PHYSIOLOGY, ENDOCRINOLOGY, AND REPRODUCTION

Effects of dietary n-6:n-3 ratio on immune and reproductive systems of pullet chicks

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ABSTRACT The objective of this study was to examine the effects of dietary n-6:n-3 ratio on immune and reproduction systems of Leghorn pullet chicks. A total of 216 Ily-Line W-36 pullet chicks (1 d old) were randomly assigned to 3 diets (n-6:n-3 ratios of 10, 6, and 2) until 22 wk of age. The Optomega-50 (Optivite International Ltd., Nottinghamshire, UK) containing 50% salmon fish oil was used as an n-3 source. Pullets were injected with SRBC suspension at 6 and 9 wk and anti-SRBC titers were measured 7 and 14 d after each immunization. The antibody (Ab) titers for Newcastle disease (ND), avian influenza, infectious bronchitis (IB), and infectious bursal disease (IBD) were determined at 4, 6, 10, 14, and 18 wk of age. Pullets fed diet with the n-6:n-3 ratio of 2 had higher feed consumption and lower BW gain ($P < 0.05$) compared those fed diets with the ratios of 6 and 10. The results demonstrated that the different dietary n-6:n-3 ratios did not have a significant effect on the anti-SRBC titers in pullets serum ($P > 0.05$). The Ab production against avian influenza vaccine was increased in pullets fed diet containing the n-6:n-3 ratio of 10 at wk 10 and 14 and increased for ND at only 14 wk ($P < 0.05$). On the other hand, the Ab response to IBD (14 wk) and IB (14 wk) vaccines was increased when pullets were fed diet containing a ratio of 6 ($P < 0.05$). The pullets fed diet with the n-6:n-3 ratio of 2 showed lower egg production and late sexual maturity ($P > 0.05$) whereas ovary weight ($P = 0.01$) and number of large yellow follicles ($P = 0.049$) were significantly decreased at first oviposition. Our results revealed that the supplementation of Optomega-50 as a source of n-3 to decrease the n-6:n-3 ratio in diet significantly increased Ab production for ND, IB, and IBD in pullet chicks with slight reduction in reproductive organs weight at early production.

Key words: pullet chick, n-6:n-3 ratio, humoral immunity, reproductive organ, vaccine titer

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INTRODUCTION

Most infectious diseases cause serious economic losses to the poultry industry. Recent investigations on the effect of nutrients on immunity are related to the increase in antibody (Ab) responses of young chickens, which have poor immunity against some diseases even with vaccination. Most of these studies have focused on dietary polyunsaturated fatty acids (**PUFA**; Sijben et al., 2000; Puthpongsiriporn and Scheideler, 2005). Two major classes of immunomodulating PUFA are n-6 and n-3 fatty acids. The principal precursor of n-3 family is α-linolenic acid (**LNA**; $C_{18:3}$), whereas linoleic acid ($C_{18:2}$) and arachidonic acid (**AA**; $C_{20:4}$) are the forms of n-6 PUFA. These PUFA compete to react with eicosanoid synthesis enzymes. Eicosanoids are important regulators and influence various immune responses (Kinsella, 1991; Sijben et al., 2000).

The type and amount of dietary PUFA dictates the fatty acids composition of lipids in egg yolk (Jia et al., 2008), bursa of Fabricius and thymus (Fritsche et al., 1991a; Puthpongsiriporn and Scheideler, 2005), and bone marrow (Friedman and Sklan, 1995) of poultry. Linoleic acid and LNA may induce immunomodulatory functions after elongation and desaturation to AA and docosahexaenoic acid (**DHA**; $C_{22:6}$), respectively, which are incorporated into phospholipid membranes in immune cells. Phospholipase causes the release of these long-chain PUFA, which change to active communicators of the immune system such as prostaglandins, leukotrienes, and thromboxanes (Guo et al., 2004).

