RE-EVALUATION OF THE DIETARY VITAMIN D_3 REQUIREMENT OF MODERN BROILER CHICKENS USING NOVEL ORAL INTUBATION

A Dissertation

by

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ABSTRACT

The studies in this dissertation were conducted to evaluate the response of modern broiler chickens to dietary D_3 and 25-OH-D_3 (25-hydroxycholecalciferol) as well as to establish a marginal vitamin-D-depleted broiler breeder flock to increase the sensitivity of the oral gavage bioassay establish previously in our laboratory. For this purpose, four experiments were conducted. In the first and second experiments, commercial broiler chickens were fed a diet devoid of D_3 for 17 and 21 d, respectively. The first 9 d of the study served as a depletion period of the maternal stores of D_3. On d-10 through the end of each trial, birds were offered commercially available sources of vitamin D_3 or 25-OH-D_3 by gavage daily. The basal diets were formulated with sub-optimal levels of calcium and non-phytate phosphorus (0.75 and 0.375% respectively). Experiment 1 results indicated no significant performance differences between dietary supplementation of either vitamin D_3 or 25-OH-D_3. However, significant ($P<0.05$) differences for body weight (BW) and weight gain (WG) between the negative control and treatment groups were observed. For experiment 2, no performance or bone mineralization differences were detected between treatment groups or the negative control. This suggests that maternal stores of D_3 in the yolk were sufficient to last the broiler chicks through the duration of the trial.

In the third trial, a commercial breeder flock was marginally depleted of vitamin D in order to run a vitamin D requirement study on the progeny chicks. A negative control response was observed between all treatment groups and suggests that establishing a marginally vitamin-D-deficient breeder flock increases the sensitivity of

establishing vitamin D requirements. Furthermore, this experiment estimated the vitamin D_3 requirement to be 73 IU/kg of feed based on bone ash. Using industry-type diets as in this trial, the dietary D_3 requirement appears to be lower than reported in the NRC (1994).

In the last trial, we compared the vitamin D requirement of two modern commercial broiler strains using the oral gavage intubation assay. Results from this study showed no significant differences in performance parameters, however, main effect differences between strains for all performance and bone mineralization parameters were observed.

DEDICATION

First and foremost, I dedicate this dissertation to my much loved and missed grandpa, Dr. Fred A. Gardner. For without his encouragement to pursue Poultry Science and furthermore a Ph.D., I would not be where I am today.

To my parents, Paul and Cindy Gardner, for their continuous moral, spiritual, and financial support. Thank you for answering my million and one phone calls, listening to me ramble on about my research and the hurdles that were involved, and then reassuring me that everything was going to work out in the end. I truly could not have done this without either of you- I love you both.

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NOMENCLATURE

D_3	Vitamin D_3
Ca	Calcium
P	Phosphorus
25-OH-D_3	25-hydroxycholecalciferol
1α(OH)D_3	1-α-hydroxycholecalciferol
FCR	Feed Conversion Ratio (Feed-to-Gain Ratio)
BW	Body Weight
nPP	Non-phytate phosphorus
FI	Feed Intake
WG	Weight Gain
FE	Feed Efficiency
BMC	Bone Mineral Content
BMD	Bone Mineral Density
DXA	Dual X-ray Absorptiometry
TBA	Tibia Bone Ash
TBS	Tibia Breaking Strength
TD	Tibial Dyschondroplasia
NRC	National Research Council
PTH	Parathyroid Hormone
RBV	Relative Bioavailability
AIUI	Adjusted IU intake

TOGU	Total orally gavaged IU’s
ER	Estimated Requirement

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1. INTRODUCTION AND LITERATURE REVIEW

1.1. Introduction

With the world population expected to approach 10 billion by 2050, the food supply will drastically need to increase to meet the demand. If current global processes continue and population growth tendencies remain unchanged, agriculture production must also keep pace with it. It is crucial that food production industries develop more sustainable practices while using scarce natural resources such as water, land, while adapting to climate changes. The approaches of USDA labeled organic and slow-growing meat broilers may not be sustainable, and the best way to reduce the carbon footprint is to grow poultry bigger and faster with less feed. Modern broilers used more recently have been selected for fast growth and high meat yield with the greatest feed efficiency (Siegel, 2014). Further, an increased need of cereal grains in broiler feed formulations and production needs is making genetic selection for maximum feed efficient broiler lines a mandate for profitable poultry production and enhanced sustainability moving forward.

Because today's feed costs can represent up to 70% of the total poultry production costs, it is necessary to seek ways to optimize the consumption and availability of nutrients for the bird in each stage of it's life. Additionally, nutritionists rely on literature references such as the Nation Research Committee (NRC) to formulate diets that meet the animal's nutritional requirements. Today's modern broiler breeds are much different genetically than they were a century ago and their diet requirements have changed correspondently. However, skeletal problems associated with improved growth

rate, such as tibial dyschondroplasia (TD) and rickets, have continued to be concerns amongst nutritionists and welfare experts. Bone metabolic disorders can often be countered by altering management, environment, and nutrition. A crucial point here is the correct balance of calcium and bioavailable phosphorus which is strongly regulated by vitamin D_3 and its metabolites. Together, the changes in genetic performance, new vaccination programs, and health requirements mandate more research to be done using new methods of evaluation under practical situations.

The goal of this research project was to evaluate modern broiler chickens response to dietary D_3 and 25-OH-D_3 as well as precisely estimate the D_3 requirement of these birds using an oral gavage bioassay established previously in our laboratory (Leyva-Jimenez, 2015). Therefore, the objectives of this dissertation research were:

1. Evaluate bioavailability of dietary D_3 and 25-OH-D_3 based on performance and bone mineralization of two commercial broiler strains.
2. Compare broiler strain growth responses to supplemental D_3 and 25-OH-D_3 and requirements to those reported by the latest Poultry NRC (1994).
3. Establish a marginal D_3-deficient breeder flock to produce marginal D_3. performing progeny to reassess the D_3 requirement using the novel oral gavage bioassay (Leyva-Jimenez, Hector, et al., 2019).
4. Evaluate the D_3 requirements of two commercial broiler strains using the novel oral gavage bioassay.

For the purpose of this dissertation, any broilers produced after the most recent published NRC publication released in 1994 will be considered “modern”.

1.2. Literature Review

1.2.1. Poultry Production in the U.S.

The consumption of poultry products in the U.S. has continued to grow due to their affordable price and nutritional value. The National Chicken Council reported that in 2020, 9.25 billion broilers weighing ~59.75 billion pounds, live weight, were produced in the U.S. The world population is estimated to reach 9.8 billion by 2050, which will bring a corresponding increase in the demand for meat, and put greater pressure on agriculture industries to be more efficient (Ruiz, I. B., 2017). The U.S. is the largest broiler chicken industry with over 16% of production being exported to other countries: China, Mexico, Canada, Vietnam, and Cuba (National Chicken Council, 2020). During a period of 50 years, methods of raising poultry have changed more than any other animal production industry. Advancements in vaccination programs, housing conditions, nutritional requirements, and genetic selection have been implemented to provide all of the conditions needed by the birds for optimum performance (McKay, J.C., 2009).

In 1925, a broiler required 112 days to reach a target weight of 2.5 kg; whereas, a commercial broiler today can reach the same weight in ~30 days (National Chicken Council, 2020). The accelerated growth rate continues through present day production and has led to higher meat yields and better feed efficiency. Genetics have been accredited with the majority of performance improvements (85-90%), while nutritional advancements have contributed only 10-15% of these improvements (Havenstein et al., 2003). However, selection problems with a narrow focus and desire for a small number

of traits bring opportunity with negative consequences for traits that are not selected. Some problems which are directly linked to the broilers fast growth rate include: cardiovascular diseases causing mortality by sudden death syndrome and ascites; leg disorders and bone deformations causing leg weakness, lameness, low locomotor activity and extended periods spent sitting or lying, and in return this can produce skin lesions due to contact with moist litter (Bradshaw, Kirkden, and Broom 2002; Bessei 2006; Knowles et al., 2008). Therefore, advancements in nutritional research must coincide with genetic improvements to ensure that the genetic potential of the modern broiler is being attained (Smith and Pesti, 1998).

1.2.2. Post 1994 NRC Modern Commercial Broilers

The latest global trend is the production of "slow-growing" chickens. Widowski (2020) found that many welfare indicators are directly related to growth rate, making slow-growing chickens an option on welfare grounds. The National Chicken Council (2017) estimated that "if one-third of the US broiler industry adapted the slow-growing broiler production, approximately 1.5 billion more broilers would be required annually to meet the current market demands". This increase in amount of broilers would only have a downstream effect and increase the amount of land and resources needed to grow the required feed ingredients. While slow-growing chickens may be beneficial from a welfare perspective, the economics and resources would not be sufficient to be a sustainable switch to slow-growing birds. The focus should be switched to evaluating and determining optimal levels of nutrients amongst modern broiler strains used in poultry production to reach market demands with lower production costs. Current

guidelines with nutritional recommendations for modern broiler crosses are provided by the primary breeder companies, containing more accurate recommended nutrient levels than those suggested by the relatively old 1994 NRC (Leeson, 1994). Even more so, nutritionists often feed diets with excess amounts of nutrients due to limited research done of individual strain requirements and variation due to bird-related factors (genetics, sex, and age) or external factors (stress, temperature, and management). However, the nutritional requirements in broiler feeding could change depending on genetic line and age. Mehaffey et al. (2006), evaluated five commonly used U.S commercial broiler strains and found significant differences in body weight between the strains at various ages. Differences in growth patterns and nutrient requirements (Figure 1-1 and 1-2) can be seen when comparing genetic lines and crosses from primary breeders, but additional differences within individual lines from a single primary breeder company are also present and should be addressed independently.

