EVALUATION OF THE DIETARY VITAMIN D_3 REQUIREMENT OF MODERN BROILER CHICKENS AND PARTIAL REPLACEMENT OF VITAMIN D_3 WITH 25-HYDROXYCHOLECALCIFEROL

A Dissertation

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HECTOR ENRIQUE LEYVA JIMENEZ

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Chair of Committee,	Christopher A. Bailey
Committee Members,	Rosemary L. Walzem
	Morgan Farnell
	Delbert M. Gatlin
Head of Department,	David J. Caldwell

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ABSTRACT

The goal of this dissertation was to explore the response of broiler chickens to dietary D_3 (vitamin D_3) and 25-OH-D_3 (25-hydroxycholecalciferol) as well as to establish a protocol that precisely estimated the D_3 requirement of modern meat-type chickens through an oral gavage bioassay. For this purpose, three research projects were conducted. For the first and second experiments, broiler chickens were fed a diet devoid of D_3 for 21 d. The first 9 d of the study served to deplete the maternal stores of D_3. On d-10 of the study, a crystalline source of D_3 was diluted in corn oil to prepare graded levels of D_3 that were directly gavaged into the crop of birds for 11 d. Broken-line regression was employed to estimate the requirement of D_3 for maximum bone mineralization. The first experiment fed a mash corn-soy diet with reduced calcium and non-phytate phosphorus. The second experiment fed a corn-soy diet as well but as crumbles, and all nutrients met or exceeded industry-type nutritional requirements. The D_3 requirement of starter broilers was estimated in experiment-1 to be close to the 500 IU/kg of feed and in experiment-2 to be around 200 IU/kg of feed for maximum bone mineralization. I concluded that nutrient restrictions increased the D_3 requirement with respect the NRC (1994), and that maternal D_3 stores in the yolk highly influenced the growth performance and bone mineralization response of broiler chickens to dietary D_3. Using industry-type nutritionally adequate diets, the dietary D_3 requirement appears to be in agreement with the NRC (1994).

In the third experiment, broiler chickens were subjected to a coccidiosis vaccine challenge and fed dietary concentrations of D_3 or a partial replacement of D_3 with 25-OH-D_3. Results suggested that the combination of both D_3 and 25-OH-D_3 was more effective than D_3 alone in promoting bone mineralization and enhancing vitamin D status as measured by total

plasma 25-OH-D_3 in young (21 d-old) broiler chickens. Additionally, the supplementation of high dietary levels of D_3, independently of the source, above NRC (1994) recommendations, yielded better performance, bone mineralization and total plasma 25-OH-D_3 in starter broiler chickens.

DEDICATION

I dedicate this dissertation to my family, especially to my much loved grandparents Macario Leyva Arellano, Marcia Rojo, Marciano Jiménez Alarcón and Isabel Cruz Coca and my parents Lorenzo Leyva Rojo and Maria Elena Jiménez for all your support and love.

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All work for this dissertation was completed independently by the student in collaboration with Dr. Bailey`s laboratory: Dr. Akram-ul Haq, Raghad Abdaljaleel, Yansoon Al-Jumaa, Akhil Alsadwi, Kimberly Gardner, Momin Khan, John Connor Padgett and Elise Voltura.

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TABLE OF CONTENTS

Page

Page

LIST OF FIGURES

Page

LIST OF TABLES

Page

Page

CHAPTER I

INTRODUCTION AND LITERATURE REVIEW

INTRODUCTION

According to the United Nations (2017), the global inhabitants are expected to reach ≈9.8 billion by 2050. Along with the increasing population, food supply from vegetal and animal origin will play a key role in preventing food insecurity around the world and especially in developing countries where poverty is still one of the biggest obstacles to overcome. Therefore, to increase the availability of animal protein at reasonable cost, production systems need to increase their efficiency and sustainability.

Since the early 1960's the commercial poultry industry has worked intensively in diverse areas such as, genetics, nutrition, flock management, feed technology, and immunology to promote efficiency and reduce production costs. As a result of these efforts, meat-type chickens have experienced an accelerated increase in growth rate (Havenstein et al., 2003). The accelerated growth continues through the present day and has led to higher meat yields and better feed efficiency when compared to the 1960's meat-type chickens. However, there are problems associated with the improvement in growth rate. Skeletal disorders, due to intensive genetic selection towards higher meat yields, are an example. Nutritional inadequacies leading to bone deformities and diseases have been a subject of concern among nutritionists and welfare experts (Edwards, 2000; Waldenstedt, 2006; Kapell et al., 2012). Bone health is an important component of animal welfare and therefore has a direct relationship with the bird's performance. Annual loses in 1994, due to skeletal problems, were calculated to be around $80 to $120 million USD

for broiler chickens (Morris, 1993; Sullivan, 1994) which, adjusted to the annual inflation (U.S. Bureau of Labor Statistics), would represent $134 to $200 million USD in 2017.

A common nutritional strategy used to reduce the incidence of skeletal disorders, such as tibial dyschondroplasia or rickets, has been the supplementation of poultry feeds with vitamin D_3 (**D_3**) and more recently, derived metabolites like 25-hydroxycholecalciferol (**25-OH-D_3**) (Ledwaba and Roberson, 2003; Waldenstedt, 2006). Vitamin D_3 is known for its role in the regulation of calcium (**Ca**) and phosphorus (**P**) homeostasis in conjunction with calcitonin and parathyroid hormones (Collins and Norman, 2001) which ultimately, promote bone health.

International units (**IU**) are used to express the biological activity of vitamin D. One IU of vitamin D is defined as the activity of 0.025 μg of crystalline cholecalciferol. The NRC (1994) established the D_3 requirement at 200 IU/kg of diet for starting growing broiler chicks. However, current industry-type poultry diets are supplemented with 10 or even 30 times the NRC (1994) recommendations (Leeson, 2007). Post NRC (1994) research done on high dietary D_3 effects on meat-type chickens reported beneficial effects on growth, bone health (Kasim and Edwards, 2000; Fritts and Waldroup, 2003; Whitehead et al., 2004; Rama Rao et al., 2006; Khan et al., 2010), and avian immunity (Aslam et al., 1998; Morris and Selvaraj, 2014; Morris et al., 2015). It is probable that modern poultry strains have altered their ability to utilize dietary nutrients after 24 years of intensive genetic selection (Havenstein et al., 2003; Schmidt et al., 2009; Applegate and Angel, 2014). Additionally, nutritionists rely on literature references such as the NRC to formulate diets that precisely meet the animal's nutritional requirements. Hence, it is necessary to develop new protocols that more accurately reflect current feeding strategies and the ability of modern poultry strains to respond to dietary nutrients such as vitamin D_3. Furthermore, the effect of dietary D_3 and derived metabolites beyond bone mineralization is rapidly gaining popularity

among the commercial poultry industry. Nutritionists are actively seeking for possible dietary "alternatives" to antibiotic growth promoters and ways to enhance animal well-being through nutrition (Ferket, 2004).

The goal of this research project was to explore the response of modern broiler chickens to dietary D_3 and 25-OH-D_3 as well as to establish a protocol that precisely estimated the D_3 requirement of meat-type chickens through an oral gavage bioassay. Thus, this research project was conducted with the following objectives: 1) evaluate the effects of high dietary levels of D_3 and 25-OH-D_3 on performance, bone mineralization and intestinal morphology of growing broiler chickens; 2) evaluate the effects of high dietary levels of D_3 and 25-OH-D_3 supplementation on broiler plasma cytokine levels after a coccidia vaccine challenge; 3) compare broiler growth response to supplemental D_3 alone versus supplemental D_3 in combination with 25-OH-D_3; 4) identify optimal supplemental levels of D_3 and 25-OH-D_3 to maximize performance, bone mineralization, intestinal morphology and immune response of broiler chickens; 5) re-evaluate the D_3 requirement for starter broiler chickens using a non-traditional methodology to deliver D_3 to the chickens; 6) compare D_3 requirements of modern broiler strains to those reported by the latest Poultry NRC (1994).

LITERATURE REVIEW

Cholecalciferol "The Sunshine Vitamin" Overview

Vitamin D is a fat-soluble vitamin known for its anti-rachitic activity in humans and animals. The early study of vitamin D dates back to the 18th century, during the industrial revolution, when the incidence of rickets among young children became a health problem known as "The English Disease." At that time, the cause of rickets was unknown. Therefore, researchers

started to investigate the factors that possibly caused rickets. Many factors were described as the cause of rickets such as, lack of fresh air or sunlight, congenital factors, age, infectious agents, lack of physical activity and malfunction of endocrine glands were described as possible factors predisposing the incidence of rickets (Norman, 1979). In 1919, Edward Mellanby undertook studies of the recently identified vitamin A in cod liver oil, which was recognized to prevent xeropthalmia and night blindness, to study the relationship of rickets and nutritional factors or vitamin factors. He conducted several studies using dogs fed low-fat diets trying to develop an animal model for rickets. Mellanby observed a potent antirachitic activity in the cod liver oil and concluded that a vitamin A deficiency will cause a higher incidence of rickets. McCollum (1922) who previously identified the nutritional importance of vitamin A, believed a different compound was responsible of the antirachitic activity of the cod liver oil. After he destroyed vitamin A in cod liver oil using oxygen and heat, the treated oil was fed to rachitic rats and chicks who successfully recovered from rickets. It was clear that a different compound was responsible of curing rickets, and he named it vitamin D.

A few years later, Goldblatt and Soames (1923) and Steenbock and Black (1925) established the fundamental relationship between UV-light and vitamin D. The chemical structure of vitamin D_3 was determined years later by the German chemist, and Nobel Prize winner Adolf Windaus in collaboration with his research group in 1936 (Collins and Norman, 2001). However, it was until the 1970´s when Kodicek et al. (1970), DeLuca (1978), Norman (1979) and other scientists clearly defined vitamin D's metabolic role and its importance in human and animal nutrition.

Two main forms of vitamin D have been described, ergocalciferol (**D_2**) or vitamin D_2 and cholecalciferol (**D_3**) or vitamin D_3 (Leeson and Summers, 2001). Ergocalciferol is widely

distributed in plants, fungi and molds while cholecalciferol is present in animals where vitamin concentration depends on dietary D_3 and exposure of the tissue to sunlight (Combs, 2012). Vitamins D_2 and D_3 have relatively the same bioactivity for cattle, sheep and pigs. However, in the case of poultry, D_2 has only 10% the bioactivity of D_3 (McDonald et al., 1995). Therefore, D_3 supplements are preferred to formulate commercial poultry vitamin premixes.

Vitamin D_3 is soluble in organic non-polar solvents and oils; it has a melting point of 84-85°C, maximum UV absorption is between 254-265 nm and can be destroyed by excess irradiation of UV-light and when exposed to air at 24°C for 72 h (Collins and Norman, 2001).

Vitamin D_3 is obtained from the diet or is synthesized *in vivo* from the provitamin 7-dehydrocholesterol in the epidermal layer of the skin after exposure to UV-light. Dietary and endogenous synthesized D_3 are considered biologically inactive and must undergo activation through hydroxylation reactions in the body. First, D_3 is transported via the blood to the liver where it is converted to 25-OH-D_3, the major circulating form of D_3. The newly formed 25-OH-D_3 must then be transported to the kidneys for conversion into 1, 25-dihydroxycholecalciferol (**1, 25(OH)$_2$ D$_3$**) which is the fully active hormonal D_3 metabolite (Norman, 1979). Vitamin D participates in the regulation of calcium and phosphorus homeostasis to assure proper metabolic functions and promote bone development (Collins and Norman, 2001). Calcium is important for bone formation and development; it is also required for proper nerve impulse transmission and muscle contraction (Leeson and Summers, 2001). Phosphorus is considered an essential nutrient for normal bone development, muscle coordination, energy, carbohydrate, amino acid and fat metabolism, nervous tissue metabolism, normal blood chemistry, and is a structural component of nucleic acids and co-enzymes (Leeson and Summers, 2001). Bone formation is indirectly stimulated by D_3. The presence of 1, 25$(OH)_2$ D_3 induces osteoclast (large multinucleated cells

responsible for bone resorption) activity from osteoblasts (bone-forming cells) stimulation (Standford, 2006). In individuals with adequate physiological functions, bone resorption and formation are tightly balanced (Norman and Hurwitz, 1993) in a process called bone remodeling. Hence, bone formation is also stimulated by active release and deposition of Ca and P in the mineral matrix of the bone. Additionally, the expression of the calbindin-D_{28K}-mRNA gene in the intestinal wall is stimulated by 1, 25$(OH)_2$-D_3 (Hall and Norman, 1990). This gene is related to the synthesis of calcium binding protein calbindin-D_{28K}. The presence of calbindin-D_{28K} reflects the ability of the intestine to absorb Ca efficiently (Combs, 2012). Other functions associated with D_3 include: regulation of intestinal absorption of P, resorption of P in the renal tubes of the kidney (Norman, 1979; Combs, 2012), and immune cell function (Aslam et al., 1998; Cantorna 2006; Wintergerst et al., 2007).

Vitamin D_3 deficiency is associated with rickets, tibial dyschondroplasia, retarded growth, poor feathering, osteomalacia (bone softening) in adult animals, and pliable beaks and claws (Leeson and Summers, 2001). On the other hand, toxicity caused by excessive intake of D_3 is associated with hypercalcemia (McDowell 1989), twisted leg, increased feed conversion ratios (**FCR**) (Cruickshank and Sim, 1987), abnormal deposition of calcium in the viscera due to increased circulating calcium and phosphorus levels, and renal damage (Leeson and Summers, 2001).

Vitamin D_3 Requirement for Growing Meat-type Chickens

Literature referenced in the Poultry NRC (1994) used to establish the D_3 requirement of young growing chickens date as early as 1963 (Table 1). Along with the development of specialized meat-type chickens in the late 19^{th} century, breeding programs have focused on improving growth, breast yield and feed conversion ratio (FCR) (Muir and Aggrey, 2003).

Intensive genetic selection has led to an accelerated increase in growth rate of broiler chickens (Havenstein et al., 2003). According to data from the National Chicken Council (2017) in 1925, it was required a grow-out period of 112-d to produce a broiler chicken with average market weight of 1.13 kg (2.5 lb) and FCR of 4.7.

Table 1 Referenced literature in the NRC (1994) to establish vitamin D requirements of growing broiler chickens.

Dietary level IU D_3/kg	Duration of study	Response criteria	Reference
200-396	28 d	Performance Tibia ash	Waldroup et al., 1963
198	28 d	Performance Tibia ash	Waldroup et al., 1965
200	54 d	Performance Tibia ash	Biely and March, 1967
≤200	14 d	Performance Bone mineralization	McAuliffe et al., 1976
198	21 d	Performance Tibia ash	McNaughton et al., 1977
400	56 d	Performance Tibia ash	Lofton and Soares, 1986

Adapted from NRC (1994)

In contrast, only 47-d are required to produce a broiler chicken with average market weight of 2.8 kg (6.2 lb) and FCR of 1.85 in 2017. Along with the intensive genetic selection, improvements in flock management, feed technology, vaccination programs, transportation routes, and animal welfare practices have contributed to the development of modern commercial

poultry strains. As previously mentioned, it is possible that the physiology of modern poultry strains has been altered due to the intensive genetic selection and therefore, their ability to respond to dietary nutrients (Havenstein et al., 2003; Schmidt et al., 2009; Applegate and Angel, 2014). Consequently, it is logical to believe that nutrient requirements of modern poultry have changed since the last revision of the NRC in 1994 which established the D_3 requirement at 200 IU/kg of diet for starting growing broiler chicks. However, current industry-type poultry diets are supplemented with 10 or even 30 times the NRC (1994) recommendations (Leeson, 2007). Vitamin requirements found in the NRC (1994) were estimated based on simple observational response criteria such as the absence of classical deficiency signs or growth performance variables (Leeson, 2006; 2007). The development of new technologies allows researchers to test more extensive or diverse response criteria such as blood chemistry, immune responses, or meat quality. The use of new technologies to evaluate modern broiler response to dietary nutrients suggest different requirements are needed to maximize growth and health (Applegate and Angel, 2014) when compared to the latest Poultry NRC (1994). Tibia dyschondroplasia scores, incidence of rickets, tibial breaking strength, serum P and Ca concentration, cell mediated and humoral immune response and mineral retention content of the liver have been employed in addition to performance evaluations to estimate dietary D_3 requirements for meat-type chickens in recent literature reports (Table 2).

High dietary concentrations of vitamin D_3 [10-20 times NRC (1994) recommendations] have been found to positively influence bird's performance, bone mineralization (Rama Rao et al., 2006), and immunity (Aslam et al., 1998), as well as enhance overall welfare by improving gait scores and tibial quality which improves walking ability reducing the contact of the foot,

hock and keel with the litter, thus reducing the development of footpad and hock dermatitis (Sun et al., 2013).

Increasing concerns among nutritionists regarding the need for re-evaluating nutrient requirements that are based on current feeding strategies have increased over the last few years (Applegate and Angel, 2014). Hence, it is necessary to adapt and develop strategies that allow nutritionist to precisely estimate nutrient requirements of modern poultry strains. Moreover, differences in growth between modern poultry strains (Figure 1) should be addressed as well. Different D_3 requirements to maximize bone mineralization, performance or immunity are possible among strains depending on their specific ability to respond to dietary nutrients.

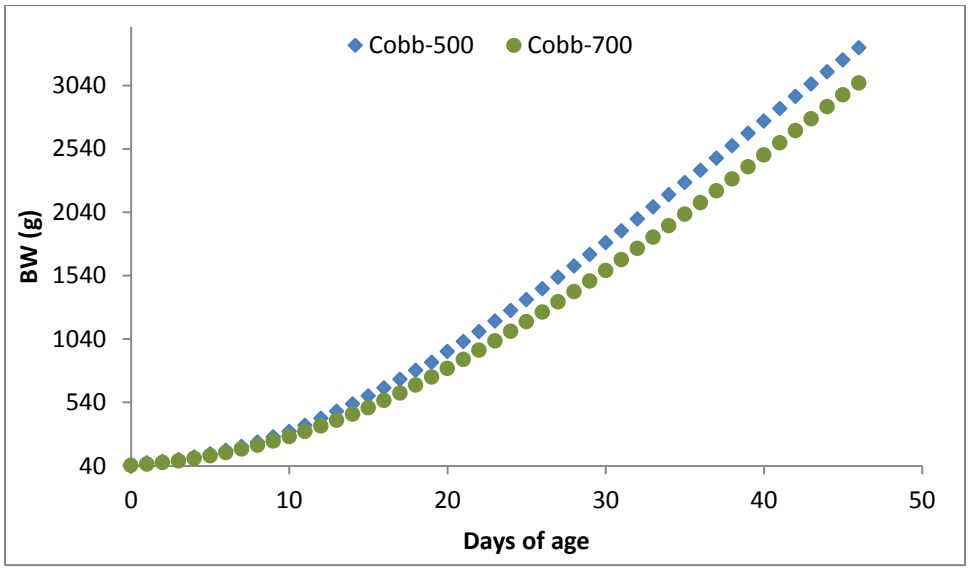

Figure 1 Growth of Cobb-500 and Cobb-700 modern broiler strains
Data source: Broiler Performance and Nutrition Supplement from Cobb-Vantress

Table 2 Literature reports published after the NRC (1994) estimating vitamin D requirements of starting growing broilers.

Form and dietary D_3 level	Strain and sex	Response criteria and estimated requirement	Reference
Cholecalciferol 200-3,000 IU/kg of feed Corn-soy-diet	Male Cornish Giant Arbor Acre	Performance Bone mineralization Blood chemistry 400 IU/kg of feed were needed to maximize weight, feed intake, bone density, bone calcium and phosphorus/cm^3 bone in a 21 d trial	Goff and Horst, 1995
Cholecalciferol 0-50,000 IU/kg of feed Corn-soy diet	Female New Hamshire x Columbian and Male Ross x Hubbard	Performance Bone mineralization 200 IU/kg appeared to be sufficient to maximize performance and bone ash in diets with adequate levels of non-phytate P (8-20 d post hatch) non-phytate P deficient diets, can increase the requirement to 1,500 IU/kg.Toxicity signs were not observed at 50,000 IU/kg	Baker et al., 1998
Cholecalciferol 0-1,200 IU/kg of feed Corn-soy diet	Straight run Peterson x Hubbard	Performance Bone mineralization Incidence of rickets Blood chemistry D_3 requirement ranged from 804-1,131 IU/kg. Estimated requirement appeared to be related with D_3 source type and properties of feed ingredients.	Kasim and Edwards, 2000
Cholecalciferol 125-4,000 IU/kg of feed Corn-soy diet	Male Cobb-500	Performance Bone mineralization Incidence of tibia dyschondroplasia Approximately 2,000 IU/kg were required to maximize tibia ash in 21 and 42 d old broilers.	Fritts and Waldroup, 2003

Table 2 Continued

Form and dietary D_3 level	Strain and sex	Response criteria and estimated requirement	Reference
Cholecalciferol 200-10,000 IU/kg of feed Wheat-soy diet	Male Ross-308	Performance Tibia ash and breaking strength Blood chemistry Incidence of tibia dyschondroplasia The D_3 requirement up to 14 d of age at sufficient dietary Ca and non-phytate P may be in the range of 1,400-2,000 IU/kg based on cortical bone quality and 10,000 IU/kg required for prevention of tibial dyschondroplasia	Whitehead et al., 2004
Cholecalciferol 200-3,600 IU/kg of feed Corn soy diet	Female Cobb-100	Performance Bone mineralization Mineral retention Performance and bone mineralization in broilers could be maintained with suboptimal concentration of Ca (0.5%) and non-phytate P (0.25%) with dietary concentrations of D_3 above the 1994 NRC (3,600 IU/kg)	Rama Rao et al., 2006
Cholecalciferol 200-3,500 IU/kg of feed Industry type diet containing rice, corn gluten, canola meal and molasses	Straight run Hubbard	Performance, bone mineralization and lymphoid organ weight/body weight ratio was significantly improved with dietary levels of D_3 at ranging from 1,500-3,500 IU/kg in broiler diets	Khan et al., 2010
Cholecalciferol 0-700 IU/kg of feed Corn-soy diet	Lingnan yellow male broilers	Performance Bone characteristics Blood chemistry Meat quality 464 IU/kg of feed to maximize tibia ash in a 21 d trial	Jiang et al., 2015

The Use of Vitamin D_3 Metabolites in Poultry Nutrition

Currently, D_3 metabolites such as 1-α-hydroxycholecalciferol (**1α(OH)D_3**) and 25-OH-D_3 are commercially available as dietary supplements. The use of these metabolites in substitution or in combination with conventional D_3 in poultry diets has become more popular among the commercial poultry industry because beneficial effects have been reported in the literature, especially as an effective strategy for the prevention of tibial dyschondroplasia in poultry (Whitehead, 1995; Ledwaba and Roberson, 2003; Atencio et al., 2005). Edwards et al. (2002) investigated the efficacy of 1α(OH)D_3 as a substitute for D_3. They reported that 1α(OH)D_3 was approximately eight times as effective as D_3 in maximizing body weight, plasma calcium, rickets, and percent bone ash in young broiler chickens. Bar et al. (2003) reported that dietary 25-OH-D_3 was beneficial to overcome moderate P restriction on weight gain and bone mineralization although, no consistent positive effects were observed in overall performance when 25-OH-D_3 replaced D_3 in broiler diets. Chou et al. (2009) reported improved intestinal morphology and enhanced humoral immunity of broiler chickens supplemented with 25-OH-D_3 although no effects were observed in performance variables. Garcia et al. (2013) compared the biological activity of 25-OH-D3, 1,25$(OH)_2$ D_3 and 1α(OH)D_3 to conventional D_3 on male broiler chickens. Their results suggested that 25-OH-D_3 and 1,25$(OH)_2$ D_3 were the most effective metabolites in maintaining performance and bone quality when compared to 1α(OH)D_3. Vignale et al. (2015) compared the activity of 25-OH-D_3 (5,520 IU/kg) to reduced (2,760 IU/kg) and high (5,520 IU/kg) D_3 supplementation in male broiler chickens raised up to 42 d of age. The supplementation of broiler diets with 25-OH-D_3 resulted in increased circulating 25-OH-D_3 and breast meat yield. Additionally, they reported that muscle of chickens under 25-OH-D_3 supplementation expressed higher concentrations of vitamin D receptor when compared to the D_3

groups. Morris et al. (2014) observed that the dietary supplementation of 25-OH-D_3 to broiler diets improved growth performance and decreased inflammatory gene IL-1β mRNA amounts in the liver following a lipopolysaccharide (**LPS**) injection. In a different study, Morris et al. (2015) reported that "high" dietary 25-OH-D_3 supplementation (100 µg/kg) to layer chick diets was effective in reducing the negative performance effects after coccidia challenge. Additionally, dietary 25-OH-D_3 decreased IL-1β mRNA and increased IL-10 mRNA amounts in the cecal tonsils, suggesting that the D_3 metabolite was effective in modulating immune response after coccidia challenge.

