ISSN 1682-8356
ansinet.org/ijps

INTERNATIONAL JOURNAL OF

POULTRY SCIENCE

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Mob: +92 300 3008585, Fax: +92 41 8815544
E-mail: editorijps@gmail.com

International Journal of Poultry Science 4 (9): 701-708, 2005
ISSN 1682-8356

Effect of Dietary Protein on Egg Production and Immunity Responses of Laying Hens During Peak Production Period

Chaiyapoom Bunchasak[1], Kanokan Poosuwan[1], Rattana Nukraew[1],
Kanchana Markvichitr[1] and Apassara Choothesa[2]
[1]Department of Animal Science, Faculty of Agriculture, [2]Department of Physiology, Faculty of Veterinary,
Kasetsart University, Bangkok, Thailand, 10900

Abstract: The study was completely randomized design. Six hundred and seventy two commercial laying hens (Babcock B-308) from 21 to 33 weeks of age were used. The hens were divided into 3 groups; each group consisted with 6 replications of sixteen layers each, and then two hens were kept in a multiple-cage located in evaporative cooling house system. Feed and water were offered *ad libitum*. According to the experimental groups, 3 levels of dietary protein (14, 16 and 18% CP) with similar energy content (2,750 ME kcal/kg) were given to the hens in order to investigate effects of dietary protein on their production performances, liver triglyceride, serum non-esterified fatty acid (NEFA) and immunity responses during peak production period. The results showed that hens received 14% CP diet had significantly poorer in production performances than the 16 and 18% CP groups. Liver weight of hens fed 14% CP diet was smaller than those of 16 and 18% CP diets ($P<0.05$). However, the protein conversion ration was significantly improved as decrease of protein consumption ($P<0.01$), while feed intake was not significantly affected by dietary protein levels. There were tendencies of increase of liver triglyceride and NEFA due to high protein consumption. For the immunological aspect, Newcastle disease (ND) titre of hens fed 18% CP diet was significantly higher than those of hens fed 16 and 18% CP diets ($P<0.05$). Except alpha-globulin and ratio of albumin : globulin that tended to decline, all serum protein fractions and serum total protein were tended to increase as protein levels increased. Spleen size was not affected by dietary protein levels.

Key words: Dietary protein, production performance, non-esterified fatty acids, immunity responses

Introduction

Thailand is located in the tropical zone. During summer season, the temperature is around 33-40°C. This high temperature can normally induce heat stress and loss of resistance to infectious diseases. Although the raising laying hens in Thailand has been commercialized and evaporative cooling system are commonly implemented in order to minimize those problems. However, in general, hens produce high egg production need more nutrients consumption and susceptible to alternation of the environments. Thus, nutrient and environment fluctuations during peak production period may have serious implications on defensive system and nutrients metabolism of the hens. Inappropriate amount of nutrients consumption can modulate quantitative and qualitative aspects of the immune response to pathogens. In poultry, it has been shown that deficiency or excess of dietary protein (Glick *et al*., 1981, 1983; Payne *et al*., 1990) or amino acids (Bhargava *et al*., 1970; Tsiagbe *et al*., 1987a,b) changes immune responses. Protein deficiency inhibited antibody production and the development of antibody-producing cells in response to T-dependent antigens (Carlomagno *et al*., 1980). Glick *et al*. (1983) showed that diet deficient in protein (33% of requirement) could reduce numbers of lymphocytes in the thymus of chickens. However, the responses are varied by strain (Manzoor *et al*., 2003), environment, stress, production state and health status. Thus, protective immune responses require a supply of nutrients at the appropriate times and amounts (Humphrey *et al*., 2002). Regarding to the measurement of protein induced immune and lipid metabolism alteration, appropriate hematological, immunological tests, liver triglyceride and NEFA in serum have been recommended (Margaret, 2001 and Poosuwan and Bunchasak, 2004). The present study, therefore, was designed to assess the effect of various dietary protein levels on production performance, liver triglyceride, serum NEFA and serum protein fractions of commercial hens during peak production period. Antibody response to ND vaccine was measured to assess humoral-mediated immunity, while the serum protein fractions were used as sensitive indicators of protein deficiency responses relevant to immune function.