Figure 1-1 Cobb-500 and Cobb-700 Growth Comparison

Data sourced from: Broiler Performance and Nutrition Supplement Cobb-Vantress

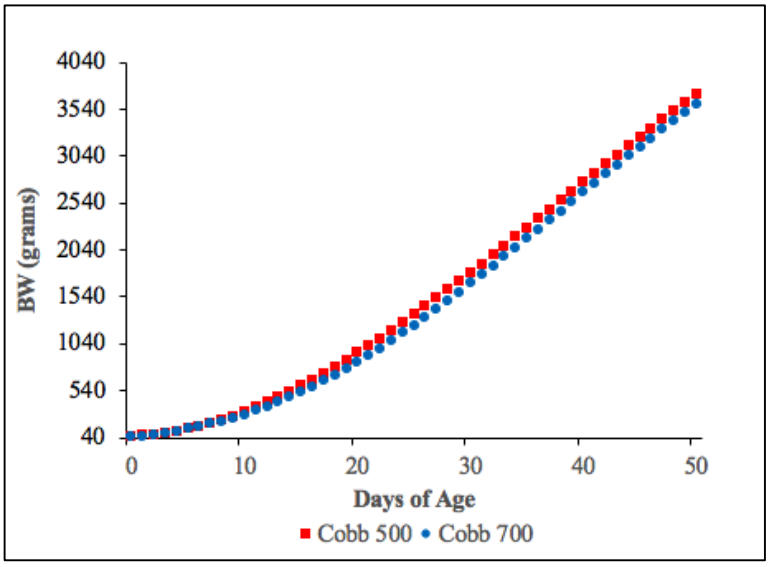
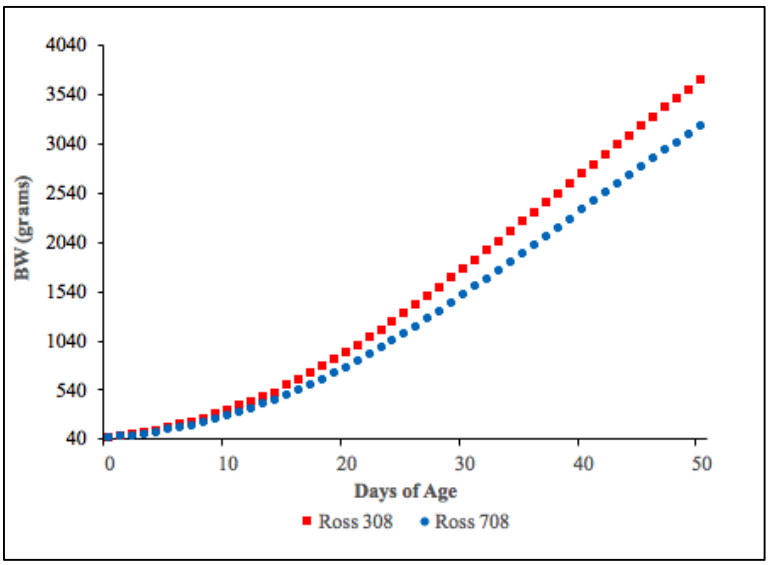


Figure 1-2 Ross-308 and Ross-708 Growth Comparison
Data sourced from: Broiler Performance and Nutrition Supplement Aviagen



The Ross-708 has been developed for large bird or roaster production and has a slower growth rate than the Ross-308 strain that is used for multipurpose production goals. Sterling et al (2006) demonstrated that Cobb broilers grew better with a lower feed conversion ratio when compared with their Ross counterparts. Due to the increase in genetic selection and crosses between strains, there is a need to evaluate the response of these new commercial broiler crosses to different nutritional specifications in order to maximize performance.

Several studies have demonstrated a positive response in broiler performance to increased dietary amino acid density regimens, depending on the strain (Kidd et al., 2004; Corzo et al., 2005; Corzo et al., 2010; Dozier et al., 2006). With significant differences reported in feed intake and feed conversion ratios between different

commercial strains, integrators are constantly looking to optimize production cost by reducing feed cost, which has and continues to be the largest cost in live production (Jackson et al., 1982; Abdullah et al., 2010; Maynard et al., 2019).

While there has been extensive work done in evaluating how modern broiler strains respond to various feeding regimens and varying nutrient densities, there has been little classical work done on vitamin requirements for any class of poultry. This leads the 9th edition of the 1994 NRC list of vitamin requirements as the most recent classical requirements established. In response, some commercial broiler producers routinely provide water-soluble vitamin mixtures (containing D_3) over the first few days of life. This procedure can provide a total vitamin D_3 intake almost double that consumed from the diet alone. This implies that under some conditions the vitamin D_3 needs of the birds may be much higher than the 1994 estimates. It should be noted a national committee of scholars has been established to produce a 10th edition of the Poultry NRC estimated to be published by 2022.

Studies done by Williams et al. (2000a,b) and Bar et al. (2003) have suggested that the calcium requirements of the most modern broiler strains are higher than earlier reported values (NRC 1994). In today's ingredient and micro ingredient pricing environment, this rogue over supplementation likely can no longer be afforded without a quantifiable performance or yield response to nutrient input variation. Ahmad and Roland (2003) and Gous (2014) have suggested that researchers should be discussing nutrient "responses" rather than requirements per se; collectively considering marginal cost of ingredient or nutrient input versus marginal returns of the product.

1.2.3. Vitamin D and It's Metabolites

Vitamin D is a fat-soluble vitamin that involves a group of steroid chemical compounds that possess antirachitic activity. The two most common forms of vitamin D are ergocalciferol (D_2) and cholecalciferol (D_3). Ergocalciferol is found most commonly in plants, fungi, and molds while cholecalciferol is present in animals where vitamin concentration is entirely dependent on dietary D_3 and exposure of the tissue to sunlight (Combs, 2012). The use of windowless houses in most commercial broiler farms does not allow the skin to produce endogenous vitamin D, while most raw ingredients contain minimal or no vitamin D.

To become active, vitamin D_2 or D_3 must undergo a double hydroxylation reaction. The first hydroxylation, results from 25-hydroxylase, which takes place in the liver and forms 25-hydroxy D_3 (25-OH-D_3. The second hydroxylation and the formation of the active form of vitamin D_3, 1,25$(OH)_2$ D_3, occurs in the kidneys and is facilitated by 1 α-hydroxylase (Jones et al., 1998). The active form of vitamin D is thus a hormone. The fundamental role of 1,25$(OH)_2$ D_3 is to control Ca and P homeostasis through direct actions of the hormone on the intestine, kidney, and bones through a feedback inhibition of parathyroid hormone (PTH) production in the parathyroid glands (Pike et al., 2007). Calcitonin also functions as an inhibitor of bone resorption thus leading to decreased plasma Ca concentration. Therefore, vitamin D metabolites play a strong role with respect to Ca and P metabolism and their effects on bone health. In fact, vitamin D_3 has been shown to improve growth of broilers by increasing phytate phosphorus utilization,

so much so that the positive effect on performance is exerted exclusively in P-deficient diets (Beihl and Baker, 1997; Edwards, 2002). Broilers that were fed high concentrations (10,000 IU/kg of D_3) had improved body weight gain, feed intake, and feed efficiency with optimal or suboptimal levels of Ca and non-phytate phosphorus (nPP) (Whitehead et al., 2004; Rao et al., 2006; Rama Rao et al., 2009). Additionally, Rao et al. (2006) recommended that 3,600 IU/kg of vitamin D_3 was needed for adequate growth performance and bone mineralization while using suboptimal levels of Ca and nPP. Increasing vitamin D_3 from 300 IU/kg to 1,200 IU/kg improved BW gain, tibia ash%, and tibia strength in broilers at 17 d and 35 d (Ramo Rao et al., 2019).

In past studies that evaluated the efficiency of commercially available vitamin D_3 sources, broiler requirements ranged from 800 - 1,000 IU/kg of feed depending on the product (Kasim and Edwards, 2000). Vitamin D_3 deficiency has long been associated with rickets, tibial dyschondroplasia, retarded growth, poor feathering, osteomalacia in adult animals, and pliable beaks and claws (Leeson and Summers, 2001). The number of studies that have been done post 1994 suggest supplementations of vitamin D at much higher doses to support rapid growth of modern poultry stains and to prevent the appearance of leg disorders or deficiency symptoms such as rickets.

1.2.4. Bone Formation and Development

There are three types of cells that are found only in bone: osteoclasts, osteoblasts, and osteocytes. Osteoclasts formed from two or more cells that fuse together and typically have more than one nucleus. They originate from the bone marrow and are classically found on the outer surface of the bone mineral next to dissolving bone.

Osteoblasts also originate from the bone marrow, however, they produce bone called "osteoid" which is made of bone collagen and other protein to help build new bone. The matrix of bone contains diverse materials, the relative proportions of which, are dependent on the species, age, and site of the bone and often even the position in a single long bone. Various proteins, glycoproteins, peptides, carbohydrates, and lipids are found in the bone but the bulk (~90%) of the organic component is made up of a single protein known as collagen (Feng, 2009). Osteoblasts regulate the passage of calcium into and out of the bone, and they respond to hormones by making proteins that activate the osteoclasts. Lastly, osteocytes are found inside the bone and originate from osteoblasts while the new bone is being formed. They are surrounded as new bone develops, but they are not isolated; they send out long branches that connect other osteocytes. These cells sense pressure or fractures in the bone and help direct where osteoclasts dissolve the bone. As long bone, the tibia for example, first contains a primary center of ossification. The epiphyses or the secondary centers of ossification appear at the two ends of the bone. In reference, the bone grows inwards from its epiphyseal ends. Once the bone of the main shaft reaches the bone of the epiphysis, growth is no longer possible.

1.2.5. Bone mineralization

Bone measurements, including bone breaking strength (Merkleu, 1981; Ruff and Hughes, 1985; Park et al., 2003; Kim et al., 2006), bone density (Watkins and Southern, 1992; Onyango et al., 2003; Kim et al., 2006), bone mineral content (Akpe et al., 1987; Onyango et al., 2003; Kim et al., (2006), and bone ash (Garlich et al., 1982; Cheng and

Coon, 1990; Park et al., 2003; Shim et al., 2008), have been used as indicators in the mineral nutrition of poultry. The use of tibia ash is recommended in the official assay method of the Association of Official Analytical Chemists for vitamin D sources (Association of Official Analytical Chemists, 1990). Tibia ash also has been considered a response criterion to Ca and P concentrations and most sensitive to deficiencies in either one of these minerals. Several other variables such as stress and strain, bending moments, moment of inertia, and modulus of elasticity can be used to evaluate the mechanical state of the bone. Bending moment is a measure of the amount of force withstood by the bone; whereas, stress is a measure of force per unit area of the bone. Bone breaking strength is measured by evaluating the reaction of the bone to stress and force. Bones with increased mineralization will result in an increase in bone stress and bending moment values. Bone ash is a measurement that can quantify the amount of mineralization in the bone. Chicks with inadequate nutrient amounts will usually have a lower percentage of bone ash than those fed adequate amounts of required nutrients. The amount of ash (inorganic material) present in bone is proportional to its degree of hardness or compression strength; the organic component of bone is important in providing tensile strength and flexibility. It is the balance of these two components that contribute to the breaking strength of the bone (Rath et al., 1999).