Precautions should be taken when using D_3 metabolites as they might increase the risk of D_3 toxicity in poultry. Yarger et al. (1995) conducted two experiments using graded dietary levels of 25-OH-D_3 in broiler diets. In the first experiment, dietary 25-OH-D_3 was supplemented from 69 (basal level) to 690 µg/kg. No toxic effects were observed with increasing 25-OH-D_3 levels in broiler diets. In the second experiment, dietary 25-OH-D_3 was supplemented from 69 (basal level) to 13,800 µg/kg. At 10 times the basal 25-OH-D_3 dietary level, evidence of renal calcification in birds was observed; whereas, dietary levels of D_3 at 50 times the basal level were required to show some evidence of renal calcification. The authors concluded that 25-OH-D_3 is 5 to 10 times more toxic than vitamin D_3.

Vitamin D_3 Beyond Bone Mineralization

Performance, bone mineralization, and incidence of tibial dyschondroplasia have been historically used to assess the effects of dietary D_3 on poultry. However, there are literature reports suggesting vitamin D_3 plays an important role in poultry immunity (Aslam et al., 1998; Gómez-Verduzco et al., 2013; Morris et al., 2014; Morris et al., 2015), and therefore, the possibility of exploring new response variables that will expand our current knowledge of D_3.

The vitamin D receptor **(VDR)** belongs to the nuclear receptor superfamily and has been detected in many cells types (Wang and DeLuca, 2012). The VDR regulates the expression of several genes whose promoters contain vitamin D response elements (Norman and Powell, 2014), and it is thought that D_3 participates in the regulation of normal function in cells expressing the VDR. Although the role of D_3 in immunity regulation is still not well understood, researchers have been exploring this area in recent years as VDR has been found in T lymphocyte and macrophage populations (Veldman et al., 2000; Deluca and Cantorna, 2001; Wang and DeLuca, 2012). Suggested regulatory points of vitamin D in immune function include: 1) regulation of T-cell mediated immunity; 2) stimulation of growth factor TGFβ-1 and IL-4 (Deluca and Cantorna, 2001); 3) inhibition of IL-2 transcription; 4) reduced expression of chemokine receptors CXCR3 and CCR2 (Lucas et al., 2014).

Evidence of beneficial effects of dietary D_3 in mammalian immunity has been reported in the literature. Wintergerst et al. (2007) reported that a deficiency in vitamin D can increase susceptibility to infections due to impaired localized innate immunity and defects in antigen-specific cellular immune response in humans. Barrera et al. (2015) studied the relation between vitamin D and cytokine expression in the human placenta of healthy and preeclamptic pregnancies. They observed that vitamin D supplementation downregulated the expression of pro-inflammatory cytokines TNF-α, IL-6 and IFN-γ in preeclamptic women. Additionally, vitamin D inhibited mRNA expression and protein levels of IL-10 in cultures obtained from normotensive pregnant women. Cantorna (2006) studied autoimmune disorders in mice and found evidence of vitamin D having a positive effect in the regulation of T-cell development and function, which should decrease the susceptibility of autoimmune diseases such as, multiple sclerosis and inflammatory bowel disease.

In the particular case of birds and more specifically poultry species, Aslam et al. (1998) found that a broiler starter diet devoid of D_3 depresses the cellular immune response in young chicks by reducing the relative weight of the thymus and the number of abdominal macrophages. Huff et al. (2000) reported vitamin D_3 having a positive effect on performance, mortality, relative weight of liver and heart, and serum levels of glucose and alanine aminotransferase (**ALT**) of turkeys subjected to dexamethasone (**DEX**) challenge. They concluded that dietary D_3 provided a protective effect to the DEX challenge and has a direct effect on health and disease resistance of turkeys. Morris et al. (2014) observed that the dietary supplementation of 25-OH-D_3 to broiler diets improved performance and decreased inflammatory gene IL-1β mRNA amounts in the liver after lipopolysaccharide (**LPS**) injection. Morris and Selvaraj (2014) studied *in vitro* supplementation of 25-OH-D_3 on chicken monocytes and a HD11 macrophage cell line subjected to LPS challenge. They found that 25-OH-D_3 treatment increased the production of nitric oxide (**NO**) and mRNA expression of IL-1β, IL-10, 1α-hydroxylase and 24-hydroxylase in HD11 cells following LPS stimulation. Morris et al. (2015) reported that 25-OH-D_3 influenced *in vitro* expression of IL-10 mRNA using HD11 chicken macrophage cell lines. Additionally, Morris and colleagues in the same study used layer-type chicks that were orally challenged with 1×10^5 live coccidia oocysts at 21 d of age and supplemented with 6.25, 25, 50 or 100 μg of 25-OH-D_3 per kg of feed. Their results revealed evidence suggesting that the D_3 metabolite has a regulatory effect on body weight, CD8+ cells, IL-10 mRNA expression and CD4+CD25+ cells. Moreover, dietary 25-OH-D_3 supplementation (100μg/kg) decreased IL-1β mRNA and increased IL-10 mRNA amounts in the cecal tonsils. Shojadoost et al. (2015) studied the effect of D_3 on chicken macrophage activity using a MQ-NCSU cell line, which is derived from chicken macrophages. Sigma-Aldrich standard 1,25$(OH)_2$ D_3 was used to treat cells at different

concentrations ranging from 0.1 to 0.001 pM and were subjected to different stimuli. They observed chicken macrophages expressed VDR and the expression was upregulated when exposed to LPS stimulation. Additionally, 1,25$(OH)_2$ D_3-treated cells increased the ability of macrophages to respond to stimuli and produce NO, but D_3 alone did not activate macrophages and resulted in downregulation of CD86, MHC-II, CXCL8, and IL-1β. The authors concluded that vitamin D_3 may have an anti-inflammatory and immunomodulatory role in chickens similar to what is seen in mammals. Further research has to be done in this area to better understand the relationship between vitamin D_3 and immunity using different diet formulations, D_3 metabolites and the prescience of pathogens such as coccidia which is commonly found in industry-type facilities raising poultry under intensive conditions.

Coccidiosis in Chickens

According to the Merck Veterinary Manual (**MVM**), coccidiosis is defined as "an acute invasion and destruction of intestinal mucosa by protozoa of the genera *Eimeria* or *Isospora*". Coccidiosis is one of the most economically important diseases affecting the commercial poultry industry. Just for the broiler industry, around $3 billion USD are spent worldwide every year in the prevention of coccidiosis (Anon., 2013). Clinical signs and lesions include intestinal hemorrhages and inflammation, bloody feces, reduced performance, listlessness, pale combs and wattles, ruffled feathers, diarrhea, and dehydration (MVM, 2010). *Eimeria* species recognized to infect chickens are: *E. acervulina, E. brunetti, E. maxima, E. mitis, E. necatrix, E. praecox,* and *E. tenella* (De Gussem, 2007). On the other hand, *E. meleagrimitis*, *E. adenoeides*, and *E. gallopavonis* are the most common *Eimeria* species affecting the turkey (Chapman, 2008).

Eimeria species are host specific parasites (Yun et al., 2000); this means that very rarely *Eimeria* species affecting the chicken will be found in other poultry species like the turkey or

duck. Additionally, intestinal lesions caused during coccidia infections have a particular location and appearance facilitating the diagnosis and treatment (Table 3). *Eimeria* is spread by the fecal-oral route and its life cycle is complex (Figure 2). Briefly, unsporulated oocysts excreted through the feces get in contact with the litter. In the presence of moisture, oxygen and warmth oocysts will sporulate and become infective. Sporulated oocysts are ingested by the bird and reach the gizzard where they get crushed and sporocysts are released. Sporocysts undergo excystment in the duodenum and sporozoites are released to invade the intestine. Sporozoites undergo two cycles of asexual reproduction and one cycle of sexual reproduction to form newly unsporulated oocysts that are excreted to repeat the cycle (Kheysin, 1972).

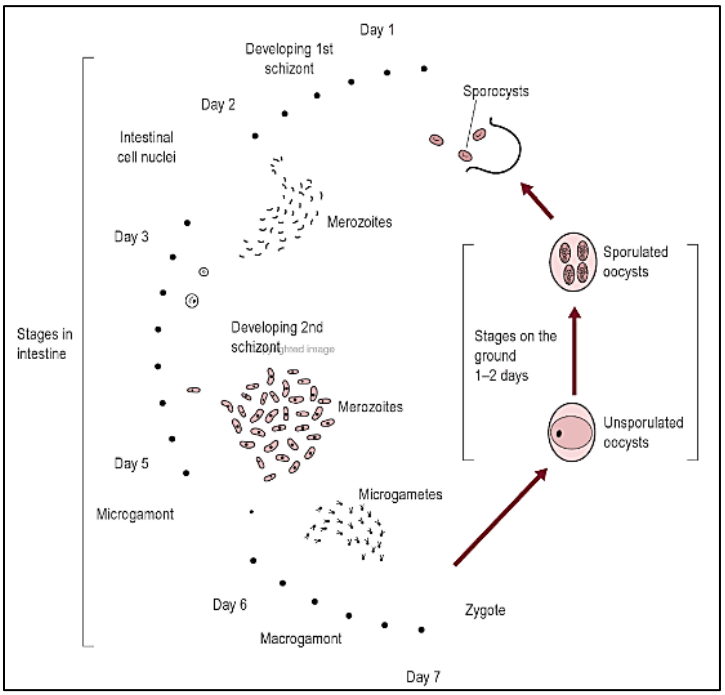

Figure 2 *Eimeria* life cycle
(Reprinted from: Pattinson et al. 2008)

Table 3 *Eimeria* spp. affecting the chicken.

Species	Lesions and diagnosis
Eimeria tenella	Localized only in the ceca. Infection cause severe morbidity, mortality and reduced weight gain. Lesions include: severe inflammation of the ceca, accumulation of blood in the ceca, and bloody droppings.
Eimeria necatrix	Infects the anterior and middle portion of the small intestine. Infection results in high mortality. Lesions include small white spots, observed on the serosal surface of the intestine. Severe enteritis with thickening, congestion (blood, mucus, and fluid), hemorrhage, and necrosis along with bloody feces.
Eimeria acervulina	Moderately severe pathogen usually affecting the anterior 1/3 portion of the small intestine. Lesions include mild to severe enteritis, numerous whitish, oval or transverse patches in the upper half of the small intestine and thickening of the mucosa. Clinically protracted disease of chicken and results in poor growth, increase in culls and increased mortality.
Eimeria maxima	Moderate pathogen which has a significant effect on weight gain and feed conversion. Develops in the middle section of the small intestine, where it causes dilatation and thickening of the wall, petechial hemorrhage and a reddish, orange, or pink viscous mucous exudate and fluid
Eimeria brunetti	Affects the lower small intestine, colon, ceca, and cloaca. Moderately severe pathogen causing debris covering intestinal mucosa and cheesy cores.

Source: Merck Veterinary Manual (2010)

The severity of coccidia infections is influenced by many factors, such as host age, genetic background and concurrent infections as well as coccidial species (Vermeulen et al., 2001). *Eimeria necatrix* and *Eimeria tenella* are the most pathogenic in domestic chickens (MVM, 2010).

Development and Applications of Anticoccidial Vaccines

Anticoccidial medications and vaccines are used to prevent and control coccidiosis in commercial poultry facilities (De Gussem, 2007). The use of live oocyst vaccines in young birds has the advantage of inducing early coccidia cycling, which results in early development of immunity against specific species of *Eimeria*. Most commercially available vaccines use one or

multiple *Eimeria* species to infect the birds with a low number of parasites that will induce protective immunity after two or three consecutive re-infections (Sharman et al., 2010). Four main types of vaccines (live virulent vaccine, live attenuated vaccine, live-tolerant to ionophores vaccine and recombinant vaccine) have been developed as a means to prevent coccidial infections (Vermeulen et al., 2001; Sharman et al., 2010). The use of live pathogens is associated with reduced performance due to the induced subclinical infection. Therefore, the attenuation techniques to reduce the pathogenicity of coccidial parasites have received special attention (Lillehoj and Trout, 1993). Live virulent vaccines are developed from laboratory or field strains without any modification that alters their natural virulence (Ahmad et al., 2016). Examples of live vaccines for coccidiosis control include Advent™, Coccivac-B52™ and Immucox™. The efficacy of live vaccines relies on the use of live parasites to induce immunity through repeated exposure to oocysts in the litter (Sharman et al., 2010). This process is called "recycling" and is critical for the development of immunity. Live virulent vaccines are more commonly used in breeder flocks rather than in broiler production (Lillehoj and Trout, 1993). The reason is that live vaccines present two major disadvantages: 1) immunovariant infection within a flock can lead to the emergence of new strains (Ahmad et al., 2016) and 2) potential excessive accumulation of oocysts in the litter may lead to disease outbreaks (Lillehoj and Trout, 1993).

Live attenuated vaccines such as Paracox-5™, Livacox-T™, and Eimerivax 3m™, are developed from parasites with artificially reduced virulence (Vermeulen et al., 2001), usually achieved by serial *in vivo* passage of parasites through embryonated eggs, by selection for precocity of oocysts (Dalloul and Lillehoj, 2005), or by parasite irradiation (Lillehoj and Lillehoj, 2000). A major advantage of live attenuated vaccines over live virulent vaccines is the reduced proliferation capacity of parasites. Consequently, there is less damage to the intestinal

lining (Vermeulen et al., 2001; Ahmad et al., 2016) and reduced mortality (Lillehoj and Lillehoj, 2000).

Live-tolerant to ionophores vaccines are developed from *Eimeria* strains tolerant to anti-coccidial drugs (Vermeulen et al., 2001). The objective of co-administering ionophore drugs and live vaccines at the same time (Ahmad et al., 2016) is to promote immune protection during the first 3-4 weeks of life (Kitandu and Juranova, 2006). However, these vaccines have the same disadvantages related to the use of live vaccines which include potential capacity of oocysts to revert to a more pathogenic state. Additionally, increased risk in ionophore resistance is also a problem (Ahmad et al., 2016). Examples of live-tolerant to ionophore vaccines include Nobilis-Cox ATM™ and Vac M™.

Recombinant vaccines are developed through recombinant DNA technology. Recombinant vaccines have received special attention as an alternative for the problems associated with live vaccines (Lillehoj and Trout, 1993). Protein recombinant vaccines are derived from purified pathogen proteins or subunits (Nascimiento and Leite, 2012). Sporozoites are the preferred parasitic form used for the development of recombinant protein vaccines due to their availability and effectiveness in preventing infection (Lillehoj and Lillehoj, 2000). Recombinant protein vaccines depend on DNA encoding an antigen that stimulates an immune response, then expressing the antigen in cells for the final purification process. Recombinant DNA vaccines employ genes encoding immunogenic proteins of pathogens rather than the proteins themselves (Lillehoj and Lillehoj, 2000) as in protein recombinant vaccine development.

Current delivery strategies of anticoccidial vaccines consist on oocysts suspensions that are directly sprayed into the feed or directly sprayed to newly-hatched chicks allowing them to

ingest the oocysts by preening themselves and each other (Dalloul and Lillehoj, 2005). Other routes of administration include ocular drops, water suspensions, and *in ovo* injection (Ahmad et al., 2016).

Selected Cytokine Secretion During Coccidiosis Infection in Chickens

Cytokines are proteins that coordinate and regulate the activity of innate and adaptive immunity (Abbas et al., 2014). Chickens that have been vaccinated or naturally infected by *Eimeria* parasites produce specific immunity responses mediated by T cells and B cells (Hong et al., 2006). These responses include the secretion of various cytokines. The possible role of secreted cytokines during coccidial infection has been an active field of research. For the purpose of this literature review the possible roles of IFN-γ, IL-1β, IL-6 and IL-10 (Table 4) during coccidial infections will be covered.

Interferon-gamma (IFN- γ) secretion has been associated to *Eimeria* infection in mice and chickens (Lillehoj and Trout, 1996). Upregulation of IFN-γ mRNA expression has been observed after *Eimeria acervulina* infection in the cecal tonsils and spleen (Lillehoj et al., 2004). Following *Eimeria tenella* infection IFN-γ transcripts were detected in spleen, cecal tonsils and intraepithelial lymphocytes (Lillehoj et al., 2004). Lymphocytes from *Eimeria*-infected chickens were reported to increase the production of IFN-γ when induced with concanavalin A compared to lymphocytes from non-infected chickens (Lillehoj and Trout, 1996). Lillehoj et al. (2005) studied the effect of *in ovo* DNA immunization with cloned 3-IE *Eimeria* gene with or without supplemental cytokine genes (IL-1, IL-2, IL-6, IL-8, IL-15, IL-16, IL-17, IL-18, IFN-γ) as adjuvants. Their results showed that *in ovo* vaccination with 3-1E gene in combination with IL-1, IL-2, IL-15 and IFN-γ genes induced higher serum antibody responses when compared with immunization with 3-1E alone. Additionally, weight gain was increased in chickens hatched

from eggs vaccinated using 3-1E gene plus IL-15 and IFN-γ when compared to chickens that hatched from eggs vaccinated with 3-1E gene alone. The authors concluded that the use of cytokine adjuvants such as IFN-γ provide protective intestinal immunity against coccidiosis.

Interleukin-1 beta (IL-1β) is associated with inflammatory response, cell differentiation and apoptosis regulation in the body (Giraldo et al., 2009). Secretion of IL-1β during coccidial infection has been reported in chickens. *In vitro* production of IL-1β was observed in macrophages obtained from *Eimeria maxima* and *Eimeria tenella* infected chickens (Byrnes et al., 1993). Interleukin-1 beta transcripts were increased 27- to 80-fold 7 d after chickens were infected with *Eimeria tenella* or *Eimeria maxima* (Laurent et al., 2001). Hong et al. (2006) reported that transcripts of pro-inflammatory cytokines (IFN-α, IL-1β, IL-6, and IL-17) were increased up to 2,000-fold following *Eimeria acervulina* and *Eimeria tenella* primary infection.

Interleukin-6 (IL-6) regulates the proliferation of antibody-producing cells (Abbas et al., 2014). Lillehoj et al. (2004) reviewed the role of IL-6 during the first hours post coccidial infection. They reviewed literature reports where bacterially expressed chicken IL-6 was biologically active inducing proliferation of the IL-6-dependent murine hybridoma cell line 7TD1. Additionally, chicken IL-6-like factor activity was detected in chickens infected with *E. tenella* during the course of a primary infection. The authors concluded that IL-6 activity during the first hours post-infection indicates a possible role in the development of acquired immunity.

Interleukin-10 (IL-10) inhibits the synthesis of proinflamatory cytokines such as, IL-1β and IL-6 (Hong et al., 2006). This relationship was observed by Morris et al. (2015) who reported that 25-OH-D_3 influenced *in vitro* expression of IL-10 mRNA using chicken macrophage cell lines. Additionally, an *in vivo* study conducted by Morris et al. (2015) using layer-type chicks orally challenged with 1×10^5 live coccidia oocysts at 21-d of age and

supplemented with 6.25, 25, or 50 μg of 25-OH-D_3 per kg of feed revealed evidence suggesting the D_3 metabolite has a regulatory effect on body weight, CD8+ cells, IL-10 mRNA expression and CD4+CD25+ cells. Moreover, dietary 25-OH-D_3 supplementation (100μg/kg) decreased IL-1β mRNA and increased IL-10 mRNA amounts in the cecal tonsils. It appears that IL-10 has an anti-inflammatory role during coccidial infection.

Table 4 Selected cytokine role in general immunity

Cytokine	Cell Source	Principal cellular targets and role in immunity
IFN-γ	T cells (T_H1, $CD8^+$ T cells), NK cells	Macrophages: classical activation (increased microbial functions) B cells: isotype switching to opsonizing and IgG subclasses T cells: T_H1 proliferation Various cells: increased expression of class I and class II MHC molecules, increased antigen processing and presentation to T cells
IL-1β	Macrophages, dendritic cells, fibroblasts, endothelial cells, keratinocytes, hepatocytes	Endothelial cells: activation (inflammation, coagulation) Hypothalamus: fever Liver: synthesis of acute-phase proteins T cells: T_H17 differentiation
IL-	Macrophages, endothelial cells, T cells	Liver: synthesis of acute-phase protein B cells: proliferation of antibody-producing cells
IL-10	Macrophages, T cells (Mainly regulatory T cells)	Macrophages, dendritic cells: inhibition of expression of IL-12, costimulators, and class II MHC

Adapted from: Abbas et al., 2014

REFERENCES

Abbas, A. K., A. H. Lichtman, and S. Pillai. 2014. Cellular and molecular immunology.8th rev. ed. Elsevier Saunders, Philadelphia, PA., USA.

