids will suppress inflammatory response of the hens

P. Maternal dietary n-3 fatty acids will suppress the inflammatory response of the offspring (broilers) following hatching.

These two hypotheses were supported by the results reported in Chapter 7. The inflammatory response as measured by delayed-type hypersensitivity was significantly suppressed in the hens fed higher levels of n-3 PUFA. This effect was strongly remained in the progeny (broilers) until 4 wk following hatching.

VI. THE RATIO OF DIETARY N-6 TO N-3 PUFA PLAYS A ROLE IN REGULATING CHICKEN IMMUNE RESPONSES TO BSA

The precise effects of dietary ratio of n-6 to n-3 PUFA on chicken immune functions were reported in Chapter 7. Specifically, it was hypothesized that:

Q. Decrease of dietary n-6 to n-3 fatty acid ratio will suppress chicken inflammatory response.

This hypothesis was supported by the results reported in Chapter 7. The inflammatory response in laying hens as measured by delayed-type hypersensitivity was significantly suppressed when the birds were given lower ratios of n-6 to n-3 PUFA.

R. Dietary ratio of n-6 to n-3 fatty acids will alter the total IgG and antibody IgG production.

This hypothesis was not supported by the results in Chapter 7. There was no effect of dietary n-6 to n-3 PUFA ratio on the total IgG concentration and antibody activity to bovine serum albumin in the hen serum.

S. Total serum IgG concentration will reflect the potential capability of chickens for producing specific antibody IgG.

This hypothesis was verified by the results in Chapter 7. There is significant correlation between the total IgG concentration and specific antibody IgG activity against bovine serum albumin in laying hen serum.

T. Maternal-fetal transfer of specific antibody IgG will be affected by maternal dietary fatty acids via yolk fatty acids.

U. Net transfer of specific antibody IgG to the circulating system of embryo will be associated with the total IgG transportation via yolk sac.

These two hypotheses were partially supported by the results in Chapter 7. With increase of dietary n-6 to n-3 PUFA ratio from 0.8 to 5.4 and to 12.4, the amounts of total IgG and antibody IgG transferred from the egg yolk to the embryo (11-d) and hatching chick were significantly decreased. However, further increase of n-6 to n-3 PUFA ratio in hen diet to 27.7, the amounts of IgG and antibody IgG in the embryo and hatching chick was backed up and became not different from the other groups. The amounts of bovine serum albumin-specific antibodies transferred from egg yolk to the circulating system of embryo and hatching chick were significantly correlated with the amounts of total IgG transferred.

8.2. General Discussion

PUFA are involved in maintaining a variety of physiological processes and therefore are viewed to be important nutrients that perform key roles in nutrition in both health and disease status (Fernandes and Venkatraman, 1993; Calder 1997b, 1998a, b). The fatty acid composition of essential fatty acids in the immune tissues or cells could be readily modified by dietary fatty acid manipulation, with strong competition between n-6 and n-3 families. The immunologically important n-6 PUFA is AA and n-3 PUFA are EPA and DHA. Inclusion of n-3 fatty acids in chicken diet resulted in an increase of these fatty acids and decrease of AA in the immune tissues or cells. The incorporation of AA into the immune tissues or cells was limited, which might be due to the limited ability of synthesizing it from its precursor or a saturation process. Feeding chickens with a diet containing 5% (w/w) of linseed oil resulted in a half amount of EPA and DHA in the immune tissues or cells compared with feeding the same amount of fish oil. This indicates that the enrichment of n-3 longer-chain n-3 PUFA depends on the forms of n-3 fatty acids in the diet.

