

# **Effect of Amino Acids and Vitamin D on Performance and Biological Responses in Poultry**

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## **Scholarly Abstract**

As productive performance is improved by breed selection, amino acid requirements may change to support this higher performance in poultry. The first objective of this dissertation was to update the valine and tryptophan requirement of small-framed laying hens and the lysine requirement of young broilers using empirical dose-response methods. The tryptophan requirement was estimated as 155.8 mg/d for egg mass, 153.2 mg/d for egg production and 140.4 mg/d for feed conversion ratio using a linear broken line model. For valine, the requirement was highest for egg mass, 597.3 mg/d, followed by egg production, 591.9 mg/d and feed conversion ratio (FCR), 500.5 mg/d. The lysine requirement of young chicks was estimated by conducting four short term experiments from 1 to 3, 3 to 5, 5 to 8 and 8 to 11 days of age, respectively. The lysine requirement from 1 to 3, 3 to 5 and 5 to 8 days of age were not able to be estimated as no dose response was observed on growth performance most likely due to an overestimation of the lysine requirement. Digestible lysine requirement from 8 to 11 days of age was 1.057%, 1.050% and 1.016% based on body weight gain, FCR and pectoralis major weight using a linear broken line model, respectively. In addition to determining amino acid requirements, research was conducted to develop a new bacterial protein meal for use in laying hens diets. The data suggested that diets containing 7.5% of the bacterial protein meal was able to at least maintain egg production in laying hens, but 15% bacterial protein meal resulted in reduced performance.

The second objective of this dissertation was to investigate the effects of various concentrations of dietary vitamin D<sub>3</sub> on pullet and laying hen performance, eggshell quality and bone health in laying hens. Pullets/hens were randomly assigned to five dietary treatments

containing vitamin D<sub>3</sub> from 1,681 to 68,348 IU/kg diet from day of hatch until 68 weeks of age. These data suggested that dietary vitamin D<sub>3</sub> fed at 68,348 IU/kg resulted in reduced egg production, but vitamin D from 8,348 to 35,014 IU/kg diet maintained egg production, increased egg vitamin D content in a dose dependent manner, and generally increased both eggshell quality and pullet and hen bone mineral status.

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## **General Public Abstract**

The goal of the poultry industry is to increase the efficiency of meat and egg production. To achieve this goal, laying hens with higher egg production and broilers with faster growth rates are genetically selected over time. By breed selection, laying hens are able to produce 2-3 additional eggs every year. The body weight of a broiler chicken raised today is approximately four times greater than one raised to the same age in 1958. This increased egg production and body growth requires a higher nutrient intake, especially amino acids, to support protein production. One objective of this dissertation was to update the requirement of three amino acids (valine, tryptophan and lysine) in poultry production to provide current and accurate information to poultry producers. Valine, tryptophan and lysine are essential amino acids that cannot be synthesized by poultry in sufficient quantities and need to be ingested from the diet. Three experiments were conducted to determine the valine and tryptophan requirement in laying hens and lysine requirement in broilers. The results of the current experiment show that a laying hen requires at least 156 mg tryptophan and 597 mg valine per day to maximize egg production from 41 to 60 weeks of age. The broiler chicks need to ingest rations containing at least 1.06% lysine to support growth from 8 to 11 days of age.

Bacterial protein meal is a feed ingredient that has been proposed for use in poultry diets. It is usually produced via the fermentation process by converting various substrates such as methane, methanol, or agriculture by-products into protein-rich biomass. The advantage of using bacterial protein meal in the poultry industry is to decrease feed cost and alleviate the demand on croplands. A novel bacterial protein meal, generated from waste water purification, was

evaluated as a feedstuff for laying hens. Two levels of bacterial protein meal, 7.5 or 15%, were added to a regular laying hen diet to replace soybean. The results indicated that replacing soybean meal with 7.5% bacterial protein meal was a feasible solution for egg production but a 15% inclusion rate may result in a decreased egg production.

During egg production, bone structural health can be reduced as laying hens age. This loss of bone structural health is due to the loss of bone mineral content, especially calcium and phosphorus, as laying hens produce the calcium rich eggshell. With age, decreased bone mineral mass will induce a higher probability of bone structural failure. Vitamin D plays an important role on calcium absorption and bone mineral deposition. In addition to benefits to skeletal health, the addition of vitamin D<sub>3</sub> in the diet will result in increased vitamin D<sub>3</sub> content in eggs used for human consumption. An experiment was conducted to evaluate the use of high concentrations of vitamin D to increase egg vitamin D content, improve eggshell quality and increase hen skeletal health. The data suggest that adding vitamin D<sub>3</sub> from 8,300 to 35,000 IU/kg diet will increase egg vitamin D content, and generally improve eggshell and bone quality; however, adding vitamin D<sub>3</sub> at 68,000 IU/kg diet resulted in negative effects on pullet growth and subsequent egg production of adult hens.

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## Table of Contents

Chapter 1:	
Introduction.....	1
References .....	4
Chapter 2: Literature Review.....	7
2.1 Amino Acid.....	7
2.1.1 Introduction.....	7
2.1.2 Protein Digestion in Poultry.....	8
2.1.3 Amino Acid Absorption and Transportation.....	9
2.1.4 Methodology to Estimate Amino Acid Requirement.....	9
2.1.5 Tryptophan.....	11
2.1.5.1 Introduction.....	11
2.1.5.2 Tryptophan Functions.....	12
2.1.5.3 Tryptophan and Niacin.....	13
2.1.5.4 Tryptophan and Other Amino Acids.....	13
2.1.5.5 Tryptophan Requirement of Laying Hens.....	14
2.1.6 Valine.....	18
2.1.6.1 Introduction.....	18
2.1.6.2 Relationship between Valine and Other Amino Acids.....	19
2.1.6.3 Excessive and Deficient Valine Intake.....	19
2.1.6.4 Valine Requirement on Laying Hen.....	20
2.1.7 Lysine.....	23
2.1.7.1 Introduction .....	23
2.1.7.2 Catabolic Pathway of Lysine.....	23
2.1.7.3 Biological Function of Lysine.....	23
2.1.7.4 Lysine Transporters.....	25
2.1.7.5 Lysine as a Limiting Amino Acid in Poultry Feed.....	25
2.1.7.6 Lysine Deficiency and Toxicity.....	26
2.1.7.7 Lysine Requirement in Poultry.....	27
2.1.7.8 Interaction between Lys and Other Amino Acids.....	28
2.1.7.9 Phase Feeding in Broiler Production.....	29
2.2 Bacterial Protein Meal.....	30
2.2.1 Introduction.....	30
2.2.2 Nutritional Evaluation of Bacterial Protein Meal.....	30
2.2.3 Effect of Bacteria Protein Meal in Poultry Ration.....	31
2.2.4 Bacteria Protein Meal and Immunology.....	32
2.3 Vitamin D.....	35
2.3.1 Introduction.....	35
2.3.2 Vitamin D <sub>3</sub> Absorption.....	36
2.3.3 Mode of Vitamin D Action on Calcium Absorption.....	36
2.3.4 Mode of Vitamin D Action on Bone Homeostasis.....	38
2.3.5 Mode of Vitamin D Action on Kidney.....	38
2.3.6 Vitamin D Toxicity.....	39
2.3.7 Vitamin D <sub>3</sub> and Egg Shell Formation.....	39
2.3.8 Effect of Increased Dietary Vitamin D Supplementation in Poultry.....	40

References.....	42
Chapter 3: Evaluation of the Tryptophan Requirement of Small-framed First Cycle Laying Hens.....	
3.1 Abstract .....	61
3.2 Introduction .....	62
3.3 Materials and Methods .....	63
3.3.1 Diets.....	63
3.3.2 Animal and Housing.....	64
3.3.3 Statistical Analysis.....	65
3.4 Results and Discussion.....	67
3.4.1 Laying Hen Performance.....	67
3.4.2 Egg Quality and Composition.....	70
3.4.3 Plasma Serotonin Concentration.....	71
3.4.4 Tryptophan Requirement.....	71
References.....	75
Chapter 4: Evaluation of the Valine Requirement of Small-framed First Cycle Laying Hens.....	
4.1 Abstract .....	85
4.2 Introduction .....	86
4.3 Materials and Methods .....	87
4.3.1 Diets.....	87
4.3.2 Animal and Housing.....	87
4.3.3 Statistical Analysis.....	88
4.4 Results and Discussion.....	91
4.4.1 Laying Hen Performance.....	91
4.4.2 Egg Quality and Composition.....	94
4.4.3 Valine Requirement.....	95
References.....	100
Chapter 5: Evaluation of the Lysine Requirement in Young Broilers from 1 to 3, 3 to 5, 5 to 8 and 8 to 11 Day of Age.....	
5.1 Abstract .....	109
5.2 Introduction .....	110
5.3 Materials and Methods .....	111
5.3.1 Diets.....	111
5.3.2 Animal and Housing.....	112
5.3.3 Data and Sample Collection.....	113
5.3.4 Relative mRNA Abundance of Cationic Amino Acid Transporter.....	113
5.3.5 Plasma Urea Nitrogen.....	114
5.3.6 Statistical Analysis.....	114
5.4 Results and Discussion.....	115
References.....	121

Chapter 6: Investigation of the Nutritional and Immune Benefits of Feeding a Bacterial Protein Meal to Laying Hens.....	129
6.1 Abstract.....	129
6.2 Introduction.....	130
6.3 Materials and Methods .....	132
6.3.1 Bacterial Protein Meal.....	132
6.3.2 Diet.....	133
6.3.3 Animal and Housing.....	133
6.3.4 Data Collection.....	134
6.3.5 Statistical Analysis.....	135
6.4 Results and Discussion.....	135
6.4.1 Nutrients in bacterial protein meal.....	135
6.4.2 Laying Hen Performance.....	136
6.4.3 Egg Quality.....	138
6.4.4 Immune Response.....	138
6.5 Conclusions.....	139
References .....	140
Chapter 7: Effect of High Concentrations of Dietary Vitamin D <sub>3</sub> on Pullet and Laying Hen Performance, Skeleton Health, Eggshell Quality and Yolk Vitamin D <sub>3</sub> Content when Fed to W36 Laying Hens from Day of Hatch until 68 Weeks of Age.....	149
7.1 Abstract .....	149
7.2 Introduction .....	151
7.3 Materials and Methods .....	152
7.3.1 Diets.....	152
7.3.2 Animal and Housing.....	153
7.3.3 Data Collection.....	154
7.3.4 Dual x-ray Absorptiometry (DXA).....	155
7.3.5 Yolk Vitamin D <sub>3</sub> Concentration.....	155
7.3.6 Eggshell and Tibia Breaking Strength.....	155
7.3.7 Fat-free tibia as content.....	156
7.3.8 Keel Bone Score.....	156
7.3.9 Statistical Analysis.....	157
7.4 Results and Discussion.....	157
7.4.1 Pullet Growth Performance.....	157
7.4.2 Pullet Bone Quality.....	158
7.4.3 Laying Hen Performance.....	159
7.4.4 Egg Characteristics.....	160
7.4.5 Laying Hen Bone Quality.....	161
7.4.6 Egg Vitamin D <sub>3</sub> Concentration.....	162
References.....	164
Chapter 8: Epilogue.....	173
References.....	179

## List of Tables and Figures

Table 2.1 Tryptophan requirement summary of previous literatures in laying hens .....	17
Table 2.2 Valine requirement summary of previous literatures in laying hens.....	22
Table 2.3 Bacterial protein meal summary of previous literature in poultry research.....	34
Figure 2.1 vitamin D <sub>3</sub> synthesis from sunlight exposure. ....	34
Table 3.1 Composition of trp deficient basal diet. Tryptophan was added at 0.02% increments to generate experimental diets that contained 0.096, 0.116, 0.136, 0.156, 0.176, 0.196% trp.....	79
Table 3.2 Hen-housed egg production (HHEP), body weight (BW) loss, feed intake, egg weight, egg mass, feed conversion ratio (FCR) and trp daily intake from 41-60 wk of birds fed diets containing 0.096, 0.116, 0.136, 0.156, 0.176 and 0.196% trp respectively.....	80
Table 3.3 Egg quality, egg composition and plasma serotonin from 41-60 wk of birds fed diets containing 0.096, 0.116, 0.136, 0.156, 0.176 and 0.196% trp.....	81
Table 3.4 Trp requirement estimated from linear broken line, quadratic broken line, quadratic polynomial and exponential model based on hen-housed egg production (HHEP), egg mass and FCR.....	82
Figure 3.1 (a) feed intake, (b) hen-housed egg production (HHEP), and (c) body weight change from 41-60 wk of birds fed diets containing 0.096, 0.116, 0.136, 0.156, 0.176 and 0.196% trp respectively. Treatment 0.096% was halted after 45 weeks of age.....	83
Figure 3.2 Trp requirement estimation using linear broken line regression based on (a) hen housed egg production (HHEP) [ $y=80.86-0.4824(x-153.2)$ , $R^2=0.89$ ], (b) egg mass [ $y=49.29-0.2891(x-155.8)$ , $R^2=0.90$ ] and (c) feed conversion ratio (FCR) [ $y=1.988+0.01598(140.4-x)$ , $R^2=0.77$ ].....	84
Table 4.1 Composition of val-deficient basal diet. Valine was added at 0.07% increments to generate experimental diets that contained 0.515, 0.585, 0.655, 0.725, 0.795, 0.865% val.....	103
Table 4.2 Hen-housed egg production (HHEP), Body weight loss, feed intake (FI), egg weight (EW), egg mass (EM), feed conversion ratio (FCR) and val daily intake from 41-60 wk of birds fed diets containing 0.515, 0.585, 0.655, 0.725, 0.795 and 0.865% val respectively.....	104
Table 4.3 Egg quality and egg composition from 41-60 wk of birds fed diets containing 0.515, 0.585, 0.655, 0.725, 0.795 and 0.865% val.....	105
Table 4.4 Val requirement estimated from linear broken line, quadratic broken line, quadratic polynomial and exponential model based on hen-housed egg production (HHEP), egg mass and FCR.....	106
Figure 4.1 (a) feed intake, (b) hen-housed egg production (HHEP), and (c) body weight change from 41-60 wk of birds fed diets containing 0.515, 0.585, 0.655, 0.725, 0.795 and 0.865% val respectively.....	107
Figure 4.2 Val requirement estimation using linear broken line regression based on (a) hen housed egg production (HHEP) [ $y = 80.24 - 0.1222(x - 591.9)$ , $R^2=0.83$ ], (b) egg mass [ $y = 48.90 - 0.07885(x - 597.3)$ , $R^2=0.87$ ], and (c) feed conversion ratio (FCR) [ $y = 1.930 + 0.004247(x-500.3)$ , $R^2=0.62$ ].....	108
Table 5.1 Composition of lys deficient basal diet. Lysine was added at 0.11% increments to generate experimental diets that contained 0.83, 0.94, 1.05, 1.16, 1.27, 1.38, 1.49 and 1.60% digestible lys.....	124
Table 5.2 Effect of various dietary digestible lys concentrations on body weight gain (BWG) and body weight without yolk sac (BWY) in broiler from 1 to 3, 3 to 5, 5 to 8, and 8 to 11 days of age, respectively.....	125

Table 5.3 Effects of various dietary digestible lys concentrations on pooled (5 birds) pectoralis major absolute (AW), relative (RW) weight in broilers from 1 to 3, 3 to 5, 5 to 8, and 8 to 11 days of age, respectively.....	126
Table 5.4 Effect of various dietary digestible lys concentrations on plasma urea nitrogen (PUN) and b <sup>0,+</sup> AT relative jejunum mRNA abundance (b <sup>0,+</sup> AT) in broiler from 8 to 11 days of age..	127
Table 5.5 Digestible lys requirements as estimated by linear broken line regression analysis of 8 to 11 days of age broilers based on body weight gain (BWG), pectoralis major absolute weight (AW).....	128
Table 6.1 Bacterial protein meal (BPM) nutrients composition and amino acid digestibility....	143
Table 6.2 Nutrients composition of experimental laying hen diets containing no bacteria protein meal (BPM) (control), 7.5% BPM and 15% BPM, respectively.....	144
Table 6.3 Laying hen performances as hens fed diets containing no bacteria protein meal (BPM) (control), 7.5% BPM and 15% BPM, respectively, from 38 to 54 weeks of age.....	145
Table 6.4 Haugh unit, egg composition and shell thickness of eggs from hens fed diets containing no bacteria protein meal (BPM) (control), 7.5% BPM and 15% BPM, respectively.....	146
Figure 6.1 Feed intake (a), egg weight (b), body weight (c) of hens fed control, 7.5% and 15% BPM diets from 38 to 54 weeks of age.....	147
Figure 6.2 Egg yolk color (a), relative spleen weight (%) (b), blood heterophil/lymphocyte ratio (c) of laying hens fed diets containing no bacteria protein meal (BPM) (control), 7.5% BPM, 15% BPM at 54 weeks of age.....	148
Table 7.1 Pullet and laying hen basal diets for wk 0-4, wk 5-8, wk 9-12, wk13-15, wk 16-17, wk 18-24, and wk 25-68.....	167
Table 7.2 Body weight, bone mineral density and content of 17 wk old pullets fed diets containing 1,681, 8,348, 18,348, 35,014 and 68,348 IU of D <sub>3</sub> /kg of diet, respectively.....	168
Table 7.3 Hen-housed egg production (HHEP), feed intake, egg weight, egg mass, feed conversion ratio (FCR) from hens fed diets containing 1,681, 8,348, 18,348, 35,014 and 68,348 IU of D <sub>3</sub> /kg of diet, respectively, during first cycle laying phase.....	169
Table 7.4 Egg characteristics from hens fed diets containing 1,681, 8,348, 18,348, 35,014 and 68,348 IU of D <sub>3</sub> /kg of diet, respectively, during first cycle laying phase.....	170
Table 7.5 Bone qualities from hens fed diets containing 1,681, 8,348, 18,348, 35,014 and 68,348 IU of D <sub>3</sub> /kg of diet, respectively.....	171
Figure 7.1 Cholecalciferol concentrations and vitamin D <sub>3</sub> transfer efficiency of eggs from hens fed diets containing 1,681, 8,348, 18,348, 35,014 and 68,348 IU of D <sub>3</sub> /kg of diet, respectively.....	172

## Chapter 1

### Introduction

As breed selection has improved productive potential of laying hens, amino acid requirements change to support higher egg production (Applegate and Angel, 2014). Continuous updates on amino acid requirements are necessary to provide accurate information to commercial nutritionists for accurate dietary formulation. Besides methionine, lysine (lys) and threonine, valine (val) and tryptophan (trp) are the next limiting amino acids in typical corn-soybean meal (SBM) based poultry diets (Ishibashi 1985, Peganova and Eder 2003, Lelis, et al., 2014). Tryptophan is not only involved in protein synthesis but also the precursor of serotonin which is a neurotransmitter associated with mood, stress response, sleep and appetite regulation (Le Floc'h and Seve, 2007). Recent research on the trp requirement of commercial laying hens has been limited. The most recent research conducted by Calderano and coworkers (2012) reported a digestible trp requirement of 142 mg per hen per day based on feed conversion ratio data set. Valine is a branch chain amino acid and primarily functions as a building block for protein synthesis in animals. As a glucogenic amino acid, val also can contribute to glutamine synthesis. Research on the val requirement of commercial laying hens has been limited. A digestible val requirement of 567.0 mg/d was estimated for Dekalb Brown laying hens from 43 to 54 weeks of age by averaging results from quadratic linear broken line and quadratic broken line models (Lelis, et al., 2014). As production cost of feed grade val and trp decrease over time, have updated requirements become more important. One objective of this dissertation was to evaluate the val and trp requirement of small-frame first cycle laying hens (Chapter 3&4).

Besides evaluating val and trp requirements in laying hens, lys requirements in young broiler chicks were also estimated. Since lys requirement has been intensively studied in broiler,

we explored a novel method to evaluate lys requirement over 2 to 3 day period. Short term lys requirement would provide useful information to a phase feeding (PF) regimen. Phase feeding is division of a traditional feeding period into multiple periods using different formula or balanced diets to more closely follow the nutrient requirement over the age of the broilers. Previous studies suggested that using a PF regimen to replace traditional industry regimens were able to reduce amino acid intake and feed cost without sacrificing growth performance or carcass yield in broilers (Emmert and Baker, 1997; Warren and Emmert, 2000; Pope and Emmert, 2001; Pope, et al., 2002; Brewer, et al., 2012a; Brewer, et al., 2012b). Using a similar approach, an experiment was conducted to estimate the digestible lys requirement in young broilers from 1 to 3, 3 to 5, 5 to 8 and 8 to 11 day of age to provide an updated estimation of lys requirements over short time periods.

As limited cropland may not be able to produce the greater amount of grain needed for feed ingredient use for animal production, bacterial protein meal (BPM), whose production does not require cropland, may potentially be an alternate protein source. In broilers, research has indicated that adding BPM up to 10% of the diet could successfully replace soybean meal in the diet (Waldroup and Payne, 1974; Skrede et al. 2003). In laying hens, limited research has been conducted to evaluate the effect of BPM on laying hens performance. The only scientific report indicated that supplementation of BPM up to 10% in laying hen diet did not reduce egg production (Whittemore et al. 1978). An experiment was conducted using a novel BPM, derived from fermentation of wastewater, to replace soybean meal in laying hen diets and investigate the effect on hen productive performance and immune response.

It has been reported that vitamin D<sub>3</sub> content in eggs can be enriched by increasing vitamin D<sub>3</sub> concentration in laying hen diets (Mattila, et al., 2011; Yao, et al 2013). This provides a

feasible approach to increase vitamin D<sub>3</sub> intake in the human population without changing eating habits (Mattila, et al., 2004). Since vitamin D<sub>3</sub> plays an important role in calcium metabolism, the effect of increased vitamin D<sub>3</sub> concentration on bone health and eggshell quality has been studied. In this chapter, an experiment was performed that fed laying hens high vitamin D<sub>3</sub> diets from day of hatch until 68 weeks of age and investigated effects on egg production, egg vitamin D content, shell quality and bone mineral status in pullets and laying hens.

Overall, the objectives of this dissertation provided needed updates to the trp and val requirements of laying hens and provided initial data for a new more efficient approach to phase feeding, potentially allowing for diet cost savings by determining the lys requirements of young broiler chicks over a short time period. In addition to direct amino acid requirement data, an experimental source of protein was evaluated for use in laying hens diets. Finally, the supplementation of laying hens diets with high concentrations of vitamin D<sub>3</sub>, was evaluated using egg production, egg vitamin D<sub>3</sub> content, egg shell quality and pullet and hen bone quality as response criteria. This dissertation provided need information around amino acid and crude protein utilization in in poultry as well as documented the beneficial effects of high vitamin D feeding in laying hens.

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## Chapter 2: Literature Review

### 2.1 Amino Acid

#### 2.1.1 Introduction

Amino acids are the building blocks of proteins. The  $\alpha$ -amino acid consists of an  $\alpha$ -carbon, an amino acid group, a carboxylic acid group, a hydrogen atom and a R group.  $\alpha$ -carbon is in the central position and bond to other groups. The R group is the side chain group, which varies by amino acid. There are two chiral forms of amino acid: L isomer and D isomer. L isomers are the only biologically active form used to synthesize proteins, D isomers of certain amino acids can be utilized by animals by conversion to L isomers. For example, D-methionine can be converted to L-methionine through deamination and transamination with the assistance of D-amino acid oxidase and ubiquitous transaminases. The ionization state of an amino acid changes with pH, as the amino acid exists as a dipolar form (zwitterions) at neutral pH. In an acidic environment, the amino group is positively charged ( $\text{NH}_3^+$ ) and carboxyl group is not charged. In a basic environment, the carboxyl group is negatively charged and amino group is not charged. The R group determines the characteristic of the amino acid. The amino acid can be classified into four categories: 1) the side chain consists only of hydrogen and carbon are hydrophobic; 2) the polar amino acids with neutral R group but the charge is not evenly distributed; 3) positively charged amino acids with R groups that have a positive charge at physiological pH; 4) negatively charged amino acids with R groups that have a negative charge at physiological pH (Tymoczko, et al., 2013). Amino acids can be classified as essential amino acid, non-essential amino acid and conditionally essential. Amino acids that cannot be synthesized by animals in sufficient quantity are called essential amino acids. Essential amino acids need to be ingested from external sources such as the diet. In contrast, non-essential amino

acid can be synthesized and are not required in the diet specifically. Conditionally essential amino acids can be synthesized but if proper precursors are not available may not meet the requirement of animals. According to their catabolic pathway, amino acid can be grouped as ketogenic or glucogenic amino acids. Glucogenic amino acids are converted to substrates such as pyruvate and  $\alpha$ -ketoglutarate that can be oxidized to generate ATP. Ketogenic amino acids are broken down into acetoacetate or acetyl-CoA.

### 2.1.2 Protein Digestion in Poultry

The gastrointestinal tract of poultry includes the esophagus, crop, proventriculus, ventriculus, duodenum, jejunum, ileum, cecum, and cloaca. The initial digestion process of protein was described by Scott (1982). The digestion of protein starts in the proventriculus as feed stimulates the secretion of gastric secretion via vagus nerves of gastric mucosa. The ventriculus or the gizzard provides mechanical grinding of the digesta. Under the acid environment of the proventriculus and ventriculus, pepsinogen is activated to pepsin, the major proteolysis enzyme. The pepsin enzyme will hydrolyze specific peptides including amino acids such as leucine and valine, resulting in denaturation of the larger proteins. As the digesta passes into the duodenum, the acid released from the proventriculus is neutralized by bicarbonate and proteases secreted by the pancreas begin to break down protein. Trypsinogen, secreted by pancreas, is cleaved by endopeptidases released from duodenal enterocytes to form active trypsin. Trypsin catalyzes the hydrolysis of protein, especially around lys or arginine. Trypsin will further activate enzymes such as chymotrypsin, elastase and carboxypeptidase. Chymotrypsin is important for the hydrolysis of protein at the aromatic amino acids. Carboxypeptidase cleaves terminal bonds in a peptide chain to remove the amino acid residues sequentially. Peptidases

located on the epithelial brush borders degrade oligopeptides into free amino acids, dipeptides and tripeptides for absorption.

### 2.1.3 Amino Acid Absorption and Transportation

The absorption of amino acids and peptides occurs at the brush border of the epithelial cell. Amino acids are absorbed by enterocytes as free amino acids, dipeptides, tripeptides. Peptide absorption is most rapid in the jejunum and amino acid absorption is most rapid in ileum. Amino acid and peptide transporters located on the membrane of enterocytes are responsible for transportation. According to Broer (2008), there are three major transporter systems: the neutral system, basic system and acidic system. The neutral system is responsible for the transportation of neutral amino acids such as methionine and branch chain amino acids. The basic system is responsible for the transportation of cationic amino acids together with cysteine. The acidic system is to transport negatively charged amino acids such as glutamate and aspartate. Among these transporters, free amino acids are transported in a  $\text{Na}^+$  dependent or independent manner. In addition, dipeptides and tripeptides are transported by PepT1 which relies on the  $\text{H}^+$  gradient for transport. In epithelial cells, most of the dipeptides and tripeptides are degraded by cytoplasmic peptidases to free amino acids. The free amino acids and small peptides are then transported through epithelial basolateral side by transporters for further utilization.

### 2.1.4 Methodology to Estimate Amino Acid Requirement

The estimation of animal amino acid requirements are critical for the efficient production of animals. Several methods have been developed to evaluate amino acid requirements (Scott, 1982; Mavromichalis et al., 2001; Pond et al., 2005; Humayun, et al., 2006).

- 1) Growth assay. By supplementing various concentrations of amino acids in the diet, growth performance or production performance can be used as a response criterion to fit

certain regression models. The basal diet should be an amino acid deficient diet and as the amino acid level increases, performance will increase until a plateau is achieved. This method requires a longer timeframe and feeding experiments with animals to get the expected response.

2) Factorial method. This method uses modeling to estimate the amino acid requirements by considering amino acid loss and gain from a theoretical standpoint. Amino acid requirement = (requirement for growth or production + requirement for maintenance + requirement for synthesis of endogenous amino acid loss – endogenous amino acid loss synthesized from body)/true digestibility. As the factorial method estimated requirement is based on a mathematical calculation, the results from this method should be verified by direct animal verification responses.

3) Direct amino acid oxidation (DAAO): This method uses labeled amino acids in the diet or direct-labeled amino acids via vein injection. The basic premise is that the oxidation of the target amino acid should not increase until the requirement for protein synthesis is exceeded. After the amino acid concentration exceeds the requirement, extra amino acids would be oxidized and excreted as CO<sub>2</sub>. The excreted CO<sub>2</sub> can be collected and to measure the labeled CO<sub>2</sub>, to allow for the generation of a regression relationship between amino acid level and level of oxidation. The amino acid requirement is the point that the regression reaches a plateau. This method allows for a short-term methodology and is sensitive. The method requires that the labeled amino acid is an essential amino acid and will not have any other carbon disposal route other than CO<sub>2</sub>. If the target AA has a big AA pool, it will be harder to generate a requirement estimation.

4) Indicator amino acid oxidation (IAAO): This method uses an indicator amino acid rather than target amino acid as the tracer. As the amino acid concentration increases before the

requirement, the indicator amino acid oxidation level will decrease as more amino acids are used for protein synthesis. After the amino acid requirement is reached, the indicator amino acid oxidation will stabilize. This method has the same advantage as the DAAO method and an essential amino acid with a small pool, such as phenylalanine, can be used as an indicator giving this method more flexibility than DAAO.

5) Plasma amino acid concentration: Excessive amino acid in diet will lead to an increased amino acid concentration in plasma. By establishing the relationship between dietary amino acid concentration and plasma amino acid, a requirement can be estimated at the point the plasma amino acid concentration starts to increase. This method requires taking frequent samples as amino acid concentrations will be affected by feeding status. One drawback to this method is a difficulty to estimate amino acids with a low plasma concentration due to low sensitivity.

6) Plasma urea method. The plasma urea concentration in the animal is measured as the target amino acid concentration increases in the diet. As the amino acid increases from deficiency to adequate, more amino acids are used to synthesize protein and nitrogen excretion will decrease. Plasma urea will decrease as well. As the amino acid reaches the requirement, the plasma urea concentration will stay stable for a period. The breakpoint is the requirement. This method needs a longer adaptation time and may not be sensitive for small changes.

## 2.1.5 Tryptophan

### 2.1.5.1 Introduction

Tryptophan (trp) is an essential amino acid that cannot be synthesized by animals. The R group of trp is an indole group. Tryptophan is a hydrophobic amino acid which is not charged under biological conditions. L-tryptophan is the active form in animals. D-tryptophan cannot be used by poultry, dogs, humans, and mice but can be used by pigs. Tryptophan is the third

limiting amino acid in corn-soybean meal laying hen diets after methionine (met) and lys (Ishibashi, 1985; Peganova and Eder, 2003). Since met, lys and thr are commonly used in feed formulation, trp would be the next limiting amino acid widely used in laying hen diets. Commercial feed grade L-trp is produced by the fermentation of raw materials of agricultural origin, such as beet molasses or hydrolyzed starch (Lima, et al., 2012).

#### 2.1.5.2 Tryptophan Functions

Tryptophan is the precursor of serotonin (also known as 5-HT). As the precursor of serotonin, an important neuromediator, dietary trp is associated with mood, stress response, sleep and appetite regulation (Le Floc'h and Seve, 2007). Increased concentrations of dietary trp may be useful in alleviating aggressive behavior in poultry via increased serotonin synthesis in brain (Laycock and Ball, 1990). Manipulations of serotonin metabolism can, under certain conditions, produce marked changes in food intake, food preferences and body weight (Blundell, 1984). Lacy and co-workers (1986b) found that trp significantly decreased both feed intake and body temperature of the birds. These results suggest that trp may inhibit feed intake via peripheral rather than central mechanisms. Treatment with trp also decreased feed intake in the *ad libitum*-fed chickens in a dose-dependent manner (Lacy, et al., 1986a). Increased concentrations of dietary trp decrease aggression in both developing and mature, socially stable flocks of broiler breeder males (Shea et al., 1990). These data suggest that dietary trp may provide benefits to well-being of poultry. Others have reported limited effects of dietary trp on regulating animal behavior. Various trp supplementation in diets did not improve the negative effect of overcrowding on laying hens performance (Rech et al., 2010). In pigs, Koopmans and co-workers (2006) found that high concentrations of trp improved neuroendocrine component and

gastrointestinal robustness but did not affect behavior reactivity in pigs during weaning and mixing.

Tryptophan may be associated with lipid metabolism. It was reported that dietary trp alleviates fatty liver in laying hens and modifies microsomal mixed function oxidase in the liver (Akiba et al., 1992). Oral trp supplementation attenuated experimental non-alcoholic fatty liver disease in mice (Ritze et al., 2014). Supplemental trp by itself caused a reduction in total liver lipid storage, but when supplemented to a diet containing aflatoxin, trp caused an increased severity of lesions associated with aflatoxicosis in laying hens (Rogers, et al., 1991).

#### 2.1.5.3 Tryptophan and Niacin

Tryptophan is a precursor of niacin, although data in poultry are mixed as to the effects of one on the other. Supplementation of laying hens with 5 mg/kg dietary niacin increased the efficiency of trp utilization and decreased the minimum trp requirement compared with supplementation of 2 mg/kg dietary niacin in young chickens (West et al., 1952). The addition of 280 mg/kg niacin decreased the body weight of broiler breeder pullets fed 0.15% trp (Powell and Gehle, 1975). The tryptophan to niacin conversion was 103:1 and 119:1 in two independent turkey experiments indicating that although trp can be used as a precursor for niacin synthesis, under practical conditions, this conversion rate is lower when compared with the reported conversion in other species (Ruiz and Harms, 1990).