Ahmad, T. A., B. A. El-Sayed, and L. H. El-Sayed. 2016. Development of immunization trials against *Eimeria* spp. Trials Vaccinol. 5:38-47.

Anon. 2013. High cost of coccidiosis in broilers. The Poultry Site. Accessed May 2018. http://www.thepoultrysite.com/poultrynews/28036/high-cost-of-coccidiosis-in-broilers.

Anon. 2017. Statistics. National Chicken Council (NCC). Washington, DC. Accessed May 2018. http://www.nationalchickencouncil.org/about-the-industry/statistics/u-s-broiler-performance/.

Anon. 2017. World population projected to reach 9.8 billion in 2050, and 11.2 billion in 2100. United Nations News. Accessed May 2018. https://www.un.org/development/desa/en/news/population/world-population-prospects-2017.html.

Applegate, T. J., and R. Angel .2014. Nutrient requirements of poultry publication: History and need for an update. J. Appl. Poult. Res. 23:567-575.

Aslam, S. M., J. D. Garlich, and M. A. Qureshi. 1998. Vitamin D deficiency alters the immune responses of broiler chicks. Poult. Sci. 77:842-849.

Atencio, A., G. M. Pesti, and H. M. Edwards Jr. 2005. Twenty-five hydroxycholecalciferol as a cholecalciferol substitute in broiler breeder hen diets and its effect on the performance and general health of the progeny. Poult. Sci. 84:1277-1285.

Baker, D. H., R. R. Biehl, and J. L. Emmert, 1998. Vitamin D_3 requirement of young chicks receiving diets varying in calcium and available phosphorus. Br. Poult. Sci. 39:413-417.

Bar, A., V. Razaphkovsky, E. Vax, and I. Plavnik. 2003. Performance and bone development in broiler chickens given 25-hydroxycholecalciferol. Br. Poult. Sci. 44:224-233.

Barrera, D., L. Díaz, N. Noyola-Martínez, and A. Halhali. 2015. Vitamin D and inflammatory cytokines in healthy and preeclamptic pregnancies. Nutrients. 7:6465-6490.

Biely, J., and B. E. March. 1967. Calcium and vitamin D in broiler rations. Poult. Sci. 46:223.

Byrnes, S., R. Eaton, and M. Kogut. 1993. In vitro interleukin-1 and tumor necrosis factor-α production by macrophages from chicken infected with *Eimeria maxima* or *Eimeria tenella*. Int. J. Parasitol. 23:639-645.

Cantorna, M. T. 2006. Vitamin D and its role in immunology: multiple sclerosis, and inflammatory bowel disease. Prog. Biophys. Mol. Biol. 92:60-64.

Chapman, H. D. 2008. Coccidiosis in the turkey. Avian Pathol. 37:205-223.

Chou, S. H., T. K. Chung, and B. Yu. 2009. Effects of supplemental 25-hydroxycholecalciferol on growth performance, small intestine morphology, and immune response of broiler chickens. Poult. Sci. 88:2333-2341.

Cobb-Vantress. 2015. Performance and Nutrition Supplement, Cobb-500. Accessed http://www.cobb-vantress.com/docs/default-source/cobb-500 guides/Cobb500_Broiler_Performance_And_Nutrition_Supplement.pdf

Cobb-Vantress. 2012. Performance and Nutrition Supplement, Cobb-700. Accessed http://www.cobb-vantress.com/.

Collins, E. D., and A. W. Norman .2001. Vitamin D: in Handbook of vitamins. 3rd ed. Edited by Rucker R. B., J. W. Suttie, D. B. McCormick, and L. J. Machlin. Marcel Dekker, New York. USA.

Combs, G. F. The vitamins: fundamental aspects in nutrition and health. 2012. 4th ed. Academic Press, ProQuest ebrary. Saint Louis, MO.USA.

Cruickshank, J. J. and J. S. Sim. 1987. Effects of excess vitamin D_3 and cage density on the incidence of leg abnormalities in broiler chickens. Avian Dis. 31:332-338.

Dalloul, R. A., and H. S. Lillehoj. 2005. Recent advances in immunomodulation and vaccination strategies against coccidiosis. Avian Dis. 49:1-8.

De Gussem, M. 2007. Coccidiosis in poultry: Review on diagnosis, control, prevention and interaction with overall gut health. Pages 253–261 in Proc. 16th Eur. Symp. on Poult. Nutr. World's Poultry Science Association, Beekbergen, The Netherlands.

DeLuca, H. F. 1978. Vitamin D metabolism and function. Arch. Intern. Med. 138:836-847.

DeLuca, H. F., and M. T. Cantorna. 2001. Vitamin D: its role and uses in immunology. The FASEB Journal. 15:2579-2585.

Edwards, Jr., H. M. 2000. Nutrition and skeletal problems in poultry. Poult. Sci. 79:1018-1023.

Edwards, Jr., H. M., R. B. Shirley , W. B. Escoe , and G. M. Pesti .2002 . Quantitative evaluation of 1α-hydroxycholecalciferol as a cholecalciferol substitute for broilers. Poult. Sci. 81:664-669.

Ferket, P. R. 2004. Alternatives to antibiotics in poultry production: Responses, practical experience and recommendations. Pages 56–67 in Re-imaging the feed industry. Proc. Alltech's 20th Annu. Symp. T. P. Lyons and K. A. Jacques, ed. Nottingham Univ. Press, UK.

Fritts, C. A., and P. W. Waldroup. 2003. Effect of Source and level Vitamin D on live performance and bone development in growing broilers. J. Appl. Poult. Res. 12:45-52.

Garcia, A. F. Q. M., A. E. Murakami, C. R. do Amaral Duarte, I. C. O. Rojas, K. P. Picoli, and M.M. Puzotti. 2013. Use of vitamin D_3 and its metabolites in broiler chicken feed on performance, bone parameters and meat quality. Asian-australas. J. Anim. Sci. 26:408-415.

Giraldo, S., J. Sanchez, Q. Felty, and D. Roy. 2009. IL-1β (interleukin 1, beta), Atlas Genet. Cytogenet. Oncol. Haematol. 13:273-275.

Goff, J. P., and R. L. Horst. 1995. Assessing adequacy of cholecalciferol supplementation in chicks using plasma cholecalciferol metabolite concentrations as an indicator. J. Nutr. 125:1351-1357.

Goldblatt, H., and K. M. Soames. 1923. A study of rats on a normal diet irradiated daily by the mercury vapour quartz lamp or kept in darkness. Biochem. J. 17:294.

Gómez, V. G., L. R. Morales, and G. E. Avila. 2013. Use of 25-hydroxycholecalciferol in diets of broiler chickens: effects on growth performance, immunity and bone calcification. J. Poult. Sci. 50:60-64.

Hall, A. K. and A. W. Norman. 1990. Regulation of calbindin-D28K gene expression in the chick intestine: Effects of serum calcium status and 1, 25-dihydroxyvitamin D_3. J. Bone Miner. Res. 5:331-336.

Havenstein, G. B., P. R. Ferket, and M. A. Qureshi .2003. Growth, livability, and feed conversion of 1957 versus 2001 broilers when fed representative 1957 and 2001 broiler diets. Poult. Sci. 82:1500-1508.

Hong, Y. H., H. S. Lillehoj, S. H. Lee, R. A. Dalloul, and E. P. Lillehoj. 2006. Analysis of chicken cytokine and chemokine gene expression following *Eimeria acervulina* and *Eimeria tenella* infections. Vet. Immunol. Immunopathol. 114:209-223.

Huff, G. R., W. E. Huff, J. M. Balog, and N. C. Rath. 2000. The effect of vitamin D3 on resistance to stress-related infection in an experimental model of turkey osteomyelitis complex. Poult. Sci. 79:672-679.

Jiang, S., Z. Jiang, K. Yang, F. Chen, C. Zheng, and L. Wang. 2015. Dietary vitamin D_3 requirement of Chinese yellow-feathered broilers. Poult. Sci. 94: 2210-2220.

Kapell, D. N., W. G. Hill, A. M. Neeteson, J. McAdam, A. N. Koerhuis, and S. Avendano. 2012. Twenty-five years of selection for improved leg health in purebred broiler lines and underlying genetic parameters. Poult. Sci. 91:3032-3043.

Kasim, A. and H.M., Jr. Edwards. 2000. Evaluation of cholecalciferol sources using broiler chick bioassays. Poult. Sci. 79:1617-1622.

Khan, S. H., R. Shahid, A. A. Mian, R. Sardar, and M. A. Anjum. 2010. Effect of the level of cholecalciferol supplementation of broiler diets on the performance and tibial dyschondroplasia. J. Anim. Physiol. Anim. Nutr. 94:584-593.

Kheysin, Y. M. 1972. Chapter I. The basic type of life cycles of representatives of the class sporozoa. Pages 149-177 in: Life cycles of coccidia of domestic animals. K. S. Todd Jr., ed. University Park Press, London, UK.

Kitandu, A., and R. Juranova. 2006. Progress in control measures for chicken coccidiosis. Acta Vet. (Brno) 75:265-276.

Kodieck, E., D. E. M. Lawson and P. W. Wilson.1970. Biological activity of a polar metabolite of vitamin D_3. Nature. 228:763-764.

Laurent, F., R. Mancassola, S. Lacroix, R. Menezes, and M. Naciri. 2001. Analysis of chicken mucosal immune response to *Eimeria tenella* and *Eimeria maxima* infection by quantitative reverse transcription-PCR. Infect. Immun. 69:2527-2534.

Ledwaba, M. F., and K. D. Roberson. 2003. Effectiveness of twenty-five-hydroxycholecalciferol in the prevention of tibial dyschondroplasia in Ross cockerels depends on dietary calcium level. Poult. Sci. 82:1769-1777.

Leeson, S. and J. D. Summers. 2001. Scott´s Nutrition of the Chicken. 4th ed. University Books, Ontario Canada.

Leeson, S. and J. D. Summers. 2005. Commercial Poultry Nutrition. 3th ed. University Books. Ontario, Canada.

Leeson, S. 2006. Defining and predicting changes in nutrient requirements. XII European Poultry Conference Verona, Italy.

Leeson, S. 2007. Vitamin requirements: Is there a basis for re-evaluating dietary specifications? World's Poult. Sci. J. 63:255-266.

Lillehoj, H. S., and J. M. Trout. 1993. Coccidia: A review of recent advances in immunity vaccine development. Avian Pathol. 22:3-21.

Lillehoj, H. S., and J. M. Trout. 1996. Avian gut-associated lymphoid tissues and intestinal immune responses to Eimeria parasites. Clin. Microbiol. Rev. 9:349-360.

Lillehoj, H. S., and E. P. Lillehoj. 2000. Avian coccidiosis. A review of acquired intestinal immunity and vaccination strategies. Avian Dis. 44:408-425.

Lillehoj, H. S., W. Min, and R. A. Dalloul. 2004. Recent progress on the cytokine regulation of intestinal immune responses to *Eimeria*. Poult. Sci. 83:611-623.

Lillehoj, H. S., X. Ding, M. A. Quiroz, E. Bevensee, and E. P. Lillehoj. 2005. Resistance to intestinal coccidiosis following DNA immunization with the cloned 3–1E *Eimeria* gene plus IL-2, IL-15, and IFN-γ. Avian Dis. 49:112-117.

Lofton, J. T., and J. H. Soares, Jr. 1986. The effects of vitamin D_3 on leg abnormalities in broilers. Poult. Sci. 65:749.

Lucas, R. M., S. Gorman, S. Geldenhuys, and P. H. Hart. 2014. Vitamin D and immunity. F1000Prime Rep. 6:118.

McAuliffe, T., A. Pietraszek, and J. McGinnis. 1976. Variable rachitogenic effects of grain and alleviation by extraction or supplementation with vitamin D, fat, and antibiotics. Poult. Sci. 55:2142.

McCollum, E. V., N. Simmonds, J. E. Becker and P. G. Shipley. 1922. Studies on experimental rickets XXI. An experimental demonstration of the existence of the vitamin which promotes calcium deposition. J. Biol. Chem. 53:293-312.

McDonald, P., R. A. Edwards, J. F. D. Greenhalg and C. A. Morgan. 1995. Animal Nutrition, 5th ed. AddisonWesley Longman, Edinburgh, UK.

McDowell, L. R. 1989. Vitamins in animal nutrition: comparative aspects to human nutrition. Academic Press Inc. USA. pp 55-87.

McNaughton, J. L., E. J. Day, and B. C. Dillworth. 1977. The chick's requirements for 25-hydroxycholecalciferol and cholecalciferol. Poult. Sci. 56:511.

Mellanby, E. 1919. The present state of knowledge concerning accessory food factors. Med Res (GB), Spec Rep Ser SRS-38.

Merck Veterinary Manual. 2010. Overview of Coccidiosis in Poultry. 10th rev. ed. Merck & Co. Inc. Whitehouse Station, NJ. USA.

Morris, M. P. 1993. National survey of leg problems. Broiler Ind., 20-24.

Morris, A., R. Shanmugasundaram, M. Lilburn, and R. K. Selvaraj. 2014. 25-Hydroxycholecalciferol supplementation improves growth performance and decreases inflammation during an experimental lipopolysaccharide injection. Poult. Sci. 93:1951-1956.

Morris, A., and R. K. Selvaraj. 2014. In vitro 25-hydroxycholecalciferol treatment of lipopolysaccharide-stimulated chicken macrophages increases nitric oxide production and mRNA of interleukin- 1beta and 10. Vet. Immunol. Immunopathol. 161:265-270.

Morris, A., R. Shanmugasundaram, J. Mcdonald, and R. K. Selvaraj. 2015. Effect of *in vitro* and *in vivo* 25-hydroxyvitamin D treatment on macrophages, T cells, and layer chickens during a coccidia challenge. J. Anim. Sci. 93:2894-2903.

Muir, W. M. and S. E. Aggrey. eds. 2003. Poultry Genetics, Breeding, and Biotechnology. CABI Publishing, Cambridge, MA. USA.

Nascimento, I. P., and L .C. Leite. 2012. Recombinant vaccines and the development of new vaccine strategies. Braz. J. Med. Biol. Res. 45:1102-11.

National Research Council .1994. Nutrients requirements of poultry. 9th rev. ed. Natl. Acad. Press, Washington, DC. USA.

Norman, A. W. 1979. Vitamin D: the calcium homeostatic steroid hormone. Academic Press, Inc. New York, USA.

Norman, A. W. and S. Hurwitz. 1993. The role of the vitamin D endocrine system in avian bone biology. J. Nutr. 123:310-316.

Norman, P. E., and J. T. Powell. 2014. Vitamin D and cardiovascular disease. Circ. Res. 114:379-393.

Pattinson, M., P. F. McMullin, J. F. Bradbury, and D. J. Alexander. 2008. Poultry diseases. 6th ed. Pages 444-449 in Parasitic Diseases, Saunders, Elsevier Limited: UK.

Rama Rao, S. V., M. V. L. N. Raju, A. K. Panda, G. Shyam Sunder, and R. P. Sharma. 2006. Effect of high concentration of cholecalciferol on growth, bone mineralization and mineral retention in broiler chicks fed sub-optimal concentrations of calcium and non-phytate phosphorus. J. Appl. Poult. Res. 15:493-501.

Schmidt, C. J., M. E. Persia, E. Feierstein, B. Kingham, and W.W. Saylor. 2009. Comparison of a modern broiler line and a heritage line unselected since the 1950s. Poult. Sci. 88:2610-2619.

Sharman, P. A., N. C. Smith, M. G. Wallach, and M. Katrib. 2010. Chasing the golden egg: Vaccination against poultry coccidiosis. Parasite Immunol. 32:590-598.

Shojadoost, B., S. Behboudi, A. I. Villanueva, J. T. Brisbin, A. A. Ashkar, and S. Sharif. 2015. Vitamin D_3 modulates the function of chicken macrophages. Res. Vet. Sci. 100:45-51.

Stanford, M. 2006. Calcium metabolism. In: Clinical avian medicine, ed. Harrison G. J. and T. L. Lightfoot. Spix Publishing, Palm Beach, FL. USA. pp 141-151.

Steenbock, H., and A. Black. 1925. Fat-soluble vitamins xxiii. The induction of growth-promoting and calcifying properties in fats and their unsaponifiable constituents by exposure to light. J. Biol. Chem. 64:263-298.

Sullivan, T. W. 1994. Skeletal problems in poultry: estimated annual cost and descriptions. Poult. Sci. 73:879-882.

Sun, Z., L. Yan, J. Zhao, H. Lin, and Y. Guo. 2013. Increasing dietary vitamin D_3 improves the walking ability and welfare status of broiler chickens reared at high stocking densities. Poult. Sci. 92:3071-3079.

United States Bureau of Labor Statistics CPI Inflation calculator Accessed Oct. 2017. http://www.bls.gov/home.htm.

Veldman, C. M., M. T. Cantorna, and H. F. DeLuca. 2000. Expression of 1, 25-dihydroxyvitamin D_3 receptor in the immune system. Arch. Biochem. Biophys. 374:334-338.

Vermeulen, A. N., D. C. Schaap, and T. P. Schetters. 2001. Control of coccidiosis in chickens by vaccination. Vet. Parasitol. 100:13-20.

Vignale, K., E. S. Greene, J. V. Caldas, J. A. England, N. Boonsinchai, P. Sodsee, E. D. Polock, S. Dridi, and C. N. Coon. 2015. 25-hydroxycholecalciferol enhances male broiler breast meat yield through the mTOR pathway. J. Nutr. 145:855-863.

Waldenstedt, L. 2006. Nutritional factors of importance for optimal leg health in broilers: A review. Anim. Feed Sci. Technol. 126:291-307.

Waldroup, P. W., C. B. Ammerman, and R. H. Harms. 1963. The relationship of phosphorus, calcium and vitamin D_3 in the diet of broiler-type chicks. Poult. Sci. 42:982.

Waldroup, P. W., J. E. Sterns, C. B. Ammerman, and R. H. Harms. 1965. Studies on the vitamin D_3 requirement of the broiler chick. Poult. Sci. 44:543.

Wang, Y., J. Zhu, and H. F. DeLuca. 2012. Where is the vitamin D receptor? Arch. Biochem. Biophys. 523:123-133.

Whitehead, C. C. 1995. The role of vitamin D metabolites in the prevention of tibial dyschondroplasia. Anim. Feed Sci. Technol. 53:205-210.

Whitehead, C. C., H. A. McCormack, L. McTier, R. H. Fleming. 2004. High Vitamin D_3 requirements in broilers for bone quality and prevention of tibial dyschondroplasia and interactions with dietary calcium, available phosphorus and Vitamin A. Brit. Poult. Sci. 45:425-436.

Windaus, A., F. R. Schenck and F. T. Werder. 1936. Über das antirachitisch wirksame Bestrahlungsprodukt ans 7-Dehydro-cholesterin. Hoppe-Seyler´ s Z. Physiol. Chem. 241:100-103.

Wintergerst, E. S., S. Maggini, and D. H. Hornig. 2007. Contribution of selected vitamins and trace elements to immune function. Ann. Nutr. Metab. 51:301-323.

Yarger, J. G., C. L. Quarles, B. W. Hollis, and R. W. Gray, 1995. Safety of 25-hydroxycholecalciferol in poultry rations. Poult. Sci. 74:1437-1446.

Yun, C. H., H. S. Lillehoj, and E. P. Lillehoj. 2000. Intestinal immune responses to coccidiosis. Dev. Comp. Immunol. 24:303-324.

CHAPTER II

DEVELOPING AN ORAL GAVAGE BIOASSAY TO RE-EVALUATE THE VITAMIN D_3 REQUIREMENT OF GROWING BROILER CHICKENS

INTRODUCTION

Cholecalciferol, or vitamin D_3 (**D_3**), has been extensively studied due to its potent antirachitic activity in poultry and other animal species. It is known that D_3 plays an important role in calcium (**Ca**) and phosphorus (**P**) homeostasis in conjunction with calcitonin and parathyroid hormones (Collins and Norman, 2001). Although D_3 can be synthesized in the skin after exposure to UV-light from the sun, current industry facilities used to raise poultry species have limited access to sun light. Therefore, to avoid D_3 deficiencies and prevent the incidence of bone problems such as, rickets or tibia dyschondroplasia, D_3 supplements are widely used in poultry vitamin premixes and feed formulations.

The Poultry NRC (1994) established the vitamin D_3 requirement at 200 IU D_3/kg of diet for starter growing broiler chicks. However, post NRC (1994) research suggests higher amounts of D_3 are required for optimal performance (Kasim and Edwards, 2000; Fritts and Waldroup, 2003; Whitehead et al., 2004; Rama Rao et al., 2006; Khan et al., 2010). Modern commercial poultry diets often include up to 20 times the minimal requirement of 200 IU D_3/kg specified in the NRC (1994). Therefore, it is possible that nutrient requirements of meat type chickens have changed since the last revision of the NRC in 1994 which referenced literature, regarding D_3 requirements, dated as early as 1963. Thus, concern among nutritionists regarding the need to re-evaluate nutrient requirements that are based on the current ability of modern poultry strains to respond to dietary nutrients has increased over the last few years.

Previous research done in our laboratory investigating relative bioavailability of D_3 sources, have found considerable variability in response to dietary D_3. Our biggest challenge is to observe a decrease in performance or bone mineralization as indicators of D_3 biological activity in young growing broilers subjected to D_3-deficient diets. Maternal D_3, found in the yolk, appears to play a key role in the early performance of young broilers and therefore, in their response to supplemental D_3 as suggested by previous literature reports (Moran, 2007; Coto et al., 2010a; Coto et al., 2010b; Saunders-Blades and Korver, 2014).

Nutrient requirements have traditionally been determined by adding graded concentrations of the nutrient in question to a deficient diet and using broken-line regression to assess the response variables. Feed mixing error, selective feeding, and other sources of error including chemical nutrient analysis of the test diets are inherently problematic with this approach. Thus, the present study focused on reducing the effect of maternal D_3 and increasing the precision of D_3 delivery method to the chickens through a precise gavage intake bioassay. Additionally, this study was useful to identify any possible pitfalls in the protocol that will possibly be corrected in a second trial. Hence, the results of the first approach to the development of the precision gavage intake bioassay will be presented in the following sections of this dissertation.