Materials and Methods

Hens and diets: The study was designed with completely randomized design. Six hundred and seventy two commercial laying hens (Babcock B-308) from 21 to 33 weeks of age were used. The hens were divided into 3 groups; each group consisted with 6 replications of

Table 1: Composition of experimental diets

Ingredients (%)	14% CP	16% CP	18% CP
Corn	67.16	65.57	62.63
Soybean meal (44% CP)	11.71	18.39	20.21
Fish meal (58% CP)	5.00	5.00	7.65
Extracted rice brand	5.72	0.77	-
L-lysine	0.11	-	-
Monocalciumphosphate (P 21%)	0.95	0.96	0.55
Oyster shell	8.61	8.57	8.27
Salt	0.24	0.24	0.19
[1/]Premix	0.50	0.50	0.50
Total (kg)	100.00	100.00	100.00
Crude Protein (%)	14.00	15.00	16.00
Metabolizable energy (kcal/kg)	2750	2750	2750

[1/]Premix: Lutavit® Mix CNK004 consist of Vitamin A 4.80 MIU; D_3 0.96 MIU; E 3.20 g; K_3 0.80 g; B_1 0.40 g; B_2 1.60 g; B_6 1.20 g; B_{12} 0.004 g; Pantothenic acid 3.80 g; Niacin 6 g; Folic acid 0.20 g; Biotin 0.036 g; Se 0.04 g; Fe 24 g; Mn 24 g Zn 16 g; Cu 2.40 g; I 0.14 g; Feed preservative substance 2.50 g; Flavor 10 g and carrier added to 1.00 kg premix.

sixteen layers each, and then hens were kept in multiple-cage located in evaporative cooling house system. Environmental temperature and relative humidity were set around 27-29°C and 75-80%, respectively. Cage dimensions were 41 cm × 46 cm equaling 1,886 cm^2 total floor space. With 2 hens per cage, each bird had approximately 943 cm^2 of floor space. The lighting period was provided for 16 hours from 05:00 to 21:00 daily. Water and feed in mash form were supplied *ad libitum* throughout the experiment.

To test the effects of dietary protein, three dietary protein levels (14, 16 and 18% CP) were given to the hens from 21 to 33 weeks of age. According to the suggestion of Poeikhampha and Bunchasak (2004), the metabolizable energy of each experimental diet was 2,750 kcal/kg diets. To eliminate the influence of methionine and amino acid pattern (relative to lysine), ratio of crude protein to methionine of each experimental group was kept at 51.62 and 0.11% of synthetic L-lysine was supplemented to the 14% CP diet.

Other nutrients in diets were adjusted according to the recommendation of NRC (1994) (Table 1 and 2). Feed samples were collected and subsequently ground using a 1-mm screen in grinder. All diets were analyzed for protein, fat, fiber, ash, calcium and phosphorus according to the AOAC (1990) methods. Gross energy contents in diets were determined by bomb-calorimeter. Amino acids composition of feedstuff and experimental diets were determined using Near-Infrared Spectroscopy (NIR) (Jianguo *et al*., 2002).

Measurements: Hen-day egg production and feed intake were recorded daily whereas egg weight were determined 4 weeks interval (3 periods), regularly in the same day of period. The hens were weighed at the beginning and final date of the experiment. Egg mass was calculated by multiplying egg weight by hen-day egg production percentage. Feed conversion ratio (FCR) and protein conversion ratio was calculated as gram feed and protein per day per hen divided by gram egg mass per day per hen, respectively.

At the end of experiment, after overnight feed deprivation, one hen from each replication that had body weight closed to the replicate mean were chosen, 30 ml of blood were taken from wing vein. Subsequently, the hens were killed to evaluate the size of spleen and liver. The livers and serum were stored at -20°C until the chemical analysis.

Sample preparation and chemical analysis: Serum was separated from the 30 ml of blood samples by centrifugation at 3,000 rpm for 15 minutes. Then, serum was kept at -20°C before chemical analysis. Serum total protein was assayed using Biuret method (Test kit; Erba Diagnostics Mannheim GmbH). Amount and fractions of serum protein such as albumin, alpha-globulin, beta-globulin and gamma-globulin were investigated by Electrophoresis (cellulose acetate technique). Hemagglutination-Inhibition test (HI-test) was used to evaluate the level of ND-titre. Non-esterified fatty acid (NEFA) in serum was determined according to the description of Smith (1975).