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Fish oil is rich in n-3 PUFA and consists mainly of eicosapentaenoic acid (**EPA**; $C_{20:5}$) and DIIA; conversely, flaxseed is rich in LNA n-3 PUFA. Calder (1996) fed mice dietary fish oil and observed decreased production of interleukins and tumor necrosis factor that are required for lymphocyte development. However, production of IgG and IgE was enhanced by using high levels of fish oil (EPA and DIIA) in rat diet. It has been demonstrated in chickens that in vitro spleen lymphocyte proliferation was significantly suppressed through n-3 PUFA, LNA, or EPA and DIIA (Fritsche et al., 1991b). In broiler chickens, feeding fish oil produced more Ab in response to SRBC compared with maize oil supplementation diets rich in n-6 PUFA (Fritsche et al., 1991b). Friedman and Sklan (1995) demonstrated that anti-BSA Ab responses developed more rapidly and were more persistent in 2 groups of chicks fed lower levels (11.7 and 23.8) of n-6:n-3 ratio than those fed high levels (27 and 32) of n-6:n-3 ratio. However, they did not add n-3 PUFA sources to make these ratios. Puthpongsiriporn and Scheideler (2005) used flaxseed and maize oil to formulate n-6:n-3 ratios. Their results showed that dietary ratio of 2 had greater Ab responses against Newcastle disease (**ND**) virus vaccine compared with those fed diets with ratios of 4, 8, and 17 .This apparent discrepancy may be attributable to the result of interaction between n-6 and n-3 PUFA, different actions of dietary PUFA between antigens, and the nature of the antigens (Sijben et al., 2001). Because the metabolisms of n-3 and n-6 are intertwined, the effect of one PUFA depends on the level of all diet fatty acids.

Two series of prostaglandins (**PG**; such as PGE_2 and PGF_2) synthesized by a cyclooxygenase-dependent pathway from n-6 PUFA AA would play a different biological function in endocrine metabolic pathways (Takahashi et al., 2004) and the immune responses (Sijben et al., 2000). Fish oil in diets of laying hens significantly decreased PGE_2 synthesis (15.65 pg/mL) by peripheral blood leukocytes compared with linseed and maize oil (18.6 and 33.9 pg/mL, respectively; Guo et al., 2004). Little is known about the effect of dietary n-3 and n-6 PUFA on female reproductive physiology of laying hens. However, feeding a diet supplemented with 60 g/kg of fish oil significantly decreased plasma estrogen concentration in laying hens (Whitehead et al., 1993). The ovarian hormones such as estrogen are thought to act on the uterus wall because the uterine tissue contains receptors for estrogen (Kawashima et al., 1984). Takahashi et al. (2004) found a positive relationship between PGF and estrogen before the oviposition action in laying hens. Therefore, it is important to know the effects and mechanisms of dietary n-6 and n-3 on reproductive physiology of laying hens at early egg production. The objective of the current study was to examine the effects of different n-6:n-3 ratios in diets supplemented with a commercially available fish oil product (Optomega-50; Optivite International Ltd., Retford, Nottinghamshire, UK) on immune and reproductive systems of pullets.

MATERIALS AND METHODS

Birds and Management

A total of 216 pullet chicks (1 d old) with average initial BW of 40.7 g were assigned to 3 dietary treatments with 6 replicates of 12 birds each. All birds were fed corn–soybean meal-based isoenergetic and isonitrogenous mash diets that were formulated to meet or exceed the nutrient requirements of pullets; diets were fed in 5 phases (NRC, 1994). A control diet was made to have 3% n-6 with no addition of Optomega-50, whereas diets 2 and 3 were adjusted by soybean oil and Optomega-50 (salmon fish oil, 50%; Σn-6, 6%; Σn-3, 24%; CP, 5%; ME, 5,250 kcal/kg; and vitamin E, 2,500 mg/kg) to produce a constant concentration of 3% n-6 PUFA (Table 1) and avert undesired interaction effects with the dietary n-3 levels. The calculated levels of n-3 of 3 experimental diets were 0.29, 0.5, and 1.5% to provide dietary n-6:n-3 ratios of 10, 6, and 2, respectively (Table 1). Prior to diet formulation, corn and soybean oil were analyzed for their fatty acid composition following the procedure of Cherian and Sim (1992). Corn oil contained 13.9 and 2.1% and soybean oil contained 52.1 and 6.5% n-6 and n-3, respectively. The fatty acids profile of Optomega-50 was provided by the manufacturer (Optivite International Ltd., Retford, Nottinghamshire, UK). All diets were supplemented with 1 mg/kg of vitamin E to prevent autoxidation of the oil mixtures in diets; fresh diets were prepared weekly and were stored in a dark, cold room in air-tight containers. Birds were housed in standard battery cages with 12 birds/cage. Each battery cage had 3 levels of 2 cages each. Supplemental heating was provided by gas heater to warm the room temperature to 32°C on arrival of the chicks. Room temperature was reduced 2°C every week after wk 1 to reach 21°C, which was kept constant thereafter. Incandescent bulbs were located between battery cages or the side walls. Light bulbs were cleaned once per week, and the intensities were adjusted with a photometer (Lutron LX-107, Lutron Electronic Enterprise Co. Ltd., Taipei, Taiwan). To ensure early access to feed and water, birds were subjected to 24 h of light for the first 3 d and then a pullet lighting program was used. The birds were raised under optimum environmental conditions as recommended by Hy-Line International (2007). During growing (0–18 wk) and laying (18–22 wk) periods, feed and water were provided ad libitum. Birds had access to jug and nipple waterers for the first 2 wk, after which the jug waterers were removed. At wk 15 of the experiment, pullets were transferred to a laying house and individually placed in standard layer cages to determine the individual sexual maturity age (first oviposition). Feed intake and BW were recorded in 3-wk intervals until wk 18 of the experiment. Weights of all dead birds were recorded before death. Chickens were vaccinated with commercial classical vaccines recommended by the local veterinary office as follows. On d 1, Marek's disease virus was