MATERIALS AND METHODS

All procedures in this experiment were approved by the Institutional Animal Care and Use Committee of Texas A&M University (AUP: IACUC 2017-0072).

Birds and General Management

A total of 300 Cobb-500 newly-hatched male broiler chickens were purchased from a commercial hatchery. At the time of arrival to the experimental facilities, the birds were weighed in groups of 20 and an average body weight (**BW**) was calculated. The average BW was used to create 48 groups of five chickens (n=240) with close to "identical" starting body weight and variance. Broilers were then allocated to two stainless steel battery brooders (5 birds per cage) inside an environmentally controlled rearing room using a completely randomized block design. Battery pen level (6 levels) was used as the blocking factor. Fluorescent 48-inch tube lamps covered with red plastic shields were used to provide 24-h constant light. The complete absence of UV-light inside the rearing rooms has been previously verified (Fowler et al., 2014) by the Texas A&M Environmental Health and Safety Office using a short wave UV meter (J-225 Blak-Ray, UVP, LLC. Upland, CA). A basal D_3-deficient corn-soy broiler starter diet was fed *ad libitum* throughout a 21 d trial period. The first nine days of the study served to deplete the maternal stores of D_3 followed by a 12-h fasting period. From d 10 to the end of the trial, birds were orally gavaged with increasing levels of vitamin D_3. Oral D_3 treatments were offered for 11 d. Water was offered *ad libitum* using nipple drinkers. Birds were monitored daily with regard to general flock condition, temperature, lighting, water, feed, and any unanticipated events inside the rearing facility.

Dietary Treatments

A basal mash corn-soy broiler starter diet devoid of D_3 was formulated using a customized vitamin / mineral premix containing no D_3 and corn oil as the fat source (Table 5). To increase the sensitivity of our response variables to the experimental treatments, the basal diet was formulated with a marginal concentration of calcium (0.75%) and non-phytate phosphorous

(**NPP**; 0.37%). Feed intake (**FI**) reference data for meat-type chickens (Cobb-500 Broiler Performance and Nutrition Supplement, 2015) was used to estimate the increasing levels of vitamin D_3 per kg of feed consumed. Daily oral gavage treatments were based on estimated intake of 0, 50, 100, 200, 400, 800, 1,600 and 3,200 IU D_3/kg of feed consumed over the last 12 days of the study (estimated to be 900 g per bird). To create the experimental treatments, a total of 30 mg of crystalline vitamin D_3 concentrate (cholecalciferol, Ref: 1131009, Sigma-Aldrich, St. Louis, MO) were diluted in 100 mL of corn oil (expected to yield 12,000 IU/mL). An aliquot of 26.2 mL was diluted again in corn oil (573.8 mL) to yield a concentration of 524 IU D_3/mL corresponding to the highest treatment dose (≈3,200 IU/kg of feed) and then serial dilutions were performed to create the other treatments so that a daily constant dose was contained in 0.5 mL. The D_3 solutions were separated in 11 daily doses (n=77) and then stored in a freezer at -20°C until required. Birds in the control group (0 IU) received 0.5 mL of corn oil without D_3 for 11 days. The oral gavage was performed per treatment group from lower to higher IU concentration using an 18-gauge stainless steel gavage needle and a 1 mL syringe graduated at 1/100 mL. The gavage needle was flushed with plain corn oil between treatments to avoid cross-contamination. The gavage procedure was performed by a single operator who gained proficiency in the delivery of a specific oil volume after repeated training (CV $\bar{x}$ =0.94%).

Chemical Analysis

The stock solution obtained from the dilution of 30 mg of the Sigma D_3 standard in 100 mL of corn oil (calculated to yield 12,000 IU/mL) was sent to a third party commercial laboratory for D_3 concentration analysis (AOAC-2002.05) to ensure a minimum concentration of D_3 in the stock solution. Analyzed concentration reported by the laboratory was 12,800 IU/mL.

Performance Evaluation

Feed intake and BW per pen were recorded on d 10, after the fasting period, and d 21 of the trial to calculate weight gain (**WG**) and feed efficiency (**FE**). The BW of dead birds was recorded daily and used to adjust FE. Feed intake data collected from d 10 to 21 were used to adjust the IU of D_3 administered through the oral gavage and expressed as IU of D_3 per kg of feed using the following equations:

1) TOGIU = S [IU/mL] * 0.5* d

Where: TOGIU = Total orally gavaged IU's

S = Solution (Corn oil + D_3) concentration (Obtained from serial dilutions)

d = number of days chickens were orally gavaged

2) AIUI= (1,000 * TOGIU) / FI

Where: AIUI = Adjusted IU intake (IU D_3/kg of feed)

FI = Feed intake (g / bird)

Bone Mineralization

On d 21 of the experiment, all birds per pen were euthanized using CO_2. Total bone mineral content (**BMC**) and bone mineral density (**BMD**) were obtained per pen using a Dual X-ray absorptiometry (**DXA**) scan (GE Lunar Prodigy Advance, GE-Healthcare, Boston, MA) located at the Applied Exercise Science Laboratory of Texas A&M University. Bone mineral content is defined as the total bone mineral found in a specific area measured in grams. Bone mineral density is derived using BMC (g) divided by an area (cm^2) of interest. Chickens were placed in prone position with their wings and legs at the sides of the body throughout the scan.

Table 5 Basal vitamin D_3 deficient broiler starter diet

Ingredient	Basal Diet [1] (%)
Yellow corn	62.7
Dehulled soybean meal	32.1
DL-Methionine	0.15
L-Lysine-HCl	0.34
Corn oil	1.81
Limestone	1.06
Monocalcium phosphate	1.19
Sodium chloride (salt)	0.46
Customized vitamin-mineral premix [2]	0.50

[1]Calculated nutritional content was as follow: 22% crude protein, 3050 kcal/kg metabolizable energy, 0.75% calcium, 0.37% non-phytate phosphorous, 0.48% methionine, 0.85% methionine+cystine, 1.2% lysine, 0.26% tryptophan, 0.82% threonine, 1.46% arginine, 3.7% crude fat, 2.17% crude fiber, 0.2% sodium , 0.93% potassium, 0.3% chloride.

[2]Vitamin-mineral premix added at this rate yields per kg of diet: 10 mg copper, 2 mg iodine, 20 mg iron, 125 mg manganese, 125 mg zinc, 0.2 mg selenium, 8,000 IU vitamin A, 40 IU vitamin E, 2 mg menadione, 4 mg thiamine, 8 mg riboflavin, 60 mg niacin, 15 mg pantothenic acid, 4 mg pyridoxine, 0.18 mg biotin, 2 mg folic acid, 0.02 mg vitamin B_{12}, 600 mg choline.

Data were analyzed using the small animal software (GE Lunar Prodigy Advanced enCore, V 16.0, GE-Healthcare, Boston, MA). After the DXA scan both tibiae were removed, labeled and stored in a freezer (-20°C) until further analysis. The right tibiae were gently boiled for 2-h in water and defatted in petroleum ether for 48-h. Defatted bones were then dried in a forced draft oven (95°C) until a constant weight was registered (≈48 h). Finally, the dried bones were ashed at 650°C for 23-h. Percent tibia bone ash (**TBA**) was calculated based on starting dry bone weight and remaining ash expressed as percent. The left tibiae were cleaned from any adhering tissue and used to assay breaking strength (**TBS**) using a texture analyzer (TA.XT Plus, Texture Technologies, Hamilton, MA,) charged with a 50-kg load cell, a crosshead speed of 100 mm/min with the tibia supported on a 3-point bending ring and a 3-cm constant span.

Statistical Analysis

Collected data were analyzed as one way-ANOVA where treatment and block were used as fixed factors in the model. Battery level was used as the blocking factor. Means were separated by Duncan's multiple range test when appropriate. Linear and quadratic effects of graded levels of D_3 were investigated by regression analysis. The 0 IU group was included as common control to investigate linear and quadratic effects. Three different regression models were used to calculate the D_3 requirement. The one-slope straight broken-line regression model consists of a line with an increasing or decreasing slope and a horizontal line. The intersection between the two lines is the breaking point. The one-slope broken-line model is as follows: $y = L + U^* (R-x)$, where (R-x) is defined as zero when $x > R$. The two-slope straight broken-line regression model is defined as $y = L + U^*(R-x) + V^*(x-R)$, where (R-x) is defined as zero when $x > R$, and (x-R) is defined as zero when $x < R$. In the two-slope model, both intersecting lines have non-zero slopes. The derived parameters for both linear models are the breaking point x value (R), an asymptote for the first segment (L), and slope for the two line segments (U, V). The point of intersection or breaking-point between the two lines was defined as the estimated D_3 requirement. Finally, the quadratic broke-line model is described as $y= L + U^*(R-x)^2$. The concentration at which the response reached 95% of the minimum response was estimated as the D_3 requirement (Robbins, 1986; Robbins et al., 2006; Jiang et al., 2015). One-way ANOVA analysis and linear regression (linear and quadratic effects) were performed by IBM SPSS software (SPSS Version 25.0, SPSS Inc., Chicago, IL) and the SAS software (SAS Version 9.4, SAS Institute, Cary, NC) was employed for the estimation of the D_3 requirement using the NLMIXED procedure. The Akaike Information Criterion (**AIC;** smaller values indicate better fit), coefficient of determination ($\mathbf{R^2}$) and adjusted R^2 where used to assess the goodness of fit for

the three models. The pen average was the experimental unit for performance and bone mineralization responses. Significant effects were accepted at $P<0.05$ for all analyses.

RESULTS AND DISCUSSION

Performance

Performance results are presented in table 6. Although BW and WG of chickens orally gavaged with graded levels of D_3 was numerically improved, no differences ($P>0.05$) were observed between treatment groups. Graded levels of D_3 did positively improve ($P<0.05$) FE. However, this improvement was detected between the 0 IU treatment and the other treatment groups only. Overall, no linear or quadratic effects ($P>0.05$) were observed for any of the performance responses but WG ($P=0.039$) which linearly increased with dietary concentrations of D_3. FI was similar among treatment groups, but was relatively low to the estimated intake of 900 g used to calculate total IU consumed over the experimental period. Thus, adjusted IU D_3/kg feed were over the estimated feed intake for all treatment groups.

While not very precise, multiple range tests are a simple and easy way to obtain an approximation or estimation of a nutritional requirement (Pesti et al., 2009). Observed differences between treatment groups will indicate where the response variable was maximized. However, this methodology was difficult to implement in the present study due to a lack of consistency in the performance response to dietary D_3 and that no differences ($P>0.05$) were detected between treatment groups.

Table 6 Effect of dietary vitamin D_3 on performance of broiler chickens

	Response[3,4]			
IU D_3/kg feed [1,2]	21 d	10-21 d	10-21 d	10-21 d
	BW	WG	FE	FI
0	711±23	504±22	0.69±0.018[b]	725±19
50 (61)	725±14	527±10	0.72±0.010[a]	736±13
100 (122)	742±13	536±11	0.73±0.006[a]	738±13
200 (242)	741±16	544±10	0.73±0.010[a]	746±19
400 (480)	722±17	530±10	0.71±0.015[ab]	753±21
800 (961)	740±9	544±9	0.72±0.004[a]	750±10
1600 (1950)	734±12	534±10	0.72±0.005[a]	740±10
3200 (3733)	754±16	557±16	0.73±0.007[a]	776±23
P value				
Linear	0.146	0.039	0.132	0.055
Quadratic	0.962	0.822	0.534	0.795

[a-b] Means within the same column without a common superscript differ ($P<0.05$) Duncan's multiple range test. Means ± SEM.

[1] Calculated IU D_3/kg feed (Adjusted IU D_3/kg feed based on feed intake data).

[2] Chemical analysis of stock solution (crystalline vitamin D_3 concentrate + corn oil) expected to yield 12,000 IU D_3/mL) was performed at Cornerstone Laboratories, LLC by AOAC-2002.05 (1775 Moriah Woods Blvd., Ste. 12 Memphis, TN 38117). Analyzed D_3 concentration was 12,800 IU D_3/mL. Serial dilutions using the stock solution were performed to create dietary D_3 treatments so that a daily constant dose was contained in 0.5 mL. D3 was administered to the chickens through a daily oral gavage.

[3] BW, Body weight (g/bird); WG, Weight gain (g/bird); FE, Mortality corrected feed efficiency (g weight gain / g feed intake), FI, feed intake (g).

[4] Values for performance responses represent the mean average of n=6 replicate pens per treatment of 5 birds each at respective age.

Despite the fact that performance responses are not very sensitive to dietary D_3 or mineral inadequacy (Waldroup et al., 1963; Edwards et al., 1994; Angel et al., 2005), early performance of growing broilers is highly influenced by maternal D_3 found in the egg yolk (Moran, 2007; Coto et al., 2010a; Coto et al., 2010b; Saunders-Blades and Korver, 2014). Performance results obtained in the present study suggest that the depletion period of 9-d prior to the introduction of the oral gavage and lowering the Ca and NPP content in the diet were not very effective in creating a severe vitamin D_3 deficiency, possibly because the chickens used in this experiment

had high maternal reserves of D_3. It is known that commercial poultry diets contain high dietary concentration of fat-soluble vitamins. In the U.S.A., vitamin D_3 is commonly supplemented at 3,000-4,000 IU D_3/kg feed in broiler breeder diets (Leeson and Summers, 2005). This is probably the biggest obstacle to overcome when evaluating vitamin D_3 requirements using "commercial" broiler chickens. Additionally, the low FI registered could have potentially reduced the growth rate of the chickens and consequently, reduced the demand for nutrients, which ultimately, decreased even further the sensitivity of the experiment.

Bone Mineralization

The effect of dietary vitamin D_3 on bone mineralization is presented in table 7. Tibia bone ash and TBS increased linearly ($P<0.05$) in response to increasing dietary concentrations of D_3. Moreover, differences ($P<0.05$) were observed among treatments groups and when compared with the 0 IU treatment group. No significant effect of dietary D_3 was observed for BMD or BMC. However, BMC appeared to have a linear response to dietary increasing concentrations of D_3. Similar to performance responses, bone mineralization results were not very consistent within the treatment groups. However, the multiple range test approach to estimate the D_3 nutritional requirement is possible. Based on Duncan's multiple range test, it appears that 480-961 IU D_3/kg feed are necessary to maximize TBA and TBS. The main disadvantage of this approach is the lack of precision (Pesti et al., 2009) and therefore, other methodologies are necessary to have a better estimate of the nutritional requirement but as previously mentioned is a good starting point to estimate nutritional requirements.

Interestingly, TBA for the 0 IU treatment group averaged 47.2 %. Compared to the other treatment levels in average this represents a ≈1.2% decrease in tibia bone ash. Previous experiments conducted by our laboratory using a similar protocol to deplete maternal stores of

D_3 and increase the sensitivity of the studied responses to dietary D_3 accomplished a 5.0% decrease in TBA (Leyva-Jimenez et al., 2018) which resulted in a more consistent performance and bone mineralization response to graded levels of D_3. Similar reductions (4-6%) in tibia ash were reported by Khan et al. (2010) and Jiang et al. (2015) using commercial broilers to estimate vitamin D_3 requirements and overall reported a consistent response to dietary D_3. These results support the hypothesis that a D_3 deficiency was not effectively induced in this experiment and that the maternal stores of D_3 in the egg yolk reduced the sensitivity of our response variables.

Table 7 Effect of dietary vitamin D_3 on bone mineralization of broiler chickens

IU D_3/kg feed[1,2]	Response[3,4] 0-21 d			
	TBA	TBS	BMD	BMC
0	47.2±0.4[c]	12.0±0.7[c]	0.063±0.003	3.54±0.24
50 (61)	48.1±0.5[ab]	13.1±0.9[bc]	0.062±0.003	3.78±0.31
100 (122)	48.3±0.3[ab]	13.8±0.7[abc]	0.062±0.004	3.67±0.25
200 (242)	48.3±0.2[ab]	13.9±0.6[abc]	0.068±0.003	4.08±0.30
400 (480)	48.2±0.5[ab]	14.4±0.8[ab]	0.065±0.002	3.88±0.17
800 (961)	48.9±0.3[a]	14.7±0.5[ab]	0.070±0.003	4.31±0.16
1600 (1950)	48.1±0.2[ab]	15.1±0.4[ab]	0.067±0.003	4.08±0.21
3200 (3733)	49.2±0.4[a]	15.8±0.5[a]	0.067±0.005	4.36±0.33
P value				
Linear	<0.001	<0.001	0.246	0.027
Quadratic	0.873	0.104	0.127	0.319

[a-c] Means within the same column without a common superscript differ ($P<0.05$) Duncan's multiple range test. Means ± SEM.

[1] Calculated IU D_3/kg feed (Adjusted IU D_3/kg feed based on feed intake data).

[2] Chemical analysis of stock solution (crystalline vitamin D_3 concentrate + corn oil) expected to yield 12,000 IU D_3/mL) was performed at Cornerstone Laboratories, LLC (1775 Moriah Woods Blvd., Ste. 12 Memphis, TN 38117). Analyzed D_3 concentration was 12,800 IU D_3/mL. Serial dilutions using the stock solution were performed to create dietary D_3 treatments so that a daily constant dose was contained in 0.5 mL. D3 was administered to the chickens through a daily oral gavage.

[3] TBA, tibia bone ash (%); TBS, tibia breaking strength (kg force); BMD, bone mineral density (g/cm^2); BMC, bone mineral content (g).

[4] Values for bone mineralization responses represent the mean average of n=6 replicate pens per treatment of 5 birds each at respective age.

Requirement Estimation Using Regression Models

To estimate the vitamin D_3 requirement with better precision than the multiple range test methodology, TBA and TBS response of growing broiler chickens to graded levels of D_3 was fitted to three different regression models are presented in table 8. Performance variables were not reported as criteria to estimate the D_3 requirement because the low response to graded levels of D_3 and also because regression models yielded very low AIC, R^2 and adjusted R^2. For TBA the one-slope broken model yielded a D_3 requirement of 491 IU/kg of feed. The two-slope broken line model resulted in an estimated D_3 requirement of 532 IU/kg of feed. The quadratic model resulted in an estimated requirement of 497 IU/kg of feed. On the other hand, D_3 requirements needed to maximize TBS were higher when compared to TBA. The one-slope broken model yielded a D_3 requirement of 574 IU/kg of feed. The two-slope broken line model estimated the D_3 requirement at 658 IU/kg of feed. Finally, the quadratic model resulted in an estimated requirement of 798 IU/kg of feed.

Overall, in this experiment the non-linear quadratic regression models resulted in better AIC, R^2 and adjusted R^2 than both linear models. The results of this experiment, suggest that the quadratic model was better to explain the response of broiler chickens to dietary D_3. In agreement with previous literature reports, quadratic models are preferred to explain nutritional response data because they provide advantages over linear models. First, they have more flexibility and allow fitting a decreasing response usually associated with toxic levels of a nutrient (Pesti et al., 2009). Additionally, non-linear models tend to accurately describe the saturation response to a nutrient as a smooth curvilinear response rather than an abrupt saturation associated with linear models (Curnow, 1973; Coma et al., 1995; Robbins et al., 2006). In contrast, quadratic models are associated with a larger standard error compared with linear

broken-line models (Robbins et al., 2006), fitting a quadratic model requires a minimum of 3 points below the estimated requirement (Robbins et al., 2006) and usually estimate bigger requirements that those estimated by linear models (Coma et al., 1995).

Table 8 Vitamin D_3 requirement estimation and model comparison

Response1	Model[2]	ER[3]	AIC[4]	R^2	Adjusted R^2
TBA	One-slope straight broken line	491	154.90	0.172	0.007
	Two-slope straight broken line	532	156.70	0.175	0.003
	Quadratic broken line	497	153.20	0.201	0.028
TBS	One-slope straight broken line	574	175.60	0.315	0.167
	Two-slope straight broken line	658	193.20	0.273	0.116
	Quadratic broken line	798	174.80	0.326	0.180

[1]TBA, tibia bone ash (%); TBS, tibia breaking strength (kg force)
[2] The one-slope straight broken-line regression model is $y = L + U^* (R- x)$, where (R-x) is defined as zero when $x > R$. The two-slope straight broken-line regression model is defined as $y = L + U^* (R- x) + V^* (x-R)$, where $(R - x)$ is defined as zero when $x > R$, and $(x - R)$ is defined as zero when $x < R$. The derived parameters for both models are the breaking point x value (R), an asymptote for the first segment (L), and slope for the two line segments (U, V). The quadratic broken-line model is $y= L+ U^*(R-x)^2$.
[3] Estimated requirement, ER (IU D_3/kg of feed).
[4] Akaike information criterion (smaller values indicate better fit).

The results of the present experiment are in agreement with post-NRC (1994) literature reports suggesting higher dietary D_3 levels over the standard 200 IU/kg of feed are required to maximize growth and bone mineralization (Kasim and Edwards, 2000; Fritts and Waldroup, 2003; Whitehead et al., 2004; Rama Rao et al., 2006; Khan et al., 2010). However, we could expect an increment in the vitamin D_3 requirement due to the reduction of Ca and NPP in the diet of this experiment (Baker et al., 1998). Our laboratory supports the lower requirement suggested by

the NRC (1994) of 200 IU/kg of feed is still adequate for starting growing broilers. In multiple research projects conducted by our laboratory team using graded levels of dietary D_3, we have noticed that the studied variables maximize before or around the 200 IU/kg of feed. For this purpose future studies will re-evaluate the D_3 requirement of broiler chickens using strain and age adequate dietary Ca and NPP. Additionally, growth will be stimulated by increasing feed intake as a result of offering the feed as crumbles. Finally, estimated D_3 IU intake from d 10 to 21 will be adjusted to better reflect the expected feed intake (reduced 50 g from 900 g).

REFERENCES

Angel, R., W. W. Saylor, A. S. Dhandu, W. Powers, and T. J. Applegate. 2005. Effect of dietary phosphorus, phytase, and 25-hydroxycholecalciferol on performance of broiler chickens grown in floor pens. Poult. Sci. 84:1031-1044.

Applegate, T. J., and R. Angel .2014. Nutrient requirements of poultry publication: History and need for an update. J. Appl. Poult. Res. 23:567-575.

Baker, D. H., R. R. Biehl, and J. L. Emmert. 1998. Vitamin D_3 requirement of young chicks receiving diets varying in calcium and available phosphorus. Br. Poult. Sci. 39:413-417.