The modification of fatty acid composition in chicken immune tissues or cells associated with significant alterations of the immune functions. The laying hens fed with diets containing higher levels of n-3 PUFA had significantly lower spleen and blood lymphocyte rates of proliferation in response to Con A stimulation. In contrast, feeding the hens with higher level of n-3 fatty acids derived from linseed oil significantly increased the serum and egg yolk content of IgG (Chapter 3). In this study, we fed the laying hens for only over 5 wk, however, for practical use, it is necessary to understand the long-term effect of dietary n-3 and n-6 PUFA on chicken immune responses. In addition, the development events important for immunocompetence begin in the embryo

and continue during the first week following hatching (Gobel, 1996; Ratcliff et al., 1996). The first week of life is a period of rapid expansion of leukocyte populations, seeding of lymphoid organs, and educational events that produce the unique clones of lymphocytes that will mediate immunity later in life (Klasing, 1998). Moreover, the immune system of young chicks may be more sensitive to the fatty acid manipulation than the adult chickens. Therefore, we conducted another experiment aimed at investigating the effect of maternal and neonatal dietary fatty acids on the lymphocyte proliferation and IgG production of the progeny. The results showed that maternal and neonatal dietary n-3 and n-6 fatty acids had similar effect on both lymphocyte proliferation and IgG production as in the hens (Chapter 5). In another word, the immunomodulatory effect of dietary n-3 PUFA on the immune responses in laying hens could be remained in a long-term (two generations).

Different immune classes perform different immune functions. It has been thought that the alteration of immune response could be a result of alterations of lymphocyte subset populations (Robinson and Field, 1998). The results of our study carried out in laying hens showed that dietary fat composition did not affect the T and B lymphocyte populations in spleen and blood (Chapter 3). Although high level of maternal and neonatal n-3 PUFA derived from linseed oil increased the proportion of spleen $CD8^+$ T cells but not the ratio of $CD4^+/CD8^+$ cells; the n-3 PUFA derived from both linseed oil and fish oil elevated IgM^+ B cells in chick spleen but only fish oil (5%, w/w) increased chick serum IgG concentration (Chapter 5). These results suggest that the effect of n-3 PUFA on chicken immune functions is not attributed to the alterations of lymphocyte subset populations, the alteration of lymphocyte function might be involved.

Maternal and neonatal dietary n-3 and n-6 PUFA affected chick immune tissue development up to 8 wk of age. The growths of the thymus, spleen, and bursa were significantly enhanced by higher levels of dietary PUFA in spite of n-6 or n-3 families in the period of 0-4 wk following hatching. However, this effect was disappeared for thymus and spleen during the period of 5-8 wk (Chapter 4). Whereas the bursa growth was slowed down from 5 to 8 wk by feeding chicks with PUFA, especially n-3 PUFA-rich diets. The bursa weight became significantly lower in the chicks fed higher levels of n-3 PUFA-rich diets, in particular, n-3 PUFA derived from fish oil diet. Anatomically, the bursas from the chicks fed fish oil diet became flat and almost withered. The altered development of three immune tissues by maternal and neonatal dietary n-3 and n-6 PUFA had not been found to be associated with the lymphocyte functions. Because the bursa is the immune site where the B-lymphocyte development and differentiation. The function of B-lymphocytes may therefore be used to assess the maturation and functionality of bursa. Although n-3 PUFA derived from fish oil increased chick serum IgG concentration at 8 wk, it is not known whether n-3 PUFA promoted the bursa growth and maturity, and thus induced early withering or inhibited growth in the course of 5 to 8 wk. The cell number and activity of B-cells in the bursa are crucial to assess the effect of dietary n-3 PUFA on the bursa development. Therefore, more research is required to determine the composition of bursa.

It is evident that the immunomodulatory effects of dietary n-6 and n-3 PUFA on the hen continued in the offspring in a similar manner as in the maternal if the progeny were given the same type of fat diets as the maternal. However, it is not known this is the maternal effect or neonatal dietary effect or the combination effect of both. It has been

reported that fat source in hen diets significantly change the fatty acid composition of egg yolk, which provides the only source of nutrients for the embryo during incubation (Cherian and Sim, 1991). The question of whether and how maternal dietary fatty acids influence the fatty acid composition of chick immune tissues was raised and examined. The results (Chapter 6) showed that the essential fatty acid composition of chick spleen reflected that of the egg yolk as reported in other tissues (Cherian and Sim, 1991,1992,1995), with significant modifications occurred. The amount of MUFA was greatly reduced but PUFA was increased, especially longer-chain n-6 PUFA, AA and longer-chain n-3 PUFA, EPA and DHA (Chapter 6). This suggested a strong demand of these fatty acids by the embryo and day-old chick.