injected subcutaneously and infectious bronchitis (**IB**) virus attenuated live vaccine was administered by fine spray. On d 7, ND virus attenuated live vaccine was administered by eye drop and infectious bursal disease (**IBD**) virus and ND virus killed vaccine were administered by subcutaneous injection. On d 14, IB virus was administered by eye drop and avian influenza (**AI**) virus killed vaccine was administered by subcutaneous injection. On d 21, IBD virus attenuated live vaccine and ND virus were administered via drinking water. On d 28, AI virus killed vaccine was given by subcutaneous injection and IBD virus attenuated live vaccine and ND virus were given via drinking water. On d 35, IBD virus attenuated live vaccine and ND virus were given via drinking water. On d 49, laryngotracheitis modified live vaccine was given via eye drop. On d 84, avian pneumonia virus attenuated live vaccine was given by wing web stab and laryngotracheitis modified live vaccine was given via eye drop. Finally, on d 126, egg deficiency syndrome, ND virus, and IB virus killed vaccine were given by thigh muscle injection.

Carcass, Reproductive, and Bone Measurements

Carcass and Reproductive Organs. At 18 wk of age, photostimulation was suddenly increased to 13L:11D; the light period was increased by 30 min every other week to reach a maximum of 16L:8D. The age at first oviposition (considered to be the time of sexual maturity) and weight of first egg for each hen were recorded. The hen in each replicate that laid the first egg was marked and feed was withdrawn overnight to facilitate gut clearance. The next morning, BW was recorded and birds were killed by cervical dislocation to measure the carcass and reproductive organs weights. The birds were dissected, and weights of whole breast, thighs, heart, gizzard, gut, spleen, liver, oviduct, ovary, and stroma were recorded. The ovary was analyzed for follicular development. Large yellow follicles (**LYF**; >10 mm diameter) and small yellow follicles (5–10 mm diameter) were counted. The LYF were sorted by order of largest to smallest, only before entry into the uterus, with the term F1 follicle given to the largest. Abdominal fat and fat surrounding the gizzard and proventriculus were cut out and weighed.

Femur. The left femur bone was removed from each bird. Femurs were defleshed, and cartilaginous caps were removed immediately after collection. They were kept frozen in plastic bags at −15°C until analysis for mineral content. Frozen femurs were thawed at room temperature for 2 h and then percentage of femur ash was measured (Hall et al., 2003). Briefly, bones were autoclaved under 1.32 Pa of pressure for 15 to 20 min. The bones were cooled, cleaned of adhering tissue, and dried at 100°C for 48 h. The length and weight of bones were measured and then the bones were ashed in a muffle furnace overnight at 550°C. Calcium and P for each femur were analyzed according to the method of AOAC (1995). Calcium and P concentrations of femurs were determined using an atomic absorption spectrophotometer (Varian SpectraAA 50 B, Agilent Technologies, Santa Clara, CA) by solubilized in HCl method and spectrophotometer (CECIL CE 1021, Cecil Instrument Ltd., Cambridge, UK) by the molybdovanadate method, respectively.

Antibody Production Determination

Two birds per replicate were bled through the wing vein at 4, 6, 10, 14, and 18 wk to determine Ab production against AI, ND, IB, and IBD in serum. Blood samples (1.0–2.0 mL/bird) were drawn and allowed to clot at room temperature for 2 h before serum collection. Serum was separated by centrifugation (1,300 × *g* for 10 min at 4°C) and stored at −20°C for further analysis.

ELISA Test. Infectious bronchitis and IBD vaccine titers were determined in serum by utilizing an ELISA method and commercial ELISA test kits according to the manufacturer's instructions (Idexx Laboratories Inc., Westbrook, ME). Positive and negative controls were provided in the kits and used for each plate. Optical density was read at a wavelength of 650 nm on an ELX800 ELISA reader (BioTek Instrument Inc., Winooski, VT). The natural log of the Ab titer ($\log_{10}$ titer) in each serum was calculated using the following equations:

$$\text{S:P} = (\text{sample mean} - \text{NC})/(\text{PC} - \text{NC}) \text{ and}$$

$$\log_{10} = 1.09\ (\log_{10} \text{S:P}) + 3.36,$$

where S:P is the sample:positive ratio, NC is the negative controls mean, and PC is the positive controls mean.