Cobb-Vantress. 2015. Performance and Nutrition Supplement, Cobb-500. Accessed http://www.cobb-vantress.com/docs/default-source/cobb-500 guides/Cobb500_Broiler_Performance_And_Nutrition_Supplement.pdf

Collins, E. D., and A. W. Norman .2001. Vitamin D in Handbook of vitamins. 3rd ed. Edited by Rucker R. B., J. W. Suttie, D. B. McCormick, and L. J. Machlin. Marcel Dekker, New York.

Coma, J., D. Carrion, and D. R. Zimmerman. 1995. Use of plasma urea nitrogen as a rapid response criterion to determine the lysine requirement of pigs. J. Anim. Sci. 73:472-481.

Coto, C., S. Cerate, Z. Wang, F. Yan, and P. W. Waldroup. 2010 a. Effect of source and level of maternal vitamin D on carryover to newly hatched chicks. Int. J. Poult. Sci. 9:613-622.

Coto, C., S. Cerate, Z. Wang, F. Yan, Y. Min, F. P. Costa, and P. W. Waldroup. 2010 b. Effect of source and level of vitamin D on the performance of breeder hens and the carryover to the progeny. Int. J. Poult. Sci. 9:623-633.

Curnow, R. N. 1973. A smooth population response curve based on an abrupt threshold and plateau model for individuals. Biometrics. 29:1–10.

Edwards, Jr., H. M., M. A. Elliot, S. Sooncharernying, and W. A. Britton. 1994 Quantitative requirement for cholecalciferol in the absence of ultraviolet light. Poult. Sci. 73:288-294.

Fowler, J., M. Hashim, A. Haq, O. Gutierrez, and C. A. Bailey. 2014. Comparing the bioactivity of 25-OHvitamin D_3 (Bio-D™) to cholecalciferol for starter broiler growth performance and bone mineralization. Poult. Sci. 93:10 (Abstr).

Fritts, C. A. and P. W. Waldroup. 2003. Effect of Source and level Vitamin D on live performance and bone development in growing broilers. J. Appl. Poult. Res. 12:45-52.

Havenstein, G. B., P. R. Ferket, and M. A. Qureshi .2003. Growth, livability, and feed conversion of 1957 versus 2001 broilers when fed representative 1957 and 2001 broiler diets. Poult. Sci. 82:1500-1508.

Jiang, S., Z. Jiang, K. Yang, F. Chen, C. Zheng, and L. Wang. 2015. Dietary vitamin D_3 requirement of Chinese yellow-feathered broilers. Poult. Sci. 94:2210-2220.

Kasim, A. and H.M., Jr. Edwards. 2000. Evaluation of cholecalciferol sources using broiler chick bioassays. Poult. Sci. 79:1617-1622.

Khan, S. H., R. Shahid, A. A. Mian, R. Sardar, and M. A. Anjum. 2010. Effect of the level of cholecalciferol supplementation of broiler diets on the performance and tibial dyschondroplasia. J. Anim. Physiol. Anim. Nutr. 94:584-593.

Leeson, S. and J. D. Summers. 2005. Commercial Poultry Nutrition. 3th ed. University Books. Ontario, Canada.

Leeson, S. 2007. Vitamin requirements: Is there a basis for re-evaluating dietary specifications? World's Poult. Sci. J. 63:255-266.

Leyva-Jimenez, H., Y. Jameel, M. N. Al-Ajeeli, A. M. Alsadwi, R. A. Abdaljaleel, and C. A. Bailey. 2018. Relative bioavailability determination of highly concentrated cholecalciferol (vitamin D_3) sources employing a broiler chick bioassay. J. Appl. Poult. Res. 0:1-8.

Moran, Jr. E. T. 2007. Nutrition of the developing embryo and hatchling. Poult. Sci. 86:1043-1049.

National Research Council .1994. Nutrients requirements of poultry. 9th rev. ed. Natl. Acad. Press, Washington, DC.

Pesti, G. M., D. Vedenov, J. Cason, and L. Billard. 2009. A comparison of methods to estimate nutritional requirements from experimental data. Br. Poult. Sci. 50:16–32.

Rama Rao, S. V., M. V. L. N. Raju, A. K. Panda, G. Shyam Sunder, and R. P. Sharma. 2006. Effect of high concentration of cholecalciferol on growth, bone mineralization and mineral retention in broiler chicks fed sub-optimal concentrations of calcium and non-phytate phosphorus. J. Appl. Poult. Res. 15:493-501.

Robbins, K. R. 1986. A method, SAS program, and example for fitting the broken-line model to growth data. Agric. Exp. Sta. Res. Rep. 86-09. Univ. of Tennessee, Knoxville.

Robbins, K. R., A. M. Saxton, and L. L. Southern. 2006. Estimation of nutrient requirements using broken-line regression analysis. J. Anim. Sci. 84(Suppl. 1):E155-E165.

Saunders-Blades, J. L., and D. R. Korver. 2014. The effect of maternal vitamin D source on broiler hatching egg quality, hatchability, and progeny bone mineral density and performance. J. Appl. Poult. Res. 23:773-783.

Schmidt, C. J., M. E. Persia, E. Feierstein, B. Kingham, and W.W. Saylor. 2009. Comparison of a modern broiler line and a heritage line unselected since the 1950s. Poult. Sci. 88:2610-2619.

Waldroup, P. W., C. B. Ammerman, and R. H. Harms. 1963. The relationship of phosphorus, calcium and vitamin D_3 in the diet of broiler-type chicks. Poult. Sci. 42:982.

Whitehead, C. C., H. A. McCormack, L. McTier, R. H. Fleming. 2004. High Vitamin D_3 requirements in broilers for bone quality and prevention of tibial dyschondroplasia and interactions with dietary calcium, available phosphorus and Vitamin A. Brit. Poult. Sci. 45: 425-436.

CHAPTER III

A RE-EVALUATION OF THE VITAMIN D_3 REQUIREMENT FOR GROWING BROILER CHICKENS USING AN ORAL VITAMIN D_3 INTAKE BIOASSAY

INTRODUCTION

Cholecalciferol is a fat-soluble vitamin (**D_3**) typically used in animal nutrition to prevent skeletal disorders due to its role in calcium and phosphorus homeostasis. In particular, commercial poultry feed formulations require D_3 to be included in the diet because endogenous synthesis of D_3 is very limited. Indoor enclosures used to grow poultry species usually do not have access to sunlight and therefore, the birds depend on dietary D_3 to have an optimal growth and development of their skeleton.

The last NRC update on the vitamin D_3 requirement for young meat-type chickens was established at 200 IU/kg of feed in 1994. Since then, very limited research has been done on the estimation of fat-soluble vitamins such as D_3 (Leeson, 2007; Świątkiewicz et al., 2017). Additionally, over the last 24 years, intensive genetic selection, flock management, precise nutrition and improvements in vaccine technology have led to the development of very efficient fast-growing modern poultry strains. Thus, it is logical to think that the NRC (1994) D_3 requirement might no longer be adequate to promote optimal performance and bone development of modern poultry strains. Moreover, post NRC (1994) literature reports (Kasim and Edwards, 2000; Fritts and Waldroup, 2003; Whitehead et al., 2004; Rama Rao et al., 2006; Khan et al., 2010) suggest that high concentrations of dietary D_3 (up to 20x the 1994 NRC) is necessary for optimal growth and prevention of skeletal disorders.

To address this problem, our research team tried to develop a protocol that would precisely estimate the vitamin D_3 requirement of young growing broilers and would adapt to the current ability of meat-type chickens to respond to dietary nutrients. This protocol focused on reducing feed mixing error, selective feeding, and other sources of error usually associated with typical methodologies of estimating nutrient requirements; as well as reducing the effect of maternal D_3 found in the egg yolk and increasing the precision of D_3 delivery to the chickens. The first approach to the protocol was covered in the previous chapter of this dissertation. The present study corresponds to a second trial where some adjustments were done to the first protocol in an attempt to continue with the development of a precision oral gavage bioassay to estimate the D_3 requirement of growing broiler chickens.

MATERIALS AND METHODS

All procedures in this experiment were approved by the Institutional Animal Care and Use Committee of Texas A&M University (AUP: IACUC 2017-0072).

Birds and General Management

A total of 300 Cobb-700 newly-hatched male broiler chickens were purchased from a commercial hatchery. At the time of arrival to the experimental facilities, birds were weighed in groups of 20 and an average body weight (**BW**) was calculated. The average BW was used to create 48 groups of five chickens (n=240) with close to "identical" starting body weight and variance. Broilers were then allocated to two stainless steel battery brooders (5 birds per cage) inside an environmentally controlled rearing room using a completely randomized block design. Battery pen level (6 levels) was used as the blocking factor. Fluorescent 48-inch tube lamps covered with red plastic shields, were used to provide 24-h constant light. The complete absence

of UV-light inside the rearing rooms has been previously verified (Fowler et al., 2015) by the Texas A&M Environmental Health and Safety Office using a short wave UV meter (J-225 Blak-Ray, UVP, LLC. Upland, CA) to prevent the endogenous synthesis of D_3. A basal D_3-deficient corn-soy broiler starter diet was fed *ad libitum* throughout a 21 d trial period. The first nine days of the study served to deplete the maternal stores of D_3 followed by a 12-h fasting period. From d 10 to the end of the trial, birds were orally gavaged with increasing levels of vitamin D_3. Oral D_3 treatments were offered for 11 d. Water was offered *ad libitum* during the whole trial using nipple drinkers. Birds were monitored daily with regard to general flock condition, temperature, lighting, water, feed, and any unanticipated events inside the rearing facility.

Dietary Treatments

A basal corn-soy broiler starter diet devoid of D_3 was formulated based on the nutrient recommendations of Cobb-700. A customized vitamin / mineral premix containing no D_3 and corn oil were used (Table 1) to formulate the basal D_3-deficient diet. The D_3-deficient basal diet was fed as crumbles throughout the trial period.

Feed intake (**FI**) reference data for meat-type chickens (Cobb-700 Broiler Performance and Nutrition Supplement, 2012) was used to estimate the increasing levels of vitamin D_3 per kg of feed consumed. Daily gavage treatments were based on estimated FI of 0, 50, 100, 200, 400, 800, 1,600 and 3,200 IU D_3/kg of feed consumed over the last 12 days of the study (estimated to be 850 g per bird). To create the experimental treatments, a total of 30 mg of the vitamin D_3 concentrate (Cholecalciferol, Ref: 1131009, Sigma-Aldrich, St. Louis, MO) were diluted in 120 mL of corn oil (expected to yield 10,000 IU/mL). An aliquot of 29.7 mL was diluted again in corn oil (570.3 mL) to yield a concentration of 494.6 IU D_3/mL corresponding to the highest treatment dose (≈3,200 IU/kg of feed) and then serial dilutions were performed to create the

other treatments so that a daily constant dose was contained in 0.5 mL. The D_3 solutions were separated in 11 daily doses (n=77) and then stored in a freezer at -20°C until required. Birds in the control group (0 IU) received 0.5 mL of corn oil without D_3 for 11 days. The oral gavage was performed per treatment group from lower to higher IU concentration using an 18-gauge gavage needle and a 1 mL syringe graduated at 1/100 mL. The gavage needle was flushed with plain corn oil between treatments to avoid cross-contamination. The gavage procedure was performed by a single operator who gained proficiency in the delivery of a specific oil volume after repeated training (CV $\bar{x}$ =0.94%).

Performance Evaluation

FI and BW per pen were recorded on d-10, after the fasting period, and d-21 of the trial to calculate weight gain (**WG**) and feed efficiency (**FE**). The BW of dead birds was recorded daily and used to adjust FE. FI data collected from d 10 to 21 were used to adjust the IU of D_3 administered through the oral gavage and expressed as IU of D_3 per kg of feed using the following equations:

1) TOGIU = S [IU/mL] * 0.5* d

Where: TOGIU = Total orally gavaged IU's

S = Solution (Corn oil + D_3) concentration (Obtained from serial dilutions)

d = number of days chickens were orally gavaged

2) AIUI= (1,000 * TOGIU) / FI

Where: AIUI = Adjusted IU intake (IU D_3/kg of feed)

FI = Feed intake (g / bird)

Table 9 Basal broiler starter diet devoid of vitamin D_3

Ingredient	Basal Diet [1] (%)
Yellow corn	62.1
Dehulled soybean meal (48% CP)	31.4
DL-Methionine	0.32
L-Lysine-HCl	0.27
L-Threonine	0.08
Corn oil	1.99
Limestone	1.31
Monocalcium phosphate	1.55
Sodium chloride (salt)	0.16
Sodium bicarbonate	0.27
Customized vitamin-mineral premix [2]	0.50

[1]Calculated nutritional content was as follow: 22% crude protein, 3050 kcal/kg metabolizable energy, 0.90% calcium, 0.45% non-phytate phosphorous, 0.65% methionine, 1.0% methionine+cystine, 1.36% lysine, 0.26% tryptophan, 0.89% threonine, 1.43% arginine, 3.7% crude fat, 2.13% crude fiber, 0.16% sodium , 0.91% potassium, 0.19% chloride.

[2]Vitamin-mineral premix added at this rate yields per kg of diet: 10 mg copper, 2 mg iodine, 20 mg iron, 125 mg manganese, 125 mg zinc, 0.2 mg selenium, 8,000 IU vitamin A, 40 IU vitamin E, 2 mg menadione, 4 mg thiamine, 8 mg riboflavin, 60 mg niacin, 15 mg pantothenic acid, 4 mg pyridoxine, 0.18 mg biotin, 2 mg folic acid, 0.02 mg vitamin B_{12}, 600 mg choline.

Bone Mineralization

On d 21 of the experiment, all birds per pen were euthanized using CO_2. Total bone mineral content (**BMC**) and bone mineral density (**BMD**) were obtained per pen using a Dual X-ray absorptiometry (**DXA**) scan (GE Lunar Prodigy Advance, GE-Healthcare, Boston, MA) located at the Applied Exercise Science Laboratory of Texas A&M University. Bone mineral content is defined as the total bone mineral found in a specific area measured in grams. Bone mineral density is derived using BMC (g) divided by an area (cm^2) of interest. Chickens were placed in prone position with their wings and legs at the sides of the body throughout the scan. Data were analyzed using the small animal software (GE Lunar Prodigy Advanced enCore, V 16.0, GE-Healthcare, Boston, MA). After the DXA scan both tibiae were removed, labeled and

stored in a freezer (-20°C) until further analysis. The right tibiae were gently boiled for 2-h in water and defatted in petroleum ether for 48-h. Defatted bones were then dried in a forced draft oven (95°C) until a constant weight was registered (≈48 h). Finally, the dried bones were ashed at 650°C for 23-h. Percent tibia bone ash (**TBA**) was calculated based on starting dry bone weight and remaining ash. The left tibiae were cleaned from any adhering tissue and used to assay breaking strength (**TBS**) using a texture analyzer (TA.XT Plus, Texture Technologies, Hamilton, MA,) charged with a 50-kg load cell, a crosshead speed of 100 mm/min with the tibia supported on a 3-point bending ring and a 3-cm constant span.

Statistical Analysis

Collected data were analyzed as one way-ANOVA where treatment and block were used as fixed factors in the model. Battery level was used as the blocking factor. Means were separated by Duncan's multiple range test when appropriate. Linear and quadratic effects of graded levels of D_3 were investigated by regression analysis. The 0 IU group was included as common control to investigate linear and quadratic effects. Three different regression models were used to calculate the D_3 requirement. The one-slope straight broken-line regression model consists of a line with an increasing or decreasing slope and a horizontal line. The intersection between the two lines is the breaking point. The one-slope broken-line model is as follows: $y = L + U^* (R-x)$, where (R-x) is defined as zero when $x > R$. The two-slope straight broken-line regression model is defined as $y = L + U^*(R-x) + V^*(x-R)$, where (R-x) is defined as zero when $x > R$, and (x -R) is defined as zero when $x < R$. In the two-slope model, both intersecting lines have non-zero slopes. The derived parameters for both models are the breaking point x value (R), an asymptote for the first segment (L), and slope for the two line segments (U, V). The point of intersection or breaking-point between the two lines was defined as the estimated D_3

requirement. Finally, the quadratic broke-line model is described as $y= L + U*(R-x)^2$. The concentration at which the response reached 95% of the minimum response was estimated as the D_3 requirement (Robbins, 1986; Robbins et al., 2006; Jiang et al., 2015). One-way ANOVA analysis and linear regression (linear and quadratic effects) were performed by IBM SPSS software (SPSS Version 25.0, SPSS Inc., Chicago, IL) and the SAS software (SAS Version 9.4, SAS Institute, Cary, NC) was employed for the estimation of the D_3 requirement using the NLMIXED procedure. The Akaike Information Criterion (**AIC;** smaller values indicate better fit), coefficient of determination ($\mathbf{R^2}$) and adjusted R^2 where used to assess the goodness of fit for the three models. The pen average was the experimental unit for performance and bone mineralization responses. Significant effects were accepted at $P<0.05$ for all analyses.

RESULTS AND DISCUSSION

Performance

Performance results are presented in table 10. No differences ($P>0.05$) were observed in BW of broiler chickens orally gavaged with D_3. The D_3 treatments positively improved ($P<0.05$) WG and FE. There was no linear or quadratic effect with increasing concentration of D_3 for any of the performance variables. However, WG linear effect approached significance ($P=0.059$) and FE quadratic response approached significance as well ($P=0.057$).

In this experiment, performance was more consistent with increasing levels of D_3 when compared with the results of the first part of this research project. An estimation of a vitamin D_3 requirement through multiple range test was possible by looking at the Duncan's means separation. Approximately, 200-400 IU/kg of feed were required to maximize BW and WG and 100-200 IU/kg of feed were required to maximize FE. Although, better results were observed in

this experiment when compared to the first trial, it appears that similarly, a vitamin D deficiency was not effectively achieved. Again, high maternal stores of D_3 in the yolk reduced the sensitivity of our experiment and also they increased the variability in the response to graded levels of D_3 (Moran, 2007; Coto et al., 2010a; Coto et al., 2010b; Saunders-Blades and Korver, 2014). Another factor that could possibly have affected our results in both experiments is the lighting system, which consisted on fluorescent tubes covered with red plastic shields with the objective of reducing the possibility of endogenous synthesis of D_3. However, lights were kept at normal intensity, and thus it was possible that some light could have potentially reached the skin of the chickens and endogenous synthesis of D_3 was slightly induced. Edwards et al. (1994) reported that chickens under fluorescent light with no protection influenced broiler response to dietary D_3 and deficiency was not successfully achieved. To investigate this further, future research projects should consider both protection against UV-light and intensity.

For the present study (second trial) FI was adjusted to better reflect the expected dietary concentrations of D_3. This was achieved by lowering the expected ≈900 g of feed consumed over the last 12 d of the study to 850 g. Additionally, in this experiment the basal diet was offered as a crumble and not in a mash form as in the first experiment. Results of FI (Table 10) suggest that we effectively corrected the discrepancy between expected feed consumption and actual feed consumption allowing to better represent the calculated dietary concentrations of D_3. Moreover, BW of birds used in the second experiment was closer ($\bar{x}$ 85 g difference) to the expected age and strain BW (Cobb-700 Broiler Performance and Nutrition Supplement, 2012). Compared to the first experiment where FI was lower, in average there was a 240 g difference (Cobb-500 broiler Performance and Nutrition Supplement, 2015) with expected BW. Forcing the birds to

reach their genetic potential for growth may have increased the sensitivity of the assay but not enough to reduce the effects of maternal D_3 stores.

Table 10 Effect of dietary vitamin D_3 on broiler performance

	Response[3,4]			
IU D_3/kg feed [1,2]	21 d	10-21 d	10-21 d	10-21 d
	BW	WG	FE	FI
0	823±13	605±10$_{ab}$	0.73±0.010^{b}	818±11
50 (53)	805±17	591±15$_{b}$	0.73±0.010^{b}	806±10
100 (104)	830±9	618±8^{ab}	0.75±0.003^{a}	815±8
200 (207)	843±10	630±7^{a}	0.76±0.005^{a}	824±15
400 (412)	844±21	625±15^{a}	0.76±0.005^{a}	827±19
800 (822)	841±13	628±11^{a}	0.76±0.005^{a}	828±14
1600 (1690)	823±13	617±9^{ab}	0.75±0.006^{a}	805±8
3200 (3202)	851±11	636±9^{a}	0.74±0.005^{ab}	850±12
P value				
Linear	0.1762	0.059	0.746	0.066
Quadratic	0.995	0.578	0.057	0.258

[a-b] Means within the same column without a common superscript differ ($P<0.05$) Duncan's multiple range test. Means ± SEM.

[1] Calculated IU D_3/kg feed (Adjusted IU D_3/kg feed based on feed intake data).

[2] Serial dilutions using the stock solution were performed to create dietary D_3 treatments so that a daily constant dose was contained in 0.5 mL D_3 was administered to the chickens through a daily oral gavage.

[3] BW, Body weight (g/bird); WG, Weight gain (g/bird); FE, Mortality corrected feed efficiency (g weight gain / g feed intake), FI, feed intake (g).

[4] Values for performance responses represent the mean average of n=6 replicate pens per treatment of 5 birds each at respective age.

Bone Mineralization

Differences (P<0.05) in TBA and TBS were observed between treatment groups and the 0 IU treatment group. Both TBA and TBS were linearly increased in response to dietary levels of D_3. A quadratic response to increasing dietary levels of D_3 was observed for TBS only.

No differences (P>0.05) were detected for BMD and BMC although BMC had a linear response to dietary D_3 levels (Table 11). Overall, BMD and BMC were not good indicators of vitamin D_3 activity in growing broilers in both experiments. Multiple range test suggest 200 IU/kg of feed were needed to maximize TBA and 400- IU/kg were required to maximize TBS.

Table 11 Effect of dietary vitamin D_3 on broiler bone mineralization

IU D_3/kg feed[1,2]	Response[3,4] 0-21 d			
	TBA	TBS	BMD	BMC
0	48.9±0.4[d]	16.9±0.6[b]	0.074±0.003	4.65±0.21
50 (53)	49.9±0.5[bcd]	17.7±0.6[b]	0.087±0.006	5.42±0.49
100 (104)	49.3±0.4[cd]	19.8±0.4[a]	0.079±0.003	5.18±0.25
200 (207)	50.7±0.4[abc]	19.4±0.7[a]	0.078±0.004	5.16±0.21
400 (412)	50.6±0.5[abc]	20.4±0.3[a]	0.082±0.003	5.58±0.22
800 (822)	50.8±0.3[ab]	20.3±0.4[a]	0.082±0.003	5.54±0.15
1600 (1690)	50.4±0.4[abc]	20.7±0.9[a]	0.084±0.003	5.51±0.30
3200 (3202)	51.3±0.5[a]	21.0±0.6[a]	0.086±0.003	6.00±0.08
P value				
Linear	0.003	<0.001	0.142	0.017
Quadratic	0.229	0.016	0.697	0.642

[a-d] Means within the same column without a common superscript differ (P<0.05) Duncan's multiple range test. Means ± SEM.