Livers were retained for histopathological study. Determination of triglyceride content in the liver was prepared according to the method of Sutton *et al*. (1984). One gram of liver was extracted with 5-fold of acetone-ethanol (1:1 v/v) by homogenization at 20,000 rpm for 1 min. After the homogenization, the samples were centrifuged at 3,000 x g at room temperature for 10 min, and then supernatant was taken for triglyceride determinations using Enzymatic colorimetric method (Erba Diagnostics Manneim GmbH's test kit).

Statistical analysis: All data were statistically analyzed using analysis of variance (ANOVA). The differences between the means of groups were separated by

Table 2: Analytical nutrients composition in experimental diets

Nutrients	14%CP	16%CP	18%CP
Moisture (%)	10.44	10.70	10.53
Dry matter (%)	89.56	89.30	89.47
Fat (%)	2.65	2.99	2.80
Fiber (%)	2.89	2.90	2.89
Ash (%)	13.39	13.30	13.77
Gross energy (Kcal/Kg)	3546	3597	3424
Protein (%)	14.59	16.47	18.57
Calcium (%)	4.16	4.09	4.01
Total phosphorus (%)	0.77	0.76	0.71
Lysine (%)	0.81	0.86	1.01
Methionine (%)	0.26	0.29	0.33
Tryptophan (%)	0.15	0.18	0.20
Threonine (%)	0.53	0.61	0.69
Arginine (%)	0.85	1.00	1.13
Isoleucine (%)	0.54	0.64	0.72
Valine (%)	0.66	0.76	0.85
Leucine (%)	1.26	1.41	1.54
Total Branch chain amino acids (%)	2.46	2.81	3.11
Phenylalanine (%)	0.67	0.80	0.87
Histidine (%)	0.42	0.47	0.52

Duncan's Multiple Range Test (Duncan, 1955). Statements of statistical significance are based on $P<0.05$. All statistical analyses were done in accordance with the method of Steel and Torrie (1980).

Results and Discussion

Production performance and amino acids intake: Effect of dietary protein on production performances and amino acids consumption are presented in Table 4 and 5, respectively. Dietary protein levels did not significantly affect to egg production, but higher protein contents (16 and 18% CP) tend to have better percentage of egg production than the 14% CP. Egg mass of hens fed the 16 and 18% CP diets were significantly higher than the 14% CP group due to heavier egg weight ($P<0.05$). There was no effect of dietary protein levels on feed, energy consumptions and mortality rate of the hens. Essential amino acids and protein intake were linearly increased as increase of protein content in diets ($P<0.01$). However, feed intake was not affected by dietary protein levels.

The results were in agreement with general phenomena that egg production of hens received dietary high in both quality and quantity are commonly increased. Comparing recommendation of NRC (1994), hens fed 14% CP diet had deficient in protein, methionine, isoleucine, valine and tryptophan consumption, while all essential amino acids consumption in the 16 and 18% CP groups were meet or higher than hens' requirement. For that reason, production performance of 16% CP group was not different from the 18% CP group.

The study showed that decrease of dietary protein levels from 18 to 14% did not affect to feed intake of the hens. Accordingly, Humphrey and Klasing (2004), Keshavarz and Jackson (1992) and Penz and Jensen (1991) reported that poultry fed diets deficient in protein, lysine, methionine, threonine, or arginine did not show any characteristic depression in feed intake. In contrast, our recent study showed that hens fed with low CP diet (14% CP) had significantly lower feed intake than the 16% CP group (Bunchasak and Silpasorn, 2005). In addition, lowering feed intake of broiler chicks due to inadequate methionine consumption also has been reported by Bunchasak *et al.* (1996; 1997). Since the present study was conducted in an evaporative cooling system, while the previous study was conventional house system. Difference results of feed consumption may caused by the environmental differences.

Liver triglyceride, serum NEFA, spleen and ND titre: Liver weight, liver triglyceride, serum NEFA, spleen weight, ND titre and total serum protein are shown in Table 6. Hens fed 14% CP diet had smaller liver weight (expressed as % of body weight) than the 16 and 18% CP diets ($P<0.05$). Consequently, liver triglyceride (mg/liver) of hens fed 18% CP diet was significantly higher than those of 14 and 16% CP diets ($P<0.05$). It seems that liver triglyceride, NEFA, spleen weight and total serum protein were elevated as the increase of dietary protein levels, although significant differences were not found. Hens received 18% CP diet had significantly higher ND titer than the hens fed 14 and 16% CP diets ($P< 0.05$).