#### 2.1.5.4 Tryptophan and Other Amino Acids

Theoretically, there may be a competition between trp and other neutral amino acids for intestinal epithelium transportation, but research in poultry under practical conditions did not observe any antagonism between trp and other neutral amino acids on performance. Bray (1970)

found that excessive leucine increased the requirement of isoleucine and valine but had no effect on the requirement of trp and lys. Other authors examined the effects of adequate and high dietary isoleucine on the trp requirement of laying hens, resulting in no trp requirement differences in egg production or egg mass, although hen body weight might have been influenced by dietary isoleucine (Peganova and Eder, 2003). In contrast, an antagonism may exist between trp and phenylalanine. Ekin and Rogler (1983) found that brain uptake of trp was inhibited by hyperphenylalaninemia as excessive dietary phenylalanine was provided to the chicks.

#### 2.1.5.5 Tryptophan Requirement of Laying Hens

A corn-corn gluten meal diet (0.12% trp) was supplemented with 0.05%, 0.1%, 0.2%, 0.3% DL-trp (although D-trp cannot be utilized by chicks, approximately 50% of the supplement should be available) and fed to Single Comb White Leghorn pullets (Ingram et al., 1951). The authors found that birds who received 0.15% (0.025% L-trp) usable trp reached maximum egg production and concluded that the trp requirement for layers did not exceed 0.15%. In the Johnson and Fisher (1958) experiment, an amino acid free basal diet was used to determine the lys requirement based on egg production and nitrogen retention. After the lys requirement was estimated, remaining amino acid requirements, including trp, was estimated according to ratio of amino acid to lys in the whole egg. The trp requirement was 0.12% according to this method. Bray (1969) conducted an experiment on trp using 210-day-old pullets fed a corn-soybean meal diet. The basal diet contained 0.075% trp, estimated to be 68% of dietary tryptophan requirement. Using this trp deficient diet, 10 different diets containing 0.075, 0.078, 0.084, 0.095, 0.111, 0.134, 0.164, 0.203, 0.251, and 0.308% trp were generated and evaluated. Least square means analysis of egg yield estimated a trp requirement of 0.110% or 117 mg/hen per day. Using the average

initial body weight and maximum egg yield, the maintenance requirement of trp was calculated as 34 mg/day and a production requirement was calculated as 90 mg/d with a total trp requirement of 124 mg/day (Bray, 1969). Morris and Wethli (1978) claimed that the NRC (1971) requirement of 0.11% trp is too low and considered 0.17% more accurate according to their experiment results using 1.5 kg mean body weight birds producing 55g egg/d and eating 110g feed/d. The author also estimated trp requirement using the equation of 2.25 mg/g egg output plus 10.25 mg/kg body weight. Daily trp requirement did not decrease during the first laying year, despite a decrease in rate of egg output (Wethli and Morris, 1978). The trp requirement for Single Comb White Leghorn hens older than 15 months based on egg production, egg mass, feed intake, and plasma trp was 210, 212, 212, 191 mg/d, respectively (Ishibashi, 1985). The trp requirement ranged from 124 to 168 mg/d for egg mass of 46 to 52 g/d in hens (Jensen et al., 1990). The lys: trp ratio was estimated to be 100:16 based on an experiment using 28 week-old laying hens (Kirchgessner et al., 1995). An experiment was conducted using White Leghorn laying hens were fed corn-soybean-gelatin diets including 0.11%, 0.13%, 0.15%, 0.17%, 0.19%, 0.21%, 0.23% trp and a corn-soybean control diet (0.19% trp; Russell and Harms, 1999). Based on a broken-line regression model, the trp requirement was 136.0 and 136.5 mg/d based on egg production and egg content, respectively. The Trp requirement for egg production and egg content was estimated as 139.8 and 149.0 mg per hen/d, respectively (Harms and Russell, 2000). An estimated daily trp intake of 198 mg per hen was generated for a daily egg mass of 49g in an adequate large neutral amino acid diet (Peganova et al., 2003). Tryptophan requirements ranged from 161 to 188 mg/day, depending on the characteristics evaluated (egg production or egg mass) and the regression model applied (polynomial, exponential or broken line; Deponti et al., 2007). Calderano and coworkers (2012) suggested that the trp requirement was 212 mg/hen per day. A

higher trp requirement (142 mg/hen per day) was estimated in an experiment conducted on 60 to 76 wk old laying hens (Cardoso, et al., 2014). Although several experiments have estimated the tryptophan requirements of laying hens over time, the data suggest that the tryptophan requirement has changed over time, most likely associated with increased egg production needs.

Table 2.1 Tryptophan requirement summary of previous literature in laying hen research.

Authors, year	Trp requirement	Trp daily requirement	Maximum feed intake	Age	Maximum egg production	Strain
	(%)	(mg)	(g)	(wk)	(%)	
Bray, 1969	0.110	117	106	N/A	83	White Leghorn
Morris and Wethli, 1978	0.170	187	110	63-73	80	White Leghorn
Ishibashi, 1985	0.189	191/212	110	>60	89	White Leghorn
Jensen, et al. 1990	0.137	123	85	38-50	69	White Leghorn
Jensen, et al. 1990	0.118	95	74-81	30-85	70	White Leghorn
Russell and Harms, 1999	0.160	136/136.5	85-90	53-59	80	Hy-Line W-36
Harms and Russell, 2000	0.15-0.17	139/149	89-92	28-36	92	Hy-Line W-36
Peganova, et al. 2003	0.180	198	110-120	31-37	82	Lohmann Brown
Cardoso et al., 2014	0.192 (dig)	212(dig)	110	60-76	94	Dekalb White
Calderano et al., 2012	0.18 (dig)	142(dig)	78	24-40	79	Hy-Line W-36

## 2.1.6 Valine

### 2.1.6.1 Introduction

Valine (val) is nonpolar and one of the most hydrophobic amino acids (Brosnan and Brosnan, 2006). Along with leucine and isoleucine, val is a branched chain amino acid (BCAA). In animals, val serves as a precursor for the synthesis of protein synthesis and other amino acids (Ferrando, et al., 1995). As a glucogenic amino acid, val is a substrate for glutamine synthesis which is involved in the Krebs cycle (Wu, 2009). Valine may be a limiting amino acids for layers, after methionine, lys, trp and threonine (Lelis, et al., 2014). As the production cost of feed grade val decreases, val may be introduced into poultry diet to decrease costs and reduce nitrogen waste by formulating diets with lower overall protein concentrations.

As building blocks for protein synthesis, BCAAs have an important impact on the structure of protein (Brosnan and Brosnan, 2006). Branched chain amino acids are crucial amino acids that determine the structure of globular proteins, membranous proteins, and coiled-coil structures. Branched chain amino acid hydrophobic residues create a nonaqueous environment that is important for oxygen binding in myoglobin and hemoglobin, and for substrate binding and catalysis in a variety of enzymes. In addition, membranous proteins require hydrophobic amino acids such as BCAAs in their transmembrane domains for interaction with the hydrocarbon chains of fatty acids.

Low concentrations of dietary val (0.5 to 0.7%) in chicks resulted in lower antibody titers than higher concentrations of dietary val (0.9 to 1.5%; Bhargava et al., 1970). In a follow up experiment, a higher val diet (1.5%) increased the antibody titer compared with a low val diet (0.5%) in chicks (Bhargava et al., 1971). Increasing one BCAA in isolation can cause a depletion

of other structurally similar amino acids in the brain, causing secondary anorexia (Harrison and Dmello, 1986).

#### 2.1.6.2 Relationship between Valine and Other Amino Acids

Benton and coworkers (1956) found that there were antagonisms between isoleucine and val, and between phenylalanine and val in rats. Excess leucine increased the requirement of val in young pullets (Bray, 1970). Allen and Baker (1972) found that val efficacy reached a minimum of 74% of normal when 5.57% excess dietary leucine was provided to young chicks. Interactions for growth and plasma BCAAs concentrations exist in turkey poult between leucine-val, leucine-isoleucine and isoleucine-val (Tuttle and Balloun, 1976).

#### 2.1.6.3 Excessive and Deficient Valine Intake

Dietary val concentrations of up to 1.06% did not cause reduced performance in laying hens (Peganova and Eder, 2002). A dietary val concentration of 1.36%, however, reduced feed consumption and daily egg mass by 5 to 10% compared with its maximum and caused a loss of body weight. High concentrations of dietary val are well tolerated and can be successfully supplemented into diets without detrimental effects on laying hen performance or immune function (Azzam et al., 2014). The supplementation of chicks with excess isoleucine and val increased plasma thyroid hormone concentrations (Carew et al., 2008). It is important to avoid excessive dietary val when estimating val requirements as the performance altering (reduced) effects of excess val might affect regression analysis.

Farran and Thomas (1992) found that val deficiency significantly decreased weight gain and feed intake, bone calcification and feather protein production. Valine deficiency also decreased the level of cysteine in feathers, but increased those of aspartic acid, glutamic acid,

methionine, tyrosine, histidine, and lys. These results suggested that reducing the level of dietary val alone was more detrimental than reducing the three BCAAs simultaneously.

#### 2.1.6.4 Valine Requirement of the Laying Hen

The val requirement of laying hens was estimated to be 0.54% with 0.50% dietary lys based on the amino acid composition of whole egg (Johnson and Fisher, 1958). The predicted val requirements for laying hens have increased over time and were reported as 550 mg/hen per day (NRC, 1977), 600 mg/hen per day (NRC, 1984), and 700mg/hen per day (NRC, 1994). The val requirement of broiler breeder hens was estimated to be 0.92% (Bornstein et al., 1979). Harms and Russell (2001) conducted an experiment on 39 wk old Hy-line W36 laying hens in corn-soybean meal based diets with dietary val concentrations of 0.525, 0.560, 0.595, 0.630, 0.665 and 0.700%. According to broken-line regression analysis, the val requirements were  $592.5 \pm 19.9$ ,  $677.7 \pm 40.0$  and  $619.0 \pm 14.6$  mg/hen per day based on egg production, egg weight and egg content, respectively. Peganova and Eder (2002) used Lohmann Brown hens to determine the val requirement and took the average of results from broken-line and exponential models. The required val concentrations were 0.615% (experiment 1, 24-32 weeks of age, daily egg mass 50 g), 0.685% (experiment 2, 25-32 weeks of age, daily egg mass 58 g) and 0.585% (experiment 3, 46-52 weeks of age, daily egg mass 55 g), corresponding to daily val intakes of 609, 782 and 651 mg, respectively. Another experiment working with Hy-line W-36 laying hens from 26-34 wk of age showed digestible val requirement of 501mg/d or digestible val to lys ratio of 93% (Bregendahl et al., 2008). The authors reduced soybean meal content to form a multi amino acid deficiency diets and set 5 true digestible val concentrations (0.47, 0.61, 0.74, 0.88, 1.02%). A broken line model was used to predict the val requirement based on egg production, egg weight and egg mass. Dekalb Brown laying hens from 42-54 wk of age were fed diets with 84, 88, 92,

96 and 100% digestive val to lys ratios (Lelis et al., 2014) Both quadratic and broken line models were used to evaluate optimum val to lys ratio. The authors concluded that 92.1% (0.607% digestible val or 567mg/d) was the val requirement based on the combined values of the different models.

Table 2.2 Valine requirement summary of previous literature in laying hen research

Authors, year	Val requirement	Val daily requirement	Maximum feed intake	Age	Maximum egg production	Strain
	(%)	(mg)	(g)	(wk)	(%)	
Bornstein et al. 1979	0.92	N/A	N/A	32	N/A	N/A
Harms and Russell, 2001	0.63-0.66	593/678/619	96	39	87	Hy-line W-36
Peganova and Eder, 2002	0.62	609	N/A	24-32	N/A	Lohmann Brown
Peganova and Eder, 2002	0.69	782	N/A	25-32	N/A	Lohmann Brown
Peganova and Eder, 2002	0.59	651	N/A	46-52	N/A	Lohmann Brown
Bregendahl, et al., 2008	0.61-0.74	501 (dig)	N/A	26-34	90	Hy-line W-36
Lelis, et al., 2014	0.607 (dig)	567 (dig)	95	43-54	83	Dekalb Brown

## 2.1.7 Lysine

### 2.1.7.1 Introduction

Lysine (lys) contains an  $\alpha$ -amino group, an  $\alpha$ -carboxylic acid group and a side chain  $(\text{CH}_2)_4\text{NH}_2$ . Under biological conditions, the amino group in the side chain exists with a positive charge ( $-\text{NH}_3^+$ ), so lys is classified as a cationic amino acid along with histidine and arginine. Lysine is a ketogenic amino acid and the product of lys catabolism is acetyl-CoA. Acetyl-CoA is not converted to glucose therefore energy generation from this molecule is through the TCA cycle. Although lys can be catabolized, it is highly conserved as demonstrated in rat and chick experiments (Benevenga and Blemings, 2007; Flodin, 1997). Lysine oxidation was significantly decreased as lys intake was reduced in young men (Meredith et al., 1986).

### 2.1.7.2 Catabolic Pathway of Lysine

There are two major catabolic pathways of lys in animals. The primary pathway is the saccharopine pathway in the liver (Hallen, et al., 2013). Lysine is converted to saccharopine by combining with  $\alpha$ -ketoglutarate ( $\alpha$ -KG) under the catalysis of lys-ketoglutarate reductase (LKR). After that, saccharopine is converted to  $\alpha$ -aminoadipic-6-semialdehyde and glutamate by saccharopine dehydrogenase (SDH) (Gatrell, et al., 2013). Alpha-aminoadipic-6-semialdehyde is subsequently converted to acetyl-CoA. In the brain, lys is catabolized through the piperolate pathway (Chang, 1976). Within this pathway, lys is converted to  $\alpha$ -keto- $\epsilon$ -aminocaproic acid by removing the  $\alpha$ -amino group.

### 2.1.7.3 Biological Function of Lysine

Similar to other amino acids, one of the major functions of lys is protein synthesis. In addition, lys plays an important role in several biologically functional peptides. It was reported

that the lys-13 residue in parathyroid hormone (PTH) of poultry is responsible for folding the active domain of the hormone structure (Zull, et al., 1987). Some poly-lysine-peptides were reported to influence the activities of membrane bound enzymes including protein kinases, phosphatidylinositol kinases, and adenylate cyclase (Gatica, et al., 1987). Lysine can also be found in biologically active small peptides containing 10 or less amino acid residues. For example, kallidin (10 amino acid residues) and bradykin (9 amino acid residues) are lys containing bioactive peptides in body fluids and tissues which function as vasodilators for the maintenance of normal blood pressure (Lafarga and Hayes, 2014).

Lysine can also be used to provide energy. When dietary lys intake exceeds the body's demand for lys, the surplus lys will be catabolized to keto acid and enter the TCA cycle for further oxidation to generate ATP (Liao, et al., 2015). The catabolism of surplus lys takes place in a cell- and tissue-specific manner (Gatrell, et al., 2013). The intestine oxidizes one-third of the total oxidized lys in growing pigs fed high protein diet (van Goudoever, et al., 2000). Liver, kidney, muscle and brain also are involved in whole body lys catabolism (Liao, et al., 2015).

Lysine is the precursor of several non-peptide molecules, which exert biochemical and physiological function in animals. Carnitine is a bioactive molecule which transports long-chain fatty acid from the cytoplasm into the mitochondria for  $\beta$ -oxidation and the regulation of intramitochondrial acyl-CoA/CoA ratio (Steiber, et al., 2004; Vaz and Wanders, 2002). Carnitine can be synthesized from lys and methionine after several steps. Vaz and Wanders (2002) found that as animals were fed a low protein diets, liver carnitine synthesis was prohibited and lipid concentrations in the liver were increased. As additional dietary lys and methionine were provided, carnitine synthesis recovered resulting in a decreased lipid in liver. Another important molecule is glutamate, which is a neurotransmitter in the mammalian brain. It is believed that the

saccaropine pathway may facilitate the synthesis of glutamate from lys in the brain (Papes, et al., 2001).

#### 2.1.7.4 Lysine Transporters

The major transporter of cationic amino acids across the apical membrane of the intestinal epithelial cell is called rBAT/b<sup>0,+</sup> AT, and this transporter is a high affinity Na<sup>+</sup> independent antiport transporter. The transporter rBAT/b<sup>0,+</sup> AT is a heterodimer which consists of two subunits, rBAT (heavy unit) and b<sup>0,+</sup> AT (light unit). The heavy subunit is a type II membrane glycoprotein, and the light subunit is an unglycosylated membrane protein bearing 12 putative transmembrane domains (Fernández, et al., 2002). In addition, rBAT/b<sup>0,+</sup> AT can also transport cystine but is inhibited by neutral amino acids. In rabbits, rBAT/b<sup>0,+</sup> has a high affinity Na<sup>+</sup> dependent symport transporter that transports cationic amino acids from the lumen side into intestinal epithelial cells (Munck and Munck., 1994).

Another transporter, 4F2hc/y<sup>+</sup> LAT1, a high affinity Na<sup>+</sup> dependent antiport, was believed to be the major cationic transporter on the basolateral side of intestine epithelial cells (Broer, 2008). The protein y<sup>+</sup> LAT1 associates with the 4F2hc to form a heteromeric transporter (Pfeiffer, et al., 1999). Other transporters which are related to lys transport in animals include: 4F2hc/y<sup>+</sup> LAT2: high affinity Na<sup>+</sup> dependent antiport transporter; SNAT4: medium affinity Na<sup>+</sup> dependent symport transporter; CAT-1: medium affinity Na<sup>+</sup> independent uniport transporter (Broer, 2008). These transporters are not important during lys absorption in poultry and will not be discussed.

#### 2.1.7.5 Lysine as a Limiting Amino Acid in Poultry Feed

Warnick and Anderson (1968) found that lys, is the next limiting amino acids after sulfur amino acids in poultry. Lysine was the third limiting amino acid in a corn-soybean meal chicken diets (Fernandez et al., 1994). They also concluded that, lys was the first limiting amino acid in corn and third limiting amino acid in soybean meal for growing chicken. Lysine was reported as first limiting amino acid in corn gluten meal (CGM) for young chickens, which makes CGM an ideal ingredient to generate lys deficient diet (Peter, et al., 2000). Lysine concentrations are higher in animal protein meals than in grain, resulting in the supplementation of protein meal in chicken feed as a source of dietary lys. Lysine was the fifth limiting amino acid in meat and bone meal (MBM) for chicken due to a higher lys concentration in MBM (Wang et al., 1997).

#### 2.1.7.6 Lysine Deficiency and Toxicity

Lysine deficiency leads to decreased body weight, feed intake and increased feed conversion ratio in broilers (Dozier and Payne, 2012; Dozier, et al., 2008; Dozier, et al., 2009; Dozier, et al., 2010; Garcia and Batal, 2005; Vazquez and Pesti, 1997). Insufficient dietary lys resulted in a decreased tissue protein content due to a inhibited protein synthesis in chicks and the pectorial major was more sensitive than the liver as fractional breakdown rates (FBR) in the pectorails major were higher in lys deficient treatment in comparison to the control (Tesseraud, et al., 1996). In addition, inadequate lys intake was reported to impair animal immune function. As a building block of protein synthesis, limitation on lys intake affects the synthesis of animal immunity-related proteins that include antibodies and cytokines (Liao, et al., 2015). Chen and others (2003) found a reduced antibody response to Newcastle disease virus as broilers fed 0.83% dietary lys in comparison to 1.24% lys. Cell-mediated immune responses were also inhibited by lys deficiency with an increased morbidity and mortality in chickens as cytokines generation and proliferation of lymphocytes were prohibited (Kidd, et al., 1997; Konashi, et al., 2000).

Edmonds and Baker (1987) conducted an experiment using 8 day old crossbred chicks supplemented with 4% excess lys in corn-soymeal meal diets. The excess dietary lys induced a 47% reduction on body weight gain. Another study reported that adding 1.6% and 3.2% lys decreased body weight gain by around 9% and 12% in crossbred chickens from one to three weeks posthatch (Han and Baker, 1993). In laying hens, 1% additional lys did not change laying hen performance indicating a considerable tolerance exists in high-producing laying hens (Koelkebeck, et al., 1991). Studies in rats indicated that diets containing lys over 5% did not induce negative effects on body weight or other clinical signs (Tsubuku, et al., 2004). The reason for high dietary lys intake tolerance may include a slower entry in to circulation, high lysine-ketoglutarate reductase activity in the liver, more time for egress of lys from circulation and more time for kidneys to respond to the increasing plasma lys level by acceleration of urinary lys excretion into muscle for temporary storage (Flodin, 1997). In comparison to the report of Edmonds and Baker (1987), young chicks seem to be more susceptible to high dietary lys, possibly associated with the relatively lower lysine-ketoglutarate reductase activity in young chicks.

#### 2.1.7.7 Lysine Requirement in Poultry

Total lys requirements for 0-3, 3-6, and 6-8 wk old broilers were estimated at 1.10%, 1.00% and 0.85%, respectively (NRC, 1994). Han and Baker (1991) estimated the lys requirement of fast growing and slow growing chicks from 8 to 21 days posthatching and concluded that digestible lys requirement was no more than 1.01% based on body weight gain and no greater than 1.21% based on feed efficiency. Vazquez and Pesti (1997) used 16 response sets of graded lys from previous publications to estimate the lys requirement of chicks from 0 to 3 wks of age. The lys requirements were  $1.04 \pm 0.02\%$  and  $1.10 \pm 0.06\%$  for gain when using linear and

quadratic broken line models, respectively. The lys requirements were  $1.21 \pm 0.06\%$  and  $1.32 \pm 0.10\%$  for feed efficiency when using linear and quadratic broken line models, respectively. In another experiment, lys requirements of chicks from 0-7 days old were evaluated and the estimates were 1.03% and 1.08% for body weight and feed efficiency using a broken line model (Sklan and Noy, 2003). Lysine requirements were estimated over the 0-4, 0-7 and 0-21d periods for broiler and were found to be similar (Garcia and Batal, 2005). Digestible lys requirements ranged from 0.95% to 1.01% based on weight gain and 0.94% to 1.10% based on feed efficiency using a broken line model (Sklan and Noy, 2003). An experiment was conducted on broilers from 49 to 63 d of age and digestible lys requirement were 0.87% and 0.81% for weight gain based on quadratic and broken line models (Dozier et al., 2008). A followup experiment was conducted on 14 to 28 d old Ross  $\times$  Ross TP16 broilers and digestible lys requirements of was 1.10% and 1.00% based on BW gain and FCR were estimated using quadratic and quadratic broken line models (Dozier, et al., 2009). Dozier and coworkers (2010) estimated digestible lys requirements for male Ross  $\times$  Ross TP16 and Cobb  $\times$  Cobb 700 broilers at 1.001% and 0.995% based upon live performance and meat yield responses using a quadratic broken line model. Digestible lys requirements of Ross  $\times$  Ross 708 and Hubbard  $\times$  Cobb 500 broilers were evaluated from 1 to 14 d of age using a quadratic broken line model (Dozier and Payne, 2012). Digestible lys requirements for female Ross  $\times$  Ross 708 were 1.35 and 1.27% from 1 to 7 d and 1 to 14 d of age for body weight gain. Digestible lys requirement for female Hubbard  $\times$  Cobb 500 were estimated to be 1.26 and 1.18% for body weight gain.

#### 2.1.7.8 Interaction between Lys and Other Amino Acids

Excess lys has been shown to impair the reabsorption of arginine in the kidney as both amino acids share common cell membrane transporters (Scott, 1982). Lysine also stimulates the

production of an arginase resulting in breakdown of arginine. In addition, there was an interaction between lys and threonine to improve weight gain and breast fillet yields (Kidd et al., 1997). At the 100% NRC lys level, optimal fillet yields were observed by feeding threonine at 83 or 92% of the NRC. At the 105% NRC lys level, optimal fillet yields were observed by feeding threonine at 100 or 108% of the NRC. This result indicates that when dietary lys concentrations increase the threonine to lys ratio need to be evaluated and potentially increased.

#### 2.1.7.9 Phase Feeding in Broiler Production

As amino acid requirements (% of diet or % of calories) decrease steadily throughout the grow-out period, phase feeding (PF) regimens have been established to reduce amino acid waste without sacrificing performance in comparison to traditional feeding in broilers (Warren and Emmert, 2000). Phase feeding divides the feeding period into multiple shorter periods based on a predicted amino acid requirements. Regression equations using the Illinois ideal chick protein (IICP) were developed for use in the PF regimen (Emmert and Baker, 1997). Warren and Emmert (2000) conducted an experiment to compare the PF regimen to NRC or IICP on broilers during the starter and finisher phases and concluded that PF facilitated reduced dietary cost without sacrificing growth or carcass yield. A follow up experiment used chicks from 43 to 71 d of age to compare evaluate PF to NRC feeding recommendations with additional experimental diets included a 10% reduction in amino acid requirements to the PF diets (PF10: Pope and Emmert, 2001). The authors concluded that PF and PF10 may lower the cost of feed consumed and the cost per unit weight gain or breast yield relative to broilers fed the NRC diet without reducing performance. A further experiment was conducted by decreasing AA requirement every other day from 42 to 63 d of age (Pope, et al., 2002). It was found that this PF regimen was able to maintain growth performance but decrease AA intake. Brewer and others (2012b) validated

the effect of PF on broilers from 18 to 32 d of age by changing feed every other day in comparison to averaged industry nutrient diets resulting in PF generally being able to maintain or improve growth performance, but strain of commercial boiler did alter these responses. A follow up experiment on 17 to 58 d old broilers showed comparable results on performance (Brewer, et al., 2012a).

## 2.2 Bacterial Protein Meal

### 2.2.1 Introduction

Bacterial protein meal (BPM) is derived from bacterial fermentation converting various substrates such as methane, methanol, or agriculture by-products into protein-rich biomass with a minimum dependence on soil, water, and climate conditions (Anupama and Ravindra, 2000).

### 2.2.2 Nutritional Evaluation of Bacterial Protein Meal

Bacterial protein meal as a protein rich ingredient contains high level of crude protein. The crude protein value ranged from 58% to 75.5% in previous scientific reports (Waldroup and Payne 1974, Plavnik, Bornstein et al. 1981, Skrede, Berge et al. 1998, Schøyen, Hetland et al. 2007). Amino acid concentrations of BPM were close to that of soybean meal and fish meal except but the cysteine concentration was extremely lower than that of soybean meal (D'Mello 1973, Skrede, Berge et al. 1998). Total tract digestibility of BPM was 80.5% when estimated in 1-year old Leghorn roosters (Skrede et al., 1998). In another experiment, both total tract and ileal amino acid digestibility on 5 week old broilers (Schøyen et al., 2007). In comparison to soybean meal, ileal arg, lys, met and phe digestibility were significantly lower in BPM while other indispensable amino acid digestibility values showed no difference. Metabolizable energy (ME) of BPM ranged from 2,282 to 2,610 kcal/kg dry matter and nitrogen-corrected ME ranged from

2,139 to 2,428 kcal/kg dry matter using 17 and 18 day of age broiler chicks (D'Mello and Acamovic, 1976).

### 2.2.3 Effect of Bacteria Protein Meal in Poultry Ration

D'Mello (1973) found no differences in growth performance of chicks fed corn-SBM control diet or a diet containing 10% BPM produced with methane. Increasing the supplementation of BPM (0, 5, 10, 15%) in all-mash broiler diets reduced both feed intake and body weight over time (Waldroup and Payne, 1974). On the other hand, pelleting the diets was effective in improving feed consumption and weight gain, allowing up to 10% BPM without detrimental effect on broiler performance. In another experiment, 10 or 20% inclusion of spray-dried methanol-grown BPM reduced growth rate and feed efficiency of broiler chickens (D'Mello and Acamovic, 1976). Higher inclusion of flash-dried methanol-grown BPM rate up to 29% may decrease the growth performance of chicks while 9.6% supplementation would marginally increase growth rate and feed efficiency (D'Mello and Acamovic, 1976). In another experiment, different nitrogen concentrations were used to investigate the effect of replacing soybean meal with spray-dried (10%) or flash-dried (15%) forms of methanol-grown BPM in chicken diet. Diets containing 10% spray-dried BPM were not able to maintain growth at 32, 36 and 39 g nitrogen/kg, and only 43 g nitrogen/kg diet containing 15% flash-dried forms of methanol-grown BPM showed a similar growth performance to corn-soybean meal diet (D'Mello, 1978). Whittemore and coworkers (1978) conducted two experiments on broilers and laying hens investigating the effect of the substitution of BPM with soybean meal. Increasing BPM inclusion (3.8, 5, 7.5 and 10%) resulted in a linear decrease in feed intake and egg production in laying hens (Whittemore et al., 1978). The addition of either 9 to 10% BPM to broiler diets resulted in a 3-5% reduction in feed intake that reduced body weight by 2-3% (Bornstein et al., 1981).

Increasing the supplementation of BPM from 9 to 15% of the diet resulted in a 14 to 16% reduction in growth rate in young chicks that was attributed to reductions in feed intake and not directly related to feed utilization (Plavnik et al., 1981). Lower concentrations of BPM were evaluated in broiler diets, as previous work has been clear as to the negative effects of feeding higher concentrations, resulting in the addition of 0, 2, 4, 6, 8 and 10% BPM and the conclusion that 6% inclusion did not decrease growth performance and could be tolerated by young birds (Skrede et al., 2003). The addition of up to 6% BPM in broiler diets did not alter energy or protein metabolism and did not change carcass composition (Hellwing et al., 2006). Up to 6% BPM or autolyzed BPM were used to replace soybean meal in broiler diets from 0 to 35 days of age without significant differences on growth performance or feed efficiency (Schøyen et al., 2007). The same report detailed the amino acid digestibility of the two BPM processes in comparison to soybean meal resulting in no difference between SBM and BPM, but autolyzed BPM was reduced. Although the nutritional characteristic of BPMs vary in different experiments, inclusion of BPM up to 10% could replace traditional protein source without negatively affecting performance in poultry.

#### 2.2.4 Bacteria Protein Meal and Immunology

Research on the effects of feeding BPM to poultry on immune response in poultry has been limited. An experiment was conducted on broilers receiving diets containing 0, 6.2, 12.4, 24.8% BPM (Farstad, 1977). The birds were inoculated with bovine serum albumin (BSA) and serum BSA-antibody was detected. The author found that a significant increase in precipitation zone diameter in 12.4 and 24.8% groups indicating a higher BSA-antibody titer. The reason for this alternation of immune response by BPM was not fully explained. As BPM may contain high

concentration of nucleic acid (Skrede, et al., 1998), this immune response could be associated with nucleic acid as nucleic acid was reported to benefit immune status in humans (Yu, 1998).

Table 2.3 Bacterial protein meal summary of previous literature in poultry research<sup>1</sup>

Laying hens			
Citation	BPM inclusion	Age	Result
Whittemore et al., 1978	3.8, 5, 7.5, 10%	28 to 60 w	a linear decrease in feed intake and egg production in laying hens
Broilers			
Citation	BPM inclusion	Age	Result
D'Mello, 1973	10%	7 to 21 d	no difference in comparison to corn-SBM diet
Waldroup and Payne, 1974	5, 10, 15% (mash)	0 to 28 d	reduce feed intake and body weight
Waldroup and Payne, 1974	5, 10, 15% (pellet)	0 to 28 d	BPM up to 10% did not affect performance
D'Mello and Acamovic, 1976	10, 20%	7 to 21 d	spray dried BPM reduced growth and feed efficiency
D'Mello and Acamovic, 1976	9.6, 29%	7 to 21 d	29% flash dried BPM reduced growth and feed efficiency but 9.6 marginally increase growth
Whittemore et al., 1978	3.8, 5, 7.5, 10%	7 to 56 d	The growth of broilers was not significantly affected although there was a significant linear trend for an increasing rate of inclusion of BPM to decrease food intake
Bornstein et al., 1981	9, 10%	35 to 56 d	3-5% reduction in feed intake; 2-3% reduction in body weight
Plavnik et al., 1981	9 to 15%	7 to 28 d	14 to 16% reduction in growth rate
Skrede et al., 2003 <sup>2</sup>	2, 4, 6, 8, 10%	0 to 35 d	up to 6% inclusion did not decrease growth performance
Schøyen et al., 2007 <sup>2</sup>	6%	0 to 35 d	6% did not affect performance

<sup>1</sup> Unless otherwise noted, source of feedstock is methanol.

<sup>2</sup> Source of feed stock is natural gas.

## 2.3 Vitamin D

### 2.3.1 Introduction

Vitamin D (VD) is a group of closely related compounds associated with antirachitic function. Vitamin D occurs as colorless crystals in pure form and they are insoluble in water but soluble in alcohol and other organic solvents (Soares, et al., 1995). The main forms of VD are ergocalciferol (D<sub>2</sub>) found in plant-based ingredients and cholecalciferol (D<sub>3</sub>) found in animal-based ingredients. Vitamin D<sub>3</sub> can be synthesized by exposure to sunlight in animals. The unit of measure for vitamin D is an International unit (IU) or more specifically an international chicken unit (ICU) as various metabolites of vitamin D have differing activities and these measurements account for that activity and can be expressed across a range of similar compounds. Vitamin D<sub>3</sub>, the animal related form has greater direct activity in birds than vitamin D<sub>2</sub> (Houghton and Vieth, 2006). One IU or ICU of D<sub>3</sub> is defined as the activity of 0.025 µg of D<sub>3</sub> (NRC, 1994).