It is interesting that the differences in the composition of each fatty acid of SFA, MUFA, and n-6 PUFA disappeared 2 wk following hatching, however, the differences in the relative compositions of n-3 fatty acids were maintained, in particular, EPA and DHA. Even 4 wk after hatching, the fatty acid composition of DHA was still significantly different among the chicks from the hens given different levels of dietary n-3 fatty acids, indicating a strong demand of growing chicks for DHA and reserving action against the depletion of DHA (Chapter 6). This may indicate that the utilizations of n-6 and n-3 PUFA by broilers are different or broilers need more n-3 PUFA than the amount supplied by the commercial broiler diets applied in this study. The DTH response was significantly suppressed in the chicks from the hens fed higher levels of n-3 PUFA at 2 or 4 wk of age (Chapter 6). The influence of maternal dietary n-3 fatty acids on the immune functions of progeny may be due to n-3 PUFA. It could also be the influence of n-3 PUFA on the immune tissue development. We found that immune tissue weights were

affected by maternal and neonatal dietary n-3 PUFA, but the functions of immune tissues (immune cell number and activity) need further investigation.

Other fatty acids except PUFA also influence the immune functions (Jeffery et al., 1997a,b; Yaqoob, 1998). The precise immunomodulatory effect of n-6 to n-3 PUFA ratio was examined in another study by feeding laying hens with the diets differing only in the amounts of LA and LNA (Chapter 7). This study demonstrated that cell-mediated immune response in laying hens was significantly suppressed with the increase of the n-3 PUFA (LNA) or the decrease of the ratio of n-6 to n-3 PUFA (LA to LNA), with the total IgG and BSA-specific antibody IgG production in serum and egg yolk unaffected. This is not in agreement with our previous studies (Chapter 3) and also different from that reported by Friedman and Sklan (1995). An interesting finding observed from this study is that the amount of antibody IgG or total IgG transferred from egg yolk to the circulating system of embryo during the incubation was associated with the yolk n-3 fatty acids, LNA. If this effect is reproducible, it should be beneficial to the early chicks because antibodies transferred from the yolk to the embryo provide the acquired immunity in early chick while its immune system is developing (Smith et al., 1994).

The induction of antibody production for assessing humoral immune response often evokes concerns about animal welfare. Significant correlations of serum total IgG with BSA-specific antibody IgG in the serum of laying hens and the sera of 11-d embryos and hatching chicks might suggest an alternative approach to assess humoral immune response in chicken. The mechanism(s) for the increased transfer of total IgG and BSA-specific antibody IgG from egg yolk to the circulating system of 11-d embryo and day-old chick by maternal n-3 PUFA during the incubation is not clear. The modification of

IgG receptor activity on the yolk sac membrane or the alteration of yolk sac membrane permeability may be involved.

The cell-mediated immunity of young chicks as measured by cell proliferation was suppressed by n-3 PUFA as reported by Fritsche et al., (1991a) and Korver and Klasing (1997). Our studies had shown that higher levels of dietary n-3 fatty acids also suppressed the mitogenic response of adult chicken (laying hen) lymphocyte proliferation. Inflammatory response is non-specific cell-mediated immune response. This response in adult (laying hen and broiler breeder) and young chicken (broilers) as measured by DTH response was suppressed by feeding higher levels of dietary n-3 PUFA. The inflammatory response is a part of innate immunity and provides the first line of the protection of the host from various pathogens, and is critically important in maintaining the health. Overreaction of inflammatory responses could cause diseases such as chronic inflammation and autoimmunity. In these cases, appropriate suppression of inflammatory responses has been reported to be beneficial (Grimble, 1998; Harbige, 1998; James et al., 2000; Kremer, 2000). Similar results have been reported in chicken by Korver and Klasing (1997) that feeding young chicks with fish oil ($\leq$ 2%) suppressed inflammatory responses and improved the production of chicks with moderate levels of coccidial infection (Korver and Klasing, 1997, Korver et al., 1997). A different approach used in our study had showed that maternal dietary n-3 fatty acids (2.5 - 5% fish oil) increased the composition of these fatty acids in the spleen of progeny up to 4 wk, but did not affect the feed intake, growth rate, feed efficiency and the mortality of healthy chicks up to 6 wk under the experimental conditions (Appendix 4-6). However, in commercial environment, there is dust, dander and feces, which may continuously stimulate the