Hemagglutination Inhibition Test. The Ab titers to AI and ND in serum were ascertained by a hemagglutination inhibition test (Qiu et al., 2007) using AI and ND virus antigen (4 hemagglutination units). The mean titers were expressed as $\log_2$ values of the highest dilution for each serum.

SRBC Inoculation. All birds were injected intramuscularly in the left breast muscle with SRBC antigen (25% suspension in PBS; 0.5 mL/bird) at 6 and 9 wk of age. Sheep red blood cells were collected in Alsever's solution (2.05% dextrose, 0.42% sodium chloride, and 0.8% sodium citrate). Cells were washed 3 times and were diluted to 25% in PBS. Blood samples were collected at 7 and 14 d after primary immunization and after secondary immunization. The serum from each sample was collected, heat inactivated at 56°C for 30 min, and analyzed for total and 2-mercaptoethanol-resistant IgG anti-SRBC antibodies as described previously (Qureshi and Havenstein, 1994; 2-mercaptoethanol from Sigma, St. Louis, MO). Serum dilutions ranged from 1:2 to

Table 1. Composition of diets with n-6:n-3 ratios[1] of 10, 6, and 2 fed to pullets during different phases

	0 6 wk of age			6 9 wk of age			9 16 wk of age			16 wk to 5% egg production			5 50% egg production		
Item	10	6	2	10	6	2	10	6	2	10	6	2	10	6	2
Ingredient															
Corn	57.13	53.99	38.25	61.56	58.18	42.69	68.06	63.70	48.21	61.18	58.35	42.75	56.24	54.9	39.28
Soybean meal	33.16	33.65	33.28	27.34	27.82	27.44	21.66	22.02	21.64	26.6	26.52	25.85	28.57	28.66	27.98
Soybean oil	3.10	3.11	2.96	2.92	2.96	2.83	2.57	2.81	2.68	0.323	3.06	2.9	3.72	3.21	3.05
Wheat bran	2.15	2.02	9.97	3.86	3.87	11.04	3.31	4.18	11.36	0.00	1.28	9.37	0.00	0.00	8.12
Optomega-50[5]	0.00	1.80	10.29	0.00	1.87	10.36	0.00	1.95	10.44	0.00	1.84	10.34	0.00	1.78	10.28
Limestone	1.22	1.22	1.22	1.32	1.32	1.32	1.39	1.39	1.39	5.89	5.89	5.89	8.18	8.19	8.19
Dicalcium phosphate	1.93	1.90	1.70	1.81	1.78	1.59	1.77	1.73	1.54	1.92	1.88	1.69	2.02	1.99	1.79
Salt	0.42	0.42	0.42	0.39	0.40	0.40	0.39	0.39	0.39	0.42	0.42	0.42	0.42	0.42	0.42
DL-Met	0.17	0.17	0.18	0.14	0.15	0.16	0.13	0.13	0.15	0.16	0.17	0.19	0.25	0.26	0.28
L-Lys	0.12	0.12	0.12	0.05	0.05	0.06	0.09	0.09	0.10	0.00	0.00	0.00	0.00	0.00	0.00
Sand	0.00	1.00	1.00	0.00	1.00	1.50	0.00	1.00	1.50	0.00	0.00	0.00	0.00	0.00	0.00
Vitamin E[2]	0.10	0.10	0.10	0.10	0.10	0.10	0.10	0.10	0.10	0.10	0.10	0.10	0.10	0.10	0.10
Vitamin premix[3]	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25
Mineral premix[4]	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25
Calculated content															
ME (kcal/kg)	3,000	3,000	3,000	3,020	3,020	3,020	3,070	3,070	3,070	2,970	2,970	2,970	2,900	2,900	2,900
CP (%)	20.00	20.00	20.09	18.00	18.00	18.00	16.00	16.00	16.00	17.00	17.00	17.00	17.50	17.50	17.50
Ca (%)	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	2.75	2.75	2.75	3.65	3.65	3.65
Available P (%)	0.50	0.50	0.50	0.47	0.47	0.47	0.45	0.45	0.45	0.48	0.48	0.48	0.50	0.50	0.50
Ether extract (%)	6.16	6.94	10.56	6.16	6.99	10.62	6.01	7.07	10.69	6.33	6.99	10.62	6.62	6.94	10.57
n-6 PUFA[6] (%)	3.00	3.00	3.00	2.99	3.00	3.00	2.88	3.00	3.00	3.07	3.00	3.00	3.24	3.00	3.00
n-3 PUFA (%)	0.29	0.50	1.15	0.28	0.50	1.50	0.25	0.50	1.50	0.29	0.50	1.50	0.32	0.50	1.50
n-6:n-3 ratio	10	6	2	10	6	2	9.12	6	2	10	6	2	10	6	2

[1]10 = 3%:0.29%; 6 = 3%:0.5%; and 2 = 3%:1.5%.

