Immunomodulation by Dietary Lipids: Soybean Oil, Menhaden Fish Oil, Chicken Fat, and Hydrogenated Soybean Oil in Japanese Quail (*Coturnix coturnix japonica*) and Bobwhite Quail (*Colinus virginianus*)

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By

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ABSTRACT

Soybean oil (SBO), menhaden fish oil (FO), chicken fat (CF) or hydrogenated soybean oil (HSBO) were incorporated at 5% of the total diet to study changes in the immunological status of both Japanese quail (JAP) and Bobwhite quail (BOB). The SBO diet, in which 66% of the total dietary fatty acids were polyunsaturated fatty acids (PUFA), was rich in linoleic acid (LA 18:2 n-6), alpha-linolenic acid (ALA 18:3 n-3) and low in saturated fatty acids (SFA). The FO diet which contained about 50% PUFA, had only 40% n-6 fatty acids and 8% n-3 PUFA. The trans fatty acid isomers and other monounsaturated fatty acids (MUFA) were high in the HSBO diet. The diet containing CF provided a relatively balanced fatty acid composition with 18% SFA, 31% MUFA and 50% PUFA. Plasma fatty acid and hepatic fatty acid profiles consistently reflected their respective dietary lipid treatments. There were no differences in the fatty acid profile between blood and liver within respective dietary treatments in the two species.

Dietary fatty acids had no effect on antibody titers against sheep red blood cells (SRBC) at 1, 2 and 8 months following the start of dietary lipid treatment in JAP.

However, female JAP fed FO had a significantly ($p < 0.05$) higher antibody production compared to the other dietary lipid treatments at 4 months following the start of fatty acids supplementation. BOB fed either FO or SBO diets had a higher immunoglobulin G production compared to birds fed the CF diet. The total antibody titer was significantly higher in BOB fed SBO compared to CF.

Dietary fatty acids had a significant effect on cell-mediated immunity (CMI) as accessed by toe web thickness 24 hours post intradermal injection of phytohemagglutinin-P (PHA) in both JAP and BOB. In general, birds fed a FO diet had a significantly higher CMI response than those fed HSBO. A diet high in n-3 PUFA increased the index of cutaneous basophil hypersensitivity (CBH), while the high trans fatty acid isomers suppressed the CBH response. By observing a CBH response over a 72-hour period in JAP, it was concluded that quail fed CF or SBO had a different peak response time (12 hours post PHA challenge) and amplitude compared with those fed FO or HSBO (24 hours post PHA challenge). Phagocytic ability was not affected by dietary lipid treatments in BOB while the quail fed FO diet had a faster carbon clearance rate.

The FO fed JAP had a significantly higher response ($p < 0.05$) to concanavalin A ensiformis (CONA) compared to HSBO fed birds. There was no difference in B lymphocyte proliferation stimulated by lipopolysacchride (LPS) in female JAP, whereas it was significantly higher in male JAP fed SBO compared to those fed FO and HSBO. Phorbol 12-myristate 13-acetate/ionomycin calcium salt (PMA/ION) was used to nonspecifically stimulate cell proliferation by increasing chromosome mitosis. Dietary FO or HSBO suppressed cell proliferation stimulated by PMA/ION. However, JAP fed SBO or CF had a significantly higher PMA/ION stimulated lymphocyte proliferation compared those fed FO or HSBO. In male BOB, the FO fed birds had the highest

response to all mitogens. In contrast, female BOB did not show any dietary effects by lymphocyte proliferation. Consistent with JAP, BOB fed HSBO had depressed lymphocytes proliferation in response to various mitogens stimulation. In general, female birds had a higher plasma total protein (PTP) and lower pack cell volume (PCV) compared to their males counterparts in both BOB and JAP.

In summary, in *in vivo* experiments, feeding a diet high in menhaden fish oil that is rich in n-3 PUFA enhanced the CMI. There was a minimal effect on antibody production caused by feeding n-3 PUFA in JAP since a significant treatment effect was only found at one sampling period, while BOB were more sensitive to dietary lipid manipulation and had a higher antibody production with SBO or FO treatments. Dietary lipids exerted different effects in the two species in *in vitro* experiments. While both BOB and JAP fed FO had higher lymphocyte proliferation to CON A mitogen compared to those fed HSBO, only male BOB showed a higher proliferation to LPS. Feeding HSBO that contained a higher content of trans fatty acid isomers, MUFA, but lower PUFA content resulted in the lowest lymphocyte proliferation to various mitogens in both BOB and JAP.

(***Key words:*** Lipid, fatty acid, antibody titer, phagocyte, lymphocyte proliferation, cell-mediated immunity, humoral immunity, Japanese quail, Bobwhite quail).

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CHAPTER I

Literature Review

General Nature of Fatty Acids

Fatty acids have several biological functions. They serve as a major storage form of energy, basic component of the cytoskeleton, form biologically isolated compartments, act as intra- and extra- cellular signals, and are precursors of hormones having a short half life. There is a variety of naturally occurring fatty acids. Different living organisms posses unique enzymes to catalyze or synthesize fatty acids and assemble them into biological structures. Since different fatty acids aid in biological complexity by modifying membrane structure, fatty acids can be a good fingerprint of one species, or even an individual. In nature, odd carbon chain, cyclic and branched fatty acids are only found in microorganisms whereas higher evolved organisms synthesize only straight carbon chain fatty acids (Numa, 1984; Alberts and Greenspan, 1984).

The skin of animals is constantly in contact with the outside environment. There are minute differences in the membrane fatty acid composition of animals from that of pathogenic microorganisms, and these differences may confer the first line of defense to repel microorganism invaders (Minnikin *et al*., 1999). For example, ticks and other blood-sucking insects are preferentially attracted by mammalian skin polyunsaturated fatty acid (PUFA) composition; changes in the membrane PUFA profile have generated counteractive effects (Stanley, 2000). In addition, the natural aroma of fatty acids and their metabolites can act as pheromone-like signals to communicate within species. Moreover, chemical properties of fatty acids such as the melting point, viscosity, and polarity contribute to each species survival capacity in different natural environments.

In plants, fatty acids are synthesized through photosynthesis. These fatty acids serve as a major source of fatty acid for animals. Polyunsaturated fatty acids are the major group of fatty acids found in most leafy vegetables. Other fatty acids, such as oleic acid (18:1) and linoleic acid (18:2) can be found in rich supply in seeds or fruits. Soybeans, a major feed source for commercial food animals, are rich in linoleic acid and oleic acid. Alpha-linolenic acid, a major precursor for long chain n-3 PUFA, is enriched in linseed and flaxseed. In general, the level of unsaturated lipids is higher in plants than animals, and they are liquid at room temperature (Drackley, 2000).

De novo synthesis of fatty acids can play a role in an animal's adipose tissue content. In ruminants, the highly saturated fatty acids are derived from *de novo* synthesis using short chain fatty acids that are major products from rumen fermentation. Tallow, produced from ruminants, is rich in saturated fat including lauric acid (12:0), myristic acid (14:0), palmitic acid (16:0) and stearic acid (18:0) (Drakeley, 2000; Ching, 2000). Different species can contain high levels of a particular fatty acid due to different enzyme systems in the guts. For example, trans fatty acid isomers are not naturally occurring fatty acids. However, ruminant fat contains a large amount of conjugated fatty acids, because rumen flora can convert cis fatty acid isomers to trans isomers (trans-11 $C_{18:1}$). This trans fatty acid is precursor for synthesis of conjugated fatty acids (cis-9, trans-11 $C_{18:2}$) under desaturase (Δ^9-desaturase) (Wonsil *et al*., 1994; Corl *et al*., 2001). Poultry is also rich in saturated fats. Both dark and white meat contain more than 35% saturated fat (SFA) and about 25% monounsaturated (MUFA) fats (Cantor *et al*., 2000). However, chicken legs and breast are lower in SFA and MUFA, and higher in PUFA compared to beef and pork (USDA, 1988).

Fish oils are very high in n-3 fatty acids. The high n-3 fatty acids come from marine

plants and algae. On the other hand, land-based plants such as soybean oil and linseed oil have also been found rich in the 18 carbons n-3 fatty acid α-linolenic acid (ALA). While the α-linolenic acid (18:3 n-3) is more abundant in land-based plants, eicosapentaenoic acid (EPA 20:3 n-3) and docosahexaenoic acid (DHA 22:5 n-3) are particular high in marine life forms. EPA and DHA are synthesized by phytoplankton at the base of the food chain (Ching, 2000). They provide abundant sources for highly unsaturated n-3 fatty acids in shellfish, fish, and sea mammals.

The majority of naturally occurring unsaturated fatty acids are in the cis configuration. The presence of the cis double bound fatty acid in plants confers a flexible structure and lower melting point. In contrast, the trans form fatty acids are usually more rigid, have a higher melting point, and are artificially produced, although trans fatty acids do occur naturally in ruminants. Microorganisms in the rumen are responsible for the transformation of trans double bond fatty acids found in tallow and milk (Jensen, 2000). Commercially, trans fatty acids are produced to suit the needs of food processing, deodorization, anti-oxidation and storage. Hydrogenation of vegetable oil or soybean oil results in a large quantity of trans monoenoic fatty acids, such as the well-known Crisco® shortening (hydrogenated soybean oil) containing a high concentration of vaccenic acid (trans-11 $C_{18:1}$). The trans fatty acid isomers are metabolized through similar enzymatic pathways as cis isomers in the rat, pig and human for energy production (Kritcheveky, 1983). However, trans isomers have also been shown to have lower digestibility and to interfere with other fatty acid utilization (Kaplan and Greenwood, 1998).

Structure, Biochemistry, Essentiality of Fatty Acids

The nomenclature or termination of fatty acids is based on the International Union of

Pure and Applied Chemistry (1978). In brief, fatty acids are named based on the number of carbons in the parental structure of the carbon chain and the number and position of unsaturated bonds relative to the carboxyl end. In addition, the orientation of the branch chain (functional group) and geometric configuration of the substituted functional groups are also designated. Fatty acids that contain no double bond are saturated fatty acids (SFA). If a double bond exists between the 7^{th} and 8^{th} carbons from methyl end and there are 16 carbons, the fatty acid is palmitic acid. Furthermore, the double bond is also termed as n-7 double bond. Another well-known example is oleic acid (18:1 n-9) that has its double bond between the 9^{th} and 10^{th} carbons. Fatty acids containing only one double bond are monounsaturated fatty acids (MUFA). Two conformations exist in the unsaturated fatty acid. Cis isomer fatty acids have their structure bend toward the same side of the double carbon bond. In contrast to cis isomers, trans isomers are relatively straight in structure and have their acyl chain and hydrogen group resides on the different side across the double bond. In membranes, trans fatty acids or saturated fatty acid make the bi-layer less permeable due to the spatial width between two fatty acids being much narrower than the structure formed by cis isomers.

Fatty acids possessing more than one double bond are polyunsaturated fatty acids (PUFA). Nomenclature of these fatty acids is based on the first double bond position from the methyl end. For example, docosahexaenoic acid (DHA, 22:6 n-3) is a 22 carbon fatty acid that contains 6 double carbon bonds with first double bond starting at the 3^{rd} carbon position from the methyl end. The degree of unsaturation (number of double bonds) and position of double bonds are associated with thermodynamic regulations. In addition, membrane fatty acids are usually structurally associated with membrane receptors. Different PUFA compositions in membrane have also been reported to affect receptor

affinity (Watkins and German, 1998).

In animal species, dietary intake is the sole source of fatty acids such as linoleic acid (LA, 18:2 n-6) and α-linolenic acids (ALA, 18:3 n-3). Due to lack of enzymes for synthesizing these parental fatty acids for n-3 and n-6 long chain unsaturated fatty acids, the LA and ALA are essential in maintenance of normal health. These two fatty acids, together with their longer derivatives that can be synthesized within the body are termed essential fatty acids (EFA). The longer derivatives synthesized from these two fatty acids are arachidonic acid (AA) from linoleic and eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) from α-linolenic acid. Other naturally occurring short chain fatty acids can also be absorbed and utilized by animals. Therefore, an animal's body fat composition usually mimics their diet. A dietary deficiency in EFA can lead to impaired growth and deleterious effects on membrane biology, nervous system, bone formation, visual function and reproduction (Watkins, 1995; Calder, 1997). Impaired biological symptoms result mainly from insufficient production of 20-carbon long-chain PUFA for synthesis of phospholipids that are primarily associated with biological membranes. In addition, lack of dietary lipids may also impair cholesterol metabolism, energy balance and utilization of lipid-soluble vitamins.

Saturated fatty acids such as stearic acid (18:0) are converted by delta-9 desaturase to become MUFA. The delta-6 and delta-5 desaturases continually facilitate the conversion of MUFA to PUFAs. The conversion of PUFA is shown in Figure 1.1. The LA and ALA are both 18-carbon fatty acids with 2 and 3 double carbon bonds starting at the 6 and 3 positions, respectively, from the methyl end. In desaturase reactions, double bonds can be added. In mammals, the n-9, n-6 and n-3 fatty acids are not able to inter-convert to each other, and the rate limiting step is the delta-6 desaturase that preferentially reacts in the

order of ALA > LA > oleic acid (Horrobin, 1993). Enzymatic activities for *de novo* synthesis of polyunsaturated fatty acids are regulated by hormones, relative concentration of precursor fatty acids, and their intermediated products (Holman, 1986; Ching, 2000). In addition, animals do not have the enzymatic capability to insert double bonds at less than the delta-7 carbon position. Therefore, palmitic acids (16:0) or stearic acid (18:0) cannot be converted to n-6 or n-3 series fatty acids by the *de novo* synthesis pathway.

Physiologically, 20 carbons PUFA are especially important due to their role in membrane synthesis and embryonic development. In addition, the 22 carbons docosahexaenoic acid (DHA, 22:6 n-3) synthesized from ALA is found in high concentrations in the retina and nervous tissue (Budowski and Crawford, 1986; Anderson *et al*., 1989). The essentiality of LA and ALA to animals therefore becomes obvious. Arachidonic acid derived from LA facilitates major components of the cytoskeleton. ALA undergoes elongation and desaturation to form n-3 PUFAs, the EPA and DHA for the retina and myelin sheath of nerves. Besides forming membrane structure, free 20 carbons n-3 and n-6 fatty acids are also substrates for cyclooxygenase (COX) and lipoxygenase (LOX) pathways to synthesize the biological active metabolites, the eicosanoids.

Metabolism of Lipids

Compared to most animals, birds are extremely efficient at absorbing fat. They are able to utilize fat at more than 50% of dietary dry mass (Place, 1996). Dietary fatty acids are absorbed mainly in the duodenum and jejunum after being emulsified by lipase and bile salt. Fatty acids cross the enterocytes in a micellular form. Micelles in mammals are transported via the lymphatic system after being bound with fatty acid binding protein secreted by the liver. The original complex with lipoprotein is called a chylomicron.

Finally, fatty acids travel through the thoracic duct into the blood circulation system.

Once in the circulatory system, triglycerides will then bind to specific proteins that control their path. The destinations of fatty acids can be energy production via β-oxidation or re-esterification to form triglycerides in the adipose tissue for storage. Acylation reaction (thiokinases) takes place before fatty acid can be oxidized for energy. Acyl-CoA fatty acids are then transported via the acyl-carnitine transferases system into mitochondria. β-oxidation occurs when there is insufficient energy from sugars and other carbohydrates. One round of β-oxidation releases a molecule of acetyl-CoA through acyl-CoA dehydrogenase. Acetyl-CoA becomes available for the TCA cycle to generate ATP.

Regulation of fatty acid metabolism depends on energy status and hormone changes such as insulin, glucagons and catacholamines. Via incorporation into cell membranes, fatty acids are substrates for *de novo* synthesis and precursors of their functional metabolites. Sometimes, cooperation of β-oxidation in removing 2 carbons is necessary for *de novo* synthesis of certain long chain PUFA (Moore *et al*., 1995). For example, DHA is synthesizing from $C_{22:5}$ n-3 to $C_{24:5}$ n-3 during an elongation, continually a desaturation by Δ-6 desaturase to become a intermediate molecule, $C_{24:6}$. Following one cycle of β-oxidation remove two carbons by one molecule of acetyl-CoA, the $C_{24:6}$ can then convert to $C_{22:6}$ n-3 (DHA). An extensive review of fat absorption and incorporation by avian species is available by Place (1996).

The fate of n-9, n-6 and n-3 fatty acids has some variations, in terms of their physiological functions. Oleic acid is the precursor of n-9 fatty acids, and through elongation and desaturation can become eicosatrienoic acid. It is usually found in trace amounts in animals, but accumulations occur during EFA deficiency due to the n-9 fatty

acids not being preferred substrates for elongases and desaturases (Holman, 1986). Linoleic acid is essential for generation of AA. Arachidonic acid is a major component of bio-membranes. Deficiency of AA causes retardation of growth, hair loss and skin damage. A deficiency of AA can be slowly corrected using dietary LA in most animal species except cats. Cats have been shown inefficient in converting LA to AA (Hassam *et al*., 1977; Macdonald *et al*., 1983). Emken *et al.* (1992) reported that the conversion rate of LA to AA in human to be only 2.3%, while the conversion of ALA to their longer derivatives was 18.5 % efficient. Therefore, if not all animals at least in human, ALA is more preferred than LA for converting to their longer derivatives. As mentioned before, n-3 fatty acids are essential for maintaining normal neuronal systems, and retinal function and fetal growth. Since both n-6 and n-3 (AA and EPA) fatty acids can be readily incorporated into cell membranes, competition in their enzymatic utilization have been demonstrated, as well as their deposition in cell membrane.

Fatty acids can be synthesized when an animal has excess energy balance. The *de novo* synthesis of fatty acids occurs when a greater amount of acetyl- CoA is being generated in the TCA cycle. Generation of malonyl-CoA from one molecule of acetyl CoA is governed by the activity of acetyl-CoA carboxylase, and requires biotin as a co-enzyme. Beginning with one molecule of malonyl-CoA and one molecule of acetyl-CoA, the end product from *de novo* synthesis of fatty acids is palmitic acid (16:0). Elongation and desaturation occurs thereafter in either the mitochondria or endoplasmic reticulum. Without desaturase below the delta-7 carbon, *de novo* synthesis of fatty acids only produces palmitic and stearic acid, which are quickly desaturated to palmitoleic acid and oleic acid respectively, the monounsaturated fatty acids. Additional desaturation and elongation can take place, but the saturated and monounsaturated fatty acids are preferred

forms for storage in adipose tissues (Drackley, 2000). The trans monoenoic fatty acids have been reported to disturb the metabolism of linoleic and linolenic acids and exacerbate essential fatty acid deficiency in experimental animals (Sugano and Ikeda, 1996). The trans fatty acid isomers are found to depress Δ-5 and Δ-6 desaturase activities in rat liver. Different fatty acid isomers may antagonize each other in their metabolism pathway.

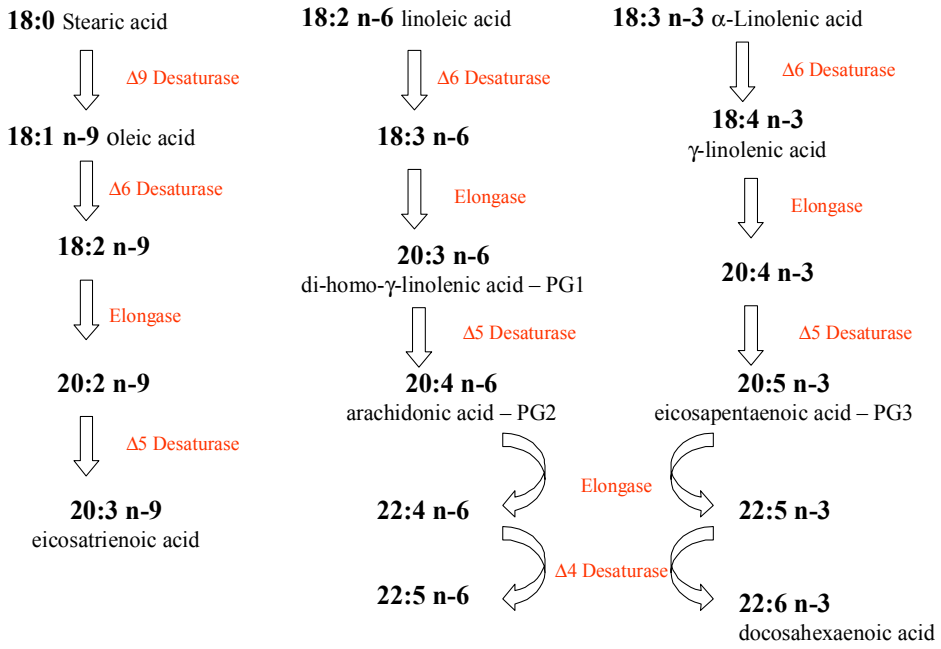

FIGURE 1.1. The pathway of conversion of essential fatty acids.

Functional Metabolites (Eicosanoids)

Both n-6 and n-3 fatty acids are substrates for the generation of eicosanoids. Eicosanoids are biologically active hormones with a short half-life, and include prostaglandins, leukotrienes, lipoxins and tromboxsans. Two major enzymes,

cyclooxygenase (COX) and lipoxygenase (LOX) catalyze the conversion of fatty acids to eicosanoids. The COX is responsible for generation of prostaglandins and thromboxsans from AA and EPA, while the LOX is responsible for the generation of leukotrienes. The prostaglandin 2 series (PGE_2) and leukotrienes 4 series (LTB_4) are metabolites from n-6 fatty acid substrates whereas the 3 series prostaglandin and 5 series leukotriene are from n-3 fatty acid substrates (Jeffcoat and James, 1984).

As mentioned earlier, n-6 and n-3 PUFA are precusors for eicosanoids, and they compete with each other for incorporation into membranes and in enzymatic reaction to generate their functional metabolites. Activation of phospholipase (A_2) is required for liberating fatty acids from cell membranes. Hormones, neurochemical stimuli, and cytokines are the major signals for the activation of phospholipases. Phospholipase A_2 preferentially liberates n-6 PUFA rather than n-3 PUFA (Sumida *et al*., 1993). Therefore, the 20 carbon fatty acid substrates liberated from the membrane should have a higher percentage of AA than EPA. The AA or EPA increases intracellularly following liberation by phospholipases. The enzymatic reactions then take place in the cytosol to produce functional metabolites. The metabolic transformation of essential fatty acids to eicosanoids is illustrated in Figure 1.2.

Both prostaglandins and leukotrienes have pro-inflammatory properties. Prostaglandin can cause vasodilation and mediate IL-1 production, while leukotrienes act as chemotactic substance to recruit neutrophils and macrophages. In general, prostaglandins and leukotrienes from the n-3 fatty acids have less potent biological effects than their n-6 counterparts (Calder, 1997). Therefore, higher levels of n-3 metabolites may be required to have the same physiological effects as n-6 metabolites. Betz *et al*. (1991) reported that PGE_2 enhances humoral immunity while suppressing cellular immunity. Interestingly, at

low concentration, PGE_2 boosts cell-mediated immunity while suppressing it at high concentrations (Mertin and Mertin, 1988; and Clissold, 1999). Therefore, conflicting results from the alteration of n-6/n-3 ratios are commonly seen in many studies due to the lack of monitoring local PGE_2 changes during a cellular event.

Prostaglandins and leukotrienes have potent immunomodulatory roles. The effects of fatty acids on immune functions are usually believed to occur through these biologically active metabolites. Therefore, eicosanoid precursor fatty acid pools are important in understanding the biology of lipid-mediated processes. In reviews by Calder (1998), and Zhou and Nilsson (2001), it is stated that about 25% to 29% of plasma phospholipids, including the membranes of immune cells, is AA. Evidence indicated that alteration of the plasma phospholipids AA pool by dietary n-3 PUFA results in production of less bioactive eicosanoids (Endress, 1996; Calder, 1997). The modulation of n-3 PUFA in immunity via competition in the substrate pool of COX and LOX explains immunosuppressive effects of fish oil in most experimental observations.

In addition, some other mechanisms have been suggested recently involving G protein-coupled receptors, termed E prostandoid (EP) receptors, which mediate signal transduction (Nataraj *et al.*, 2001). There are four subtypes of EP receptors that have been identified, the EP1, EP2, EP3 and EP4. Distinct EP subtypes mediate different signaling pathways to either activate G stimulatory or G inhibitory proteins (Hwang, 2001). Since both T and B cells express various EP receptors, the non-lymphoid cells producing prostaglandins, acting in a paracrine fashion, may activate T and B cells functions with various responses determined by their EP receptors.

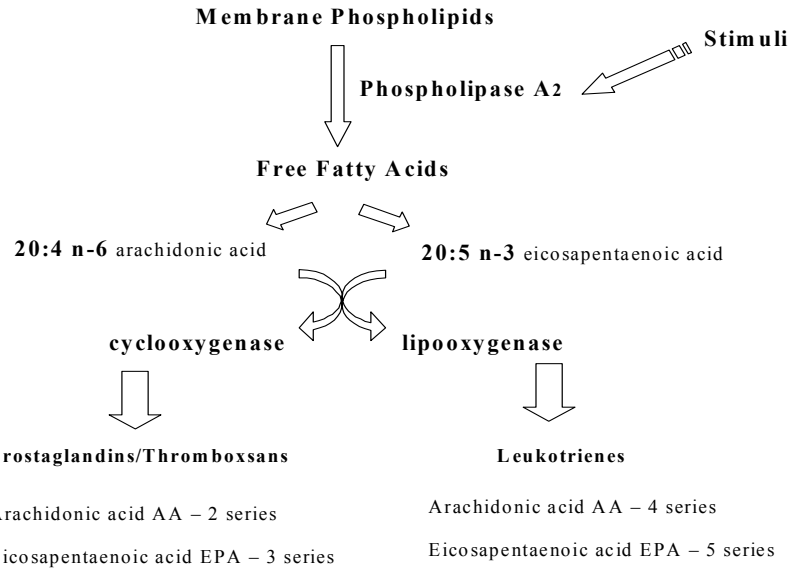

FIGURE 1.2. The metabolic transformation of essential fatty acids to eicosanoids.

Membrane and Phospholipids

Fatty acids are basic components in the composition of phospholipids. Triglycerides contain different fatty acids, thus changing the biochemical properties of the phospholipids. The sn-2 acyl chain position of phospholipids is highly variable in fatty acid composition whereas the other two acyl chains usually contain palmitic or steric acids. Highly unsaturated fatty acids in the phospholipids maintain the membrane fluidity and polarity. In addition, trans-membrane protein receptors are structurally associated with membrane fatty acid (De Pablo *et al.*, 2000). The degree of unsaturation of fatty acids could affect the affinity of receptors.

Activation of phospholipase generates sn 1,2 diacylglerol (DG) and inositol 1,4,5

trisphosphate (IP3), which activates protein kinase and increases cytosolic Ca^{2+} concentration (Hornstra *et al*., 1992). In addition, DG lipase can further act on DG to free arachidonic acid and sustain the phospholipases activity (Sumida *et al*., 1993). Phospholipase A_2 is particularly important for the lipid mediator signal transduction pathway during immunological processes. The role of distinct fatty acids as a second signal is not quite clear. Different fatty acids may trigger various signal transduction pathways that could be an important factor explaining fatty acid in modulation of immunity (Bi and Altman, 2001).

Innate Immunity and Acquired Immunity

Immunity depends on the distinction between self and non-self for the protection of the individual. Like mammalian counterparts, the immune system of avian species has both the innate immune system and acquired immune system (Fig 1.3). The innate immune system includes non-specific immunity as well as host defense mechanisms present at birth. In higher vertebrates, components of innate immunity include skin, mucosal membranes, pH, neuronal reflexes, and secreted fatty acids which act as a barrier against invaders (Benjamini and Leskowitz, 1991). Fatty acid composition can alter the function of innate immunity by affecting its various components. For example, the degrees of unsaturation, and the configurations of the double bonds of PUFA, are associated with the inhibition of the growth of bacteria containing a penicillinase plasmid (Butcher *et al*., 1976; Raychowdhyry *et al*., 1985). Fungi species such as *Candida albicans* are also sensitive to fatty acid profiles in phospholipids (Mago and Khuller, 1989). Long chain unsaturated fatty acids have a detergent cleansing property. Sebaceous secretions of various fatty acids and acidic pH of sweat apparently serve as a protection to

pathogenic microorganisms.