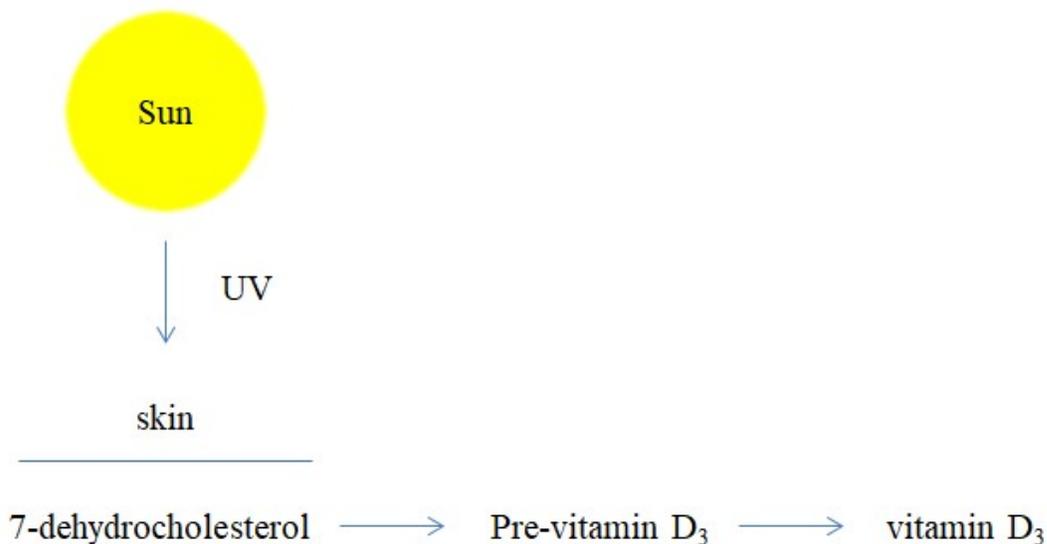


Figure 2.1 vitamin D<sub>3</sub> synthesis from sunlight exposure.

Early reports on vitamin D in poultry demonstrated that 11 to 45 minutes of sunlight exposure was sufficient to allow for the conversion of 7-dehydrocholesterol to vitamin D by growing chickens to prevent rickets and maximize growth rates (Heuser and Norris, 1929). Feather cover of skin is an important consideration as Tian and coworkers (1994) reported that the 7-dehydrocholesterol content in skin of legs (no feather cover) is about 30 times more than that in body skin in poultry. Whole body exposure of chickens to ultraviolet B (UVB) radiation resulted in the production of pre-vitamin D<sub>3</sub> in the skin of the legs and feet, whereas no pre-vitamin D<sub>3</sub> was detected in the back skin that was covered by feathers. The combination of body feather cover and the majority of the poultry raised in commercial conditions being raised in side under strict light control has resulted in vitamin D supplementation in the diet as the preferred method to ensure both vitamin D required for normal growth and maintenance and vitamin D applied to improve skeletal health.

### 2.3.2 Vitamin D<sub>3</sub> Absorption

Dietary Vitamin D<sub>3</sub> is mainly absorbed in ileal portion of the intestinal tract due to the retention time of feed in ileum (Norman and DeLuca, 1963). Vitamin D is a fat soluble compound and the absorption of vitamin D will be with lipids and other fat-soluble molecules also requiring the involvement of bile salts (Braun, 1986). Previous reports have estimated that only 50% of dietary D<sub>3</sub> can be absorbed in rats (Schachter, 1964). Some authors have speculated that the body has not evolved a more efficient mechanism for VD absorption as typical sufficient concentrations of VD can be obtained by sunlight exposure (Rucker, et al., 2007).

### 2.3.3 Mode of Vitamin D Action on Calcium Absorption

Calcium is absorbed either by an active transcellular pathway, which is energy dependent, or by a passive paracellular pathway through tight junctions (Christakos, et al., 2011). The active form of VD,  $1,25\text{-(OH)}_2\text{-D}_3$ , promotes intestinal calcium absorption by regulating gene transcription process. In the intestine,  $1,25\text{-(OH)}_2\text{-D}_3$  mediates calcium influx into the enterocyte through the transient receptor potential vanilloid type 6 (TRPV6), intracellular calcium transfer through effects on calbindin and cellular calcium release by the plasma membrane calcium ATPase (Ca pump). Vitamin D receptor (VDR) is expressed in all segments of the small and large intestine and active  $1,25\text{-(OH)}_2\text{-D}_3$  calcium absorption has been reported in the distal as well as the proximal intestine (Christakos, 2012). Transient receptor potential vanilloid type 6 is the apical calcium channel, which is involved in transferring calcium from the lumen into the enterocyte. Calbindin, a calcium binding protein, facilitates calcium diffusion throughout the cell. In a vitamin D-deficient state, both mammals and birds have decreased intestinal absorption of calcium and no detectable concentrations of calbindin (Rucker, et al., 2007). Plasma membrane calcium ATPase (PMCA1b) is the Ca pump that moves Ca out of the enterocyte via the basolateral membrane (Wasserman and Ayala, 2005). In addition to Ca absorption and cellular transport, there is increasing evidence that  $1,25\text{-(OH)}_2\text{-D}_3$  can enhance paracellular calcium diffusion by regulating tight junction proteins (Kutuzova and Deluca, 2004).

It has been reported that  $1,25\text{-(OH)}_2\text{-D}_3$  treatment alters the biochemical and morphological characteristics of intestinal cells in chicks (Putkey, et al., 1982). Treatment with  $1,25\text{-(OH)}_2\text{-D}_3$  has been shown to increase the height of the villus and the number of microvilli in chicks (Spielvogel, et al., 1972).

#### 2.3.4 Mode of Vitamin D Action on Bone Homeostasis

Although VD is reported as an antirachitic compound during animal growth, its primary function is to stimulate bone resorption to increase or maintain calcium and phosphorus plasma concentrations (Rucker, et al., 2007). The main function of  $1,25\text{-(OH)}_2\text{-D}_3$  in the mobilization of bone calcium is by stimulation of osteoclast differentiation (Walters, 1992). In bone, VDR was detected in normal osteoblasts (Walters, et al., 1982) and in osteoblast-like osteosarcoma cells, but not in mature osteoclasts (Rucker, et al., 2007). This indicates that  $1,25\text{-(OH)}_2\text{-D}_3$  may stimulate bone resorption by activating osteoblast differentiation in the osteoclast.  $1,25\text{-(OH)}_2\text{-D}_3$  increased alkaline phosphatase production and the proliferation of cultured osteoblasts (Kurihara et al., 1984). The result of increased synthesis of  $1,25\text{-(OH)}_2\text{-D}_3$  is elevated serum calcium. When serum calcium returns to normal, calcitonin, released from the thyroid gland, inhibits osteoclast activity. Mobilization of bone calcium occurs when dietary calcium is unavailable to meet daily metabolic and productive needs (Maierhofer, et al., 1981).

#### 2.3.5 Mode of Vitamin D Action on Kidney

The kidney is the major organ which generates  $1,25\text{-(OH)}_2\text{-D}_3$ , but is tightly controlled by a negative feedback mechanism as  $1,25\text{-(OH)}_2\text{-D}_3$  also inhibits the activity of  $25\text{(OH)D}_3\text{-}1\alpha\text{-hydroxylase}$  (Henry and Norman, 1984). Calcium reabsorption in the proximal tubule is passive and follows a sodium gradient, whereas calcium reabsorption in the distal tubule involves an active transcellular mechanism and resembles intestinal calcium absorption (Christakos, et al., 2016). In the distal tubules, calcium absorption is regulated by  $1,25\text{-OH-D}_3$  and PTH. The presence of  $1,25\text{-(OH)}_2\text{-D}_3$  increases the expression of calbindin-D 9k and calbindin-D 28k and to a lesser extent of TRPV5.

The proximal tubules of the kidney are also the major site of 1,25-(OH)<sub>2</sub>-D<sub>3</sub> synthesis and of phosphate absorption (Christakos, et al., 2016). Cytochrome P450 family 27 subfamily B member 1 (CYP27B1) expression is suppressed by 1,25-(OH)<sub>2</sub>-D<sub>3</sub> and Fibroblast growth factor 23 (FGF23) (Christakos, et al., 2016). FGF23 also stimulates phosphate excretion by decreasing the expression of the phosphate transporters NPT2a/c in the apical membrane (Bacic et al., 2006). FGF23 may signal by binding to the few FGFR1-Klotho complexes in the proximal tubules or by inducing a paracrine factor (factor X) in the distal tubules where abundant FGFR1-Klotho complexes are present (Li et al., 2004).

### 2.3.6 Vitamin D Toxicity

Poultry can tolerate high concentrations of D<sub>3</sub> in the diet. There were no toxic responses reported in laying hens fed either 39,000 or 66,000 IU D<sub>3</sub> /kg (Garlich and Wyatt, 1971; Griminger, 1966). Renal tubular calcification was observed when D<sub>3</sub> was fed at the rate of 400,000 IU /kg of diet (Morrissey et al., 1977) Elevated serum calcium is a common result of the vitamin D toxicity. Consequently, extensive soft tissue calcification and inflammatory responses with cellular degeneration can result.

### 2.3.7 Vitamin D<sub>3</sub> and Egg Shell Formation

Ca<sup>2+</sup> transportation in shell gland was a multi-factor dependent process (Bar, 1999). This process was regulated by calbindin and calbindin gene expression in the shell gland is predominantly calcium-mediated (Bar, 1999). Defect in vitamin D metabolism or its expression is a typical situation in thin-shell-forming hens indicating important role of vitamin D during shell calcification (Bar, 1999). Circulating 1,25(OH)<sub>2</sub>-D<sub>3</sub> concentration was associated with ability of egg shell calcification (Soares et al., 1988). Hens producing eggs with superior egg

shell quality had higher concentrations of circulating 1,25-(OH)<sub>2</sub>-D<sub>3</sub> than similar hens producing poor egg shells (Soares et al., 1988).

### 2.3.8 Effect of Increased Dietary Vitamin D Supplementation in Poultry

In laying hens, high dietary D<sub>3</sub> resulted in increased D<sub>3</sub> concentration in eggs produced. Supplementation of 1,720, 4,280, 11,200, 12,000 IU/kg D<sub>3</sub> in laying hen diets increased D<sub>3</sub> concentration in eggs without negatively affecting egg sensory, functional properties, fatty acid composition or hen health (Mattila et al., 2003). In another experiment, 2,200, 9,700, 17,200, 24,700, and 102,200 IU/kg D<sub>3</sub> also increased D<sub>3</sub> concentration in eggs (Yao et al., 2013). In this experiment, the peak D<sub>3</sub> concentrations in egg yolk occurred at 3 weeks after initiation of experiment and were 865, 1641, 2411, and 34815 IU/100 g egg yolk (wet basis) from diets containing 9,700, 17,200, 24,700, 102,200 IU D<sub>3</sub>/kg diet, respectively. Transfer efficiency of hens fed 9,700 to 24,700 IU D<sub>3</sub> diets was between 11% to 14% but much lower than the 45 to 50% transfer efficiency in the 102,200 IU D<sub>3</sub> fed hens (Yao et al., 2013).

In addition to increased D<sub>3</sub> concentration in eggs, research was conducted to investigate the effects of increased dietary D<sub>3</sub> on egg shell quality and bone status in laying hens. Eggshell specific gravity and eggshell strength of laying hens fed 6,000 and 15,000 IU/kg D<sub>3</sub> were not different from hens fed 2,500 IU/kg D<sub>3</sub> (control) from 20 to 65 wk of age (Mattila et al., 2004). In this scientific report, tibia breaking strength was significantly higher in the 6,000 and 15,000 IU/kg D<sub>3</sub> treatments in comparison to control fed hens. No differences in egg specific gravity or laying hen tibia bone ash was reported as laying hens fed high D<sub>3</sub> diets from 2,200 to 102,200 IU from 19 to 58 wk of age (Persia et al., 2013). Feeding 12,000 IU/kg D<sub>3</sub> to laying hens did not improve eggshell strength from 38 to 62 wk of age (Mattila et al., 2003). In another experiment,

supplementation of D<sub>3</sub> up to 20,000 IU/kg in laying hen diets did not improve eggshell strength from 87 to 91 wk of age (Park et al., 2005).

In broiler experiments, body weight gain was improved with the supplementation of 4,000 IU/kg D<sub>3</sub> in comparison to those fed 1,000 IU/kg D<sub>3</sub> (Fritts and Waldroup, 2005). Body weight and tibia breaking strength were maximized at 14d by the supplementation of 10,000 IU D<sub>3</sub> /kg, tibia ash was maximized with 5,000 IU D<sub>3</sub>/kg (Whitehead et al., 2004). Supplementation of 200, 1,500, 2,500 and 3,500 IU/kg D<sub>3</sub> in broiler diets increased growth performance and bone mineralization (Khan et al., 2010). Increased dietary concentration of D<sub>3</sub> (2,000 or 4,000 IU/kg) improved bone quality and walking ability and decreased the intensity of footpad and hock dermatitis in broilers (Sun et al., 2013). Increased D<sub>3</sub> (7,500 or 15,000 IU/kg) in the diet increased P retention without improving performance in broilers (Green and Persia, 2012). In Chinese yellow chicken, supplementation of 100, 200, 300, 400, 500, 600 and 700 IU/kg D<sub>3</sub> resulted in a linear response on growth performance, bone breaking strength/density/mineralization, serum Ca/P/25-OH-D<sub>3</sub>/calcitonin, and meat quality (Jiang et al., 2015). Increased dietary D<sub>3</sub> (3,500 and 5,000 IU/kg) were reported to alleviate tibial dyschondroplasia (TD) in broilers (Whitehead et al., 2004; Khan et al., 2010). In contrast to limited effect of high D<sub>3</sub> on laying hens, beneficial effect on performance, bone mineralization, bone quality has been will established in broilers. The data generated in growing birds may indicate a more significant effect of D<sub>3</sub> during the bone development phase with limited results once those bones are mature.

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## Chapter 3: Evaluation of the Tryptophan Requirement of Small-framed First Cycle Laying Hens

### 3.1 Abstract

An experiment was conducted to evaluate the total tryptophan (trp) requirement of first cycle laying hens from 41 to 60 weeks of age. In total, 270 Hy-Line W36 laying hens were randomly allocated to 6 treatments with 15 replicate groups of 3 birds for each experimental unit. A trp deficient basal diet was formulated with corn, corn gluten meal and soybean meal with determined trp, lysine and crude protein concentrations of 0.096, 0.873, and 15.0%, respectively. Synthetic L-trp was supplemented to the basal diet in 0.020% increments to generate experimental diets containing 0.096, 0.116, 0.136, 0.156, 0.176, and 0.196% trp respectively. Hens were provided a controlled amount of feed resulting in approximately 95g/hen/d. The diet containing the lowest concentration of trp resulted in reduced egg production and was halted at 45 weeks of age due to low performance with all other dietary treatments reaching the conclusion of the experiment at 60 weeks of age. Blood samples were taken and plasma serotonin concentrations were measured at 60 weeks of age. Linear broken line, quadratic broken line, quadratic polynomial and exponential models were used to estimate trp requirement based on hen-housed egg production (HHEP), egg mass (EM), and feed conversion ratio (FCR). Hen-housed egg production ranged from 50.7 to 81.0%, dependent upon dietary concentration of trp. Using the linear broken line model, trp requirement was highest for egg mass, 155.8 mg/d, followed by egg production, 153.2 mg/d and lowest for FCR, 140.4 mg/d. Data suggested that plasma serotonin is not a good candidate for trp requirement estimation.

Key words: laying hen, tryptophan, egg production, first cycle, serotonin

### 3.2 Introduction

Tryptophan is an essential amino acid, required for protein synthesis, and several other metabolic processes in laying hens. Although required, the literature is mixed if trp is considered the third or fourth limiting amino acid for laying hens. Research on the trp requirement in laying hens has been limited (Ishibashi, 1985; Peganova and Eder, 2003). As an important essential amino acid, trp is involved in body protein synthesis as well as protein production to support egg production (Wu, 2009). In addition to protein synthesis, trp is also involved in several metabolic processes. As the precursor of serotonin, an important neuromediator, dietary trp is associated with mood, stress response, sleep and appetite regulation (Le Floc'h and Seve, 2007). Tryptophan is also involved in the Kynurenine pathway, which is associated with nicotinamide adenine dinucleotide (NAD<sup>+</sup>), niacin and picolinic acid metabolism.

As the productive potential of laying hens is improved by breed selection, amino acid requirements may change to support the higher productive performance (Applegate and Angel, 2014). Recent research on the trp requirement of commercial white-egg laying hens has been limited. The NRC (1994) suggests white-egg laying hens during peak production require 160 mg total trp per hen per day, but this number is based on limited data sets that all predate 1994. Russell and Harms (1999) estimated total trp requirement of 53 to 59 week old Hy-Line W36 laying hens as 136.5 mg/d based on 43.5 g of egg content production. Another experiment using young Hy-Line W36 laying hens estimated the total trp requirement from 28 to 36 weeks of age to be 149.0 mg/d at 45.4 g of egg content (Harms and Russell, 2000). Recent research on small framed white-egg laying hens indicated a digestive trp requirement of 142 mg/d based on FCR (Calderano, et al., 2012). The objective of the current experiment was to update the total trp

requirement of first cycle white laying hens from 41 to 60 weeks of age using HHEP, egg mass and FCR as response criteria.

Linear broken line models have been the most widely used estimate for trp requirement experiments (Bray, 1969; Russell and Harms, 1999; Harms and Russell, 2000; Peganova, et al., 2003; Bregendahl, et al., 2008; Calderano, et al., 2012), but quadratic polynomial and exponential models can also be used (Morris and Wethli, 1978; Ishibashi, 1985; Peganova, et al., 2003; Cardoso, et al., 2014). In this experiment, four models (linear broken line, quadratic polynomial, quadratic broken line and exponential model) were fitted to the performance dataset to estimate trp requirement in small-framed first-cycle laying hens.

### 3.3 Materials and Methods

#### 3.3.1 Diets

Corn, corn gluten meal and soybean meal were selected as major ingredients to generate a trp deficient basal diet. Amino acid concentrations of corn, corn gluten meal and soybean meal were determined before dietary formulation (University of Missouri AESCL, Columbia, MO 65211). After the basal diet was mixed, basal diet samples were sent for amino acid analysis (Table 3.1) (Evonik GMBH, Hanau, Germany). In the basal diet, trp concentration was 0.096% and this result confirmed a trp deficient diet in comparison with trp requirement of 0.160% in NRC (1994). Branch chain amino acid (BCAA) concentrations were 0.680%, 1.819% and 0.768% for isoleucine, leucine and valine, respectively. Based on previous research, the concentration of BCAAs in this test diet was not expected to generate an antagonism with trp or impact requirements (Peganova, et al., 2003). Synthetic L-trp (98% feed grade, PT Cheil Jedang Indonesia) was supplemented to the basal diet in 0.020% increments, resulting in experimental

diets containing 0.096%, 0.116%, 0.136%, 0.156%, 0.176% and 0.196% trp, respectively. In the current experiment, all trp values were expressed as total trp. Over the duration of the experiment, experimental diets were mixed every two weeks from the same group of experimental ingredients.

### 3.3.2 Animal and Housing

All animal work was approved by the Institutional Animal Care and Use Committee at Virginia Tech (Blacksburg, VA). In total 270, 41 wk old Hy-Line W36 laying hens were selected from a flock of 600 birds. Birds selected were healthy and in high production (90.2%). Birds were randomly assigned to six treatments with 15 replicates of 3 birds/cage (464.5 cm<sup>2</sup>/cage) over two A-frame battery cage units. Hens were provided with approximately 95g/hen/d feed and ad libitum access to water. Hens were housed in a dark-out house conditions and provided a 16:8 lighting schedule. House temperature was controlled via a fan and air inlet ventilation system and ranged from 70 to 80 °F over the duration of the experiment. The hen-housed egg production for the two week period before initiation of the experiment (wk 39 and 40) ranged from 90.2-90.3% across the treatments (P=0.99).

Over the duration of the experiment, laying hens were monitored at least twice daily and abnormalities, culled or mortality were noted and removed if needed. Eggs were collected and recorded and feed provided at approximately 10am daily. Feed intake, HHEP and FCR were calculated to correspond with bi-weekly feed manufacture periods and were analyzed over two week periods. Each week, two consecutive days of egg production were collected for egg weight measurements and were calculated on a bi-weekly basis to again correspond with feed manufacture periods. Two eggs, one from each day, per experimental unit from the above

collected eggs were stored for seven days before Haugh unit, relative albumen weight, relative yolk weight, relative shell weight and egg shell thickness measurement. Egg quality indices were analyzed on a bi-weekly basis. Body weights were measured and recorded every four weeks.

At 45 and 60 weeks of age, one hen was randomly selected from each experimental unit and 1ml of blood was collected from the brachial vein to determine plasma serotonin concentrations. The plasma serotonin concentration was measured by ELISA serotonin kit (Mybiosource, Inc. San Diego, CA)

### 3.3.3 Statistical Analysis

SAS 9.4 (SAS Institute Inc, Cary, NC) was used to analyze hen housed egg production, feed intake, egg weight, egg mass, feed conversion ratio (FCR), daily trp intake, Haugh unit, relative albumen weight, relative yolk weight, relative shell weight, shell thickness and plasma serotonin using a one-way ANOVA with means separated by repeated measures using a Tukey's adjustment. Body weight loss at the end of 60 weeks of age was analyzed using a one-way ANOVA as means were separated by Fisher's LSD test. Significance was accepted at  $P \leq 0.05$ . Laying hen trp requirement was calculated using linear broken line, quadratic broken line, quadratic polynomial and exponential models based on HHEP, egg mass, and FCR. The regression was analyzed by JMP non-linear model option of JMP Pro 11.0.0 (SAS Institute Inc, Cary, NC) with daily trp intake as the independent variable in the linear broken line (Bregendahl, et al., 2008), quadratic broken line and quadratic polynomial models (Pesti, et al., 2009). Tryptophan concentration was the independent variable to estimate requirement in exponential model. In both the quadratic polynomial and exponential models, trp requirement was estimated when 95% of maximum response was achieved. In published reports, 90 to 100% of the

maximum quadratic response represents the typical range to quantify a response and 95%, the median level, is widely used (Kidd and Tillman, 2016).

The equation of the linear broken line model was:

$$y = a - b * (x - c) \text{ for } x \leq c \text{ and}$$

$$y = a \text{ for } x > c$$

where  $y$  = response criteria,  $a$  = ordinate of the breakpoint,  $b$  = slope of the line for  $x \leq c$ ,  $c$  = abscissa of the breakpoint (= requirement), and  $x$  = trp daily intake.

The equation of the quadratic broken line model was:

$$y = a - b (x - c)^2 \text{ for } x \leq c \text{ and}$$

$$y = a \text{ for } x > c$$

where  $y$  = response criteria,  $a$  = ordinate of the breakpoint,  $b$  = coefficient of quadratic term for  $x \leq c$ ,  $c$  = abscissa of the breakpoint (= requirement), and  $x$  = trp daily intake.

The equation of the quadratic polynomial model was:

$$y = ax^2 + bx + c$$

where  $y$  = response criteria,  $a, b, c$  are the coefficients of quadratic equation,  $x$  = trp daily intake.

The equation of the exponential model was:

$$y = a + b * (1 - e^{-c * (x - d)})$$

where  $y$  = response criteria,  $a$  = intercept (performance of the lowest trp concentration diet in the model. Since 0.096% trp treatment was removed, the lowest trp concentration was 0.116%),  $b$  = maximum response due to increased trp concentration,  $c$  = slope,  $d$  = trp concentration of the lowest trp concentration diet, and  $x$  = trp concentration of the diet.

## 4. Results and Discussion

### 3.4.1 Laying Hen Performance

Dose response on laying performance observed in this experiment was consistent with those previously published (Bray, 1969; Ishibashi, 1985; Russell and Harms, 1999; Bregendahl, et al., 2008). The HHEP dropped below 40% in the 0.096% treatment, therefore this entire

treatment was discontinued and birds returned to a trp sufficient diet (Figure 3.1). For the remaining treatments, HHEP increased significantly as trp concentration increased from 0.116% to 0.176%, with a plateau occurring after the 0.176% treatment throughout the nineteen-week period (Table 3.2). After an initial drop, HHEP and feed intake somewhat recovered (Figure 3.1) in the 0.116 and 0.136% trp fed hens suggesting an adaptation of the laying hens to the deficient trp diets over time, but no ability to fully overcome the low concentration of trp in the feed.

Feed intake responded to dietary trp concentration similar to HHEP (increasing with increased trp concentration). Reduced feed intake has been associated with low dietary trp concentration (Bray, 1969; Ishibashi, 1985; Jensen, et al., 1990; Russell and Harms, 1999; Peganova, et al., 2003). In contrast, there have been reports of a reduced sensitivity of feed intake to low dietary trp concentration (Lima, et al., 2012; Cardoso, et al., 2014; Dong and Zou, 2017). Although these authors reported decreased sensitivity of feed intake to trp concentration, the lowest trp concentration in these experiments was set from 0.150 to 0.167% trp, which was close to the break point concentration when feed intake was about to reach plateau in the dose response. According to the observation in the current experiment, reduced feed intake was observed when trp concentration was 0.156% or lower. This may suggest that decreased trp concentration only results in reduced feed intake when dietary trp concentrations are below 0.150 to 0.156% possibly due to unbalanced dietary amino acid concentrations.

Over the 19 wk experimental period, body weights were reduced resulting in a body weight loss in all treatments. This response is not unexpected and is most likely due to the change to experimental diets from control diets and the controlled feeding strategy applied over the experimental period. This controlled feeding strategy was used to ensure feed intake similar to commercial hens (Hy-Line Breeder Management Guide, 2015) and to minimize differences in

feed intake due to hen over consumption. The 0.116% dietary trp concentration resulted in the highest body weight loss of 402.5 g throughout nineteen-week period and body weight loss was decreased as dietary trp concentration increased. Even with the body weight loss, body weights of hens fed the diets that contained sufficient trp (0.176 and 0.196%) were at least equal to or above the breeder recommendations, while those diets below the trp requirement resulted in hens with body weights at or below the breeder recommendations. In hens fed ad libitum feed, an increase in body weight was reported as dietary trp concentration increased to sufficiency (Wethli and Morris, 1978; Bregendahl, et al., 2008). When dietary trp was sufficient, no change or slightly increased body weight were observed at the end of these experiments. These data support the current experiment despite the differences in feed availability as body mass was sacrificed in hens fed trp deficient diets not sufficient to support egg production and body mass.

Increasing dietary trp resulted in a quadratic response on egg weight ( $P \leq 0.01$ ). Although egg weights from birds fed the 0.136% trp diets were numerically lower than that in 0.116% treatment, egg weight generally increased as dietary trp concentration increased from 0.116 to 0.176% and reached a plateau thereafter. Similar observations concerning egg weights were reported with increasing dietary trp (Jensen, et al., 1990; Russell and Harms., 1999). In contrast, other reports have indicated no significant difference on egg weight across different dietary trp concentrations (Bregendahl, et al., 2008; Cardoso, et al., 2014). The differences in the literature may be explained by the magnitude of the trp deficiency as a wider dietary trp concentration range is more likely to induce significant difference on egg weight.

The differences in egg mass are more consistent with the differences on HHEP than the egg weight differences that were only slightly altered by dietary trp. Egg mass responded in a quadratic fashion as dietary trp increased. Increased dietary trp led to an improved FCR until the

dietary trp concentration reached 0.156% and no further improvements were observed. Similar observations were reported on egg mass and FCR in previous studies (Bregendahl, et al., 2008; Cardoso, et al., 2014).

### 3.4.2 Egg Quality and Composition

Haugh unit, an indicator of egg quality, decreased linearly ( $P \leq 0.05$ ) as dietary trp concentration increased (Table 3.3), although these responses were somewhat mixed as eggs from the 0.116 and 0.156% trp fed hens resulted in significantly higher Haugh unit than eggs from hens fed 0.136, 0.176 and 0.196% trp. No linear relationship between dietary trp concentration and Haugh unit has been reported previously. A mixed result as Haugh units were significantly increased by the 0.17 and 0.19% trp in comparison to the 0.15, 0.21 and 0.23% trp in diets (Dong and Zou, 2017). No differences on Haugh unit with hens fed different trp concentration diets (Mousavi et al., 2017). Although there was a linear response, the mixed data in the current experiment are consistent with previous reports in that there is no consistent relationship between dietary trp concentration and egg Haugh units. As all Haugh unit values were above 83, differences are not of practical significance. Relative yolk weight showed a quadratic response ( $P \leq 0.01$ ) as dietary trp concentration increased. Relative yolk weights in treatment 0.176 and 0.196% were significantly higher than that in treatments 0.116, 0.136 and 0.156%. A quadratic response on yolk weight as trp to lysine ratio increased from 19% to 27% was reported (Lima et al., 2012). In contrast, no differences in yolk weight were observed with various dietary trp concentrations in other reports (Calderano, et al., 2012; Cardoso, et al., 2014; Mousavi, et al., 2017). Relative shell weights in hens fed 0.156% trp diets were significantly lower than eggs from hens fed the 0.136% diets. There were no significant differences across treatments on relative albumen weight and egg shell thickness. Overall, mixed results from

current study and previous research may suggest little or no effect of dietary trp concentration on egg component weights.

#### 3.4.3 Plasma Serotonin Concentration

Both linear and quadratic responses were fitted to the plasma serotonin concentration as dietary trp concentration increased. Birds fed diets containing 0.176% trp showed significantly higher plasma serotonin concentration than those fed diets containing 0.116%, and 0.136% trp (Table 3.3). Previous research indicated that adding 0.5% trp in laying hens diet could double plasma serotonin concentrations (Laycock and Ball, 1990). Since the dietary trp inclusion rate was much lower in current experiment than that in the Laycock and Ball (1990)'s report, the current approach might indicate a deficiency response while the former experiment suggests an increase in plasma serotonin with trp concentrations above the requirement. The current data do suggest that the plasma serotonin response is a deficient/sufficient response and not a good candidate for requirement determination.

#### 3.4.4 Tryptophan Requirement

Tryptophan requirement was estimated using linear broken line, quadratic broken line, quadratic polynomial and exponential model and all results are shown in Table 3.4. The linear broken line model is most commonly applied to estimate amino acid requirements of poultry (Pesti, et al., 2009). Nevertheless, this model has been criticized as it fails to describe a real biological response by assuming an abrupt cessation of response after a linear response (Mack, et al., 1999). Linear broken line models typically result in a lower amino acid requirement in comparison with other nonlinear curve models (Fisher, et al., 1973). In the current experiment, the trp requirement based on linear broken line model was lower than that from other models

evaluated including 153.2, 155.8 and 140.4 mg of trp per day based on HHEP, egg mass and FCR, respectively. Although mg of trp per hen per day is a common expression of the requirement, mg per gram egg mass produced has also been reported and these data suggest that 3.16 mg of trp is required per g egg mass. In similar research reports, linear broken line regression in Hy-Line W-36 laying hens (Harms and Russell, 2000) resulted in trp intake of 3.28 mg/g egg mass. In 1999, Russell and Harms reported a trp intake 3.10 mg/g egg mass in Hy-Line W-36 laying hens from 53 to 59 weeks of age. Calderano and coworkers (2012) estimated the trp requirement as 3.53 mg/g egg mass (these numbers were converted from a digestible value of 3.28 mg trp/g egg mass, as originally reported). Although several experiment agree with the current data, the literature is not in full agreement as in two other experiments, a lower trp requirement of 2.52 and 2.91 mg/g egg mass has been reported (Bray, 1969; Bregendahl, et al., 2008). Hen age was not reported in these two studies and digestible trp requirement was originally reported as 2.71 mg of trp/g egg mass (tryptophan digestibility: 93%) by Bregendahl and coworkers (2008). In Bray's (1969) experiment, single comb white leghorn laying hens were used and highest egg mass was 46.4 g/hen/d, which was 2.89 g/hen/d lower than 49.29 g/hen/d in current study. This lower trp requirement could be explained by lower laying hen performance of the experiment conducted in the 1960's. Similarly, in Bregendahl and coworkers' (2008) report, 48.68 g/hen/d egg mass may explain a relatively lower trp requirement in relationship to the current experiment.

The Quadratic broken line model features a smooth breakpoint and a plateau (Pesti, et al., 2009). The quadratic broken line model is a compromise between the linear broken line and quadratic polynomial models and resulted in a trp requirement of 182.3, 199.5 and 163.9 mg per day based on HHEP, egg mass and FCR, respectively. This model showed the best fit ( $R^2$ )

among the different models evaluated in this experiment. When a quadratic broken line model was fitted to the data, the break point was 182.3 mg per day of trp and the corresponding HHEP was 79.91%. Estimated trp requirement by quadratic broken line model was higher than that from the linear broken line model but close to quadratic polynomial model. Based on egg mass, estimated trp requirement was 4.02 mg trp/g of egg mass. No previous literature has reported trp requirement of laying hens using a quadratic broken line model.

Quadratic polynomial models represent a response with a curved ascending portion at a decreasing rate as nutrient concentrations increase and reach a maximum response without any plateau. This model reflects the diminishing gains in performance as the diets draw closer to the bird's requirement (Pesti, et al., 2009). In this experiment, trp requirement was estimated when 95% of the maximum response was achieved, resulting in a requirement estimate of 180.2, 186.9 and 182.1 mg trp/day based on HHEP, egg mass and FCR, respectively. In comparison with estimated trp requirement by linear broken line and quadratic broken line model, trp requirement by quadratic polynomial analysis based on FCR was higher. Ishibashi (1985) used quadratic polynomial models and estimated the trp requirement of white Leghorn laying hens as 212 mg per day based on egg mass from 60 to 64 weeks of age. In Dekalb White laying hens from 60 to 76 weeks of age, digestible trp requirement was 212 mg per day based on egg production (maximum egg mass was not reported) using the quadratic polynomial model (Cardoso, et al., 2014). In both of the above reports, 100% of maximum response was applied. By converting to 95% of maximum response, the trp requirement was 201 and 201 (digestible) mg per day, respectively. When comparing trp requirement on per gram of egg mass basis, the trp requirement in current study was 3.74 mg trp/g of egg mass which is higher than 3.66 mg trp/g of egg mass in Ishibashi's (1985) report.

Exponential models account for the response by the law of diminishing return and are able to determine small changes in the response (Mack, et al., 1999). Although, there is some belief that this model overestimates the requirement (Fuller and Garthwaite, 1993). In the current experiment, the exponential model estimated the trp requirement of 41 to 60 week old laying hens to be 0.235, 0.253 and 0.199% based on HHEP, egg mass and FCR, respectively (95% of the asymptotic response was applied). The exponential model indicates that laying hens need 4.41 mg of trp to produce one gram of egg mass. In comparison with other models used in this experiment, the exponential model overestimated the trp requirement even though a 95% asymptotic response was applied to help moderate the trp requirement estimate. No trp requirement data were estimated based on exponential model in previous laying hen research for comparison.