1.2.6. Bone Abnormalities in Broilers

Sullivan (1994) estimated the annual losses in the United States due to skeletal issues in broilers to be around $80 to $120 million USD which, if adjusted to annual inflation (U.S. Bureau of Labor Statistics) would represent $147,000,000 to

$221,000,000 in 2020. It is difficult to accurately determine the cost of skeletal problems in poultry due to the cause of these losses. Leg problems seen in broilers can increase mortality and the number of on-farm culls, increase condemnations from septicemia-toxemia, and increase the amount of trimming downgrades of the breasts and legs. The common bone-related conditions, which include TD, rickets, lameness, femoral head necrosis, valgus-varus or "twisted leg", osteomalacia, and ruptured tendons have been reported to be concerns economically and of welfare importance to the industry.

Tibial Dyschondroplasia

This disorder is a common skeletal abnormality found in young rapidly growing meat-type poultry (ducks, chickens, and turkeys) influenced by a combination of genetic selection and nutrition. TD is a bone abnormality at the growth plate arising from a growth disorder in endochondral long bone and lesions associated with TD were first observed by Leach and Nesheim (1965). The onset of TD in broilers is distinct in the pattern of osteoblasts and osteoclasts differentiation and their growth. Jansen's metaphyseal dysplasia is a human disease with a cartilage lesion similar to TD. A mutation in the parathyroid hormone (PTH)/PTH related peptide (PTHrP) receptor gene results in constitutive activation of the receptor (Schipani et al., 1995). It is well established that diets imbalanced for calcium phosphate (marginal calcium and excess phosphate) can induce hyperparathyroidism. Thus, in addition to acting on the kidney and bone tissues, systemic PTH could be influencing chondrocytes through the stimulation of PTH/PTHrP receptors in the cartilage. While mild and moderate lesions may not cause lameness, the proximal end of the tibia may be enlarged. Severe lesions

cause weakening of the proximal tibia which is compressed by the body weight of the bird, causing painful lameness. Reluctance in walking often results in change in feeding behavior and body weight is negatively affected. In addition, the bone is more prone to deformities and breakage especially during processing (Rath et al., 2000); This can lead to downgrading of the carcass or condemnations.

Rickets

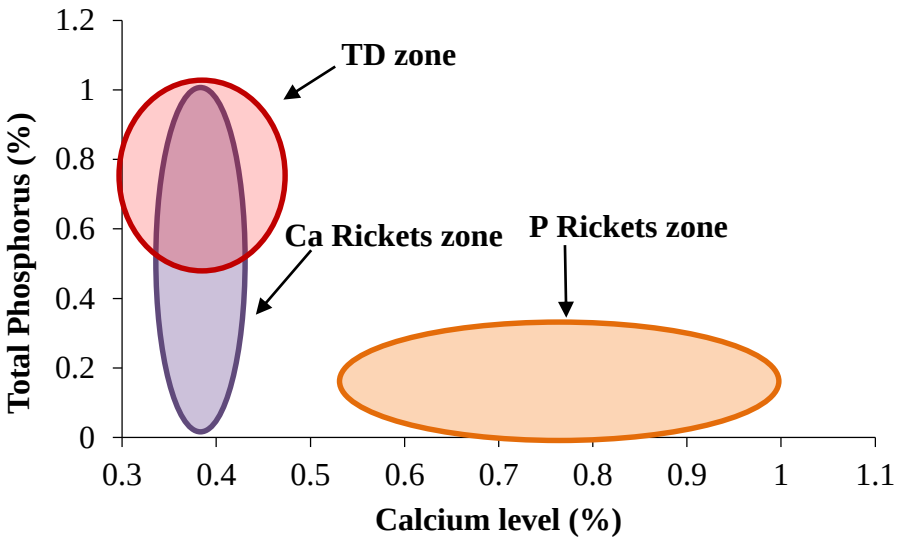
Rickets is characterized by the generalized failure of endochondral ossification or the failure of mineralization of the growth plate. The occurrence of rickets is normally related to disturbances in the levels of Ca, P, or vitamin D and inadequately mixed dietary ingredients that can affect nutrient balance. Illustrated in Figure 1-3 is the combinations of dietary calcium and phosphorus that results in high (over 90%) incidences if these leg problems. There are two types of rickets; hypocalcemic rickets is characterized by an accumulation of proliferating chondrocytes (Jande and Dickson, 1980); and hypophosphatasemia (phosphorus deficiency) rickets in which the hypertrophic chondrocytes accumulate with normal metaphyseal vessel invasion (Lacey and Huffer, 1982).

Calcium deficiency rickets occurs when chicks are fed low-calcium diets (especially under 0.4%), and or diets deficient in vitamin D_3. Phosphorus rickets occur when chicks are fed diets low in phosphorus regardless of the calcium level. Mohammed et al (1991) reported an increase in phytate degradation from 51 to 59% when the cholecalciferol level of a Ca-adequate (1.00%) and P-deficient (0.50%) broiler diet was increased from 12.5 (500 IU/kg) to 1,250 (1,000 IU/kg) μg/kg, respectively. Reducing

the Ca level to 0.50% and adding 12.5 µg/kg of supplemented cholecalciferol resulted in phytate hydrolysis of 65%, while supplementation of 1,250 µg/kg cholecalciferol resulted in a phytate hydrolysis of 77%.

Many studies have shown the beneficial effects of supplemental 1-αOH D_3, 1,25$(OH)_2$ D_3, ultraviolet light exposure or high levels of supplemental vitamin D_3 and 25-OH-D_3, on the reduction of TD and Ca rickets incidence in rapid-growing broilers (Edwards, 1989, 1990; Thorp et al., 1993; Rennie and Whitehead, 1996; Xu et al., 1997; Mitchell et al., 1997a,b; Aburto et al., 1998; Edwards et al., 2004; McComark et al. 2004).

Figure 1-3 Representation from Pesti et al (2005) Calcium and phosphorus combinations that produce incidents of TD, Ca rickets, and P rickets



1.2.7. Calcium and Phosphorus roles in Poultry

Calcium and phosphorus are essential nutrients involved in many biological processes. Both of these minerals are abundant elements within the body, with 99% of

288 Ca and 80% of P stored in the skeleton as hydroxyapatite (Veum, 2010). Both are crucial
289 in bone development and mineralization. Plasma total and ionized Ca concentrations in
290 growing chicken are similar to those observed in mammals (10 mg/dL, 1.2-1.3 mmol,
291 respectively) (Tamin et al., 2004).

292 Chickens fed a Ca-deficient diet had lower plasma Ca and calcitonin
293 concentrations in comparison to chickens fed an adequate-Ca diet (Eliam et al., 1988). In
294 contrast to these findings, Williams et al. (2000) found that selected fast-growing strains
295 showed lower bone-ash content than slow-growing stains. This suggests that current
296 industry diets should be higher in Ca and P than current recommendations (10 g Ca/kg
297 and 4.5 g non-phytate P (nPP)/kg at ages 1 to 21 d) (NRC, 1994) in order to support
298 skeletal development of modern strains. However, most commercial diets are formulated
299 with less than 1% Ca and with a desired Ca and nPP ratio of 2:1. When Ca intake is
300 either high or at adequate concentrations, passive transport of Ca dominates due to
301 inhibition of active Ca transport by high plasma Ca concentrations (Proszkowiec-
302 Weglarz and Angel, 2013). However, the ability to release Ca from the bones is vital for
303 maintaining constant levels of Ca in the blood.

304 ***1.2.8. Units for D_3 and 25-OH D_3***

305 The Poultry NRC (1994) recommends 200 International Units (IU) of vitamin D_3
306 for starter poultry feeds (1 to 21 d). International Units are used to express the biological
307 activity of vitamin D with one IU of vitamin D defined as the activity of 0.025 μg of
308 crystalline cholecalciferol. Thus, a supplement of 200 IU/kg of feed is equivalent to 5 μg
309 of vitamin D_3/kg of feed. Currently, 25-OH-D_3 is used as a commercial feed additive and

supplemented to diet formulations as a source of cholecalciferol, however, there is still disagreement on the equivalence of 25-OH-D_3 in terms of IU activity. The development in technology allowed for the synthesis of 25-OH-D_3 in kilogram quantities in the 1990's. Amoco Bioproducts Corporation began formulating 25-OH-D_3, into hydrogenated vegetable oil-based beadlets and mixing it into a premix using ricehulls as the carrier (Yargar et al., 1995). The basal recommendation level for 25-OH-D_3 was between 50 to 70 µg/kg. However, 69 µg/kg was chosen as the optimal level based on studies conducted by Yargar et al. (1995) which demonstrated that supplementation of 25-OH-D_3 at 69 µg/kg was adequate for maximal WG and feed efficiency when the basal diet contained no cholecalciferol. Differences observed between D_3 sources could be due to particle size which could result in segregation during mixing (Nir, 1996), type of coating material to protect during the pelleting process which could potentially prevent D_3 digestion and absorption (Gribbs et al., 1999; Gharasallaoui et al., 2007)), or lower biological activity than the chemical activity reported by the manufacture (Yang et al., 1973).

1.2.9. Vitamin D_3 in the Maternal Diet and its Effect on the Progeny

Nutritional status of the broiler breeder is important for the performance of the hens but even more so for the transfer of nutrients from the dam to the egg. Mattila et al. (1999) demonstrated the correlation between vitamin D_3 status in the egg and the amount of vitamin D_3 supplied in the diet to the hen. Their studies evaluated three levels of vitamin D_3 fed to laying hens (26.6, 62.4, and 216 µg/kg), and the amount of D_3 found in the eggs at 6 weeks were 1.4, 3.4, and 23 µg/100 g of egg yolk, respectively. The

corresponding values of 25-OH-D_3 in the egg yolk were 0.5, 1.0, and 1.5 µg/100 g of egg yolk. Moreso, Coto et al. (2010) demonstrated the presence of a distinct carryover effect from broiler breeders when fed different combinations of D_3 (0, 300, 600, 1,200, and 2,400 IU D_3/kg) and 25-OH-D_3 (0, 68 µg/kg), which influenced the overall performance, bone development, and incidence of tibia dyschondroplasia in newly hatched chicks. Another study done by Edwards (1995) used the progeny of laying hens fed various levels of vitamin D_3 (0 to 2000 IU/kg). Results showed that when the maternal diet had 500 IU/kg of vitamin D_3, the chicks could not reach maximum growth or bone ash when fed a diet supplemented with various levels of vitamin D_3 (0 to 200 IU/kg). However, when the maternal diet contained 2,000 IU/kg, chicks had increased growth and bone ash when fed the diet supplemented with vitamin D_3 (0-200 IU/kg). However, limited research has been conducted using the progeny of broiler breeders, even though bone abnormalities are still a problem in the poultry industry. Studies have been done on the manipulation of young broiler chick diets with the objective of reducing bone disease; however, further research is needed to determine the role of maternal vitamin D status of the hens on the performance of the progeny.