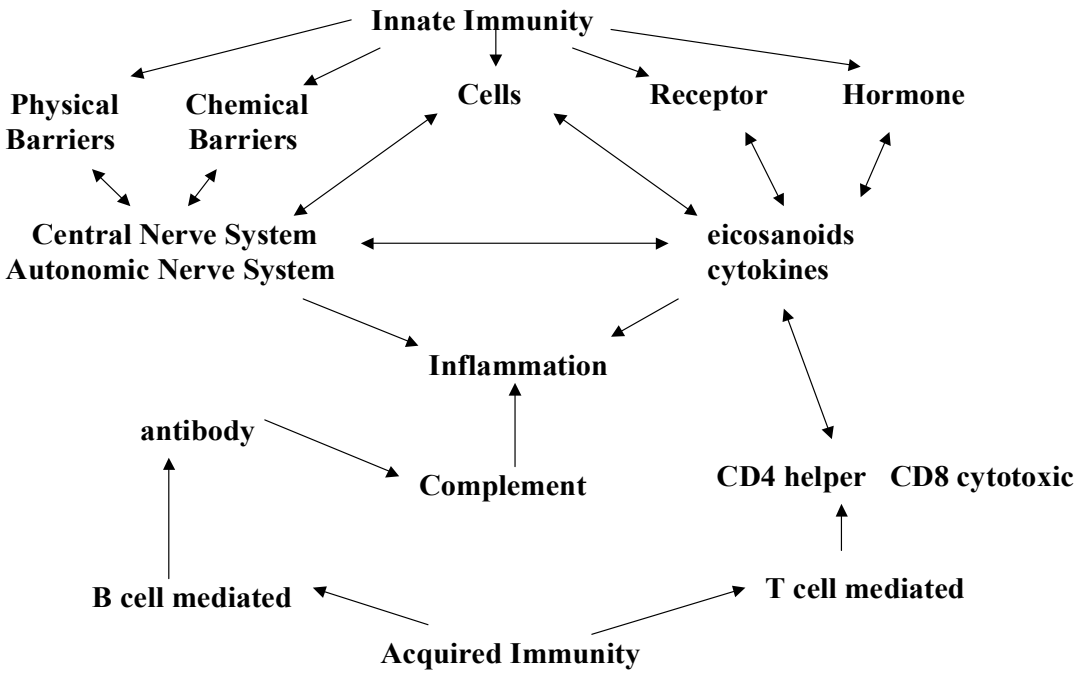

FIGURE 1.3. The scheme of immune systems.

Fatty acids incorporated into phospholipids can greatly affect membrane property. Highly unsaturated fatty acids modulate the fluid permeability of cell membranes. In immunology, recognition of foreign materials distinguished from self has long been conferred as the initial step of an immune response. Until recently, the innate immune response which distinguishes non-infectious self and infectious self via specific receptors was considered as primary immunity rather than the acquired immunity. The pathogen associated molecular patterns (PAMPs) are suggested as active defense mechanisms of innate immune systems in which the majority of non-immune cells possess PAMP receptors

(Medzhitov and Janeway Jr., 2000). These receptors of the innate immune system are encoded in the germ lines that are expressed without gene rearrangement, unlike receptors of immuno-competent cells in adaptive immunity which are encoded in rearranging gene segment. The modulation of the membrane physical or chemical characteristics by fatty acids could truncate cellular signal transduction as well as the binding affinity of PAMP receptors that would have a great influence in innate immune functions.

Moreover, phagocytic cells such as macrophages are essential for innate host defense. Macrophages are responsible for processing antigens for presentation to T lymphocytes, and they produce principal inflammatory cytokines including interleukin 1 (IL-1) and tumor-necrosis factor -α (TNF-α). IL-1 induces collagenase production, fever, CAM expression, T cells proliferations, etc. (Endres, 1996). TNF-α is produced only by monocyte, and has a cytotoxic effect on tumor cells. Both IL-1 and TNF-α are essential for the inflammatory response, and their production is regulated by eicosanoids. By acting as autocrines, prostaglandins produced from macrophages bind to their EP receptors to self regulate IL-1 production.

Adaptive (acquired) immunity is more limited in the sense of fighting off pathogenic materials than its innate counterpart. Two of the major participants in acquired immunity are T lymphocytes and B lymphocytes. They are progenitors of the two cell types originating from bone marrow, which differentiate and mature in the thymus and bursa of fabricius, respectively. Acquired immunity mediated by T cells is termed cell-mediated immunity, whereas B cell mediated is called humoral immunity. Both cell-mediated and humoral immunity are triggered by antigen associated with MHC (major histocompatibility complex) molecules.

Cell-mediated immunity requires recognition of the MHC molecule. T cells can be

divided into two subset families determined by their cell surface markers (CD; cluster-determinant) including CD4 T cells (T helper cell) and CD8 T cells (T cytotoxic cell). CD4 T cells recognize the antigen presented by MHC II molecules, whereas CD8 T cells are specific to antigens that associate with the MHC I molecules. The specific interaction between T cells and MHC surface molecules is to prevent self-destruction during an immune response. In general, MHC I molecules occur in all giving cells, while the MHC II molecules are only found on the surfaces of immuno-competent cells (Toivanen and Toivanen, 1987). CD8 T cytotoxic cell has ability to kill invaders such as bacteria or viruses via perforins that puncture a hole in the membrane. Activated CD4 T cells produce various cytokines in cooperation with many immune processes including antibody production, immunoglobulin class switching and cell proliferation.

When categorized by the cytokines they produce, CD4 T helper cells contain two subset populations called T helper 1 (T_{H1}) and T helper 2 (T_{H2}) cells. Cytokines from T helper 1 cells such as interleukin 2 (IL-2) and interferon-γ (IFN-γ) are essential during cell-mediated immunity. On the other hand, T helper 2 cells produce interleukin 4 (IL-4) and interleukin 5 (IL-5) assisting in antibody production in B cell mediated humoral immunity. Because cytokines from T_{H1} and T_{H2} cells are antagonistic to each other in their specific cytokine productions (Benjamini and Leskowitz, 1991), acquired immunity usually favors either cell-mediated or humoral immunity. The cytokines from T_{H1} or T_{H2} cells direct the shifting of the two major arms of acquired immunity.

In humoral immunity, immunoglobulins are produced by B cells in response to antigens. Different classes of immunoglobulin are produced; they are immunoglobulin A (IgA), immunoglobulin E (IgE), immunoglobulin G (IgG) and immunogloculin M (IgM). Like T cells, B cells possess memory to specific antigens that have been encountered. In

the primary immune response (first encounter of antigen), IgM is the major class of antibody found in serum. Following IgM production, IgG production occurs in response to persisting antigens. The production of IgG is larger in quantity and more prolonged in the secondary immune response compared to primary immune response. Humoral immunity needs synergistic action with T_{H2} cells. IL-4 and IL-5 are required for B cell differentiation and proliferation, as well as antibody secretion and class switching.

Cytokines, which are produced by immuno-competent cells during immune responses, serve as transduction signals. Cytokines include families of interleukins, interferon, colony-stimulating factor, transforming growth factor, and tumor necrosis factor (Roitt *et al*., 1994). The hormonal network can regulate cytokine production. For example, corticosterone has been shown to block interleukin-1 (IL-1) transcription leading to suppression of inflammation (Cook, 1991). Polyunsaturated fatty acids derived prostaglandins and leukotrienes act locally during an immune response. Lymphocytes do not produce either prostaglandins or leukotrienes (Calder, 1997); therefore, the eicosanoids play an important role as signals between the innate immune system and acquired immune system.

The inflammatory response involves both the innate and acquired immune systems. Therefore, it is a complicated process. In general, inflammatory responses are categorized by the cell type involved and the time to sensitize into type I – IV hypersensitivities. The four types of inflammatory reactions include swelling, redness, heat, and pain. Although inflammation is one of the protective mechanisms of the body, it commonly brings about discomfort and becomes harmful to normal tissues when the source of antigen is persistent and occurs over a broad range. Acute inflammatory responses which are usually associated with allergy and antibody mediated inflammation belong to type I to type III

hypersensitivity that are different from type IV hypersensitivity. The type IV hypersensitivity is also termed delayed type hypersensitivity due to the response usually occurring 24 hours after initiation. Mast cells and other polymorphonuclear cells (PMN) play an important role in acute inflammation by producing histamine (vasoactive amines) and lysosomal enzymes when activated by IgE (Bacon, 1992). In contrast to acute inflammation, the chronic inflammatory response involves PMN, macrophages and T lymphocytes. The delayed type hypersensitivity requires interactions between antigen presentation cells and T cells through cytokine communications and it is more sophisticated. The macrophages are major effectors which produce IL-1 and TNF in the chronic inflammation unlike mast cells produce histamine in the acute inflammation (Corrier and DeLoach, 1990).

Inflammation begins with the activation of cascade of eicosanoids synthesis. The locally induced production of prostaglandin E_2 in response to injury is mediated by cyclooxygenase 2 (COX 2), distinguished from the tissue-protective prostaglandin produced from cyclooxygenase 1 (COX 1) (Clissold, 1999; Kelly, 2001). PGE_2 is a powerful dialator, increasing blood flow, and enhancing capillary permeability. It is also the ligand to EP receptors on immune cells for activating cytokine production. Another powerful inflammatory mediator from eicosanoids is catalyzed by lipooxygenase is LTB_4. It is a potent chemotactic substance produced by PMN and macrophages which recruits more immunocompetent cells, especially neutrophils (hetrophils in birds) to the inflammatory site. Excess levels of PGE_2 and LTB_4 are harmful and occur in autoimmune disease patients. Aspirin and indomethacin are non-steroidal anti-inflammatory drugs, which have an effect in ameliorating inflammation by inhibition of cyclooxygenase activity.

Recently, the discovery of drugs that selectively inhibition COX enzyme isomers has

helped in understanding COX 1 and COX 2- independent mechanisms (Tegeder *et al*., 2001). Further knowledge in this area could reveal more evidence in the role of COX enzyme isomers in inflammatory responses.

Sanderson and Calder (1998) reported that dietary fatty acids alter the cell trafficking by modulation of cell adhesion molecules gene expression. Cell trafficking is controlled by cell surface molecules termed cell adhesion molecules (CAM). CAMs are associated with localization and migration of leukocytes to infected sites (Roitt *et al*., 1994). There are three major categories of CAM which are differentiated by their protein structures. Integrin and lymphocyte function associated antigen (LFA) are transmembrane proteins consisting of herodimeric α- and β- subunits responsible for their binding specificity and affinity (Mousa, 1998; Eble and Kuhn, 1997). Expression of CAMs on both leukocytes and endothelial cells is necessary for recruiting immune cells to the inflammatory site. The expression of CAMs is regulated by eicosanoids, cytokines and free radicals (Sanderson and Calder, 1998). Diminished expression of CAMs may benefit in relief of symptoms of both acute and chronic inflammation.

Role of Fatty Acids in Immunity

The status of the immune system closely aligns with the health of an individual. Malnutrition and metabolic disorders commonly result in decreased food intake, decreased feed efficiency, growth retardation, low uniformity and high mortality. Nutrients from diets provide basic needs to maintain sound animal health. In animal husbandry, genetic selection for rapid growth has compromised immunity in animals since nutrients are allocated for growth (Cook, 2000). Therefore, manipulation of specific nutrients to enhance immunity is of great interest. Fat composition of the diets, along with protein,

has a significant role in health through its content of fatty acids, cholesterol, and fat-soluble vitamins.

The profound effect of fatty acids on immunity is best exemplified by the Greenland Eskimo paradox (PUFA newsletter). Consumption of large quantities of EPA and DHA from marine products has greatly decreased the incidence of coronary heart disease and stroke among this population (Carlson, 1996; Kremer, 1996; Bruckner, 1997). In addition, evidences have shown that EPA and DHA reduce cholesterol and inhibit atherosclerosis (Chamberlain *et al*. 1991; Sadi *et al*. 1996), inhibit the blood coagulation pathway (Miller, 1998; Mutanen and Freese, 2001), ameliorate symptoms of lung disease by decreasing production of bronchoconstrictive leukotrienes (Schwartz, 2000) and modulates bone growth in animals (Liu and Denbow, 2001). In all cases, eicosanoids and the cytokine, interleukin-1 (IL-1), have been associated with these effects.

Dietary supplement of fish oil rich in EPA and DHA has been shown to suppress IL-1 production and TGF-α in various animal models (Endres, 1996). Suppression of IL-1 causes a decrease in lymphocyte proliferation, hampering the expression of cell adhesion molecules, and down regulates inflammatory gene (NF-κB) expression (Toborek *et al*., 2002), which leads to decreased indices of inflammation. Among evidence that supports n-3 PUFA ameliorating inflammatory symptoms in autoimmune diseases, the EPA in marine lipids that are metabolized to the less bioactive eicosanoids is the most promising evidence. The 3 series of prostaglandin and 5 series of leukotriene are produced from EPA. PGE_3 has pro-inflammatory activity similar to that of PGE_2, but it synthesized with a very low efficiency, and LTB_5 has little inflammatory activity compared to LTB_4 (James *et al*., 2000). The significance of substitutes of PG and LT families to less bioactive EPA metabolites has been reviewed (Kremer, 1996). Kremer has suggested that

supplementation with n-3 PUFA in rheumatic arthritis patients enables the discontinuation of nonsteroidal anti-inflammatory drugs (NSAID).

Competition of EPA and AA for metabolism by the COX and LOX enzyme systems has also demonstrated a preference for the use of EPA as substrate (Whelan *et al*., 1991; Denzlinger *et al*., 1995). Both AA and EPA are tightly bound in the sn-2 position of phospholipids of cell membrane. Liberation of membrane bound EPA and AA requires activation of phospholipase A2, C, and D. According to Ribardo *et al*. (2000) the level of prostaglandin produced by macrophages is controlled by PLA2-activating protein. Synthesis of eicosanoids could be limited by the availability of the substrates that are directly controlled by the activity of phospholipases. Since the phospholipases are involved in the liberation of fatty acid from membrane, regulation of these enzymes determines the fatty acid mediated intra- and extra- cellular signals. Secretory PLA_2 ($sPLA_2$) is different from cytosolic PLA_2. Lymphocytes produce $sPLA_2$ in response to cell injury. Since lymphocytes do not produce prostaglandins, and PGE_2 primes the activation of lymphocytes, the indirect stimulation of PGE_2 production by lymphocytes depends on releasing fatty acids from membrane by producing $sPLA_2$.

Free fatty acids may act as secondary messengers mediating various signal transductions that are independent of PG and LT activities. Free fatty acids positively or negatively regulate the cyclic AMP signal pathway and protein kinase C activity in the presence of cyclooxygenase and lipooxygenase inhibitors (Sumida, 1993). Fatty acid mediated signal pathways are complex, and usually combined with different protein kinase cascades to regulate cell growth, differentiation and apoptosis. Fatty acids and oxidized metabolites are ligands for peroxisome proliferator-activated receptors (PPAR) that regulate gene transcription (Strum *et al*. 1997; Bi and Altman, 2001). Analysis of the structure of

PPAR binding domains has revealed that the nuclear receptor sufficiently binds to different conformations of fatty acids (Nolte *et al*., 1998). The binding affinity of PPAR to fatty acids is PUFA > MUFA > SFA (Kliewer *et al*., 1997; Hwang, 2000). It has been shown that PUFA bind to PPAR inhibiting inflammatory cytokine, IL-1, production by monocytes and macrophages (Jiang *et al*., 1998; Ricote *et al*., 1998). Moreover, ligands binding to PPAR have been shown to inhibit production of IL-1, IL-6 and expression of COX2 in smooth muscle cells (Staels *et al*., 1998). Differential binding to PPAR by specific fatty acids could have potential effects on inflammation and immune function.

Among a variety of fatty acids, n-3 and n-6 PUFA have been most extensively studied for their functions in immunity. However, research on the effects of monounsaturated fatty acids on immunity is incomplete. With the popularity and amount being consumed by humans, MUFA has become a center of attention in human health. In Western diets, more than 40% of commercial fats are trans MUFA isomers (Craig-Schmidt *et al*. 2000). A recent study investigating the uptake of individual fatty acids into adipose tissue in relation to their presence in the human diet indicated that MUFA was preferentially absorbed and stored in adipose tissues (Summers *et al*. 2000). The quantity stored in relation to type of fatty acids was MUFA > n-6 PUFA > SAF > n-3 PUFA. It is reasonable to speculate that MUFA could have a greater influence than other classes of fatty acids.

Moreover, it has been shown that the consumption of olive oil rich in oleic acid (cis $C_{18:1}$) decreases the risk for developing rheumatoid arthritis in humans (Linos *et al*., 1991). In animals, the PUFA content in basal diets usually masks the effects of lipid treatment containing high MUFA such as lard or chicken fat. Therefore, MUFA diets have typically been used as control treatments, or have been reported not to affect various immunological indices. In 1997, Jeffery *et al*. reported an inversed relationship between splenocytes

stimulation index and the ratio of oleic to linoleic acid in the diet. Moreover, Yaqoob (1998) reported that MUFA consumption resulted in suppression of proliferation of peripheral blood mononuclear cell stimulated by CON A. The host versus graft response to injection of allogenic cells into the footpad of a host was suppressed by fish oil rich in n-3 PUFA, while feeding olive oil rich in oleic acid had a similar but weaker effect in rats (Sanderson *et al*., 1995). In humans, consumption of a MUFA rich diet decreased CAM expression on peripheral blood mononuclear cells and suppressed NK cell activity and cell proliferation (Yaqoob *et al*., 1994). The immunosuppressive effect by dietary lipids is not limited to fish oil containing n-3 PUFA. The n-9, 18:1 oleic acid also exerts an anti-inflammatory property, at least when incorporated into the diet using olive oil.

In poultry, the majority of studies have used animal fat, lard, as the experimental treatment in studying dietary lipid effects on immunity. Lard containing MUFA as more than 40% of the total fatty acid content had been shown to induce a significantly higher splenocyte proliferation stimulated by CON A compared to linseed oil and fish oil treatments (Wang *et al*., 2000). In addition, the lard diet used by Fritsche and Cassity (1992) caused a higher antibody-dependent cellular cytotoxicity of splenocytes compared to corn oil, fish oil and flaxseed oil diets in chickens. Research conducted by the same group in 1991 indicated that the lard diet did not cause any difference in antibody or lymphocyte proliferation compared to corn oil treatment (Fritsche *et al*., 1991). Unlike the results from animal studies using olive oil enriched in MUFA, poultry fed a lard diet have shown either unchanged or elevated immunity.

The naturally occurring fat sources that enrich MUFA are cis isomers including 16:1, 18:1, and 20:1 fatty acids with cis 18:1 oleic acid being the majority. Trans MUFA isomers are not a conventional content in animal diets nor naturally occurring fat sources.

Even though there is still discrepancy in digestibility and utilization of trans fatty acid as energy in a recent paper (Kaplan and Greenwood, 1998), as reviewed earlier, trans fatty acids did not differ in metabolic property from saturated fat in animals (Kritcheveky, 1983). Trans fat, a significant compound of modern diets, may adversely affect health. It has been shown that dietary trans fat has adverse effects on the cardiovascular system due to increasing low-density lipoproteins, decreasing high-density lipoproteins and increasing in platelet aggregation and cholesterol level (Kamei *et al*., 1995; Chiang and Lu, 1996; Lichtenstein *et al*, 1999). Apparently, trans MUFA had many bad characteristics in arteriosclerosis and chronic heart disease. Royce *et al*. (1984) used swine as the experimental model demonstrated that hydrogenated fat was similar to lard decreased eicosanoids production. However, dietary hydrogenated fat containing large quantity of trans fatty acid isomers did not affect PGE_2 production but increased inflammatory cytokines, IL-6 and TNF-α, production compared to soybean oil diet in humans (Han *et al*., 2002). Moreover, studies have also shown that hydrogenated fat did not affect cellular immunity as assessed by DTH or lymphocyte proliferation (Yaqoob *et al*, 1994; Han *et al*., 2002). A well-controlled study having the same level of total PUFA in the diets, investigated cis- and trans- MUFA effects on immune indices in rats (Koga *et al*., 1997). They reported that trans fatty acids (elaidic acid) decreased splenic PGE_2 production but not leukotrienes, and increased the ratio of $CD4^+$ to $CD8^+$ T lymphocytes, but this effect was only when perilla oil was used as PUFA source, not safflower oil. Taken together, trans MUFA did not show an adverse effect on immunity, but decreased eicosanoids production and increased inflammatory cytokines.

Conjugated fatty acids that naturally exist in milk have been shown to affect carcinogenesis (MacDonald, 2000). The effect on suppression of tumor growth could be

due to modulation of some aspects of the immune system. Kelly *et al*. (2001) reported that dietary conjugated linoleic acid did not affect eicosanoids, cytokine production or lymphocyte proliferation in human. More research should be conducted to further understand immunomodulatory functions of this specific class of fatty acids.

Clearly, there is still discrepancy in the results making it difficult to draw conclusions in roles of specific classes of fatty acids on immunity. Although the availability of fatty acid substrates greatly affects eicosanoids production, consequently altering immune functions, it is certain that fatty acids also exert their roles independent to eicosanoids.

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CHAPTER II

The Effect of Dietary Lipids on Immunity of Japanese Quail (Coturnix coturnix japonica)

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ABSTRACT

The effects of soybean oil (SBO), menhaden fish oil (FO), hydrogenated soybean oil (HSBO) and chicken fat (CF) on both *in vivo* and *in vitro* immune functions were studied. Japanese quail were randomly assigned to four dietary lipid treatment groups after reaching sexual maturity at 4 weeks-of-age. Total antibody titers against sheep red blood cells (SRBC) and cutaneous basophil hypersensitivity (CBH) were measured at 1, 2, 4 and 8 months after beginning dietary lipid supplementation. At the end of the experiment, quail were sacrificed to measure *in vitro* lymphocyte proliferation using a non-radioactive assay. Plasma and liver were collected for fatty acid analysis. Quail fed FO had a higher plasma and hepatic n-3 PUFA and lower total n-6 PUFA in liver compared to other lipid treatments. There was no difference in plasma n-6 PUFA among different treatments. The level of trans fatty acid isomers was highest in quail fed HSBO. In general, plasma fatty acids reflected the dietary fatty acid profile. However, the hepatic total saturated fatty acid (SFA) and monounsaturated fatty acids (MUFA) were not affected by dietary fatty acid manipulation. There was no significant treatment effect on antibody production except in female quail fed FO at the 4-month sampling period. These birds exhibited a significantly ($p< 0.05$) higher antibody titer when compared to the other dietary treatments. Moreover, quail fed fish oil (rich in n-3 fatty acids) had an elevated CBH response ($P < 0.05$)

measured at 24 hours after intradermal injection of 50μg/10μL phytohemagglutinin (PHA) across all sampling periods except at 1 month after dietary treatments began. While the CBH response of the FO, CF and SBO groups remained relatively consistent across different sampling period, the HSBO treatment had a negative impact on the index of cell-mediated immunity after prolonged supplementation. By 8 month after HSBO diet supplementation, the index of CBH response was lowest among all treatments. The CBH response was also measured over a 72 hours period at 12, 24, 48 and 72 hours after injection. Monitoring the CBH response across time revealed that quail fed SBO and CF had their peak toe web thickness (12 hour) earlier than those fed FO and HSBO (24 hour). In addition, the swelled toe web normalized faster in quail fed SBO compared to those fed FO. At the end of the study, i*n vitro* evaluation of lymphocyte proliferation was assessed by the Alamar Blue™ lympho-pro assay. Lymphocyte blastogenesis stimulated by concanavalin A (CON A), lipopolysacchride (LPS) or phorbol 12-myristate 13-acetate + ionomycin mitogens (PMA) were significantly depressed ($P < 0.05$) in birds fed HSBO compared to those fed SBO or CF. In addition, birds fed FO rich in n-3 polyunsaturated fatty acids (PUFA) had a significantly higher ($p< 0.05$) T cell response in both male and female quail. Dietary SBO enhanced T- and B- cell proliferation by CON A and LPS respectively. In contrast, HSBO, that is rich in trans isomers of monounsaturated fatty acids (MUFA) impaired both T and B lymphocyte proliferation. Females had a higher plasma total protein (PTP), but lower pack cell volume (PCV), than those of males. In addition, CF fed quail had a higher PTP than SBO fed female quail. Both FO and SBO fed female quail had a higher ($p< 0.05$) PCV than those fed HSBO. Microscopic examination of blood cytology revealed no differences between dietary treatments. The current study showed that different dietary lipids altered various indices of immunity in

Japanese quail, and these effects depended on the distinctive fatty acid profile facilitated by different dietary lipids. Feeding quail menhaden fish oil containing large amounts of n-3 PUFA, particularly eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), appeared to have beneficial effects on immunity. In contrast, quail consuming HSBO with large quantity of trans fatty acid isomers had relatively impaired immune responses.
(***Key words***: Japanese quail, lipids, fatty acid, cell-mediated immunity, humoral immunity)

Introduction

Broilers currently reach market weight of 2 kg in less than 6 weeks. Selection for better feed-efficiency and growth rate has resulted in an allocation of the majority of resources to accommodate these traits (Cook, 2000). Simultaneously, selection for high body weight causes a decrease in immune function (Siegel *et al.*, 1982; Miller *et al.*, 1992). To accommodate the decline in host defense caused by selection for growth, it is of great interest for nutritionist to improve immune functions via manipulation of dietary nutrients. In addition, the poultry industry may soon be forced to abolish the application of antibiotics due to growing consumer pressure (Levy, 1998; Cook, 2000). To find an alternative to antibiotics while boosting immunity in rapidly growing poultry is of great concern to commercial animal industries. Nutritionally manipulating animal immunity may be a cost-effective solution to this problem in animal industry.

Avian species are extremely efficient in fat absorption, and are capable of utilizing fat exceeding 50% of dietary dry mass (Place, 1996). Therefore, birds are potentially more susceptible to dietary manipulation of fatty acid composition. In birds, a deficiency in linoleic acid (18:2), α-linolenic acid (18:3) and arachidonic acid (20:4) reduces the production of eicosanoids and hampers normal growth and health (Watkins, 1991). The quantity and quality of fat are critical in maintaining normal animal health. In poultry, increasing the ratio of n-3/n-6 fatty acids in diets has been shown to either increase or decrease immunological parameters of both humoral and cell-mediated immunity (Fritsche *et al.*, 1991; Kover and Klasing, 1997; Friedman, and Sklan, 1997; Wang *et al*., 2000; Sijben *et al.*, 2001). Suppression of the inflammatory response could be beneficial to poultry in minimizing the catabolic effect from both pathogens and environmental immunogens. However, the suppressed inflammatory response could also increase

susceptibility to bacterial and viral infections (Kover and Klasing, 1997). Therefore, adequately adjusting immune status to fit environmental conditions and disease challenges would bring the maximum benefits to animal producers.

Eicosanoids are metabolites derived from 20-carbon PUFA that possess immunological properties. Particularly, the prostaglandins and leukotrienes produced by cyclooxygenase (COX) and lipooxygenase (LOX), respectively, are potent immunomodulators. Both n-6 and n-3 PUFAs are precursors for synthesis of their distinct prostaglandins and leukotrienes. The 2-series prostaglandin (PGE_2) and the 4 series leukotriene (LTB_4) are derived from the n-6 arachidonic acid (AA). On the other hand, the n-3 eicosapentaenoic acid is the precursor for the less bio-active metabolites, the 3-series prostaglandins (PGE_3) and 5-series leukotrienes (LTB_5). Based on the Greenland Eskimo paradox, consumption of a high level of marine products enriched in eicosapentaenoic acid (EPA, 20:4 n-3) and docosahexaenoic acid (DHA, 22:5 n-3) has been associated with a decrease in inflammatory diseases (Connor, 2000). The ratio of dietary n-3/n-6 fatty acids could partially explain this paradox through its effect on altering products from the COX and LOX pathways. Eicosanoids govern the production of pro-inflammatory cytokines such as IL-1, TNF and IL-6 (Endress, 1996). In the majority of mammalian studies, diets rich in EPA or DHA have consistently demonstrated decreased PGE_2 and LTB_4 derived from arachidonic acid (AA) leading to a reduction in pro-inflammatory cytokines (Meydani *et al.*, 1993; Calder, 1996; Endress, 1996).

Substrate availability is a rate-limiting factor in both COX and LOX pathways. Replacing AA with EPA in the membrane fatty acid pool decreases metabolites from AA due to EPA competing with AA to generate its less bio-active metabolites, PGE_3 and LTB_5. Higher concentrations of PGE_3 or LTB_5 would be necessary to induce the same biological

action as those primed by PGE_2 and LTB_4. Moreover, the local concentration of these eicosanoids greatly affects their biological actions. It has been shown that prostaglandins enhance cell-mediated immunity at low to medium physiological concentrations while suppressing immunity when given in a pharmacological dose (Mertin and Mertin, 1988; Clissold, 1999).

Via autocrine or paracrine actions, PGE_2 and LTB_4 regulate cytokine production and shift the balance favoring T helper-2 from T helper-1 cell cytokine profiles (Betz and Fox, 1991). Birds have been demonstrated to have a similar dimorphic response of T helper cells (Vandaveer *et al.*, 2001). However, whether cytokines from T helper-2 cells are upregulated by PGE_2 in avian species is still not clear. The 20-carbon PUFAs can alter the potency of PGs by switching classes of PGs. The immune system can favor a T helper 2 mediated humoral immunity with increased PGE_2 production, while PGE_3 derived from n-3 PUFA could have less effect on such events.

Changing of the dietary fatty acid profile has been shown to affect cell characteristics and functions by altering membrane permeability, generating secondary messengers as intracellular signal, and modulating gene transcription (Calder, 1998; Kelly, 2001). Dietary PUFA has been shown to inhibit expression of cell adhesion molecules (CAMs) of both endothelial and immunocompetent cells (Caterina and Libby, 1996; Sanderson and Calder, 1998). Expression of CAMs during inflammation by activated cells is essential for leukocyte localization and migration to the site of inflammation. Diminished CAM expression by n-3 PUFA, or a lower degree of saturated fatty acids provides another mechanism of action of fatty acids in immunity at the cellular level. Recent studies on innate immunity have identified a series of common receptors on all or most cells that are termed pathogen associated molecular patterns (PAMPs) (Medzhitov, and Janeway Jr.,

2000). PAMPs are encoded in the germ line of all sorts of cells, and serve as receptors which recognize common structures of pathogens. Membrane- incorporated fatty acids could alter the basic physical structure of trans membrane proteins that could further affect affinity of PAMPs systemically.