In this experiment, trp requirement of small-framed white leghorn egg laying hens was in the same range as reported in previous literature. Nevertheless, the dietary trp requirement varies as different models are used. From a fit ( $R^2$ ) perspective, HHEP and egg mass presented a higher  $R^2$  than FCR in all models. Among different models, quadratic broken line model presented a better fit than the other three models. No preference among the different models exists in the current experiment and analyzed data are provided to help people build their own opinion on trp requirement in laying hens. For producing one gram of egg mass, a white leghorn laying hen needs 3.16, 4.02, 3.74 and 4.41 mg trp based on linear broken line, quadratic broken line, quadratic polynomial and exponential model, respectively.

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Table 3.1 Composition of trp deficient basal diet. Tryptohan was added at 0.02% increments to generate experimental diets that contained 0.096, 0.116, 0.136, 0.156, 0.176, 0.196% trp.

Ingredient	(%)
Corn	73.33
Corn gluten meal	9.87
Soybean meal	1.87
Soy oil	0.41
Biolys <sup>® 1</sup>	1.00
L-arginine	0.38
DL-methionine	0.22
L-threonine	0.20
L-valine	0.20
L-isoluecine	0.20
Oyster shell (large particle)	4.71
Limestone (small particle)	4.71
Dicalcium Phosphate	1.53
Salt	0.27
Choline chloride	0.10
Mineral and vitamin premix <sup>2</sup>	0.50
Celite	0.50
<hr/>	
Analysis of basal diet	(%)
Crude protein	15.01
ME, kcal/kg (calculated)	2,975
Calcium (calculated)	4.00
Available phosphorus (calculated)	0.35
Methionine	0.458
Cysteine	0.254
Lysine	0.873
Histine	0.306
Tryptophan	0.096
Threonine	0.613
Arginine	0.854
Isoleucine	0.680
Leucine	1.819
Phenylalanine	0.734
Valine	0.768
Glycine	0.438
Serine	0.652

<sup>1</sup> Contains 54.6% L-lysine

<sup>2</sup> Provided per kg of diet: vitamin A, 4,403 IU; vitamin D<sub>3</sub>, 1,457 ICU; vitamin E, 1.10 IU; menadione, 0.77 mg; vitamin B<sub>12</sub>, 4.40 µg; choline, 254.79 mg; niacin, 13.21 mg; pantothenic acid, 4.05 mg; riboflavin, 2.75 mg; Cu, 2.70 mg; Fe, 33.75 mg; I, 0.67 mg; Mn, 42.90 mg; Zn, 32.50 mg; Co, 0.17mg.

Table 3.2 Hen-housed egg production (HHEP), body weight (BW) loss, feed intake, egg weight, egg mass, feed conversion ratio (FCR) and trp daily intake from 41-60 wk of birds fed diets containing 0.096, 0.116, 0.136, 0.156, 0.176 and 0.196% trp.

Dietary trp %	HHEP %	BW loss <sup>2</sup> g/hen	Feed intake g/hen/d	Egg weight g/egg/d	Egg mass g/hen/d	FCR g/g	Trp daily intake mg/hen/d	
0.096 <sup>1</sup>	-	-	-	-	-	-	-	
0.116	50.7 <sup>d</sup>	402.5 <sup>a</sup>	78.5 <sup>d</sup>	59.5 <sup>ab</sup>	31.4 <sup>d</sup>	2.76 <sup>a</sup>	91.1 <sup>e</sup>	
0.136	61.4 <sup>c</sup>	242.2 <sup>b</sup>	85.1 <sup>c</sup>	59.2 <sup>b</sup>	37.5 <sup>c</sup>	2.40 <sup>b</sup>	115.7 <sup>d</sup>	
0.156	74.4 <sup>b</sup>	211.2 <sup>bc</sup>	90.0 <sup>b</sup>	60.4 <sup>ab</sup>	44.9 <sup>b</sup>	2.04 <sup>c</sup>	140.3 <sup>c</sup>	
0.176	80.7 <sup>a</sup>	123.1 <sup>d</sup>	96.0 <sup>a</sup>	61.0 <sup>a</sup>	49.3 <sup>a</sup>	1.97 <sup>c</sup>	168.9 <sup>b</sup>	
0.196	81.0 <sup>a</sup>	152.4 <sup>cd</sup>	97.2 <sup>a</sup>	61.0 <sup>a</sup>	49.3 <sup>a</sup>	1.99 <sup>c</sup>	190.5 <sup>a</sup>	
Pooled SEM	0.85	25.5	0.55	0.42	0.45	0.034	1.38	
			P Value					
Tukey's	≤ 0.01	≤ 0.01	≤ 0.01	≤ 0.01	≤ 0.01	≤ 0.01	≤ 0.01	
Quadratic	≤ 0.01	≤ 0.01	≤ 0.01	≤ 0.01	≤ 0.01	≤ 0.01	≤ 0.01	

<sup>1</sup>The 0.096 treatment was removed from the experiment due to low egg production.

<sup>2</sup>This is the body weight loss at 60 weeks of age in comparison with initial body weight at 41 weeks of age. P value came from Fisher's LSD test.

<sup>a-d</sup>Least square means without a common superscript differ significantly ( $P \leq 0.05$ ).

Table 3.3 Egg quality, egg composition and plasma serotonin from 41-60 wk of birds fed diets containing 0.096, 0.116, 0.136, 0.156, 0.176 and 0.196% trp.

Dietary trp	Haugh unit <sup>1</sup>	Yolk weight	Albumen weight	Shell weight	Egg shell thickness	Plasma serotonin
%		%	%	%	mm	ng/ml
0.096 <sup>2</sup>	-	-	-	-	-	-
0.116	85.9 <sup>a</sup>	29.2 <sup>b</sup>	53.6	9.2 <sup>ab</sup>	0.31	3.14 <sup>b</sup>
0.136	83.1 <sup>b</sup>	29.3 <sup>b</sup>	54.6	9.3 <sup>a</sup>	0.32	3.22 <sup>b</sup>
0.156	84.9 <sup>a</sup>	29.1 <sup>b</sup>	55.4	9.1 <sup>b</sup>	0.31	3.70 <sup>ab</sup>
0.176	83.5 <sup>b</sup>	30.0 <sup>a</sup>	55.1	9.2 <sup>ab</sup>	0.31	3.87 <sup>a</sup>
0.196	83.1 <sup>b</sup>	30.1 <sup>a</sup>	54.6	9.3 <sup>ab</sup>	0.32	3.76 <sup>ab</sup>
Pooled SEM	0.38	0.21	0.55	0.04	0.002	0.172
			P Value			
Tukey's	≤0.01	≤0.01	0.24	≤0.01	0.29	≤0.01
Quadratic	0.06	≤0.01	0.06	0.85	0.96	≤0.01
Linear	0.02	≤0.01	0.02	0.70	0.94	≤0.01

<sup>1</sup> Haugh unit =  $100 \cdot \log(h - 1.7 \cdot w^{3.7} + 7.6)$ ; h = observed height of the albumen in millimeters; w = egg weight in grams

<sup>2</sup> The 0.096 treatment was removed from the experiment due to low egg production.

<sup>a-b</sup> Least square means without a common superscript differ significantly ( $P \leq 0.05$ ).

Table 3.4 Trp requirement estimated from linear broken line, quadratic broken line, quadratic polynomial and exponential model based on hen-housed egg production (HHEP), egg mass and FCR.

Data set	Model	Independent variables	Trp requirement*	Ordinate value of requirement	Regression	R <sup>2</sup>
HHEP (%)	linear broken line	trp daily intake	153.2	80.86	$y = 80.86 + 0.4824(x - 153.2)$	0.89
	quadratic broken line	trp daily intake	182.3	79.91	$y = 79.91 - 0.003533(x - 182.3)^2$	0.92
	quadratic polynomial	trp daily intake	180.2 <sup>#</sup>	81.70	$y = -0.003202x^2 + 1.214x - 33.57$	0.89
	exponential	trp concentration	0.235% <sup>#</sup>	87.20	$y = 50.27 + 36.93(1 - e^{-2518(x - 0.116\%)})$	0.88
Egg mass(g/hen/d)	linear broken line	trp daily intake	155.8	49.29	$y = 49.29 + 0.2891(x - 155.8)$	0.90
	quadratic broken line	trp daily intake	199.5	49.68	$y = 49.68 - 0.001627(x - 199.5)^2$	0.91
	quadratic polynomial	trp daily intake	186.9 <sup>#</sup>	50.04	$y = -0.001765x^2 + 0.6766x - 17.06$	0.89
	exponential	trp concentration	0.253% <sup>#</sup>	54.55	$y = 30.25 + 24.30(1 - e^{-2179(x - 0.116\%)})$	0.86
FCR (g/g)	linear broken line	trp daily intake	140.4	1.99	$y = 1.988 + 0.01598(140.4 - x)$	0.77
	quadratic broken line	trp daily intake	163.9	2.01	$y = 2.012 + 0.0001437(163.9 - x)^2$	0.77
	quadratic polynomial	trp daily intake	182.1 <sup>#</sup>	1.95	$y = 4.670 - 0.02842x + 0.00007415x^2$	0.74
	exponential	trp concentration	0.199% <sup>#</sup>	1.89	$y = 2.787 - 0.8929(1 - e^{-3611(x - 0.116\%)})$	0.74

\* Unit is mg/d when using trp daily intake and % when using trp concentration.

<sup>#</sup> Abscissa of the point on the fitted curve whose ordinate was 95% of the upper asymptote

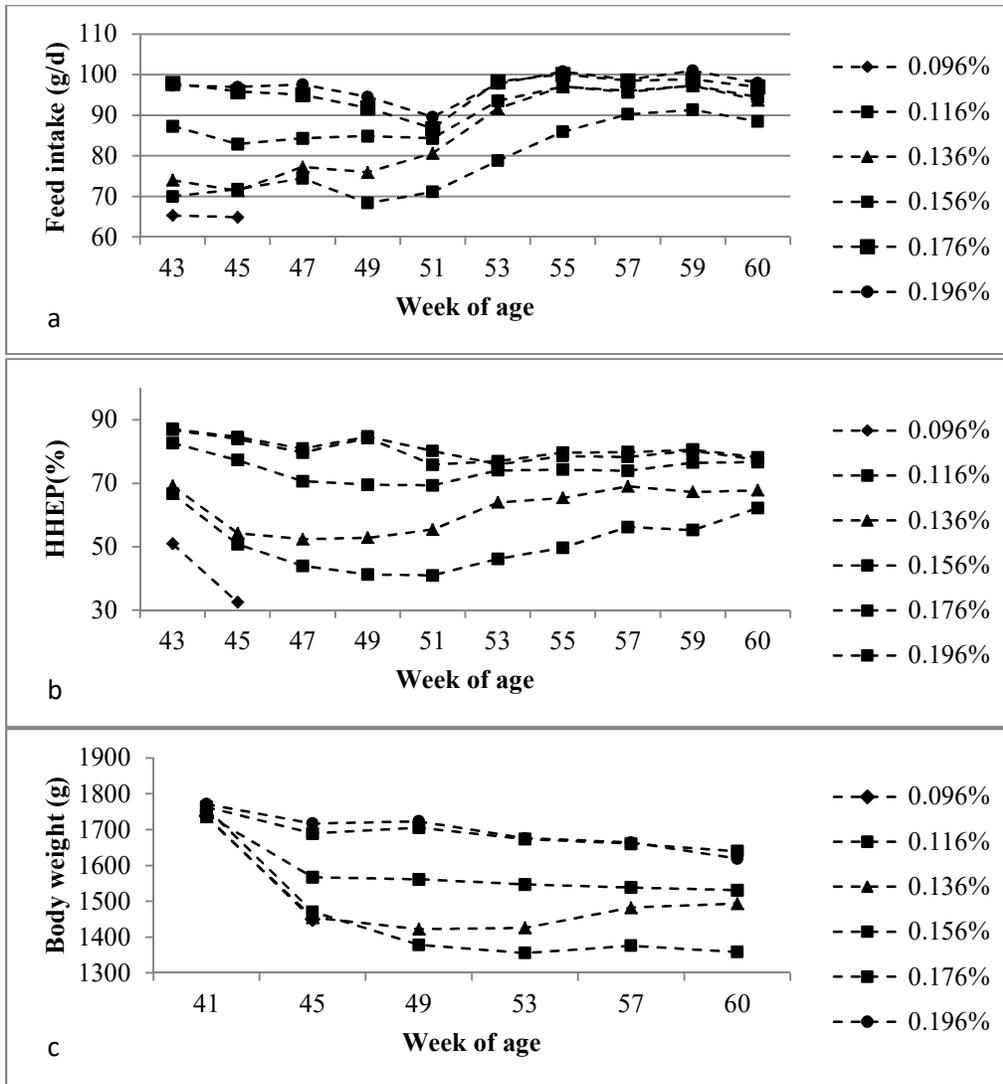


Figure 3.1 (a) feed intake, (b) hen-housed egg production (HHEP), and (c) body weight change from 41-60 wk of birds fed diets containing 0.096, 0.116, 0.136, 0.156, 0.176 and 0.196% trp respectively. Treatment 0.096% was halted after 45 weeks of age.

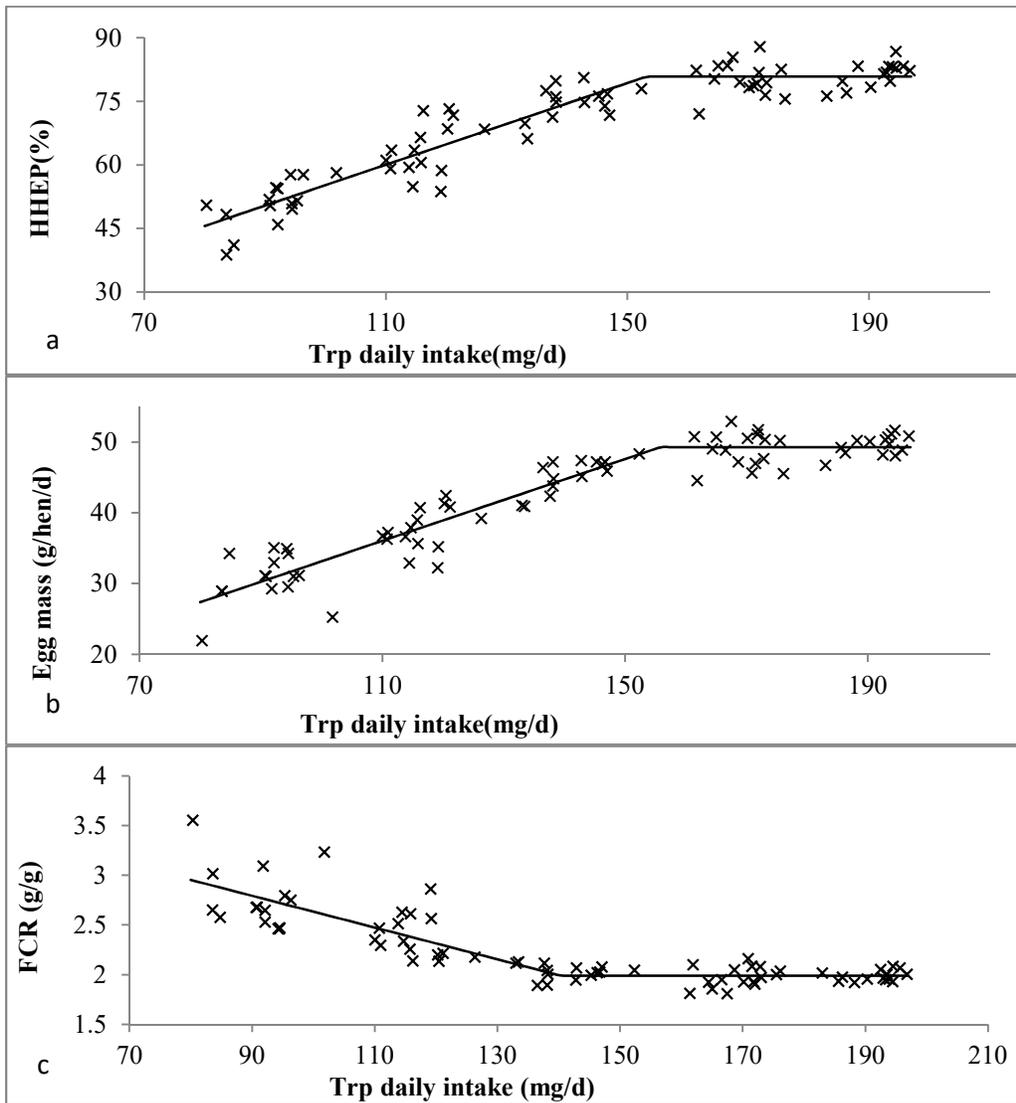


Figure 3.2 Trp requirement estimation using linear broken line regression based on (a) hen housed egg production (HHEP) [ $y=80.86 + 0.4824(x - 153.2)$ ,  $R^2=0.89$ ], (b) egg mass [ $y=49.29 + 0.2891(x - 155.8)$ ,  $R^2=0.90$ ] and (c) feed conversion ratio (FCR) [ $y=1.988 + 0.01598(140.4 - x)$ ,  $R^2=0.77$ ].

## Chapter 4: Evaluation of the Valine Requirement of Small-framed First Cycle Laying Hens

### 4.1 Abstract

An experiment was conducted to evaluate the total valine (val) requirement of first cycle laying hens from 41 to 60 weeks of age. A total of 270 Hy-line W-36 laying hens were randomly assigned to 6 treatments with 15 replicate groups of 3 birds for each experimental unit. A val deficient basal diet was formulated with corn and peanut meal with analyzed val, lysine and crude protein concentrations of 0.515, 0.875, and 13.38%, respectively. Synthetic L-val was supplemented to the basal diet in 0.070% increments to generate experimental diets containing 0.515, 0.585, 0.655, 0.725, 0.795, and 0.865% val respectively. A controlled feeding program was applied during the experiment resulting in approximately 95 g feed intake per hen per day. Linear broken line, quadratic broken line, quadratic polynomial and exponential models were used to estimate the val requirement of the hens based on hen-housed egg production (HHEP), egg mass (EM), and feed conversion ratio (FCR). Hen-housed egg production ranged from 48.3 to 81.4%, dependent upon dietary concentration of val. Valine requirements estimated by linear broken line, quadratic broken line, quadratic polynomial and exponential models were reported. Using the linear broken line model, the val requirement was highest for egg mass, 597.3 mg/d, followed by egg production, 591.9 mg/d and lowest for FCR, 500.5 mg/d.

Key words: laying hen, valine, egg production, first cycle, requirement

## 4.2 Introduction

As a nonpolar amino acid, val is one of the most hydrophobic amino acids (Brosnan and Brosnan, 2006). Valine, leucine and isoleucine are called branched chain amino acids (BCAA) due to the branched chain structures of their R groups. In animals, val serves as a precursor for the synthesis of protein and other amino acids (Ferrando, et al., 1995). As a glucogenic amino acid, val is a substrate for glutamine synthesis which is involved in the Krebs cycle (Wu, 2009).

It has been reported that val may potentially be the next limiting amino acids for laying hens, after methionine, lysine, tryptophan and threonine (Lelis, et al., 2014). Nevertheless, research on the val requirement of commercial white-egg laying hens has been limited. The NRC (1994) suggests 700 mg total val per hen per day is required for white-egg laying hens during peak production. Harms and Russell (2001) estimated total val requirement of 41 to 47 weeks old Hy-Line W-36 laying hens as 619.0 mg/d based on 47.2 g of egg content production. Digestible val requirement was 501.3 mg/d based on 49.4 g of egg mass in Hy-line W-36 laying hens from 26 to 34 weeks of age (Bregendahl, et al. 2008). In brown laying hens, total val requirements were 585, 735 and 634 mg/d in Lohmann Brown hens from 25 to 32, 24 to 32 and 46 to 54 weeks of age using linear broken line model in their consecutive experiments Peganova and Eder (2002). Digestible val requirement in Dekalb Brown laying hens from 43 to 54 weeks of age was 567.0 mg/d by averaging results from 100% response quadratic, 95% response quadratic, linear broken line and quadratic broken line models (Lelis et al., 2014). In this experiment, the total val requirement in small-framed first-cycle laying hens (41 to 60 weeks of age) was estimated based upon performance using linear broken line, quadratic polynomial, quadratic broken line and exponential models.

## 4.3 Materials and methods

### 4.3.1 Diets

Corn and peanut meal were selected as major ingredients to generate a val deficient basal diet. Amino acid concentrations of corn and peanut meal were determined before dietary formulation (University of Missouri AESCL, Columbia, MO 65211). After the basal diet was mixed, basal diet samples were sent for amino acid analysis (Table 4.1) (Evonik GMBH, Hanau, Germany). In the basal diet, val concentration was 0.515% and this result confirmed a val deficient diet in comparison with val requirement of 0.740% as reported by NRC (1994). Dietary leucine and isoleucine concentrations were determined as 1.024% and 0.699%, respectively. The concentrations of leucine and isoleucine were not expected to generate an antagonism with val or impact the requirement estimation (Bray, 1970; Allen and Baker, 1972). Synthetic L-val (96.5% feed grade, Ajinomoto Heartland, Inc.) was supplemented to the basal diet in 0.070% increments, resulting in experimental diets containing 0.515, 0.585, 0.655, 0.725, 0.795 and 0.865% val, respectively. During the experiment, experimental diets were mixed every two weeks from the same group of experimental ingredients.

### 4.3.2 Animals and Housing

All animal work undertaken in this experiment was approved by the Institutional Animal Care and Use Committee at Virginia Tech (Blacksburg, VA). A laying hen flock of 600 Hy-line W-36 laying hens were reared from day of hatch to mature laying hens using multiphase diets and management as recommended in the Hyline W-36 Commercial Layers Management Guide (2015). At 41 weeks of age, 270 hens were evaluated for overall good health and high production (average egg production: 90.8%) before being selected for inclusion in the experiment. Hens

were assigned to 6 treatments with 15 replicates of 3 birds/cage (464.5 cm<sup>2</sup>/cage) over A-frame battery cage units. Hens were provided with approximately 95g/hen/d feed and ad libitum access to water. Birds were housed in dark-out housing conditions and provided a 16:8 lighting schedule for the duration of the experiment. House temperatures were controlled via a fan and air-inlet ventilation system and ranged from 70 to 80 °F over the experiment. The hen-housed egg production (HHEP) for the two week period before initiation of the experiment (39 and 40 weeks of age) ranged from 90.3-90.5% across the randomly allocated treatments (P=0.99).

Over the 19 week experimental period, laying hens were visually inspected at least twice daily, at the morning check, eggs were collected and feed provided at approximately 10am, and a follow up check was provided after 4pm each day. Egg numbers and mortality were recorded daily, but feed intake, HHEP and FCR were calculated to correspond with bi-weekly feed manufacture periods. Each week, all eggs produced on Tuesday and Wednesday were collected for egg weight measurements, again calculated on a bi-weekly basis to correspond with feed manufacture periods. Meanwhile, thirty eggs from each treatment (two eggs from two consecutive days in each experimental unit) were stored for Haugh unit, relative albumen weight, relative yolk weight, relative shell weight and egg shell thickness determination and egg quality indices. Body weight data were collected every four weeks.

#### 4.3.3 Statistical Analysis

SAS 9.4 (SAS Institute Inc, Cary, NC) was used to analyze hen housed egg production (HHEP), feed intake, egg weight, egg mass, feed conversion rate (FCR), daily val intake, Haugh unit, relative albumen weight, relative yolk weight, relative shell weight and shell thickness using a one-way ANOVA with means separated by repeated measures using a Tukey ' s

adjustment. Body weight losses at the end of 60 weeks of age were analyzed using a one-way ANOVA with Fisher's LSD test. Significance was accepted at  $P \leq 0.05$ . Laying hen val requirements were calculated by fitting regression based on average HHEP, egg mass and FCR over the entire duration of the experiment using nonlinear models (linear broken line, quadratic broken line, quadratic polynomial and exponential model) in JMP (SAS Institute Inc, Cary, NC) described by Bregendahl, et al. (2008). Daily val intake was the independent variable in linear broken line, quadratic broken line and quadratic polynomial models and val concentration was the independent variable in exponential model to estimate requirement. The reason why percentage val was applied in exponential model was due to the limitation of the exponential model which can only estimate val requirement on a percentage basis. In both quadratic polynomial and exponential models, val requirements were estimated when 95% of maximum response was achieved. As 90 to 100% of the maximum quadratic response was typically presented to quantify a response in published reports, we chose the median level, 95%, to estimate requirements (Peganova and Eder, 2002; Kidd and Tillman, 2016).

The equation of the linear broken line model was:

$$y = a - b * (x - c) \text{ for } x \leq c \text{ and}$$

$$y = a \text{ for } x > c$$

where  $y$  = response criteria,  $a$  = ordinate of the breakpoint,  $b$  = slope of the line for  $x \leq c$ ,  $c$  = abscissa of the breakpoint (= requirement), and  $x$  = val daily intake.

The equation of the quadratic broken line model was:

$$y = a - b(x - c)^2 \text{ for } x \leq c \text{ and}$$

$$y = a \text{ for } x > c$$

where  $y$  = response criteria,  $a$  = ordinate of the breakpoint,  $b$  = coefficient of quadratic term for  $x \leq c$ ,  $c$  = abscissa of the breakpoint (= requirement), and  $x$  = val daily intake.

The equation of the quadratic polynomial model was:

$$y = ax^2 + bx + c$$

where  $y$  = response criteria,  $a, b, c$  are the coefficients of quadratic equation,  $x$  = val daily intake.

The equation of the exponential model was:

$$y = a + b * (1 - e^{-c * (x - d)})$$

where  $y$  = response criteria,  $a$  = intercept (performance of the lowest val concentration diet in the model),  $b$  = maximum response due to increased val concentration,  $c$  = slope,  $d$  = val concentration of the lowest val concentration diet, and  $x$  = val concentration of the diet.

## 4.4 Results and Discussion

### 4.4.1 Laying Hen Performance

In the current experiment, quadratic responses were observed on HHEP, body weight loss, feed intake, egg mass, FCR and val daily intake by feeding different concentration val diets to laying hens (Table 4.2). Average feed intake increased from 66.5 to 92.9 g/hen/d as val concentration increased from 0.515 to 0.725% and a plateau was reached after 0.725% val concentration. After an initial drop from 41 to 50 weeks of age, feed intake in 0.515 and 0.585% treatments recovered after 51 weeks of age but could not fully overcome the val deficiency (Figure 1-a). Laying hens fed 0.725, 0.795 and 0.865% val diets maintained a relatively stable feed consumption at approximate 92 g/hen/d throughout the duration of experiment. The dose response of feed intake in the current experiment was consistent with those reported previously (Harms and Russell, 2001; Peganova and Eder, 2002; Lelis, et al., 2014). Feed intake reduction is a typical response of laying hens to val deficient diets. Harms and Russell (2001) fed deficient to adequate val diets (0.525 to 0.765%) to Hy-line W-36 laying hens and feed intake ranged from 81.2 to 96.2 g/hen/d. In Peganova and Eder's three brown layer trials (2002), the lowest val diets, 0.44% val, induced a low feed intake at 68 g/hen/d in hens from 24 to 32 weeks of age during their first experiment. In the second and third experiment, the lowest val concentration of 0.51% led to feed intakes between 109 to 110 g/h/d which was 4 to 7 g lower than the maximum feed intake. Although the lowest val concentrations were only slightly reduced between their first trial

and subsequent experiments (0.44 vs 0.51%), feed intake was more adversely affected in the first experiment. In current experiment, the lowest val concentration diet (0.515%) led to a feed intake of 66.5 g/hen/d. This value was lower than that in Harms and Russell's experiment (2001) and Peganova and Eder's experiments (2002) when similar val concentration diets were fed to laying hens. Differences in macro ingredients (peanut meal v. soybean meal) and crude protein of the basal diets may explain these feed intake differences.

Average HHEP increased significantly as dietary val increased from 0.515% to 0.865%, with a plateau occurring after the 0.725% treatment over the 19 week period. After the onset of experiment, HHEP of 0.515% val treatment dropped dramatically during the first 10 weeks to lower than 40% (Figure 4.1-b). An adaptation on deficient val was observed in 0.515% treatment as HHEP rebounded after 51 weeks of age; however, no such adaptation occurred in 0.585% or 0.665% val treatments. Decreased egg production caused by a val deficient diet has been observed in previous experiments. Harms and Russell (2001) supplemented dietary val from 0.525 to 0.765% in Hy-line W-36 laying hens rations from 39 to 46 weeks of age and egg production was lowest in 0.525% val treatment at 77.9%. Egg production was around 50% (exact egg production was not reported) in Hy-line W-36 laying hens fed 0.47% digestible val diet from 26 to 34 weeks of age (Bregendahl et al.,2008). Since val is an important component for protein synthesis in egg, insufficient dietary val reducing egg production is not surprising (Lewis, et al., 1950).

Body weight loss was observed in all treatments as compared to initial body weight. The 0.515% dietary val concentration induced the highest body weight loss of 516.7 g/hen by the end of the experiment and this was decreased as dietary val concentration increased from 0.515 to 0.725%. Birds were fed diets containing 0.795 and 0.865% val, body weight loss showed

numeric increases but no significant differences in comparison to the hens fed the 0.725% val treatment. Body weight loss was found in all treatments, most likely due to the change to experimental diets and controlled feeding strategy applied over the experimental period. This controlled feeding was used to ensure feed intake similar to commercial hens (in the Hyline W-36 Commercial Layers Management Guide, 2015). Body weight of laying hens fed 0.515% val diet dropped dramatically after receiving experimental diet and no further decrease was observed after 53 weeks of age (Figure 4.1-c). Meanwhile, an increase of both feed intake and HHEP were observed after 53 weeks of age indicating the possibility of adaptation to the low val diets may be occurring. The improvement in HHEP before an increase in body weight gain may indicate that egg production is conserved before body weight at least under the body weights observed in this experiment.

Egg weight increased from 56.5 to 62.1 g/egg as dietary val increased from 0.515 to 0.725%. It is interesting to note that a reduced egg weight was observed as birds were fed diets containing 0.795 and 0.865% val. The quadratic response on egg weight as increased dietary val concentration was consistent with the response reported by Peganova and Eder (2002). In their experiment, egg weight kept increasing as dietary digestible val concentration increased to 0.74%. When additional val was supplemented in these diets (digestible val of 0.80 and 0.86%, respectively), a numeric decrease in egg weight was observed. Harms and Russell (2001) reported a reduced egg weight by feeding diets containing 0.525, 0.560, 0.595, 0.630% val to laying hens and egg weight was improved as dietary val increased to 0.665, 0.700 and 0.765%. Unlike current and other reported data, egg weights were not reduced with excess val in this experiment. Valine requirements based on egg weight were estimated in previous studies (Harms

and Russell, 2001; Bregendahl, et al., 2008). In the current experiment, although egg weight fitted a quadratic response, val requirement was not reported due to a poor fit ( $R^2=0.37$ ).

Egg mass and FCR responded quadratically as dietary val increased. Laying hens fed 0.515% val produced 27.9 g egg mass per day. As hens were fed diets with additional val, egg mass significantly increased until 0.725% val was reached. A slight decrease in egg mass was observed as dietary val concentrations climbed to 0.795 and 0.865%, most likely due to the numerically reduced egg weights associated with these treatments. Laying hens fed diet containing 0.515% val showed a FCR of 2.62. FCR was improved from 2.62 to 1.89 as dietary val concentration increased from 0.515 to 0.725%. A plateau was achieved as more val was added to diets after 0.725% val on FCR. Similar responses were reported previously for both egg mass and FCR (Bregendahl, et al., 2008; Harms and Russell, 2001; Lelis, et al., 2014; Peganova and Eder, 2002).

#### 4.4.2 Egg Quality and Composition

Egg Haugh unit responded quadratically as dietary val increased. Eggs from hens fed 0.515% val showed the highest Haugh unit across all treatments (Table 4.3). As val concentration increased, Haugh unit decreased from 86.6 to 82.6. In contrast, Peganova and Eder (2002) found no difference on Haugh unit by various dietary val concentrations. Haugh unit, an egg quality indicator, can be affected by factors such as bird age, molting, temperature, lighting and housing system (Travel, et al., 2011); however, various dietary val concentrations have not been reported to affect Haugh unit. Since all Haugh unit values were above 82, the differences are noted, but might be questionable from a practical standpoint as these were all high protein quality eggs. Higher Haugh unit was observed when laying hens were fed diets containing 14.7%

crude protein than those fed 16.7% crude protein (Harms and Douglas, 1960). There were small differences in dietary crude protein in this experiment, but the relative differences were small enough to discount dietary protein as a major factor in Haugh unit response. Relative yolk weight result of eggs generated by hens fed various val concentrations was mixed. Eggs from 0.515 and 0.795% val treatments showed a significant higher Relative yolk weight than those in 0.725% with that of other eggs being intermediate. Relative albumen weight in 0.515% val treatment was significantly lower than those in other five treatments and a quadratic response was observed as dietary val concentration increased. Egg relative shell weight in treatment 0.515% was significantly higher than those in other treatments. Shell thickness in the eggs from hens fed 0.585% val was lower than those in other treatments ( $P \leq 0.01$ ). No significant difference on relative yolk, relative shell weight and relative albumen weight were reported when digestible val concentration varied from 0.555 to 0.666% in laying hen diets Lelis and coworkers (2014). In the current experiment, egg weight in the lowest val concentration treatment was significantly lower than that in the other treatments, while there were no differences in egg weight with different dietary concentration in Lelis and coworkers (2014)'s study. A dramatic impact on egg weight by the most deficient val treatment in the current experiment may explain the increased relative shell weight and reduced relative albumen weight. As the quantity of shell deposited on the shell membrane is generally not related to overall egg weight/size (Roland, 1980), the lower egg weight probably resulted in a relatively higher shell weight. These data may also indicate that albumen weight is a more sensitive indicator of val status than relative yolk weight.