In avian species, fat synthesis and accumulation in the liver are mainly affected by hormone estrogen which synthesized from an ovary (Akiba *et al.*, 1982). In the 18%

Table 3: Analysis of chemical nutrients composition of feedstuff

	Corn	Soybean meal	Fish meal	Extracted rice bran
Moisture (%)	12.56	11.58	6.96	10.16
Protein (%)	7.73	43.55	58.55	15.01
Calcium (%)	0.02	0.31	5.98	0.10
Total phosphorus (%)	0.19	0.66	3.28	1.50
Ash (%)	1.20	6.22	24.28	11.0
Lysine (%)	0.24	2.64	4.07	0.69
Methionine (%)	0.16	0.58	1.51	0.20
Tryptophan (%)	0.06	0.60	0.58	0.12
Threonine (%)	0.28	1.68	2.25	0.50
Isoleucine (%)	0.25	1.96	2.22	0.59
Arginine (%)	0.38	3.16	3.32	1.07
Leucine (%)	0.93	3.29	3.87	0.97
Valine (%)	0.37	2.07	2.58	0.80

Table 4: Effects of dietary protein on production performances of laying hens during 21-33 weeks of age

	CP (%)			
	14	16	18	P-value
Final body weight (kg)	1700±44.3	1670±90.0	1643±6.6	0.77
Body weight change (g)	390±0.9	381±25.9	381±25.1	0.31
Egg production (%)	91.9±1.5	94.5±1.2	95.0±0.9	0.21
Egg weight (g)	56.72^{b}±0.5	58.96^{a}±0.8	59.34^{a}±0.5	0.03
Egg mass	51.8^{b}±0.7	54.9^{a}±1.1	56.1^{a}±0.5	0.008
Feed intake (g/hen/d)	110.1±0.7	110.7±0.7	111.2±0.7	0.55
Energy intake (kcal/hen/d)	302.72±1.9	304.43±2.0	305.84±1.9	0.55
Feed conversion (feed intake : egg mass)	2.12^{a}±0.03	2.02^{ab}±0.05	1.98^{b}±0.02	0.03
Protein conversion ratio (protein intake : egg mass)	0.29^{C}±003	0.32^{B}±0.01	0.35^{A}±0.01	0.001
Mortality (%)	0.33±0.31	0.11±0.03	0.27±0.19	0.526

[a, b, c] Means within a row with no common superscripts differ significantly ($p<0.05$). Values reported represent an average± SE.

CP group, increasing liver triglyceride content may be caused by high estrogen synthesis in ovary in order to support high egg production. However, the lipolysis (amount of NEFA) was tended to increase; this can be hypothesized that during peak production period, lipid turnover of high productive hens (18% CP group) may be higher than those gave lower egg productions. In addition, body weight was inversely related with the values of NEFA. This can be postulated that since feed and energy intake of all experimental groups were not significantly different, high lipolysis rate may be stimulated in order to supply energy for high egg production and triglyceride synthesis.

Spleen is identified as the secondary lymphoid tissue (Glick, 2000), while all plasma proteins, except immunoglobulins, are manufactured in the liver. Spleen size was not significantly affected by dietary treatments; this indicated that deprivation of protein level in diets from 18% to 14% did not give any harmful effect on the secondary lymphoid tissue, whereas higher protein intake may induce bigger liver size due to high protein synthesis (serum protein fractions) and fat synthesis (triglyceride). These phenomena are confirmed by reported of Bunchasak and Slipasorn (2005) and Bunchasak *et al.* (1996, 1997). Conversely, Payne *et al.* (1990) reported that deprivation of protein in chickens reduced numbers of lymphocytes in the circulation and the spleen. Kenney *et al.* (1968) studied effect of chronic protein deprivation on antibody forming capacity and reported that spleen size was decreased with fewer cells, less RNA and more DNA per cell, and amounts of circulating antibodies was also depressed. Moreover, Konashi *et al.* (2000) reported that amino acid deficiencies may preferentially affect cell-mediated immune responses relative to development of the lymphoid organs and antibody production in chickens. However, the results may be varied according to the duration of protein deprivation and the severity of the deficiency.