By serving as secondary messengers, cytosolic free fatty acids can bind to nuclear peroxisome proliferator-activated receptors (PPARs) and regulate gene expression (Hwang, 2000). Free fatty acids have been shown to have different binding affinities to different subclasses of PPAR family by their degree of unsaturation (Kliewer *et al*., 1997; Hwang, 2000). Fatty acid ligands bound to PPARs activate lipid metabolism, cell differentiation, proliferation, apoptosis and inflammatory response. Apparently, fatty acids differing in their chemical structure specifically modulate diverse biological events.

In poultry, there are conflicting results on the effect of dietary lipids on immune functions (Fritsche *et al*., 1991; Korver, and Klasing, 1997; Wang *et al*., 2000; Sijben *et al*., 2001). Therefore, the goal of current study was to investigate the effect of long-term supplementation of specific fatty acids on immunity in Japanese quail.

Materials and Methods

Animals and Diets (See Appendices; APP. I.)

Newly hatched quail were fed a starter diet with 3.5% blended fat until gender could be determined at approximately 4 weeks-of-age. The starter diet formulation is shown in Table 2.1. Once gender was determined, quail were randomly assigned to four treatment groups and switched to experimental diets, which were fed for 8 months. The experimental diets contained 5% menhaden fish oil, soybean oil, hydrogenated soybean oil or chicken fat (Table 2.2). All nutrients were supplied to meet or exceed NRC

requirements (1994). Birds were housed in a temperature controlled environment in cages, and feed and water were provided *ad libitum* throughout the entire experiment. Total 120 quail per sampling period were used for both assays of antibody titer and CBH response. Each of thirty birds per treatment per sex was used to evaluate the effect of dietary lipids on antibody titer and cutaneous basophil hypersensitivity at 1, 2, 4 and 8 months. Lymphocyte proliferation assays were performed at the end of experiment and birds were sacrificed thereafter for tissues fatty acid analysis.

Fatty Acid Analysis

Lipids from plasma, liver, and feed were extracted using a Folch wash of 2:1 (v/v) chloroform:methanol (Folch *et al*., 1957). Fatty acids in dietary oils and lipid extracts were methylated by base-catalyzing by transesterification 0.5 N methanolic NaOH followed by 14% boron triflouride in methanol (Park and Goins, 1994). A 10-undecenoate as an internal standard was added prior to methylation. Fatty acid methylated ester (FAME) were separated with Chrompack 100m CP-Sil 88 column (0.25mm ID, 0.2um film thickness) (Vaian Associates, Walnut Creek, CA, 94595) in an Agilent 6890 gas chromatograph fitted with an autosampler and a flame ionization detector. For liver, feed, and oils, split injection (80:1) of 0.5 µl was used. Injector and detector temperature were 250^{o}C and 300^{o}C, respectively. Initial oven temperature (70^{o}C) was held for 1 minute, increased to 100^{o}C at rate of 10^{o}C/min, held for 3 minutes, increased to 175^{o}C at rate of 10^{o}C/min, held for 40 minutes, increased to 220^{o}C at a rate of 5^{o}C/min and held for 17 minutes. Total time for each sample was 80.5 minutes. Splitless injection was used for separation of plasma FAME. Injection volume was 0.5 µl and purge valve closure time was 0.6 minutes. Column temperature was maintained at 40^{o}C during valve

closure, increased to 100°C at a rate of 40°C/min, increased to 175°C at a rate of 25°C/min, held for 45 minutes, increased to 220°C at a rate of 10°C/min, and held for 25 minutes. Total analysis time for each sample was 89.6 minutes. Ultra pure hydrogen was used as the carrier gas for all analyses. Injections were made using a constant pressure of 20.19 psi. Column flow was 1.4 ml/min at 70°C. Integration and quantification was made using a ChemData Station (Agilent Technologies, Wilmington, DE, 19801).

Antibody Titer to Sheep Red Blood Cells

Thirty birds per treatment per sex per sampling period were used. Antibody titers were assayed at 1, 2, 4 and 8 months after starting the dietary treatment supplementation. The dosage of SRBC for inoculation was pre-determined by a separate trial. Birds were immunized with 0.1 ml of a 2.5% SRBC via the brachial vein. Antiserum to SRBC was collected 7 days post challenge. One ml of blood with one drop of EDTA (Sigma® Chemicals Co, St Louis, MO, 63178) was refrigerated to allow red blood cells to settle. If sedimentation was not complete, samples were centrifuged (DuPont Instrument®, Wilmington, DE, 19880) for 1 to 2 min at 3000 rpm to separate plasma and erythrocytes, and the supernatant was collected.

Antibody titers were determined by the method of Wegmann and Smithies (1966). Briefly, 96-well plates were first filled with 25 µl of saline in each well. Then 25 µl of antiserum was pipetted into the first well in duplicates after which 25 µl from the first well was pipetted into the second well, and so forth using an automatic titrater (Flow Laboratories, Titerteck® Medimixer, UK). Finally, a 0.75% of SRBC solution was added to each well. Plates were incubated at 37°C for 3 hours and then examined visually for agglutination. The last well that showed agglutination was determined and recorded.

Cutaneous Basophil Hypersensitivity

The same animals were used to test cutaneous basophil hypersensitivity (CBH) as those for the antibody titer trial. In order to avoid possible interference from injection of SRBC, a two week interval for recovery was allowed.

Phytohemoaglutinin-P (PHA, Sigma® Chemicals Co, St Louis, MO, 63178) was used as the mitogen to evaluate the CBH response that was categorized as type IV hypersensitivity (delayed-type hypersensitivity). Sterile saline (0.9% NaCl) was used to reconstitute the PHA to a concentration of 50 µg/10µL. The proper dosage of PHA was determined by a pre-trial (APP. I). The preparation of the PHA solution was completed immediately prior to the first injection, to prevent possible degradation of the mitogenic level of PHA. A 10 µL syringe (Hamilton®, Reno, NV, 89520) was used to deliver a total of 50 µg of PHA subcutaneously into the toe web between the second and third digit. The toe web of the other leg served as a control and was injected with the same volume of sterile saline. The methodology was similar to that described for young chicks (Corrier and DeLoach, 1990). In each trial, injections were performed within 2 hours.

The response of CBH to PHA mitogen was calculated by the following formula:

Response = Diff (PHA) – Diff (Saline)
Diff (PHA) = Increment of thickness of toe web post PHA injection.
Diff (Saline) = Increment of thickness of toe web post saline injection.

The increment of skin thickness was determined by the difference in measurement of the web thickness prior to injection and 24 hours post injection of the same foot. All measurements of toe web thickness were performed using a spring micrometer with an accuracy of ± 0.001mm.

The CBH response over a 72 hours time period was also conducted using the same

method described above. A total of 8 birds per sex per treatment at 5 month post dietary lipids treatment were used. The measurements were taken at 12, 24, 36, and 72 hours post PHA challenge.

In vitro Non-Radioactive Lymphocyte Blastogenesis Assay

A total of 80 quail at 10 months-of-age (9 month lipids supplementation) were used for the lymphocyte proliferation assay. A 3 ml sample of blood was collected from the jugular vein with a 25-gauge needle and placed in heparinized 6 mm x 10 mm glass tube. A small aliquot of blood was used for a hematological profile which included cells stained using the Natt-Herrick Blood Stain (Natt, and Herrick, 1952) for examining lymphocyte population, packed cell volume and plasma total protein via a refractometer (Refractometer Handheld A 300 CL, Fisherbrand cat# 142116, Fisher's Scientific, PA, 15275). A whole blood smear stained by Giemsa Blood Stain (Crolina Biological Co. Burlington, NC, 27215) was examined under microscope.

Isolation of peripheral leukocytes and assessment of *in vitro* lymphocyte proliferation was conducted based on the technique described by Gogal *et al.* (1997). Blood was layered on 3 mL of Lymphoprep™ (Density 1.077; Nycomed, Oslo, Norway) after a 1:1 dilution of blood with RPMI 1640 (Sigma®; Sigma Chemicals Co, St Louis, MO, 63178). Samples were then centrifuged at 250 x *g* at 4^{o}C for 20 min. The suspension of peripheral leukocytes in the buffy layer was collected and washed 3 times with fresh RPMI 1640. The final pellet was then resuspended in 3 ml of cold complete RPMI media containing 10% fetal bovine serum (Sigma® Co, St. Louis, MO, 63178), 2 mM L-glutamine, 50 IU/ml penicillin and 50 mg/ml streptomycin (Sigma®; Sigma Chemicals Co, St Louis, MO, 63178). Then 10 µl of the re-suspended sample (stock leukocyte solution) was taken for

total cell count using a cell counter (CASY® Schärfe System GmbH, Emil-Adolff-Straße 14, D-72760 Reutlingen, Germany), and live/dead percentage was determined using Trypan Blue (Sigma®; Sigma Chemicals Co, St Louis, MO, 63178) stain at a dilution of 10µl stock / 990µL stain. The stock cell solution was quantitated to a concentration to 5 x 10^6 cells/ml of actual live peripheral blood leukocyte (PBL) via the following formulas,

Life/Death Adjustment

$$\textit{Counted PBMC/ml x (1 - } \frac{\textit{Live}}{\textit{Death}} \textit{ Live/Death ratio) = Actual Live PBL/ml}$$

$$\frac{5\times10^6}{ActualLivePBMC} = K$$

Make up a working cell solution with 5 x 10^6 by combining the following preparations -

$$\textit{The amount to pipette from counted PBL solution} = \frac{1000\mu L}{K} = S$$

$$\textit{The amount to pipette from complete RPMI media} = 1000\ \mu L - S$$

A 96-well round bottom plate (Corning Glass Works, Corning, NY) was used for cell culture. A 100 µl aliquot of cells at a concentration of 5 x 10^6 stock solution was added to quadruplicate wells containing 100 µl of either medium alone, concanavalin A (CON A, 25 µg/ml, Sigma®), lipopolysaccharide (LPS, 20ng/ml, Sigma®), or phorbol 12-myristate 13-acetate (PMA, 400ng/ml, Sigma®) + ionomycin (ION, 10 ng/ml, Sigma®). The plate was then incubated in a humidified incubator at 37°C and 5% CO_2. After 24 hours, 25 µL of Alamar Blue Dye™ (Accumed International Inc., Chicago, IL, 60610) was added into each well and the plate was returned to the incubator for another 24 hours. The dye is in a blue oxidized form and is reduced to a red color as cells proliferate. Alamar Blue Dye™ also has a fluorescent property when it is reduced. A CytoFluor II Fluorescence

Multi-Well Microplate Reader (Perceptive Biosystems, Inc., Framingham, MA, 01701) was used to measure reduced form at excitation of 530/25 nm and the oxidized form at an emission 590/35 nm, gain = 30. The level of lymphocyte proliferation was determined by the change in absorbency (Δ specific absorbency) obtained by the difference between the mean values of absorbancies of triplicates from unstimulated cell medium and mitogen stimulated cell medium.

Statistical Analysis

The statistical analysis for antibody titers was performed using the GLM model procedure of SAS (SAS Institute, 1996). The main effects include gender, lipid treatments, and time post lipid diet supplementation. Significance between treatments was determined using Tukey's test. All titers were transformed to the square root of the log2 before analysis. Cutaneous basophil hypersensitivity (delayed-type hypersensitivity) was analyzed by a three- way ANOVA with main effects including dietary lipid treatments, sex, and length of supplementation. Significance between treatments was tested by Tukey's method. The statistical model is as follows:

$$Y_{ijkl} = \mu + Treatment_i + Sex_j + Treatment*Sex_{ij} + Length_k + Treatment*Length_{ik} + e_{ijkl}$$

In the analysis of mitogen stimulated PBL proliferation, the three- way ANOVA was performed with main effects being treatment, sex and batch of assay performed.

Results

Fatty Acid Composition of Diets, Plasma and Liver

The fatty acid analysis of the diets and lipid sources is shown in Table 2.3. As expected, the FO diet contained a higher amount of total n-3 PUFA, particularly DHA.

The fatty acid profile of the SBO diet was rich in linoleic acid (LA 18:2 n-6) and α-linolenic acid (ALA) that were at least 2 times higher than in the FO and HSBO diets, while the CF diet had intermediated concentrations. Total n-6 PUFA was about two times higher in the CF and SBO diet than in the FO and HSBO diets. Large amounts of MUFA were found in the HSBO diet. The majority of MUFA in the HSBO diet was trans $C_{18:1}$, whereas the CF diet contained large amounts of cis $C_{18:1}$. Similar to the CF diet, the FO diet was also rich in MUFA, particularly cis $C_{16:1}$, $C_{18:1}$, and $C_{24:1}$. The percentage of PUFA in each diet was 66.6%, 50.23%, 50.2%, and 34.63% in the SBO, FO, CF, HSBO diet respectively. The calculated percentage of PUFA, SFA, MUFA, trans isomers, n-6 PUFA and n-3 PUFA of total fatty acids is shown in Figure 2.1.

Plasma and hepatic fatty acid composition are shown in Tables 2.4 and 2.5. Interestingly, while plasma fatty acids reflected the dietary fatty acid profile, the proportion of hepatic SFA and MUFA did not differ among treatments even after long-term supplementation. In addition, plasma $C_{18:2}$, $C_{20:3}$, and $C_{22:4}$ n-6 PUFA were not affected by feeding different dietary lipids. Both plasma and hepatic AA ($C_{20:4}$) were significantly changed in the order SBO > CF > HSBO > FO diet. It was noteworthy that EPA and DHA were significantly higher in the plasma of FO fed birds, but there was no difference in hepatic EPA concentration among all treatments. Only trace amounts of EPA were detected in the liver of FO fed birds. This resulted in a much higher (three times) n-3/n-6 ratio in the plasma than in the liver of FO fed birds. It is a reasonable assumption that circulating EPA may be quickly metabolized in the liver of Japanese quail.

Total Antibody Production

The SBO and CF treatments, which had 61% and 46% n-6 PUFA, respectively, in total

dietary fatty acids did not affect antibody titers against SRBC, nor did the FO diet rich in n-3 PUFA or the HSBO diet rich in MUFA (Fig 2.2). At the 4-month sampling period, female quail fed the FO diet had a significantly ($P < 0.05$) higher serum total antibody compared to other treatments. However, there were no differences found in other sampling periods. Antibodies initially produced against an antigen are primarily immunoglobulin M, while in response to a second exposure are primarily immunoglobulin G. The secondary antibody response was significantly ($p< 0.01$) higher than the primary response in all treatments (data not shown). However, there were no dietary effects on either the primary or secondary antibody response. In general, the overall mean antibody titer was at least one log unit higher than the other treatments in FO-fed female quail.

Cutaneous Basophil Hypersensitivity

There was a significant ($p< 0.05$) treatment effect on *in vivo* evaluation of cell-mediated immunity. While the overall CBH response peaked at 24 hr post PHA injection ($p< 0.01$), the birds fed FO and HSBO had a peak CBH response at 24-hour post PHA injection compared to that of CF and SBO which had highest response at 12 hour (Figure 2.3). At 12 hr post challenge, the SBO group had a significantly greater ($P < 0.05$) CBH response than that of the FO and HSBO groups. Birds fed CF had an intermediated response and peaked at 12 hr, which was similar to the SBO group. By 24-hour post PHA injection, the CBH response in birds fed HSBO and FO started to peak and resulted in a significantly higher toe web thickness in the FO fed birds compared to the CF fed birds. The CBH response did not differ among treatments at 48 hr. At the last sampling period, 72 hr, SBO and CF fed birds had significantly lower skin thickness than that of FO fed birds, which indicates a faster recovery from PHA- induced inflammation.

Since there was no significant sex effect on the CBH response, data from both sexes were pooled. The effect of dietary lipids on the CBH response is shown in Figure 2.4. No significant treatment effects were observed on CBH response at one-month post dietary lipid treatment supplementation. However, there were significantly higher CBH responses in quail fed FO at 2, 4 and 8 months after beginning dietary treatment. Quail fed HSBO had a higher CBH response at 2 month, but decreased with increasing length of supplementation. CF and SBO fed birds did not have a consistent elevation or suppression in the *in vivo* index of cell-mediated immunity, possibly due to measurements not being taken from their peak response.

In vitro Non-radioactive Lymphocyte Proliferation

Since this was the first time a non-radioactive lymphocyte proliferation assay was conducted on Japanese quail, a preliminary trial to optimize mitogen concentrations for CON A, LPS and PMA+ION was performed. The optimal dose for CON A mitogen to stimulate T lymphocyte proliferation was 25 µg/ml (APP. II), and the LPS to stimulate B lymphocyte proliferation was 200 ng/ml (APP. III). For the proliferation of both T and B cells stimulated by PMA + ION, 400 ng/ml PMA and 10 ng/ml ION was used (APP. IV). CON A specifically stimulates the T-cell population and was used to evaluate cell-mediated immunity whereas LPS was used to specifically stimulate the B lymphocyte population. Birds fed HSBO, which is high in MUFA and trans fatty acid isomers, had depressed ($p < 0.05$) T and B cell proliferation compared to other treatments (Fig. 2.5). In general, Japanese quail lymphocytes did not respond to PMA+ION stimulation very well. However, there was better ($p < 0.05$) proliferation in CF and SBO fed birds compared to that of FO and HSBO. Female quail had a significantly ($p < 0.05$) higher T cell proliferation

induced by CON A than males. Male and female bird lymphocyte proliferations by mitogens are shown separately because of a significant sex effect. In both sexes, quail fed FO had a significantly higher ($p< 0.05$) T cells response compared to those fed HSBO. There was no significant difference in B cell proliferation stimulated by LPS in females. However, male quail fed SBO had a better B cell response than those fed FO and HSBO.

The blood profile of Japanese quail is shown in Table 2.6. Female birds had a significantly ($p <0.05$) higher plasma total protein compared to males. The PCV was significantly ($p <0.05$) higher in males than females. There was no significant difference across all treatments in the blood cytology (data not shown).

Discussion

The current study demonstrated that the levels of dietary n-3, n-6 fatty acids, monounsaturated fatty acids, trans fatty acid isomers and degree of saturation of fatty acids differentially affected immunity in Japanese quail. Incorporation of SBO and FO oils into the basal diet resulted in highly unsaturated fatty acid diets (66% and 50% of PUFA in total fatty acids, respectively). Furthermore, among the proportion of total PUFA in each diet, the ratio of n-3/n-6 PUFA was FO (0.419) > SBO (0.07) > CF (0.06) > HSBO (0.05). The respective lipid diets resulted in plasma n-3/n-6 PUFA ratio FO (0.78) > CF (0.18) > SBO (0.06) = HSBO (0.06), whereas hepatic ratio was FO (0.87) > SBO (0.13) > CF (0.10) > HSBO (0.08).

In general, the plasma and hepatic fatty acid profile reflected their dietary fatty acid composition. Although the SBO diet contained a large quantity of n-6 PUFA, the plasma n-6 PUFA level was not significantly different from each other. This suggested a homoeostasis of the n-6 PUFA was maintained in the blood of Japanese quail. On the

other hand, hepatic SFA and MUFA were only slightly affected by dietary lipid manipulation and remain relatively unchanged across different treatments. Moreover, the HSBO diet contained 29% of n-6 PUFA that was much less than that of SBO (61%), FO (40%) and CF (46%). The lower n-6 PUFA content in the HSBO diet did not result in significantly lower plasma and hepatic n-6 PUFA concentrations, while birds consuming the FO diet had the lowest plasma and hepatic n-6 PUFA contents. The FO contained large amount of n-3 PUFA which may interfere with the absorption, desaturation and elongation of n-6 fatty acids. Other interference in fatty acid metabolisms by feeding different type lipids was also studied in broiler chicks by Friedman and Sklan (1995). They found that decrease the amount of SFA in diet result in increasing serum $C_{18:2}$ level, while increasing the amount of $C_{18:2}$ in diet does not alter serum AA level. The 20 carbon n-3 and n-6 PUFA are precursors for eicosanoids synthesis. Quail fed FO had a significantly lower level of plasma and hepatic AA that could decrease the capacity to synthesize PGE_2 and LTB_4 while the higher concentration of EPA in the plasma may provide for the production of less bio-active PGE_3 and LTB_5. Fritsche et al. (1991) reported that autogolous serum from FO-fed chicks had negative effects on lymphocyte proliferation indicating that certain contents in plasma may play a role in the immuno-suppressive effect of feeding fish oil.

Trans fatty acid isomers were readily absorbed in quail fed HSBO resulting in a large quantity of trans $C_{18:1}$ in plasma and liver. An accumulation of hepatic trans isomers, particularly trans $C_{18:2}$, was also found in quail fed the CF and FO diet. Conjugated linoleic acids (CLA) are those $C_{18:2}$ fatty acids with 2 double bonds conjugated. Quail fed HSBO diet, which contain non-detectable cis-9, trans-11 CLA but is rich in trans-11 $C_{18:1}$ had significantly increased plasma and hepatic cis-9, trans-11 CLA levels. Therefore,

feeding quail hydrogenated oil containing trans-11 $C_{18:1}$ may have potential benefit for providing a CLA fortified meat product as opposed to feeding conventional CLA sources which include solely beef and dairy products. Another well-known CLA, trans-10, cis-12 CLA was found in trace amounts in CF diet but not detectable in the other lipid diets, plasma or liver samples. The trans-10, cis-12 CLA has potent properties in inhibiting de novo fatty acid synthesis. CLA had been shown to decrease fat production by inhibiting de novo fatty acid synthesis (Loor and Herbein, 1998), exerting an anticarcinogenic effect (McGuire and McGuire, 1999) and decreasing PGE_2 synthesis (Liu and Belury, 1998; Li and Watkins, 1998). The presence of CLA in plasma and liver of quail fed HSBO may have partially contributed to our current findings.

Studies on the effect of dietary lipid in immune functions are more extensive in mammalian than avian species. As reviewed by Calder, (1997) mostly based on mammalian research, diets high in n-3 PUFA have a suppressive property on rat and mouse spleno-lymphocyte proliferation by a diet containing 7% to 20% FO compared with either animal fat or a diet high in n-6 PUFA. In addition, dietary FO depressed natural killer cell activity, cytokine production, and antigen presentation in mice fed 10 to 20% FO (Meydani *et al*. 1988; Fritsche and Johnstone, 1990), while also enhancing some parameters of phagocytic cell function and antibody production (Prickett *et al*., 1982; Fritsche *et al*. 1991). Conclusions made in mammalian studies were based on feeding much higher lipid contents in the diet compared to the current study in birds. Feeding 5 % fish oil in a corn-soybean basal diet in the current study resulted in a relatively moderate change in the ratio of n-3 and n-6 PUFA. Dramatically increasing n-3 PUFA level in the diet may provide a pharmaceutical effect on health rather than a nutritional modulation.

There was a significant increase in total antibody titer against a non-replicating antigen,

SRBC, in female quail fed the FO diet for 4 month. However, there were no other dietary differences in total antibody titers against SRBC across different sampling periods. Consistent with the *in* v*ivo* results in antibody production, LPS-induced B lymphocyte proliferation was not affected by dietary lipid treatments in females, whereas male quail fed FO and HSBO had significantly lower B-cell proliferation compared to SBO fed birds. Although the antibody titer was not different, the overall titer in birds fed FO and HSBO were lower than in those fed CF or SBO. Wang *et al*. (2000) reported a similar result in which birds fed a high in n-3 PUFA (FO diet) had a significantly higher antibody production than those fed SBO, linseed oil or animal fat at 8 weeks-of-age, but not 4 weeks-of-age. Similarly, Korver and Klasing (1997) reported that circulating levels of antibody against the infectious bronchitis virus were not significantly affected by dietary lipid treatments. They used corn oil and fish oil in combination with either cereal grain or corn based diets to provide 9 different combinations of n-3/n-6 ratio ranging from 0.07 to 0.98. The antibody titer was not changed by altering the n-3/n-6 PUFA ratio could probably due to the relatively short supplementation period. Moreover, it has also been reported that the n-6 linoleic acid enhanced antibody responses to SRBC while the n-3 α-linolenic acids decreased antibody responses to bovine serum albumin in chickens genetically selected for humoral immunity (Parmentier *et al*., 1997).

The conflicting results of the effects of dietary n-3 PUFA on humoral immunity could be due to different level of incorporation of dietary fatty acid in the diets and the sources of n-3 PUFA being either ALA or other n-3 PUFA. Other factors such as the length of the dietary lipid supplementation and the immune-assay used could account for the different findings.

According to Sijben *et al*. (2000; 2001), the effect of dietary linoleic acid (LA) and

α-linolenic acid (ALA) on antibody production, delayed-type hypersensitivity and lymphocyte proliferation was antigen dependent. They immunized growing layer hens fed 16 different levels of combinations of LA and ALA with either keyhole limpet hemocyanin (KLH, T helper 2 dependent) or *Mycobacterium butyricum* (MB, T helper 1 dependent) particles. Dietary LA was negatively correlated to antibody response to MB, but not KLH, and ALA exerted a positive correlation to MB. In order to mount a humoral immune response, B cells need to go through differentiation to become plasma cells, which produce antibodies. The process is under regulation of cytokines such as IL-4 and IL-6 mediated by T helper cells ($CD4^+$), especially those cells from T_{H2} subfamily. Therefore, the effect of dietary lipid on humoral immunity may be indirectly influenced by altered T cell functions. Although, the concept of the mammalian T helper cells dimorphic effects is still not fully elucidated in avian species, a recent finding (Vandaveer *et al*., 2001) of shifting T_{H1}/ T_{H2} balance by antigen delivery to scavenger receptors could give some insights in this area in birds. To evaluate the effect of fatty acid on humoral immunity is more difficult since humoral immunity requires a synergistic effort by both T and B cells.

It is a strongly believed that n-3 PUFA depress PGE_2 synthesis, which can further decrease production of a variety of cytokines (Liu and Denbow, 2000; Kelley, 2001; Calder *et al*., 2002). In mammalian models, decreasing pro-inflammatory cytokines such as IL-1 and TNF by n-3 PUFA was shown to decrease T and B cell proliferation and *in vivo* measure of cell-mediated immunity thereby ameliorating symptoms of autoimmune diseases (Ziboh *et al*., 1986; Kremer *et al*., 1990). In the present study, both *in vivo* and *in vitro* evaluation of cell-mediated immunity in Japanese quail showed that an enriched n-3 PUFA diet resulted in an increased CBH response and T lymphocyte proliferation stimulated by CON A. This result was consistent with other reports (Korver and Klasing,

1997; Sijben *et al.*, 2001). In addition, our result indicated that long-term supplementation with high SFA and MUFA diet (HSBO group) had a negative impact on both CBH and lymphocyte proliferation.

Cutaneous basophil hypersensitivity is presently the best *in vivo* evaluation of cell-mediated immune response. PGE_2 upregulates production of pro-inflammatory cytokines leading to increases in the DTH response and T cell proliferation. However, this mechanism does not apply in avian species, or at least is not the sole explanation for the present study using healthy quail. Quail fed FO had significantly increased plasma EPA levels and lowered AA levels, which was opposite to that of CF and SBO fed birds. Therefore, production of a less bio-active PGE_3 from EPA, and decreasing substrate (AA) for PGE_2 production may be expected in FO fed birds. However, the reduction of PGE_2 by inclusion of n-3 PUFA in the diet did not suppress the CBH response in this study. Only the time frame in the peak CBH response and resolution of the inflammation were slower than that of SBO and CF fed birds. Since it had been reported that n-3 PUFA inhibited expression of various cell adhesion molecules (Caterina and Libby, 1996), suppression in these molecules hampers cells trafficking and migration. The slow reaction time in cell-meditated immunity may partially explain the immunosuppressive effects of n-3 PUFA.

The HSBO group with the highest SAF, MUFA and lowest essential fatty acids content had a significantly ($P < 0.05$) lower CBH and T cell proliferation after long-term supplementation. This could be due to an insufficient production of eicosanoids. Interesting, measurement of CBH was conducted 24 hours post PHA injection across all sampling periods. However, our study on the CBH response over a period of 72 hours indicated that SBO and CF fed bird had a different temporal response to FO and HSBO

groups. Like in birds fed FO, birds fed HSBO diet had a late peak CBH response compared to SBO and CF groups. However, different from effect of n-3 PUFA, the amplitude of the CBH response was significantly lower which is consistent with *in vitro* results that lymphocytes did not proliferate in response to stimulation by various mitogens in birds fed HSBO. In humans (hypercholesterolemia patients), it has been reported that consumption of a diet high in hydrogenated soybean oil did not adversely affect lymphocyte proliferation and DTH responses while increasing production of IL-6 and TNF (Han *et al*., 2002). The short period of supplementation in the experiment may have negated the deleterious effects of HSBO in specific immunity.

Long-term feeding HSBO diet in Japanese quail has impaired lymphocyte proliferation by different mitogens. The HSBO diet contained the lowest PUFA (19%) and the highest MUFA (44%) and SFA (21%) of total fatty acids. Highly saturated fatty acid diets have detrimental effects on immunity due to impaired PGE_2 synthesis and decreased membrane permeability (Calder, 1997). Previously, it was demonstrated that maternal diets enriched in high trans-18:1 fatty acids significantly ($p< 0.01$) decreased *ex vivo* bone marrow cell PGE_2 production in neonatal quails (Liu and Denbow, 2001). Since bone marrow contains pluripotent stem cells for differentiation of all haemopoietic cells, it is reasonable to speculate that reduction of PGE_2 by dietary HSBO could affect the both function and population of immunocompetent cells and their differentiation.