#### 4.4.3 Valine Requirement

Antagonism may exist among branch chain amino acids due to their similar molecule structure. Excess dietary leucine induces growth-depressing effect in several species (Harper, et

al., 1984). High leucine intake increases the activity of branched chain  $\alpha$ -keto acid dehydrogenase, the key enzyme involved in degradation of leucine, isoleucine and val (Harris et al., 2001). Increased activity of branched chain  $\alpha$ -keto acid dehydrogenase leads to an increased oxidation of val and isoleucine resulting in a reduced efficacy of these amino acids (Smith and Austic, 1978). In the current experiment, the leucine concentration in experimental diets was 1.024%, which was higher than the NRC leucine requirement at 0.86% (adjusted feed intake as 95 g/d). Increased dietary leucine resulted in decreased val efficiency of utilization in chicks and that val efficacy reached a minimum value of 74% of normal with a 5.57% increase in dietary leucine in broilers (basal diet contained 1.20% leucine) (Allen and Baker, 1972). The leucine concentration in the current experiment was lower than the leucine concentrations that generate antagonism and an impact on val requirement by dietary leucine should not be expected. Therefore, we conclude that no performance depression was observed by dietary leucine concentration on HHEP, feed intake or egg weight in val sufficient treatments in the current experiment.

The val requirement was estimated based on HHEP, egg mass and FCR using linear broken line, quadratic broken line, quadratic polynomial and exponential model. In the linear broken line model, optimum val requirement was 591.9 mg per day as the maximum HHEP was 80.24% (Table 4.4). Based on egg mass and FCR, the optimum requirement was 597.3 and 500.5 mg per day. Across all models, the val requirement estimated by linear broken line was lowest. This agrees with the demonstration that linear broken line model typically resulted in a lower amino acid requirement in comparison with other nonlinear curve models (Fisher, et al., 1973). Daily egg mass has been of particular interest in the determination of the val requirement of laying hens due to its economic importance (Peganova and Eder, 2002). In the current

experiment, after conversion to val requirement per gram egg mass production, the requirement was 12.2 mg/g egg mass. In similar research reports, linear broken line regression in Hy-line W-36 laying hens (Harms and Russell, 2001) resulted in val intake of 11.0 mg/g egg mass (originally reported as 13.1 mg/g egg content). Bregendahl and others (2008) estimated val requirement as 11.7 mg/g egg mass in Hy-line W-36 laying hens from 26 to 34 weeks of age (digestible value was originally reported as 10.1 mg/g egg mass and 86% digestibility was applied to calculate total requirement). The estimated requirement from our study was close but slightly higher than requirements from other studies evaluating Hy-line W-36 val requirement. The slightly differences on val requirement may be explained by different laying hen performance and hen age. In brown layers, Valine requirement was 13.3, 22.7 and 11.1 mg/g egg mass in three different trials at different ages (experiment 1: 24 to 32 weeks of age; experiment 2: 25 to 32 weeks of age; experiment 3: 46 to 52 weeks of age) (Peganova and Eder, 2002). With the exception of the second experiment, the 13.3 and 11.1 mg/g egg mass requirement was similar to the estimation in the current experiment.

Using a quadratic broken line model, optimum val requirement was estimated as 691.4, 719.7 and 650.0 mg per day based on HHEP, egg mass and FCR, respectively. After conversion, val requirement was 14.5 mg/g egg mass. As expected, this value was higher than that estimated from linear broken line model. The quadratic feature of the model tended to overestimate the requirement.  $R^2$  was 0.92 indicating the best fit across different models. Lelis and others (2014) estimated val to lysine ratio as 0.9028 in Dekalb brown layers from 43 to 54 weeks of age using the quadratic broken line model. The val requirement was 648 mg per day based on egg production (86% digestibility was applied from Bregendahl, et al.; 2008) which was slightly lower than the current experiment.

Using a quadratic polynomial model, optimum val requirement was 697.5, 675.2 and 648.3 mg per day based on HHEP, egg mass and FCR, respectively. Lelis and others (2014) estimated val requirement as 651 mg based on egg production (86% digestibility was applied from Bregendahl, et al., (2008)).

An exponential model was fitted using the HHEP, egg mass and FCR data sets resulting in a val requirement estimates of 0.746, 0.744 and 0.646%, respectively. The val requirement was 693.0, 691.2 and 600.1 mg per hen per day (using a feed intake of 92.9 g per day in this calculation). The val requirement estimated from egg mass was close to 0.72% in Peganova and Eder's (2002) second experiment but higher than 0.65% and 0.60% in their first and third experiments. Requirements estimated from exponential models were higher than those from using a linear broken line approach. The higher requirements generated by fitting exponential models were in agreement with that from Peganova and Eder (2002). As reported previously, the val requirement for optimum egg mass using the exponential model, was 8, 13 and 5% higher than the same estimate using broken line models. In the current experiment, the val requirement from an exponential model was 15.7% higher than a linear broken line model when based on egg mass.

Valine requirement estimated from FCR was lower than those from HHEP and egg mass in all models. A reduced val requirement was reported based on FCR data in comparison to other performance parameters (Peganova and Eder, 2002).

In conclusion, different dietary val concentrations induce a dose response on HHEP, body weight loss, feed intake, egg weight, egg mass and FCR. Hy-line W-36 laying hens from 41 to 60 weeks of age need 12.2, 14.5, 13.6 and 14.0 mg val per day to produce one gram of egg

mass using linear broken line, quadratic broken line, quadratic polynomial and exponential models, respectively.

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Table 4.1 Composition of Val-deficient basal diet. Valine was added at 0.07% increments to generate experimental diets that contained 0.515, 0.585, 0.655, 0.725, 0.795, 0.865% Val.

Ingredient	(%)
Corn	69.24
Peanut meal	13.61
Soy oil	3.31
Biolys <sup>® 1</sup>	0.94
DL-methionine	0.35
L-threonine	0.25
L-tryptophan	0.09
L-isoleucine	0.27
Oyster shell (large particle)	4.73
Limestone (small particle)	4.73
Dicalcium Phosphate	1.55
Salt	0.33
Choline chloride	0.10
Mineral and vitamin premix <sup>2</sup>	0.50
Analysis of basal diet	
	(%)
Crude protein	13.38
ME, kcal/kg (calculated)	2,975
Calcium (calculated)	4.00
Available phosphorus (calculated)	0.35
Methionine	0.511
Cysteine	0.194
Lysine	0.875
Histine	0.293
Tryptophan	0.189
Threonine	0.591
Arginine	1.015
Isoleucine	0.658
Leucine	1.024
Phenylalanine	0.576
Valine	0.515
Glycine	0.590
Serine	0.561

<sup>1</sup>Contain 54.6% L-lysine

<sup>2</sup>Provided per kg of diet: vitamin A, 4,403 IU; vitamin D<sub>3</sub>, 1,457 ICU; vitamin E, 1.10 IU; menadione, 0.77 mg; vitamin B<sub>12</sub>, 4.40 µg; choline, 254.79 mg; niacin, 13.21 mg; pantothenic acid, 4.05 mg; riboflavin, 2.75 mg; Cu, 2.70 mg; Fe, 33.75 mg; I, 0.67 mg; Mn, 42.90 mg; Zn, 32.50 mg; Co, 0.17mg.

Table 4.2 Hen-housed egg production (HHEP), Body weight loss, feed intake (FI), egg weight (EW), egg mass (EM), feed conversion ratio (FCR) and val daily intake from 41-60 wk of birds fed diets containing 0.515, 0.585, 0.655, 0.725, 0.795 and 0.865% val respectively.

Dietary Val %	HHEP %	BW loss <sup>1</sup> g/hen	Feed intake g/hen/d	Egg weight g/egg/d	Egg mass g/hen/d	FCR g/g	Val daily intake mg/hen/d	
0.515	48.3 <sup>d</sup>	516.7 <sup>a</sup>	66.5 <sup>d</sup>	56.5 <sup>c</sup>	27.9 <sup>e</sup>	2.62 <sup>a</sup>	342.3 <sup>f</sup>	
0.585	69.1 <sup>c</sup>	391.2 <sup>b</sup>	80.5 <sup>c</sup>	59.6 <sup>b</sup>	41.1 <sup>d</sup>	2.05 <sup>b</sup>	471.1 <sup>e</sup>	
0.655	73.9 <sup>b</sup>	285.5 <sup>c</sup>	86.0 <sup>b</sup>	60.4 <sup>b</sup>	44.6 <sup>c</sup>	1.98 <sup>bc</sup>	563.0 <sup>d</sup>	
0.725	79.4 <sup>a</sup>	144.9 <sup>d</sup>	92.9 <sup>a</sup>	62.1 <sup>a</sup>	49.7 <sup>a</sup>	1.89 <sup>c</sup>	673.2 <sup>c</sup>	
0.795	81.4 <sup>a</sup>	188.7 <sup>d</sup>	92.2 <sup>a</sup>	60.0 <sup>b</sup>	48.8 <sup>ab</sup>	1.91 <sup>c</sup>	732.9 <sup>b</sup>	
0.865	80.2 <sup>a</sup>	207.0 <sup>d</sup>	91.6 <sup>a</sup>	60.0 <sup>b</sup>	47.3 <sup>b</sup>	1.92 <sup>bc</sup>	792.4 <sup>a</sup>	
Pooled SEM	0.85	24.9	0.60	0.31	0.55	0.031	6.03	
			P Value					
Tukey's	≤0.01	≤0.01	≤0.01	≤0.01	≤0.01	≤0.01	≤0.01	
Quadratic	≤0.01	≤0.01	≤0.01	≤0.01	≤0.01	≤0.01	≤0.01	

<sup>1</sup>This is the body weight loss at 60 weeks of age in comparison with initial body weight at 41 weeks of age. P value came from Fisher's LSD test.

<sup>a-f</sup>Least square means without a common superscript differ significantly ( $P \leq 0.05$ ).

Table 4.3 Egg quality and egg composition from 41-60 wk of birds fed diets containing 0.515, 0.585, 0.655, 0.725, 0.795 and 0.865% val.

Dietary Val	Haugh Unit <sup>1</sup>	Yolk weight	Albumen weight	Shell weight	Egg shell thickness
%		%	%	%	Mm
0.515	86.6 <sup>a</sup>	30.2 <sup>a</sup>	51.0 <sup>b</sup>	9.6 <sup>a</sup>	0.32 <sup>a</sup>
0.585	85.9 <sup>a</sup>	30.3 <sup>ab</sup>	54.8 <sup>a</sup>	9.1 <sup>b</sup>	0.30 <sup>b</sup>
0.655	84.1 <sup>b</sup>	30.1 <sup>ab</sup>	54.6 <sup>a</sup>	9.2 <sup>b</sup>	0.31 <sup>a</sup>
0.725	82.9 <sup>bc</sup>	29.6 <sup>b</sup>	54.8 <sup>a</sup>	9.2 <sup>b</sup>	0.32 <sup>a</sup>
0.795	82.6 <sup>c</sup>	30.5 <sup>a</sup>	55.0 <sup>a</sup>	9.3 <sup>b</sup>	0.32 <sup>ab</sup>
0.865	82.6 <sup>c</sup>	30.2 <sup>ab</sup>	55.3 <sup>a</sup>	9.2 <sup>b</sup>	0.31 <sup>a</sup>
Pooled SEM	0.33	0.14	0.51	0.07	0.002
			P Value		
Tukey's	≤0.01	≤0.01	≤0.01	0.04	≤0.01
Quadratic	≤0.01	0.52	≤0.01	≤0.01	0.61
Linear	≤0.01	0.98	≤0.01	0.04	0.56

<sup>1</sup> Haugh Unit =  $100 \cdot \log(h - 1.7 \cdot w^{3.7} + 7.6)$ ; h = observed height of the albumen in millimeters; w = egg weight in grams

<sup>a-c</sup> Least square means without a common superscript differ significantly ( $P \leq 0.05$ ).

Table 4.4 Val requirement estimated from linear broken line, quadratic broken line, quadratic polynomial and exponential model based on hen-housed egg production (HHEP), egg mass and FCR.

Data set	Model	Independent variables	Val requirement*	Ordinate value of requirement	Regression	R <sup>2</sup>
HHEP (%)	linear broken line	val daily intake	591.9	80.24	$y = 80.24 - 0.1222(591.9 - x)$	0.83
	quadratic broken line	val daily intake	691.4	79.81	$y = 79.81 - 2.535 \cdot 10^{-4}(x - 691.4)^2$	0.90
	quadratic polynomial	val daily intake	697.5 <sup>#</sup>	81.15	$y = -2.084 \cdot 10^{-4}x^2 + 0.3060x - 31.18$	0.85
	exponential	val concentration	0.746% <sup>#</sup>	81.15	$y = 48.52 + 32.64(1 - e^{-1296(x-0.515\%)})$	0.82
Egg mass(g/hen/d)	linear broken line	val daily intake	597.3	48.90	$y = 48.90 - 0.07885(597.3 - x)$	0.87
	quadratic broken line	val daily intake	719.7	49.49	$y = 49.49 - 1.490 \cdot 10^{-4}(x - 719.7)^2$	0.92
	quadratic polynomial	val daily intake	675.2 <sup>#</sup>	49.41	$y = -1.596 \cdot 10^{-4}x^2 + 0.2268x - 31.18$	0.88
	exponential	val concentration	0.744% <sup>#</sup>	49.31	$y = 28.03 + 21.28(1 - e^{-1309(x-0.515\%)})$	0.84
FCR (g/g)	linear broken line	val daily intake	500.5	1.93	$y = 1.930 + 0.004247(500.3 - x)$	0.62
	quadratic broken line	val daily intake	650.0	1.88	$y = 1.880 + 7.347 \cdot 10^{-6}(x - 650.0)^2$	0.65
	quadratic polynomial	val daily intake	648.3 <sup>#</sup>	1.87	$y = 6.009 \cdot 10^{-6}x^2 - 0.008201x + 4.670$	0.60
	exponential	val concentration	0.646% <sup>#</sup>	1.92	$y = 2.626 - 0.7110(1 - e^{-2282(x-0.515\%)})$	0.64

\*Unit is mg/d when using val daily intake and % when using val concentration.

<sup>#</sup>abscissa of the point on the fitted curve whose ordinate was 95% of the upper asymptote.

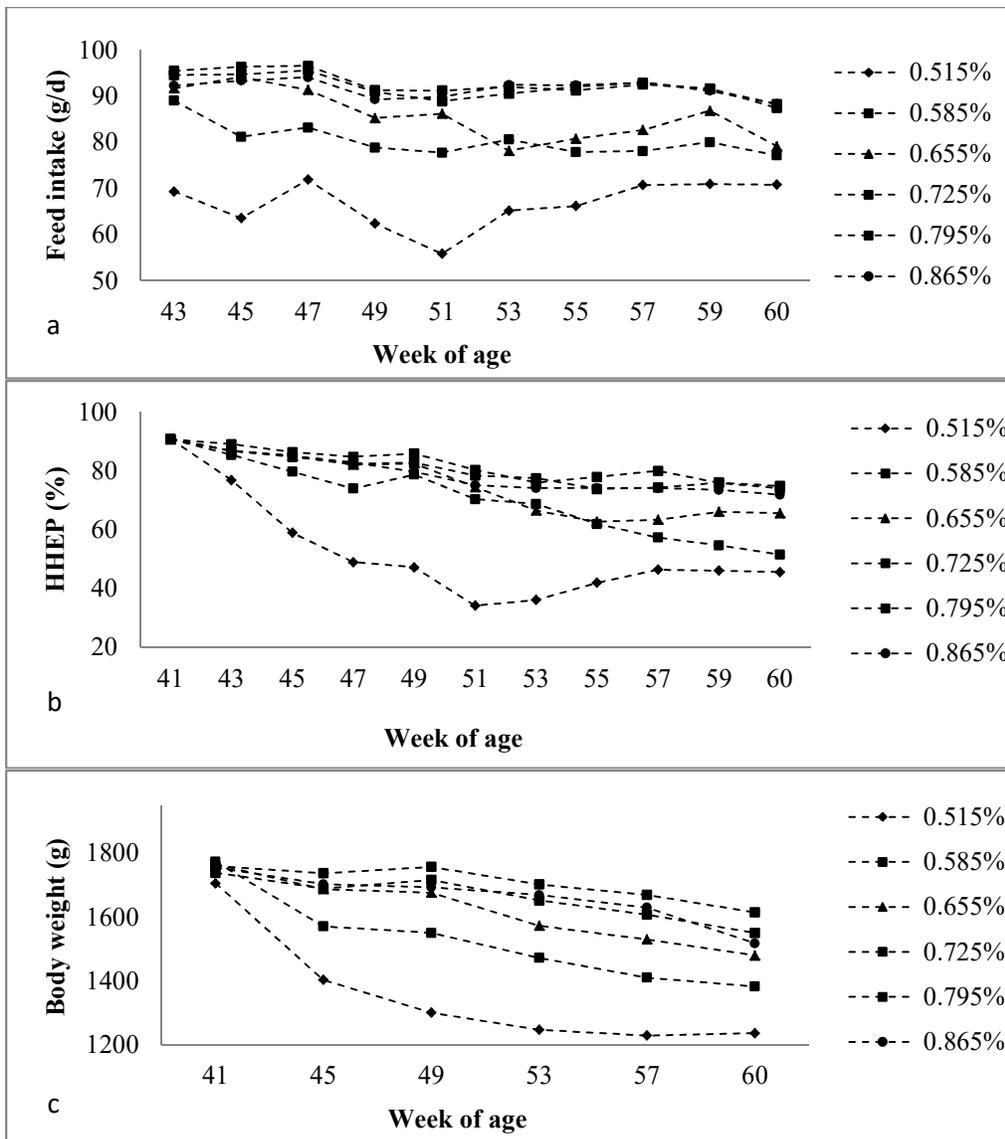


Figure 4.1 (a) feed intake, (b) hen-housed egg production (HHEP), and (c) body weight change from 41-60 wk of birds fed diets containing 0.515, 0.585, 0.655, 0.725, 0.795 and 0.865% val respectively.

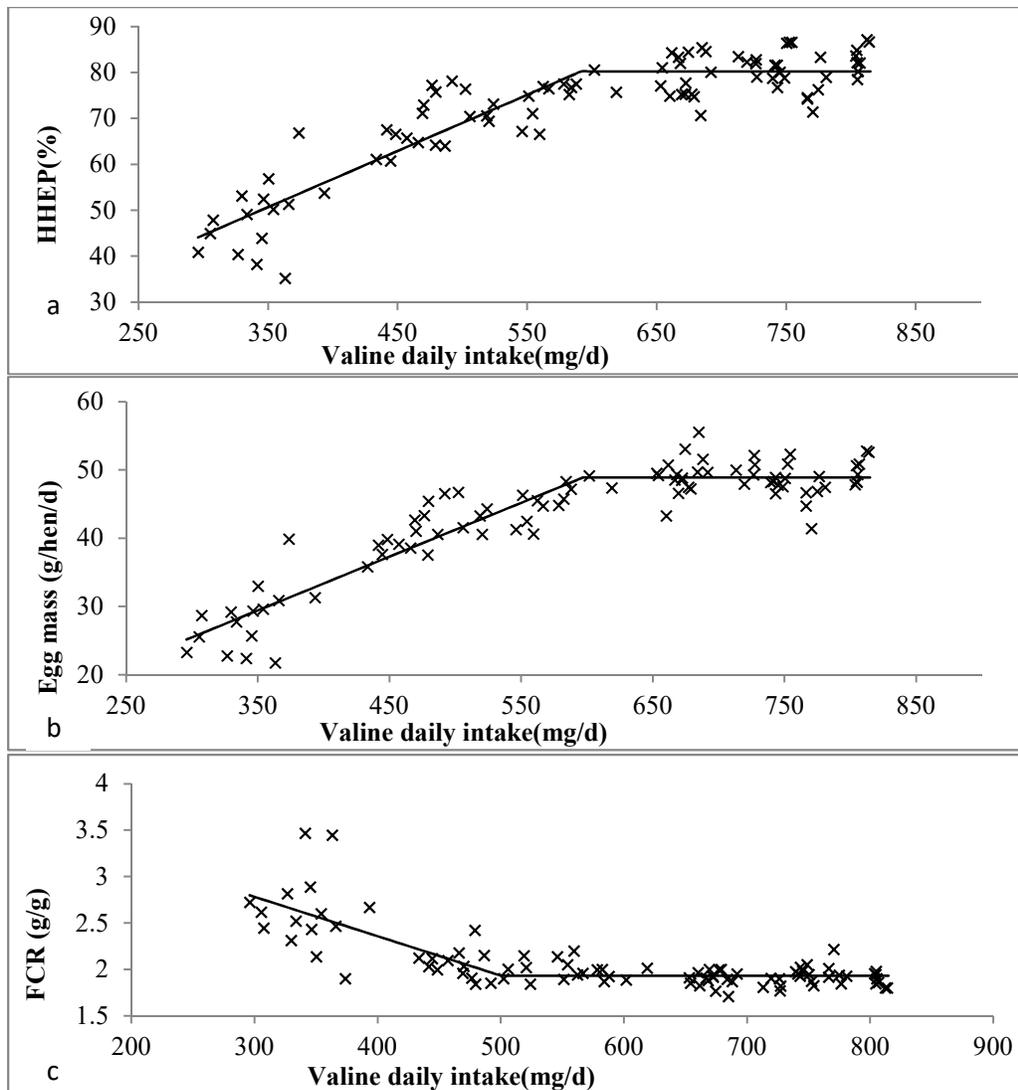


Figure 4.2 Val requirement estimation using linear broken line regression based on (a) hen housed egg production (HHEP) [ $y = 80.24 - 0.1222(591.9 - x)$ ,  $R^2=0.83$ ], (b) egg mass [ $y = 48.90 - 0.07885(597.3 - x)$ ,  $R^2=0.87$ ], and (c) feed conversion ratio (FCR) [ $y = 1.930 + 0.004247(500.3 - x)$ ,  $R^2=0.62$ ].

## Chapter 5: Evaluation of the Lysine Requirement in Young Broilers from 1 to 3, 3 to 5, 5 to 8 and 8 to 11 Day of Age

### 5.1 Abstract

Experiments were conducted to evaluate the lysine (lys) requirement of straight-run Hubbard x Cobb 500 broiler chicks from 1 to 3 (E1), 3 to 5 (E2), 5 to 8 (E3) and 8 to 11 (E4) day of age, respectively. In each experiment, 490 chicks were allocated, by weight, into 7 treatments with 10 replicates of 7 birds in each pen. In E1, E2 and E3, birds from 7 treatments received diets containing 0.94%, 1.05%, 1.16%, 1.27%, 1.38%, 1.49% and 1.60% digestible lys, respectively. In E4, the 7 treatment diets contained 0.83%, 0.94%, 1.05%, 1.16%, 1.27%, 1.38% and 1.49% digestible lys, respectively. Birds were provided *ad libitum* access to feed and water. In each experiment, body weight gain, feed intake, pectoralis major weights were measured. In E1 and E2, body weights were recorded without the yolk sac weight to increase the accuracy of the measurement. In E4, plasma urea nitrogen and cationic amino acid transporter ( $b^{0,+}$  AT) mRNA abundance in the jejunum was measured. There were no dose responses with growth performance due to digestible dietary lys concentration in E1, E2 and E3 as measured responses were not altered due to dietary lys concentration. Digestible lys of broilers from 8 to 11 days of age did result in a dose-dependent response with a linear broken line estimate of 1.057 and 1.016% digestible lys based on body weight gain and pectoralis major absolute weight, respectively. No differences in jejunum  $b^{0,+}$  AT relative mRNA abundance were observed in 8 to 11 day old chicks fed diets containing digestible lys 0.83, 1.05, 1.27 and 1.49%, respectively. These data suggest that commercial concentrations of digestible lys estimates in starter diets may overestimate the requirement as it was not until the digestible lys concentration of the diets was reduced to well below the expected requirement that a response was generated.

Key words: broiler, digestible lysine, growth performance, plasma urea nitrogen

## 5.2 Introduction

Lysine is an essential amino acid in poultry. It is believed to be the second or third limiting amino acid in corn-soybean meal diets for poultry (Warnick and Anderson, 1968; Fernandez, et al., 1994; Dozier and Payne, 2012). It is also used as the standard amino acid to calculate an amino acid: lys ratio using the ideal protein model (Baker and Han, 1994). The lys requirement in young broilers has been reported previously. Han and Baker (1991) estimated the lys requirement of fast growing and slow growing chicks from 8 to 21 days posthatch and concluded that digestible lys requirement was no more than 1.01% based on body weight gain and no greater than 1.21% based on feed efficiency. In another experiment, lys requirements of chicks from 0-7 days old were evaluated and the broken line estimates were 1.03% and 1.08% for body weight and feed efficiency using a broken line model (Sklan and Noy, 2003). Garcia and Batal (2005) conducted an experiment in broilers from 0-21 days old and the requirements of 0-4 days, 0-7 days and 0-21 days of age were similar. Broken line regression models for digestible lys requirements ranged from 0.95% to 1.01% based on weight gain and 0.94% to 1.10% based on feed efficiency. A recent experiment indicated that the quadratic broken line model for digestible lys requirement for the BW of female Ross × Ross 708 broilers was 1.35 and 1.27% and 1.26 and 1.18% for female Hubbard x Cobb 500 chicks when evaluated from 1 to 7 d and 1 to 14 d of age, respectively (Dozier and Payne, 2012).

As amino acid requirements (% of the total diet or % of calories fed) decrease throughout the grow-out period, phase feeding (PF) regimens had been researched to reduce amino acid waste without sacrificing performance in comparison to commercial feeding regimens in broilers (Warren and Emmert, 2000). Phase feeding divides or splits the feeding period into multiple periods based on a predicted amino acid requirement over the difference ages of broilers. Several

experiments have indicated that using a PF regimen to replace traditional industry regimens was able to reduce amino acids intake and feed cost without sacrificing growth performance or carcass yield in broilers (Emmert and Baker, 1997; Warren and Emmert, 2000; Pope and Emmert, 2001; Pope, et al., 2002;. Brewer, et al., 2012a; Brewer, et al., 2012b). In the experiments above, amino acids requirements were based on regression equations using Illinois ideal chick protein (IICP) (Baker and Han, 1994). No experimental data were available to provide lys requirements over a short period, such as every two to three days.

Effects of dietary L-lys on cationic amino acid transporters gene expression in weaned pigs were reported but no such research has been conducted in broilers (He et al., 2013). As verification of the performance data, digestible lys requirement will be estimated based on plasma urea nitrogen which is an indicator of amino acid utilization for poultry (Donsbough, et al., 2010). The objective of the current experiment is to determine the lys requirement for young broilers every two or three days over the starter phase (day 1 to 11) in contrast to previous experiments that have looked at nutrient requirements over a longer timeline (generally at least 7 days). Secondary objectives include investigation of the effects of lys deficiency on the mRNA abundance of cationic amino acid transporter on the lumen side of intestinal epithelial cells and evaluation of PUN as verification of lys requirements based on performance data.

## 5.3 Materials and Methods

### 5.3.1 Diets

A lys deficient broiler starter diet was formulated as a basal diet used to manufacture all experimental diets. To generate the lys deficient diet, corn, corn gluten meal, DDGS and soybean meal were selected as the major ingredients. The basal diet was formulated to contain a deficient

concentration of digestible lys (0.83%) and sufficient concentrations of all other essential amino acids (Table 5.1). The nutrition requirement follows the Cobb 500 nutritional recommendation (Cobb 500 Broiler Performance and Nutrition Supplement, 2013). Experimental diets containing 0.94, 1.05, 1.16, 1.27, 1.38, 1.49 and 1.60% digestible lys were manufactured by supplementation of feed grade lys•HCl on top of the basal diet. Basal and experimental diets were sampled for amino acid composition analysis (University of Missouri AESCL, Columbia, MO 65211). A starter diet was manufactured following Cobb 500 nutritional recommendations (Cobb 500 Broiler Performance and Nutrition Supplement, 2013). This diet was utilized to maintain all extra birds before they were transferred to experimental diets.

### 5.3.2 Animals and Housing

All animal work was approved by the Institutional Animal Care and Use Committee at Virginia Tech (Blacksburg, VA). Four independent experiments were conducted to estimate lys requirement of young chicks from 1 to 3 (E1), 3 to 5 (E2), 5 to 8 (E3), 8 to 11 (E4) day of age, respectively. In each experiment, 490 chicks were sorted into three body weight ranges, high, medium and low to minimize differences in initial body weights among pens. This resulted in a similar body weight in each experiment unit and birds were randomly assigned to the 7 dietary treatments. Each treatment consists of 10 replicates with 7 birds per replicate. In E1, E2 and E3, birds from 7 treatments received diets containing 0.94%, 1.05%, 1.16%, 1.27%, 1.38%, 1.49% and 1.60% digestible lys, respectively. In E4, the 7 treatment diets contained 0.83%, 0.94%, 1.05%, 1.16%, 1.27%, 1.38% and 1.49% digestible lys, respectively, due to an expected reduced lys requirement. No significant differences in initial average body weight were observed in any of the experiments. Chicks were placed into floor pens with clean pine shavings at a stocking density of 208 sq inch/bird. Temperature was set to 30°C from 1 to 8 days of age and then

28.3°C from 8 to 11 days of age. Continuous lighting and supplemental heat were provided from 1 to 8 days of age and then a lighting program of 20 hours of light and 4 hours of darkness was implemented according to the commercial broiler management guide. Birds were provided *ad libitum* access to feed and water. Feed was placed on a paper tray in each pen for each experiment.

### 5.3.3 Data and Sample Collection

Pen body weight and pen feed offered and refused were recorded at the over the duration of each experiment. FCR was calculated in each experiment. At the end of each experiment, birds were euthanized and the pectoralis major of five birds per pen were weighed to generate a pooled sample weight. In E1 and E2, pooled yolk sac weight of the five birds per pen was recorded. In E4, 0.5ml blood samples were collected using EDTA coated 4ml tubes from one bird per pen for urea nitrogen determination. In E4, 3 cm jejunum samples (before Meckel's diverticulum) were collected from one bird per pen from birds receiving 0.830, 1.050, 1.270 and 1.490% digestible lys for cationic amino acid transporter ( $b^{0,+}$  AT) mRNA abundance measurement.

### 5.3.4 Relative mRNA Abundance of Cationic Amino Acid Transporter

A sample of 25 to 50 mg of frozen jejunum tissue were homogenized in Tri-Reagent (Molecular Research Center, Cincinnati, OH) using 5mm stainless steel beads (Qiagen) and a Tissue Lyser II (Qiagen). After RNA isolation, RNA was purified by the Direct-zol RNA MiniPrep Kit (Zymo Research, Irvine, CA). The total RNA concentration and purity were measured by spectrophotometry at 260/280/230 nm with a NanoDrop 1000 (Thermo Scientific, Waltham, MA). The RNA concentration was adjusted to 200 ng/ml before transcription. First-

strand cDNA was synthesized from 500 ng total RNA with a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Grand Island, NY). Transcription reactions were performed under the following conditions: 25° C for 10 min, 37° C for 120 min, and 85° C for 5 min. mRNA abundance of b<sup>0,+</sup> AT and reference gene  $\beta$ -actin were assayed by relative quantification real time PCR followed the procedure of Zhang et al. (2017). The cycle threshold (CT) value of target gene and reference gene in each experimental unit were averaged by duplicated.  $\Delta$ CT of each experiment unit were calculated by subtracting CT of reference gene from CT of target gene.  $\Delta\Delta$ CT were calculated by subtracting average  $\Delta$ CT of 0.83% digestible lys treatment from  $\Delta$ CT of each experiment unit. Finally, the  $2^{-\Delta\Delta CT}$  were calculated for statistic analysis. Primer sequences information were reported by Gilbert et al. (2010) and listed below.

b<sup>0,+</sup> AT (5' - CAGTAGTGAATTCTCTGAGTGTGAAGCT - 3'; forward; 5' - GCAATGATTGCCACAACCTACCA - 3', reverse)

$\beta$  -actin (5' - GTCCACCGCAAATGCTTCTAA - 3'; forward; 5' - TGCGCATTATGGGTTTTGTT - 3', reverse)

### 5.3.5 Plasma Urea Nitrogen

Plasma urea nitrogen (PUN) was measured by urea assay kit using enzymatic method (Sigma-Aldrich, St. Louis, MO). Plasma samples were diluted 10 times before measurement. Linear regression was fitted on urea standards. 50  $\mu$ L diluted plasma samples and 50  $\mu$ L reaction mixes were added to each well of a clear 96 well plate (Cytoone, Orlando, FL). Plates were incubated for 60 minutes at 37 °C. The absorbance was measured by colorimeter at 570 nm (A570). Urea concentration was calculated based on regression equation of urea standards.

### 5.3.6 Statistical Analysis

JMP Pro 13.0.0 (SAS Institute Inc, Cary, NC) was used to analyze body weight gain, pectoralis major weight, body weight without yolk sac, plasma urea nitrogen using a one-way ANOVA with means separated using a Fisher's LSD test. Jejunum b<sup>0+</sup> AT relative mRNA abundance was analyzed using a nonparametric test. Significance was accepted at  $P \leq 0.05$ . Digestible lys requirement was calculated using the linear broken line model based on body weight gain and pectoralis major absolute weight. Regression analysis was analyzed by JMP non-linear model option of JMP Pro 13.0.0 (SAS Institute Inc, Cary, NC) with digestible lys concentration as the independent variable.

The equation of the linear broken line model was:

$$y = a - b * (x - c) \text{ for } x \leq c \text{ and}$$

$$y = a \text{ for } x > c$$

where  $y$  = response criteria,  $a$  = ordinate of the breakpoint,  $b$  = slope of the line for  $x \leq c$ ,  $c$  = abscissa of the breakpoint (= requirement), and  $x$  = digestible lys concentration.