Moderate protein deficiency has been intensively examined for its effect on immune response and infectious disease resistance. Payne *et al.* (1990) reported that susceptibility to Newcastle disease virus was increased dramatically in protein-deprived chicks,

Table 5: Protein and amino acids intake of the hens

	CP (%)			P- value
	14	16	18	
	(g/hen/d)			
Protein	15.41^{C}±0.101	17.71^{B}±0.120	20.12^{A}±0.127	<0.001
Methionine	0.30^{C}±0.002	0.34^{B}±0.002	0.37^{A}±0.002	<0.001
Lysine	0.89^{C}±0.005	0.95^{B}±0.006	1.12^{A}±0.007	<0.001
Isoleucine	0.59^{C}±0.005	0.71^{B}±0.005	0.80^{A}±0.004	<0.001
Leucine	1.33^{C}±0.009	1.56^{B}±0.011	1.71^{A}±0.011	<0.001
Valine	0.73^{C}±0.005	0.84^{B}±0.006	0.94^{A}±0.006	<0.001
Branch-chain amino acids	2.65^{C}±0.017	3.11^{B}±0.021	3.45^{A}±0.022	<0.001
Threonine	0.58^{C}±0.003	0.67^{B}±0.004	0.77^{A}±0.005	<0.001
Arginine	0.93^{C}±0.006	1.11^{B}±0.007	1.26^{A}±0.008	<0.001
Histidine	0.46^{C}±0.003	0.52^{B}±0.004	0.58^{A}±0.004	<0.001
Phenylalanine	0.74^{C}±0.005	0.89^{B}±0.006	0.97^{A}±0.006	<0.001
Tryptophan	0.16^{C}±0.001	0.19^{B}±0.001	0.22^{A}±0.001	<0.001

$^{A, B, C}$ Means within a row with no common superscripts differ significantly ($p<0.01$). Values reported represent an average± SE.

Table 6: Effect of dietary protein levels on liver triglyceride, NEFA, ND titer and total protein in serum at 33 weeks of age

	CP (%)			P-value
	14	16	18	
Serum non-esterified fatty acid (micro mole/liter)	1210±211	1243±157	1338±177	0.84
Liver weight (% of body weight)	1.67^{b}±0.07	1.98^{a} ±0.10	2.02^{a} ±0.05	0.015
Liver triglyceride (mg/liver)	934 ^{a}±46.4	1049 ^{a}±89.2	1299 ^{b}±89.2	0.024
Liver triglyceride (mg/g liver)	32.7±2.6	33.8±2.2	35.4±1.0	0.288
Fatty degeneration score*	1	0	0	0.263
Spleen weight (% of body weight)	0.10±0.02	0.12±	0.14±	0.734
ND Titre (HI-test)	2.20^{b}±0.58	2.50^{b}±0.34	4.17^{a}±0.20	0.036
Total serum protein (mg/ml)	3.38 ± 0.02	3.55 ± 0.24	3.91 ± 0.14	0.209

^{a,b,c} Values within a row with different superscripts are significantly different ($p<0.05$). Fatty degeneration score; 0 = normal, 1 = mild, 2 = abundant. * Cochran-Mentel-Haenzel Statistic

the same as serum antiviral antibody responses were also reduced significantly. Similarly, the present study also showed that hens received high protein (18% CP) had significantly higher antibody titre of ND than the 14 and 16% CP diets. Reduction of this humoral immunity may be caused by the deficiencies of methionine, isoleucine, valine and tryptophan in the 14% CP group. Several investigators have reported the negative effects of inadequate essential amino acids in diets on immune responses. For example, Bhargava *et al.* (1970) and Mazija *et al.* (1982) showed that the shortage of valine (it makes 7% of poultry gamma-globuline), lysine, methionine or methionine + cysteine had significant negative effects on the immune response to ND vaccine (HI titre). Among amino acids deficiency, Konashi *et al.* (2000) reported that branch-chain amino acids (BCAA) had the greatest potential to modulate immune responses in chickens. In current study, although valine and isoleucine were insufficient for the requirement of hens fed 14% CP diet, but total BCCA was higher than hens' requirement due to high level of leucine. Therefore, we suggest that deficient in methionine may mainly affected to negative immunity responses of the hens. Likewise, it seems that amino acids requirement for egg production is lower than the requirement for humoral immune responses.