In conclusion, the present study used Japanese quail to evaluate the effect of dietary fatty acid on immunity. Diets enriched with n-3 PUFA increased both *in vivo* and *in vitro* cell-mediated immunity, and slightly increased total antibody production in female quail. In addition, n-3 PUFA divergently affected T and B cells proliferation. Dietary FO increased CON A stimulated lymphocyte population but either did not affect or decreased

LPS stimulated lymphocyte proliferation. The n-3 PUFA tended to have more effect on cell-mediated immunity and less influence on humoral immunity. Moreover, the HSBO diet contained a large quantity of MUFA with dominantly trans fatty acid isomers which should draw attention to its detrimental effect on various immunological indices after a long term feeding. Finally, our results indicate that different dietary lipids changed both the response time and amplitude in the *in vivo* CBH assay. Since the inflammatory reaction can be divided into acute (T cell independent) and chronic (T cell dependent) responses controlled by different effectors and mediators, different fatty acids could differentially affect those inflammatory conditions by altering factors such as membrane property, receptor affinity, cell trafficking, and mediator productions.

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TABLE 2.1. Feed ingredients of quail starter diet. [1]

Ingredients	%
Yellow corn	42.20
Soybean meal	38.00
Blended fat	3.50
Fish meal	7.00
Meat and bone scrap	5.00
Dehydrated alfalfa meal	1.25
Defluorinated phosphate	1.00
Limestone	0.50
Vitamin premix [2]	1.00
Trace mineral premix [3]	0.05
Salt	0.50

[1] Starter quail feed was fed to quail from hatch until 4 weeks-of-age. The feed was calculated to contain 29.6% crude protein, 2970 kcal/kg metabolizable energy, 5.5% crude fat, 2.7% crude fiber, 1.4% calcium, and 0.7% phosphorous.

[2] Vitamin premix from Big Springs Mill (Springfield, VA). Guaranteed analysis per kilogram: selenium (sodium selenite): 18.2 ppm; vitamin A (vitamin A acetate): 454,000 IU; vitamin E: 1362 IU; vitamin B_{12} (pyridoxine hydrochloride): 0.68 mg; menadione (menadione sodium bisulfite complex): 48.12 mg; biotin: 2.27 mg; choline (choline chloride): 13393 mg; folic acid: 45.4 mg; niacin: 1816 mg; d-pantothenic acid (d-calcium pantothenate): 626.52 mg; vitamin B_6 (pyridoxine): 37.23 mg; riboflavin: 272.4 mg; thiamine (thiamine mononitrate): 40.41 mg.

[3] Trace mineral premix from Big Springs Mill (Springfield, VA). Guaranteed analysis per kilogram: zinc (zinc sulfate): 120 g; manganese (manganese sulfate): 120 g; iron (ferrous sulfate): 40 g; copper (copper sulfate): 5 g; iodine (calcium iodate): 2 g; cobalt (cobalt carbonate): 0.45 g; calcium (calcium carbonate): 280 g.

TABLE 2.2. Feed ingredients and formulation of experimental diet in each treatment.[1]

Ingredients (%)	CF	FO	HSBO	SBO
Yellow corn	53.525	53.525	53.525	53.525
Soybean meal	32.00	32.00	32.00	32.00
Soybean oil	..	..	..	5.00
Hydrogenated soybean oil	..	..	5.00	..
Chicken fat	5.00	..	..	..
Menhaden fish oil	..	5.00	..	..
Limestone	6.50	6.50	6.50	6.50
Dicalcium phosphate	1.50	1.50	1.50	1.50
Vitamin premix [2]	1.00	1.00	1.00	1.00
Trace mineral premix [3]	0.10	0.10	0.10	0.10
Salt	0.35	0.35	0.35	0.35
Ethoxyquin (antioxidant)	0.025	0.025	0.025	0.025

[1] The experimental quail feed was fed to quail after starter diet at 1 month of age. The feed was calculated to contain 21% crude protein, 2997 to 3007 kcal/kg metabolizable energy, 7.36% crude fat, 2.43% crude fiber, 2.81% calcium, and 0.67% phosphorous.

[2] Vitamin premix from Big Springs Mill (Springfield, VA). Guaranteed analysis per kilogram: selenium (sodium selenite): 18.2 ppm; vitamin A (vitamin A acetate): 454,000 IU; vitamin E: 1362 IU; vitamin B_{12} (pyridoxine hydrochloride): 0.68 mg; menadione (menadione sodium bisulfite complex): 48.12 mg; biotin: 2.27 mg; choline (choline chloride): 13393 mg; folic acid: 45.4 mg; niacin: 1816 mg; d-pantothenic acid (d-calcium pantothenate): 626.52 mg; vitamin B_6 (pyridoxine): 37.23 mg; riboflavin: 272.4 mg; thiamine (thiamine mononitrate): 40.41 mg.

[3] Trace mineral premix from Big Springs Mill (Springfield, VA). Guaranteed analysis per kilogram: zinc (zinc sulfate): 120 g; manganese (manganese sulfate): 120 g; iron (ferrous sulfate): 40 g; copper (copper sulfate): 5 g; iodine (calcium iodate): 2 g; cobalt (cobalt carbonate): 0.45 g; calcium (calcium carbonate): 280 g.

TABLE 2.3. Fatty acid analysis of the diets and lipids.[1]

	Diets				*Lipids*			
µg/mg	CF	FO	HSBO	SBO	CF	FO	HSBO	SBO
14:0	0.1346	0.7154	0.0446	0.0177	2.6380	19.8873	0.5154	0.2779
14:1	0.0060	0.0045	0.0000	0.0000	0.0000	0.1458	0.0000	0.0000
15:0	0.0124	0.1270	0.0091	0.0048	0.1877	2.9929	0.1069	0.0580
16:0	4.2029	4.5803	3.4038	2.8682	40.8168	76.5801	40.5275	31.7009
t9 16:1	0.0021	0.0537	0.0016	0.0000	0.1089	1.2209	0.0560	0.0000
c9 16:1	0.2026	2.2137	0.0420	0.0192	3.2051	52.4840	0.1815	0.2718
17:0	0.0446	0.1069	0.0374	0.0271	0.6794	2.2885	0.6439	0.4131
18:0	1.6744	0.8488	3.1240	1.1614	27.6821	14.0475	73.0041	19.9927
t6 & t7 18:1	0.1361	0.0098	1.1109	0.0050	2.8164	0.1696	27.2111	0.0000
t9 18:1	0.1747	0.0097	1.3882	0.0079	3.5236	0.2729	34.9593	0.0317
t10 18:1	0.3582	0.0024	2.1353	0.0101	7.2682	0.0000	52.9094	0.0000
t11 18:1	0.3284	0.0051	2.1123	0.0126	6.6747	0.1055	53.2283	0.0901
t12 & c7 18:1	0.1202	0.2428	1.0210	0.0022	2.4350	5.8212	25.5114	0.0000
t13&c6 18:1	0.1710	0.0000	1.5014	0.0000	3.1225	0.0000	39.3251	0.0000
c9 18:1	8.0059	3.3742	6.6990	6.1515	114.3876	27.8374	86.4639	98.5158
c11 18:1	0.3741	0.4433	0.5576	0.2628	6.1723	8.9397	12.1562	4.8908
c12 18:1	0.4730	0.0044	1.8633	0.0077	9.4811	0.2981	46.5922	0.0000
c13 18:1	0.0436	0.0210	0.2812	0.0163	0.7228	0.3780	7.1605	0.2701
t16 18:1	0.0245	0.0009	0.1668	0.0000	0.2720	0.3921	1.7894	0.0000
c15 18:1	0.0284	0.0230	0.0929	0.0000	0.0000	0.1421	0.7923	0.0000
t9,t12 18:2	0.0167	0.0309	0.0258	0.0055	0.3007	0.7149	0.6333	0.0000
c9,t12 18:2	0.1541	0.0418	0.0454	0.1938	2.9980	0.7370	1.3740	3.0368
t9,c12 18:2	0.1166	0.0655	0.0260	0.1640	2.4480	0.1247	1.1176	2.7368
18:2n6	15.4107	7.1282	7.4201	19.4368	155.8825	6.0801	1.1450	306.1599
20:0	0.1144	0.0663	0.1295	0.0984	1.4423	0.7760	1.2953	1.6330
18:3n3	0.9420	0.5331	0.3033	1.2909	12.7569	5.9820	0.5297	26.7336
c9,t11 18:2	0.0114	0.0000	0.0000	0.0000	0.2465	0.0474	0.0000	0.0000
c11,t13 18:2	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
t10,c12 18:2	0.0116	0.0000	0.0000	0.0000	0.2451	0.0000	0.0000	0.0000
t11,t13 18:2	0.0034	0.0275	0.0028	0.0000	0.0981	0.6253	0.0000	0.0000
other t/t 18:2	0.0331	0.5631	0.0015	0.0189	0.9450	13.1357	0.0000	0.3435
20:3n6	0.0053	0.0396	0.0009	0.0000	0.1094	0.9614	0.0000	0.0000
22:1	0.0074	0.0673	0.0030	0.0019	0.1483	1.5601	0.0000	0.0000
20:4n6	0.0092	0.1805	0.0043	0.0016	0.1455	4.2605	0.0000	0.0000
24:1	0.0025	2.7705	0.0376	0.0040	0.0248	66.1658	0.0000	0.0000
20:5n3 EPA	0.0396	0.0254	0.0471	0.0329	0.5807	0.1868	0.6491	0.3545
24:0	0.0011	0.0554	0.0010	0.0000	0.0510	1.3193	0.0000	0.0000
22:4n6	0.0023	0.0401	0.0000	0.0000	0.0494	0.9544	0.0000	0.0000
22:5n3	0.0121	0.4562	0.0172	0.0045	0.1539	10.7558	0.0969	0.0000
22:6n3 DHA	0.0017	2.0801	0.0295	0.0017	0.0183	49.8156	0.0000	0.0000
Total fatty acid µg/mg	33.4128	26.9582	33.6873	31.8294	410.8383	378.2062	509.9753	497.5111
SFA [2]	6.1844	6.5001	6.7493	4.1775	73.4973	117.8915	116.0931	54.0757
MUFA [3]	10.4586	9.2461	19.0142	6.5013	160.3631	165.9331	388.3365	104.0704
PUFA [4]	16.7698	11.2119	7.9239	21.1506	176.9779	94.3816	5.5457	339.3650
Total trans fatty acid [5]	1.6621	1.0532	9.5389	0.4200	33.5025	23.3671	238.1149	6.2388
N-6 [6]	15.4275	7.3884	7.4253	19.4384	156.1868	12.2565	1.1450	306.1599
N-3 [7]	0.9954	3.0947	0.3970	1.3300	13.5098	66.7402	1.2757	27.0881
n-3:n-6 ratio [8]	0.0644	0.4187	0.0537	0.0683	0.0865	5.4453	1.1141	0.0885

[1]The dietary lipids include chicken fat (CF oil), menhaden fish oil (FO oil), hydrogenated soybean oil (HSBO oil) and soybean oil (SBO oil). The dietary treatments are chicken fat (CF), menhaden fish oil (FO), hydrogenated soybean oil (HSBO) and soybean oil (SBO). Fatty acid was calculated in the unit of μg/mg of sample.

[2] Sum of total saturated fatty acids of $C_{14:0}$, $C_{15:0}$, $C_{16:0}$, $C_{17:0}$, $C_{18:0}$, $C_{20:0}$, and $C_{24:0}$.

[3] Sum of total monounsaturated fatty acids of $C_{14:1}$, $C_{16:1}$, $C_{18:1}$, $C_{22:1}$, and $C_{24:1}$.

[4] Sum of total polyunsaturated fatty acids of $C_{18:2,}$ $C_{18:3}$, $C_{20:3}$, $C_{20:4}$, $C_{20:5}$, $C_{22:4}$, and $C_{22:6}$.

[5] Sum of total trans fatty acid isomers of t9$C_{16:1}$, t6t7$C_{18:1}$, t9$C_{18:1}$, t10$C_{18:1}$, t11$C_{18:1}$, t12c7$C_{18:1}$, t13c6$C_{18:1}$, t16$C_{18:1}$, t9t12$C_{18:2}$, c9t12$C_{18:2}$, t9c12$C_{18:2}$, c9t11$C_{18:2}$, t11t13$C_{18:2}$, and other trans $C_{18:2}$.

[6] Sum of total n-6 fatty acids of $C_{18:2}$, $C_{20:3}$, $C_{20:4}$, and $C_{22:4}$.

[7] Sum of total n-3 fatty acids of $C_{18:3}$, $C_{20:5}$, and $C_{22:6}$.

[8] Ratio of Σn-3 PUFA to Σn-6 PUFA.

FIGURE 2.1. The calculated percentage of saturated fatty acid (SFA), polyunsaturated fatty acid (PUFA), monounsaturated fatty acid (MUFA), total trans fatty acid (total trans FA), n-6 PUFA (N-6) and n-3 PUFA (N-3) in different dietary lipid treatments.

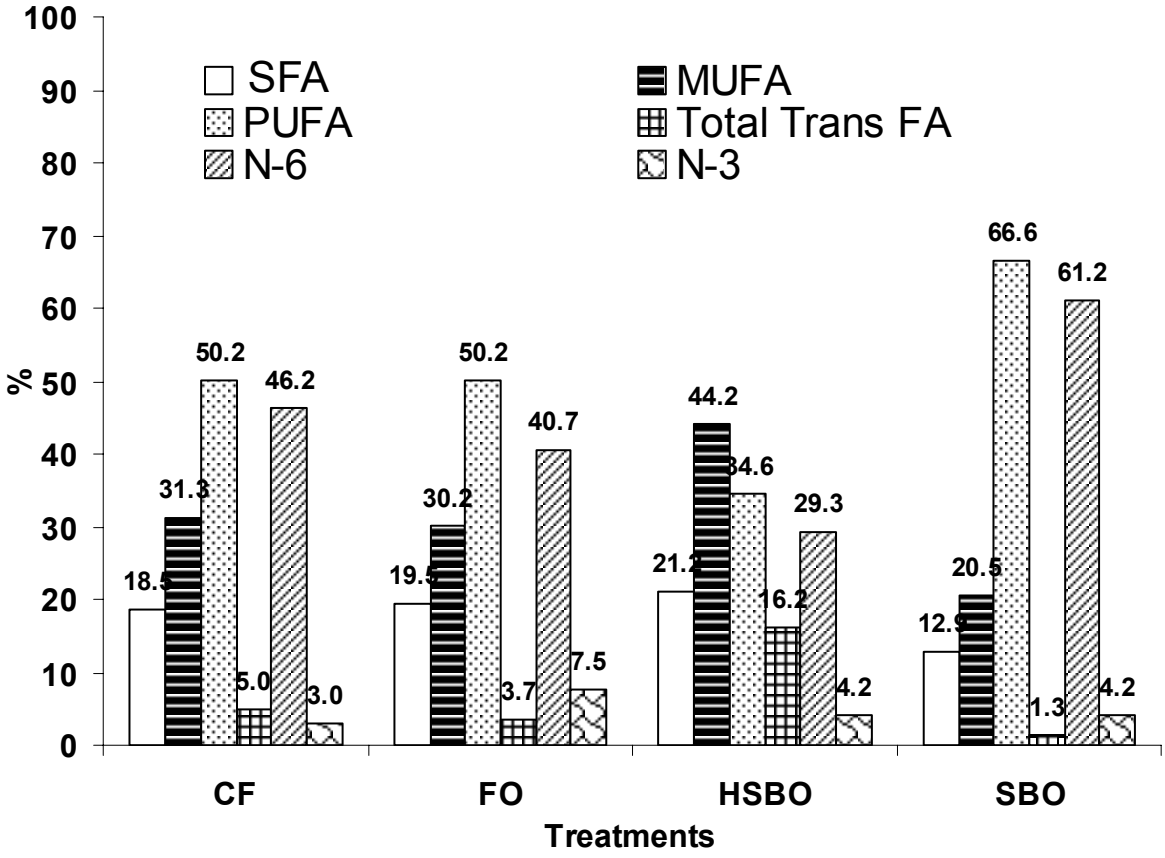


TABLE 2.4. Effect of feeding different lipids on plasma fatty acid composition in Japanese quail.[1]

Fatty Acid (μg/ml)	CF	FO	HSBO	SBO	Pooled SEM	P-value
14:0	12.14^{b}	31.73^{a}	6.78^{b}	5.85^{b}	2.96	0.0001
14:1	0.64	1.65	1.74	0.81	0.72	0.5895
15:0	1.95^{b}	5.18^{a}	1.03^{b}	1.17^{b}	0.45	0.0001
16:0	560.20^{b}	1185.84^{a}	502.53^{b}	557.47^{b}	100.87	0.0001
t9 16: 1	2.94	1.85	1.14	0.13	1.00	0.2356
c9 16:1	33.24^{bc}	118.85^{ab}	33.30^{c}	29.88^{bc}	12.31	0.0001
17:0	5.26^{b}	13.46^{a}	3.00^{b}	4.15^{b}	1.11	0.0001
18:0	597.27^{ab}	893.72^{a}	512.77^{b}	627.62^{ab}	83.62	0.0154
t6t7 18:1	5.22^{b}	0.98^{b}	23.60^{a}	0.32^{b}	2.47	0.0001
t9 18:1	8.36^{b}	5.75^{b}	51.40^{a}	3.95^{b}	4.47	0.0001
t10 18:1	8.52^{b}	4.64^{b}	53.93^{a}	1.76^{b}	4.32	0.0001
t11 18:1	9.11^{b}	3.82^{b}	49.69^{a}	0.51^{b}	3.58	0.0001
t12c7 18:1	7.19^{b}	6.75^{b}	35.19^{a}	0.06^{b}	2.88	0.0001
t13c6 18:1	6.30^{b}	4.35^{b}	47.64^{a}	1.49^{b}	3.62	0.0001
t16 18:1	0.84^{b}	0.30^{b}	5.05^{a}	0.00^{b}	0.42	0.0001
c9 18:1	674.37^{b}	1756.47^{a}	746.99^{b}	705.17^{b}	197.93	0.0006
c11 18:1	42.97^{b}	100.36^{a}	48.48^{b}	28.49^{b}	10.22	0.0001
c12 18:1	12.31^{b}	4.25^{bc}	69.24^{a}	0.38^{c}	5.70	0.0001
c13 18:1	2.14^{b}	3.79^{b}	8.63^{a}	1.25^{b}	0.78	0.0001
c15 18:1	3.42^{b}	4.62^{ab}	8.87^{a}	3.25^{b}	1.42	0.0244
t9t12 18:2	1.29^{ab}	1.54^{a}	1.57^{a}	0.79^{b}	0.20	0.0326
c9t12 18:2	10.49^{ab}	9.47^{b}	7.78^{b}	14.50^{a}	1.36	0.0090
t9c12 18:2	10.29^{a}	4.71^{b}	4.08^{b}	13.22^{a}	1.16	0.0001
18: 2 (n-6)	1359.93	1290.86	955.82	1474.42	145.36	0.0845
18: 3 (n-3)	28.08^{a}	27.88^{a}	11.17^{b}	28.37^{a}	3.39	0.0013
20:0	8.22	9.11	6.44	6.29	0.96	0.1173
c9t11 18:2	2.07^{b}	2.94^{b}	14.85^{a}	0.70^{b}	1.60	0.0001
t11t13 18:2	1.55	2.00	1.62	0.74	0.38	0.1356
Other t/t 18:2	4.53^{b}	16.84^{a}	1.90^{b}	1.36^{b}	2.21	0.0001

20:3 (n-6)	56.26	29.30	47.88	37.98	10.25	0.2590
22:1	2.57^{ab}	1.88^{b}	4.45^{ab}	5.37^{a}	0.93	0.0378
20:4 (n-6)	713.80^{ab}	437.31^{b}	582.68^{ab}	784.03^{a}	90.50	0.0465
20:5 (n-3) EPA	251.16^{b}	1143.88^{a}	15.99^{b}	27.20^{b}	133.71	0.0001
22:4 (n-6)	21.72	11.55	17.58	22.58	3.10	0.0578
22:6 (n-3) DHA	48.30^{b}	147.32^{a}	12.01^{b}	35.58^{b}	21.30	0.0003
SFA[2]	1186.32^{b}	2142.34^{a}	1036.03^{b}	1204.17^{b}	184.55	0.0004
MUFA[3]	826.63^{b}	2017.77^{a}	1188.17^{ab}	782.35^{b}	227.00	0.0010
PUFA[4]	2509.47^{ab}	3125.60^{a}	1674.93^{b}	2441.47^{ab}	299.58	0.0147
Σ n-6 PUFA[5]	2095.45	1739.72	1556.08	2281.03	231.42	0.1207
Σ n-3 PUFA[6]	383.80^{b}	1348.38^{a}	87.05^{b}	129.13^{b}	149.93	0.0001
n-3 to n-6 ratio[7]	0.18^{b}	0.78^{a}	0.06^{b}	0.06^{b}	0.10	0.0001

[1] The dietary treatments are chicken fat (CF), menhaden fish oil (FO), hydrogenated soybean oil (HSBO) and soybean oil (SBO). Fatty acid concentration was expressed in unit of μg/ml of plasma.

[a-d] Means within rows with no common superscripts are significantly different ($p < 0.05$) by Tukey's test of SAS.

[2] Sum of total saturated fatty acids of $C_{14:0}$, $C_{15:0}$, $C_{16:0}$, $C_{17:0}$, $C_{18:0}$, and $C_{20:0}$.

[3] Sum of total monounsaturated fatty acids of $C_{14:1}$, $C_{16:1}$, $C_{18:1}$, and $C_{22:1}$.

[4] Sum of total polyunsaturated fatty acids of $C_{18:2,}$ $C_{18:3}$, $C_{20:3}$, $C_{20:4}$, $C_{20:5}$, $C_{22:4}$, and $C_{22:6}$.

[5] Sum of total n-6 fatty acids of $C_{18:2}$, $C_{20:4}$, and $C_{22:4}$.

[6] Sum of total n-3 fatty acids of $C_{18:3}$, $C_{20:3}$, $C_{20:5}$, and $C_{22:6}$.

[7] Ratio of Σn-3 PUFA to Σn-6 PUFA.

TABLE 2.5. Effect of feeding different lipids on hepatic fatty acid composition in Japanese quail.[1]

Fatty Acid (µg/mg)	CF	FO	HSBO	SBO	Pooled SEM	p-value
14:0	0.0804	0.1507	0.0913	0.0625	0.0333	0.2865
14:1	0.0086	0.0051	0.0089	0.0059	0.0032	0.7987
15:0	0.0089^{b}	0.0238^{a}	0.0054^{b}	0.0087^{b}	0.0027	0.0001
16:0	5.1928	6.2006	4.6863	4.2647	0.9314	0.5018
t9 16:1	0.0028	0.0059	0.0066	0.0033	0.0026	0.6690
c9 16:1	0.3902	0.4384	0.4620	0.3232	0.1271	0.8731
17:0	0.0332^{b}	0.0574^{a}	0.0169^{b}	0.0254^{b}	0.0050	0.0001
18:0	3.2911	3.8719	2.8231	3.3267	0.4019	0.3481
t6t7 18:1	0.0098^{b}	0.0000^{b}	0.0333^{a}	0.0000^{b}	0.0049	0.0001
t9 18:1	0.0222^{b}	0.0071^{b}	0.1067^{a}	0.0116^{b}	0.0137	0.0001
t10 18:1	0.0354^{b}	0.0088^{b}	0.1336^{a}	0.0017^{b}	0.0142	0.0001
t11 18:1	0.0295^{b}	0.0020^{b}	0.1240^{a}	0.0010^{b}	0.0094	0.0001
t12c7 18:1	0.0214^{b}	0.0022^{b}	0.1312^{a}	0.0000^{b}	0.0104	0.0001
t13c6 18:1	0.0300^{b}	0.0032^{b}	0.1009^{a}	0.0034^{b}	0.0089	0.0001
c9 18:1	4.5563	3.8349	3.8085	3.0947	0.7560	0.6052
c11 18:1	0.1574	0.1978	0.1697	0.1447	0.0263	0.5351
c12 18:1	0.0526^{b}	0.0000^{c}	0.1869^{a}	0.0000^{c}	0.0140	0.0001
c13 18:1	0.0091^{b}	0.0092^{b}	0.0259^{a}	0.0076^{b}	0.0029	0.0002
t16 18:1	0.0037^{b}	0.0000^{b}	0.0132^{a}	0.0000^{b}	0.0012	0.0001
c15 18:1	0.0131^{ab}	0.0111^{b}	0.0178^{a}	0.0093^{b}	0.0017	0.0102
t9t12 18:2	0.0020	0.0101	0.0047	0.0003	0.0028	0.0852
c9t12 18:2	0.0294^{a}	0.0096^{b}	0.0188^{ab}	0.0227^{a}	0.0030	0.0006
t9c12 18:2	0.0189^{a}	0.0051^{b}	0.0107^{b}	0.0004^{c}	0.0016	0.0001
18:2n6	3.4168^{ab}	2.5835^{b}	2.5261^{b}	3.9972^{a}	0.2547	0.0005
20:0	0.0103	0.0113	0.0096	0.0114	0.0015	0.8054
18:3n3	0.0532^{ab}	0.0507^{ab}	0.0250^{b}	0.0730^{a}	0.0093	0.0093
c9t11 18:2	0.0217^{b}	0.0034^{b}	0.1038^{a}	0.0035^{b}	0.0094	0.0001
c11t13 18:2	0.0051^{b}	0.0000^{b}	0.0228^{a}	0.0000^{b}	0.0025	0.0001
t10c12 18:2	0.0000	0.0007	0.0000	0.0000	0.0002	0.0557
t11t13 18:2	0.0000	0.0004	0.0008	0.0000	0.0004	0.3634
other t/t 18:2	0.0023^{b}	0.0081^{a}	0.0004^{b}	0.0068^{a}	0.0015	0.0030
20:3n6	0.0754^{ab}	0.0495^{b}	0.0961^{a}	0.0955^{a}	0.0080	0.0006
22:1	0.0021	0.0071	0.0021	0.0014	0.0010	0.7064
20:4n6	1.4826^{ab}	0.6496^{c}	1.4085^{b}	1.7295^{a}	0.0703	0.0001
24:1	0.0247^{b}	0.6460^{a}	0.0157^{b}	0.0317^{b}	0.0346	0.0001
20:5n3 EPA	0.0010	0.0010	0.0008	0.0001	0.0003	0.1378
24:0	0.0000^{b}	0.0000^{b}	0.0038^{a}	0.0006^{b}	0.0008	0.0031
22:4n6	0.0409^{a}	0.0133^{b}	0.0398^{a}	0.0504^{a}	0.0037	0.0001
22:5n3	0.0408^{b}	0.2844^{a}	0.0201^{b}	0.0646^{b}	0.0334	0.0001
22:6n3 DHA	0.4164^{b}	2.5157^{a}	0.2631^{b}	0.6448^{b}	0.1641	0.0001

SFA [2]	8.6181	10.3138	7.6367	7.7002	1.3503	0.4749
MUFA [3]	5.3693	5.1733	5.3462	3.6379	0.9353	0.5059
PUFA [4]	5.6071^{ab}	6.1852^{ab}	4.5402^{b}	6.7131^{a}	0.4702	0.0177
Total Trans FA [5]	0.2333^{ab}	0.0674^{b}	0.8124^{a}	0.0762^{b}	0.0704	0.0001
n-6 [6]	5.0167^{b}	3.2967^{c}	4.0700^{bc}	5.8733^{a}	0.2986	0.0001
n-3 [7]	0.5098^{b}	2.8514^{a}	0.3095^{b}	0.7836^{b}	0.2023	0.0001
n-3 to n-6 ratio [8]	0.1036^{bc}	0.8660^{a}	0.0767^{c}	0.1338^{b}	0.0186	0.0001

[1] The dietary treatments are chicken fat (CF), menhaden fish oil (FO), hydrogenated soybean oil (HSBO) and soybean oil (SBO). Fatty acid content was expressed in unit of μg/mg of tissue sample.

[a-d] Means within rows with no common superscripts are significantly different ($p < 0.05$) by Tukey's test of SAS.

[2] Sum of total saturated fatty acids of $C_{14:0}$, $C_{15:0}$, $C_{16:0}$, $C_{17:0}$, $C_{18:0}$, $C_{20:0}$, and $C_{24:0}$.

[3] Sum of total monounsaturated fatty acids of $C_{14:1}$, $C_{16:1}$, $C_{18:1}$, $C_{22:1}$, and $C_{24:1}$.

[4] Sum of total polyunsaturated fatty acids of $C_{18:2,}$ $C_{18:3}$, $C_{20:3}$, $C_{20:4}$, $C_{20:5}$, $C_{22:4}$, and $C_{22:6}$.

[5] Sum of total trans fatty acid isomers of t9$C_{16:1}$, t6t7$C_{18:1}$, t9$C_{18:1}$, t10$C_{18:1}$, t11$C_{18:1}$, t12c7$C_{18:1}$, t13c6$C_{18:1}$, t16$C_{18:1}$, t9t12$C_{18:2}$, c9t12$C_{18:2}$, t9c12$C_{18:2}$, c9t11$C_{18:2}$, t11t13$C_{18:2}$, and other trans $C_{18:2}$.

[6] Sum of total n-6 fatty acids of $C_{18:2}$, $C_{20:3}$, $C_{20:4}$, and $C_{22:4}$.

[7] Sum of total n-3 fatty acids of $C_{18:3}$, $C_{20:5}$, and $C_{22:6}$.

[8] Ratio of Σn-3 PUFA to Σn-6 PUFA.