1.2.10. Nutritional Requirements of Vitamin D_3 and 25-OH- D_3 for chickens

Historically, vitamin D inclusion levels for commercial broilers far exceed what is typically reported as the requirement by the NRC (200 IU/kg of feed). However, the intensive production growout systems impose higher metabolic stress on the bird. In turn, this has led to increased vitamin deficiencies or increased vitamin requirements. Nutritionists typically formulate diets with vitamins in accordance with the following

criteria: (a) determining factors on live production growout which impact vitamin supplementation; (b) determining appropriate vitamin nutrition with minimum feed cost; (c) determining a "safety factor" for vitamin levels (Coelho and McNaughton, 1995).

Currently, D_3 metabolites such as 1- α-hydroxycholecalciferol (1α(OH) D_3) and 25-OH-D_3 are readily used as dietary supplements. At comparable levels of D_3, the 25-OH-D_3 isomer has been generally shown to improve performance and skeletal health (Cantor and Bacon, 1978; Fritts and Waldroup, 2003; Yager et al., 1995). Additionally, supplementation with 25-OH D_3 has been reported to significantly increase tibial mineralization of broilers (Ferket et al., 2009; Santiago et al., 2016; Wideman et al., 2015). A possible candidate mechanism for the increase in bone mass is by the well-known vitamin D stimulation of intestinal absorption of Ca and P. Additionally, supplementation of 25-OH-D_3 at 2,760 IU/kg has been shown to increase villi length and decrease crypt depth in broilers (Chou et al., 2009). Increasing villi length is linked to increased nutrient absorption and decreased crypt depth is associated with less frequent epithelial turnover (Yang et al., 2008). Ledwaba and Roberson (2003) studied the efficacy of 25-OH-D_3 in low-Ca diets; the inclusion of 70 μg/kg 25-OH-D_3 had a positive effect in preventing TD in birds fed diets marginally deficient in Ca.

Not all of the vast amounts of commercially available D_3 supplements will have the same potency nor will they be equally bioavailable to the bird. Comparison of biological activity between given nutrient sources is usually expressed as relative bioavailability (RBV), which is "the ratio between the amount of the standard and testing source required to produce equivalent response" (Littell et al., 1995; Finney,

1978). When comparing the RBV of vitamin D_3 (Qian et al., 1997) and 1α-OH-D_3 (Han et al., 2012), both have high activity at low levels of dietary Ca and P. However, when D_3 is used in poultry, the maternal D_3 carryover effect appears to play a key role in the response of the progeny to D_3 supplementation.

2. EVALUATION OF DIETARY CHOLECALCIFEROL (D_3) AND HIGHLY CONCENTRATED 25-OH-D_3 SOURCES ON COBB-700 BROILER PERFORMANCE, TIBIA ASH, AND TIBIA BREAKING STRENGTH

2.1. INTRODUCTION

Continuous genetic selection in meat-type chickens has helped to increase productivity due to improvements in growth potential and feed efficiency. However, the improvement to performance has inadvertently caused broilers to suffer from bone abnormalities and disorders, which results in high economic loss (Abbasi et al. 2017). It is well acknowledged that vitamin D enhances Ca and P absorption in the small intestine and helps to maintain optimal Ca and P homeostasis (Haussler et al. 2013). This relationship between vitamin D to help regulate calcium (Ca) and phosphorus (P) metabolism plays a crucial role in the prevention of rickets and tibial dyschondroplasia (Edwards, 2000).

Vitamin D supplementation is most commonly provided as cholecalciferol or vitamin D_3. Once absorbed, D_3 is hydroxylated in the liver to form 25-hydroxycholecalciferol (25-OH-D_3) which is transported via the blood to various tissues. Once it reaches the kidney it must undergo further hydroxylation into 1,25 dihydroxycholecalciferol (1,25-$(OH)_2$-D_3), which is considered the “most active” hormonal form of the vitamin. With current industry operations, poultry species have limited access to sunlight, therefore, limiting the amount of D_3 that can be synthesized in the skin after being exposed to UV-light from the sun. Since the commercialization of 25-hydroxycholecalciferol (25-OH-D_3), there have been growing interests in the use of

406 this vitamin D metabolite as a source of vitamin D activity in poultry feed formulations.
407 The use of 25-OH-D_3 circumvents the 25-hydroxylation reaction in the liver and has
408 demonstrated a higher bio-potency compared with cholecalciferol, which has been
409 confirmed among others for humans, pigs, and broilers (Cashman et al. 2012; Coffey et
410 al. 2012; Han et al. 2016).

411 Previous research done in our laboratory on the bioavailability of D_3 sources have
412 found considerable variability in commercial broiler chick responses to dietary D_3
413 (Leyva-Jimenez, 2015). Dietary vitamin D_3 in the breeder flock will affect egg shell
414 thickness, egg productions and egg mass that ultimately will affect the performance of
415 the hatched progeny (Coto *et* al. 2010a). Maternal D_3, found in the yolk, appears to play
416 a key role in the early performance of young broilers and therefore, in their response to
417 supplemental D_3 as suggested by previous literature reports (Moran, 2007; Coto et al.,
418 2010a; Coto et al., 2010b; Saunders-Blades and Korver, 2014). Another study done
419 previously in our lab using diets with normal levels of Ca and P but devoid of vitamin D
420 suggests that maternal reserves will deplete around day 10 of age but still observed high
421 variability when percent bone ash was used to evaluate vitamin D activity (Leyva-
422 Jimenez, 2015).

423 Hence, the present study was conducted with the objective of comparing the
424 effect of dietary levels of commercially available D_3 and two commercially available
425 sources of 25-OH-D_3 supplementation on performance, bone mineralization, and vitamin
426 D status in Cobb-700 broilers when fed a vitamin-D-devoid diet with a marginal
427 concentration of Ca (0.75%) and nPP (0.365%).

2.2. MATERIALS AND METHODS

2.2.1. Birds, Diets and Management

All methods in this experiment were approved by the Institutional Animal Care and Use Committee of Texas A&M University (AUP: IACUC 2017-0072). Three hundred newly-hatched off sex male Cobb-700 broiler chicks were purchased from a commercial hatchery, individually wing banded, and allocated in 2 stainless steel battery brooders (~10 birds per cage). For the first 9 days, chicks were fed a basal D_3-deficient corn-soy broiler starter diet *ad libitum* to serve as a depletion phase of the maternal stores of D_3 followed by a 12-h fasting period. On day 10 of the trial, the birds were weighed in groups of 20 and an average body weight (BW) was calculated. The average BW was then used to create 48 groups of five chickens (n=240) with close to "identical" starting body weight and variance. Broilers were re-allocated into two stainless steel battery brooders (5 birds per cage) using a completely randomized block design. Battery pen level (4 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 environmentally controlled 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). From d 10 to the end of the trial, birds were offered one of the ten dietary treatments. Dietary treatments were offered for 7 days and water was offered *ad libitum* using nipple drinkers. Birds were monitored daily with regards to general flock condition,

449 temperature, lighting, water, feed, and any unanticipated events inside the rearing
450 facility.

451 ***2.2.2. Dietary Treatments***

452 A basal D_3-deficient corn-soy broiler starter diet was formulated based on the
453 nutrient recommendations of Cobb-700 (Cobb-Vantress. 2012) and a custom vitamin/
454 mineral premix containing no vitamin D_3 and corn oil as the fat source (Table 2-1). Diets
455 were formulated with a marginal concentration of Ca (0.75%) and (0.375%) non-phytate
456 phosphorus (nPP) to increase the sensitivity of our response variables to the
457 supplemental D_3 and 25-OH-D_3 products. The basal diet was then subdivided into 10
458 equally sized batches and supplemented with 0, 100, 200, or 400 IU D_3/kg (0, 2.5, 5, or
459 10 μg D_3/kg) of diet based on the labeled concentration of two sources of 25-OH-D_3
460 (Rovimix Hy-D; DSM Nutritional Products, Parsippany, NJ or 25-
461 hydroxycholecalciferol; Orffa, Henderson, NJ) and one source of D_3 (Rovimix D_3 500;
462 DSM Nutritional Products, Parsippany, NJ) which was used as the D_3 control. To create
463 each treatment diets, a specific amount of either 25-OH-D_3 or D_3 from each source was
464 weighed and directly mixed with the basal diet for 12 minutes using a stainless steel
465 mixer (model L-800, Hobart Corp. Troy, Ohio, U.S.A). The 0 IU D_3/kg of diet treatment
466 served as the common negative control (NC) group for all sources.
467
468
469
470

Table 2-1 Basal D_3 Deficient Diet

Ingredient	Basal Diet[1] (%)
Yellow corn	63.76
Dehulled soybean meal	31.15
DL-methionine	0.32
L-Lysine HCl	0.28
L-Threonine	0.08
Corn oil	1.28
Limestone	1.07
Monocalcium phosphate	1.20
Sodium chloride (salt)	0.36
Customized vitamin-mineral premix[2]	0.50

[1]Calculated nutritional content was as follow: 22% crude protein, 3,035 kcal/kg metabolizable energy, 0.75% calcium, 0.37% non-phytate phosphorus, 0.65% methionine, 1.01% methionine + cystine, 1.36% lysine, 0.26% tryptophan, 0.89% threonine, 1.43% arginine, 3.19% crude fat, 2.14% crude fiber, 0.16% sodium, 0.91% potassium, 0.31% 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.