inflammation of the chickens. Under this situation, the inclusion of certain amount of n-3 PUFA may be beneficial to the chicken health and productivity (Korver and Klasing, 1997). However, the suppression of inflammatory response could also place the chicken at a risk of increasing susceptibility to infectious challenges. Therefore, for practical use, more investigations are warranted.

The IgG is the major antibodies secreted in the second humoral response, and is the only class of immunoglobulins capable of crossing the placental barrier (Nysather et al. 1976). The enhanced antibody production by feeding n-3 PUFA is assumed to strengthen the humoral defense of the chicken though there is no evidence has been reported yet. On the other hand, in avian (except pigeon), all the maternal immunoglobulins needed to protect hatching chick must be present in the egg laid, and transported from the yolk across the yolk sac to the circulation of the developing chick (Brambell, 1969). The increase of maternal antibody transfer from yolk to the circulating system of embryo and hatching chick is critically important for the early chicks while their immune systems are developing.

In our study, a significant heterogeneity responses to dietary fatty acids were observed among the immune tissues or between the different origins of lymphocytes, indicating that dietary lipids may modify the fatty acid composition of various immune tissues or cells in different patterns (Chapter 4). This effect could also be a result of different composition of immune tissues. The mechanisms for this heterogeneity have not been established. The proposed mechanisms have been discussed in chapter 4. The heterogeneity of fatty acid incorporation into different immune tissues or different origins of immune cells may implicate that dietary fatty acids have different modulations on the

immune functions at different immune tissue sites. The differences of immune cell subset populations among the immune tissues could be an important factor.

In conclusion, dietary fatty acids had profound effects on chicken immune system including the fatty acid composition and immune functions. At 5% (w/w) in the diet, the level of PUFA and the ratio of n-6 to n-3 PFUA significantly affected the immune tissue development, which might impact the immune functions later in life. The ratio of n-6 to n-3 PFUA played a greater role in modulating chicken immune responses. N-3 PUFA suppressed cell-mediated immunity as measured by *in vitro* thymus, spleen and blood lymphocyte proliferation, and *in vivo* inflammatory response such as DTH response. N-3 PUFA appeared to enhance the IgG production and antibody formation. The immunoregulatory effect of dietary fatty acids on chickens can be remained in the progeny up to 8 wk of age if the progeny were fed the same type of diets as the maternal. The solely maternal dietary fatty acids affected the fatty acid profile of immune system (such as spleen) in the progeny and this effect lasted until 4 wk of age. As a consequence, the immune response (DTH) in the progeny was suppressed by the maternal n-3 PUFA. The serum total IgG concentration was significantly correlated with specific antibody production and significantly associated with amount of antibody transferred from egg yolk to the embryo or hatching chick. The increased antibody or total IgG transfer from yolk to the embryo by n-3 PUFA is likely to improve the passive immunity of early chick while its immune system is developing. However, for practical use of immunomodulatory effect of dietary n-3 fatty acids in poultry industry, more investigations are required for the following aspects:

1) The level of fat or oil supplementation in the diet that is suitable economically, immunologically and physiologically. To date, three levels have been tested: 7% (Fritsche, et al., 1991a), 5% in our studies (our studies), and ≤ 2% (Korver and Klasing, 1997).

2) Whether n-3 PUFA suppressed cell-mediated immunity has deleterious effect on the immune defense of the host in protecting infectious pathogens has not been well documented in avian and mammals. With experiments that more specific cell-mediated immune responses are measured, the appropriate level of n-3 in the diet will be recommended for different physiological conditions.