[1] Calculated IU D_3/kg feed (Adjusted IU D_3/kg feed based on feed intake data).

[2] Serial dilutions using the stock solution were performed to create dietary D_3 treatments so that a daily constant dose was contained in 0.5 mL D_3 was administered to the chickens through a daily oral gavage.

[3] TBA, tibia bone ash (%); TBS, tibia breaking strength (kg force); BMD, bone mineral density (g/cm^2); BMC, bone mineral content (g).

[4] Values for bone mineralization responses represent the mean average of n=6 replicate pens per treatment of 5 birds each at respective age.

In average, a reduction of 1.5 % in TBA was obtained for this experiment. Between the lowest level (0 IU/kg) and the highest (3,202 IU/kg) a 2.4% reduction was achieved. Although, bone mineralization showed consistent responses to graded dietary levels of D_3, the low reduction in TBA supports the results of the first experiment regarding the effect of high maternal stores of D_3 in the egg yolk.

Requirement Estimation Using Regression Models

Using three different regression models when possible, the present study determined different D_3 requirements of chickens depending on the variable used as estimation criteria. WG and FE performance responses were able to fit regression models in contrast to the previous experiment. The vitamin D_3 requirement, using the one-slope regression model, for maximum WG and FE was calculated at 185 and 116 IU/kg of feed respectively. Two-slope model was not able to fit WG but FE resulted in an estimated D_3 requirement of 135 IU/kg. The quadratic model yielded 253 and 201 IU /kg for maximum WG and FE respectively. It is necessary to note that R^2 and adjusted R^2 were relatively low compared to bone mineralization responses (Table 12) which reported nutritional requirements using the one-slope model of 231 (TBA) and 251 (TBS) IU/kg of feed. The two-slope model estimated the D_3 requirement at 466 and 111 for TBA and TBS respectively. Finally, the quadratic model reported in agreement with the first experiment higher requirements than the linear models. TBA was found to be maximized at 350 IU/kg and TBS was maximized at 300 IU/kg.

Compared to the requirements calculated in the first experiment, this study generally supports the NRC (1994) D_3 requirement of 200 IU /kg of feed. A reduction in the D_3 requirement was expected as Ca and non-phytate phosphorus (**NPP**) were not reduced in the diet offered to the chickens in this experiment. In agreement with Baker et al. (1998) when broiler

chickens are fed adequate amounts of Ca and NPP, the D_3 requirement will be around the 200 IU/kg of feed between 1 and 21 days post-hatch. However, changes in NPP in the diet can increase the D_3 requirement up to 1,500 IU/kg (Baker et al., 1998). Similarly, Rama Rao et al. (2006) concluded that performance and bone mineralization in broilers could be maintained with suboptimal concentration of Ca and NPP (0.5% and 0.25% respectively) with high dietary concentrations of D_3 (3,600 IU/kg) in the diet.

Table 12 Estimated vitamin D_3 requirement and model comparison

Response[1]	Model[2]	ER[3]	AIC[4]	R^2	Adjusted R^2
WG	One-slope straight broken line	185	455.40	0.158	-0.015
	Quadratic broken line	253	456.00	0.148	-0.027
FE	One-slope straight broken line	116	-247.5	0.212	0.050
	Two-slope straight broken line	135	-247.4	0.243	0.087
	Quadratic broken line	201	-246.7	0.199	0.034
TBA	One-slope straight broken line	231	143.10	0.317	0.177
	Two-slope straight broken line	466	147.20	0.286	0.140
	Quadratic broken line	350	143.40	0.313	0.172
TBS	One-slope straight broken line	251	178.80	0.438	0.322
	Two-slope straight broken line	111	176.60	0.486	0.380
	Quadratic broken line	300	177.40	0.454	0.342

[1] WG, weight gain (g); FE, feed efficiency (g/g); TBA, tibia bone ash (%); TBS, tibia breaking strength (kg force)
[2] The one-slope straight broken-line regression model is $y = L + U^* (R- x)$, where (R-x) is defined as zero when $x > R$. The two-slope straight broken-line regression model is defined as $y = L + U^* (R- x) + V^* (x-R)$, where $(R - x)$ is defined as zero when $x > R$, and $(x - R)$ is defined as zero when $x < R$. The derived parameters for both models are the breaking point x value (R), an asymptote for the first segment (L), and slope for the two line segments (U, V). The quadratic broken-line model is $y= L+ U^*(R-x)^2$.
[3] Estimated requirement, ER (IU D3/ kg of feed).
[4] Akaike information criterion (smaller values indicate better fit).

In contrast, Kasim and Edwards (2000) estimated the D_3 requirement for growing broiler chickens ranging from 800-1,100 IU/kg of feed to maximize final BW, feed conversion ratio, plasma Ca and bone ash in broiler chickens fed adequate amounts of C and NPP. Whitehead et al. (2004) reported that the D_3 requirement of growing broilers up to 14 days of age at sufficient dietary Ca and available P may be in the range of 1,400-2,000 IU/kg of feed based on maximum cortical bone quality and 10,000 IU/kg of feed required for prevention of tibial dyschondroplasia.

There is a considerable variation in the published D_3 requirement for broiler chickens. Whether some literature reports base their estimation of the D_3 requirement on multiple range test only and other use very complex regression models, the resulting D_3 requirement appear to be very different. The development of this protocol had the objective of reducing the errors associated with classical methodologies to estimate nutritional requirements. From our perspective, the present protocol allows an estimation of the vitamin D_3 requirement. However, it is highly influenced by the maternal reserves of D_3 found in the egg yolk. If a D_3 deficiency is not observed the sensitivity of the assay will be highly reduced. Therefore, our future efforts will be placed on developing new protocols to obtain broiler chickens with a more homogenous vitamin D background and estimate the vitamin D_3 requirement with a minimum carryover effect from the breeder flock. A good approach could be obtaining a breeder flock and depleting the birds from D_3 for a period of time, expecting the resulting progeny will have a more homogenous and lower D_3 reserve in the egg yolk that birds obtained directly from a commercial hatchery.

REFERENCES

Baker, D. H., R. R. Biehl, and J. L. Emmert. 1998. Vitamin D3 requirement of young chicks receiving diets varying in calcium and available phosphorus. Br. Poult. Sci. 39:413-417.

Cobb-Vantress. 2012. Performance and Nutrition Supplement, Cobb-700. Accessed http://www.cobb-vantress.com/.

Coto, C., S. Cerate, Z. Wang, F. Yan, and P. W. Waldroup. 2010 a. Effect of source and level of maternal vitamin D on carryover to newly hatched chicks. Int. J. Poult. Sci. 9:613-622.

Coto, C., S. Cerate, Z. Wang, F. Yan, Y. Min, F. P. Costa, and P. W. Waldroup. 2010 b. Effect of source and level of vitamin D on the performance of breeder hens and the carryover to the progeny. Int. J. Poult. Sci. 9:623-633.

Edwards, Jr., H. M., M. A. Elliot, S. Sooncharernying, and W. A. Britton. 1994 Quantitative requirement for cholecalciferol in the absence of ultraviolet light. Poult. Sci. 73:288-294.

Fowler, J., M. Hashim, A. Haq, O. Gutierrez, and C. A. Bailey. 2014. Comparing the bioactivity of 25-OHvitamin D_3 (Bio-D™) to cholecalciferol for starter broiler growth performance and bone mineralization. Poult. Sci. 93:10 (Abstr).

Fritts, C. A. and P. W. Waldroup. 2003. Effect of Source and level Vitamin D on live performance and bone development in growing broilers. J. Appl. Poult. Res. 12:45-52.

Jiang, S., Z. Jiang, K. Yang, F. Chen, C. Zheng, and L. Wang. 2015. Dietary vitamin D_3 requirement of Chinese yellow-feathered broilers. Poult. Sci. 94:2210-2220.

Kasim, A. and H. M., Jr. Edwards. 2000. Evaluation of cholecalciferol sources using broiler chick bioassays. Poult. Sci. 79:1617-1622.

Khan, S. H., R. Shahid, A. A. Mian, R. Sardar, and M. A. Anjum. 2010. Effect of the level of cholecalciferol supplementation of broiler diets on the performance and tibial dyschondroplasia. J. Anim. Physiol. Anim. Nutr. 94:584-593.

Leeson, S. and J. D. Summers. 2005. Commercial Poultry Nutrition. 3th ed. University Books. Ontario, Canada.

Leeson, S. 2007. Vitamin requirements: Is there a basis for re-evaluating dietary specifications? World's Poult. Sci. J. 63:255-266.

Moran, Jr. E. T. 2007. Nutrition of the developing embryo and hatchling. Poult. Sci. 86:1043-1049.

National Research Council .1994. Nutrients requirements of poultry. 9th rev. ed. Natl. Acad. Press, Washington, DC.

Pesti, G. M., D. Vedenov, J. Cason, and L. Billard. 2009. A comparison of methods to estimate nutritional requirements from experimental data. Br. Poult. Sci. 50:16–32.

Rama Rao, S. V., M. V. L. N. Raju, A. K. Panda, G. Shyam Sunder, and R. P. Sharma. 2006. Effect of high concentration of cholecalciferol on growth, bone mineralization and mineral retention in broiler chicks fed sub-optimal concentrations of calcium and non-phytate phosphorus. J. Appl. Poult. Res. 15:493-501.

Robbins, K. R. 1986. A method, SAS program, and example for fitting the broken-line model to growth data. Agric. Exp. Sta. Res. Rep. 86-09. Univ. of Tennessee, Knoxville.

Robbins, K. R., A. M. Saxton, and L. L. Southern. 2006. Estimation of nutrient requirements using broken-line regression analysis. J. Anim. Sci. 84(Suppl. 1):E155-E165.

Saunders-Blades, J. L., and D. R. Korver. 2014. The effect of maternal vitamin D source on broiler hatching egg quality, hatchability, and progeny bone mineral density and performance. J. Appl. Poult. Res. 23:773-783.

Świątkiewicz, S., A. Arczewska-Wlosek, D. Bederska-Lojewska, and D. JÓzefiak. 2017. Efficacy of dietary vitamin D and its metabolites in poultry - review and implications of the recent studies. World's Poult. Sci. J. 73:57-68

Whitehead, C. C., H. A. McCormack, L. McTier, R. H. Fleming. 2004. High Vitamin D_3 requirements in broilers for bone quality and prevention of tibial dyschondroplasia and interactions with dietary calcium, available phosphorus and Vitamin A. Brit. Poult. Sci. 45: 425-436.

CHAPTER IV

EVALUATION OF DIETARY CHOLECALCIFEROL AND 25-HYDROXYCHOLECALCIFEROL ON BROILER CHICKENS SUBJECTED TO A COCCIDIOSIS VACCINE CHALLENGE

INTRODUCTION

Traditionally, cholecalciferol or vitamin D_3 (**D_3**) has been supplemented to poultry feeds as a strategy to prevent skeletal disorders in commercial meat-type poultry. Active participation in P and Ca homeostasis confers vitamin D_3 a potent antirachitic activity (Collins and Norman, 2001). However, vitamin D_3 is not considered biologically active until it undergoes a series of hydroxylation reactions in the body. First, D_3 obtained from the diet or synthesized in the skin, after UV-irradiation, is transported to the liver where it is converted into 25-hydroxycholecalciferol (**25-OH-D_3**), the major circulating metabolite of D_3. Newly synthesized 25-OH-D_3 is then transported to the kidneys where it undergoes a second hydroxylation and it is converted into 1,25-dihydroxycholecalciferol, the fully active form of D_3 (Leeson and Summers, 2001).

There are literature reports suggesting that dietary 25-OH-D_3 in replacement or in addition to D_3 is effective in promoting performance, enhancing bone mineralization, reducing incidence of tibial dyschondroplasia (Whitehead, 1995; Yarger et al., 1995; Fritts and Waldroup, 2003; Ledwaba and Roberson, 2003; Atencio et al., 2005; Han et al., 2016), improving intestinal morphology (Chou et al., 2009), and regulating avian immunity (Gómez-Verduzco et al., 2013; Morris et al., 2014; Morris et al., 2015). Thus, the use of commercially available D_3 metabolites such as 25-OH-D_3 in poultry diets has increased over the last few years.

Coccidiosis in poultry is a protozoal infection caused by coccidia from the genus *Eimeria* and it is characterized by the destruction of the intestinal mucosa, hemorrhages, dehydration, and reduced performance (Merck Veterinary Manual, 2010; Peek and Landman, 2011). Coccidiosis is one of the most economically important diseases affecting the commercial poultry industry. Just for the broiler industry, around $3 billion USD are spent worldwide every year in the prevention of coccidiosis (Anon., 2013). Live anticoccidial vaccines are widely used to prevent and control coccidiosis in commercial poultry facilities (De Gussem, 2007). Current delivery strategies of live vaccines consist on oocysts suspensions that are directly sprayed into the feed or directly sprayed into newly-hatched chicks allowing them to ingest the oocysts by preening themselves and each other (Dalloul and Lillehoj, 2005). The use of live vaccines is associated with the development of sub-clinical infection and therefore, potential reduction in performance (Peek and Landman, 2011; Ahmad et al., 2016). Recent studies done in our laboratory suggested that the stress caused by the live cocci vaccine during the first weeks of life could be reduced with supplemental prebiotic feed additives (Al-Ajeeli et al., 2017). Due to the known effects of D_3 on performance, bone mineralization, and avian immunity, a similar approach may be taken using D_3 and 25-OH-D_3 at high dietary levels, usually used in the commercial poultry industry, to compare their effectiveness in ameliorating the negative effects of live coccidial vaccines. Hence, the present study was conducted with the objective of comparing the effect of high dietary levels of D_3 and partial replacement of D_3 with 25-OH-D_3 on performance, bone mineralization, intestinal integrity, vitamin D status, and plasma cytokine levels of broiler chickens after a live coccidiosis vaccine challenge.

MATERIALS AND METHODS

All procedures in this experiment were approved by the Institutional Animal Care and Use Committee of Texas A&M University (AUP: IACUC 2017-0072).

Birds and General Management

A total of 300 Cobb-700 1-d-old male broiler chickens were procured from a commercial hatchery. At the time of arrival to the experimental facilities, the birds were weighed in groups of 20 and an average body weight (**BW**) was calculated. The average BW was used to create 45 groups of six chickens (n=288) with close to "identical" starting body weight and variance. Broilers were then allocated to two stainless steel battery brooders (6 birds per cage and 5 pen replicates per treatment) inside an environmentally controlled rearing room using a completely randomized block design. Battery pen level (5 levels) was used as the blocking factor. Fluorescent 48-inch tube lamps covered with red plastic shields, were used to provide 24-h constant light. The complete absence of UV-light inside the rearing rooms has been previously verified (Fowler et al., 2014) by the Texas A&M Environmental Health and Safety Office using a short wave UV meter (J-225 Blak-Ray, UVP, LLC. Upland, CA). Treatment diets were offered *ad libitum* throughout the 21-d trial period. Water was offered *ad libitum* using nipple drinkers. Birds were monitored daily with regard to general flock condition, temperature, lighting, water, feed, and any unanticipated events inside the rearing facility.

Dietary Treatments

A basal D_3-deficient corn-soy broiler starter diet was formulated using a custom made vitamin / mineral premix containing no D_3 and corn oil as the fat source (Table 13). To increase the sensitivity of our response variables to the supplemental D_3 and 25-OH-D_3 products, the basal diet was formulated to yield 0.75% Ca and 0.37% non-phytate phosphorus (**NPP**).

Table 13 Basal vitamin D_3 deficient diet

Ingredient	Basal Diet [1] (%)
Yellow corn	63.07
Dehulled soybean meal	31.46
DL-Methionine	0.29
L-Lysine-HCl	0.21
L-Threonine	0.05
Corn oil	1.67
Limestone	1.07
Monocalcium phosphate	1.19
Sodium chloride (salt)	0.44
Sodium bicarbonate	0.03
Customized vitamin-mineral premix [2]	0.50

[1]Calculated nutritional content was as follow: 22% crude protein, 3,050 kcal/kg metabolizable energy, 0.75% calcium, 0.37% non-phytate phosphorous, 0.61% methionine, 0.98% methionine+cystine, 1.32% lysine, 0.26% tryptophan, 0.86% threonine, 1.44% arginine, 3.57% crude fat, 2.14% crude fiber, 0.2% sodium , 0.91% potassium, 0.35% chloride.

[2]Vitamin-mineral premix added at this rate yields per kg of diet: 10 mg copper, 2 mg iodine, 20 mg iron, 125 mg manganese, 125 mg zinc, 0.2 mg selenium, 8,000 IU vitamin A, 40 IU vitamin E, 2 mg menadione, 4 mg thiamine, 8 mg riboflavin, 60 mg niacin, 15 mg pantothenic acid, 4 mg pyridoxine, 0.18 mg biotin, 2 mg folic acid, 0.02 mg vitamin B_{12}, 600 mg choline.

The basal diet was then subdivided into nine equally sized batches to create the experimental treatments. Treatments consisted of the basal diet plus the supplementation of a commercially available D_3 source (D3-30 [30,000 IU D_3/g or 750 μg D_3/g], Animal Science Products Inc., Nacogdoches, TX) at 200 (control diet; **CON**), 2,000 , 4,000, 6,000 and 8,000 IU D_3 per kg of feed (5, 50, 100, 150, 200 μg D_3/kg of feed) or the basal diet supplemented with the same D_3 source at 1,000, 2,000, 3,000 and 4,000 IU D_3 per kg of feed (25, 50, 75, 100 μg D_3/kg of feed) in combination with 25, 50, 75 and 100 μg 25-OH-D_3 (Bio-D, Huvepharma Inc., Peachtree, GA) per kg of feed (Table 14) respectively, and calculated to be equivalent to 1,000, 2,000, 3,000 and 4,000 IU of D_3 activity based on the conversion of 0.025 μg of cholecalciferol

into one IU (NRC, 1994). All treatment diets were offered *ad libitum* as crumbles throughout the 21 d trial period.

Coccidia Vaccine Challenge

On d 7 of the study, all birds were orally gavaged with a commercially available live coccidiosis vaccine (ADVENT-Vaccine, Huvepharma Inc., Peachtree, GA) containing a mixture of viable sporulated oocysts (*E. acervulina*, *E. maxima* and *E. tenella*) at 7x recommended dose. Oocysts were delivered using a 20-gauge stainless steel gavage needle and a 3 mL syringe. A total of 0.5 mL containing the oocysts where delivered directly into the crop to induce coccidial infection.

Performance Evaluation

Feed intake (**FI**) and BW per pen were recorded on d 7, 14 and 21 to calculate weight gain (**WG**), and feed conversion ratio (**FCR**). Mortality and body weight of dead birds were recorded daily and used to adjust FCR.

Vitamin D Status and Plasma Cytokine Levels

Blood samples were drawn from one bird per pen at d 14 (7-d post-challenge). Blood samples were collected within Vacutainer EDTA tubes (BD, Franklin Lakes, NJ) and centrifuged for 15 min at 1000 x *g* and 5°C immediately after collection. Two plasma aliquots were collected within 1 mL micro centrifuge tubes. After processing, the plasma samples were immediately stored at -20 °C until further analysis. Vitamin D status (**VDS**) was determined by total plasma 25-OH-D_3 concentration using a commercially available ELISA kit (25-OH-Vitamin D Kit Eagle Biosciences, Nashua, NH, Ref:VID91-K01; Assay range 9-150 ng/mL). Plasma samples were run in duplicate and results were expressed as nanogram per mL.

Table 14 Experimental treatment details

TRT	µg D_3/kg feed	25-OH-D_3 µg /kg feed*	Analyzed D_3 µg / kg of feed**	Analyzed 25-OH-D_3 µg /kg feed
TI (Control)[1]	5		12	<5
T2	50		68	<5
T3	100		64	<5
T4	150		97	<5
T5	200		138	<5
T6	25	25	52	19.6
T7	50	50	50	37.4
T8	75	75	66	55.0
T9	100	100	90	81.9

[1] NRC (1994) Cholecalciferol requirement for starting meat-type chicken (200 IU/kg feed or 5 µg/kg of feed)
*Bioactivity of all products is based on 1:1 conversion of 1 IU D_3= 0.025µg cholecalciferol
** Experimental treatments were chemically analyzed at Heartland Assays Laboratories (2711 s. Loop Dr. Suite 4400 Ames, Iowa 50010) via liquid chromatography mass-spectrometry (LC/MS/MS).

Plasma cytokine levels were determined using commercially available ELISA kits (Novatein Biosciences, Inc., Woburn, MA) for chicken specific IFNγ (Ref:NB-E60048; Assay range 62.5-1,000 pg/mL), IL-1β (Ref:NB-E60024; Assay range 20-640 pg/mL), IL-6 (Ref:NB-E60049; Assay range 15.6-1,000 pg/mL) and IL-10 (Ref:NB-E60092; Assay range 15.6-1,000 pg/mL) cytokines. Plasma samples were run in triplicate and results were expressed as picograms per mL.

Intestinal Morphology

At d 21 (14-d post-challenge) one bird per pen was randomly selected and euthanized using CO_2. Approximately 3 cm sections of duodenum, jejunum and ileum were obtained and gently flushed with cold autoclaved PBS and fixed in 10% neutral buffered formalin. The

duodenum samples were taken at the middle point between the gizzard and the duodenal loop. The jejunum samples were taken 5 cm proximal to the Meckel's diverticulum. The ileum samples were taken 5 cm proximal to the ileo-cecal junction. Intestinal samples were then embedded using standard paraffin wax procedures and hematoxylin-eosin stain. Villi height and crypt depth were measured from five well-oriented villi and villus-associated crypts for each sample. Villus height (**VH**) was measured from the tip of the villus to the villus-crypt junction. Crypt depth (**CD**) was defined as the depth of the invagination between adjacent villi (Feng et al., 2007). Both measurements were used to estimate villi height over crypt depth ratio (**VH:CD**). Histology slides were scanned using Leica DFC550 camera and Leica DM2500 LED optical microscope (Leica Microsystems, Wetzlar, Germany). Image processing and analysis was performed using the LAS software (Version 4.0, Leica Microsystems, Wetzlar, Germany).