Table 2.6 Hematological profile of Japanese quail fed different dietary lipids.[1]

	PCV[4]	PTP[5]
CF		
M[2]	49.0±1.0	3.80±0.60
F[3]	44.5±4.5	6.50±0.10
FO		
M	51.3±2.3	3.90±0.10
F	48.5±3.5	4.75±0.25
HSBO		
M	51.5±1.0	3.85±0.15
F	44.0±3.5	4.95±1.25
SBO		
M	53.3±3.3	4.50±0.10
F	48.8±1.3	5.30±0.30

[1] The dietary treatments are chicken fat (CF), menhaden fish oil (FO), hydrogenated soybean oil (HSBO) and soybean oil (SBO).

Mean comparisons of PCV and PTP were significantly ($P < 0.05$) different between males and females.

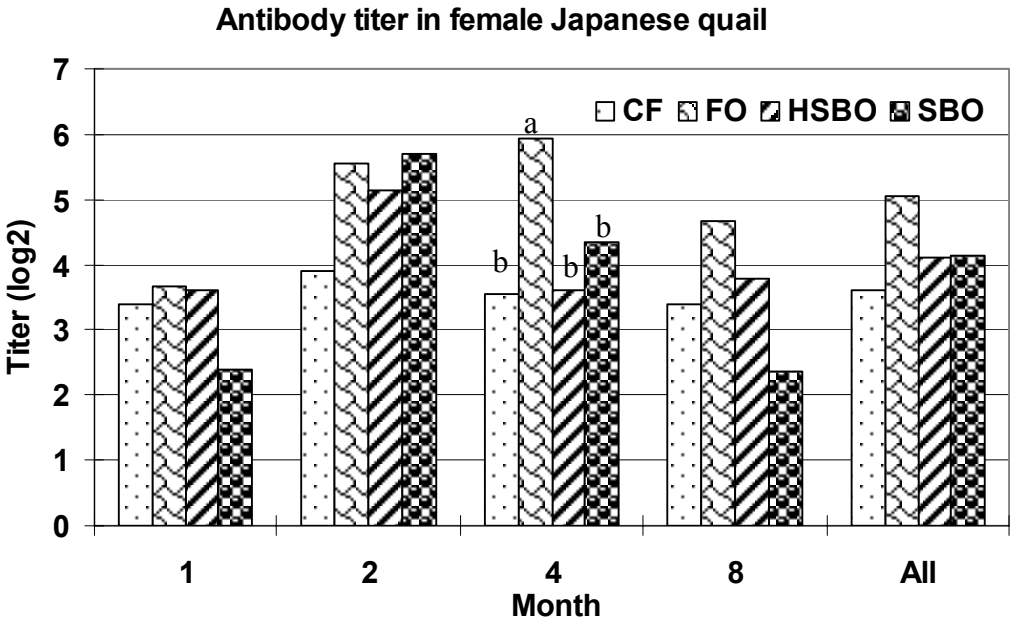
[2] Male = 12/treatment

[3] Female = 8/treatment

[4] Pack cell volume.

[5] Plasma total protein.

FIGURE 2.2. Effect of different dietary lipid, chicken fat (CF), menhaden fish oil (FO), hydrogenated soybean oil (HSBO) and soybean oil (SBO) on antibody production 7 days after an initial injection of SRBC at different sampling periods after starting of treatment supplementation. Different letters indicate treatment means differ significantly, ($P < 0.05$). N = 30/sex/treatment/period time.



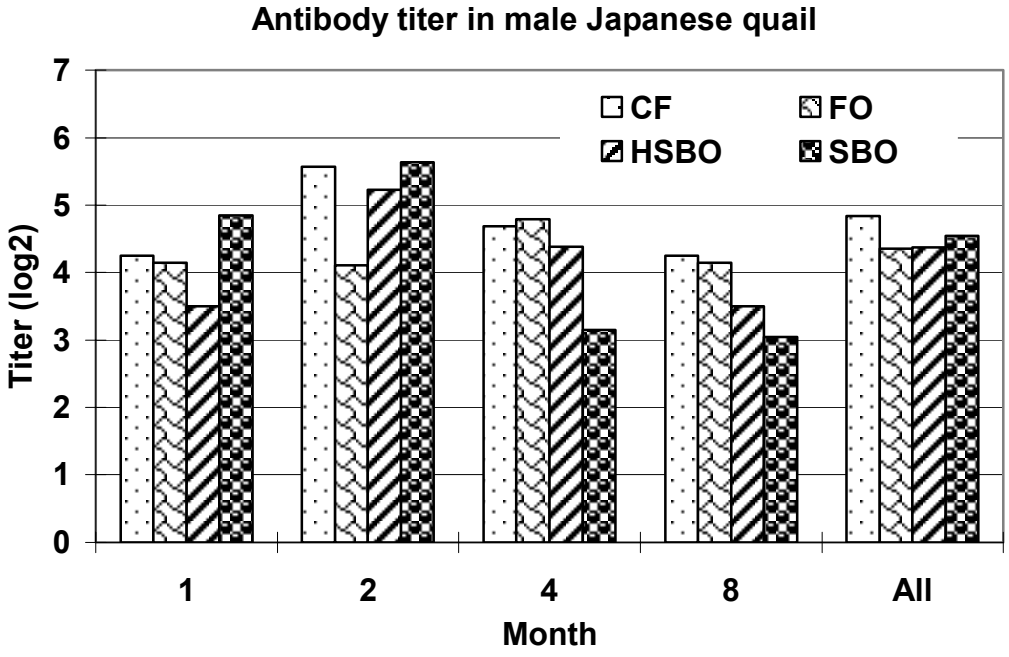


FIGURE 2.3. Effect of dietary lipids including chicken fat (CF), menhaden fish oil (FO), hydrogenated soybean oil (HSBO) and soybean oil (SBO) on the CBH response over a 72-hour period. A total 15/sex/treatment was used. Mean comparison between sex was not different. Different letters indicate treatment means differ significantly, ($P < 0.05$). N = 15/sex/treatment.

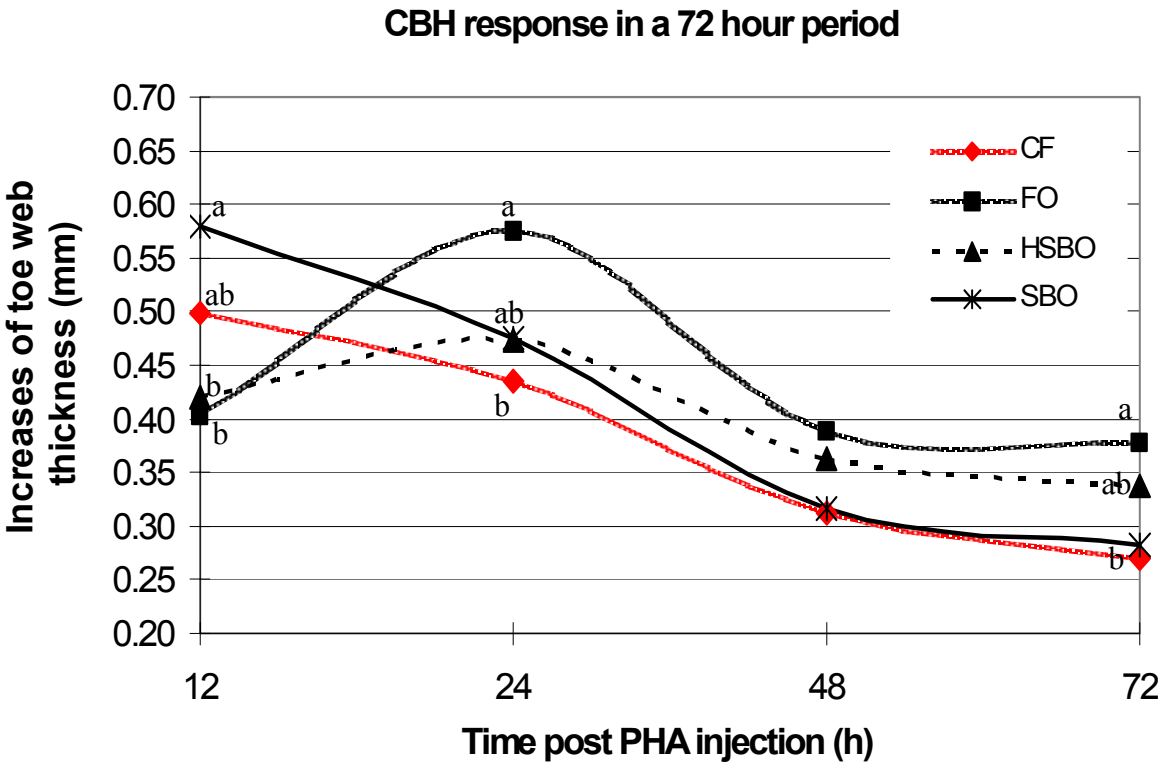


FIGURE 2.4. Different dietary lipids including chicken fat (CF), menhaden fish oil (FO), hydrogenated soybean oil (HSBO) and soybean oil (SBO) affect on the CBH response sampled during different length of treatment supplementation. Different letters indicate treatment means differ significantly, ($P < 0.05$). N = 60 /treatment/time period.

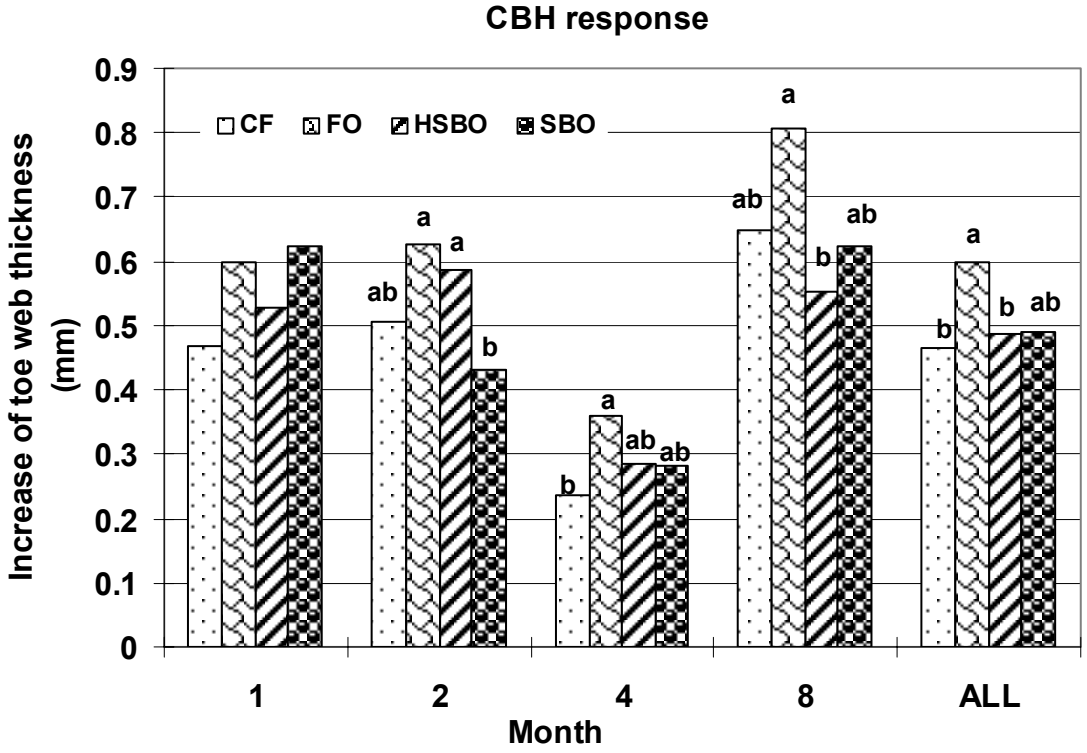
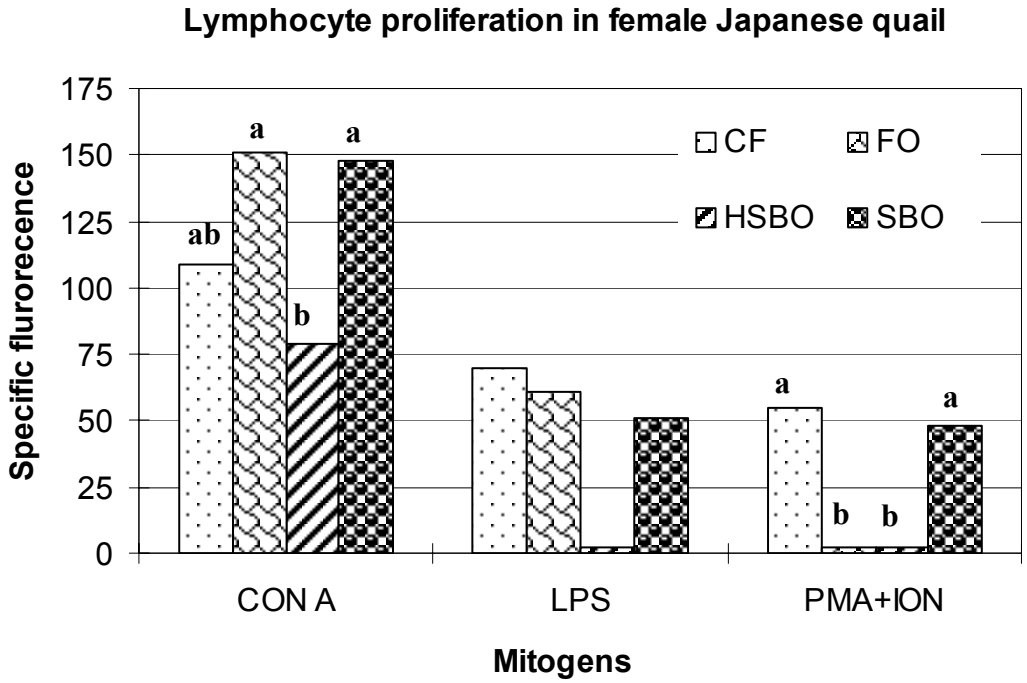
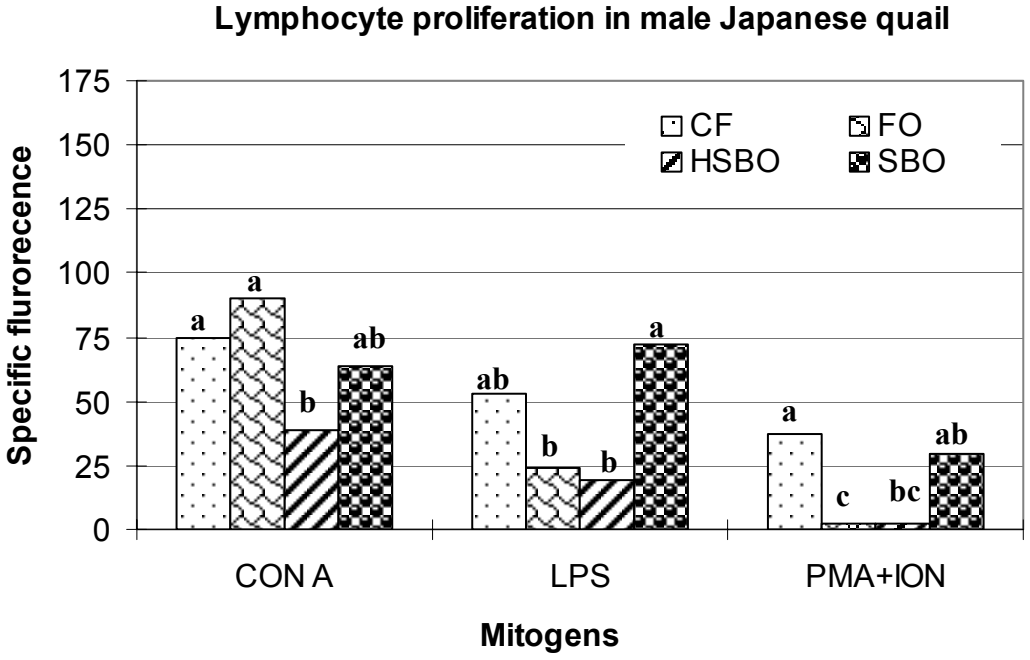


FIGURE 2.5. Effect of dietary lipids including chicken fat (CF), menhaden fish oil (FO), hydrogenated soybean oil (HSBO) and soybean oil (SBO) on lymphocyte proliferations stimulated by CON A, LPA and PMA/ION. Different letters indicate treatment means differ significantly, ($P < 0.05$). N = 16/sex/treatment.





CHAPTER III.

Dietary Lipids Affect Both in vivo and in vitro Cell-Mediated and Humoral Immunity in Bobwhite Quail (Colinus virginianus)

B.C. Weng and D.M. Denbow

Abstract

This study was conducted to investigate the effects of dietary lipids on immunity of bobwhite quail. Experiments were designed to study the effect of inclusion of 5% dietary lipid as either menhaden fish oil (FO), soybean oil (SBO), chicken fat (CF), or hydrogenated soybean oil (HSBO) on antibody production, phagocytic ability and cell-mediated immunity. The experimental diets were started when quail were 2 months-of-age and the experimental measurements were made at 6 month-of-age. Quail fed fish oil enriched with n-3 PUFA had a higher cutaneous basophil hypersensitivity (CBH) response as measured by the toe web skin thickness following injection of phytohemoaglutinin-P (PHA) than quail fed highly saturated diets (CF and HSBO). Moreover, feeding FO and SBO that contained higher levels of polyunsaturated fatty acids (PUFA) resulted in an increase in antibody titer against SRBC compared to feeding diets with a higher level of saturated fatty acids (SFA). Although there was no dietary effect on phagocytic ability as assessed by the clearance rate of peripherally injected carbon ink, there was a faster rate of carbon clearance in quail fed FO. There was an enhanced *in vitro* proliferation of T and B lymphocytes in male quail fed FO which is enriched with n-3 PUFA, but there was no difference found in females. Dietary HSBO, rich in trans isomers of monounsaturated fatty acid (MUFA), suppressed all immunological parameters studied.

It is concluded that the 5 % dietary FO or SBO which enhanced humoral immunity probably resulted from consumption of either n-3 PUFA or n-6 PUFA. Dietary FO also increased both *in vivo* and *in vitro* cell-mediated immunity. Although there was no significant difference in phagocytic ability, quail fed diets high in PUFA had a faster rate of carbon clearance compared to those fed low PUFA. It appears that moderately increasing the n-3/n-6 PUFA ratio or degree of unsaturation of fatty acids in the quail diet enhanced phagocytic ability, humoral and cell-mediated immunity. In addition, feeding highly saturated dietary fatty acids, (CF and HSBO) especially the HSBO diet containing large quantities of trans isomers, had detrimental effects on indices of immunity.

(**Key words:** Bobwhite quail, lipids, immunity, cell-mediated immunity, humoral immunity, phagocytic ability)

Introduction

The effect of dietary lipid on immunity was previously investigated in Japanese quail (Chapter II). In the current study, bobwhite quail were used as an experimental model to provide further evidence of the potential effects of a high dietary ratio of n-3/n-6 PUFA on immunity in birds. Genetically, bobwhite quail are more related to the pheasant family rather than quail species, although they have been named incorrectly as "quail" (Toschi, 1959).

Based on a meager number of studies on the effect of dietary lipids on immunity in avians, discrepancies when compared to mammalian models are numerous. Enhancing dietary n-3 PUFA by feeding menhaden fish oil has been shown to have profound effects in diminishing inflammatory responses in mammalian species (Calder, 1998), and in decreasing inflammatory cytokine (interleukin-1) production in chickens (Korver and Klasing, 1997). This effect has been associated with decreasing membrane arachidonic acid (AA; 20:4 n-6) availability for generation of metabolites through the cyclooxygenase and lipooxygenase pathways. In addition, natural killer cell activity, cytotoxic T lymphocyte activity and antigen presentation were all down- regulated by n-3 PUFA in most experiments using mammals as models (Calder, 1997).

Recently, specific fatty acids such as AA, eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) and their metabolites have been shown to activate peroxisome proliferator- activated receptors (PPAR) (Hwang, 2000). Fatty acids bound to PPAR inhibit inflammatory cytokine production and regulate proliferation and differentiation of immune cells. The binding of fatty acids to PPAR may be a method for their immunomoulatory mechanism.

In avian species, dietary fish oil has been shown to increase delayed-typed

hypersensitivity (Korver and Klasing, 1997; Sijben *et al*., 2001). It has also been reported to either enhance (Fritsche *et al*., 1991; Wang *et al*., 2000) or not affect (Korver and Klasing, 1997) antibody production. Conflicting results have also shown that dietary n-3 PUFA either increase or decrease concanavalin A (CON A) stimulated lymphocyte proliferation (Wang *et al*., 2000; Sijben *et al*., 2001). Different fatty acid sources, duration of supplementation, and different states of subjects are possibly causes for the inconsistent outcomes, and imply that manipulation of dietary fatty acid composition can alter specific immunity in birds. The objective of the current study was to investigate whether dietary lipids containing different proportion of fatty acids alter specific immunity in bobwhite quail.

Materials and Methods

Animals and Diets

In the present study, 20 quail per treatment per sex were used. They were fed a starter diet (Table 3.1) until 1 month-of-age, and then switched to experimental diets containing 5% of either chicken fat (CF), menhaden fish oil (FO), hydrogenated soybean oil (HSBO) or soybean oil (SBO) (Table 3.2). All nutrient levels satisfied or exceed NRC requirements (1994). Hatchlings were fed a starter diet until 4 week-of-age when treatments were assigned. Both sexes were housed together in wire-floored batteries in a temperature-controlled environment. Feed and water were provided *ad libitum*. Assays of antibody titer, cutaneous basophil hypersensitivity and phagocytic ability were conducted at 5 months-of-age and lymphocyte proliferation assays were performed at 8 months-of-age, when quail were sacrificed to collect tissue samples for fatty acid analysis.

Fatty Acid Analysis

Lipids from plasma, liver, and feed were extracted using a Folch wash of 2:1 (v/v) chloroform:methanol (Folch *et al*., 1957). Fatty acids in the dietary oils and lipid extracts were methylated by base-catalyzed using 0.5 N methanolic NaOH followed by transesterification and 14% boron triflouride in methanol (Park and Goins, 1994). An internal standard 10-undecenoate was added prior to methylation. Fatty acids methylated ester (FAME) were separated with a Chrompack 100m CP-Sil 88 column (0.25 mm ID, 0.2 um film thickness) (Vaian Associates, Walnut Creek, CA, 94595) in an Agilent 6890 gas-chromatograph fitted with an autosampler and a flame ionization detector. For liver, feed, and oils, split injection (80:1) of 0.5 µl was used. Injector and detector temperature were 250^{o}C and 300^{o}C, respectively. Initial oven temperature (70^{o}C) was held for 1 minute, increase to 100^{o}C at rate of 10^{o}C/min, held for 3 minutes, increased to 175^{o}C at rate of 10^{o}C/min, held for 40 minutes, increased to 220^{o}C at a rate of 5^{o}C/min and held for 17 minutes. Total time for each sample was 80.5 minutes. Splitless injection was used for separation of plasma FAME. Injection volume was 0.5 µl and purge valve closure time was 0.6 minutes. Column temperature was maintain at 40^{o}C during valve closure, increased to 100^{o}C at a rate of 40^{o}C/min, increased to 175^{o}C at a rate of 25^{o}C/min, held for 45 minutes, increased to 220^{o}C at a rate of 10^{o}C/min, and held for 25 minutes. Total analysis time for each sample was 89.6 minutes. Ultrapure hydrogen was used as the carrier gas for all analyses. Injections were made using a constant pressure of 20.19 psi. Column flow was 1.4ml/min at 70^{o}C. Integration and quantification was made using a ChemData Station (Agilent Technologies, Wilmington, DE, 19801).

Antibody Titer to Sheep Red Blood Cells

Antibody titers against the sheep red blood cell antigens were determined by the method of Wegmann and Smithies (1966). The dosage of SRBC for immunization was determined in a preliminary trial. Birds were injected with 0.1 ml of a 2.5% SRBC solution into the brachial vein. Antiserum to SRBC was collected 7 days post injection by withdrawing 1 ml of blood with one drop of EDTA (Sigma® Chemicals Co, St Louis, MO, 63178). The blood sample was refrigerated overnight to allow the red blood cells to settle. If sedimentation was not complete, samples were centrifuged to separate plasma and erythrocytes. The supernatant was then collected for assay.

Briefly, 96-well plates were first filled with 25 µl of saline in each well. Then 25 µl of antiserum was pipetted into the first well in duplicate after which 25 µl from the first well was pipetted into the second well, and so forth using an automatic titrater (Flow Laboratories, Titerteck® Medimixer, UK). Finally, a 25 µl of a 0.75% of SRBC solution was added into each well. Plates were then incubated at 37^{o}C for 3 hours and then examined visually for agglutination. The last well that showed agglutination was determined and recorded.

The measurement of titers of antibody subclasses including IgM and IgG were performed by a similar methodology as described above. 2-mercaptoethanol (2-ME) (Sigma® Chemicals Co, St Louis, MO, 63178) was used to halt the binding affinity of multivalent IgM antibody whereas agglutination by IgG was not affected. Plates were prepared as described above. Additionally, 25 µl of 0.15 M of 2-ME were added to the first well of each row. Plates contained 2-ME were used to determine the titer by IgG only. The titration of IgM was obtained by total antibody titer minus 2-ME resistant titers.

Cutaneous Basophil Hypersensitivity

The same quail as those used to measure the response to SRBC were used to measure the cutaneous basophil hypersensitivity (CBH). In order to avoid possible interference from injection of SRBC, a two week interval was given before the CBH assay.

Phytohemoaglutinin-P (Sigma® Chemicals Co, St Louis, MO, 63178) was used as the mitogen to evaluate the CBH response that is categorized as a delayed- type hypersensitivity. Sterile saline was used to reconstitute the phytohemoaglutinin-P (PHA) into the concentration of 50 µg/10 µL immediately prior to injection. The response to various doses of PHA was evaluated in a separated trial to determine optimal dosage. A 10 µl syringe (Hamilton®, Reno, NV, 89520) was used to deliver a total of 50 µg of PHA subcutaneously into the toe web between the second and third digit. The toe web of the other leg served as a control, and was injected with the same volume of sterile saline. The methodology was similar to that describe for young chicks (Corrier and DeLoach, 1990). In each trial, injections were finished within 2 hours.

The response to PHA mitogen was calculated by the following formula:

$$\text{Response} = Diff\,(PHA) - Diff\,(Saline)$$

Diff(PHA) = Increment of thickness of toe web post PHA injection.

Diff(Saline) = Increment of thickness of toe web post saline injection.

The increment of skin thickness was determined by the difference in measurement of the web thickness prior to injection and 24 hours post injection of the same foot. All measurements of toe web thickness were made by using a spring micrometer with accuracy in ± 0.001mm.

Carbon Clearance Assay

A total of 64 quail at 5 month-of-age (4 month after dietary lipid treatments

supplementation) were used to evaluate phagocytic ability. The carbon clearance assay was based on the method of Lamont (1986) and Heller *et al.* (1992). Prior to use, the India ink (Pelikan 518.21A 862) was centrifuged at 300 x g for 20 minutes. The homogenous carbon particles were injected into the right jugular vein at a dose of 1 ml/kg body weight. A 0.5 ml blood sample was collected from the right brachial vein, left brachial vein and finally the left jugular vein at 10, 20 and 30 minutes, respectively, after carbon solution was injected. Blood samples were quickly transferred to a 5 ml centrifuge tubes with 2 ml of 1.5% of sodium citrate. Samples were then centrifuged at 50 x g for 5 minutes at room temperature. The absorbance of the supernatant was measured at 675 nm using a Spectrophotometer (Bousch&Lomb spectronic 1001; Spectronic® Instrument, Inc. UK). The spectrophotometer was zeroed with carbon free supernatant collected from each bird prior to carbon injection.

In vitro Non-Radioactive Lymphocyte Blastogenesis Assay

Quail fed the experimental diets for 6 month were used for the study. A 3 ml blood sample was collected from the jugular vein with a 25-gauge needle and placed in heparinized 6 mm x 10 mm glass tubes. A small aliquot of blood was used for a hematological profile which included cells stained using the Natt-Herrick Blood Stain (Natt and Herrick, 1952) for examining the lymphocyte population, packed cell volume and plasma total protein via a refractometer (Refractometer Handheld A 300 CL, Fisherbrand cat# 142116). A whole blood smear stained by Giemsa Blood Stain (Carolina Biological Co. Burlington, NC, 27215) was examined under the microscope for the cytology of whole blood.