3) The ratio of n-6 to n-3 PUFA plays a role in regulating immune functions. It is quite necessary to conduct more experiments to determine if down-regulating cell proliferation or DTH in a healthy bird is not deleterious. There may have a range for the ratio of n-6 to n-3 PUFA within which the n-3 PUFA can reduce the negative effect of an inflammatory response without significant deleterious effect on the overall immune defense.

4) Effects of dietary n-3 PUFA on cytokine production. For the crucial roles of cytokines in regulating immune responses, the measurement of cytokine production is important for better understanding the immunoregulatory functions of dietary n-3 PUFA on chicken immunity.

5) Oleic acid (C18:1) may be used to modify and balance between specific and non-specific cell-mediated immune responses. It has been reported that oleic acid can maintain strong specific cell-mediated immunity while suppress

inflammatory response in mammals (Yaqoob, 1998). However, no intensive studies have been conducted.

6) A significant financial loss in the broiler industry comes from a variety of mortality including those from infections and cardiac muscle failure, the fatty acid manipulation may provide a new approach to reduce the mortality by increasing the humoral immunity and suppress inflammatory response. Moreover, along with stabilizing heart physiology, the suppression of inflammatory response by n-3 PUFA may be used to reduce the mortality caused by sudden death syndrome (SDS).

9. REFERENCES

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10. APPENDIXES

APPENDIX 1. Broiler starter diet

Ingredients	g/kg
Wheat shorts	75.0
Oats	50.0
Soy meal	173.4
Corn	141.4
Wheat	442.3
Barley	50.0
Tallow	20.0
Limestone	16.5
Dicalcium phosphate	15.8
Iodized salt	3.8
Methionine	1.4
Lysine	0.3
Broiler premix[1]	5.0
Choline premix[2]	5.0
Monensin[3]	0.8
Protein (%)	18.2
ME (kcal/kg)	2783

[1]Broiler premix provides per kg diet: vitamin A, 2,000,000 IU; vitamin D3, 500,000 IU; vitamin E, 7000 IU; pantothenic acid, 2,800 mg; riboflavin, 1,000 mg; folacin, 1.0 mg; niacin, 13,000 mg; thiamine, 400 mg; menadione, 400 mg; folic acid, 160 mg; pyridoxine, 800 mg; vitamin B12, 3 mg; biotin, 36 mg; iodine, 100 mg; manganese, 14,000 mg; copper, 1,700 mg; zinc, 16,000 mg; selenium, 20 mg; iron, 20,000 mg.

[2]50 kg choline chloride premix contains choline chloride (60%) 1.7 kg, wheat shorts 48.3 kg.

[3]Protecting broilers from coccidial infection.

APPENDIX 2. Broiler grower diet

Ingredients	g/kg
Wheat	344.2
Corn	164.4
Oats	125.0
Barley	100.0
Wheat shorts	150.0
Soy meal	73.7
Limestone	17.2
Dicalcium phosphate	8.6
Tallow	0.7
Iodized salt	3.3
Lysine	1.6
Methionine	1.3
Broiler premix[1]	5.0
Choline premix[2]	5.0
Monensin[3]	0.5
ME (kcal/kg)	2711
Protein (%)	15.0

[1]Broiler premix provides per kg diet: vitamin A, 2,000,000 IU; vitamin D3, 500,000 IU; vitamin E, 7000 IU; pantothenic acid, 2,800 mg; riboflavin, 1,000 mg; folacin, 1.0 mg; niacin, 13,000 mg; thiamine, 400 mg; menadione, 400 mg; folic acid, 160 mg; pyridoxine, 800 mg; vitamin B12, 3 mg; biotin, 36 mg; iodine, 100 mg; manganese, 14,000 mg; copper, 1,700 mg; zinc, 16,000 mg; selenium, 20 mg; iron, 20,000 mg.

[2]50 kg choline chloride premix contains choline chloride (60%) 1.7 kg, wheat shorts 48.3 kg.

[3]Protecting broilers from coccidial infection.