Lesion Scores and Relative Organ Weight

At d 21 (14-d post-challenge) of the trial, four birds per pen were weighed and euthanized using CO_2 to score lesions in the upper (duodenum), middle (jejunum) and lower (ileum) small intestine and ceca. Lesions were scored from 0-4 as described by Johnson and Reid (1970). Where 0 score represents no gross lesions and 4 indicates severe damage to the intestine. Intestinal lesion scores were performed by Dr. Michael H. Kogut (USDA-ARS, College Station, TX) who was blinded to the experimental treatments. Lesion scores were analyzed together and adjusted per specific area of intestine (Perez-Carbajal, 2010).

To calculate relative organ weight, the liver, bursa of Fabricius, and spleen were removed from the same four birds. Pen average relative organ weights were calculated as percentage of average body weight per pen (g/100 g body weight).

Bone Mineralization

At d 21 (14-d post-challenge) after birds were sampled for intestinal and organ measurements (n=5) both tibias were removed. The right tibiae were gently boiled in water for 2-h and defatted in petroleum ether for 48-h. Defatted bones were then dried in a forced draft oven (95°C) until a constant weight was registered (≈48-h). Finally, the dried bones were ashed at 650°C for 23-h. Percent tibia ash (**TBA**) was calculated based on starting dry bone weight and remaining ash. The left tibiae were cleaned from any adhering tissue and used to assay breaking strength (**TBS**) using a texture analyzer (TA.XT Plus, Texture Technologies, Hamilton, MA,) charged with a 50-kg load cell, a crosshead speed of 100 mm/min with the tibia supported on a 3-point bending ring and a 3-cm constant span.

Statistical Analysis

Collected data were analyzed by one way-ANOVA where treatment and block were used as fixed factors in the model. Battery level (five levels) was used as the blocking factor. Lesion score and plasma cytokine data were analyzed using the Kruskal-Wallis test as the data were not normally distributed. Means were separated by Duncan's multiple range test when appropriate. Linear and quadratic effects of graded levels of D_3 and D_3 plus 25-OH-D_3 treatments were investigated by regression analysis. The 200 IU control group was included as common control for both D_3 alone and D_3 plus 25-OH-D_3 groups to investigate linear and quadratic effects. Additionally, orthogonal contrasts were employed to investigate particular effects of interest. The pen average was the experimental unit for performance, bone mineralization, lesion scoring and relative organ weight responses. Individual birds were the experimental unit for vitamin D status, plasma cytokine level and intestinal morphology responses. Statistical analyses were

performed using IBM SPSS software (SPSS Version 25.0, SPSS Inc., Chicago, IL) and significant differences were accepted at $P<0.05$ for all analyses.

RESULTS AND DISCUSSION

Performance

Performance results are presented in table 15. The BW and WG of 7-d broiler chickens was not different ($P>0.05$) across the D_3 and D_3+25-OH-D_3 dietary treatments. However, FCR of birds consuming dietary D_3 or D_3+25-OH-D_3 was improved when compared with the CON group (5 μg D_3/ kg). A quadratic response was observed in FCR of broiler chickens being fed increasing dietary levels of D_3. The BW, WG and FCR of chickens linearly improved with increasing dietary levels of D_3+25-OH-D_3 after 7 d. Similar results were observed after 14 d of the trial. The WG of broiler chickens consuming the CON diet was significantly lower than all other D_3 and D_3+25-OH-D_3 dietary treatments. After 14-d on treatment diets, linear responses were found for FCR of chickens fed D_3 alone, and BW and WG of chickens offered the D_3+25-OH-D_3 combination treatments. Quadratic responses were observed for WG of chickens fed D_3 alone and the FCR of chickens fed the combined D_3+25-OH-D_3 dietary treatments. Finally, at the end of the trial (21-d) differences ($P<0.05$) were observed in all performance variables. The BW, WG and FCR of broilers were improved compared to the CON group. The WG and FCR of 21-d chickens were linearly improved with increasing dietary levels of D_3 while the D_3+25-OH-D_3 dietary treatments had a quadratic response for the same response variables. The BW of 21-d chickens had a linear and quadratic response to graded dietary levels of D_3+25-OH-D_3.

Table 15 Effect of dietary vitamin D_3 and 25-hydroxycholecalciferol on performance of broiler chickens

Vit. D_3 (µg/kg)	25-OH-D_3[1] (µg/kg)		Response[2,3]								
			7d BW	0-7d WG	0-7 d FCR	14 d BW	7-14 d WG	7-14 d FCR	21 BW	14-21 d WG	14-21 d FCR
5 (Control)		T1	160±3	114±3	1.26±0.03^{a}	440±7	280±5^{b}	1.32±0.01^{a}	858±20^{b}	418±14^{b}	1.44±0.01^{a}
50		T2	172±4	127±4	1.16±0.01^{b}	476±9	304±5^{a}	1.28±0.01^{b}	956±9^{a}	479±8^{a}	1.35±0.01^{b}
100		T3	166±6	121±6	1.16±0.01^{b}	466±10	300±5^{a}	1.28±0.01^{b}	924±21^{a}	458±13^{a}	1.38±0.01^{b}
150		T4	165±3	119±3	1.20±0.01^{b}	464±8	299±6^{a}	1.28±0.01^{b}	927±11^{a}	463±6^{a}	1.35±0.01^{b}
200		T5	167±5	121±5	1.18±0.01^{b}	466±9	299±5^{a}	1.28±0.01^{b}	935±8^{a}	470±2^{a}	1.36±0.02^{b}
25	25	T6	167±6	122±6	1.20±0.03^{b}	469±14	302±9^{a}	1.27±0.01^{b}	944±20^{a}	475±10^{a}	1.36±0.02^{b}
50	50	T7	169±1	124±1	1.19±0.01^{b}	475±3	306±4^{a}	1.28±0.01^{b}	955±10^{a}	481±8^{a}	1.38±0.01^{b}
75	75	T8	174±4	128±4	1.15±0.02^{b}	479±9	305±5^{a}	1.30±0.01^{ab}	949±23^{a}	470±6^{a}	1.39±0.03^{b}
100	100	T9	176±2	130±3	1.17±0.01^{b}	485±8	309±6^{a}	1.30±0.01^{ab}	934±18^{a}	449±14^{ab}	1.38±0.02^{b}
P value											
Vit. D_3											
Linear			0.716	0.730	0.082	0.222	0.094	0.006	0.055	0.036	0.007
Quadratic			0.386	0.355	0.017	0.093	0.037	0.063	0.047	0.075	0.024
Vit. D_3 + 25-OH-D_3											
Linear			0.002	0.002	0.003	0.002	0.004	0.689	0.039	0.291	0.282
Quadratic			0.507	0.373	0.086	0.145	0.075	0.038	0.005	0.001	0.010
Contrasts											
$\bar{x}$ T1-T5 vs $\bar{x}$ T6-T9			0.056	0.046	0.130	0.024	0.024	0.892	0.037	0.167	0.699
$\bar{x}$ T2-T5 vs $\bar{x}$ T6-T9			0.179	0.160	0.928	0.179	0.235	0.312	0.430	0.909	0.333

[a-b] Means within the same column without a common superscript differ ($P<0.05$) Duncan's multiple range test; Means± SEM.

[1]25-hydroxycholecalciferol (25-OH-D_3) supplemented levels were calculated to be equivalent to 1,000, 2,000, 3,000 and 4,000 IU of D_3 activity based on the conversion of 0.025 µg of cholecalciferol into one IU (NRC, 1994).

[2] BW, Body weight (g/bird); WG, Weight gain (g/bird); FCR, Mortality corrected feed conversion ratio (g feed intake / g weight gain).

[3] Values for performance responses represent the mean average of n=5 replicate pens per treatment of 6 birds each at respective age.

In this experiment, overall performance of the birds was improved with the supplementation of D_3 or D_3+25-OH-D_3 dietary treatments above the NRC (1994) recommendations of 5 μg D_3/ kg of diet. However, no consistent differences were detected between dietary sources of D_3. Our results are agreement with Bar et al. (2003) who conducted five experiments to compare the effects of dietary 25-OH-D_3 to D_3 alone on broiler chickens. From the five experiments, only one resulted in an improvement in performance of 22-d old broilers. In the other experiments, the growth response was similar between dietary D_3 sources. Interestingly, the experiment where performance was positively influenced by 25-OH-D_3 used a Ca and NPP restriction in the diet similar to the one used in this experiment. These results support the hypothesis of Baker et al (1998) that lowering the Ca and NPP in the diet in fact increases the dietary requirement of D_3. Moreover, in agreement with the results of this experiment and Baker et al. (1998) conclusions, Ledwaba and Roberson (2003) reported that the effectiveness of dietary 25-OH-D_3 to positively influence performance and bone characteristics was higher when diets were fed with suboptimal Ca levels (0.80 to 0.85%). Therefore, the use of 25-OH-D_3 as partial replacement of D_3 in poultry diets could provide a more effective margin of safety in preventing performance reductions in the presence of low Ca or NPP diets.

A second factor influencing the bird's need for higher dietary D_3 is the presence of intestinal parasites such as coccidia (Leeson and Summers, 2001). In this experiment, a live coccidiosis vaccine was used to challenge the immune and digestive system of the bird. The use of live vaccines is usually associated with reduced performance due to the induced subclinical infection after vaccination (Lillehoj and Trout, 1993). Thus, the results of this experiment suggest that dietary supplementation of D_3, independent of the source, is effective in ameliorating the negative effects of intestinal parasites introduced after coccidiosis vaccination.

Vitamin D Status and Plasma Cytokines

Vitamin D status (VDS) of 14-d broiler chickens increased linearly ($P<0.05$) with increasing concentrations of D_3 and D_3+25-OH-D_3 combination treatments. A quadratic response was observed for the D_3+25-OH-D_3 treatments only. Additionally, VDS of the CON group was different ($P<0.05$) from all other treatments. The 50, 100, and 150 μg of D_3 alone treatment groups were significantly lower than all D_3+25-OH-D_3 treatments. Moreover, D_3+25-OH-D_3 dietary treatments (Contrast [$\bar{x}$ T2-T5 vs $\bar{x}$ T6-T9]) were found to be significantly higher than all equivalent μg of D_3 treatments offered as D_3 alone (Table 16).

Vitamin D status of 14-d old broiler chickens increased linearly for both D_3 and D_3+25-OH-D_3 dietary treatments. Additionally, plasma 25-OH-D_3 concentration was higher in all D_3+25-OH-D_3 dietary treatments compared to their equivalent D_3 treatments. In agreement with the results of this experiment, Clark and Potts (1977) found that there was a linear relationship between the intake of vitamin D_3 and the serum levels of 25-OH-D_3. Also, Yarger et al. (1995a) compared the effect of feeding D_3 or 25-OH-D_3 at different concentration levels and found a significant dose response with serum 25-OH-D_3 concentrations increasing more rapidly in birds fed 25-OH-D_3 than in birds fed D_3 alone. Plasma or serum 25-OH-D_3 is considered to be the best indicator of VDS because of its ability to respond to the source and dietary level of D_3.

The improvement of VDS appears to have a relationship with improved BW and FCR (Yarger et al., 1995a). Although this relation was not very clear in this experiment, previous literature reports indicate that dietary 25-OH-D_3 is more effective than D_3 in improving overall performance of broiler chickens (Yarger et al., 1995b; Han et al., 2016).

Table 16 Effect of dietary vitamin D_3 and 25-hydroxycholecalciferol on plasma vitamin D status and cytokines of 14-d-old broiler chickens

Vit. D_3 (µg/kg)	25-OH-D_3[1] (µg/kg)		Response (7-d post-cocci challenge)[2,3]				
			VDS	Cytokines[4]			
				IFN-γ	IL-1β	IL-6	IL-10
5(Control)		T1	25.1±6.3^{f}	306±86	43±16	294±258	190±90
50		T2	55.1±8.1^{e}	291±248	62±20	4±1	40±23
100		T3	92.5±8.7^{d}	637±459	91±17	589±477	396±213
150		T4	96.0±8.2^{d}	367±219	74±32	256±180	222±172
200		T5	116.4±2.1^{c}	870±421	405±294	295±277	369±221
25	25	T6	125.9±6.2^{bc}	95±62	60±29	206±202	51±47
50	50	T7	138.4±3.4^{ab}	105±97	63±30	92±88	61±30
75	75	T8	146.9±0.3^{a}	692±469	22±12	53±30	419±317
100	100	T9	149.6±0.9^{a}	185±109	62±31	397±193	86±65
P value							
Vit. D_3							
Linear			<0.001				
Quadratic			0.081				
Vit. D_3 + 25-OH-D_3							
Linear			<0.001				
Quadratic			<0.001				
Contrasts							
$\bar{x}$ T1-T5 vs $\bar{x}$ T6-T9			<0.001				
$\bar{x}$ T2-T5 vs $\bar{x}$ T6-T9			<0.001				

[a-f] Means within the same column without a common superscript differ ($P<0.05$) Duncan's multiple range test. Means± SEM.

[1] 25-hydroxycholecalciferol (25-OH-D_3) supplemented levels were calculated to be equivalent to 1,000, 2,000, 3,000 and 4,000 IU of D_3 activity based on the conversion of 0.025 µg of cholecalciferol into one IU (NRC, 1994).

[2] VDS, Vitamin D status (ng/mL) is defined as the total plasma concentration of circulating 25-OH-D_3; IFN-γ (pg/mL); IL-1β (pg/mL); IL-6(pg/mL); IL-10 (pg/mL);

[3] Values for vitamin D status and cytokines represent the mean of n=5 replicate birds per treatment.

[4] Cytokine data were analyzed by Kruskal-Wallis test.

This relationship was further investigated by Hutton et al. (2014), who compared the efficacy of D_3 alone (125 µg/kg) or a D_3 (56 µg/kg) + 25-OH-D_3 (69 µg/kg) combination on

broiler chickens. They found higher circulating levels of D_3 in the broilers fed the D_3+25-OH-D_3, although overall performance was not improved among treatments.

The ability of D_3+25-OH-D_3 treatments to increase VDS higher than D_3 alone after the coccidiosis vaccine challenge could represent a better strategy to reduce the negative effects of live coccidiosis vaccines in young growing broilers.

Plasma cytokine concentration assayed by the ELISA kits resulted in high variability across D_3 treatments (Table 16). No differences (P>0.05) were detected between D_3 sources for any of the plasma cytokines assayed in this experiment. However, IL-1β, a cytokine associated with inflammatory response, cell differentiation and apoptosis regulation in the body (Giraldo et al., 2009), appeared to have numerically lower concentrations than all other cytokines measured in this experiment (IFN-γ, IL-6, and IL-10) except for the 200 μg of D_3 treatment which reported a high concentration with respect other treatment groups. No differences in plasma cytokines were observed between the CON group and the graded D_3 treatments either.

IL-10 is known to inhibit the synthesis of pro-inflammatory cytokines such as, IL-1β and IL-6 (Hong et al., 2006). A concentration increase of IL-10 in the plasma would indicate an anti-inflammatory response of dietary D_3. This relationship was observed by Morris et al. (2015) who reported that 25-OH-D_3 influenced *in vitro* expression of IL-10 mRNA using chicken macrophage cell lines. Additionally, an *in vivo* study conducted by the same research team using layer-type chicks orally challenged with 1×10^5 live coccidia oocysts at 21-d of age and supplemented with 6.25, 25, or 50 μg of 25-OH-D_3 per kg of feed revealed evidence suggesting dietary 25-OH-D_3 has a regulatory effect on body weight, CD8+ cells, IL-10 mRNA expression and CD4+CD25+ cells. Moreover, dietary 25-OH-D_3 supplementation (100μg/kg) decreased IL-1β mRNA and increased IL-10 mRNA amounts in the cecal tonsils. The results of this

experiment did not provide enough information to either support or not support the immunomodulatory role of D_3 after cocci vaccine challenge.

Intestinal Integrity and Relative Organ Weight

No differences (P>0.05) in gross coccidial lesions were observed after 14-d of the coccidiosis vaccine across dietary treatments or the upper (duodenum), middle (jejunum), lower intestine (ileum) and the ceca of 21-d broiler chickens. Comparing the different intestine sections, very mild lesions were detected in the medium and lower intestine and almost no lesions were detected in the ceca. The majority of lesions were detected in the upper section of the intestine but were not affected by dietary treatment (Table 17).

The presence of lesions confirms that I effectively delivered the coccidiosis vaccine to the chickens and therefore there was a digestive and immune challenge to the birds. However, the low severity of the lesions observed across the different sections of the intestine and that no treatment differences were detected in this experiment suggests that there was a mild non-clinical challenge to the birds. This is expected because the caging system did not allow recycling of the oocysts. Further research projects investigating similar effects of vitamin D_3 using facilities that do not allow recycling of the oocysts may consider increasing the dose of the coccidiosis vaccine challenge.

Intestine histology results are presented in table 18. Differences (P<0.05) between dietary treatments were observed in VH:CD ratio of the duodenum only. A linear increase (P<0.05) in duodenum villi height was observed with increasing dietary levels of D_3+25-OH-D_3 and a quadratic response (P<0.05) to graded level of D_3+25-OH-D_3 was observed in ileum villi height. No differences (P>0.05) were observed in any villi height, crypt depth or associated ratio in the jejunum of 21-d broiler chickens.

Table 17 Effect of dietary vitamin D_3 and 25-hydroxycholecalciferol on lesion scores of 21-d-old broiler chickens

Vit. D_3 (µg/kg)	25-OH-D_3[1] (µg/kg)	Response (14-d post- cocci challenge)[3,4]				
			Lesion scores[2]			
			U	M	L	C
5 (Control)		T1	0.50±0.18	0.30±0.15	0.06±0.06	0.00±0.00
50		T2	0.55±0.15	0.10±0.61	0.00±0.00	0.05±0.05
100		T3	0.75±0.14	0.10±0.61	0.15±0.10	0.00±0.00
150		T4	0.32±0.12	0.00±0.00	0.10±0.06	0.00±0.00
200		T5	0.80±0.27	0.21±0.15	0.12±0.07	0.00±0.00
25	25	T6	0.55±0.15	0.20±0.01	0.05±0.05	0.00±0.00
50	50	T7	0.65±0.17	0.05±0.05	0.10±0.10	0.00±0.00
75	75	T8	0.35±0.17	0.25±0.08	0.05±0.05	0.00±0.00
100	100	T9	0.73±0.28	0.10±0.06	0.27±0.08	0.00±0.00

Means± SEM.

[1]25-hydroxycholecalciferol (25-OH-D_3) supplemented levels were calculated to be equivalent to 1,000, 2,000, 3,000 and 4,000 IU of D_3 activity based on the conversion of 0.025 µg of cholecalciferol into one IU (NRC, 1994).

[2] Chickens were vaccinated (7x recommended dose) on day 7 of the trial. Lesions were scored from 0-4 as described by Johnson and Reid (1970). Where 0 score represents no gross lesions and 4 indicates severe damage to the intestine. Data were analyzed by Kruskal-Wallis test.

[3] U, Upper intestine (duodenum); M, Medium intestine (jejunum); L, Lower intestine (ileum); C, Ceca;

[4] Values for lesion scores represent the mean average of n=5 replicate pens per treatment of 4 birds each.

Although not consistent, villi height in the duodenum of chickens fed D_3 and D_3+25-OH-D_3 diets was numerically higher. Also, V:C ratio in the duodenum and in the ileum had a similar response, suggesting an increase in nutrient absorption in the upper and lower sections of the intestine. Similar results were found by Chou et al. (2009) who reported that supplemental 25-OH-D_3 positively influenced small intestine morphology; villi height of the duodenum and jejunum were longer. Also, a better ratio of villi height to crypt depth was observed in duodenum and jejunum.

In the experiment of Chou et al. (2009), intestinal morphology was evaluated in the absence of a pathogen or parasite challenge. It is possible that the coccidiosis vaccine caused an increased variability in the intestinal response of the chickens to supplemental D_3. Even when the same vaccine dose was administered to all chickens, some of them were likely more resistant than others to infections and parasite infestations. Moreover, the presence of coccidia in the intestine could have resulted in alterations in the intestinal morphology. There are some literature reports (Yamauchi, 2002; Khambualai et al., 2009; de Verdal et al., 2010) that suggest the intestine is highly reactive to nutritional or histological alterations. Yamauchi (2002) described how a compensatory enlargement of ileal villi is induced after a disturbance of the natural histology of the intestine. Further research investigating histological variables in the presence of pathogens or parasites is recommended to better understand the role of D_3 and 25-OH-D_3 in the intestinal morphology.

Overall, no differences (P<0.05) were observed in relative organ weight of broiler chickens after 21-d (Table 19). Differences in the relative weight of the bursa of Fabricius were observed between D_3 and D_3+25-OH-D_3 treatments. In average (Contrast [$\bar{x}$ T2-T5 vs $\bar{x}$ T6-T9]) the D_3+25-OH-D_3 treatment groups had smaller bursas of Fabricius and bigger livers than the D_3 treatments.

Relative weight of immune organs such as the spleen, bursa of Fabricius and liver is a method of evaluating overall immune status of the chicken (Heckert et al., 2002). Reduced growth of these organs could be thought of compromised immunity (Zhou et al., 2009). Relative weight of livers in this experiment was higher for the D_3+25-OH-D_3 treatments on average ($\bar{x}$ T2-T5 vs $\bar{x}$ T6-T9) suggesting improved immunity.