Table 2-2 Experimental Treatments

TRT	Source	Product Type	IU D_3/kg	µg/kg of 25-OH D_3	n
T1	DSM	D_3	100	2.5	25
T2	DSM	D_3	200	5	25
T3	DSM	D_3	400	10	25
T4	HY-D	25-OH D_3	100	2.5	25
T5	HY-D	25-OH D_3	200	5	25
T6	HY-D	25-OH D_3	400	10	25
T7	ORFFA	25-OH D_3	100	2.5	25
T8	ORFFA	25-OH D_3	200	5	25
T9	ORFFA	25-OH D_3	400	10	25
T10 (Control)	--	--	--	--	15

NRC (1994) Cholecalciferol requirement for starting meat-type chicken (200 IU/kg feed or 5 ug/kg of feed)

*Bioactivity of all products is based on a 1:1 conversion of 1 IU D_3=0.025 ug cholecalciferol

2.2.3. Performance Evaluation

Feed intake (FI) and body weight (BW) per pen were recorded on day 10, after the fasting period, and on day 17 of the trial 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.

2.2.4. Vitamin D Status

Blood samples were drawn from two birds per pen on day 17 of the trial. Blood samples were collected within 3-ml Vacutainer tubes and centrifuged at 2,000 x *g* for 15 min at 5°C immediately after collection. The serum samples were aliquoted within Eppendorf tubes and stored at -20°C until 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 0-150 ng/mL). Plasma samples were run in triplicate and results are reported as ng/mL.

2.2.5. Bone Mineralization and Analysis

On day 17 of the experiment, three birds per pen were euthanized using CO_2, labeled, and immediately transported to the Texas A&M Applied Exercise Science Laboratory to perform a whole-body analysis using a Prodigy Dual X-ray absorptiometry (DXA) scan (GE Lunar Prodigy Advance bone densitometer, General Electric-Healthcare, Boston, Massachusetts, U.S.A.). Chickens were placed in prone position with their wings and legs at the sides of the body. Data were analyzed using the small animal software (GE Lunar Prodigy Advanced enCore, V 16.0, GE-Healthcare,

Boston, MA), which is specifically designed for animals <20 kg. Bone mineral content (BMC) is defined as the total bone mineral that is found in a specific area and measured in grams. Bone mineral density (BMD) is derived by using BMC (g) and dividing by an area (cm^2) of interest. At completion of the DEXA scans total BMC and BMD per pen were calculated. After the DEXA scan and serum collection, both tibias from all birds (n=5) were removed, labeled, and stored in a freezer (-20°C) until further analysis. Right tibiae were defatted in petroleum ether for 48 h. Defatted bones were then dried in a force draft oven (95°C) until they reached constant weight (~48 h). The dried bones were ashed overnight at 650°C for 23 h. Percent tibia ash (TBA) was calculated using the starting dry bone weight and remaining ash weight. The left tibiae were cleaned from any remaining tissue and used to assay (raw) 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 3-cm constant span.

2.2.6. Statistics

All data was analyzed as a (3 x 3) factorial using the GLM procedure of SPSS. Source, concentration, and source*concentration was used as fixed factors in the model. Main effects were analyzed using a 2-way ANOVA. Means were separated by Duncan's multiple range test when appropriate. Linear and quadratic effects of graded levels of D_3 and 25-OH-D_3 were investigated by regression analysis. Broken-line regression was used to analyze the tibia breaking strength and percentage bone ash using the "Nutritional Response Model Program" Version 1.01 from Gene Pesti and Dmitry

Vedenov, University of Georgia. The 0 μg/kg D_3 treatment served as our control reference treatment and used to establish a common baseline for the regression analysis on bioavailability evaluation. 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.

2.3. RESULTS AND DISCUSSION

2.3.1. Performance

Performance results are presented in Table 2-3 and Table 2-4. With respect to weight gain, feed conversion, and feed intake, no significant differences ($P>0.05$) were found between source or level. This suggests that independently of the source of vitamin D_3, the supplementation of 100, 200 and 400 IU 25-OH/kg feed had a positive effect in the performance of growing broiler chicks. There also were no significant source by level interactions for any of the measured performance variables.

Performance results obtained in this study suggests that the 9-d depletion prior to the introduction of the dietary treatments and lowering the Ca and nPP content was not effective in creating a severe D_3 deficiency, possibly due to the high maternal reserves of D_3. The 9-day depletion period was established based on previous chick depletion experiments (Leyva-Jimenez, 2015), and results were in agreement with Aslam el al. (1998) who reported that maternal D_3 stores were depleted by 7 days of age. With commercial breeder diets containing high dietary concentrations of fat-soluble vitamins, this poses the biggest challenge when evaluating vitamin D_3 requirements using "commercial" broiler chicks.

Table 2-3 Effect of Dietary Vitamin D_3 and 25-OH-D_3 on Performance

		Response[1,2]			
Treatment IU D_3/kg	**n**	**17d BW***	**10-17d WG****	**10-17d FI**	**10-17d FCR**
DSM: 100	5	493 ± 13.5	304 ± 13.7	409 ± 16.9	1.35 ± 0.07
DSM: 200	5	496 ± 13.2	306 ± 12.2	383 ± 38.0	1.34 ± 0.07
DSM: 400	4	489 ± 8.2	302 ± 9.9	401 ± 23.1	1.32 ± 0.03
Hy-D: 100	5	494 ± 17.3	305 ± 16.7	415 ± 7.4	1.31 ± 0.05
Hy-D: 200	5	493 ± 4.0	304 ± 5.4	406 ± 6.7	1.35 ± 0.02
Hy-D: 400	5	488 ± 16.6	300 ± 14.0	401 ± 25.9	1.39 ± 0.07
Orffa: 100	5	494 ± 9.4	306 ± 9.8	379 ± 30.4	1.38 ± 0.12
Orffa: 200	5	500 ± 7.8	311 ± 6.8	391 ± 34.0	1.33 ± 0.04
Orffa: 400	5	500 ± 18.1	312 ± 18.1	425 ± 29.5	1.30 ± 0.06
Main Effects					
Source					
DSM	14	492 ± 13.1	304 ± 12.7	398 ± 28.1	1.34 ± 0.06
Hy-D	15	492 ± 13.3	303 ± 12.2	407 ± 16.0	1.35 ± 0.06
Orrfa	15	498 ± 12.0	309 ± 11.8	399 ± 35.2	1.33 ± 0.08
Pvalue		*0.174*	*0.191*	*0.526*	*0.731*
Level					
100	15	494 ± 12.8	305 ± 12.7	401 ± 24.9	1.35 ± 0.08
200	15	496 ± 9.0	307 ± 8.5	393 ± 29.1	1.34 ± 0.05
400	14	492 ± 16.6	304 ± 15.7	409 ± 27.3	1.33 ± 0.07
Pvalue		*0.340*	*0.490*	*0.310*	*0.804*
Source*Level					
Pvalue		*0.535*	*0.620*	*0.082*	*0.119*

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

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

[2]Values for performance responses represent the mean average of n=5 replicate pens per treatment of 5 birds each at day 17.

*All treatments significantly different from the negative control (P=0.0178).

**All treatments significantly different from the negative control (P=0.0244).

Negative Control n=3 BW, WG, FI, and FCR were 456, 269, 426, 1.39, respectively.

2.3.2. Bone Mineralization

The effect of dietary vitamin D_3 and 25-OH-D_3 on broiler bone mineralization is presented in Table 2-4. Differences ($P<0.05$) in TBS were observed between source with the two sources of 25-OH-D_3 having an increased TBS compared to the D_3 source. No significant interaction was found between sources at any of the three increasing concentrations with regard to BMD, BMC, or TBA. Overall, BMD and BMC were not good indicators of D_3 activity in growing broilers based on the TBA for the 0 IU treatment group which averaged 48.9%. Compared to the other treatment groups, this equates to <1% decrease in tibia bone ash. The 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-Jimeneze et al., 2018) which resulted in a more consistent performance and bone mineralization response to graded levels of D_3. The results of this study suggest that a D_3 deficiency was not effectively reached in this experiment and that the maternal stores of D_3 in the yolk hindered the sensitivity of the response variables.

Table 2-4 Effect on Dietary Vitamin D3 and 25-OH-D_3 on Bone Mineralization of 17d Old Broiler Chickens

		Response[1,2]			
Treatment IU D_3/kg	**n**	**TBA**	**TBS**	**BMD**	**BMC**
DSM: 100	5	48.4 ± 1.3	8.9 ± 8.9^{cd}	0.04 ± 0.01	1.52 ± 0.59
DSM: 200	5	48.3 ± 0.8	8.7 ± 0.5^{d}	0.05 ± 0.00	2.12 ± 0.33
DSM: 400	4	49.1 ± 3.0	9.6 ± 1.9^{d}	0.05 ± 0.00	1.92 ± 0.34
Hy-D: 100	5	49.4 ± 1.1	9.0 ± 0.6^{cd}	0.05 ± 0.00	2.04 ± 0.34
Hy-D: 200	5	50.0 ± 1.9	10.2 ± 0.7^{ab}	0.10 ± 0.09	2.20 ± 0.41
Hy-D: 400	5	50.4 ± 2.0	10.8 ± 0.8^{ab}	0.06 ± 0.01	2.42 ± 0.44
Orrfa: 100	5	49.0 ± 2.7	9.9 ± 0.8^{bc}	0.06 ± 0.01	1.86 ± 0.34
Orrfa: 200	5	52.0 ± 1.5	10.9 ± 0.7^{ab}	0.05 ± 0.02	2.08 ± 0.74
Orrfa: 400	5	50.0 ± 2.5	11.3 ± 1.0^{a}	0.06 ± 0.01	2.36 ± 0.78
Main Effects					
Source					
DSM	14	48.6 ± 2.28^{b}	9.1 ± 0.67^{c}	0.05 ± 0.01	1.85 ± 0.49
Hy-D	15	50.0 ± 1.64^{ab}	10.0 ± 1.05^{b}	0.07 ± 0.06	2.22 ± 0.37
Orrfa	15	50.2 ± 2.39^{a}	10.7 ± 0.97^{a}	0.05 ± 0.01	2.10 ± 0.64
Pvalue		*0.064*	*0.000*	*0.177*	*0.132*
Level					
100	15	49.0 ± 2.25	9.3 ± 0.88^{b}	0.04 ± 0.01	1.81 ± 0.44^{b}
200	15	49.9 ± 2.11	10.0 ± 1.14^{ab}	0.07 ± 0.06	2.13 ± 0.49^{ab}
400	14	49.8 ± 2.35	10.6 ± 1.32^{a}	0.05 ± 0.01	2.23 ± 0.58^{a}
Pvalue		*0.458*	*0.003*	*0.257*	*0.079*
Source*Level					
Pvalue		*0.663*	*0.057*	*0.562*	*0.764*

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

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

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

Negative Control n=3 TBA, TBS, BMD, and BMC were 48.9 ± 0.46, 7.9 ± 0.8, 0.03 ± 0.01, and 1.47 ± 0.32, respectively.