#### 5.4 Results and Discussion

There were no differences in body weight gain of broilers fed diets containing various concentration of digestible lys (0.94, 1.05, 1.16, 1.27, 1.38, 1.49 and 1.60%) from 1 to 3, 3 to 5 and 5 to 8 days of age, respectively (Table 5.2). These same diets did not alter body weight without yolk sac in broilers from 1 to 3 and 3 to 5 days of age. No dose response relationship on

growth performance could be fitted because the digestible lys requirement was lower than 0.94%. This was not consistent with our estimated digestible requirement of 1.15 to 1.28% based on previous literature. Garcia and Batal (2005) fed day old Cobb500 broilers with diets containing digestible lys from 0.88 to 1.28% and the diets that contained less than 0.98% digestible lys resulted in a slower growth and reduced feed efficiency at 4 and 7 of age. Reduced body weight and feed intake were observed in another experiment when two strains of boilers (Ross × Ross 708 and Hubbard × Cobb 500) were fed diets containing digestible lys from 0.95 to 1.43% from 1 to 14 weeks of age. The body weight and feed intake increased as digestible lys increased from 0.95 to 1.27% in both strains from 1 to 7 days of age. The commercial broiler guide (Cobb 500 Broiler Performance and Nutrition Supplement, 2013) suggests that body weight gain for straight-run or as hatched performance is 24, 34 and 77g from 1 to 3, 3 to 5 and 5 to 8 days of age. The body weight gain in current experiment was about 25 to 40% lower than recommended body weight gain in Cobb500 management guide. Inferior growth of chicks in the current experiment resulted in a lower digestible lys requirement, therefore, no dose response on growth was observed on chicks from 1 to 8 days of age. The reason for relative slower growth is unknown but corn gluten meal was added to these diets and this might have inadvertently reduced growth, in addition the number of chicks initially placed into pens was relatively low (seven birds) and could have altered growth responses (Pesti et al., 1983).

There were significant differences on body weight gain ( $P < 0.01$ ) of birds fed the diets containing 0.83, 0.94, 1.05, 1.16, 1.27, 1.38 and 1.49% digestible lys from 8 to 11 days of age. Body weight gain and feed intake of broilers fed diets containing 0.83 and 0.94% were significantly lower than those from the other treatments. The digestible lys requirement of broilers from 8 to 11 days of age was estimated as 1.057% based on body weight gain. As no

experiment has been conducted to look at the lys requirement from 8 to 11 days of age, no direct comparison can be made. In an experiment evaluating broiler digestible lys from 8 to 21 days of age, Digestible lys requirement was no more than 1.01% based on body weight gain and feed efficiency (Han and Baker, 1991). Two scientific reports concluded that digestible lys requirements were 1.03% or 0.95-1.10% based on body weight using broken line model (Sklan and Noy, 2003; Garcia and Batal, 2005). The reduced body weight gain of the chicks noted over the first 8 d of growth was again noted over the 8 to 11 d period and may suggest that the lys requirement from current experiment might be lower than lys requirement if the birds were growing to commercial expectations.

In the current experiments, feed intake from 1 to 3, 3 to 5, 5 to 8 and 8 to 11 days of age was higher than commercial recommendation (Cobb 500 Broiler Performance and Nutrition Supplement, 2013). This is most likely due to the feed wastage. This would result in increased feed disappearance (paper trays were used throughout the experiment), but would not reflect true feed intake. The increased feed intake resulted in an inaccurate FCR in the current experiment and neither feed intake nor FCR will be reported.

As birds age, both the absolute weight (AW) and relative weight of pectoralis major increased. This indicated the development of breast muscle occurred during the first week post-hatch. Absolute breast weight of birds fed 1.16% digestible lys was significantly lower than birds fed 1.27%, 1.38% and 1.49% digestible lys with the other treatments being intermediate from 1 to 3 days of age ( $P < 0.05$ ) (Table 5.3). Relative breast weight of birds fed 1.16% digestible lys was significantly lower than birds fed 1.27% and 1.38% digestible lys from 1 to 3 days of age ( $P < 0.05$ ). These mixed responses during the short-term feeding periods would need to be verified in future experiments to ensure these are not artificial. Relative breast weight of birds fed 0.94%

digestible lys was significantly lower than 1.16%, 1.38% and 1.60% digestible lys treatment from 5 to 8 days of age ( $P < 0.05$ ). This may indicate that 0.94% digestible lys diet limited breast muscle growth even if it did not depress body weight. The AW in 0.94% digestible lys treatment was also numerically lower than the other treatments. This may suggest that breast muscle is more sensitive to dietary lys deficiency than body weight. Absolute breast weight increased as broilers were fed dietary digestible lys from 0.83 to 1.16% and AW of 0.83% digestible lys treatment was significantly lower than those of 1.05, 1.16, 1.27, 1.38 and 1.49% treatments. A digestible lys requirement of 1.016% was estimated from AW from 8 to 11 days of age. This requirement was lower than requirement based on body weight gain indicating that breast meat weight was less sensitive than body weight gain to reduced dietary lys concentrations. It appears that this was contradictory to the observation on broilers from 5 to 8 days. Although no reports were found in young broilers, previous experiments did report the effects of lys concentration on body weight gain and breast meat weight in broilers at market weight. Digestible requirements were slightly higher for breast meat weight than body weight gain in male broilers from 49 to 63 days of age using quadratic polynomial and quadratic broken line models; however, the same digestible lys requirements were estimated for both body weight gain and breast meat weight using broken line model (Dozier et al., 2008). In another experiment, mixed results were reported as digestible lys requirements (quadratic broken line models) for breast weight was lower than that for body weight gain in the Ross×Ross TP16 strain but higher for breast weight than body weight gain in the Cobb×Cobb 700 strain from 28 to 42 days of age (Dozier, et al., 2010). The mixed results from the current experiment and the literature suggest that multiple factors might be limited either body or breast muscle growth.

Plasma urea nitrogen (PUN) was reported as an indicator of amino acid utilization in poultry (Donsbough, et al., 2010). In the current experiment, no differences on PUN were observed across dietary lys concentrations and the lys requirement estimate could not be generated. No responses on blood urea nitrogen by dietary lys concentration have been reported in previous experiments (Dozier, et al., 2009; Dozier, et al., 2010). In contrast, Dozier and Payne (2012) observed a quadratic response on blood urea nitrogen and estimated a digestible lys requirement at 1.088%. The lack of significant differences in the current data might suggest that limited feed intake associated with early growth may reduce the sensitivity of PUN measurements and a wider array of dietary lys concentrations may be needed.

No differences in jejunum  $b^{0,+}$  AT relative mRNA abundance were observed in 8 to 11 day old chicks fed diets containing digestible lys concentrations of 0.83, 1.05, 1.27 and 1.49%. The  $b^{0,+}$  AT relative mRNA abundance increased as weaning pigs were fed diets containing 1.35% lys in comparison to a zein-based diet free of lys (He et al., 2013). In the current experiment, jejunum relative  $b^{0,+}$  AT mRNA abundance was not affected by dietary digestible lys concentration from 0.83 to 1.49%. The differences in dietary lys between the swine data (1.35% v. none) and the broiler data (1.49 to 0.83%) most likely explain the contrary results between the swine and poultry reports.

In conclusion, the lys requirement of young broiler chicks (1 to 8 days) was not able to be estimated based on body weight in diets containing 0.94, 1.05, 1.16, 1.27, 1.38, 1.49, 1.60% digestible lys, respectively. It could be concluded that a relatively lower growth rate than commercial recommendation would be the one reason that smaller birds required less lys than estimated lys requirement. Consequently, even the lowest digestible lys concentration (0.94%) was able to provide sufficient lys to broiler chicks and no dose response relationship was

observed. In addition, lys deficiency may not be reflected in this experiment due to the relative short duration of only 2 or 3 days of feeding. Chicks with deficient lys intake may still utilize lys from protein reserves over the short time period to support protein synthesis. Theoretically, the lys requirement (%) should decrease as chicks age and consume more feed. The linear broken line estimate for digestible lys of broilers from 8 to 11 days of age were estimated at 1.057 and 1.016% based on body weight gain and pectoralis major absolute weight, respectively. Dietary lys concentrations ranging from 0.83 to 1.49% did not alter the mRNA abundance of the cationic amino acid transporter (b<sup>0,+</sup> AT) in broilers from 8 to 11 days of age. These data suggest that commercial recommendation may over-estimate the requirement of lys in starter diets and cationic amino acid transporters on apical intestinal epithelial cells may not respond to short term difference in dietary lys concentrations.

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Table 5.1 Composition of lys deficient basal diet. Lysine was added at 0.11% increments to generate experimental diets that contained 0.83, 0.94, 1.05, 1.16, 1.27, 1.38, 1.49 and 1.60% digestible lys<sup>1</sup>.

Ingredient	(%)
Corn	65.55
Soybean meal	23.40
Corn gluten meal	3.66
DDGS	1.25
Soy oil	0.72
L-arginine	0.21
DL-methionine	0.34
L-threonine	0.20
L-valine	0.13
L-isoleucine	0.10
Limestone	0.95
Dicalcium Phosphate	1.91
Salt	0.21
Sodium bicarbonate	0.15
Choline chloride	0.10
Mineral and vitamin premix <sup>2</sup>	0.63
Celite (acid insoluble ash)	0.50
Calculated composition	(%)
Crude protein	20.43
ME, kcal/kg	3,048
Calcium	0.90
Available phosphorus	0.45
Digestible methionine	0.64
Digestible methionine + cysteine	0.88
Digestible lysine	0.83
Digestible tryptophan	0.18
Digestible threonine	0.77
Digestible arginine	1.24
Digestible isoleucine	0.79
Digestible leucine	1.68
Digestible valine	0.89
Sodium	0.16
Chloride	0.17
Potassium	0.78

<sup>1</sup>Analyzed lysine concentrations in experimental diets were 0.92, 1.01, 1.10, 1.18, 1.32, 1.37, 1.41 and 1.51%, respectively.

<sup>2</sup> Provided per kg of diet: vitamin A, 5,548 IU; vitamin D<sub>3</sub>, 1,859 ICU; vitamin E, 1,386 IU; menadione, 0.97 mg; vitamin B<sub>12</sub>, 5.54 µg; choline, 321.04 mg; niacin, 16.64 mg; pantothenic acid, 5.10 mg; riboflavin, 3.47 mg; Cu, 3.40 mg; Fe, 42.53 mg; I, 0.84 mg; Mn, 54.05 mg; Zn, 40.95 mg; Co, 0.21 mg.

Table 5.2 Effect of various dietary digestible lys concentrations on body weight gain (BWG) and body weight without yolk sac (BWY) in broiler from 1 to 3, 3 to 5, 5 to 8, and 8 to 11 days of age, respectively.

Digestible lys %	1 to 3 day <sup>1</sup>		3 to 5 day <sup>2</sup>		5 to 8 day <sup>3</sup>	8 to 11 day <sup>4</sup>
	BWG g	BWY g	BWG g	BWY g	BWG g	BWG g
0.83	-	-	-	-	-	65.8 <sup>b</sup>
0.94	13.5	52.5	24.4	79.4	45.6	69.2 <sup>b</sup>
1.05	14.2	53.7	23.7	79.0	48.1	77.2 <sup>a</sup>
1.16	13.4	53.1	23.3	77.4	46.6	76.2 <sup>a</sup>
1.27	14.4	53.6	25.3	80.6	47.5	78.1 <sup>a</sup>
1.38	14.4	54.9	25.7	79.6	49.4	77.8 <sup>a</sup>
1.49	14.1	54.2	23.1	77.5	45.8	75.3 <sup>a</sup>
1.60	13.4	53.6	25.2	80.5	48.4	-
Pooled SEM	0.55	0.81	1.15	1.51	1.50	1.97
P Value	0.65	0.53	0.58	0.65	0.52	<0.01

<sup>a-c</sup> Least square means without a common superscript differ significantly ( $P \leq 0.05$ ).

<sup>1</sup> Initial average weights were 41.3, 41.3, 41.2, 41.5, 41.3, 41.4 and 41.3g per bird for treatment 0.94%, 1.05%, 1.16%, 1.27%, 1.38%, 1.49% and 1.60% digestible lys, respectively ( $P=0.99$ ).

<sup>2</sup> Initial weights were 55.9, 56.0, 55.4, 55.6, 55.4, 55.1 and 55.7g per bird for treatment 0.94%, 1.05%, 1.16%, 1.27%, 1.38%, 1.49% and 1.60% digestible lys, respectively ( $P=0.11$ ).

<sup>3</sup> Initial weights were 83.5, 84.1, 83.8, 83.9, 84.1, 83.8 and 84.3 g per bird for treatment 0.94%, 1.05%, 1.16%, 1.27%, 1.38%, 1.49% and 1.60% digestible lys, respectively ( $P=0.90$ ).

<sup>4</sup> Initial weights were 141.0, 142.0, 140.8, 141.2, 141.5, 141.0, and 141.0 g per bird for treatment 0.83%, 0.94%, 1.05%, 1.16%, 1.27%, 1.38%, and 1.49% digestible lys, respectively ( $P=0.82$ ).

Table 5.3 Effects of various dietary digestible lys concentrations on pooled (5 birds) pectoralis major absolute (AW), relative (RW) weight in broilers from 1 to 3, 3 to 5, 5 to 8, and 8 to 11 days of age, respectively.

Dietary lys	1 to 3 day		3 to 5 day		5 to 8 day		8 to 11 day	
	AW	RW	AW	RW	AW	RW	AW	RW
%	g	%	g	%	g	%	g	%
0.83	-	-	-	-	-	-	63.74 <sup>c</sup>	6.13
0.94	3.71 <sup>abc</sup>	1.38 <sup>abc</sup>	9.42	2.36	29.15	4.51 <sup>c</sup>	68.40 <sup>bc</sup>	6.45
1.05	3.75 <sup>abc</sup>	1.36 <sup>abc</sup>	9.29	2.32	31.62	4.76 <sup>abc</sup>	69.93 <sup>ab</sup>	6.46
1.16	3.49 <sup>c</sup>	1.28 <sup>c</sup>	8.86	2.27	31.85	4.94 <sup>a</sup>	71.13 <sup>ab</sup>	5.94
1.27	3.97 <sup>a</sup>	1.45 <sup>a</sup>	9.77	2.40	30.57	4.68 <sup>bc</sup>	72.13 <sup>ab</sup>	6.57
1.38	3.95 <sup>a</sup>	1.41 <sup>ab</sup>	9.56	2.39	32.79	4.92 <sup>ab</sup>	73.25 <sup>a</sup>	6.67
1.49	3.82 <sup>ab</sup>	1.37 <sup>abc</sup>	8.94	2.29	30.53	4.73 <sup>abc</sup>	71.70 <sup>ab</sup>	6.66
1.60	3.60 <sup>bc</sup>	1.31 <sup>bc</sup>	9.00	2.22	32.25	4.84 <sup>ab</sup>	-	-
Pooled SEM	0.113	0.037	0.432	0.086	0.924	0.090	1.683	0.241
P Value	<0.05	<0.05	0.71	0.78	0.11	<0.05	<0.01	0.29

<sup>a-c</sup>Least square means without a common superscript differ significantly ( $P \leq 0.05$ ).

Table 5.4 Effect of various dietary digestible lys concentrations on plasma urea nitrogen (PUN) and b<sup>0,+</sup> AT relative jejunum mRNA abundance (b<sup>0,+</sup> AT) in broiler from 8 to 11 days of age.

Dietary lys	PUN	b <sup>0,+</sup> AT <sup>1</sup>
%	mg/dL	-
0.83	1.32	1.27
0.94	1.25	-
1.05	1.23	0.87
1.16	1.19	-
1.27	1.19	0.75
1.38	1.25	-
1.49	1.32	0.79
1.60	-	-
Pooled SEM	0.132	-
P Value	0.98	0.89

<sup>1</sup>β-actin is the reference gene.

Table 5.5 Digestible lys requirements as estimated by linear broken line regression analysis of 8 to 11 days of age broilers based on body weight gain (BWG), pectoralis major absolute weight (AW).

Data set	Independent variables	Digestible lys requirement	Ordinate value of requirement	Regression	R <sup>2</sup>
BWG (g)	digestible lys (%)	1.057	76.85	$y = 76.85 - 52.097(x - 1.057)$	0.34
AW (g)	digestible lys(%)	1.016	14.33	$y = 14.33 + 8.472(x - 1.016)$	0.23

## Chapter 6: Investigation of the Nutritional and Immune Benefits of Feeding a Bacterial Protein Meal to Laying Hens

### 6.1 Abstract

The objective of this experiment was to determine the effects of feeding a bacterial protein meal (BPM) on performance and immune status in first cycle laying hens. In total, 144 Hy-Line W36 laying hens (38 weeks of age) were randomly assigned to 3 treatments with 12 replicates of 4 birds. The control fed birds consisted of a corn-soybean meal (SBM) diet while treatment fed birds consumed corn-SBM based diets with 7.5% or 15% BPM formulated to replace both SBM and corn. The BPM was analyzed to contain 46.7% crude protein, 3,241 kcal/kg gross energy, 5.6% crude fiber, 31.5% non-fiber carbohydrate, 0.9% crude fat and 20.9% ash. A controlled feeding program was applied resulting in 95 g/hen/d feed intake. Laying hens fed the control and 7.5% BPM diets resulted in significantly higher hen-housed egg production (HHEP) (86.7 and 88.7%, respectively) than those fed 15% BPM diet (83.5%). As expected, feed intake was similar due to controlled feeding and there was no mortality over the 16 weeks experimental period. Body weight loss was greater in the 15% BPM in comparison to both the control and 7.5% BPM fed birds. Egg yolk color from hens fed BPM included diet was significantly darker than the control fed hens ( $P \leq 0.01$ ). There were no difference in relative spleen weights or blood heterophil to lymphocyte (H/L) ratio across the three treatments. In summary, 7.5% of the BPM was able to maintain egg production in laying hens, but 15% BPM resulted in reduced feed intake and egg production.

Key words: bacterial protein meal, laying hen, egg production, immune response

## 6.2 Introduction

Increased oil seed and grain demand by the animal production industry may result in a potential burden on limited cropland worldwide. Alternative protein ingredients produced without utilizing croplands may become more desirable in the future to help relieve the pressure on limited cropland. Bacterial protein meal is derived from bacterial fermentation by converting various substrates such as methane, methanol, or agriculture by-products into protein-rich biomass with minimum dependence on soil, water, and climate conditions (Anupama and Ravindra, 2000).

Bacterial protein meal can provide considerable amounts of crude protein and other nutrients as a feed ingredient. The crude protein of BPM ranged from 58.0% to 75.5% as described in previous experiments (Waldroup and Payne, 1974; Plavnik, et al., 1981; Skrede, et al., 2003; Schøyen, et al., 2007). Although the protein concentration was high, protein quality may not be ideal due to a high concentrations of nucleic acid in the BPM (Skrede et al., 1998). In total, 19% of the nitrogen in BPM was from nucleic acids and 71% from amino acids in a methanol derived BPM (Stringer, 2007). It has been reported that amino acid concentrations of BPM derived from methane were close to that of soybean meal except for a relatively lower cysteine concentration (D'Mello, 1973). In another experiment, amino acid composition of BPM derived from natural gas was reported to be similar to fish meal and soybean meal (Schøyen, et al., 2007). The authors reported that total tract and ileal amino acid digestibility of BPM in 5-week-old broilers were significantly reduced for arg, lys, met and phe while the other indispensable amino acid showed no difference when compared to soybean meal. Besides serving as a protein source, the carbohydrate and lipid in BPM can provide energy for animal production. D'Mello and Acamovic (1976) evaluated the metabolic energy of BPM using broiler

chickens and the values ranged from 2,282 to 2,610 kcal/kg dry matter and nitrogen-corrected values ranged from 2,139 to 2,428 kcal/kg dry matter.

A limited BPM inclusion rate in poultry rations was suggested to generate comparable growth rates and egg production to corn-soybean diets in previous reports. A BPM experiment in laying hens showed no significant differences in egg production as BPM was supplemented up to 10% (Whittemore, et al., 1978). In broiler diets, BPM derived from methanol was reported to replace approximately up to 10% of soybean meal without depressing growth performance when diets were pelleted in broilers from 1 to 28 days of age (Waldroup and Payne, 1974). The same experiment showed that replacing 15% soybean meal with BPM resulted in a reduced body weight gain. Another study suggested that BPM derived from natural gas could be supplemented up to 6% in broiler diets without affecting performance from 1 to 35 days of age (Skrede, et al., 2003). Reduced body weight gain was observed when BPM inclusion rate was higher than 6% especially during the first two weeks of the experiment. The reduced performance by high dietary BPM concentration might be associated with its powdery texture, bulkiness, or the adhesive properties of BPM (Waldroup and Payne, 1974; D'Mello and Acamovic, 1976).

As BPM contains high concentrations of nucleic acids, it has been suggested as an immune modifier (Yu, 1998; Anupama and Ravindra, 2000). Limited experiments have been conducted to determine the effects of BPM on the immune response of poultry species. It was reported that supplementation of 12.4 and 24.8% BPM in broiler diet induced a higher BSA-antibody level in serum indicating an activated immune response (Farstad, 1977). Nevertheless, this response was not noted with rats fed 20 % BPM in the diet (Steinmann, et al., 1990).

Unlike BPM derived from methane, methanol or natural gas, a novel BPM has been produced by treating waste water with a microbial organism. Nutrient composition and utilization is different among BPM with consistent feedstocks, but this product is proposed with waste water as a feedstock making further evaluation necessary. The objective of this experiment was to evaluate the effect of substituting soybean meal and corn with a new bacterial protein product on laying performance and to investigate the immune response of hens fed diets containing up to 15% BPM.

## 6.3 Materials and Methods

### 6.3.1 Bacterial Protein Meal

Two independent batches of BPM (ProFloc<sup>™</sup>, Nutrinsic Corporation, Glendale, CO) were received from a pilot production plant. These BPMs were granular and light tan in color. The nutrients composition of the two batches of BPM were analyzed (Table 6.1) (BPM batch No.1: University of Missouri AESCL, Columbia, MO 65211; BPM batch No.2: Cumberland Valley Analytical Service, Hagerstown, MD 21742). Mineral content of BPM batch No.2 was analyzed by inductively coupled plasma atomic emission spectrometer (Virginia Tech Department of Crop and Soil Environmental Sciences, Blacksburg, VA 24061). Apparent ileal amino acid digestibility of BPM from batch No.1 was evaluated by precision feeding assay (Kim, et al., 2011). Ten broiler chicks were reared from day 1 to day 21 by feeding a nutrient sufficient diet. Before the assay began, all birds were fasted overnight (10h). Approximately 10 g of BPM sample was precision fed to the crop of each bird. After 4 hours, ileal contents were collected for amino acid analysis. Amino acid digestibility was calculated based on BPM and ileal amino acid analysis.

### 6.3.2 Diets

Formulation of the three treatment diets is presented in Table 6.2. Based on the proximate analysis and amino acid digestibility data of the BPM, three experimental diets were generated from a basal diet. The treatment diets were generated by the addition of corn, soybean meal and BPM to the basal diets. The AME<sub>n</sub> of BPM was not available and an estimated value of 2,730 was used based on previous literature. The AME<sub>n</sub> and amino acids concentration of control and 15% BPM were formulated and generated to the same requirements and were similar concentrations. The 7.5% BPM diet was manufactured by mixing 50% of the control diet and 50% of the 15% BPM diets. During the experiment, experimental diets were mixed every two weeks from the same group of experimental ingredients.

### 6.3.3 Animals and Housing

The Institutional Animal Care and Use Committee at Virginia Tech approved the protocol. In total, 144 Hy-line W36 laying hens were selected from a flock of 600 healthy birds. At 34 weeks of age, birds were assigned to three treatments with 12 replicates of four birds per replicate. Birds were reared in A-frame battery cages. Each experimental unit (EU) consisted of two consecutive cages with two birds in each cage (4 total hens) at a density of 108 square inches per bird. Experimental treatments were randomly assigned across the pens.

The 0, 7.5 and 15% BPM diets were fed starting at 38 weeks of age. Egg production was monitored the week before the experiment and was not different (control: 93.7%; 7.5% BPM: 90.1%; BPM 15%: 89.1%; P=0.33). Although egg production was not different, body weights were different across treatments with the 0, 7.5 and 15% BPM treatments weighing 1658.2, 1490.7 and 1529.9 g, respectively. Hens were housed in dark-out house conditions and provided

a 16:8 lighting schedule over the duration of the experimental period. House temperature was controlled via a fan and air inlet ventilation system and ranged from 21 to 27 °C. Hens were provided approximately 95g/hen/d feed and had *ad libitum* access to water. The controlled amount of feed was placed in the feed trough at approximately 9:00am each morning. Laying hens were monitored at least twice daily and cull birds or mortality were noted and removed as needed.

#### 6.3.4 Data Collection

Egg production was monitored daily and feed offered and refused was recorded weekly. Feed intake, HHEP and FCR were calculated from the collected data to correspond with the bi-weekly feed manufacture period. Birds were weighed by experimental unit (EU) at 38, 46 and 54 weeks of age. At 42, 46, 50, 54 weeks of age, eggs were weighed by EU and two eggs from each EU were collected and stored in 4°C refrigerator for 7 days before determination of Haugh unit, egg composition and egg shell thickness. At 54 weeks of age, two eggs from each EU were used to measure yolk color using Roche yolk color fan (DSM, Heerlen, Netherlands). At the end of the experiment (54 weeks of age), 1 ml blood was drawn from the wing vein of one bird per EU for H/L ratio measurement. Three of the 4 hens from each EU were euthanized and spleen weights were recorded.

Plasma H/L ratio was measured according to previous reports (Zhang et al., 2017). To measure the H/L ratio, blood smears were prepared on a glass slide. Slides were stained by Wright-Giemsa differential. Nikon Eclipse 80i microscope and DS-Ri1 color camera (Nikon, Japan) was used to capture white blood cells on slides. In three different areas of each slide, the

numbers of eosinophil and heterophil, lymphocyte, basophil, and monocyte were counted per 100 white blood cells in each area and the average percentages were calculated.

### 6.3.5 Statistical Analysis

Hen-housed egg production, feed intake, FCR, egg weight, egg mass, Haugh unit, relative albumen weight, relative yolk weight, relative shell weight, shell thickness were analyzed using one-way ANOVA and repeated measures with means separated using a Tukey's adjustment (SAS 9.4, SAS Institute Inc, Cary, NC). Body weight gain, egg yolk color, relative spleen weight, H/L ratio were analyzed using one-way ANOVA with means separated using Fisher's LSD test. Significance was accepted at  $P \leq 0.05$ .

## 6.4 Results and Discussion

### 6.4.1 Nutrients in bacterial protein meal

There were differences in the nutrient composition of the two BPM samples received over the duration of this experiment. Gross energy, and amino acid and protein concentrations of the second batch of BPM was higher than the first batch and ash concentration was reduced. The ileal amino acid digestibility of BPM from batch No.1 was determined and ranged from 71.73 to 84.45% but methionine and cysteine digestibility was only 64.36 and 61.69%, respectively. Schøyen and coworkers (2007) determined apparent ileal amino acid digestibility of two BPMs in 35 days old broilers and most of the amino acids digestibility values ranged from 80 to 90% while cysteine digestibility was the lowest (76-77%).

### 6.4.2 Laying Hen Performance

Hen-housed egg production of hens fed the 7.5% BPM diet were similar to the control fed hens, but hens fed the 15% BPM diet resulted in reduced HHEP ( $P \leq 0.05$ ; Table 6.3). Similar to these data, feeding concentrations of BPM up to 10% did not result in negative effects on egg production in laying hens from 28 to 60 weeks of age (Whittemore et al., 1978).

No difference was observed on feed intake in laying hens fed diets containing up to 10% BPM in comparison to a diet without BPM inclusion again from 28 to 60 weeks of age (Whittemore et al., 1978). In broilers, 10% and 15% BPM diets induced a significant decrease in feed intake in comparison to diets with no BPM or 5% BPM inclusion (Waldroup and Payne, 1974). The same authors found pelleting improved the utilization of BPM allowing 10% BPM to maintain feed intake, but was not able to overcome a 15% BPM inclusion. In another broiler experiment, feed intake decreased to 31.0 and 23.4 g dry matter per day in birds fed 10% and 20% BPM in comparison to feed intake of 35.7 g dry matter per day of birds fed a corn-soybean control diet (D'Mello and Acamovic, 1976). It was hypothesized that the reduced feed intake associated with BPM might be the result of its powdery nature (Waldroup and Payne, 1974) or the adhesive properties when moistened or bulkiness (D'Mello and Acamovic, 1976). When the BPM was provided in granular or pelleted form, its acceptability would be enhanced (Plavnik, et al., 1981). In the current experiment, feed intake was close among birds fed control, 7.5% and 15% BPM over the duration of experiment (Table 6.3). An interaction between treatment and time was observed on feed intake ( $P \leq 0.05$ ). In the first 6 week of the experiment, hens fed 7.5% BPM had slightly higher feed intake than control and 15% BPM treatments; however, feed intake of 7.5% BPM was reduced after 6 wk resulting in the treatment x time interaction, but not a treatment effect (Figure 6.1-a).

Egg weights of the 7.5% BPM treatment were lower than that of the control treatment ( $P \leq 0.05$ ) with 15% treatment being intermediate (Table 6.3). An interaction between treatment and time occurred, for egg weight, but in general the egg weights of the 7.5% fed hens were reduced over the duration of the experiment although sometimes they were not significant from period to period (Figure 6.1-b). Hen body weight is another factor that has been shown to alter egg size (Scott, 1982). Since the body weights of 7.5% and 15% BPM fed hens were initially reduced in comparison to the control fed hens, the reduced egg weights in BPM fed hens could have resulted from the reduced body weights of those hens rather than a direct effect of BPM. Previous research is in agreement with the current data as no differences in egg weight were noted in diets with up to 10% BPM in laying hens (Whittemore, et al., 1978).

No differences due to BPM were observed on egg mass; however, there was a trend of reduced egg mass when hens fed the diet containing 15% BPM ( $P=0.06$ ) (Table 6.3). The decreased egg mass was driven by a lower HHEP as the egg weight was close to control treatment. As expected, feed conversion ratio (FCR) of 15% BPM treatment was significantly lower than control treatment.

Hens fed control and 7.5% BPM diets maintained a stable body weight over the first 8 weeks of the experiment; however, body weight of hens fed 15% BPM diet dramatically decreased by around 60 g per hen (Figure 1-c). A further decrease in body weight was observed over the following 8 weeks in 15% BPM treatment, while a slight body weight decrease was observed during this period in control and 7.5% BPM treatments. Overall, body weight loss of hens fed 15% BPM diets at the end of the experiment was significantly greater than those of hens fed control and 7.5% BPM diets ( $P \leq 0.01$ ) (Table 6.3). In another laying hen report, feeding various concentration of BPM did not lead to body weight difference (Whittemore, et al., 1978).

In the current experiment, body weight loss of 15% BPM fed hens could be related to inadequate calorie consumption as the BPM used contained nearly 20% ash. This additional ash could have reduced the actual AME content of the meal in comparison to the previous data used to estimate the formulated AME value during formulation.

#### 6.4.3 Egg Quality

Eggs from hens fed BPM presented a darker yolk color than those from hens fed control diet ( $P \leq 0.01$ ) (Figure 6.2). Eggs from hens fed diets containing 7.5 and 15% BPM received a yolk color of 8.87 and 9.48, respectively, in comparison to 6.73 from control treatment. Similar color response have been reported by Brazilian researchers (Polonio, et al., 2010). In their experiment, inclusion of bacterial (*Rubrivivax gelatinosus*) biomass in Dekalb White laying hen diet was able to darken and increase redness of yolks but did not affect yellowness over 30 days feeding period. Another experiment indicated that dried biomass obtained from stressed cells of *Chlorella vulgaris* contained canthaxanthin, astaxanthin and its esters, lutein and  $\beta$ -carotene (Gouveia, 1996). These carotenoids were transferred to yolk resulting in comparable yolk pigmentation with commercial feed pigments. Although the pigment compounds were not analyzed in the current experiment, darker yolk color by BPM supplementation would suggest that BPM may contain fat-soluble compounds and these pigments can be transferred to the egg yolk. No differences on Haugh unit, relative yolk weight, relative albumen weight, relative shell weight and shell thickness were observed by feeding BPM diets to laying hens. These data suggest that adding BPM to laying hen diet does not affect egg quality (Table 6.5).

#### 6.4.4 Immune Response

No difference in relative spleen weights or H/L ratio were observed across the treatments at 54 weeks of age, although a numerical increase on relative spleen weights were observed (Figure 6.2). This increase in spleen weight may be explained by differences on body weight at 54 weeks of age. These findings would suggest that inclusion of BPM up to 15% in the diet was not able to stimulate an immune response based on spleen weight and plasma H/L ratio. In contrast, BPM was reported to alter the immune response of chicks (Farstad, 1977). In this previous report, blood from chicks fed diets containing 12.4 and 24.8% BPM showed an increase in precipitation zone diameter (indicator of higher serum antibodies) when compared with that from broilers fed 0 or 6.2% BPM. The reason for this alternation of the immune status by BPM was not fully explained, but one possible mechanism could be high nucleic acid concentration in BPM. A BPM grown on natural gas was reported to contain 9.5% nucleic acid (Skrede, et al., 1998). In humans, dietary nucleotides have been suggested to have beneficial immunological effects (Yu, 1998). Based on results from relative spleen weight and H/L ratio, no evidence was found to support the hypothesis that BPM could trigger an immune response in laying hens.

## 6.5 Conclusions

Supplementation of 7.5% BPM in laying hen diets was able to at least maintain egg production in laying hens, but 15% BPM resulted in reduced performance and body weight gain. Supplementation of 7.5% BPM in laying hens diets reduced average egg weight by 1.8 g but was still able to maintain similar egg mass and FCR in comparison to a corn-soybean diet. Hens fed 15% BPM resulted in a significantly reduced egg production and increased FCR. No evidence was obtained to support the hypothesis that adding BPM to laying hen diet can alter the immune response.