Table 18 Effect of dietary vitamin D_3 and 25-hydroxycholecalciferol on intestinal morphology of 21-d-old broiler chickens

Vit. D_3 (µg/kg)	25-OH-D_3[1] (µg/kg)		Response (14-d post- cocci challenge)[2,3]								
			VH-D	CD-D	V:C-D	VH-J	CD-J	V:C-J	VH-I	CD-I	V:C-I
5 (Control)		T1	2500±87	194±20	13.4±1.2^{bc}	1434±89	180±15	8.1±0.7	949±90	171±29	6.1±0.9
50		T2	2628±128	138±7	19.2±2.0^{a}	1459±151	180±10	8.1±1.6	827±41	158±11	5.3±0.1
100		T3	2508±93	198±15	12.9±1.2^{bc}	1599±73	193±17	8.5±0.8	927±67	168±15	5.7±0.7
150		T4	2708±129	157±18	18.2±2.2^{a}	1360±126	156±8	8.7±0.6	865±68	153±10	5.7±0.2
200		T5	2635±58	203±16	13.2±0.7^{bc}	1467±109	184±20	8.4±1.2	932±60	148±7	6.3±0.3
25	25	T6	2455±123	209±19	12.2±1.6^{c}	1239±125	170±17	7.6±1.2	844±50	128±16	7.0±0.8
50	50	T7	2621±58	156±8	16.9±0.8^{ab}	1314±60	170±13	8.0±0.9	766±46	159±15	5.0±0.3
75	75	T8	2754±40	183±17	15.5±1.3^{abc}	1348±48	158±12	8.6±0.5	798±32	130±14	6.4±0.5
100	100	T9	2714±90	212±19	13.0±0.7^{bc}	1374±90	165±13	8.5±0.8	954±53	135±14	7.3±0.7
P value											
Vit. D_3											
Linear			0.250	0.612	0.907	0.932	0.744	0.671	0.961	0.300	0.619
Quadratic			0.845	0.203	0.188	0.742	0.786	0.825	0.413	0.892	0.248
Vit. D_3 + 25-OH-D_3											
Linear			0.010	0.791	0.611	0.884	0.289	0.504	0.735	0.251	0.582
Quadratic			0.823	0.095	0.051	0.223	0.675	0.756	0.007	0.625	0.223
Contrasts											
$\bar{x}$ T1-T5 vs $\bar{x}$ T6-T9			0.358	0.524	0.333	0.067	0.228	0.647	0.103	0.031	0.180
$\bar{x}$ T2-T5 vs $\bar{x}$ T6-T9			0.240	0.825	0.157	0.068	0.279	0.573	0.209	0.209	0.154

[a-c] Means within the same column without a common superscript differ ($P<0.05$) Duncan's multiple range test; Means± SEM.

[1]25-hydroxycholecalciferol (25-OH-D_3) supplemented levels were calculated to be equivalent to 1,000, 2,000, 3,000 and 4,000 IU of D_3 activity based on the conversion of 0.025 µg of cholecalciferol into one IU (NRC, 1994).

[2] VH, villi height (µm); CD, crypt depth (µm); V:C, villi height /crypt depth ratio; D, duodenum; J, jejunum; I, ileum.

[3] Values for histology responses represent the mean of n=5 replicate birds per treatment.

Table 19 Effect of dietary vitamin D_3 and 25-hydroxycholecalciferol on relative organ weight of 21-d-old broiler chickens

Vit. D_3 (µg/kg)	25-OH-D_3[1] (µg/kg)		Response (14-d post-cocci challenge)[2,3]		
			Liver	Spleen	Bursa
5 (Control)		T1	2.98±0.05	0.12±0.01	0.16±0.01^{abc}
50		T2	2.82±0.08	0.13±0.01	0.17±0.01^{ab}
100		T3	2.86±0.05	0.12±0.01	0.18±0.01^{a}
150		T4	2.90±0.06	0.11±0.01	0.15±0.01^{abcd}
200		T5	3.00±0.06	0.13±0.01	0.15±0.01^{abcd}
25	25	T6	3.11±0.07	0.12±0.01	0.12±0.01^{d}
50	50	T7	3.03±0.07	0.11±0.01	0.14±0.01^{cd}
75	75	T8	3.02±0.06	0.12±0.01	0.15±0.01^{abcd}
100	100	T9	3.03±0.06	0.11±0.01	0.14±0.01^{abcd}
P value					
Vit. D_3					
Linear			0.576	0.934	0.282
Quadratic			0.051	0.628	0.210
Vit. D_3 + 25-OH-D_3					
Linear			0.930	0.583	0.785
Quadratic			0.464	0.993	0.171
Contrasts					
$\bar{x}$ T1-T5 vs $\bar{x}$ T6-T9			0.003	0.250	<0.001
$\bar{x}$ T2-T5 vs $\bar{x}$ T6-T9			0.002	0.223	<0.001

[a-d] Means within the same column without a common superscript differ ($P<0.05$) Duncan's multiple range test; Means± SEM.

[1]25-hydroxycholecalciferol (25-OH-D_3) supplemented levels were calculated to be equivalent to 1,000, 2,000, 3,000 and 4,000 IU of D_3 activity based on the conversion of 0.025 µg of cholecalciferol into one IU (NRC, 1994).

[2] Liver, spleen and bursa are expressed as g/100g body weight

[3] Values for relative organ weight represent the mean average of n=5 replicate pens per treatment of 4 birds each.

In contrast, the D_3+25-OH-D_3 treatments also tended to have smaller bursas of Fabricius ($\bar{x}$ T2-T5 vs $\bar{x}$ T6-T9) and may indicate reduced immune capacity. Unfortunately, with the lack of non-vaccinated controls in this experiment to have a reference of "normal growth," we cannot determine if the D_3+25-OH-D_3 treatments induced some kind of stress in the chickens or not.

Bone Mineralization

Bone mineralization results are presented in table 20. Both TBA and TBS differences (P<0.05) were observed across dietary treatment groups. Overall the supplementation of D_3 alone or D_3+25-OH-D_3 improved (P<0.05) TBA and TBS with respect to the CON treatment. TBS of 21-d broiler chickens had a linear and quadratic response to increasing concentrations of D_3 or D_3+25-OH-D_3. TBA resulted in a linear increase to dietary D_3 and both a linear and quadratic effect was observed for D_3+25-OH-D_3 treatment groups. For both bone mineralization responses (TBA & TBS), D_3+25-OH-D_3 dietary treatments (Contrast [$\bar{x}$ T2-T5 vs $\bar{x}$ T6-T9]) were found to be significantly higher than all equivalent treatments offered as D_3 alone.

Bone mineralization results of the present experiment are in agreement with literature reports suggesting that dietary D_3 alone or in combination with 25-OH-D_3 enhances bone health (Kasim and Edwards, 2000; Fritts and Waldroup, 2003; Whitehead et al., 2004; Rama Rao et al., 2006; Khan et al., 2010; Vazquez et al., 2018). In average, TBA was improved ≈3.5% when the D_3 (>50 µg D_3/kg) and D_3+25-OH-D_3 treatment groups where compared to the CON group. Between D_3 and D_3+25-OH-D_3 sources TBA was improved an average of ≈1.3%. Similarly, TBS was improved ≈6 kg of force when all the D_3 and D_3+25-OH-D_3 treatment groups where compared to the CON group and ≈2 kg of force when the D_3 alone was compared to the D_3+25-OH-D_3 treatment groups. The effectiveness of dietary 25-OH-D_3 in promoting bone mineralization is related with its higher bioactivity which is reported to be around 1.6 to 2.0 times the activity of D_3 (Han et al., 2016)

Table 20 Effect of dietary vitamin D_3 and 25-hydroxycholecalciferol on bone mineralization of 21-d-old broiler chickens

Vit. D_3 (µg/kg)	25-OH-D_3[1] (µg/kg)		Response (14-d post-cocci challenge)[3]	
			[2]TBA	TBS
5 (Control)		T1	44.2±0.5^{d}	14.1±0.5^{c}
50		T2	47.3±0.4^{abc}	19.3±0.7^{b}
100		T3	47.7±0.7^{abc}	19.7±0.8^{ab}
150		T4	46.3±0.4^{c}	20.3±1.0^{ab}
200		T5	46.7±0.4^{bc}	19.8±0.6^{ab}
25	25	T6	48.0±0.6^{ab}	20.9±0.7^{ab}
50	50	T7	48.5±0.6^{a}	21.8±0.8^{a}
75	75	T8	48.4±0.6^{a}	22.0±0.7^{a}
100	100	T9	48.3±0.6^{ab}	22.1±0.7^{a}
P value				
Vit. D_3				
Linear			0.086	0.001
Quadratic			0.004	0.001
Vit. D_3 + 25-OH-D_3				
Linear			0.002	<0.001
Quadratic			0.001	<0.001
Contrasts				
$\bar{x}$ T1-T5 vs $\bar{x}$ T6-T9			<0.001	<0.001
$\bar{x}$ T2-T5 vs $\bar{x}$ T6-T9			<0.010	<0.010

[a-d] Means within the same column without a common superscript differ ($P<0.05$) Duncan's multiple range test; Means± SEM.

[1]25-hydroxycholecalciferol (25-OH-D_3) supplemented levels were calculated to be equivalent to 1,000, 2,000, 3,000 and 4,000 IU of D_3 activity based on the conversion of 0.025 µg of cholecalciferol into one IU (NRC, 1994).

[2] TBA, tibia bone ash (%); TBS, tibia breaking strength (kg force).

[3] Values for bone mineralization responses represent the mean average of n=5 replicate pens per treatment of 6 birds each.

In conclusion, the combination of both D_3 and 25-OH-D_3 was more effective than D_3 alone in promoting bone mineralization and enhancing VDS in young broiler chickens fed diets with suboptimal levels of Ca and NPP after a live vaccine coccidiosis challenge. The supplementation of D_3, independent of the source, above NRC (1994) recommendations yielded better performance, bone mineralization and VDS in starter broiler chickens fed a low Ca and NPP diet and subjected to a live coccidia vaccine challenge.

REFERENCES

Ahmad, T. A., B. A. El-Sayed, and L. H. El-Sayed. 2016. Development of immunization trials against *Eimeria* spp. Trials Vaccinol. 5:38-47.

Al-Ajeeli, M., T. Gaydos, R. Abdaljaleel, H. Leyva-Jimenez, J. Corley, and C. A. Bailey. 2017. Evaluation of Safmannan on full term broiler performance with coccidiosis vaccinated broilers on used litter. International Poultry Scientific Forum Atlanta, Georgia. Poult. Sci. 96:278. (Abstr).

Anon. 2013. High Cost of coccidiosis in broilers. The Poultry Site. Accessed May 2018. http://www.thepoultrysite.com/poultrynews/28036/high-cost-of-coccidiosis-in-broilers.

Atencio, A., G. M. Pesti, and H. M. Edwards Jr. 2005. Twenty-five hydroxycholecalciferol as a cholecalciferol substitute in broiler breeder hen diets and its effect on the performance and general health of the progeny. Poult. Sci. 84:1277-1285.

Baker, D. H., R. R. Biehl, and J. L. Emmert. 1998. Vitamin D_3 requirement of young chicks receiving diets varying in calcium and available phosphorus. Br. Poult. Sci. 39:413-417.

Bar, A., V. Razaphkovsky, E. Vax, and I. Plavnik. 2003. Performance and bone development in broiler chickens given 25-hydroxycholecalciferol. Br. Poult. Sci. 44:224-233.

Chou, S. H., T. K. Chung, and B. Yu. 2009. Effects of supplemental 25-hydroxycholecalciferol on growth performance, small intestine morphology, and immune response of broiler chickens. Poult. Sci. 88:2333-2341.

Clark, M. B., and J. T. Potts, Jr., 1977. 25-hydroxyvitamin D_3 regulation. Calcif. Tissue Res. 22:29-34.

Collins, E. D., and A. W. Norman .2001. Vitamin D in Handbook of vitamins. 3rd ed. Edited by Rucker R. B., J. W. Suttie, D. B. McCormick, and L. J. Machlin. Marcel Dekker, New York. USA.

Dalloul, R. A., and H. S. Lillehoj. 2005. Recent advances in immunomodulation and vaccination strategies against coccidiosis. Avian Dis. 49:1-8.

De Gussem, M. 2007. Coccidiosis in poultry: Review on diagnosis, control, prevention and interaction with overall gut health. Pages 253–261 in Proc. 16th Eur. Symp. on Poult. Nutr. World's Poultry Science Association, Beekbergen, The Netherlands.

De Verdal, H., S. Mignon-Grasteau, C. Jeulin, E. Le Bihan-Duval, M. Leconte, S. Mallet, C. Martin, and A. Narcy. 2010. Digestive tract measurements and histological adaptation in broiler lines divergently selected for digestive efficiency. Poult. Sci. 89:1955-1961.

Feng, J., X. Liu, Z. R. Xu, Y. Z. Wang, and J. X. Liu. 2007. Effects of fermented soybean meal on digestive enzyme activities and intestinal morphology in broilers. Poult. Sci. 86:1149-1154.

Fowler, J., M. Hashim, A. Haq, O. Gutierrez, and C. A. Bailey. 2014. Comparing the bioactivity of 25-OHvitamin D_3 (Bio-D™) to cholecalciferol for starter broiler growth performance and bone mineralization. Poult. Sci. 93:10 (Abstr).

Fritts, C. A., and P. W. Waldroup. 2003. Effects of source and level of vitamin D on live performance and bone development in growing broilers. J. Appl. Poult. Res. 12:43-52.

Giraldo, S., J. Sanchez, Q. Felty, and D. Roy. 2009. IL-1β (interleukin 1, beta), Atlas Genet. Cytogenet. Oncol. Haematol. 13:273-275.

Gómez, V. G., L. R. Morales, and G. E. Avila. 2013. Use of 25-hydroxycholecalciferol in diets of broiler chickens: effects on growth performance, immunity and bone calcification. J. Poult. Sci. 50:60-64.

Han, J. C., G. H. Chen, J. G.Wang, J. L. Zhang, H. X. Qu, C. M. Zhang, Y. F. Yan, and Y. H. Cheng. 2016. Evaluation of relative bioavailability of 25-hydroxycholecalciferol to cholecalciferol for broiler chickens. Asian Australas. J. Anim. Sci 29:1145-1151.

Heckert, R. A., I. Estevez, E. Russek-Cohen, and R. Pettit-Riley. 2002. Effects of density and perch availability on the immune status of broilers. Poult. Sci. 81:451-457.

Hutton, K. C., M. A. Vaughn, G. Litta, B. J. Turner, and J. D. Starkey. 2014. Effect of Vitamin D Status Improvement with 25- Hydroxycholecalciferol on Skeletal Muscle Growth Characteristics and Satellite Cell Activity in Broiler Chickens. J. Anim. Sci. 92:3291-3299.

Johnson, J., and W. M. Reid. 1970. Anticoccidial drugs: Lesion scoring techniques in battery and floor pen experiments with chickens. Exp. Parasitol. 28:30-36.

Kasim, A. and H.M., Jr. Edwards. 2000. Evaluation of cholecalciferol sources using broiler chick bioassays. Poult. Sci. 79:1617-1622.

Khambualai, O., K. Yamauchi, S. Tangtaweewipat, and B. Cheva-Isarakul. 2009. Growth performance and intestinal histology in broiler chickens fed with dietary chitosan. Br. Poult. Sci. 50:592–597.

Khan, S. H., R. Shahid, A. A. Mian, R. Sardar, and M. A. Anjum. 2010. Effect of the level of cholecalciferol supplementation of broiler diets on the performance and tibial dyschondroplasia. J. Anim. Physiol. Anim. Nutr. 94:584-593.

Ledwaba, M. F., and K. D. Roberson. 2003. Effectiveness of twenty-five-hydroxycholecalciferol in the prevention of tibial dyschondroplasia in Ross cockerels depends on dietary calcium level. Poult. Sci. 82:1769-1777.

Leeson, S. and J. D. Summers. 2001. Scott´s Nutrition of the Chicken. 4th ed. University Books, Ontario Canada.

Lillehoj, H. S., and J. M. Trout. 1993. Coccidia: A review of recent advances in immunity vaccine development. Avian Pathol. 22:3-21.

Merck Veterinary Manual. 2010. Overview of Coccidiosis in Poultry. 10th rev. ed. Merck & Co. Inc. Whitehouse Station, NJ., USA.

Morris, A., R. Shanmugasundaram, M. Lilburn, and R. K. Selvaraj. 2014. 25-Hydroxycholecalciferol supplementation improves growth performance and decreases inflammation during an experimental lipopolysaccharide injection. Poult. Sci. 93:1951-1956.

Morris, A., R. Shanmugasundaram, J. Mcdonald, and R. K. Selvaraj. 2015. Effect of *in vitro* and *in vivo* 25-hydroxyvitamin D treatment on macrophages, T cells, and layer chickens during a coccidia challenge. J. Anim. Sci. 93:2894-2903.

National Research Council .1994. Nutrients requirements of poultry. 9th rev. ed. Natl. Acad. Press, Washington, DC.

Peek, H. W., and W. J. M. Landman. 2011. Coccidiosis in poultry: anticoccidial products, vaccines and other prevention strategies. Vet. Q. 31:143-161.

Perez-Carbajal, C., D. Caldwell, M. Farnell, K. Stringfellow, S. Pohl, G. Casco, A. Pro-Martinez, and C. A. Ruiz-Feria. 2010. Immune response of broiler chickens fed different levels of arginine and vitamin E to a coccidiosis vaccine and Eimeria challenge. Poult. Sci. 89:1870-1877.

Rama Rao, S. V., M. V. L. N. Raju, A. K. Panda, G. Shyam Sunder, and R. P. Sharma. 2006. Effect of high concentration of cholecalciferol on growth, bone mineralization and mineral retention in broiler chicks fed sub-optimal concentrations of calcium and non-phytate phosphorus. J. Appl. Poult. Res. 15:493-501.

Vazquez, J. R., G. V. Gómez, C. C. López, A. C. Cortés, A. C. Díaz, S. R. T. Fernández, E. M. Rosales, and A. G. Avila. 2018. Effects of 25-hydroxycholecalciferol with two D_3 vitamin levels on production and immunity parameters in broiler chickens. J. Anim. Physiol. Anim. Nutr. 102:493-497.

Whitehead, C. C., 1995. The role of vitamin D metabolites in the prevention of tibial dyschondroplasia. Anim. Feed Sci. Technol. 53:205-210.

Whitehead, C.C., H. A. McCormack, L. McTier, R. H. Fleming. 2004. High Vitamin D_3 requirements in broilers for bone quality and prevention of tibial dyschondroplasia and interactions with dietary calcium, available phosphorus and Vitamin A. Brit. Poult. Sci. 45:425-436.

Yamauchi, K. 2002. Review on chicken intestinal villus histological alterations related with intestinal functions. J. Poult. Sci.39:229–242.

Yarger, J. G., C. A. Saunders, J. L. McNaughton, C. L. Quarles, B. W. Hollis, and R. W. Gray. 1995a. Comparison of dietary 25-hydroxycholecalciferol and cholecalciferol in broiler chickens. Poult. Sci. 74:1159-1167.

Yarger, J. G., C. L. Quarles, B. W. Hollis, and R. W. Gray, 1995b. Safety of 25-hydroxycholecalciferol in poultry rations. Poult. Sci. 74:1437-1446.

Zhou, T. X., Y. J. Chen, J. S. Yoo, Y. Huang, J. H. Lee, H. D. Jang, S. O. Shin, H. J. Kim, J. H. Cho, and I. H. Kim. 2009. Effects of chitooligosaccharide supplementation on performance, blood characteristics, relative organ weight, and meat quality in broiler chickens. Poult. Sci. 88:593-600.

CHAPTER V

CONCLUSION

The accelerated growth of the commercial poultry industry over the last 20 years has led to the development of modern poultry strains that are genetically selected for improved feed efficiency and higher meat yields. Therefore, it is possible that commercial poultry strains have altered their physiology and consecutively, their ability to respond to dietary nutrients. Thus, an increasing concern among nutritionists over the last few years regards the need for adapting or developing new protocols to evaluate nutrient requirements that are based on current feeding strategies and genetic bird strains.

Nutritionist and researchers use literature references such as the NRC to establish nutritional requirements of poultry. The last revision of the NRC was in 1994 and many of the literature referenced can date as early as the 1960s. Therefore, in many cases the NRC nutrient recommendations will no longer reflect the current nutritional needs of modern poultry strains.

As an example, the Vitamin D_3 requirement of growing broiler chickens in the NRC (1994) was estimated at 200 IU/kg of feed. However, post NRC literature reports suggest that dietary supplementation of D_3 (up to 20x the 1994 NRC) is necessary for optimal growth and prevention of skeletal disorders.

To further investigate if the vitamin D_3 requirement has changed from the last NRC revision in 1994, this dissertation focused on developing a protocol that would precisely estimate the vitamin D_3 requirement of young growing broilers and would adapt to the current ability of meat-type chickens to respond to dietary nutrients. This protocol focused on reducing feed mixing error, selective feeding, and other sources of error usually associated with typical

methodologies of estimating nutrient requirements; as well as reducing the effect of maternal D_3 found in the egg yolk and increasing the precision of D_3 delivery to the chickens.

Furthermore, the effect of dietary D_3 metabolites such as 25-hydroxycholecalciferol (25-OH-D_3) is rapidly gaining popularity among the commercial poultry industry as they are actively seeking for possible dietary "alternatives" to antibiotic growth promoters and ways to enhance animal well-being through nutrition. The NRC has very limited information regarding the use of feed additives such as exogenous enzymes, probiotics, prebiotics, herbal mixtures, essential oils or vitamin metabolites such as 25-OH-D_3. Therefore, the second objective of this dissertation was to compare the effect of dietary levels of D_3 and partial replacement of D_3 with 25-OH-D_3 on performance, bone mineralization, intestinal integrity, vitamin D status, and plasma cytokine levels of broiler chickens after a live coccidiosis vaccine challenge.

The vitamin D_3 requirement for starter broilers was estimated to be around 500 IU/kg of feed when a low Ca (0.75%) and non-phytate phosphorus (0.37%) diet was fed. When an industry type nutritionally adequate feed was offered to the chickens the D_3 requirement was estimated to be around 200 IU/kg of feed for maximum growth and bone mineralization. We concluded that nutrient restrictions increased the D_3 requirement with respect the NRC (1994) but if the diet is nutritionally adequate the vitamin D_3 requirement of modern poultry strains is still in agreement with the 1994 NRC recommendations.

It can be concluded that the vitamin D_3 requirement of starter broiler chickens is highly influenced by the maternal reserves of D_3 found in the egg yolk. Due to the high supplementation of D_3 to broiler breeders in commercial facilities, the deposition in the egg yolk is high. If a D_3-deficient state is not induced in the chickens the response to dietary D_3 will be reduced and ultimately, will decrease the sensitivity of the protocol. Future research should be focused on

ensuring the chickens used in D_3 requirement studies have a homogenous and preferably low vitamin D status so a deficiency can be achieved with a practical broiler diet.

For the second part of the dissertation it can be concluded that the combination of both D_3 and 25-OH-D_3 is more effective than D_3 alone in promoting bone mineralization and enhancing vitamin D status in young broiler chickens subjected to a live coccidiosis vaccine challenge. The use of 25-OH-D_3 as a partial replacement of D_3 in poultry diets could provide a more effective margin of safety in preventing skeletal disorders in poultry and enhance performance. The supplementation of D_3, independently of the source, above NRC (1994) recommendations yielded better performance, bone mineralization and vitamin D status in starter broiler chickens subjected to a live coccidiosis vaccine challenge. Thus, dietary supplementation of D_3 or a combination of D_3 plus 25-OH-D_3 is an effective nutritional strategy to ameliorate the negative effects of intestinal parasites introduced after coccidiosis vaccination.