Isolation of peripheral leukocytes and assessment of *in vitro* lymphocyte proliferation

was conducted based on the technique described by Gogal *et al.* (1997). Blood was layered on 3 mL of Lymphoprep™ (Density 1.077; Nycomed, Oslo, Norway) after 1:1 dilution of blood with RPMI 1640 (Sigma®; Sigma Chemicals Co, St Louis, MO, 63178). Samples were then centrifuged at 250 x *g* at 4°C for 20 min. The suspension of peripheral leukocytes in the buffy layer was collected and washed 3 times with fresh RPMI 1640. The final pellet was then resuspended in 3 ml of cold complete RPMI media containing 10% fetal bovine serum (Sigma® Co, St. Louis, MO, 63178), 2 mM L-glutamine, 50 IU/ml penicillin and 50 mg/ml streptomycin (Sigma®; Sigma Chemicals Co, St Louis, MO, 63178). Then 10 µl of the re-suspended sample (stock leukocyte solution) was taken for total cell count via a cell counter (CASY® Schärfe System GmbH, Emil-Adolff-Straße 14, D-72760 Reutlingen, Germany), and live/dead percentage was determined using Trypan Blue (Sigma®; Sigma Chemicals Co, St Louis, MO, 63178) stain at a dilution of 10 µl stock / 990 µL stain. The stock cell solution was made up in a basis of 1000 µl by adjusting to the concentration to 5 x 10^6 cells/ml of actual live peripheral blood leukocyte (PBL) via following formulas,

Live/Dead Adjustment

$$\textit{Counted PBL/ml x (1 - } \frac{\textit{Live}}{\textit{Dead}} \textit{ Live/Dead ratio) = Actual Live PBL/ml}$$

$$\frac{5\times10^6}{ActualLivePBL} = K$$

Make up a working cell solution with 5 x 10^6 by combining the following preparations -

$$\textit{The amount to pipette from counted PBL solution} = \frac{1000\mu L}{K} = S$$

$$\textit{The amount to pipette from complete RPMI media} = 1000\ \mu L - S$$

A 96-well round bottom plate (Corning Glass Works, Corning, NY) was used for cell culture. A 100 µl aliquot of cells at a concentration of 5 x 10^6 stock solution was added to quadruplicate wells containing 100 µl of either medium alone, concanavalin A (CON A, 25 µg/ml, Sigma®), lipopolysaccharide (LPS, 20 ng/ml, Sigma®), or phorbol 12-myristate 13-acetate (PMA, 400ng/ml, Sigma®) + ionomycin (ION, 10 ng/ml, Sigma®). The plate was then incubated in a humidified incubator at 37°C and 5% CO_2. After 24 hours, 25 µL of Alamar Blue Dye™ (Accumed International Inc., Chicago, IL, 60610) was added into each well and the plate was returned to the incubator for another 24 hours. The dye is in an oxidized form (blue color) and is reduced to a red color as cells proliferate. Alamar Blue Dye™ also has a fluorescent property when it is reduced. A CytoFluor II Fluorescence Multi-Well Microplate Reader (Perceptive Biosystems, Inc., Framingham, MA, 01701) was used to measure reduced form at excitation of 530/25 nm and the oxidized form at an emission 590/35 nm, gain = 30. The level of lymphocyte proliferation was determined by the change in absorbency (Δ specific absorbency) obtained by the difference between mean values of absorbencies of triplicates from unstimulated cell medium and mitogen stimulated cell medium.

Statistics

Statistical analysis of antibody titers was performed using the GLM model procedures of SAS (SAS Institute, 1996). Treatment effects were compared using Tukey's test (Steel and Torrie, 1980). All titers were transformed to the square root of the log2 before analyzing. Cutaneous basophil hypersensitivity was analyzed by a two-way ANOVA with main effects including dietary lipid treatments and gender. The statistical model was as follows:

$Y_{ijk} = \mu + Treatment_i + Sex_j + Treatment*Sex_{ij} + e_{ijk}$ where $_{i = 1, 2, 3, 4 ; j = 1, 2}$.

The carbon clearance assay was analyzed by repeat measure with Proc Mixed model procedures of SAS (SAS institute, 1996). In addition, regression analysis was performed to determine a linear or quadratic responses among treatments. The statistical model is as following:

$Y_{ijk} = \mu + Treatment_i + Time_j + Treatment*Time_{ij} + Time*Time + Time*Time*Treatment + e_{ijk}$ where $_{i = 1, 2, 3, 4 ; j = 1, 2, 3}$.

In the analysis of mitogen stimulated PBL proliferation, a three-way ANOVA was performed with main effects including treatment, sex and batch of assay performed. Comparisons of treatment effects and interactions were performed by Tukey's test at the 0.05 probability level.

Results

Fatty Acids Analysis of Diets, Plasma and Liver

Bobwhite quail fed a diet containing FO had increased plasma and liver n-3 PUFA, and the n-3/n-6 PUFA ratio was 1.49 in the plasma and 0.6 in the liver. Dietary CF, HSBO and SBO treatments resulted in an n-3/n-6 PUFA ratio in the plasma of ~ 0.12 and liver of ~ 0.07. (Table 3.4 and 3.5). In comparison with our previous study in Japanese quail (Chapter II), the same dietary fatty acid manipulations more dramatically altered fatty acid composition in bobwhite quail. The n-3/n-6 PUFA in the FO diet was only 0.4 but resulted a much higher plasma and hepatic n-3/n-6 PUFA ratio in quail fed FO. This observation could indicate a higher absorption and mobilization of n-3 PUFA in this specie. Interestingly, quail fed FO or SBO had significantly lower plasma MUFA level than to that of the CF and HSBO groups. However, unlike SBO fed birds, quail fed FO had a

significantly higher hepatic MUFA accumulation. The accumulated MUFA in liver from fish oil was the short length fatty acid $C_{16:1}$. The HSBO diet contained 16.2% trans fatty acid isomers and resulted in significantly higher levels of total trans fatty acid isomers in both plasma and liver than the other lipid diets. The CF diet also resulted in an intermediate plasma level of total trans fatty acid isomers that were significantly higher than FO and SBO but lower than that of HSBO. Although the plasma level of $C_{18:2}$ n-6 (LA) and $C_{20:5}$ n-3 (EPA) were significantly altered by dietary lipid treatments, hepatic levels of LA and EPA were not different among treatments. It is important to note that fish oil and the FO diet did not have high EPA, but quail fed the FO diet had a significantly higher plasma EPA. Enzymatic activities in conversion of ALA to EPA could be stimulated in quail fed FO since it was not observed in those quail fed SBO. Moreover, quail fed FO had the highest hepatic total saturated fatty acid (SFA), monounsaturated fatty acid (MUFA), and polyunsaturated fatty acid (PUFA) among all treatments, which was different from our previous analysis in which Japanese quail fed FO had no differences in hepatic SFA and MUFA levels compared to other treatments (Chapter II). Overall, plasma and hepatic fatty acid composition reflected what the dietary lipid manipulations.

Antibody Production

The different dietary treatments fed for 5 month altered the humoral immunity of quail (Fig. 3.1). Quail fed SBO had a significantly higher total antibody titer than the CF and HSBO groups, while quail given the FO treatment were intermediate and not different from the other groups. In addition, both SBO and FO fed birds which were provided a lower saturated fatty acid diet and higher dietary PUFA content had enhanced 2-ME resistant antibody (IgG) production. The significantly lower antibody titer in CF and HSBO

compared to FO and SBO were consistent with elevated plasma MUFA levels. Antibody harvested at day 7 post-injection of SRBC did not contain a large quantity of IgM, since less than 1 log titer unit difference was observed between total antibody and 2-ME resistant antibody levels.

Cutaneous Basophil Hypersensitivity

Feeding diets with higher n-3 PUFA caused a significantly ($p< 0.05$) higher cell-mediated response than that of quail fed HSBO enriched with monoenoic acids and trans fatty acid isomers (Fig 3.2). Quail fed CF and SBO diets had an intermediate CBH index, which was higher than the HSBO group, but not statistical significant. This result was similar to that of Japanese quail study (Chapter II). Since we had found that different dietary lipids affect the time to peak CBH response in Japanese quail (Chapter II), it is reasonable to speculate that the CBH response to the FO treatment did not differ from that of the CF and SBO would also due to their different peak CBH response time. Moreover, dietary HSBO suppressed the *in vivo* index of cell-mediated immunity in bobwhite quail similarly to that in Japanese quail. In general, PHA (50 µg/10µl) caused less swelling in bobwhite quail compared to Japanese quail.

Carbon Clearance Assay

The function of phagocytosis facilitates the first line of defense in innate immunity. There was no significant difference in the level of plasma carbon particles among treatments at each sampling point. The carbon clearance from plasma showed a linear ($p< 0.0001$) decrease in all treatments. FO fed birds had significantly faster carbon clearance rate as seen in Figure 3.3. The supernatant collected at 10 min post ink injection was not

different among all dietary lipid treatments. At both 20 min and 30 min post ink injection, the remained carbon particles were much lower in FO fed birds; whereas bird fed SBO had a lower circulating carbon particle at 30 min compared to those fed high saturated lipid diets (CF and HSBO). In general, a faster carbon clearance rate was found in those fed FO and SBO containing high PUFA.

In Vitro Non-radioactive Lymphocyte Proliferations

The diet enriched in n-3 PUFA significantly increased T and B cell proliferation in response to various mitogens in male quail (Fig 3.4). Manipulation of dietary lipid composition, however, did not result in any difference in lymphocyte proliferation of female quail. Feeding the 5% HSBO diet for 6 months suppressed lymphocyte proliferations by CON A , LPS and PMA. Male quail were more susceptible to the effect of dietary lipids than females. Male quail fed FO had significantly enhanced T and B lymphocyte proliferations, a result only partially similar to that of Japanese quail. In conflict with Japanese quail (Chapter II), bobwhite quail fed SBO did not exhibit a better B lymphocyte proliferation stimulated by LPS. In addition, PMA (400pg/ml) + ionomycin (20pg/ml) did not confer the best mitogenic response. This was probably due to either a sub-optimal mitogen concentration or that fatty acids had changed the cell membrane permeability which consequently altered the affinity of membrane receptors to mitogens. In female quail, the overall mitogenic responses were slightly higher than in males. Moreover, female quail fed HSBO diet decreased T lymphocyte proliferation while FO fed females increased the index.

In male quail, neither the blood cytological profile, pack cell volume (PCV), nor plasma total protein (PTP) were affected by dietary lipid treatments (Table 3.6). In

addition, the whole blood cytology was not significantly different in female quail. In general, female quail had significantly lower PCV but higher PTP than males. In addition, females fed either FO or SBO diet had significantly higher PCV than that of HSBO fed birds. In females, CF fed birds had a significantly higher PTP than those quail fed SBO.

Discussion

Plasma and hepatic fatty acid composition generally reflected the dietary fatty acid profile. The six-month supplementation of CF, FO, HSBO or SBO diets to bobwhite quail resulted in plasma n-3/n-6 PUFA ratio of 0.09, 1.49, 0.09 and 0.06, respectively. The hepatic n-3 to n-6 ratio were 0.08, 0.6, 0.06 and 0.08, respectively. In general, quail fed SBO diet had a significantly higher total n-6 PUFA in both plasma and liver than those fed FO and HSBO. FO-fed quail had significantly higher n-3 PUFA than other dietary treatments. Consistent with that of Japanese quail (Chapter II), the hepatic EPA level was not affected by the dietary lipid treatments indicating that EPA might be metabolized at a fast rate in liver. Unlike linoleic acid $C_{18:2}$ which remained constant in the plasma without being affected by different dietary lipid treatment in Japanese quail (Chapter II), the plasma linoleic acid $C_{18:2}$ was significantly higher in bobwhite quail fed CF and SBO while the hepatic linoleic acid was not different among lipid treatments. Linoleic acid $C_{18:2}$ n-6 is the precursor for AA, which is essential component for membrane phospholipids. Plasma (Japanese quail) or hepatic (Bobwhite quail) linoleic acid remained constant and was not affected by dietary lipid treatments. Some essential fatty acids may reach a homeostasis in different tissues even after a long term supplementation of different dietary lipids in Japanese and bobwhite quail. Moreover, although the FO diet contained the highest n-6

$C_{20:4}$ AA level (at least 20 times above other treatments), quail fed FO had the lowest plasma and hepatic AA level. Depletion of n-6 $C_{20:4}$ AA level in quail fed FO may be induced by a high dietary intake of n-3 PUFA or substituted by the n-3 $C_{20:5}$ EPA. However, Friedman and Sklan (1995) reported that serum AA level was marginally affected by various lipid diets in broiler chicken. Indeed, in the case of quail species, total n-6 PUFA including AA was relatively low in plasma and liver in quail fed FO diet. The decreased n-6 PUFA level in quail fed FO exaggerated the higher n-3 to n-6 PUFA ratio, together with the high n-3 PUFA content. The reciprocal relationship between n-3 PUFA and n-6 PUFA in plasma and liver was also observed in those quail fed CF, and SBO. Therefore, the plasma and hepatic n-3 to n-6 ratio was contributed by both n-3 PUFA and n-6 PUFA level in bobwhite quail, whereas the ratio was mainly altered by the level of n-3 PUFA in Japanese quail (Chapter II).

Both n-3 and n-6 PUFA were significantly lower in quail fed HSBO. In addition, quail fed the HSBO diet had elevated plasma MUFA levels. Unlike quail fed CF, in which the major MUFA was cis-9 $C_{18:1}$, the MUFA found in the plasma of HSBO-fed quail was trans monoenoic fatty acid isomers. Higher intake of trans fatty acids has been associated with a higher risk of cardiac vascular diseases in humans (Denke *et al*., 2000). Moreover, Watkins (1991) reported that partially hydrogenated vegetable oil intensified EFA deficiency symptoms and depressed AA formation in chicks. Specifically, trans $C_{18:1}$ depressed Δ-5 and Δ-6 desaturase activity in rat liver microsomes leading to impaired *de novo* fatty acid synthesis (De Schrijver and Privett, 1982). Feeding HSBO to quail has also been shown to decrease PGE_2 production in bone (Liu and Denbow, 2000). The effect of trans fatty acids on immunity is not clear, however, we previously demonstrated that Japanese quail fed HSBO enriched in trans fatty acid isomers had decreased

cell-mediated and humoral immunity (Chapter II). The present results further indicate that long-term consumption of HSBO containing high levels of trans fatty acid isomers showed deleterious effects on immune functions, and the effects were at least due to the decreased substrates for eicosanoid production.

In addition, conjugate linoleic acid (CLA), particularly the cis-9, trans-11 $C_{18:2}$, was significantly higher in HSBO fed birds compared to other treatments. CLA has been shown to depress *de novo* fatty acid synthesis (Loor and Herbein, 1998) and decrease PGE_2 synthesis (Li and Watkins, 1998). Different from that of FO fed birds, which also resulted in a low physiological level of n-6 PUFA, HSBO fed birds did not have a high n-3 PUFA content to compensate for the depleted eicosanoids production. Therefore, in healthy subjects, dietary HSBO enriched with trans fatty acid isomers and CLA may have negative effects on the immune system.

N-3 and n-6 PUFA are particularly important for immunity because they are precursors of eicosanoids necessary for priming or triggering immune responses. Little is known about the pathways for fatty acids acting on immunity independent to eicosanoids. So far, eicosanoids, especially prostaglandins, have been most studied. In the majority of human and mice studies, consumption of n-3 PUFA ranging from 7% to 20% of the diet decrease plasma AA level and AA derived eicosanoids (Calder, 1998). Downregulated PGE_2, and LTB_4 productions from AA precursors has been determined as the primary mechanism to modulate immunological indices in a suppressive manner. Moreover, inhibition of cyclooxygenase products by non-steroids anti-inflammatory drugs (ie. indomethacin, aspirin) ameliorates symptoms of inflammation by reduction of pro-inflammatory cytokines (Clissold, 1999). However, PGE_2 is necessary for many biological processes. PGE_2 causes smooth muscle contraction during labor and is also a vasodialator and

bronchodilator. In immunity, PGE_2 primes many immunological processes and plays a major role in initiation of responding to tissue injury. Therefore, reduction of eicosanoids during inflammatory diseases may be beneficial in relieving symptoms, while the same effects could hamper normal immunity in healthy subjects.

PGE_2 has been widely shown to inhibit cytokine production, lymphocyte proliferation, natural killer cell and cytotoxic T cells activities, consumption of n-3 PUFA decreased substrate for PGE_2 production by substitution of membrane AA, and it has been shown to suppress the above immunological parameters in most mammalian models (Calder, 1997). The definitive role of PGE_2, and n-3 PUFA in modulation of immunity needs further investigation. Moreover, it had been reported that PGE_2 suppresses cell-mediated immunity and favors humoral immunity (Betz and Fox, 1991; Phipps *et al*., 1991). Reduction in PGE_2 production could enhance overall indices of cell-mediated immunity.

Indices of cell-mediated immunity were significantly increased in response to PHA as measured by the CBH response in toe web, and *in vitro* lymphocyte proliferation stimulation by CON A mitogen. Consistent with our previous study using Japanese quail (Chapter II), dietary FO had beneficial effects on the cell-mediated immunity. The results were similar to other studies using avian species as experimental models. In those avian studies, high dietary n-3/n-6 PUFA ratio has been shown to increase antibody production (Wang *et al*., 2000; Sijben *et al*., 2000), and cell-mediated immunity (Sijben *et al*., 2000; Kover and Klasing, 1997). It appears that feeding avian species a diet high in the n-3/n-6 PUFA ratio has a different effect on immunity from mammals. This discrepancy could be due to a number of factors such as level of dietary fat used in study, duration of supplementation, differences in fatty acid utilization and metabolism, and genetic variation.

In the current study and a previous study with Japanese quail (Chapter II), birds fed a

diet high in monoenoic fatty acids and trans fatty acid isomers had an overall suppression on both humoral and cell-mediated immunity. The HSBO used in the current experiment was Crisco® shortening containing 25% trans fatty acid isomers of total fatty acid content. The total monounsaturated fatty acids were more than 55% of the total fatty acids in the diet. There was a consistent decrease in immune parameters including antibody titer, CBH response and lymphocyte proliferation.

Previous studies showed that incorporation of olive oil containing high oleic acid decreased natural killer cell activity (Yaqoob *et al*., 1994), and the index of lymphocyte proliferation by CONA (Jeffery *et al*., 1996). However, the CF diet containing 40% cis MUFA isomers did not depress cell-mediated immunity in the present study. The relatively high n-6 PUFA in the CF diet could counteract the suppressive effect from the cis 18:1 mentioned in the above studies. Unlike quail fed CF, the decreased indices of cell-mediated immunity observed when feeding HSBO diet may not be solely due to the chemical structure of the trans fatty acid isomers since the HSBO diet contained PUFA level was only half of the CF diet. In addition, Sung *et al*. (2002) reported that humans consuming stick margarine (hydrogenated soybean oil) had significantly higher TNF-α, IL-6 production when compared to consumption of soybean oil. Whether the increased TNF-α and IL-6 could benefit antibody production or suppression of tumor growth would be interesting to study. A butter diet with high saturated fat was shown to suppress the DTH response compared to a SBO diet (Sung *et al*., 2002). In the current study, poultry fat is similar to HSBO high in saturated fat but contains less than 3% of trans fatty acid isomers. Birds fed CF diet did not show a decreased *in vivo* or *in vitro* cell-mediated immunity like that of HSBO-fed birds. Therefore, it is reasonable to speculate that the suppression of cell-mediated immunity could be due to the high dietary trans fatty acid

isomers and low total PUFA content in HSBO diet. Previously, it was demonstrated that maternal diets enriched in high trans-18:1 fatty acids significantly decreased *ex vivo* bone marrow cell PGE_2 production in neonatal quails (Liu and Denbow, 2001). Since bone marrow contains pluripotent stem cells for differentiation of all haemopoietic cells, it is reasonable to speculate that reduction of PGE_2 by dietary HSBO could also affect the population of immunocompetent cells and their differentiation. Indeed, the current study found that the pack cell volume of female quail fed HSBO diet was significantly lower than those of SBO and FO fed birds. Therefore, the lowered peripheral blood cells in quail fed HSBO may attribute to the lower immunological responsiveness by decreasing number of effector cells.

Recently, cyclooxygenase has been functionally classified into two isomers, COX I and COX II enzymes. The COX I derived PGs are more likely involved in maintenance of normal homeostasis such as renal filtration, blood vessel permeability, bone re-absorption, and reproduction, while COX II derived PGs are inducible during inflammatory responses (Dubois *et al*., 1998; Gilroy *et al*., 1999; Tegeder *et al*., 2001). The finding of differential activation of COX enzyme subclasses during an immune process may further elucidate the immunological roles of specific fatty acids. Moreover, free fatty acids can serve as ligands of the nuclear PPAR to initiate a gene transcription for apoptosis, differentiation and proliferation of immune cells (Hwang, 2000). With more new findings in eicosanoids centered on immunological cascades, as well as eicosanoids independent mechanisms, the role of diverse dietary fatty acids in modulation of immune functions should be able to be better elucidated.

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TABLE 3.1. Feed ingredients of quail starter diet [1]

Ingredients	%
Yellow corn	42.20
Soybean meal	38.00
Blend fat	3.50
Fish meal	7.00
Meat and bone scrap	5.00
Dehydrated alfalfa meal	1.25
Defluorinated phosphate	1.00
Limestone	0.50
Vitamin premix [2]	1.00
Trace mineral premix [3]	0.05
Salt	0.50

[1] Starter quail feed was fed to quail from hatch until 4 weeks of age. The feed was calculated to contain 29.6% crude protein, 2970 kcal/kg metabolizable energy, 5.5% crude fat, 2.7% crude fiber, 1.4% calcium, and 0.7% phosphorous.

[2] Vitamin premix from Big Springs Mill (Springfield, VA). Guaranteed analysis per kilogram: selenium (sodium selenite): 18.2 ppm; vitamin A (vitamin A acetate): 454,000 IU; vitamin E: 1362 IU; vitamin B_{12} (pyridoxine hydrochloride): 0.68 mg; menadione (menadione sodium bisulfite complex): 48.12 mg; biotin: 2.27 mg; choline (choline chloride): 13393 mg; folic acid: 45.4 mg; niacin: 1816 mg; d-pantothenic acid (d-calcium pantothenate): 626.52 mg; vitamin B_6 (pyridoxine): 37.23 mg; riboflavin: 272.4 mg; thiamine (thiamine mononitrate): 40.41 mg.

[3] Trace mineral premix from Big Springs Mill (Springfield, VA). Guaranteed analysis per kilogram: zinc (zinc sulfate): 120 g; manganese (manganese sulfate): 120 g; iron (ferrous sulfate): 40 g; copper (copper sulfate): 5 g; iodine (calcium iodate): 2 g; cobalt (cobalt carbonate): 0.45 g; calcium (calcium carbonate): 280 g.

TABLE 3.2. Feed ingredients and formulation of experimental diet in each treatment. [1]

Ingredients	CF	FO	HSBO	SBO
Yellow corn	53.525	53.525	53.525	53.525
Soybean meal	32.00	32.00	32.00	32.00
Soybean oil	…	…	…	5.00
Hydrogenated soybean oil	…	…	5.00	…
Chicken fat	5.00	…	…	…
Menhaden fish oil	…	5.00	…	…
Limestone	6.50	6.50	6.50	6.50
Dicalcium phosphate	1.50	1.50	1.50	1.50
Vitamin premix [2]	1.00	1.00	1.00	1.00
Trace mineral premix [3]	0.10	0.10	0.10	0.10
Salt	0.35	0.35	0.35	0.35
Ethoxyquin (antioxidant)	0.025	0.025	0.025	0.025

[1] The experimental diet was fed to quail after the starter diet at 1 month of age. The feed was calculated to contain 21% crude protein, 2997 to 3007 kcal/kg metabolizable energy, 7.36% crude fat, 2.43% crude fiber, 2.81% calcium, and 0.67% phosphorous.

[2] Vitamin premix from Big Springs Mill (Springfield, VA). Guaranteed analysis per kilogram: selenium (sodium selenite): 18.2 ppm; vitamin A (vitamin A acetate): 454,000 IU; vitamin E: 1362 IU; vitamin B_{12} (pyridoxine hydrochloride): 0.68 mg; menadione (menadione sodium bisulfite complex): 48.12 mg; biotin: 2.27 mg; choline (choline chloride): 13393 mg; folic acid: 45.4 mg; niacin: 1816 mg; d-pantothenic acid (d-calcium pantothenate): 626.52 mg; vitamin B_6 (pyridoxine): 37.23 mg; riboflavin: 272.4 mg; thiamine (thiamine mononitrate): 40.41 mg.

[3] Trace mineral premix from Big Springs Mill (Springfield, VA). Guaranteed analysis per kilogram: zinc (zinc sulfate): 120 g; manganese (manganese sulfate): 120 g; iron (ferrous sulfate): 40 g; copper (copper sulfate): 5 g; iodine (calcium iodate): 2 g; cobalt (cobalt carbonate): 0.45 g; calcium (calcium carbonate): 280 g.

TABLE 3.3. Fatty acid analysis of experimental diets and lipid sources.[1]

	Diets				Lipids			
Fatty Acids (μg/mg)	CF	FO	HSBO	SBO	CF	FO	HSBO	SBO
14:0	0.1346	0.7154	0.0446	0.0177	2.6380	19.8873	0.5154	0.2779
14:1	0.0060	0.0045	0.0000	0.0000	0.0000	0.1458	0.0000	0.0000
15:0	0.0124	0.1270	0.0091	0.0048	0.1877	2.9929	0.1069	0.0580
16:0	4.2029	4.5803	3.4038	2.8682	40.8168	76.5801	40.5275	31.7009
t9 16:1	0.0021	0.0537	0.0016	0.0000	0.1089	1.2209	0.0560	0.0000
c9 16:1	0.2026	2.2137	0.0420	0.0192	3.2051	52.4840	0.1815	0.2718
17:0	0.0446	0.1069	0.0374	0.0271	0.6794	2.2885	0.6439	0.4131
18:0	1.6744	0.8488	3.1240	1.1614	27.6821	14.0475	73.0041	19.9927
t6&t7 18:1	0.1361	0.0098	1.1109	0.0050	2.8164	0.1696	27.2111	0.0000
t9 18:1	0.1747	0.0097	1.3882	0.0079	3.5236	0.2729	34.9593	0.0317
t10 18:1	0.3582	0.0024	2.1353	0.0101	7.2682	0.0000	52.9094	0.0000
t11 18:1	0.3284	0.0051	2.1123	0.0126	6.6747	0.1055	53.2283	0.0901
t12&c7 18:1	0.1202	0.2428	1.0210	0.0022	2.4350	5.8212	25.5114	0.0000
t13&c6 18:1	0.1710	0.0000	1.5014	0.0000	3.1225	0.0000	39.3251	0.0000
c9 18:1	8.0059	3.3742	6.6990	6.1515	114.3876	27.8374	86.4639	98.5158
c11 18:1	0.3741	0.4433	0.5576	0.2628	6.1723	8.9397	12.1562	4.8908
c12 18:1	0.4730	0.0044	1.8633	0.0077	9.4811	0.2981	46.5922	0.0000
c13 18:1	0.0436	0.0210	0.2812	0.0163	0.7228	0.3780	7.1605	0.2701
t16 18:1	0.0245	0.0009	0.1668	0.0000	0.2720	0.3921	1.7894	0.0000
c15 18:1	0.0284	0.0230	0.0929	0.0000	0.0000	0.1421	0.7923	0.0000
t9,t12 18:2	0.0167	0.0309	0.0258	0.0055	0.3007	0.7149	0.6333	0.0000
c9,t12 18:2	0.1541	0.0418	0.0454	0.1938	2.9980	0.7370	1.3740	3.0368
t9,c12 18:2	0.1166	0.0655	0.0260	0.1640	2.4480	0.1247	1.1176	2.7368
18:2n6 LA	15.4107	7.1282	7.4201	19.4368	155.8825	6.0801	1.1450	306.1599
20:0	0.1144	0.0663	0.1295	0.0984	1.4423	0.7760	1.2953	1.6330
18:3n3 ALA	0.9420	0.5331	0.3033	1.2909	12.7569	5.9820	0.5297	26.7336
c9,t11 18:2	0.0114	0.0000	0.0000	0.0000	0.2465	0.0474	0.0000	0.0000
c11,t13 18:2	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
t10,c12 18:2	0.0116	0.0000	0.0000	0.0000	0.2451	0.0000	0.0000	0.0000
t11,t13 18:2	0.0034	0.0275	0.0028	0.0000	0.0981	0.6253	0.0000	0.0000
other t/t 18:2	0.0331	0.5631	0.0015	0.0189	0.9450	13.1357	0.0000	0.3435
20:3n6	0.0053	0.0396	0.0009	0.0000	0.1094	0.9614	0.0000	0.0000
22:1	0.0074	0.0673	0.0030	0.0019	0.1483	1.5601	0.0000	0.0000
20:4n6 AA	0.0092	0.1805	0.0043	0.0016	0.1455	4.2605	0.0000	0.0000
24:1	0.0025	2.7705	0.0376	0.0040	0.0248	66.1658	0.0000	0.0000
20:5n3 EPA	0.0396	0.0254	0.0471	0.0329	0.5807	0.1868	0.6491	0.3545
24:0	0.0011	0.0554	0.0010	0.0000	0.0510	1.3193	0.0000	0.0000
22:4n6	0.0023	0.0401	0.0000	0.0000	0.0494	0.9544	0.0000	0.0000
22:5n3	0.0121	0.4562	0.0172	0.0045	0.1539	10.7558	0.0969	0.0000
22:6n3 DHA	0.0017	2.0801	0.0295	0.0017	0.0183	49.8156	0.0000	0.0000
Total ugFA/mg sample	33.4128	26.9582	33.6873	31.8294	410.8383	378.2062	509.9753	497.5111
SAF [2]	6.1844	6.5001	6.7493	4.1775	73.4973	117.8915	116.0931	54.0757
MUFA [3]	10.4586	9.2461	19.0142	6.5013	160.3631	165.9331	388.3365	104.0704
PUFA [4]	16.7698	11.2119	7.9239	21.1506	176.9779	94.3816	5.5457	339.3650
Total Trans FA [5]	1.6621	1.0532	9.5389	0.4200	33.5025	23.3671	238.1149	6.2388
N-6 [6]	15.4275	7.3884	7.4253	19.4384	156.1868	12.2565	1.1450	306.1599
N-3 [7]	0.9954	3.0947	0.3970	1.3300	13.5098	66.7402	1.2757	27.0881

n-3:n-6 ratio [8]	0.0644	0.4187	0.0537	0.0683	0.0865	5.4453	1.1141	0.0885

[1] The dietary lipids include chicken fat (CF oil), menhaden fish oil (FO oil), hydrogenated soybean oil (HSBO) and soybean oil (SBO oil). The dietary treatments are chicken fat (CF), menhaden fish oil (FO), hydrogenated soybean oil (HSBO) and soybean oil (SBO). The unit for fatty acid composition was μg of fatty acid per mg of sample.