[2]Provided per kilogram of diet: α-tocopherol acetate, 46 IU.

[3]Provided per kilogram of diet: retinyl acetate, 13,200 IU; cholecalciferol, 5,720 IU; α-tocopherol acetate, 17.6 mg; riboflavin, 13.2 mg; pantothenic acid, 18.7 mg; niacin, 55 mg; folic acid, 0.44 mg; vitamin B_{12}, 0.022 mg.

[4]Provided per kilogram of diet: Mn, 140 mg; Cu, 12 mg; Fe, 79 mg; Zn, 140 mg; Se, 0.08 mg; I, 0.37 mg; Ca, 133 mg.

[5]Optivite International Ltd. (Nottinghamshire, UK). Contained salmon fish oil, 50%; ether extract, 50%; n-6, 6%; n-3, 24%; CP, 5%; ME, 5,250 kcal/kg; and vitamin E, 2,500 mg/kg.

[6]PUFA = polyunsaturated fatty acid.

Table 2. Feed intake and BW of pullets fed diets with n-6:n-3 ratios[1] of 10, 6, and 2 from 0 to 18 wk of age

Variable	1 3 wk	4 6 wk	7 9 wk	10 12 wk	13 15 wk	16 18 wk
Feed intake (g/d per chick)						
10	15.2	24.7[ab]	44.5	51.5	56.6	60.9[ab]
6	15.3	24.1[b]	45.8	50.9	54.1	58.7[b]
2	15.2	25.7[a]	45.6	50.1	56.6	62.9[a]
±SEM	0.18	0.39	0.8	1.66	1.39	0.98
P-value	0.829	0.028	0.449	0.84	0.369	0.031
BW[2] (g)						
10	151	405[ab]	674	869	1,107[a]	1,221[a]
6	155	410[a]	694	905	1,038[ab]	1,191[ab]
2	148	391[b]	658	858	1,010[b]	1,148[b]
±SEM	2.71	5.09	12.91	15.99	21.96	15.2
P-value	0.208	0.048	0.18	0.132	0.019	0.013

[a,b]Means within a column and variable with no common superscript differ significantly.

[1]10 = 3%:0.29%; 6 = 3%:0.5%; and 2 = 3%:1.5%.

[2]Body weight measured at end of each 3 wk of age.

1:2,048. Titration was assessed on the same day and using 96-well microtiter U-shape plates.

Statistical Analysis

The data were analyzed by 1-way ANOVA using the GLM procedure of SAS software (SAS Institute Inc., 2001). The means for treatments showing significant differences in the ANOVA were compared using Tukey test at $P < 0.05$ (Tukey, 1953).

RESULTS

Pullet Performance

Dietary effects on pullet performance are shown in Tables 2 and 3. Feed intake and BW changed significantly as a result of increase in dietary n-6:n-3 ratio (Table 2). The feed intake of pullets fed diet with the ratio of 2 (highest n-3 level) increased during 4 to 6 and 16 to 18 wk of age, whereas the BW decreased at the end of 6, 15, and 18 wk of age ($P < 0.05$). In addition, the BW was higher in birds fed diet with n-6:n-3 ratio of 6 at 6 wk and higher in birds fed diet with ratio of 10 (lowest n-3 level) at 15 and 18 wk of age ($P < 0.05$).

The combination of higher feed consumption and lower BW gain in pullets fed diet with ratio of 2 resulted in a significant increase in feed conversion of these chicks during the growing period (Table 3). Dietary n-6:n-3 ratio did not affect early egg production; however, the percentage eggs produced decreased in hens fed diet with n-6:n-3 ratio of 2 during 21 wk of age ($P = 0.145$).

Immunological Responses

Effect of different dietary n-6:n-3 ratios on Ab production against AI, ND, IB, and IBD are shown in Table 4. A decrease in dietary n-6:n-3 ratio influenced Ab production against AI vaccine at 10 and 14 wk, whereas the ND vaccine titer was significantly affected when determined at 14 wk of age. A higher Ab production was observed not only for AI vaccine but also for ND vaccine in pullets fed diet with n-6:n-3 ratio of 10 at 14 wk of age. Pullets fed diet with ratio of 2 had a greater Ab production for IB vaccine titer (3.24) than those fed diets with low and intermediate levels of n-3 (2.919 and 2.714, respectively; $P = 0.041$) measured at 4 wk of age. Significantly higher IB and IBD vaccines titers were also achieved in pullets fed diet with ratio of 6 relative to those fed diets with ratios of 10 or 2 ($P = 0.026$) at 14 wk of age.