Serum protein fractions: Effect of dietary protein on serum albumin and globulin are presented in Table 7. Dietary protein levels did not significantly affect to amount of serum albumin. For globulin fractions, beta-globulin was highest in hens fed 18% CP diet ($P<0.05$). Gamma-globulin and total serum globulin were increased when protein levels in diets were increased (not significant). In contrast, hens fed 14% CP diet tend to have higher alpha-globulin than the hens fed 16 and 18% CP diets, whilst protein intake seems to reduce the ratio of albumin : globulin.

Grandhi *et al.* (1975) reported that serum protein profiles were varied with breed, strain and age; for example, the percentage of gamma-globulin fraction was greater in dwarf 1 wk. old and laying hens of the White Leghorn

Table 7: Effect of dietary protein levels on albumin and serum protein fractions of laying hens during 21-33 weeks of age

	CP (%)			P-value
	14	16	18	
Albumin (g/dl)	1.14±0.16	1.31±0.13	1.31±0.09	0.581
Alpha-globulin (g/dl)	0.55±0.02	0.48±0.09	0.48±0.07	0.790
Beta-globulin (g/dl)	1.74^{ab}±0.13	1.39^{b}±0.17	1.94^{a}±0.14	0.050
Gamma-globulin (g/dl)	0.05±0.01	0.10±0.05	0.12±0.06	0.740
Globulin (g/dl)	2.20±0.40	2.66±0.17	2.74±0.38	0.547
Albumin : Globulin	0.60±0.26	0.49±0.03	0.47±0.05	0.750

$^{a, b, c}$ Means within a row with no common superscripts differ significantly ($p<0.05$).
Values reported represent an average± SE.

breed when compared with the normal hens. However, the serum protein profiles in present study were in agreement with the report of Odunsi (2005) who showed that albumin, globulin and albumin : globulin of broiler chicks were around 1.08-1.32, 2.06-2.49 and 0.50-0.55, respectively.

In avian species, serum total proteins are consisted with albumin and globulins and these parameters are commonly used in nutritional studies. Since the quantities of essential amino acids and protein consumption of 18% CP group were higher than the requirements of brown laying hens (110 g/d of feed intake) that recommended by NRC (1994). Thus, the results of high serum proteins of this group are not surprised. Although Agbede and Aletor, (2003) have reported that total serum protein, albumin and globulin syntheses were not affected by sources of dietary protein (quality of protein). Current study agrees with Eggum, (1989) and Tewe, (1985) who stated that total serum protein, globulin and albumin were directly responsive to both protein quantity and quality.

Albumin is serves as the major reservoir of protein and involved in colloidal osmotic pressure, acid-base balance, and it acts as a transport carrier for small molecules such as vitamins, minerals, hormones and fatty acids (Margaret, 2001). The results of production performance that associated with serum albumin confirmed the general knowledge. The globulins are composed of three fractions, designated alpha, beta and gamma. In this study, serum beta and gamma-globulin were increased as increase of protein levels, while the result of alpha-globulin seems to be contrasted.

Alpha-globulins are a group of proteins manufactured almost entirely by the liver. Normally, these proteins increase with acute nephritis, severe active hepatitis, active, usually systemic inflammation, malnutrition and in nephrotic syndromes (Margaret, 2001). Thus, the tendency of increase of alpha-globulin in the 14% CP group indicated an incidence of malnutrition (deficient in protein and amino acids). The gamma-globulin fraction contains most of the immunoproteins, including IgM, IgA, IgE and IgG. These usually elevate with ongoing antigenic stimulation, usually from infectious agents (Margaret, 2001). Chao and Lee (2001) have examined the association between the level of serum gamma-globulin and reproductive performance or immunity of Taiwan Country chicken. They found that the high serum gamma-globulin level is genetically associated with low fertility. According to Pearson and Heron (1982) who reported that high protein intake induced low fertility and hatchability of broiler breeder hens. In agreement with our study, the data also showed that hens received 18% CP had highest level of the gamma-globulin.

Acknowledgements

The authors gratefully acknowledge that its primary funding comes through the National Center for Genetic Engineering and Biotechnology (BIOTEC), Thailand. We also appreciate the support of several staff from Department of Animal Science at Khamphengsean Campus, Kasetsart University.

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