[2] Sum of total saturated fatty acids of $C_{14:0}$, $C_{15:0}$, $C_{16:0}$, $C_{17:0}$, $C_{18:0}$, $C_{20:0}$, and $C_{24:0}$.

[3] Sum of total monounsaturated fatty acids of $C_{14:1}$, $C_{16:1}$, $C_{18:1}$, $C_{22:1}$, and $C_{24:1}$.

[4] Sum of total polyunsaturated fatty acids of $C_{18:2,}$ $C_{18:3}$, $C_{20:3}$, $C_{20:4}$, $C_{20:5}$, $C_{22:4}$, and $C_{22:6}$.

[5] Sum of total trans fatty acid isomers of t9$C_{16:1}$, t6t7$C_{18:1}$, t9$C_{18:1}$, t10$C_{18:1}$, t11$C_{18:1}$, t12c7$C_{18:1}$, t13c6$C_{18:1}$, t16$C_{18:1}$, t9t12$C_{18:2}$, c9t12$C_{18:2}$, t9c12$C_{18:2}$, c9t11$C_{18:2}$, t11t13$C_{18:2}$, and other trans $C_{18:2}$.

[6] Sum of total n-6 fatty acids of $C_{18:2}$, $C_{20:3}$, $C_{20:4}$, and $C_{22:4}$.

[7] Sum of total n-3 fatty acids of $C_{18:3}$, $C_{20:5}$, and $C_{22:6}$.

[8] Ratio of Σn-3 PUFA to Σn-6 PUFA.

TABLE 3.4. Effect of feeding different lipids on plasma fatty acid composition in bobwhite quail.[1]

Fatty Acid (µg/ml)	CF	FO	HSBO	SBO	Pooled SEM	p-value
14:0	4.03^{b}	9.40^{a}	2.56^{b}	3.11^{b}	0.66	0.0001
14:1	0.52	0.79	0.63	1.44	0.54	0.6344
15:0	1.15^{b}	2.71^{a}	0.87^{b}	1.00^{b}	0.11	0.0001
16:0	373.16	340.28	294.81	292.84	26.79	0.1132
t9 16: 1	0.32^{b}	0.88^{a}	1.14^{a}	0.05^{b}	0.09	0.0001
c9 16:1	17.77^{ab}	23.48^{a}	9.58^{b}	7.87^{b}	2.98	0.0017
17:0	3.28^{b}	5.35^{a}	2.33^{b}	3.10^{b}	0.27	0.0001
18:0	511.58^{a}	357.49^{b}	354.07^{b}	451.72^{b}	33.83	0.0036
t6t7 18:1	5.30^{b}	2.21^{b}	21.00^{a}	1.54^{b}	1.31	0.0001
t9 18:1	8.08^{b}	1.78^{b}	39.88^{a}	1.48^{b}	2.23	0.0001
t10 18:1	6.34^{b}	0.09^{c}	31.90^{a}	0.93^{bc}	1.55	0.0001
t11 18:1	7.68^{b}	0.67^{b}	38.05^{a}	0.56^{b}	2.13	0.0001
t12c7 18:1	4.68^{b}	1.48^{b}	30.48^{a}	0.13^{b}	1.57	0.0001
t13c6 18:1	8.35^{b}	1.30^{b}	42.70^{a}	0.91^{b}	2.10	0.0001
t16 18:1	0.64^{b}	0.03^{b}	4.23^{a}	0.04^{b}	0.21	0.0001
trans 18:1	41.07^{b}	7.56^{b}	208.24^{a}	5.59^{b}	1.59	0.0001
c9 18:1	547.33^{a}	284.26^{b}	425.61^{ab}	260.83^{ab}	47.72	0.0002
c11 18:1	36.04^{a}	37.15^{a}	33.61^{a}	19.79^{b}	3.33	0.0016
c12 18:1	16.55^{b}	0.71^{bc}	83.26^{a}	0.17^{c}	4.32	0.0001
c13 18:1	2.14^{b}	1.22^{b}	7.46^{a}	0.78^{b}	0.42	0.0001
c15 18:1	2.90	2.82	5.40	3.90	1.16	0.3605
cis 18:1	604.96	326.16	555.34	285.47	11.39	0.0700
t9t12 18:2	1.09^{b}	0.67^{c}	1.66^{a}	0.64^{c}	0.10	0.0001
c9t12 18:2	8.20^{b}	3.44^{c}	7.26^{b}	10.53^{a}	0.58	0.0001
t9c12 18:2	13.27^{a}	2.19^{c}	6.97^{b}	12.24^{a}	0.86	0.0001
18: 2 (n-6) LA	1198.33^{a}	565.09^{c}	901.32^{b}	1163.01^{a}	71.84	0.0001
18: 3 (n-3) ALA	14.75^{a}	8.75^{b}	7.18^{b}	14.41^{a}	1.21	0.0001
20:0	4.15	3.89	3.06	3.40	0.36	0.1552
c9t11 18:2	3.28^{b}	0.40^{b}	15.95^{a}	0.18^{b}	1.09	0.0001
t11t13 18:2	1.28^{ab}	1.35^{a}	1.50^{a}	0.52^{b}	0.21	0.0097
Other t/t 18:2	1.53^{b}	5.52^{a}	2.89^{b}	0.82^{b}	0.52	0.0001
20:3 (n-6)	82.77^{a}	17.74^{d}	57.90^{b}	38.74^{bc}	5.64	0.0001
22:1	4.58^{b}	0.92^{c}	8.53^{a}	5.55^{b}	0.69	0.0001

20:4 (n-6) AA	434.9[a]	213.73[b]	318.44[b]	532.52[a]	31.15	0.0001
20:5 (n-3) EPA	39.11[b]	1042.59[a]	26.65[b]	28.72[b]	75.63	0.0001
22:4 (n-6)	18.73[a]	6.23[b]	13.56[a]	15.70[a]	1.44	0.0001
22:6 (n-3) DHA	19.01[b]	103.12[a]	20.50[b]	22.52[b]	8.34	0.0001
SFA[2]	898.39[a]	720.70[ab]	658.96[b]	758.05[ab]	60.63	0.0480
MUFA[3]	669.22[a]	359.79[b]	783.46[a]	305.97[b]	61.63	0.0001
PUFA[4]	1836.25[ab]	1970.82[a]	1381.78[b]	1840.55[ab]	151.48	0.0449
Σ n-6 PUFA[5]	1651.96[a]	785.05[c]	1233.32[b]	1711.23[a]	97.90	0.0001
Σ n-3 PUFA[6]	155.64[b]	1172.20[a]	112.23[b]	104.39[b]	84.09	0.0001
n-3 to n-6 ratio[7]	0.09[b]	1.49[a]	0.09[b]	0.06[b]	0.07	0.0001

[1] The dietary treatments are chicken fat (CF), menhaden fish oil (FO), hydrogenated soybean oil (HSBO) and soybean oil (SBO). Fatty acid concentration was expressed in unit of μg/ml of plasma.

[a-d] Means within rows with no common superscripts are significantly different ($p < 0.05$) with Tukey's test of SAS.

[2] Sum of total saturated fatty acids of $C_{14:0}$, $C_{15:0}$, $C_{16:0}$, $C_{17:0}$, $C_{18:0}$, and $C_{20:0}$.

[3] Sum of total monounsaturated fatty acids of $C_{14:1}$, $C_{16:1}$, $C_{18:1}$, and $C_{22:1}$.

[4] Sum of total polyunsaturated fatty acids of $C_{18:2,}$ $C_{18:3}$, $C_{20:3}$, $C_{20:4}$, $C_{20:5}$, $C_{22:4}$, and $C_{22:6}$.

[5] Sum of total n-6 fatty acids of $C_{18:2}$, $C_{20:4}$, and $C_{22:4}$.

[6] Sum of total n-3 fatty acids of $C_{18:3}$, $C_{20:3}$, $C_{20:5}$, and $C_{22:6}$.

[7] Ratio of Σn-3 PUFA to Σn-6 PUFA.

TABLE 3.5. Effect of feeding different lipids on hepatic fatty acid composition in bobwhite quail.[1]

Fatty Acid (μg/mg)	CF	FO	HSBO	SBO	Pooled SEM	p-value
14:0	0.1126^{b}	0.3723^{a}	0.3781^{b}	0.0466^{b}	0.0428	0.0001
14:1	0.0120^{ab}	0.0213^{a}	0.0002^{b}	0.0011^{b}	0.0044	0.0030
15:0	0.0172^{b}	0.0706^{a}	0.0104^{b}	0.0107^{b}	0.0065	0.0001
16:0	6.1931^{ab}	8.7654^{a}	3.1121^{b}	3.2081^{b}	0.9495	0.0001
t9 16:1	0.0035^{b}	0.0221^{a}	0.0138^{a}	0.0002^{b}	0.0026	0.0001
c9 16:1	0.4601^{ab}	0.8947^{a}	0.1299^{b}	0.1596^{b}	0.1275	0.0002
17:0	0.0406^{b}	0.1150^{a}	0.0264^{b}	0.0331^{b}	0.0089	0.0001
18:0	4.2449^{ab}	5.6286^{a}	2.9356^{b}	3.6479^{b}	0.3944	0.0001
t6t7 18:1	0.0256^{b}	0.0023^{c}	0.1136^{a}	0.0000^{c}	0.0061	0.0001
t9 18:1	0.0610^{b}	0.0243^{b}	0.2555^{a}	0.0120^{b}	0.0147	0.0001
t10 18:1	0.0419^{b}	0.0000^{c}	0.1656^{a}	0.0006^{c}	0.0096	0.0001
t11 18:1	0.0433^{b}	0.0046^{b}	0.2413^{a}	0.0785^{b}	0.0391	0.0004
t12c7 18:1	0.0335^{b}	0.0034^{b}	0.2562^{a}	0.0004^{b}	0.0114	0.0001
t13c6 18:1	0.0403^{b}	0.0023^{bc}	0.2173^{a}	0.0011^{c}	0.0107	0.0001
c9 18:1	6.1390^{ab}	6.9338^{a}	3.1546^{ab}	2.5908^{b}	1.0754	0.0118
c11 18:1	0.2949^{b}	0.5476^{a}	0.1958^{b}	0.1487^{b}	0.0552	0.0001
c12 18:1	0.1057^{b}	0.0002^{c}	0.4156^{a}	0.0000^{c}	0.0191	0.0001
c13 18:1	0.0208^{bc}	0.0256^{b}	0.0431^{a}	0.0081^{c}	0.0045	0.0001
t16 18:1	0.0052^{b}	0.0000^{c}	0.0188^{a}	0.0000^{c}	0.0013	0.0001
c15 18:1	0.0172^{ab}	0.0225^{a}	0.0221^{a}	0.0108^{b}	0.0019	0.0001
t9t12 18:2	0.0060	0.0044	0.0089	0.0064	0.0013	0.1105
c9t12 18:2	0.0374^{a}	0.0177^{b}	0.0301^{ab}	0.0305^{ab}	0.0037	0.0047
t9c12 18:2	0.0545^{a}	0.0129^{c}	0.0255^{b}	0.0370^{b}	0.0034	0.0001
18:2n6 LA	6.7286	6.2611	4.8676	7.1589	0.6547	0.0865
20:0	0.0114^{b}	0.0184^{a}	0.0059^{b}	0.0104^{b}	0.0018	0.0001
18:3n3 ALA	0.1287^{ab}	0.1462^{a}	0.0542^{b}	0.1358^{a}	0.0219	0.0176
c9t11 18:2	0.0411^{b}	0.0073^{bc}	0.1314^{a}	0.0028^{c}	0.0097	0.0001
c11t13 18:2	0.0079^{b}	0.0000^{b}	0.0183^{a}	0.0000^{b}	0.0025	0.0001
t10c12 18:2	0.0000	0.0000	0.0005	0.0000	0.0003	0.3997
t11t13 18:2	0.0051^{b}	0.0129^{a}	0.0016^{b}	0.0000^{b}	0.0018	0.0001
other t/t 18:2	0.0048^{b}	0.0224^{a}	0.0029^{b}	0.0054^{b}	0.0037	0.0011
20:3n6	0.2688^{a}	0.1021^{c}	0.1953^{b}	0.1396^{c}	0.0139	0.0001
22:1	0.0060^{b}	0.0146^{a}	0.0018^{b}	0.0035^{b}	0.0019	0.0001
20:4n6 AA	1.6908^{ab}	0.7824^{c}	1.4744^{b}	1.7678^{a}	0.0685	0.0001
24:1	0.0493^{b}	1.2400^{a}	0.0381^{b}	0.0396^{b}	0.0967	0.0001
20:5n3 EPA	0.0006	0.0020	0.0003	0.0008	0.0005	0.0969
24:0	0.0168^{a}	0.0014^{b}	0.0150^{a}	0.0089^{ab}	0.0028	0.0009
22:4n6	0.0411^{a}	0.0276^{ab}	0.0149^{b}	0.0323b^{ab}	0.0061	0.0301
22:5n3	0.0566^{b}	0.7162^{a}	0.0337^{b}	0.0680^{b}	0.0754	0.0001
22:6n3 DHA	0.4599^{b}	3.3316^{a}	0.2770^{b}	0.5257^{b}	0.2121	0.0001

SFA [2]	10.6369^{ab}	14.6719^{a}	6.1438^{b}	6.9669^{b}	1.3608	0.0001
MUFA [3]	7.3588^{ab}	9.7600^{a}	5.2819^{ab}	3.0563^{b}	1.3399	0.0062
PUFA [4]	9.5319^{ab}	11.4481^{a}	7.1356^{b}	9.9106^{a}	0.9603	0.0224
Total Trans FA [5]	0.4100^{b}	0.1363^{b}	1.5006^{a}	0.1750^{b}	0.0852	0.0001
N-6 [6]	8.7287^{ab}	7.1731^{b}	6.5531^{b}	9.0987^{a}	0.7066	0.0389
N-3 [7]	0.6463^{b}	4.1956^{a}	0.3650^{b}	0.7300^{b}	0.2995	0.0001
n-3:n-6 Ratio [8]	0.0750^{b}	0.6006^{a}	0.0563^{b}	0.0819^{b}	0.0161	0.0001

[1] The dietary treatments are chicken fat (CF), menhaden fish oil (FO), hydrogenated soybean oil (HSBO) and soybean oil (SBO). The fatty acid content was expressed in μg/mg of liver sample.

[a-d] Means within rows with no common superscripts are significantly different ($p < 0.05$) by Tukey's test of SAS.

[2] Sum of total saturated fatty acids of $C_{14:0}$, $C_{15:0}$, $C_{16:0}$, $C_{17:0}$, $C_{18:0}$, $C_{20:0}$, and $C_{24:0}$.

[3] Sum of total monounsaturated fatty acids of $C_{14:1}$, $C_{16:1}$, $C_{18:1}$, $C_{22:1}$, and $C_{24:1}$.

[4] Sum of total polyunsaturated fatty acids of $C_{18:2,}$ $C_{18:3}$, $C_{20:3}$, $C_{20:4}$, $C_{20:5}$, $C_{22:4}$, and $C_{22:6}$.

[5] Sum of total trans fatty acid isomers of $t9C_{16:1}$, $t6t7C_{18:1}$, $t9C_{18:1}$, $t10C_{18:1}$, $t11C_{18:1}$, $t12c7C_{18:1}$, $t13c6C_{18:1}$, $t16C_{18:1}$, $t9t12C_{18:2}$, $c9t12C_{18:2}$, $t9c12C_{18:2}$, $c9t11C_{18:2}$, $t11t13C_{18:2}$, and other trans $C_{18:2}$.

[6] Sum of total n-6 fatty acids of $C_{18:2}$, $C_{20:3}$, $C_{20:4}$, and $C_{22:4}$.

[7] Sum of total n-3 fatty acids of $C_{18:3}$, $C_{20:5}$, and $C_{22:6}$.

[8] Ratio of Σn-3 PUFA to Σn-6 PUFA.

FIGURE 3.1. Effect of dietary chicken fat (CF), menhaden fish oil (FO), hydrogenated soybean oil (HSBO), and soybean oil (SBO) on total (IgM + IgG) and 2-ME resistant (IgG) antibody production in against SRBC. Different letters indicate treatment means differ significantly, ($P < 0.05$). N = 40/treatment.

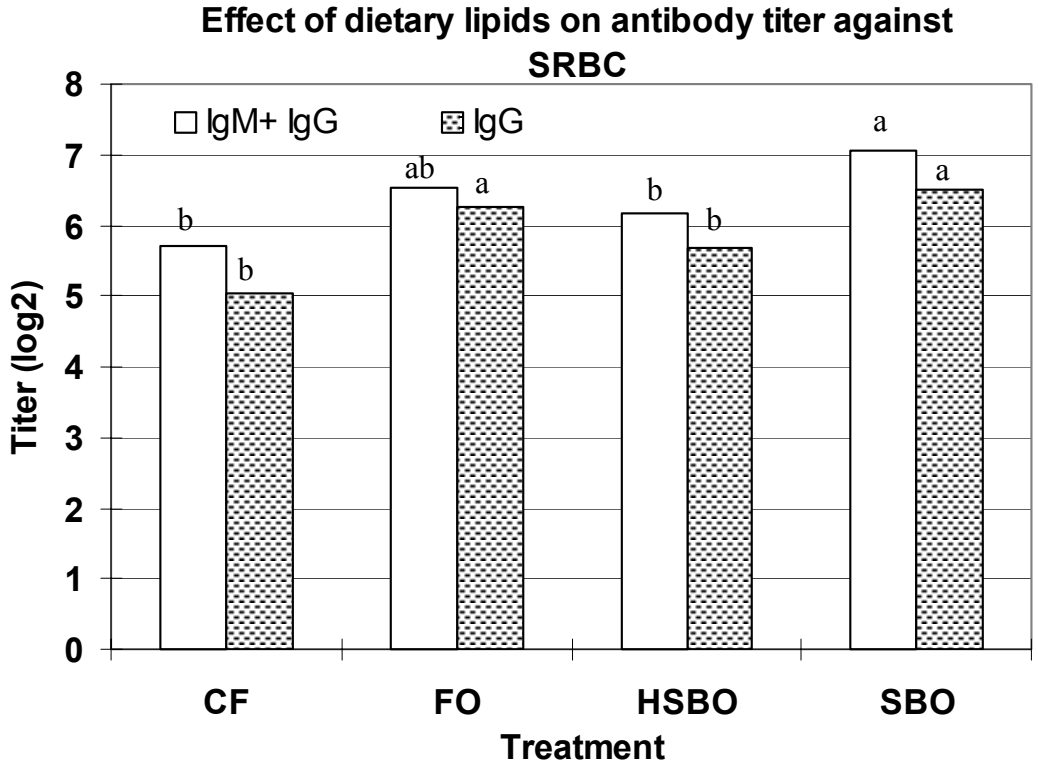


FIGURE 3.2. Effect of dietary lipids including chicken fat (CF), menhaden fish oil (FO), hydrogenated soybean oil (HSBO) and soybean oil (SBO) on cutaneous basophil hypersensitivity. Different letters indicate treatment means differ significantly, ($P < 0.05$). N = 40/treatment.

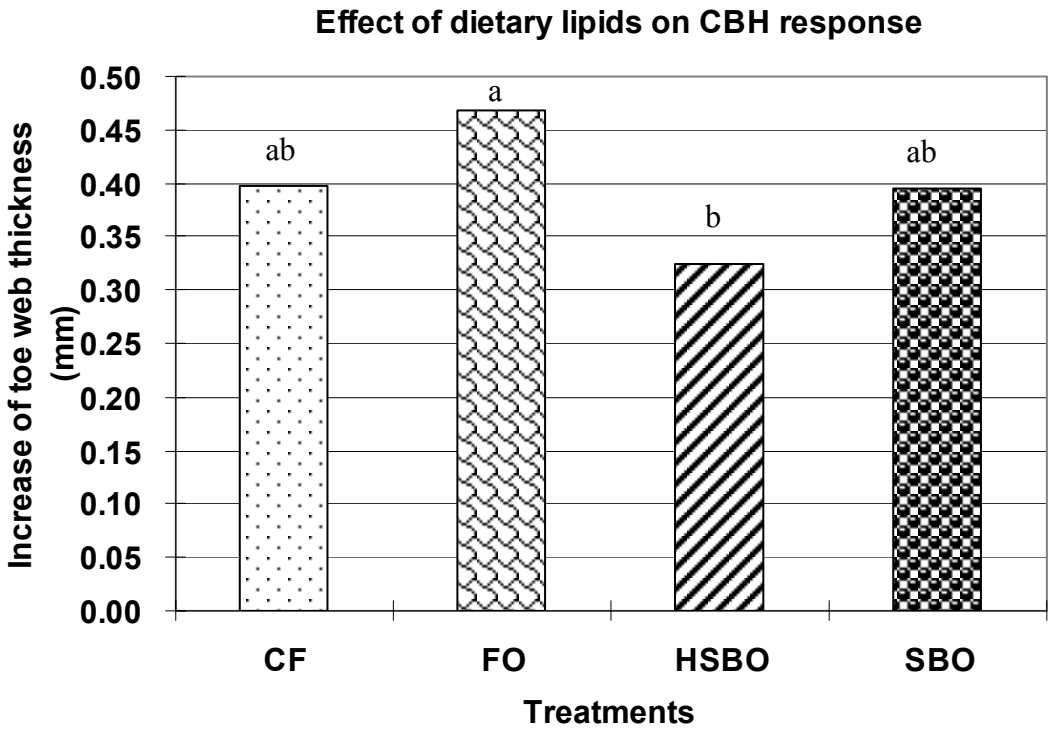


FIGURE 3.3. Effect of dietary lipids including chicken fat (CF), menhaden fish oil (FO), hydrogenated soybean oil (HSBO) and soybean oil (SBO) on phagocytic ability assessed by the rate of carbon clearance after a peripheral injection of carbon ink. Different letters indicate treatment means differ significantly, ($P < 0.05$). N = 16/treatment.

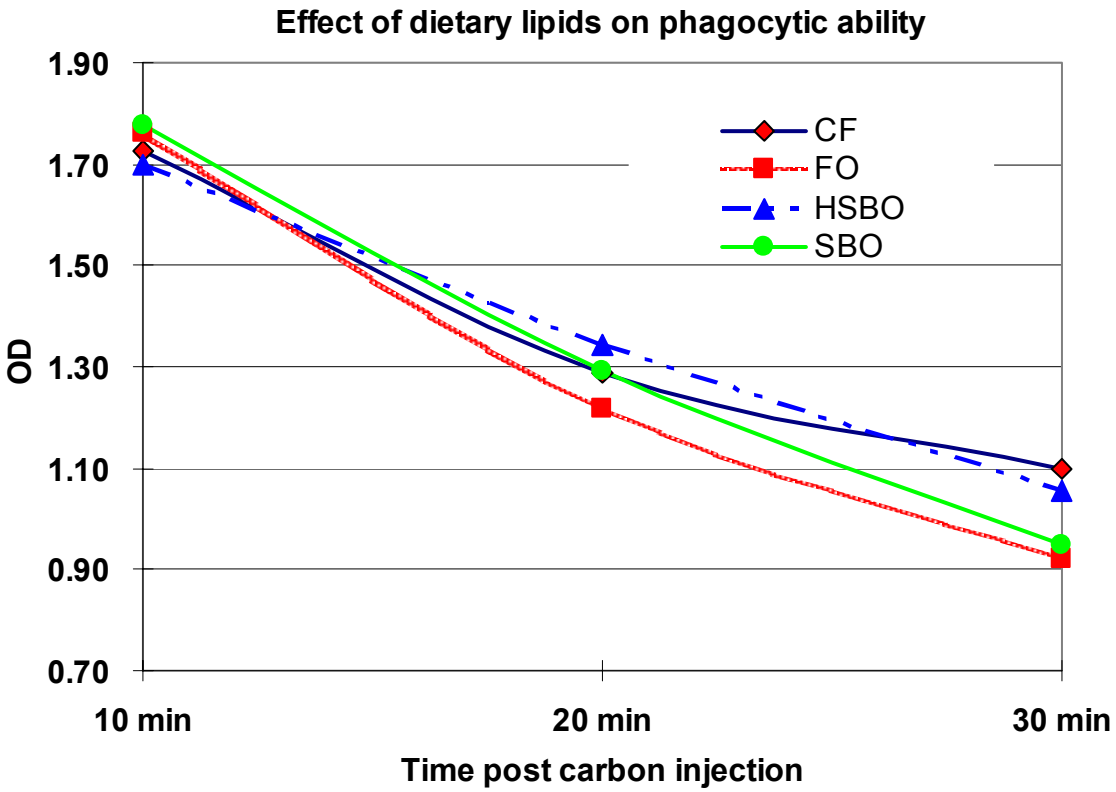
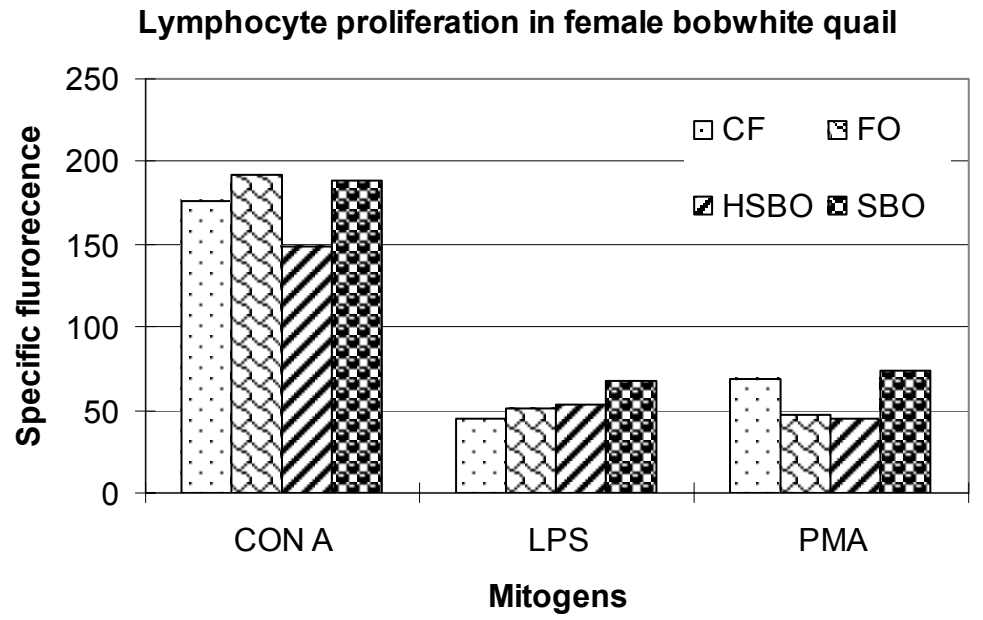


FIGURE 3.4. Effect of dietary lipids on lymphocyte proliferations stimulated by CON A, LPS and PMA/ION mitogens. Different letters indicate treatment means differ significantly, ($P < 0.05$). N = 24/sex/treatment.



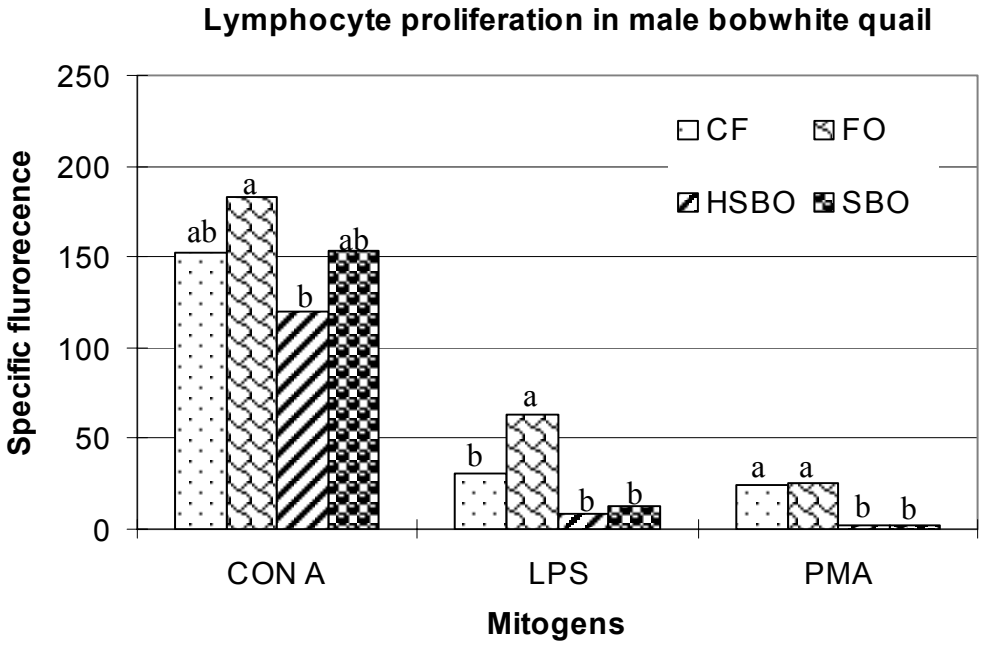


TABLE 3.6. Microscopic examination of blood cytology, pack cell volume (PCV), and plasma total protein (PTP) in quail fed either chicken fat (CF), menhaden fish oil (FO), hydrogenated soybean oil (HSBO) or soybean oil (SBO) diets.