APPENDIX 3. Fatty acid composition of broiler starter and grower diets

Fatty acids (%)[1]	Broiler starter	Broiler grower
C14:0	0.28	0.09
C15:0	0.10	0.08
C16:0	11.67	9.32
C17:0	0.12	0.11
C18:0	3.50	2.32
C20:0	0.42	0.55
C22:0	0.23	0.32
SFA	16.32	12.79
C14:1	0.05	0.00
C16:1	0.69	0.31
C17:1	0.08	0.06
C18:1	43.30	50.15
C20:1	0.79	1.05
C24:1	0.09	0.14
MUFA	47.10	51.90
C18:2n-6	28.07	27.80
C18:3n-6	0.06	0.06
C20:2n-6	0.06	0.07
n-6 PUFA	28.19	27.93
C18:3n-3	6.74	7.26
n-3 PUFA	6.74	7.26
PUFA	34.93	35.19
n-6/n-3	4.18	3.85

[1]SFA, saturated fatty acids; MUFA, monounsaturated fatty acids; PUFA, Polyunsaturated fatty acids; n-6/n-3, the ratio of n-6 to n-3 PUFA.

APPENDIX 4. Feed intake (g/d/chick) of broilers fed diets containing different ratios of n-6 to n-3 polyunsaturated fatty acids[1]

	Week					
Diet	1	2	3	4	5	6
5% SO	18.1 ± 0	42.2 ± 0.4	75.0 ± 0.3	108.7 ± 1.1	154.6 ± 2.3	178.4 ± 3.6
2.5 % SO + 2.5% FO	18.2 ± 0.3	42.1 ± 0.3	71.6 ± 0.4	108.4 ± 1.1	154.0 ± 1.6	164.3 ± 5.6
5% FO	16.8 ± 0.2	41.5 ± 0.4	69.3 ± 0.6	107.0 ± 0.8	155.3 ± 1.8	168.8 ± 4.7

[1]Values are means ± SEM from 5 replications (5 pens, 100 broilers/pen).

In each wk, there was no significant difference among the diets ($P > 0.05$).

APPENDIX 5. Body weight gain (BWG, g/chick) and feed efficiency (FE) of broilers fed diets containing different ratios of n-6 to n-3 polyunsaturated fatty acids[1]

	Day-old	0-3 wk		4-6 wk	
Diet	BW	BWG	FE	BWG	FE
5% SO	45.6 ± 0.1	634 ± 10	1.50 ± 0.02	1626 ± 30	1.95 ± 0.04
2.5 % SO + 2.5% FO	43.6 ± 0.1	616 ± 10	1.50 ± 0.04	1574 ± 40	1.95 ± 0.07
5% FO	42.0 ± 0.3	588 ± 10	1.51 ± 0.01	1602 ± 20	1.94 ± 0.09

[1]BW, body weight; FE, the ratio of feed intake to body weight gain; PUFA, polyunsaturated fatty acids. Values are means ± SEM from 5 replications (5 pens, 100 broilers/pen).

There was no significant difference in the hatching weight among the three maternal dietary treatments ($P > 0.05$). From 0-3 wk and 4-6 wk, there was not significant difference in the body weight gain and feed efficiency among the dietary treatments, respectively ($P > 0.05$).

APPENDIX 6. Mortality of broilers from breeders fed diets with different ratios of n-6 and n-3 fatty acids[1]

Diet	Ascites	SDS	Culled	Dyschondroplasia	Valgus	Dehydration	Osteomyelitis	Cardiac Tomanade
5% SO	4	6	1	1	2	5	0	0
2.5 % SO + 2.5% FO	4	6	1	0	3	4	0	0
5% FO	3	8	2	1	3	2	1	1

Appendix 6. Continued

	Necrotic Enteritis	Triculaproventriculus Dialation	Septicemia	Infectious Arthritis	Hepatitis	Oophalitis	Rickets	Total
5% SO	0	1	0	0	0	3	0	23
2.5 % SO + 2.5% FO	1	0	1	0	0	2	0	22
5% FO	0	0	0	1	1	8	1	32

[1]For each cause, the mortality was the number of dead chicks from five pens (100 chicks per pen) during 6 wk of feeding period.