The Ab response data against SRBC as measured by total and IgG levels is shown in Table 5. Diet with ratio

Table 3. Growth and laying performance of pullets fed diets with n-6:n-3 ratios of 10, 6, and 2 from 0 to 22 wk of age

Dietary n-6:n-3 ratio[1]	Feed intake[2] (g)	BW gain[2] (g)	Feed conversion ratio[2]	Egg weight[3] (g)	Days from PS to SM[4]	Egg production (%) 21 wk	Egg production (%) 22 wk
10	5,324	1,181[a]	4.5[b]	43.67	12.2	50.95	77.12
6	5,235	1,150[ab]	4.55[b]	42.01	14.5	48.07	78.9
2	5,384	1,108[b]	4.86[a]	39.72	14.8	34.04	64.66
±SEM	72.91	15.18	0.05	1.53	1.16	6.06	5.86
P-value	0.371	0.013	0.001	0.202	0.269	0.145	0.202

[a,b]Means within a column with no common superscript differ significantly.

[1]10 = 3%:0.29%; 6 = 3%:0.5%; and 2 = 3%:1.5%.

[2]From 1 to 18 wk of age (growing period).

[3]Egg weight at sexual maturity.

[4]Days from photostimulation to sexual maturity.

Table 4. Effect of dietary n-6:n-3 ratios[1] (10, 6, and 2) on antibody production against infectious bronchitis (IB), infectious bursal disease (IBD), Newcastle disease (ND), and avian influenza (AI) vaccine

	Dietary n-6:n-3 ratio				
Item	10	6	2	±SEM	*P*-value
IB vaccine titers (log_{10})					
4 wk	2.919[ab]	2.714[b]	3.243[a]	0.131	0.041
6 wk	3.571	3.357	3.449	0.1	0.348
10 wk	3.405	3.545	3.437	0.08	0.743
14 wk	3.061[ab]	3.39[a]	2.909[b]	0.113	0.026
18 wk	4.135	4.111	4.113	0.015	0.512
IBD vaccine titers (log_{10})					
4 wk	3.344	3.303	3.342	0.053	0.834
6 wk	3.654	3.671	3.667	0.026	0.881
10 wk	3.78	3.8	3.748	0.024	0.323
14 wk	3.759[ab]	3.828[a]	3.685[b]	0.031	0.009
18 wk	ND[2]	ND	ND		
ND vaccine titers (log_2)					
4 wk	7.8	8	8.5	0.49	0.625
6 wk	9.6	9.5	10.1	0.29	0.464
10 wk	8.6	8.1	9	0.39	0.491
14 wk	8.2[a]	8.1[ab]	7.6[b]	0.35	0.045
18 wk	11.1	10.6	11.1	0.34	0.223
AI vaccine titers (log_2)					
4 wk	ND	ND	ND		
6 wk	6.6	7.1	6.8	0.73	0.888
10 wk	8.3[a]	7.1[b]	7.5[b]	0.25	<0.001
14 wk	8.3[a]	7.1[b]	7.3[b]	0.28	<0.001
18 wk	8.1	6.6	7.3	0.41	0.065

[a,b]Means within a row and trait with no common superscript differ significantly.

[1]10 = 3%:0.29%; 6 = 3%:0.5%; and 2 = 3%:1.5%.

[2]ND = not determined.

of 2 did not exhibit a significantly different Ab production compared with diets with ratios of 10 or 6 at any time during the trial.

Carcass and Reproductive Organs

Body weight, carcass, and reproductive organs of birds at sexual maturity are shown in Table 6. The BW of pullets at maturity did not differ significantly as the dietary n-6:n-3 ratios changed. The weight of spleen and liver and the relative weight of breast, thigh, abdominal fat pad, heart, gizzard, and gut were not influenced by n-6:n-3 ratio. Dietary n-6:n-3 ratio of 10 numerically increased pullet oviduct weight ($P = 0.094$) at first oviposition. However, a dietary n-6:n-3 ratio of 2 significantly decreased ovary weight and number of LYF ($P < 0.05$).

Femur Parameters

The results of the femur bone parameters are shown in Table 7. Long-term dietary PUFA supplementation with various n-6:n-3 ratios did not have a pronounced effect on femur bone length, weight, ash, Ca, and P content of birds at first oviposition ($P > 0.05$).