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Table 6.1 Bacterial protein meal (BPM) nutrients composition<sup>1</sup> and amino acid digestibility

	BPM	
	Batch No.1	Batch No.2
<b>Proximate analysis</b>		
Gross energy, kcal/kg	3001.7	3240.5
Crude protein, %	33.11	40.63
Crude fiber, %	5.79	4.87
Crude fat, %	0.95	0.74
Non-fiber carbohydrates, %	ND <sup>2</sup>	27.41
Ash <sup>3</sup> , %	25.37	18.19
<b>Amino acid profile (unit: %)</b>		
Arginine	ND	1.88
Histidine	ND	0.79
Isoleucine	1.20(76.35) <sup>4</sup>	1.60
Leucine	2.19(78.16)	2.69
Lysine	1.41(71.73)	1.79
Methionine	0.62(64.36)	0.73
Phenylalanine	ND	1.61
Threonine	1.52(80.04)	1.86
Tryptophan	ND	0.45
Valine	1.91(82.35)	2.37
Alanine	2.31(84.45)	2.79
Aspartic Acid	2.91(80.17)	3.48
Cysteine	0.32(61.69)	0.35
Glutamic Acid	3.62(81.40)	4.22
Glycine	1.80(81.81)	2.23
Proline	1.28(79.01)	1.54
Serine	ND	1.27
Tyrosine	ND	1.32

<sup>1</sup> Nutrients composition data are on as is basis.

<sup>2</sup> ND means not determined.

<sup>3</sup> Mineral concentration of BPM batch No.2: aluminum 0.056%; calcium 1.161%; copper: 0.007; iron: 0.146%; potassium: 0.466%; magnesium: 0.246%; manganese: 0.011%; sodium: 0.373; phosphorus: 1.126%; zinc: 0.028%.

<sup>4</sup> Amino acid digestibility (%) of BPM batch No.1 was reported in parenthesis.

Table 6.2 Nutrients composition of experimental laying hen diets containing no bacteria protein meal (BPM) (control), 7.5% BPM and 15% BPM, respectively.

Ingredient, %	Control	7.5% BPM	15% BPM
Corn	57.575	55.805	54.025
Soybean meal	20.66	14.72	8.79
Bacterial protein meal	0.00	7.50	15.00
DDGS	5.00	5.00	5.00
Poultry byproduct meal	3.00	3.00	3.00
Soy oil	2.63	2.67	2.71
Salt	0.35	0.35	0.36
DL-methionine	0.13	0.15	0.17
L-lysine HCl	0.00	0.07	0.13
L-tryptophan	0.00	0.01	0.02
Oyster shell (large particle)	4.75	4.75	4.75
Limestone (small particle)	4.75	4.75	4.75
Dicalcium Phosphate	0.53	0.60	0.67
Choline chloride	0.10	0.10	0.10
Vitamin and mineral premix <sup>1</sup>	0.500	0.500	0.500
Phytase <sup>2</sup>	0.025	0.025	0.025

Calculated composition (analyzed)

	Control	7.5% BPM	15% BPM
Metabolizable energy, kcal/kg	2,900	2,900	2,900
Crude protein, %	17.9 (18.4)	18.6 (18.1)	19.3 (16.5)
Calcium, %	3.9	3.9	3.9
Phos, %	0.48	0.45	0.42
Available Phos, %	0.25	0.25	0.25
Crude fat, %	5.90(3.28)	5.79(3.28)	5.69(4.03)
Crude fiber, %	2.48(3.62)	2.23(6.22)	1.99(5.26)
Digestible Met + Cys, %	0.72	0.72	0.72
Digestible Lys, %	0.86	0.86	0.86

<sup>1</sup> Provided per kg of diet: vitamin A, 4,409 IU; vitamin D<sub>3</sub>, 1,147 IU; vitamin E, 1.1 IU; menadione, 0.8 mg; vitamin B<sub>12</sub>, 4 µg; choline, 255 mg; niacin, 13.2 mg; pantothenic acid, 4.1 mg; riboflavin, 2.8 mg; Cu, 2.7 mg; Fe, 34 mg; I, 670 µg; Mn, 43 mg; Zn, 33 mg, Fe, 34mg.

<sup>2</sup> OptiPhos<sup>®</sup> 5000 G (Huvepharma, Bulgaria). Minimal activity per kg of complete feedstuff is 125 OTU/g.

Table 6.3 Laying hen performances as hens fed diets containing no bacteria protein meal (BPM) (control), 7.5% BPM and 15% BPM, respectively, from 38 to 54 weeks of age.

Treatments	HHEP <sup>1</sup>	Feed intake	Egg weight	Egg mass	FCR	Body weight gain 38-46w	Body weight gain 38-54w	
	%	g/hen/d	g/egg/d	g/hen/d	g/g	g/hen	g/hen	
Control	86.7 <sup>a</sup>	94.0	60.0 <sup>a</sup>	51.8	1.832 <sup>a</sup>	3.9 <sup>a</sup>	-17.4 <sup>a</sup>	
7.5% BPM	88.7 <sup>a</sup>	94.5	58.2 <sup>b</sup>	51.3	1.859 <sup>ab</sup>	3.7 <sup>a</sup>	-43.0 <sup>a</sup>	
15% BPM	83.5 <sup>b</sup>	94.7	59.6 <sup>ab</sup>	49.1	1.943 <sup>b</sup>	-59.9 <sup>b</sup>	-127.4 <sup>b</sup>	
Pooled SEM	0.78	0.31	0.45	0.81	0.031	17.68	19.36	
			P Value					
Treatment	≤ 0.01	0.11	≤ 0.05	0.06	≤ 0.05	≤ 0.05	≤ 0.01	
Week	≤ 0.01	≤ 0.01	≤ 0.01	≤ 0.01	≤ 0.01	-	-	
Treatment×week	0.53	≤ 0.05	≤ 0.05	0.06	0.10	-	-	

<sup>1</sup>HHEP: hen-housed egg production

Table 6.4 Haugh unit, egg composition and shell thickness of eggs from hens fed diets containing no bacteria protein meal (BPM) (control), 7.5% BPM and 15% BPM, respectively.

Treatments	Relative yolk weight	Relative albumen weight	Relative shell weight	Shell thickness	Haugh unit <sup>1</sup>
	%	%	%	mm	-
Control	29.49	55.91	9.21	0.32	84.25
7.5% BPM	29.48	55.85	9.13	0.31	84.97
15% BPM	29.52	55.59	9.33	0.33	84.26
Pooled SEM	0.21	0.26	0.10	0.006	0.74
		P Value			
Treatment	0.99	0.66	0.15	0.27	0.74
Week	≤ 0.01	≤ 0.01	0.06	≤ 0.01	≤ 0.01
Treatment×week	0.42	0.31	0.79	0.31	0.17

<sup>1</sup> Haugh unit =  $100 \cdot \log(h - 1.7 \cdot w^{3.7} + 7.6)$ ; h = observed height of the albumen in millimeters; w = egg weight in grams

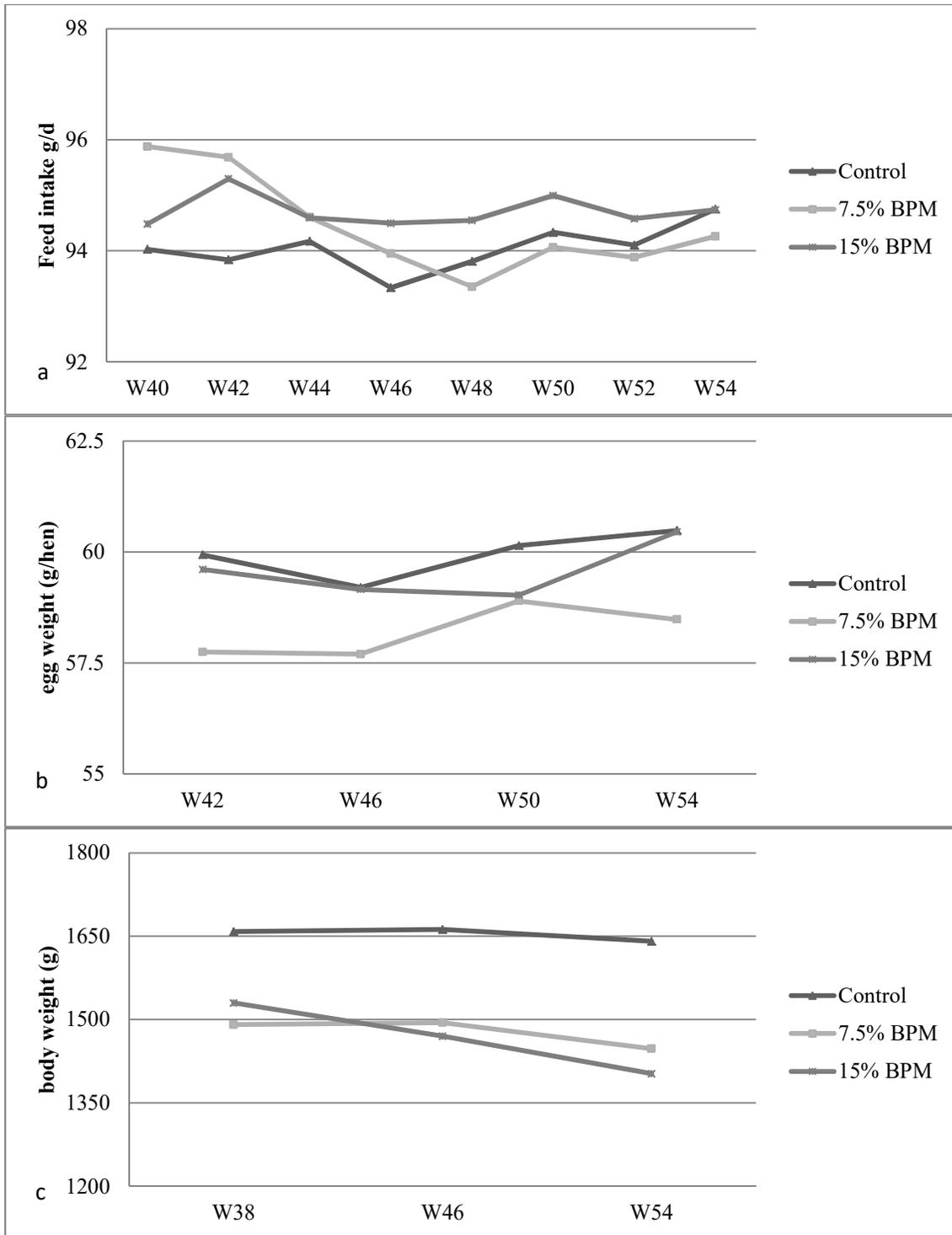


Figure 6.1 Feed intake (a), egg weight (b), body weight (c) of hens fed control, 7.5% and 15% BPM diets from 38 to 54 weeks of age.

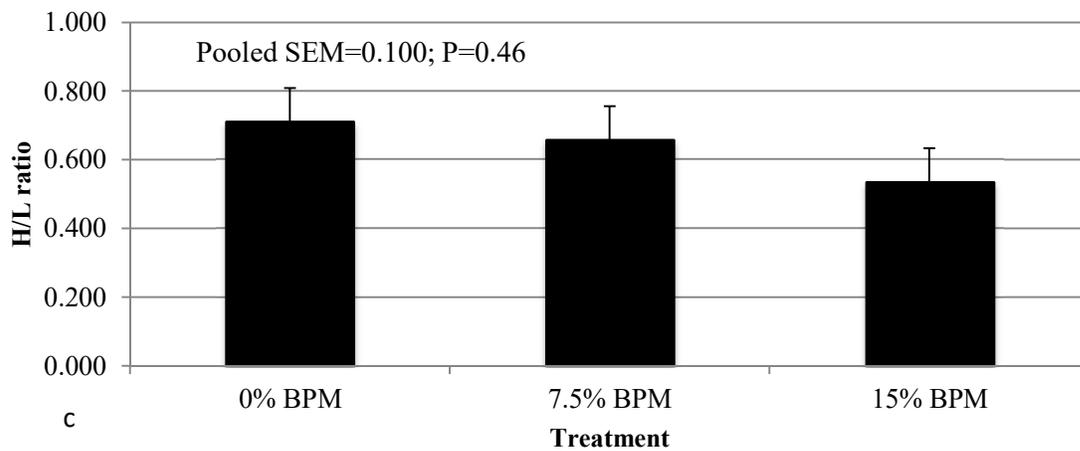
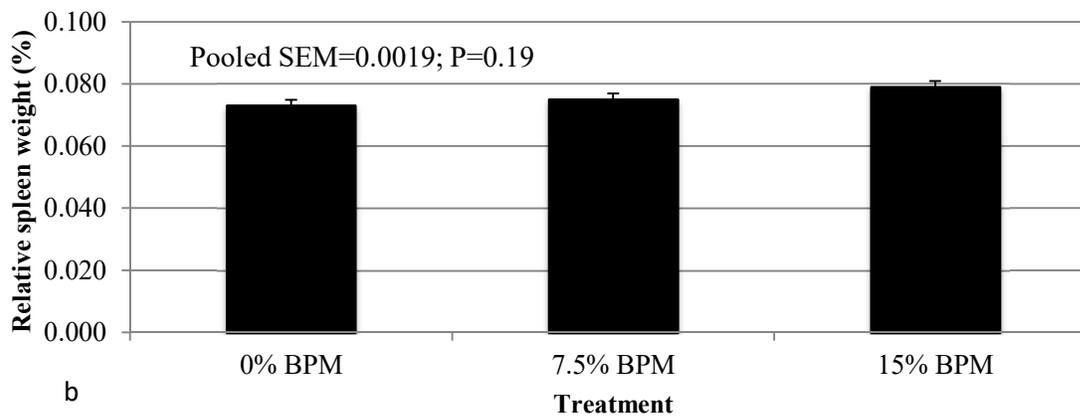
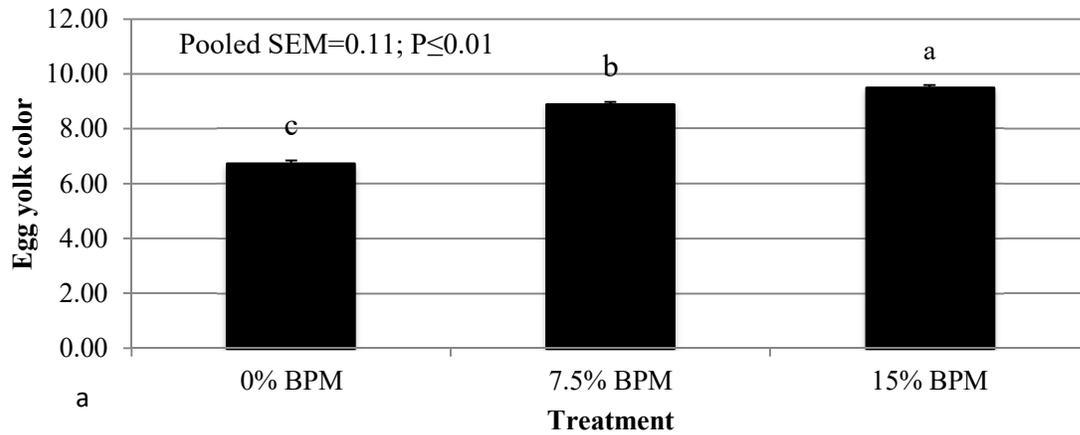


Figure 6.2 Egg yolk color (a), Relative spleen weight (%) (b), blood heterophil/lymphocyte ratio (c) of laying hens fed diets containing no bacteria protein meal (BPM) (control), 7.5% BPM, 15% BPM at 54 weeks of age.

## Chapter 7: Effect of High Concentrations of Dietary Vitamin D<sub>3</sub> on Pullet and Laying Hen Performance, Skeleton Health, Eggshell Quality and Yolk Vitamin D<sub>3</sub> Content when Fed to W36 Laying Hens from Day of Hatch until 68 Weeks of Age

### 7.1 Abstract

The objective of this experiment was to investigate the effects of various concentrations of dietary vitamin D<sub>3</sub> (D<sub>3</sub>) on pullet and laying hen performance, eggshell quality, bone health and yolk D<sub>3</sub> content in white laying hens from day of hatch until 68 weeks of age. Initially, 440 Hy-line W36 day-old pullets were randomly assigned to five dietary treatments. At 17 weeks of age, pullets were moved into a multi-tiered A-frame cage system resulting in five treatments with twelve replicates of six birds per experiment unit. From day of hatch to 68 weeks of age, birds were provided 1 of 5 experimental diets: control (1,681 IU of D<sub>3</sub>/kg), control + 6,667 IU of D<sub>3</sub>/kg (8,348 IU of D<sub>3</sub>/kg), control + 16,667 IU of D<sub>3</sub>/kg (18,348 IU of D<sub>3</sub>/kg), control + 33,333 IU of D<sub>3</sub>/kg (35,014 IU of D<sub>3</sub>/kg), control + 66,667 IU of D<sub>3</sub>/kg (68,348 IU of D<sub>3</sub>/kg). Dietary calcium and phosphorus concentrations from day of hatch to 17 weeks of age were set at 95% of the NRC recommendation, while calcium and phosphorus concentrations of 100% of the NRC recommendation were fed during the egg laying phase. At 17 weeks of age, pullets fed diets containing 8,348 and 35,014 IU D<sub>3</sub>/kg of diet resulted in a higher bone mineral density in comparison to the control fed birds ( $P \leq 0.01$ ). Body weights of pullets fed the diet with the highest concentration of D<sub>3</sub> (68,348 IU D<sub>3</sub>/kg) were lower than those from the other treatments ( $P \leq 0.01$ ). During the egg laying phase, hen-housed egg production (HHEP) of hens fed diets containing 68,348 IU D<sub>3</sub>/kg of diet was significantly reduced ( $P \leq 0.01$ ). No differences in HHEP of laying hens fed diets containing 8,348 and 18,348 IU D<sub>3</sub>/kg were observed in comparison to control treatment, but 35,014 IU D<sub>3</sub>/kg resulted in increased HHEP in comparison to the control ( $P \leq 0.01$ ). Specific gravity of eggs from hens fed 68,348 IU/kg of D<sub>3</sub> of diet was greater than

hens fed control and 18,348 IU D<sub>3</sub> diets ( $P \leq 0.01$ ). Shell breaking strength of eggs from hens fed 8,348 IU, 35,014 IU and 68,348 IU D<sub>3</sub> diet was higher than control treatment ( $P \leq 0.01$ ). Fat-free tibia ash content was increased with 8,348, 18,348, 35,014 and 68,348 IU D<sub>3</sub> fed hens in comparison to the control ( $P \leq 0.05$ ). Yolk D<sub>3</sub> content was increased linearly as dietary D<sub>3</sub> increased. The D<sub>3</sub> transfer efficiency of control, 8,348 IU, 18,348 IU, 35,014 IU, 68,348 IU D<sub>3</sub> treatments from diet to yolk was 8.24%, 10.29%, 11.27%, 12.42%, 12.06%, respectively. These data suggest that supplementation of dietary D<sub>3</sub> up to 35,014 IU/kg of diet was able to maintain if not increase laying hen performance and enhance pullet and laying hen skeletal quality as well as eggshell quality. Feeding hens 68,348 IU of D<sub>3</sub> resulted in reduction of pullet growth and decreased laying hen performance during first laying cycle. Feeding higher concentrations of D<sub>3</sub> had the benefit of improving performance/hen health/egg shell quality and resulting in an egg with increased D<sub>3</sub>.

Key words: laying hen, vitamin D<sub>3</sub>, skeleton health, eggshell quality, yolk vitamin D<sub>3</sub> concentration

## 7.2 Introduction

Vitamin D is a group of closely related compounds that have been shown to have antirachitic activity. It has been estimated that nearly 50% of the United States population is at risk for vitamin D deficiency or insufficiency (Holick, et al., 2011). Vitamin D<sub>3</sub> deficiency can cause rickets in young children or increase the risk of osteoporosis and osteomalacia in adults (Holick, 2005). One approach to increase vitamin D intake in a population without changing eating habits is to produce vitamin D-fortified eggs (Mattila, et al., 2004). As a fat-soluble vitamin, D<sub>3</sub> from a laying hen diet is absorbed into the blood stream and transferred to egg yolk via the ovary. A linear increase on egg D<sub>3</sub> concentration with increasing dietary D<sub>3</sub>, while physical, functional properties and sensory quality were not affected in the high D<sub>3</sub> eggs (Yao et al., 2013).

In addition to fortification of eggs, high dietary D<sub>3</sub> may potentially affect calcium and phosphorus metabolism and related biological characteristics in laying hens. Tibia breaking strength increased by supplementation of 6,000 and 15,000 IU D<sub>3</sub>/kg in comparison to control fed birds (2,500 IU D<sub>3</sub>/kg) in laying hens from 20 to 67 weeks of age (Mattila et al., 2004). In contrast to tibia breaking strength, no differences were observed on egg specific gravity or eggshell strength with the supplementation of dietary D<sub>3</sub> from 2,500 to 15,000 IU/kg. Egg specific gravity and eggshell breaking strength increased as hens were fed diets containing 2,400 IU D<sub>3</sub>/kg in comparison to a diet containing 300 IU D<sub>3</sub>/kg (Zang et al., 2001). Another experiment was conducted to define the effects of supplementation of D<sub>3</sub> from 2,200 to 102,200 IU/kg of diet on eggshell and bone quality from 19 to 58 weeks of age in Hy-line W36 laying hens; however, no significant differences were observed on egg specific gravity or tibia ash content (Persia, et al. 2013). As the focus of previous research has been improving bone health or

egg shell quality of laying hen by increasing D<sub>3</sub> in the egg laying phase, limited research has been conducted on increased dietary D<sub>3</sub> in pullet bone development and the subsequent impact on laying hen skeletal health and eggshell quality. The objective of the current experiment is to determine the effects of feeding various concentrations of dietary D<sub>3</sub> to pullets/hens (day of hatch to 68 wks) on pullet and laying hen bone health, laying hen performance and eggshell quality.

### 7.3 Materials and Methods

#### 7.3.1 Diets

In total, seven phases of diets were formulated including starter 1 (wk 0-4), starter 2 (wk 5-8), grower (wk 9-12), developer (wk 13-15), pre-laying (wk 16-17), peak (wk 18-24) and post peak diets (wk 25-68), respectively. Basal diets for each phase were supplemented with a commercial laying hen vitamin and mineral premix (Big Spring Mill Inc, Elliston, VA) resulting in diets containing 1,681 IU D<sub>3</sub>/kg of diet (Table 7.1). Experimental diets were prepared by adding D<sub>3</sub> (DSM Nutritional Products Inc, Parsippany, NJ) at a concentration of 6,667, 16,667, 33,333, 66,667 IU/kg resulting in diets containing 1,681, 8,348, 18,348, 35,014, 68,348 IU D<sub>3</sub>/kg, respectively. Vitamin D<sub>3</sub> premix was stored in a 4°C refrigerator throughout experimental period to maintain viability. Starter 1, starter 2, grower, developer, pre-laying diets were manufactured as single diets while peak and post peak diets were manufactured every two weeks due to increased feed intake and longer feeding period. Diet samples from each phase were collected for D<sub>3</sub> concentration analysis. Nutrient recommendations of basal diets followed Hy-Line W-36 Commercial Layer Management Guidelines (2016). Calcium and phosphorus concentration for starter 1, starter 2, grower, developer, pre-laying diets were set at 95% of NRC (1994) recommendation to mimic potential limited calcium and phosphorus availability that can become

an issue in the commercial industry. Calcium and phosphorus concentrations were set at 100% of NRC (1994) recommendation for peak and post peak diets.

### 7.3.2 Animals and Housing

All animal work was approved by the Institutional Animal Care and Use Committee at Virginia Tech (Blacksburg, VA). Initially, 440 Hy-line W36 day-old pullets were randomly assigned to five treatments with two replicates (84.5 cm<sup>2</sup>/bird) per treatment. The initial chick body weights were between 39.1 and 39.6 g ( $P > 0.05$ ). Birds in each treatment were split into four replicates at 2 weeks of age (169.0 cm<sup>2</sup>/bird) and into eight replicates at 6 weeks of age (338.0 cm<sup>2</sup>/bird) to meet bird density requirements as body weight increased, but to maintain critical body mass per cage early in life. In the pullet house, the environment was controlled according to Hy-Line W-36 Commercial Layer Management Guide (2016). The temperature was 32 to 33 °C at day of hatch and gradually decreased to 21 °C until the pullets were 36 days of age. Light length was 20 hours at day of hatch and gradually decreased to 12 hours at 11 weeks of age. Pullets were allowed *ad libitum* access to feed and water. At 14 weeks of age, pullets received a killed *salmonella enteritis* vaccination (subcutaneous injection, 0.25 ml/bird). At 17 weeks of age, the 72 pullets (closest to the average body weight within each treatment) were moved into a multi-tiered A-frame cage system resulting in twelve replicates of six birds per experimental unit (154.8 cm<sup>2</sup>/bird) for each of the five dietary treatments. Each experimental unit consisted of two consecutive cages with three birds per cage. The remaining 16 pullets from each treatment were euthanized, defeathered and the intact carcasses were stored in a -20°C freezer for bone mineral analysis. During the egg laying phase, 95 to 97g of feed was provided per hen per day and feed was provided at approximately 10:00am. Hens were housed in dark-out house conditions and length of light increased from 12 to 16 h over 17 to 32 weeks of age. House temperature was

controlled via a fan and air inlet ventilation system and ranged from 21 to 27 °C over the duration of the experiment.

### 7.3.3 Data Collection

Birds were monitored at least twice daily in both the morning and afternoon. Pullets were weighed at 2, 4, 6, 8, 12, 15 and 17 weeks of age. Feed offered and refused was recorded weekly in both the pullet and egg production phases. After the onset of lay, hens were weighed every 4 weeks and at the end of the experiment. Egg numbers and gross egg weight per pen were recorded daily. Feed intake, HHEP, egg weight, egg mass and FCR were calculated to correspond with the bi-weekly feed manufacture periods. Starting at 22 weeks of age, internal and external egg quality was determined every 4 weeks. Two eggs per experimental unit were collected and stored in a refrigerator for 7 days before egg quality determination. Egg quality measurements included Haugh unit, egg composition, eggshell thickness, egg specific gravity and eggshell breaking strength. Over two consecutive days of week 38, one egg from each pen was collected for yolk vitamin D<sub>3</sub> analysis. At 68 weeks of age, five hens from each experimental unit were euthanized for keel bone and tibia (both right and left) collection. Once collected, keel bones were stored in plastic bags and refrigerated at 4°C for keel bone scoring the next day and both tibia were frozen at -20°C and stored for later analysis.

### 7.3.4 Dual x-ray Absorptiometry (DXA)

The pullets euthanized at 17 weeks of age were thawed and arranged on the target pad on the DXA platform for analysis. Ten carcasses were scanned using a single pass of the Lunar Prodigy machine (GE Lunar, GE Healthcare, Waukesha, WI) using the small animal mode for bone mineral density and bone mineral content measurements.

The tibia bones collected from the hens at 68 weeks of age were thawed. Tibias were defleshed of adhering tissue and placed on the platform for DXA scanning to measure bone mineral density and bone mineral content using small animal mode. When scanning, five tibias from each experimental unit were pooled together as one replicate value.

#### 7.3.5 Yolk Vitamin D<sub>3</sub> Concentration

Eggs collected at 38 weeks of age were stored in a 4°C refrigerator no more than 3 days before they were analyzed. Yolks were separated from albumen by yolk separator. All yolks from the same treatment were pooled and homogenized using a spatula resulting in five samples. Homogenized yolk samples were lyophilized and vacuum packed. 100 g of yolk from each treatment were sent for D<sub>3</sub> analysis by liquid chromatography–mass spectrometry (LC-MS) method (Heartland Assay LLC, Ames, Iowa). Vitamin D<sub>3</sub> transfer efficiency was calculated (Yao, et al., 2013) and the equation is: transfer efficiency (%) = 100 × [yolk cholecalciferol concentration (IU/g) × yolk mass (g) × egg production (% per hen per day)] / [feed cholecalciferol concentration (IU/g) × feed intake (g/d per hen)].

#### 7.3.6 Eggshell and Tibia Breaking Strength

Eggshell and tibia breaking strength were measured by Instron Universal Testing Machine Model 1011 (Instron Corp, Canton, MA). Each egg was placed on a base with the air cell or blunt end of the egg located to the bottom of the machine. A round steel head was used and lowered to the top of the egg (<1mm) before compressing. During testing, the steel head was advanced towards the egg at 10 mm/min to begin and after the compression load reached 0.3 kg, the speed was reduced to 2 mm/min until the extension of the steel was 1.5mm. Maximum compression load during this process was recorded.

Bone breaking strength was measured using a 3 point bending method. Tibia length was measured and middle point was marked. The tibia was placed on two paralleled vertical holders with a 42 mm span and the middle point of tibia was underneath the bottom of loading head. Before testing, the loading head was lowered to barely touch the middle point of tibia with a preload of around 0.05 kg. The loading head descended at rate of 5 mm/min until the bone fractured. Maximum compression load before fracture was recorded.

#### 7.3.7 Fat-free tibia ash content

Right tibias were wrapped with aluminum foil and autoclaved for 20 mins. Tibias were de-fleshed and cartilage was removed. Tibias were placed beaker and dried at 100°C for 24 hours. Dried tibias were wrapped with cheese cloth and placed in soxhlet apparatus. Hexanes were used to extract fat of tibias for 48 hours. Tibias were taken out of soxhlet apparatus and allowed for evaporation of hexanes. Tibias were dried at 100°C for 24 hours and placed into muffle furnace at 600°C for 24 hours. Fat-free tibia ash content was calculated as below.

$$\text{Fat-free tibia ash content (\%)} = (\text{tibia ash weight} / \text{fat-free tibia dry weight}) * 100\%$$

#### 7.3.8 Keel Bone Score

Keel bones collected from five hens per experimental unit were scored using method described by Donaldson, et al. (2012). Bones were scored from 0 (no damage) to 4 (severe damage and multiple fractures). A bone with one fracture, usually toward the tip of keel, was scored 1. A bone with between two and three fractures or callus formation was scored 2. A bone

with multiple fractures or callus formation plus slight deformation at the fracture sites was scored 3. A bone with multiple fractures or callus formation and severe deformations of the ventral edge was scored 4.

### 7.3.9 Statistical Analysis

SAS 9.4 (SAS Institute Inc, Cary, NC) was used to analyze HHEP, feed intake, egg weight, egg mass, feed conversion ratio (FCR), Haugh unit, relative albumen weight, relative yolk weight, relative shell weight, shell thickness, egg specific gravity, eggshell breaking strength using a one-way ANOVA by repeated measures, with means separated using a Tukey's adjustment. Body weight, pullet bone mineral density, pullet bone mineral content, laying hen tibia mineral density, laying hen tibia mineral content, laying hen bone breaking strength, laying hen fat-free tibia ash content were analyzed using a one-way ANOVA with were separated by Fisher's LSD test. Keel bone scoring was analyzed using a nonparametric test. Significance was accepted at  $P \leq 0.05$ .

## 7.4 Results and Discussion

### 7.4.1 Pullet Growth Performance

Generally, dietary  $D_3$  concentrations did not affect pullet growth performance and feed intake except for pullets fed 68,348 IU  $D_3$ /kg. Feed intake of pullets from current experiment was generally in the recommended range from Hy-Line W-36 Commercial Layer Management Guide, 2016. At 17 weeks of age, body weight of pullets fed the 68,348 IU  $D_3$ /kg was lower than the other treatments ( $P \leq 0.05$ ). The average body weight of the 68,348 IU  $D_3$  fed hens was 1164 g/bird and was lower than the recommended body weight of 1,230 to 1,270g (Hy-Line W-36 Commercial Layer Management Guide, 2016) while body weights of other treatments were

1,225 to 1,241g. At 15 weeks of age, feed intake of all treatments dropped below recommended feed intake from Hy-Line W-36 Commercial Layer Management Guide (2016) but recovered at 16 weeks of age. This reduction in feed intake at 15 wk of age is most likely associated with the SE vaccination at 14 wk. Morrissey and coworkers (1977) studied the toxicity of feed high D<sub>3</sub> diets (400, 4,000, 40,000, 400,000, 4,000,000 IU D<sub>3</sub>/kg) from 14 to 28 d of age. These authors found reduced growth rate and feed intake as chicks fed 400,000 and 4,000,000 IU D<sub>3</sub>/kg at 6 and 14 days after the initiation of experiment, but no inhibited growth was observed in chicks fed 40,000 IU D<sub>3</sub>/kg or less. Increased serum calcium was observed in the 400,000 and 4,000,000 IU treatments, which was defined as a sign of toxicity. In the current experiment, serum calcium was not measured, but the reduced body weight of pullets fed 68,348 IU D<sub>3</sub> diet may suggest a toxic effect on pullet growth.

#### 7.4.2 Pullet Bone Quality

Bone mineral density of 17 weeks old pullets fed high D<sub>3</sub> diets was increased in comparison to the control diet fed pullets ( $P \leq 0.01$ ; Table 7.4). Bone mineral density of pullets fed 35,014 IU D<sub>3</sub>/kg of diet was significantly higher than hens fed the control, 8,348 IU, 18,348 IU and 68,348 IU D<sub>3</sub>/kg diets. Pullets fed the 18,348 and 35,014 IU D<sub>3</sub>/kg showed an increased bone mineral content in comparison to those fed control and 68,348 IU D<sub>3</sub>/kg diets ( $P \leq 0.01$ ). Although no previous reports were found on the effect of high concentration dietary D<sub>3</sub> on pullet skeletal health, results from broiler experiments may provide some indications. Tibia breaking strength was maximized when 10,000 IU D<sub>3</sub> /kg diet was fed to broilers from day of hatch to 14 days of age, and tibia ash was maximized in 5000 IU D<sub>3</sub> treatment (Whitehead et al., 2004). In another experiment, tibia ash and toe ash were increased as broilers were fed increased D<sub>3</sub> diets from 200 to 3000 IU D<sub>3</sub>/kg diet from day of hatch to 42 days of age (Khan, et al., 2010). The

data from the broiler chicks indicates that increased dietary D<sub>3</sub> concentration could enhance bone mineral deposition during skeletal development in laying hen pullets.