2.3.3. Vitamin D Status

Vitamin D status (VDS) in terms of serum 25-OH-D_3 concentration was found significant between level and source for 17-d broiler chickens. Chicks fed the Hy-D source (T4-T6) had a higher concentration of D_3 than source 1 or 3. However, the

supplementation levels of either 200 or 400 IU 25-OH/kg of feed significantly increased serum concentration status. 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. While not significant, we did observe a linear increase in sera vitamin D status for each treatment source.

Table 2-5 17d Broiler Serum Status

Treatment IU D_3/kg	N	Serum status (ng/mL)
DSM: 100	5	13.9 ± 4.7
DSM: 200	5	16.8 ± 4.7
DSM: 400	4	14.8 ± 1.9
Hy-D: 100	5	17.2 ± 3.5
Hy-D: 200	5	20.1 ± 6.0
Hy-D: 400	5	25.0 ± 2.3
Orffa: 100	5	14.3 ± 3.7
Orffa: 200	5	17.6 ± 3.9
Orffa: 400	5	20.6 ± 1.9
Main Effect		
Source		
DSM	14	15.2 ± 1.0[b]
Hy-D	15	20.8 ± 1.0[a]
Orrfa	15	17.5 ± 1.0[b]
Pvalue		*0.001*
Level		
100	15	15.2 ± 1.0[b]
200	15	18.2 ± 0.9[a]
400	14	20.1 ± 1.0[a]
Pvalue		*0.004*
Source*Level		
Pvalue		*0.214*

[a–c]Means within the same column without a common superscript differ ($P < 0.05$) by Duncan's multiple range test.
Negative control n=3 Serum was 14.7 ± 1.8.

2.3.4. Regression Estimation Using Regression Models

To estimate the vitamin D_3 requirements with better precision, TBA and TBS response of growing broiler chickens to graded levels of D_3 and 25-OH-D_3 was fitted to two different regression models and presented in table 2-6. The performance variables were not reported as criteria to estimate the D_3 requirement due to the low response to graded levels of dietary D_3 and 25-OH-D_3. TBA was unable to fit the linear broken line for vitamin D_3 but the linear broken line for 25-OH-D_3 resulted in a requirement of 469 IU/kg. The quadratic model yielded 252 and 251 IU/kg for TBA for vitamin D_3 and 25-OH-D_3 respectively. The linear broken line for TBS yielded 273 and 218 IU/kg for vitamin D_3 and 25-OH-D_3, with an improvement in R^2 observed for the highly concentrated source of 25-OH-D_3. An increase in the vitamin D_3 requirement is to be expected with a reduced Ca and non-phytate phosphorus (nPP) in the diets offered during this experiment. Changes in nPP in broiler diets can increase the D_3 requirement up to 1,500 IU/kg (Baker et al., 1998.).

Table 2-6 Vitamin D_3 and 25-OH D_3 Requirements and Model Comparison

	Response	Model	ER[1]	R^2(%)	P value
Vitamin D_3	TBA	Linear Broken Line	N/A	N/A	N/A
		Quadratic	N/A	N/A	N/A
	TBS	Linear Broken Line	273	26.9	0.0604
		Quadratic	250	29.7	0.1648
25-OH D_3	TBA	Linear Broken Line	N/A	N/A	N/A
		Quadratic	251	8.70	0.2458
	TBS	Linear Broken Line	218	63.9	0.0000
		Quadratic	250	66.5	0.0000

[1]Estimated requirement, ER (IU D_3/kg of feed)

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613 **Figure 2-1 25-OH-D_3 Bone Ash Quadratic Broken Line**

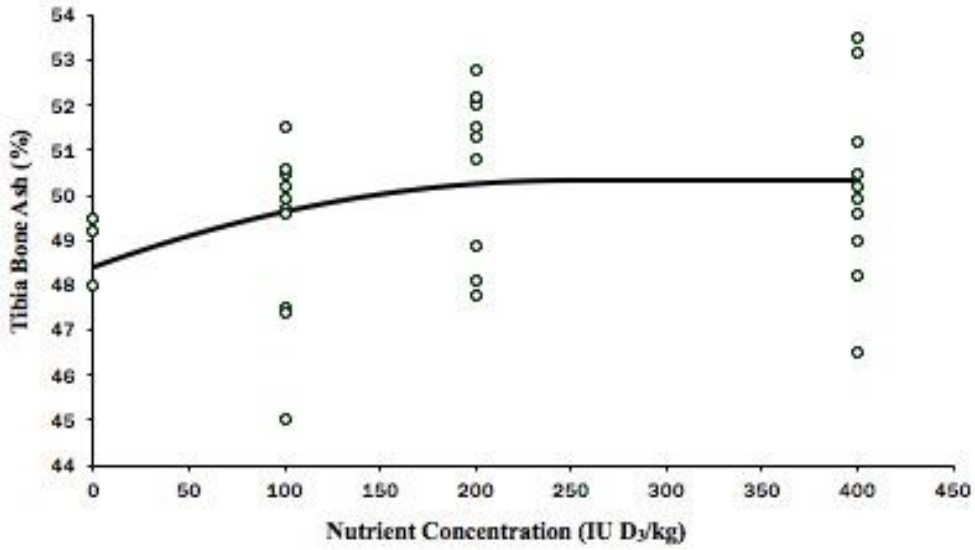

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Figure 2-2 Vitamin D_3 Breaking Strength Linear Broken Line

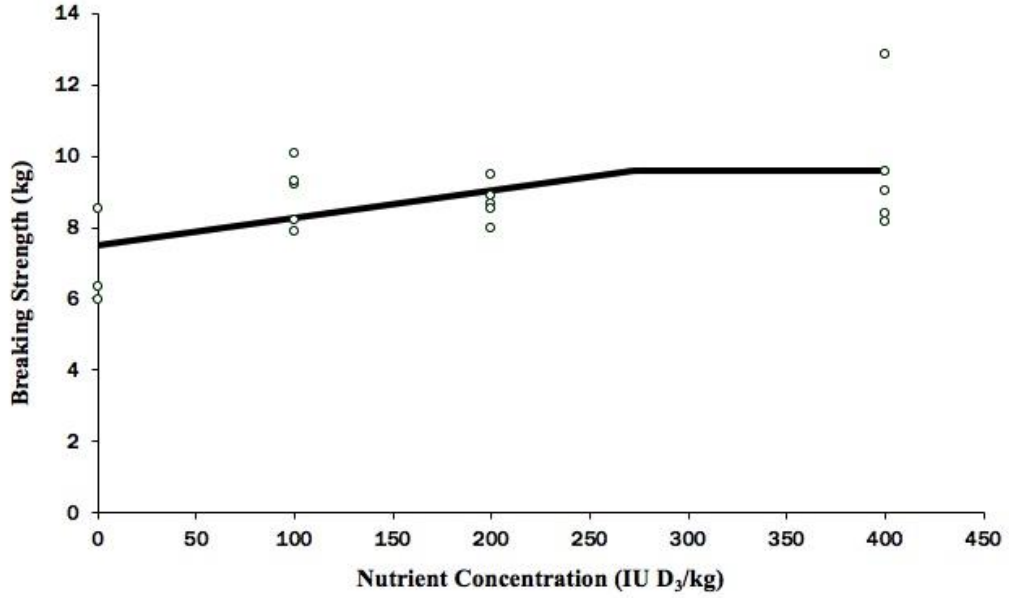
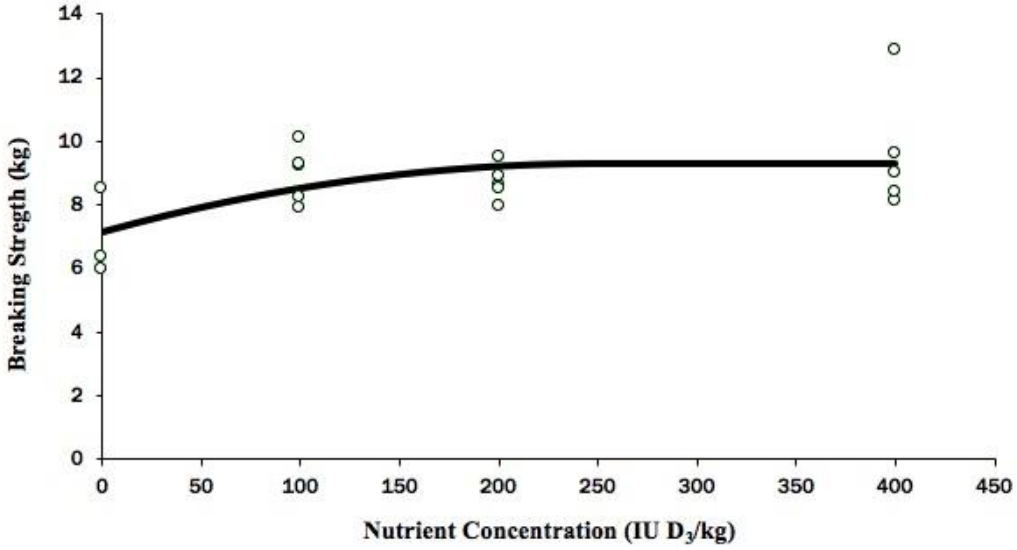
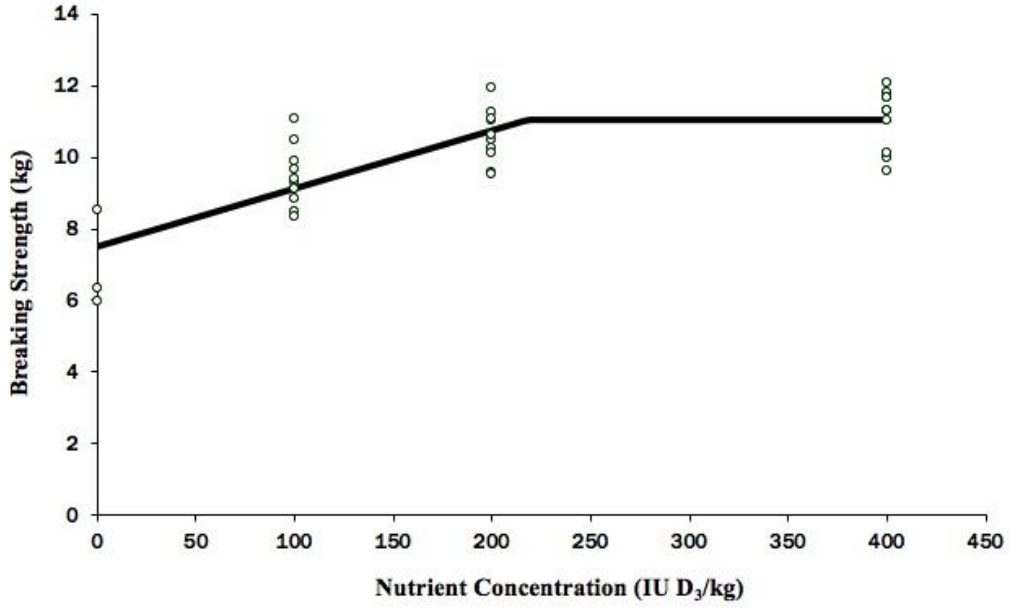