DISCUSSION

Our study revealed that the high fish oil diet increased feed intake and decreased BW of pullets ($P <$

Table 5. Effect of dietary n-6:n-3 ratio on total anti-SRBC and IgG antibody titers at primary and secondary injection

	Days after primary injection				Days after secondary injection			
	7		14		7		14	
Dietary n-6:n-3 ratio[1]	Total anti-SRBC	IgG	Total anti-SRBC	IgG	Total anti-SRBC	IgG	Total anti-SRBC	IgG
10	8.8	6.1	6.8	4.6	10	6.8	7.6	5
6	10.6	7.6	6.1	4.3	10	6	6.6	4.5
2	11.1	8.1	6.4	4.2	8.6	7.3	6.5	4.3
±SEM	0.86	0.64	0.52	0.41	0.87	0.61	0.55	0.33
P-value	0.166	0.104	0.668	0.258	0.43	0.329	0.157	0.306

[1]10 = 3%:0.29%, 6 = 3%:0.5%, and 2 = 3%:1.5%.

Table 6. Carcass and reproductive organs of pullets at first lay fed diets with n-6:n-3 ratios[1] of 10, 6, and 2 from 0 to 22 wk of age

	Dietary n-6:n-3 ratio				
Variable	10	6	2	±SEM	*P*-value
BW at first egg (g)	1,263	1,331	1,281	75.82	0.843
Breast (% of BW)	14.68	16.04	14.45	0.81	0.379
Thigh (% of BW)	15.87	17.37	16.57	0.51	0.228
Abdominal fat pad[2] (% of BW)	4.14	4.2	4.37	0.41	0.904
Heart (% of BW)	0.33	0.33	0.34	0.013	0.631
Gizzard (% of BW)	1.69	1.71	1.88	0.074	0.152
Gut[3] (% of BW)	4.34	4.7	4.85	0.29	0.146
Spleen weight (g)	2.65	1.92	1.47	0.33	0.141
Liver weight (g)	24.71	23.11	23.47	1.4	0.751
Oviduct weight (g)	55.07	45.61	39.39	4.03	0.094
Ovary weight (g)	37.03[a]	28.18[ab]	19.04[b]	4.12	0.01
Stroma weight[4] (g)	4.13	3.5	3.63	0.65	0.809
SLF[5] (no.)	5	4.33	3.66	1.03	0.706
LYF[6] (no.)	7.6[a]	4.5[b]	5.42[b]	0.71	0.049
F1 weight[7] (g)	6.52	8	7.86	0.58	0.209

[a,b]Means within a row and trait with no common superscript differ significantly.

[1]10 = 3%:0.29%; 6 = 3%:0.5%; and 2 = 3%:1.5%.

[2]Abdominal fat = fat surrounding the gizzard and proventriculus.

[3]Gut = without gizzard and liver weight.

[4]Stroma = ovary with the LYF removed.

[5]SLF = small yellow follicles (5 10 mm diameter).

[6]LYF = large yellow follicles (>10 mm diameter).

[7]F1 = LYF on the ovary with the highest weight.

0.05). Fritsche et al. (1991b) reported that feeding pullets diet containing 7% fish oil promoted growth. This discrepancy may be attributable to the high level of wheat bran (10%) used in our diet to adjust the energy. Parsons et al. (1983) reported that the level of dietary fiber significantly increased amino acids excretion. The insoluble fiber acts primarily through the nutrient dilutions, increasing digesta passage rate and reducing nutrient digestibility (Hetland et al., 2004). In general, birds counteract these negative effects by an increase in feed intake, as was marginally observed in birds fed diet containing 1.5% n-3 and 10% wheat bran. Similarly, Guo et al. (2004) have reported that diet containing 5% fish oil did not significantly influence egg production of hens.

This study supports previous findings in pullets (Puthpongsiriporn and Scheideler, 2005) and turkeys (Friedman and Sklan, 1997) that the immune responses developing after vaccination may be augmented by diets supplemented with PUFA. All of the experimental diets in the present study were formulated to have 3% of n-6. Our results corroborate with Friedman and Sklan (1995) who revealed that the inclusion of about 2 and 4% n-6 in diet increased humoral immune response of broilers more than supplementation with 6 and 7% n-6. These data and the present study indicated that the dietary n-6 levels of 4 and 3% may have a positive effect on Ab responses. Possibly, the higher contents of long-chain PUFA result in a noneicosanoid-related phenomenon that may be attributable to membrane phospholipid fluidity or oxidative damage (Yaqoob and Calder, 1993). These are in parallels with findings that showed an increase in PUFA intake inhibited immune response in general (Calder et al., 1992) and Ab production in particular (Friend et al., 1980). Our study indicated that decreasing dietary n-6:n-3 ratio decreased Ab production. However, Puthpongsiriporn and Scheideler (2005) reported that dietary n-6:n-3 ratio of 2 increased ND vaccine titers in birds, but not in those fed diet with ratio of 10 in the present study. We suggest that the n-3 and n-6 levels should be balanced to meet suitable Ab responses. Immune response