#### 7.4.3 Laying Hen Performance

Hens started to produce eggs at 19 weeks of age. From 19 to 24 weeks of age, HHEP dramatically increased from 0% to 95% at peak production. During this period, the increase of HHEP of hens fed 68,348 IU D<sub>3</sub>/kg of diet was reduced in comparison to the other high D<sub>3</sub> treatments and the control fed birds, but reached the level of egg production of the other treatments at 24 weeks of age. This is likely explained by the reduced body weight of those hens as light hens produce fewer eggs at the start of the laying period (Leeson, et al., 1997; Perez-Bonilla, et al., 2012). Hen-housed egg production was similar across treatments during the early to peaking production; however, HHEP started to separate after 54 weeks of age resulting in a differences across treatments. From 19 to 68 weeks of age, HHEP of hens fed 35,014 IU D<sub>3</sub> diet was significantly higher than control fed birds with treatments 8,348 and 18,348 IU D<sub>3</sub> being intermediate. Hen-housed egg production of hens fed 68,348 IU D<sub>3</sub> diet was significantly lower than all other treatments. Egg mass of hens fed 35,014 IU D<sub>3</sub> diet was significantly higher than that of hens fed 68,348 IU D<sub>3</sub> diet with the other treatments being intermediate. Feed conversion ratio of control and 35,014 IU D<sub>3</sub> fed birds was lower than that of 68,348 IU treatment ( $P \leq 0.01$ ). The differences noted in laying hen performance by feeding high D<sub>3</sub> diets are inconsistent with previous reports (Persia et al., 2013). In the previous experiment, dietary D<sub>3</sub> was supplemented up to 102,200 IU/kg resulting in no difference in laying hen performance from 19 to 58 weeks of age. The major difference in the current experiment and previous reports is the time that vitamin D supplementation started, as in the previous work vitamin D supplementation did not start until the point of lay, but in the current experiment, vitamin D supplementation occurred at day of

hatch. Egg weight decreased by feeding laying hens 100,000 or 200,000 IU D<sub>3</sub> diet in comparison to 960 IU D<sub>3</sub> diet, although HHEP was not altered (Ameenuddin et al., 1986). In the current experiment, reduced HHEP and egg mass in the 68,348 IU D<sub>3</sub> fed hens may be explained by the reduced body weight of the pullets as they entered egg production. This phenomenon has been noted before as pullets with reduced body weight produced less total egg mass to 70 weeks of age (Leeson, et al., 1997).

#### 7.4.4 Egg Characteristics

Haugh unit measurement of eggs from the 8,348 IU and 68,348 IU D<sub>3</sub> treatments were lower than Haugh units from control, 18,348 IU and 35,014 IU D<sub>3</sub> fed hens ( $P \leq 0.01$ ). Previous reports have indicated no difference in Haugh unit by different dietary D<sub>3</sub> concentrations from 2,200 IU to 102,200 IU /kg of diet and are contrary to current research (Persia et al., 2013). Haugh unit decreased as dietary D<sub>3</sub> increased from 2,000 to 20,000 IU/kg (Park et al., 2005). These contradictory responses with various concentrations of dietary D<sub>3</sub> among different experiments on egg Haugh unit may indicate that Haugh units are not directly associated with dietary D<sub>3</sub> or only slightly related and these differences noted are not biologically relevant as no effects of vitamin D have been proposed on the protein quality of the egg. No differences were observed on egg weight, relative shell weight, relative yolk weight, relative albumen weight and eggshell thickness across treatments. Specific gravity of eggs from the 68,348 IU D<sub>3</sub> fed hens was reduced in comparison to the control and 18,348 IU D<sub>3</sub> treatments with the other treatments being intermediate ( $P \leq 0.01$ ). Eggshell breaking strength of 8,348, 35,014 and 68,348 IU D<sub>3</sub> treatments was significantly higher than the control fed birds. As eggshell quality indicators, both egg specific gravity and eggshell breaking strength increased in high dietary D<sub>3</sub> treatments in comparison to the control treatment indicating that increased dietary D<sub>3</sub> was able to improve

eggshell quality. In contrast, there were no differences on specific gravity by feeding enriched D<sub>3</sub> feed to laying hens (Mattila et al., 2004; Persia et al., 2013). No differences were observed on eggshell breaking strength by high dietary D<sub>3</sub> (Mattila et al., 2003; Park et al., 2005). These experiments fed hens high D<sub>3</sub> diets after the onset of egg production with the pullets receiving diets with lower dietary D<sub>3</sub> concentrations. In the current experiment, the hens received high D<sub>3</sub> diets throughout pullet and egg laying phases and this difference likely explains differences in the responses on eggshell quality. Pullets with increased dietary D<sub>3</sub> had increased bone mineral density/content, and this might have promoted calcium deposition during eggshell formation as marginal calcium and phosphorus were provided to pullets rather than adequate diets provided in previous reports.

#### 7.4.5 Laying Hen Bone Quality

No differences in keel bone damage were observed across treatments. At the conclusion of the egg laying phase of the experiment (68 wk of feeding), tibia bone mineral content of the 68,348 IU D<sub>3</sub> treatment was increased in comparison to the control, 8,348 IU, and 18,348 IU D<sub>3</sub> fed hens. Generally this would be viewed as a positive, but remembering egg production reductions with the 68,348 IU D<sub>3</sub> treatments, this increased tibia mineral content is expected due to reduced Ca and P need with reduced egg production allowing for increased skeletal ash. Fat-free tibia ash content of the 8,348 IU, 18,348 IU, 35,014 IU and 68,348 IU D<sub>3</sub> treatments were increased in comparison to the control fed hens ( $P \leq 0.05$ ). Unlike the response in this experiment, there was no difference on tibia ash content by feeding laying hens D<sub>3</sub> up to 102,200 IU/kg of diet from 19 to 58 weeks of age (Persia et al., 2013). 34 weeks old white leghorn were fed dietary D<sub>3</sub> up to 200,000 IU/kg of diet for 16 weeks and reported no difference in bone ash among treatments (Ameenuddin et al., 1986). The contrary response of laying hen bone ash by

feeding high dietary D<sub>3</sub> between the current experiment and previous reports suggest that bone development and pullet skeletal status may play a more important role on maintaining skeleton bone health than supplying higher dietary D<sub>3</sub> during the egg production phase. No differences in tibia bone breaking strength were observed across treatments; however, there was a trend that high dietary D<sub>3</sub> could improve the tibia breaking strength (P=0.09). This agrees with previous experiment reports that laying tibia breaking strength was significantly increased as hens fed 6,000 IU and 15,000 IU dietary D<sub>3</sub> diets in comparison to fed 2,500 IU D<sub>3</sub> diet from 20 to 65 weeks of age (Mattila et al., 2004). In addition, no differences were observed on tibia bone mineral density as hens fed different concentration D<sub>3</sub> diets (P=0.13); however, a linear increase was observed on tibia bone mineral density as dietary D<sub>3</sub> concentration increase (P≤0.05). A quadratic response and a linear response were observed on tibia bone mineral content and tibia bone breaking strength, respectively. It should be noted that the R<sup>2</sup> of the above relationships were only from 0.06 to 0.13. In contrast, fat-free tibia ash did not follow a quadratic or linear pattern but just a low vs. high concentration response. Therefore, fat-free tibia ash could be a better candidate than other bone quality indices to distinguish the difference between low and high dietary D<sub>3</sub> in laying hen diets.

#### 7.4.6 Egg Vitamin D<sub>3</sub> Concentration

Egg D<sub>3</sub> concentration increased linearly as dietary D<sub>3</sub> increased. The D<sub>3</sub> concentration of eggs from the control, 8,348 IU, 18,348 IU, 35,014 IU, 68,348 IU D<sub>3</sub> treatments was 12.6, 88.6, 214.3, 435.5 and 872.6 IU D<sub>3</sub>/egg, respectively (Figure 7.5). The linear increase of yolk D<sub>3</sub> concentration with increased dietary D<sub>3</sub> in the current experiment was consistent with previous scientific reports (Yao et al., 2013). Although dietary D<sub>3</sub> concentrations in the current experiment were different from earlier experiments, similar yolk D<sub>3</sub> concentrations were observed when

similar dietary D<sub>3</sub> feed was provided to the laying hens. Vitamin D<sub>3</sub> transfer efficiency of the control, 8,348 IU, 18,348 IU, 35,014 IU, 68,348 IU D<sub>3</sub> treatments was 8.24%, 10.29%, 11.27%, 12.42%, 12.06%, respectively. This agreed with the D<sub>3</sub> transfer efficiency reported by Yao and coworkers (2013). In the previous report, transfer efficiency of hens fed 9,700 to 24,700 IU D<sub>3</sub> diets was between 11% to 14% which was 4 to 6 percent higher than control (2,200 IU D<sub>3</sub>) but much lower in the 102,200 IU D<sub>3</sub> diet. No dramatic increased D<sub>3</sub> transfer efficiency was observed in hens fed the highest D<sub>3</sub> concentration diet (68,348 IU D<sub>3</sub>/kg of diet) in the current experiment. This suggests that 102,000 IU was toxic to the hens and caused mismetabolism of the vitamin D as the hens tried to reduce vitamin D exposure, but this phenomenon was not observed in the current experiment with 68,348 IU D<sub>3</sub>. Taken together these data suggest that 68,348 would be safe for adult laying hens, although it has been shown to be detrimental to pullets.

Data from current experiment indicate that feeding pullets and hens (day old to 68 weeks of age) high dietary D<sub>3</sub>, up to 35,014 IU /kg, would improve pullet skeletal quality and further enhance skeletal health and eggshell quality of the laying hens during first cycle production without decreasing laying hen performance. Feeding pullet diets containing 68,348 IU D<sub>3</sub>/kg decreased body weight of pullets negatively affected laying hen performance from 19 to 68 weeks of age.

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Table 7.1 Pullet and laying hen basal diets for wk 0-4, wk 5-8, wk 9-12, wk13-15, wk 16-17, wk 18-24, and wk 25-68<sup>1</sup>.

Item	wk0-4	wk5-8	wk9-12	wk13-15	wk16-17	wk18-24	wk 25-68
Ingredient (%)							
Corn	65.65	67.14	67.99	69.35	64.65	48.47	59.49
DDGS	-	1.00	6.00	8.00	8.00	8.00	5.00
Poultry byproduct meal	2.00	1.98	3.00	2.78	2.50	2.00	2.00
Soybean meal	28.73	26.27	19.84	16.83	18.51	27.55	22.79
Soy oil	0.20	0.20	0.30	0.30	0.30	2.92	0.94
Salt	0.40	0.40	0.40	0.40	0.40	0.43	0.38
DL-methionine	0.18	0.20	0.14	0.08	0.14	0.25	0.20
L-lysine HCL	0.07	0.05	0.06	-	-	-	-
L-threonine	0.06	0.06	0.03	-	0.01	0.03	0.03
Limestone (small particle)	1.03	1.03	1.00	1.30	2.19	4.68	4.14
Oyster shell (big particle)	-	-	-	-	2.19	4.68	4.14
Dicalcium phosphate	1.02	1.02	0.59	0.31	0.47	0.34	0.23
Choline chloride	0.10	0.10	0.10	0.10	0.10	0.10	0.10
Vitamin & mineral premix <sup>2</sup>	0.50	0.50	0.50	0.50	0.50	0.50	0.50
Phytase <sup>3</sup>	0.06	0.06	0.06	0.06	0.06	0.06	0.06
Calculated composition							
Crude protein (%)	20.00	19.24	18.26	17.29	17.55	20.18	18.04
ME (kcal/kg)	3019	3029	3050	3055	2935	2844	2844
Calcium (%)	0.78	0.77	0.68	0.72	1.92	3.79	3.34
Available phosphorus (%)	0.32	0.32	0.27	0.22	0.24	0.22	0.18
Fat (%)	3.42	3.54	4.17	4.34	4.14	6.03	4.26
Digestible Lys (%)	1.05	0.98	0.88	0.76	0.79	0.97	0.85
Digestible Met (%)	0.48	0.49	0.43	0.36	0.42	0.54	0.47
Digestible Met+Cys (%)	0.74	0.74	0.67	0.60	0.66	0.80	0.71
Digestible Thr (%)	0.69	0.66	0.60	0.54	0.55	0.67	0.59

<sup>1</sup> Cholecalciferol was added to basal diets to obtain dietary cholecalciferol concentrations of 8,348, 18,348, 35,014, 68,348 IU of D<sub>3</sub>/kg of diet. Average cholecalciferol concentration was 1,681 IU of D<sub>3</sub>/kg of diet based on analyzed value of basal diets.

<sup>2</sup> Provided per kg of diet: vitamin A, 4,403 IU; vitamin D<sub>3</sub>, 1,457 IU; vitamin E, 1.10 IU; menadione, 0.77 mg; vitamin B<sub>12</sub>, 4.40 µg; choline, 254.79 mg; niacin, 13.21 mg; pantothenic acid, 4.05 mg; riboflavin, 2.75 mg; Cu, 2.70 mg; Fe, 33.75 mg; I, 0.67 mg; Mn, 42.90 mg; Zn, 32.50 mg; Co, 0.17mg.

<sup>3</sup> Supplementation of 0.06% phytase to release 0.08% calcium and 0.08% available phosphorus in complete diet.

Table 7.2 Body weight, bone mineral density and content of 17 wk old pullets fed diets containing 1,681, 8,348, 18,348, 35,014 and 68,348 IU of D<sub>3</sub>/kg of diet, respectively.

Dietary cholecalciferol (IU of D <sub>3</sub> /kg of diet)	Body weight	Pullet bone mineral density	Pullet bone mineral content
	(g)	(g/cm <sup>2</sup> )	(g)
1,681 (control)	1241.4 <sup>a</sup>	0.195 <sup>c</sup>	15.08 <sup>c</sup>
8,348	1225.3 <sup>a</sup>	0.205 <sup>b</sup>	16.35 <sup>ab</sup>
18,348	1241.3 <sup>a</sup>	0.200 <sup>bc</sup>	17.24 <sup>a</sup>
35,014	1236.1 <sup>a</sup>	0.213 <sup>a</sup>	17.87 <sup>a</sup>
68,348	1163.6 <sup>b</sup>	0.199 <sup>bc</sup>	15.96 <sup>b</sup>
Pooled SEM	13.19	0.0028	0.553
P-value	≤0.01	≤0.01	≤0.01

Table 7.3 Hen-housed egg production (HHEP), feed intake, egg weight, egg mass, feed conversion ratio (FCR) from hens fed diets containing 1,681, 8,348, 18,348, 35,014 and 68,348 IU of D<sub>3</sub>/kg of diet, respectively, during first cycle laying phase.

Dietary cholecalciferol (IU of D <sub>3</sub> /kg of diet)	HHEP (%)	Feed intake (g/h/d)	Egg mass (g/h/d)	FCR (g/g)
1,681 (control)	83.5 <sup>b</sup>	93.1 <sup>b</sup>	51.2 <sup>ab</sup>	1.865 <sup>b</sup>
8,348	83.8 <sup>ab</sup>	93.5 <sup>ab</sup>	51.0 <sup>ab</sup>	1.878 <sup>ab</sup>
18,348	83.8 <sup>ab</sup>	93.8 <sup>a</sup>	51.0 <sup>ab</sup>	1.888 <sup>ab</sup>
35,014	85.2 <sup>a</sup>	93.6 <sup>ab</sup>	51.8 <sup>a</sup>	1.850 <sup>b</sup>
68,348	81.9 <sup>c</sup>	93.7 <sup>ab</sup>	50.4 <sup>b</sup>	1.918 <sup>a</sup>
Pooled SEM	0.37	0.19	0.24	0.0101
	P Value			
Treatment (Tukey adj)	≤0.01	≤0.05	≤0.01	≤0.01
Time	≤0.01	≤0.01	≤0.01	≤0.01
Treatment×time	0.14	≤0.05	0.18	≤0.01

<sup>a-c</sup> Least square means without a common superscript differ significantly ( $P \leq 0.05$ ).

Table 7.4 Egg characteristics from hens fed diets containing 1,681, 8,348, 18,348, 35,014 and 68,348 IU of D<sub>3</sub>/kg of diet, respectively, during first cycle laying phase.

Dietary cholecalciferol	Egg weight	Haugh unit <sup>1</sup>	Relative yolk weight	Relative albumen weight	Relative shell weight	Eggshell thickness	Egg specific gravity	Eggshell breaking strength
(IU of D <sub>3</sub> /kg of diet)	(g/h)		(%)	(%)	(%)	(mm)		(kg)
1,681 (control)	59.0	85.8 <sup>a</sup>	27.2	57.7	9.5	0.399	1.072 <sup>b</sup>	4.32 <sup>b</sup>
8,348	58.5	84.5 <sup>b</sup>	27.3	57.7	9.7	0.415	1.073 <sup>ab</sup>	4.56 <sup>a</sup>
18,348	58.5	86.5 <sup>a</sup>	27.5	57.6	9.6	0.402	1.073 <sup>b</sup>	4.38 <sup>ab</sup>
35,014	58.5	85.8 <sup>a</sup>	27.4	57.7	9.6	0.397	1.074 <sup>ab</sup>	4.51 <sup>a</sup>
68,348	59.1	84.5 <sup>b</sup>	27.3	57.9	9.7	0.405	1.075 <sup>a</sup>	4.56 <sup>a</sup>
Pooled SEM	0.19	0.31	0.10	0.15	0.04	0.0057	0.0004	0.045
			P Value					
Treatment (Tukey adj)	≤0.05	≤0.01	0.22	0.69	≤0.05	0.28	≤0.01	≤0.01
Time	≤0.01	≤0.01	≤0.01	≤0.01	≤0.01	≤0.01	≤0.01	≤0.01
Treatment×time	≤0.01	0.76	0.89	0.65	0.32	0.55	≤0.05	0.45

<sup>1</sup> Haugh unit =  $100 \cdot \log(h - 1.7 \cdot w^{3.7} + 7.6)$ ; h = observed height of the albumen in millimeters; w = egg weight in grams

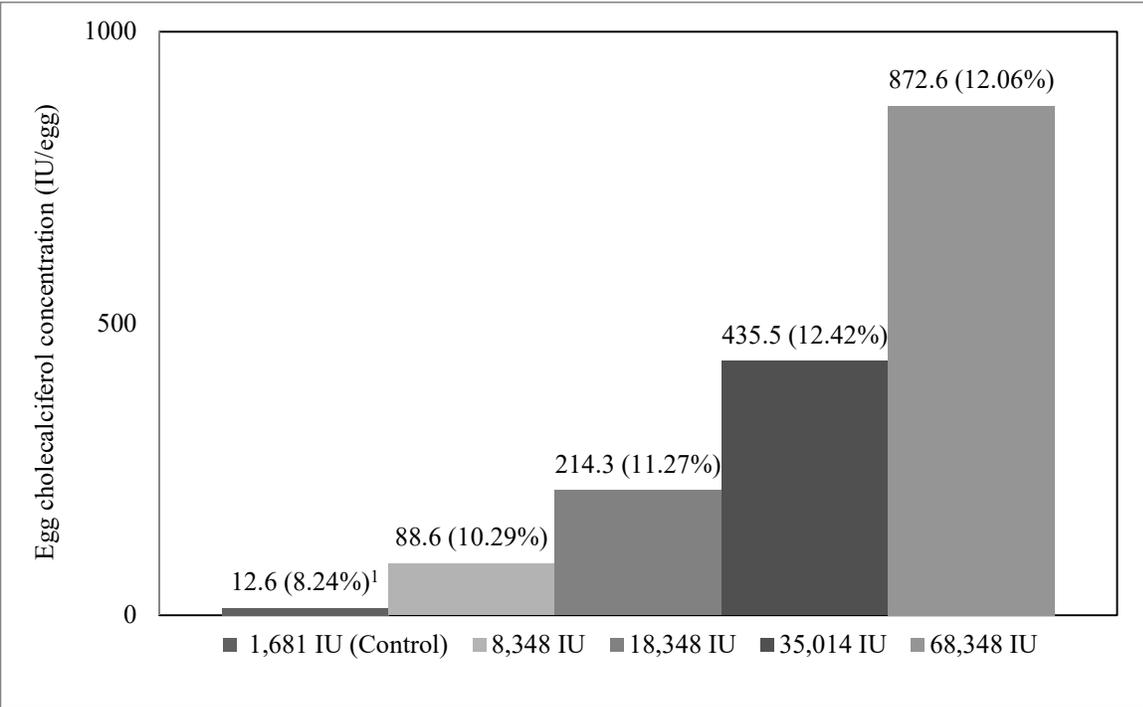
<sup>a-b</sup> Least square means without a common superscript differ significantly ( $P \leq 0.05$ ).

Table 7.5 Bone qualities from hens fed diets containing 1,681, 8,348, 18,348, 35,014 and 68,348 IU of D<sub>3</sub>/kg of diet, respectively.

Dietary cholecalciferol (IU of D <sub>3</sub> /kg of diet)	Keel bone score <sup>1</sup>	Right tibia bone mineral density (g/cm <sup>2</sup> )	Right tibia bone mineral content (g)	Left tibia bone breaking strength (kg)	Fat-free left tibia ash (%)
1,681 (control)	0.43	0.226	1.41 <sup>b</sup>	17.99	60.97 <sup>b</sup>
8,348	0.32	0.236	1.46 <sup>b</sup>	18.55	61.57 <sup>a</sup>
18,348	0.40	0.233	1.46 <sup>b</sup>	19.21	61.49 <sup>a</sup>
35,014	0.27	0.235	1.46 <sup>ab</sup>	18.49	61.74 <sup>a</sup>
68,348	0.37	0.241	1.55 <sup>a</sup>	19.22	61.58 <sup>a</sup>
Pooled SEM	0.093	0.0042	0.030	0.361	0.161
P-value (ANOVA)	0.74	0.13	≤0.05	0.09	≤0.05
Quadratic	-	0.08 (R <sup>2</sup> =0.08)	≤0.05(R <sup>2</sup> =0.13)	0.10(R <sup>2</sup> =0.08)	0.20(R <sup>2</sup> =0.05)
Linear	-	≤0.05 (R <sup>2</sup> =0.08)	≤0.01(R <sup>2</sup> =0.12)	≤0.05(R <sup>2</sup> =0.06)	0.17(R <sup>2</sup> =0.02)

<sup>a-c</sup> Least square means without a common superscript differ significantly ( $P \leq 0.05$ ).

<sup>1</sup> Score 1: a bone with one fracture, usually toward the tip of keel; Score 2: a bone with between two and three fractures or callus formation. Score 3: a bone with multiple fractures or callus formation plus slight deformation at the fracture sites. Score 4: a bone with multiple fractures or callus formation and severe deformations of the ventral edge.



<sup>1</sup> Vitamin D<sub>3</sub> transfer efficiency presented in parenthesis.

Figure 7.1 Cholecalciferol concentrations and vitamin D<sub>3</sub> transfer efficiency of eggs from hens fed diets containing 1,681, 8,348, 18,348, 35,014 and 68,348 IU of D<sub>3</sub>/kg of diet, respectively.

## Chapter 8: Epilogue

The first objective of this dissertation was to evaluate the valine (val) and tryptophan (trp) requirement of small-framed laying hens in first laying cycle. Two independent experiments were conducted to estimate the val and trp requirements of 41 to 60 wk old laying hens. In the val experiment, 270 Hy-line W-36 laying hens were randomly assigned to 6 dietary treatments including 0.515%, 0.585%, 0.655%, 0.725%, 0.795% and 0.865% valine. Four models (linear broken line, quadratic broken line, quadratic polynomial and exponential) were used to estimate total val requirements based on hen-housed egg production (HHEP), egg mass (EM) and feed conversion ratio (FCR). Valine requirement estimates using linear broken line models were lower than those from the other models. Valine requirements estimated for FCR were lower than those for either HHEP or EM. This lower val requirement FCR could be explained by the different responses of feed intake and egg mass to valine concentration. As dietary val increased from 0.515% to the estimated FCR requirement, the rate of egg mass increased faster than feed intake resulting in a decreased FCR. After the dietary val concentration exceeded the requirement, both feed intake and egg mass were altered at similar rates, resulting in a plateau for FCR. Valine requirements were estimated at 12.2, 14.5, 13.6 and 14.0 mg/g egg mass/d using linear broken line model, quadratic broken line model, quadratic polynomial model and exponential model, respectively. Val requirement per g egg mass indicates how efficient val is utilized by laying hen. As previous experiments were reviewed, no clear trends of decreased val requirements (per g egg mass) were noted as these values have varied over time (Harms and Russell, 2001; Peganova and Eder, 2002; Bregendahl, et al., 2008; Lelis, et al., 2014). This lack of reduction of val requirement over time, may indicate that val is used primarily for egg production with only minor alterations due to body maintenance requirements. The trp requirement experiment was designed to mirror the val experiment and used 0.096%, 0.116%,

0.136%, 0.156%, 0.176% and 0.196% trp diets. At 43 weeks of age, the 0.096% trp (lowest concentration of trp) treatment was stopped due to low HHEP. Dose responses were observed on HHEP, FI, EW, EM, FCR and plasma serotonin concentration in the remaining 5 treatments. Tryptophan requirements using linear broken line regression were lower than other models. Tryptophan requirements based on FCR were lower than those based on either HHEP or EM. Tryptophan requirements were estimated at 3.2, 4.0, 3.7, and 4.4 mg per g egg mass per day using linear broken line, quadratic broken line, quadratic polynomial and exponential, respectively. Similar to val, previous experiments could not provide evidence to support the assumption of increased trp utilization efficiency over time (Bregendahl, et al., 2008; Harms and Russell, 2000; Peganova, et al., 2003; Russell and Harms, 1999). Plasma serotonin concentrations suggested that plasma serotonin is not a good candidate for trp requirement estimation. Overall val and trp requirements estimated from the current experiments provide updated information on val and trp requirement to egg producers and nutritionists. Dose response experiments answer how much val is needed during a period of time but val requirements for various periods of time are still unknown as val requirement changes over various production (hen age) periods. Adaptations to the low amino acid diets (increased feed intake) in the current experiment were observed around 10 weeks after the initiation of the experiments. This trend for adaptation calls into question the accuracy of the amino acid requirements as experiments shorter than this adaptation period will result in different values for regression analysis. Adjusted experiment duration across experiments to standardize amino acid requirement could be an interesting topic in the future. In commercial poultry feed formulation, digestible amino acid requirements are more commonly used. Digestibility assays could provide additional information to determine digestible val and trp for laying hens. Another limitation of the current experiment

is that val or trp to lys ratios were not determined as the ratios are useful information for poultry nutritionist during diet formulation.

A third chapter was conducted to evaluate the digestible lysine (lys) requirement of broiler chicks over a short term feeding experiment. Four short-term independent experiments were conducted from 1 to 3 (E1), 3 to 5 (E2), 5 to 8 (E3) and 8 to 11 (E4) days of age. In each experiment, 490 chicks were allocated by weight into 7 treatments with 10 replicates of 7 birds in each replicate. In E1, E2 and E3, birds from 7 treatments received diets containing 0.94%, 1.05%, 1.16%, 1.27%, 1.38%, 1.49% and 1.60% digestible lys, respectively. In E4, 7 treatment diets contained 0.83%, 0.94%, 1.05%, 1.16%, 1.27%, 1.38% and 1.49% digestible lys, respectively. In E1, E2 and E3, no dose response was observed on body weight gain. This could be explained by an overestimated digestible lys requirement when selecting dietary lys concentrations as even the lowest lys concentration was still able to provide sufficient lys to growing broiler chicks. It should be noted that body weight gains were lower than recommendations from the commercial broiler management guide and birds with slower growth require less dietary lys to maximize growth rate. There was a dose response observed on body weight gain and pectoralis major absolute weight in 8 to 11 d old broiler chicks. Digestible lys requirements of broilers from 8 to 11 days were estimated at 1.057% and 1.016% based on body weight gain and pectoralis major absolute weight, respectively. In E4, two dietary lys concentrations resulted in performance below the break point possibly limiting the accuracy of lys requirement estimation as the ascending line was fitted by only two points. The position of a potential third point on the ascending line may dramatically change the slope if it is not located on the line fitted by the other two points. Therefore, the number of amino acid treatments below has the potential to impact the requirement estimation. Although digestible lys estimates were

expressed in the current experiments, lys digestibility was not directly determined, but estimated based on book values of the feed ingredients used.

A new bacterial protein meal (BPM) generated using fermentation of waste water to both generate the biomass and clean the waste water was investigated as a feed ingredient for laying hens. Response criteria included laying hen performance and immune response caused by replacing soybean meal and corn with BPM. In total, 144 Hy-line W-36 laying hens were randomly allocated into 3 treatments with 12 replicates; including control fed birds (corn-SBM diet) and a similar diet with 7.5 and 15.0% BPM. Hen-housed egg production of laying hens was reduced in the 15% BPM fed hens in comparison to the control and 7.5% BPM fed birds ( $P \leq 0.01$ ). The yolk color score of eggs from hens fed 7.5% and 15% BPM was darker than those from control treatment ( $P \leq 0.01$ ). There was no evidence to support the assumption that BPM was an immune modifier in the current experiment as no differences were observed on relative spleen weight or blood heterophil/lymphocyte ratio of laying hens. Upon proximate analysis of the BPM, the ash content was determined to be approximately 20% or higher and could be a factor limiting performance in the 15% BPM fed birds. Further analysis of the ash did not fully quantify the high ash content as the mineral profile (Ca, P, Cu, Fe, Mg, Zn etc. al) concentrations were not elevated. The unknown mineral in high concentrations may be reducing performance of the laying hens. Differences between estimated AMEn and real AMEn of the BPM in the birds could also confound the performance results. Traditionally high nucleic acid concentrations have been observed in BPM. Although nucleic acid concentrations were not measured in the current experiment, but could be a potential confounding factor.

The last objective of this dissertation was to investigate the effect of feeding high vitamin D<sub>3</sub> diets on pullet and laying hen performance, skeletal health, eggshell quality and yolk vitamin

D<sub>3</sub> concentration in day of hatch to 68 wk old, small-framed laying hens. Chicks received one of five experimental diets including a control diet containing 1,681 IU D<sub>3</sub>/kg diet, and the same diet supplemented to contain 8,348, 18,348, 35,014 and 68,348 IU D<sub>3</sub>/kg diet, respectively. Pullets fed 68,348 IU D<sub>3</sub>/kg diet showed a significantly lower body weight from 6 to 17 weeks of age. This reduced body weight disappeared after 21 weeks of age at the expense of delayed egg production in 68,348 IU/kg fed hens. The HHEP in 68,348 IU/kg fed hens was reduced in comparison to hen fed the other dietary treatments indicating that diets containing 68,348 IU D<sub>3</sub>/kg diet would not be an appropriate choice for commercial producers. These results might seem inconsistent with previous reports, which indicated that the addition of vitamin D<sub>3</sub> at concentrations up to 102,200 IU D<sub>3</sub>/kg when supplementation occurred once egg production had begun and had no negative effects on laying hen performance (Persia, et al., 2013). In the current experiment, reduced egg production by 68,348 IU treatment could be associated with the reduced body weights of the pullets. Laying hens fed diets containing 8,348, 18,348, 35,014 IU D<sub>3</sub>/kg diet were able maintain similar HHEP, EM, EW and FCR to the control fed laying hens. Increased dietary D<sub>3</sub> (8,348, 18,348 and 35,014 IU D<sub>3</sub>/kg) generally increased bone mineral density of pullets at 17 weeks of age with a significant increase in the 8,348 and 35,014 IU/kg treatments. Eggs from 8,348 and 35,014 IU/kg treatments showed a higher shell breaking strength than control treatment ( $P \leq 0.01$ ). Feeding birds high D<sub>3</sub> concentration diets significantly increased tibia ash content of hens at 68 weeks of age. This may indicate that tibia ash was a more precise measurement of skeletal quality. Previous experiments have reported limited effects of high dietary vitamin D<sub>3</sub> on eggshell quality and hen skeletal health (Ameenuddin, et al., 1986; Mattila, et al., 2003; Mattila, et al., 2004; Park, et al., 2005; Persia, et al., 2013). In the current experiment, dietary D<sub>3</sub> supplementation occurred at day of hatch and that concentrations of D<sub>3</sub>

from 8,348 to 35,014 IU/kg increased the combination of shell quality and bone mineral status in laying hens. The contrary responses on egg shell quality and maintenance of bone health could be explained by the important role of bone development and pullet skeletal status on maintaining bone mineral content during the egg laying phase. The last few weeks before the onset of lay is believed to be critical as the diameter of key long bones that are high in medullary tissue dramatically increased (Riddell, 1992). These higher diameters of pullet structural bone would facilitate the bone mineralization process and allow for the increase of future medullary bone production in the bone cavity (Whitehead, 2004).

In conclusion, total val requirements for Hy-line W-36 laying hens from 41 to 60 weeks of age were estimated to be 12.2, 14.5, 13.6 and 14.0 mg val per g egg mass per day per hen using linear broken line, quadratic broken line, quadratic polynomial and exponential models, respectively. Total trp requirements were estimated to be 3.16, 4.02, 3.74 and 4.41 mg trp per g egg mass per day per hen using linear broken line, quadratic broken line, quadratic polynomial and exponential models, respectively. Digestible lys requirements in the current experiment suggest that commercial concentrations of digestible lys in starter diets may over-estimate the requirement. Bacterial protein meal could be supplemented into laying hen diet as a protein source but the concentration should be limited below 7.5%. High dietary vitamin D<sub>3</sub> concentrations (8,000 to 35,000 IU/kg diet) are recommended to enrich vitamin D<sub>3</sub> in eggs and benefit pullet/laying hen skeletal health as well as eggshell quality.

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