Figure 2-3 Vitamin D_3 Breaking Strength Quadratic Broken Line



622 **Figure 2-4 25-OH-D_3 Breaking Strength Linear Broken Line**



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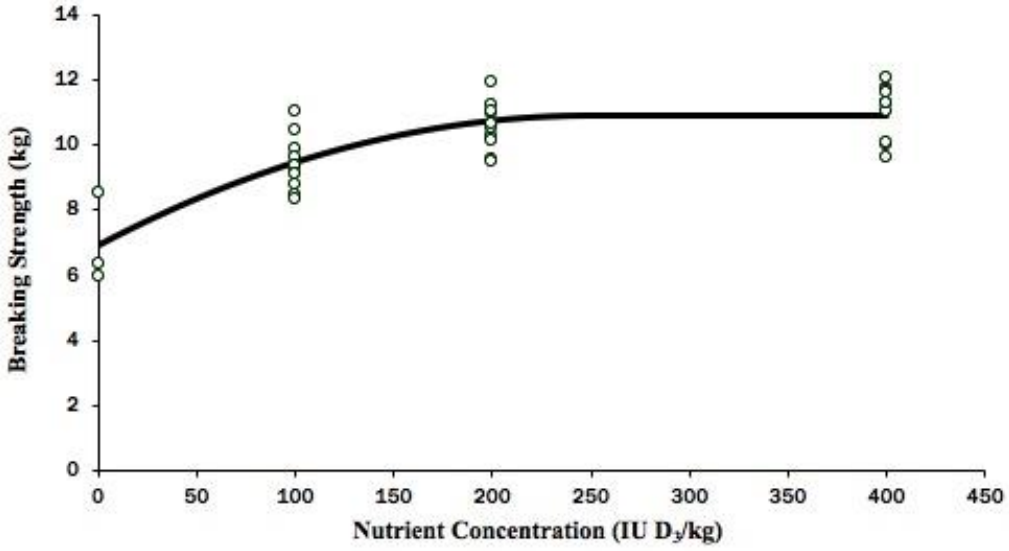
624 **Figure 2-5 25-OH-D_3 Breaking Strength Quadratic Broken Line**



625

626 ***2.3.5. Discussion***

627 The present study was conducted to compare vitamin D_3 and 25-OH-D_3 as
628 sources of vitamin D activity when supplemented to growing broiler chicks. Historically,
629 bioavailability determination of D_3 supplementation has relied on the AOAC (932.16)
630 chick bioassay (AOAC International, 1990). However, changes in current feeding
631 practices and nutritional responses of modern broiler strains has led to necessary re-
632 evaluation of not only nutrient requirements but modern broiler strain responses as well.
633 In this experiment, overall performance was not negatively affected by supplementation
634 of the diet with vitamin D_3 or 25-OH-D_3. Similar results were seen by Bar et al. (2003)
635 who conducted five experiments comparing supplementation of dietary 25-OH-D_3 to D_3
636 alone on broiler performance. Only one of these experiments improved performance,
637 while growth response in the other experiments was similar between the dietary D_3
638 sources. The experiment resulting in improved performance, utilized a diet with reduced
639 Ca and nPP which positively influence performance with supplemental dietary 25-OH-
640 D_3. Similar to results in this study, dietary 25-OH-D_3 had no effects on BW, WG or FCR
641 relative to vitamin D_3 at the same level of activity (Vignale et al., 2015; Angel et al.,
642 2006; Fitts and Waldroup, 2005; Roberson et al., 2005 and Bar et al., 2003). In contrast,
643 dietary 25-OH-D_3 increased BW (Yagar et al., 1995; Fitts and Waldroup, 2003) and
644 decreased FCR compared to vitamin D_3 at 2,760 IU/kg of feed (Yarger et al., 1995).
645 Differences in growth performance among studies is not clear but high maternal reserves
646 of D_3 found in the egg yolk highly influences the vitamin D_3 requirement in the progeny.
647 Additionally, the lowered Ca and available P (0.75 and 0.37% respectively), with the

intent of increasing the pressure of dietary D_3 and 25-OH-D_3 treatment, resulted in similar responses and no deficiency signs were observed.

Studies have shown that vitamin D_3 (Rao et al., 2009) and 25-OH-D_3 (Aburto et al., 1998) increase bone wight, length, and ash in broilers which further indicate that vitamin D metabolites increase bone growth and mineral deposition. Furthermore, Fritts and Waldroup (2003) showed that 25-OH-D_3 is more potent than D_3, however, when 25-OH-D_3 is compared to vitamin D_3, its potency depends on the levels of vitamin D_3 being tested. In agreement with these studies, data collected from this trial demonstrated that 25-OH-D_3 significantly improved TBS when compared to D_3 at increasing levels. However, a D_3 deficiency was not observed in our control group which highly reduced the sensitivity of the assay.

In conclusion, this study showed that supplementation level of 25-OH-D_3 resulted in higher TBS than that of the vitamin D_3 source. Differences due to level were also observed with 400 IU 25-OH/kg having significantly higher TBS than the two other levels. With respect to BW, WG, and FCR, no significant differences were found between sources or level. This suggest that independently of the source of vitamin D_3, the supplementation of 100, 200, or 400 IU 25-OH/kg feed had a positive effect in the performance of growing broiler chicks.

3. EVALUATION OF DIETARY CONCENTRATED 25-OH-D_3 SOURCES ON COBB-500 BROILER PERFORMANCE, TIBIA ASH, AND TIBIA BREAKING STRENGTH

3.1. INTRODUCTION

With a consumer driven market there have been changes in poultry meat market trends that have shifted from the purchase of whole chicken to carcass parts, especially further processed broiler breast meat (Mehaffey et al., 2006; Abdullah et. al., 2010). This has impacted the poultry industry to put emphasis on improving breast meat yield and muscle mass development. The United States commercial broilers are dominated by birds originating from either Ross-708 and Cobb-500 female lines. Commercial broilers can be categorized into fast- and slow-feathering, however, independent of feathering rate, broiler strain plays a significant role in growth rate. Research was conducted by Zhai et al. (2013) evaluating amino acid needs showing Cobb-500 boilers had higher body weight and lower FCR than Cobb-700 broilers at 14 and 28 days when fed the same diet. This indicates Cobb-500 broilers have a higher early growth rate; whereas, the Cobb 700 broilers have a more rapid growth in the later period of their growth curve.

When comparing the Cobb-500 Broiler Performance & Nutrition Supplement Guides (Cobb-Vantress, 2013, 2015, 2018) at day 42, they projected a live body weight at 6.02, 6.30, and 6.51lbs, respectively. However, with the increased body weight and stress on the structural frame of the Cobb-500 bird, the recommended vitamin D_3 has not changed from the minimal amount of 5 million international units (MIU) per ton during

this 5-year window. 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) are necessary for optimal growth and prevention of skeletal disorders. The objective of this study was to evaluate the relative bioavailability of three commercially available sources of 25-OH-D_3 supplementation on performance and bone mineralization in Cobb-500 broilers when fed a vitamin-D-devoid diet with a marginal concentration of Ca (0.75%) and nPP (0.375%).

3.2. MATERIALS AND METHODS

3.2.1. Birds, Diets and Management

All methods in this experiment were approved by Texas A&M Institution Animal Care and Use Committee (AUP: IACUC 2017-0072). Three hundred newly-hatched off sex male Cobb-500 broiler chickens were purchased from a commercial hatchery, individually wing banded, and allocated in 2 stainless steel battery brooders (~10 birds per cage). For 9 days chicks were fed a basal D_3-deficient corn-soy broiler starter diet *ad libitum* to serve as a depletion phase of the maternal stores of D_3 followed by a 12-h fasting period. On day 10 of the trial, the birds were weighed in groups of 20 and an average body weight (BW) was calculated. The average BW was then used to create 48 groups of five chickens (n=240) with close to "identical" starting body weight and variance. Broiler were grouped and placed in two stainless steel battery brooders (5 birds per cage) using a completely randomized block design. Battery pen level (4 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 environmentally controlled 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). From day 10 to the end of the trial, birds were offered one of the ten dietary treatments. Dietary treatments were offered for 11 days and water was offered *ad libitum* using nipple drinkers. Birds were monitored daily with regards to general flock condition, temperature, lighting, water, feed, and any unanticipated events inside the rearing facility.

3.2.2. Dietary Treatments

A basal D_3-deficient corn-soy broiler starter diet was formulated based on the nutrient recommendations of Cobb-500 (Cobb-500 Broiler Performance and Nutrition Supplement, 2015) and a customized vitamin/mineral premix containing no D_3 and corn oil as the fat source (Table 3-1). Diets were formulated with a marginal concentration of Ca (0.75%) and (0.375%) non-phytate phosphorus (nPP) to increase the sensitivity of our response variables to the supplemental 25-OH-D_3 products. The basal diet was then subdivided into 10 equally sized batches and supplemented with 0 (control), 15, 30, or 75 μg/kg of 25-OH-D_3 (600, 1,200 or 3,000 IU D_3/kg) of one of the three products (Rovimix Hy-D; DSM Nutritional Products, Parsippany, NJ., Bio-D; Huvepharma Inc., Peachtree, GA., or Provitas Hy-D; Provitas LLC, Plano, TX) based on the labeled concentration of the 25-OH-D_3 sources. To create each treatment diet, a specific amount of 25-OH-D_3 from each source was weighed and directly mixed with the basal diet for

12 minutes using a stainless steel mixer. The 0 IU D_3/kg of diet treatment served as the common negative control (NC) group for all sources.