Table 7. Femur bone parameters at first oviposition of pullets fed diets with n-6:n-3 ratios of 10, 6, and 2

Dietary n-6:n-3 ratio[1]	Femur length (mm)	Femur weight (g)	Relative weight[2] (%)	Ash[3] (%)	Ca[3] (%)	P[3] (%)
10	107	4.25	0.33	55.34	21.8	9.07
6	108	4.46	0.33	54.19	23.87	8.62
2	105	4.14	0.32	54.57	22.22	5.53
±SEM	1.3	0.18	0.01	1.08	3.4	0.48
P-value	0.354	0.503	0.565	0.795	0.848	0.717

[1]10 = 3%:0.29%; 6 = 3%:0.5%; and 2 = 3%:1.5%.

[2]Femur weight/BW × 100.

[3]Percentage of dry weight.

as measured by Ab-dependent cell cytotoxicity of splenocytes was decreased in broilers fed 7% fish oil (Fritsche and Cassity, 1992). On the other hand, Korver and Klasing (1997) demonstrated that the 2% fish oil diet increased Ab-dependent cell cytotoxicity. Thus, similar to our result, the high levels of fish oil apparently have more immunosuppressive effects than lower levels. The present data indicated that the efficacy of commercial vaccines, as determined by Ab production, can be augmented by the alteration of diet. In chickens, diets high in n-3 PUFA were reported to either stimulate (Fritsche et al., 1991b) or have no effect on Ab responses of SRBC (Fritcshe and Cassity, 1992; Phetteplace and Watkins, 1992) and reduce proliferative responses to mitogens (Wang et al., 2000). In our study the Ab response to SRBC was not as effecive as that for vaccine titers (IB and IBD). Quantification of Ab titers via ELISA is more sensitive than agglutination assays to SRBC (Parmentier et al., 1997). Other possibilities may be the time of sampling and route of inoculation for each injection (Boa-Amponsem et al., 2006) or concentration of SRBC-inoculated chicks (Praharaj et al., 1997). Unlike the Fritsche et al. (1991b) study with pullet chicks, the pullets in our study did not show significant differences in their primary or secondary humoral responses across treatments. The absence of a dietary n-3 effect on humoral response may in part be attributable to the chicks that were 3 wk older at the time of challenge in our experiment compared with that reported by Fritsche et al. (1991b). Furthermore, both the concentration and the volume of the SRBC differed and, finally, the 3-wk interval between the first and second injections may eliminate the significant difference at secondary response.

To our knowledge, no studies have been conducted concerning the effects of different dietary n-6:n-3 ratios on reproductive physiology of poultry. In fact, in the current trial the oviduct and ovary weight and number of LYF in pullets were negatively affected by the low n-6:n-3 ratio at the commence of egg production. These decreases may be a result of hormonal changes. In laying hens, corn oil (rich in n-6) and fish oil (rich in n-3) resulted in a significant increase and decrease, respectively, in PGE_2 synthesis by peripheral leukocytes (Guo et al., 2004). In addition, Whitehead et al. (1993) observed that the inclusion of fish oil decreased estrogen levels compared with corn oil (297 vs. 386 pg/mL). A positive relationship between PGF and estrogen concentrations in the uterus of hens has been demonstrated (Takahashi et al., 2004). Therefore, it is likely that feeding pullets diet containing n-6:n-3 ratio of 2 during the growing period decreased reproductive organs weight and yield at sexual maturity because of PGF and decrease in estrogen level. No effect was found of dietary n-6:n-3 ratio on bone mineralization in our study, which is in contrast with other studies (Liu et al., 2003a,b) that found that bone ash, mineral content, Ca, and P were increased in quails fed diet with 5% fish oil.

In conclusion, the present investigation demonstrated that various n-6:n-3 ratios in diets of pullet chicks did not affect bone or reproductive organs except ovary weight and LYF number, which was significantly decreased as the n-6:n-3 ratio decreased in diets. It is also revealed that supplementing Optomega-50 as an n-3 source to decrease the n-6:n-3 ratio in diet significantly increased Ab production for ND, IB, and IBD in pullet chicks. Thus, further studies are suggested to reduce the negative effects of high dietary fish oil on immune and reproductive systems of pullets.

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