	PMN[1]	Thromb[2]	Mono[3]	Lymph[4]	PCV[5]	PTP[6]
CF						
M[7]	8.55±1.79	66.64±4.91	2.55±0.66	22.36±3.65	52.6±2.92	3.95±0.71
F[8]	8.50±1.21	57.17±3.32	2.75±0.45	27.96±2.47	46.5±3.74[ab]	6.08±0.47[a]
FO						
M	8.79±1.58	65.86±4.35	2.07±0.59	22.79±3.23	52.2±2.42	3.68±0.41
F	9.09±1.26	53.91±3.47	2.86±0.47	32.45±2.58	48.7±2.71[a]	5.40±0.86[ab]
HSBO						
M	7.00±1.87	71.1±5.15	1.80±0.69	20.20±3.83	52.8±2.24	3.65±0.64
F	10.52±1.29	54.52±3.55	3.00±0.48	30.52±2.64	43.8±3.12[b]	5.28±1.09[ab]
SBO						
M	7.92±1.71	68.58±4.70	1.83±0.63	21.67±3.49	53.4±3.64	3.84±0.49
F	9.58±1.36	61.42±3.73	2.58±0.50	26.42±2.78	48.7±2.04[a]	4.97±0.41[b]

[a-d] Means within a column with no common superscripts are significantly different ($P < 0.05$) within a sex.

[1] Polymorphonuclear cells.
[2] Thrombocyte.
[3] Monocyte.
[4] Lymphocyte.
[5] Pack cell volume.
[6] Plasma total protein.
[7] Male = 24/sex/treatment.
[8] Female = 24/sex/treatment.

CHAPTER IV.

Summary and Discussion

Dietary fatty acids are known to affect immunity depending on their concentration ratio, degree of unsaturation, configuration of structure, position of double bonds, and functional metabolites. In addition, they are carriers for other essential nutrients such as lipid-soluble vitamins. Consumption of lipids containing various fatty acids can affect health.

In our studies, dietary lipid treatments including CF, FO, HSBO and SBO resulted in fatty acid profiles distinctive in saturation, configuration, and proportion. The respective lipid treatments resulted in a distinctive fatty acid profile in the plasma and liver of both JAP and BOB. In JAP, the plasma $C_{18:2}$ n-6, $C_{22:4}$ n-6, total n-6 PUFA and hepatic $C_{18:1}$ n-9, $C_{18:0}$, total SFA, total MUFA were not affected by dietary lipid treatment. On the other hand, the plasma $C_{18:1}$ n-9, $C_{16:0}$, $C_{20:0}$ and hepatic $C_{18:2}$ n-6 reached a homeostasis in BOB. In both species, it may be important to maintain a constant level of linoleic acid $C_{18:2}$ n-6 and oleic acid $C_{18:1}$ n-9. Moreover, it is noteworthy to mention that feeding the FO diet to JAP and BOB elevated plasma EPA level, although the hepatic EPA was found in only trace amounts that did not differ from other treatments. Since both species were fed the same formulation and lipid level, the fatty acid homeostasis in plasma or liver indicated a fundamental difference in fatty acid metabolism between these two avian species.

The plasma and hepatic n-3/n-6 PUFA ratios were significantly higher in quail fed the FO diet compared to the other dietary lipid treatments. Fatty acid analysis of the FO diet revealed that the n-3/n-6 ratio was 0.42. In both species fed the FO diet, the plasma and hepatic n-3/n-6 ratio were at least 50% higher than that in the diet. Therefore, a

preferential uptake and deposition of n-3 PUFA may have occurred in the two species. Moreover, quail fed SBO had also elevated plasma and hepatic α-linolenic acid ($C_{18:3}$ n-3). The CF diet contained a large amount MUFA with the highest $C_{18:1}$ n-9 content. In contrast, the HSBO diet contained a high amount of MUFA but rich in trans monoenoic fatty acids. Moreover, conjugated fatty acid (c9, t11 $C_{18:2}$) was also found in significantly higher concentrations in quail fed HSBO, while the CLA was not detectable in the HSBO diet. The enrichment of CLA in tissues can be achieved in quail by dietary supplementation of trans fatty acids by delta-9 desaturase.

Our four experimental diets with fatty acid compositions varied in levels of saturation, amount of trans isomers, ratio of n-3 to n-6 PUFA has shown a multi-fashionable influence in immunity. Both Japanese quail and bobwhite quail fed diets that contained a higher amount of PUFA, particularly with high ratio of n-3/n-6 PUFA, had an overall beneficial effect on bird health by elevating indices of humoral and cellular immunity. The total antibody titer was only moderately affected by dietary lipid treatments in JAP, while the levels of IgG+IgM and IgG were significantly higher in SBO and FO fed BOB compared to those fed CF or HSBO. The same trend, but lower degree of influence in humoral immunity of JAP, was probably due to the steady n-6 PUFA level in plasma. In BOB, the depressed antibody production in CF and HSBO groups probably resulted from high plasma MUFA level, since both groups had significantly higher plasma MUFA content than those in the SBO and FO groups. Since fatty acid composition of MUFA in the CF and HSBO diets are different in their configuration, the higher cis MUFA in CF group was not like the higher trans MUFA in the HSBO that also suppressed the *in vitro* B lymphocyte proliferation. This extended detrimental effect of HSBO diet could be due to a higher incorporation of trans fatty acid into cell membrane. Membrane trans fatty acid isomers

have their spatial distance close to each other in the lipid bi-layer compared to their cis counterparts; this decreases in membrane permeability and flexibility. The decreased membrane permeability and flexibility may lead to lower cell livability.

Cell-mediated immunity in both JAP and BOB fed a 5% menhaden fish oil diet was consistently showed enhancement that was demonstrated in both *in vivo* and *in vitro* assays. FO diet rich in N-3 PUFA antagonize the PGE_2 biosynthesis by competitive with AA incorporation. Since PGE_2 has been reported to favor T_{H2} mediated immunity in the T_{H1}/T_{H2} dimorphic immunity (Betz and Fox, 1991; Phipps *et al*., 1991) and a high level of PGs suppresses cell-mediated immunity (Clissold, 1999), the less potent n-3 PUFA derived PGE_3 may be responsible for the enhanced cell-mediated immunity.

Our studies show that the peak CBH response varied by dietary lipid manipulation. The CBH response measured 24 hours post PHA inoculation had a significant higher index in birds fed FO and the lowest index was also consistently observed in birds fed HSBO of JAP and BOB. However, with measurements taken over a 72 hours period, the SBO and CF fed birds had a faster peak CBH response (12 hours) than that of FO and HSBO fed birds. A faster recovery back to the original CBH index by 72 hours post PHA challenge was also demonstrated in quail fed SBO and CF. This observation greatly explained why some inconsistent observations on CBH response measuring at 24-hour across different sampling period in SBO and CF fed JAP, as well as those non-significantly differences in CBH response of BOB. One of the most important explanations could be the local production of eicosanoids during an inflammatory response. The polymorpho- nuclear cells (PMN) usually dominate at early stage and following with other mononuclear cells in later stage. Recruiting of effector cells relies on eicosanoids including prostaglandins and leukotrienes by PMN. Prostaglandins are required for activation of cell adhesion

molecules and leukotrienes are potent chemoattractive substances.

Recently, two isomers of cyclooxygenase were described by Hwang (2000). COX 1 is constitutively expressed in almost all tissues and helps maintain normal physiological events, while the COX 2 is activated during the inflammatory responses (Hwang, 2000; Tegeder *et al*., 2001). The specific inhibition on COX-2 activity is favored to decrease unwanted inflammation but maintain the normal requirement of PGE_2. Interestingly, our results partially (indirectly) indicate that n-3 PUFA may selectively inhibit the COX-2 activity. The COX 2 (inducible) mediated PGE_2 synthesis has been shown to peak twice at early and late stages of cell-mediated immune response. Indomethacin inhibits inflammation at early stages, while selective inhibition of COX 2 enzyme activity in later stage exacerbates the inflammatory response (Gilroy *et al*., 1999). In our result, the high n-3 PUFA diet (FO) or the low n-6 PUFA diet (HSBO) mimics the NSAID effect on COX 1 and 2 enzymes by suppressing the acute inflammation (slower peak response) and delayed inflammatory resolution. However, the highest amplitude of CBH response of FO fed birds was significantly higher than HSBO fed birds and not differs to those fed SBO or CF. This indicates that the effect of dietary FO may have selectively acted on the inducible COX 2 enzyme rather than the constitutive COX 1 enzyme. In addition, Kover and Klasing (1997) have also suggested that reduction in inflammatory cytokines such as IL-1 by n-3 PUFA may also attribute the blunted acute inflammatory response. Therefore, multiple factors may directly result the slower onset of inflammation in n-3 PUFA fed birds.

Furthermore, it has been shown that PUFA inhibits cell adhesion molecules expression depending on the number of double bonds (Caterina *et al*., 1998). Trafficking of immunocomeptent cells to the site of antigen invasion (especially heterophils and

monocytes in avian) is greatly dependent on cell adhesion molecules such as endothelial cell adhesion molecules (ECAM). Quail fed SBO or CF diets that contained a higher quantity of n-6 PUFA (about 46%) had similar amplitude of toe web thickness to those fed FO but the time of peaked response was different in response to PHA. N-3 PUFA suppresses expression of cell adhesion molecules and therefore may interfere with the recruiting mechanism and the number of effectors cells in response to injury. Combination of the mentioned factors could have resulted in the slower peak response time. In a similar manner to FO, HSBO-fed birds had their CBH response peak at 24 hours, but with a significantly lower in amplitude than FO fed birds. The slower and lower response amplitude in HSBO fed birds could be solely due to hampered immunity.

Cell integrity and skin swelling would be compromised by a rigid cell membrane produced by trans monounsaturated fatty acid enriched in HSBO diet. By analyzing fatty acid compositions between CF and HSBO diets, the CF diet contained a similar proportion of saturated fat and monounsaturated fatty acid but in cis configuration; it did not have a negative influence in the immunocompetence measured in our study. Therefore, we proposed that dietary HSBO resulted in depressed immunity mainly due to alteration of cell membrane physical properties rather than the level of lipid mediators (eicosanoids). In addition, discrepancy in metabolism of HSBO has been addressed. Kaplan *et al*. (1998) concluded that in rats, hydrogenated fats contributed much less of utilizable fat and energy than conventional fat. However, Kritchevsky, (1999) stated that cis and trans fats are metabolized in a similar manner. In the current study, the feed intake was not measured but average body weight was similar across all treatments. Since the HSBO had less utilizable energy; birds could eat more feed and indirectly increase other nutrients intake to compensate the low calories diet. The use of trans fat to alter feed intake could have other

potential implication in animal production.

Calder (1997) and Hwang (2000) summarized that n-3 PUFA significantly decreased inflammatory responses and lowered cell-mediated immunity by inhibiting lymphocyte proliferation and decreasing cytokine production. In addition, dietary n-3 PUFA had been shown to decrease PGE_2 and LTB4 productions that derived from n-6 AA in mouse macrophages (Lokesh *et al*, 1988). Previously from our lab, Liu and Denbow (2001) reported that *ex vivo* PGE_2 production in quail bone tissue was in the descending order of SBO>CF>HSBO>FO fed quail. Since lymphocytes do not produce prostaglandin or leukotriene (Calder, 1997; Huang, 2000), production of PGE_2 or LTB_4 during an immune process is responsible by the non-lymphoid derived cells. Therefore, the *in vitro* evaluation of lipid mediated immune processes such as lymphocyte proliferation or cytokine production may depend greatly on the purity of the cultured cell population.

Our *in vitro* assessment of lymphocyte proliferation was performed using a non-radioactive colorometric assay that indirectly measures the rate of lymphocyte proliferation. The flurometric change in Alamar Blue Dye (Lymphopro®) via a redox reaction caused by metabolizable proliferated lymphocytes was validated to be similar to the results using the [^{3}H] thymidine incorporation assay (Gogal et al., 1997). Since the Lymphopro® assay is validated by cell metabolism, the purity of lymphocyte culture is critical. Isolation of peripheral blood lymphocytes in avian is relatively more difficult than in mammals. This is because avian species have nucleated erythrocytes and platelets (thrombocytes) and conventional gradient isolation techniques show a high contamination of nucleated erythrocytes and platelets. Slow speed centrifugation for harvest of avian lymphocytes has been described (Barta *et al*., 1992; Kasper *et al*., 1993; Martin *et al*., 1994). Our studies were the first to attempt using this method for isolating peripheral

blood lymphocytes from Japanese quail. Due to the size of thrombocyte being very close to that of lymphocytes, we were unable to obtain a highly purified lymphocyte population by any method including the slow speed centrifugation or ficoll density gradient separation. The purity of the lymphocyte population for cell culture is a major concern. Culture containing cell populations other than lymphocytes especially thrombocytes lower the number of lymphocytes. Thus, the metabolic rate calculated to be in a particular cell culture preparation is biased. In addition, contaminated erythrocytes and thrombocytes could present physical and biological blockage where the interaction between mitogen and lymphocyte is required to activate lymphocytes. Lowered readings of lymphocyte proliferation would then occur. Since, dietary lipid treatments did not appear to affect lymphocytes' size and we did not find a significant difference in whole blood cytology in different dietary lipid treatments, the ficoll density gradient separation may provided more consistent cell population in every isolation from different treatment groups and comparisons made across different lipid treatments by using lymphocyte proliferation seemed more accurate than using the slow speed centrifugation method.

As mentioned earlier, the Lymphopro® assay is designed to determines lymphocyte proliferation by measuring cell metabolism. Application of this technique in nutritional studies may be flawed since it is impossible to eliminate the potential effects of such treatments on cell metabolism. The evaluation of the effects of dietary lipids on lymphocyte proliferation may modify cell metabolism rather than immunocompetence. In addition, lipid mediators (eicosanoids) are only produced by non-lymphoid derived cells. Thus, contamination of lymphocyte culture by non-lymphoid derived cells could affect the overall lymphocyte proliferation in response to mitogens, by the amount of eicosanoids in the culture media.

Nevertheless, recent studies have shown that fatty acids bind to PPAR inhibiting IL-1, IL-6, and COX 2 enzyme expression independently of their metabolite, eicosanoids (Jiang *et al*., 1998; Ricote *et al*., 1998). The binding affinity of PPAR to fatty acids is PUFA > MUFA > SFA (Kliewer *et al*., 1997; Nolte *et al*., 1998). This differential binding to PPAR by different fatty acids provides an additional mechanism for the effect of fatty acids on immunity.

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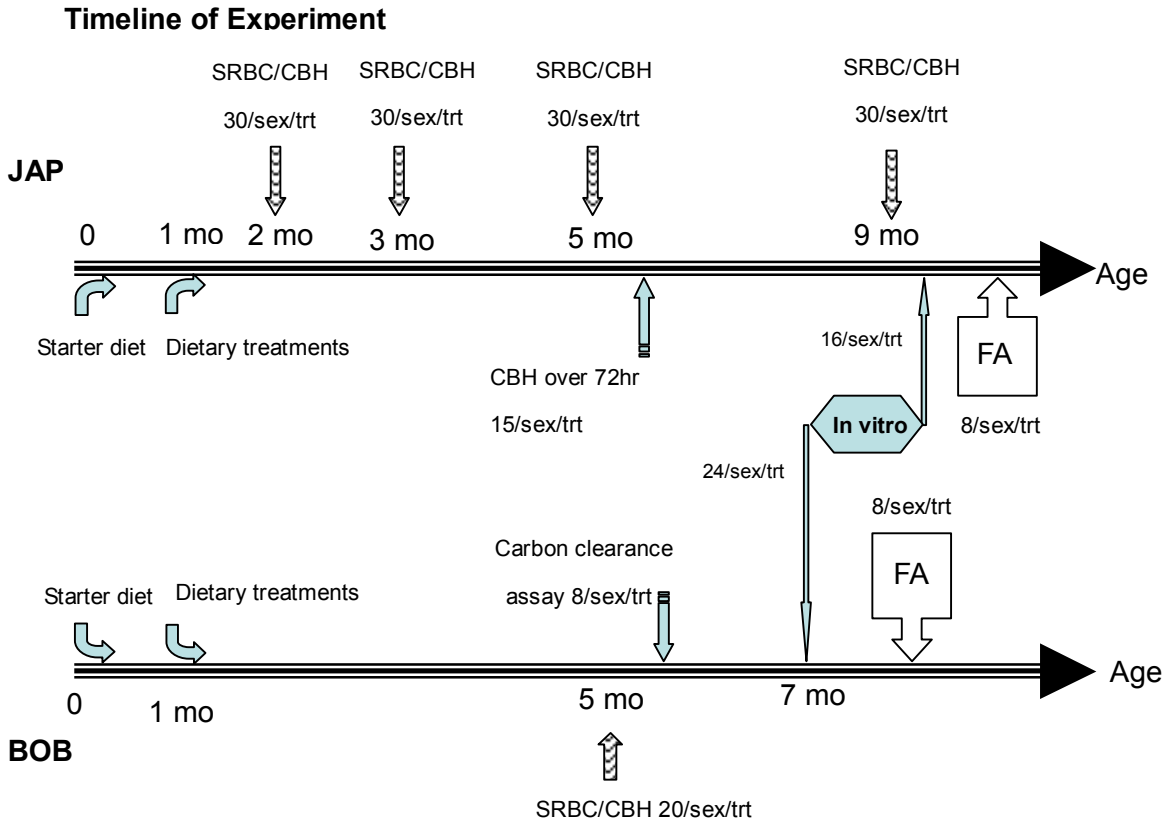
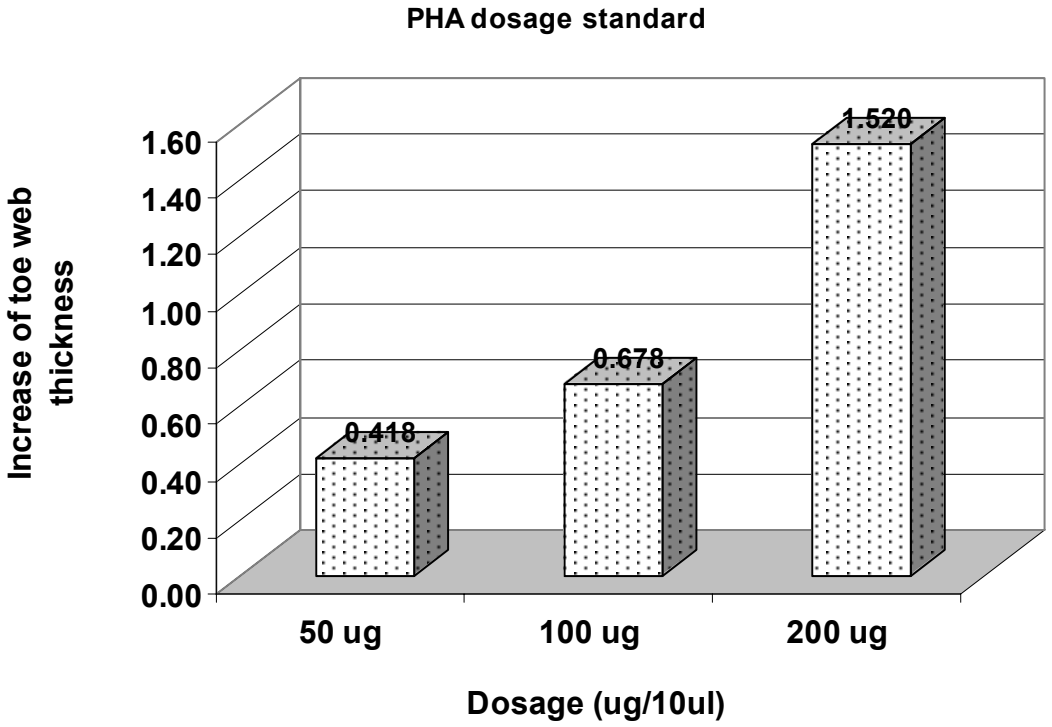
Appendices

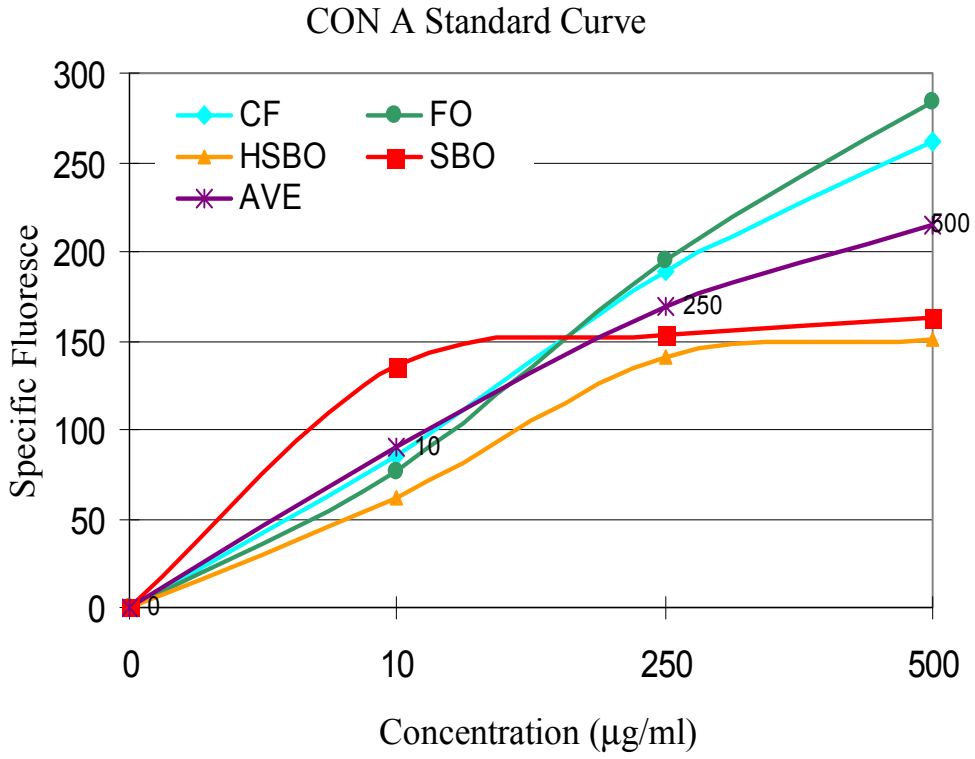
APP. I. Timeline of experiment.



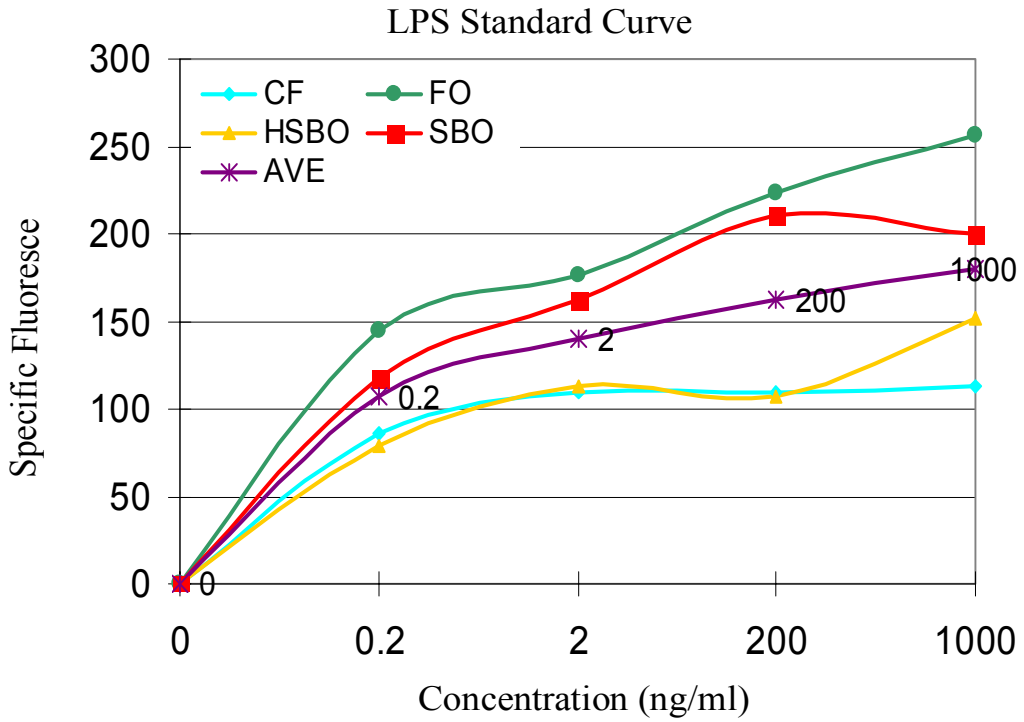
APP. II. The cutaneous basophil hypersensitivity response measured at 24 hours post injecting different dosage of phytohemagglutinin-P (PHA).



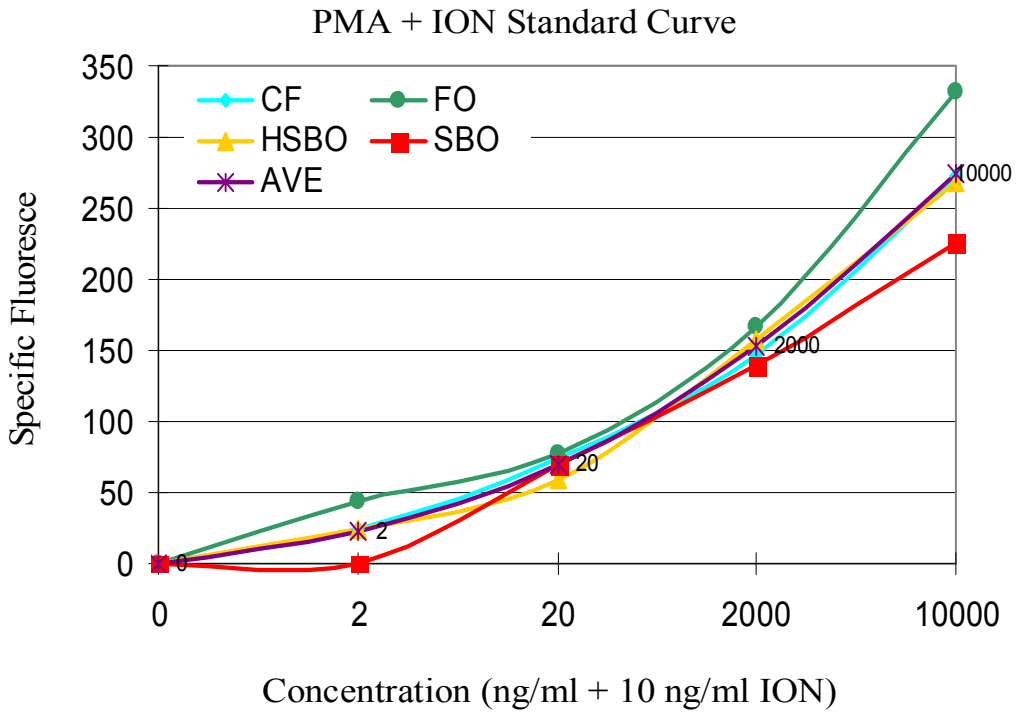
APP. III. Concanavalin A (CON A) standard curve in determination of optimum dosage for in vitro stimulation of lymphocytes of Japanese quail.



APP. IV. Lipopolysacchride (LPS) standard curve in determination of optimum dosage for in vitro stimulation of lymphocytes of Japanese quail.



APP. V. Phorbal 12-myristate 13-acetate (PMA) standard curve with a fixed 10 ng/ml ionomycine (ION) in determination of optimum dosage for *in vitro* stimulation of lymphocytes of Japanese quail.



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Researching on the mechanism of different dietary fatty acids in modulation of humoral and cell-mediated immunity by evaluation of:

- antibody agglutination against sheep red blood cells
- cell-mediated immunity via subcutaneous injection of several mitogens; fatty acids and prostaglandins quantitative assays (GC;ELISA)
- immune cell differentiation via cell counter and non-radioactive lymphocyte proliferation assay and cell culture
- analysis of lymphocyte sub-population via flow-cytometry assay.

Experience Summary:

- Extensive working experiences in animal immunology, reproductive physiology, and nutrition. Specific research areas include dietary lipids in both *in vivo* and *in vitro* immunity of domestic fowls; and the biological functions of carotenoids in immunity and reproduction of domestic carnivores. Study on leukocytes migration in against uterine infection (endometritis) in ewes.

- Hands-on experience and knowledge in animal behavior and experimental design and researches with a variety of laboratory animals

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Researching biological functions of carotenoids on immunity and reproduction of domestic carnivores. Identifying various carotenoids and isomers by high – performance liquid chromatography (HPLC), and several immunity parameters by assays such as specific antibody titers and delayed- type hypersensitivity skin test, evaluation of lymphocyte proliferation and T cell subsets population via flow cytometry assay. Measuring steroid hormones by RIA and uterine protein production by Lowary's analysis.

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Publications

Abstract

Weng, B.C., B.P. Chew, T.S. Wong, J.S. Park, H.W. Kim, and A.J. Lepine. β-Carotene uptake by corpus luteum and uterus and changes in ovarian steroids and uterine proteins during the estrous cycle in dogs. 1998. FASEB J. 12:A967.

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