Table 3-1 Basal Vitamin D_3-Deficient Diet

Ingredient	Basal Diet[1] (%)
Yellow corn	63.10
Dehulled soybean meal	31.82
DL-methionine	0.29
L-Lysine HCl	0.21
L-Threonine	0.04
Corn oil	1.40
Limestone	1.06
Monocalcium phosphate	1.19
Sodium chloride (salt)	0.36
Customized vitamin-mineral premix[2]	0.50

[1]Calculated nutritional content was as follow: 22% crude protein, 3,035 kcal/kg metabolizable energy, 0.75% calcium, 0.37% non-phytate phosphorus, 0.62% methionine, 0.98% methionine+cystine, 1.33% lysine, 0.26% tryptophan, 0.86% threonine, 1.45% arginine, 3.30% crude fat, 2.16% crude fiber, 0.16% sodium, 0.92% potassium, 0.30% 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.

Table 3-2 Experimental Treatments

TRT	Source	µg/kg of 25-OH D_3	IU/kg of D_3	n
T1	DSM	15	600	25
T2	DSM	30	1,200	25
T3	DSM	75	3,000	25
T4	Bio-D	15	600	25
T5	Bio-D	30	1,200	25
T6	Bio-D	75	3,000	25
T7	Provitas	15	600	25
T8	Provitas	30	1,200	25
T9	Provitas	75	3,000	25
T10	--	--		15

NRC (1994) Cholecalciferol requirement for starting meat-type chicken (200 IU/kg feed or 5 ug/kg of feed)

*Bioactivity of all products is based on a 1:1 conversion of 1 IU D_3=0.025 ug cholecalciferol

3.2.3. Performance Evaluation

Feed intake (FI) and body weight (BW) per pen were recorded on day 10, after the fasting period, and on day 17 of the trial to calculate average weight gain (WG) and feed efficiency (FE). The BW of dead birds was recorded daily and used to adjust FE.

3.2.4. Bone Mineralization and Analysis

On day 21 of the experiment, all birds were euthanized and both tibias from all birds (n=5) were removed, labeled, and stored in a freezer (-20°C) until further analysis. Right tibiae were defatted in petroleum ether for 48 h. Defatted bones were then dried in a force draft oven (95°C) until they reached constant weight (~48 h). The dried bones were ashed overnight at 650°C for 23 h. Percent tibia ash (TBA) was calculated using the starting dry bone weight and remaining ash weight. The left tibiae were cleaned from any adhering tissue and used to assay (raw) 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 3-cm constant span.

3.2.5. Statistics

All data was analyzed as a (3 x 3) factorial using the GLM procedure of SPSS. Source, concentration, and source*concentration will be used as fixed factors. Main effects were analyzed using a 2-way ANOVA. Means were separated by Duncan's multiple range test when appropriate. Significance was accepted at $P< 0.05$. The 0 ug/kg D_3 treatment served as our control reference treatment and used to establish a common baseline for analysis of bioavailability evaluation.

3.3. RESULTS AND DISCUSSION

3.3.1. Performance

The performance results for this trial are presented in Table 3-3 and Table 3-4. For this particular trial I was not able to deplete the negative control birds of vitamin D_3 sufficiently enough to demonstrate any symptoms of rickets even after 21 days of being fed the diet devoid of vitamin D_3. While not precise, multiple range tests are a simple way to obtain an approximation or estimation of a nutritional requirement (Pesti et al., 2009). In this particular trial there were no ANOVA difference observed in any performance variable measure on d 17 or d 21; therefore, the preferred techniques of broken line and regression models were not implemented. High maternal stores of D_3 in the yolk reduced the sensitivity of the experiment and increased the variability in response to the supplemented 25-OH-D_3 (Moran, 2007; Coto et al., 2010a; Coto et al., 2010b; Saunders-Blades and Korver, 2014). Additionally, dietary treatments were fed as a mash which may have led to some of the discrepancy between expected feed consumption and actual feed consumption. While reducing contamination between treatments and ensuring expected amounts of dietary 25-OH-D_3 by not crumbling the diets, I did not see the increase in feed intake that is stimulated by offering the feed as a crumble.

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794 **Table 3-3 Day 10-17 Performance**

Treatment µg/kg of 25-OH-D_3	**n**	**17d BW**	**10-17d WG**	**10-17d FCR**
DSM: 15	5	503 ± 18.7	273 ± 21.1	1.44 ± .06
DSM: 30	5	536 ± 18.7	306 ± 18.8	1.44 ± .05
DSM: 75	5	530 ± 18.7	298 ± 18.8	1.39 ± .05
Bio-D: 15	5	541 ± 18.7	311 ± 18.8	1.47 ± .05
Bio-D: 30	5	523 ± 18.7	294 ± 18.8	1.45 ± .05
Bio-D: 75	5	518 ± 18.7	288 ± 21.1	1.45 ± .06
Provitas: 15	5	518 ± 18.7	308 ± 18.8	1.42 ± .05
Provitas: 30	5	551 ± 18.7	321 ± 18.8	1.42 ± .05
Provitas: 75	5	507 ± 18.7	278 ± 18.8	1.47 ± .05
Main Effect				
Source				
DSM	15	523 ± 11.4	292 ± 11.4	1.43 ± .03
Bio-D	15	527 ± 11.4	298 ± 11.4	1.46 ± .03
Provitas	15	532 ± 10.9	302 ± 11.0	1.44 ± .03
Pvalue		*0.926*	*0.952*	*0.811*
Level				
15	15	528 ± 11.4	297 ± 11.4	1.45 ± .03
30	15	536 ± 10.9	307 ± 11.0	1.44 ± .03
75	15	518 ± 11.4	288 ± 11.4	1.44 ± .03
Pvalue		*0.450*	*0.438*	*0.987*
Source*Level				
Pvalue		*0.831*	*0.853*	*0.873*

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

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

[2]Values for performance responses represent the mean average of n=5 replicate pens per treatment of 5 birds each at day 17.

Negative Control n=3 BW, WG, FCR were 559 ± 24.1, 326 ± 24.3, and 1.33 ± .07, respectively.

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800 **Table 3-4 Day 10-21 Performance**

Treatment µg/kg of 25-OH-D_3	**n**	**21d BW**	**10-21d WG**	**10-21d FCR**
DSM: 15	5	694 ± 99.8	201 ± 38.4	1.69 ± 0.32
DSM: 30	5	745 ± 39.6	209 ± 36.4	1.52 ± 0.09
DSM: 75	5	748 ± 59.5	218 ± 8.9	1.49 ± 0.08
Bio-D: 15	5	751 ± 20.5	210 ± 11.1	1.55 ± 0.16
Bio-D: 30	5	738 ± 67.7	215 ± 10.4	1.41 ± 0.11
Bio-D:75	5	709 ± 85.6	232 ± 26.7	1.39 ± 0.14
Provitas: 15	5	751 ± 20.6	212 ± 9.1	1.54 ± 0.13
Provitas: 30	5	767 ± 49.8	216 ± 20.1	1.51 ± 0.28
Provitas: 75	5	749 ± 41.8	223 ± 4.2	1.41 ± 0.06
Main Effect				
Source				
DSM	15	729 ± 70.4	201 ± 38.4	1.57 ± 0.20
Bio-D	15	733 ± 62.1	219 ± 19.0	1.45 ± 0.15
Provitas	15	749 ± 39.7	217 ± 12.9	1.49 ± 0.18
Pvalue		*0.616*	*0.447*	*0.179*
Level				
15	15	732 ± 62.1	208 ± 22.4	1.59 ± 0.21bc
30	15	750 ± 51.3	213 ± 23.1	1.48 ± 0.17ab
75	15	729 ± 62.2	224 ± 16.3	1.43 ± 0.10a
Pvalue		*0.597*	*0.130*	*0.049*
Source*Level				
Pvalue		*0.831*	*0.853*	*0.873*

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

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

[2]Values for performance responses represent the mean average of n=5 replicate pens per treatment of 5 birds each at day 17.

Negative Control n=3 BW, WG, FCR were 559 ± 24.1, 326 ± 24.3, and 1.33 ± .07, respectively.

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3.3.2. Bone Mineralization

The effect of dietary 25-OH-D_3 on bone mineralization is presented in table 3-5. No significant ($P<0.05$) were observed among treatments groups and when compared to the negative control, 0 IU treatment group. Interestingly, TBA for the NC group averaged 48.4%. Compared to the other treatment levels, in average this is a ~0.4% increase in tibia bone ash. Leyva-Jimenez et al. (2018) used a similar protocol to deplete the maternal stores of D_3 and increased the sensitivity of the studied responses which resulted in a 5.0% decrease in TBA. Moreover, similar results were seen by Gurel et al. (2013) where he compared two doses of 25-OH-D_3 without the addition of vitamin D_3 which resulted in no contribution on bone development. In contrast, literature reports suggest that dietary 25-OH-D_3 in replacement or in addition to D_3 is effective in promoting performance, enhancing bone mineralization, and reducing tibial dyschondroplasia (Yarger et al., 1995; Atencio et al., 2005; Han et al., 2016).

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827 **Table 3-5 Effect of dietary 25-OH-D_3 on**
828 **Bone Mineralization of 21-d Old Broiler chickens**

Treatment μg/kg of 25-OH D_3	**n**	**TBS**	**TBA**
DSM: 15	5	19.1 ± 5.0	48.0 ± 1.5
DSM: 30	5	17.1 ± 5.8	47.6 ± 0.5
DSM: 75	5	19.2 ± 6.9	48.2 ± 1.1
BIO-D: 15	5	19.2 ± 5.5	48.1 ± 0.8
BIO-D: 30	5	16.1 ± 2.5	47.5 ± 2.4
BIO-D: 75	5	15.3 ± 1.9	48.2 ± 1.2
Provitas: 15	5	16.9 ± 4.4	48.2 ± 1.1
Provitas: 30	5	13.4 ± 4.4	48.0 ± 1.2
Provitas: 75	5	17.8 ± 8.1	47.0 ± 0.3
Main Effects			
Source			
DSM	15	18.5 ± 5.6	47.9 ± 1.0
Bio-D	15	16.9 ± 3.9	47.9 ± 1.5
Provitas	15	16.1 ± 5.8	47.8 ± 1.0
Pvalue		*0.472*	*0.912*
Level			
15	15	18.4 ± 4.7	48.1 ± 1.0
30	15	15.5 ± 4.4	47.7 ± 1.5
75	15	17.5 ± 6.2	47.8 ± 1.1
Pvalue		*0.348*	*0.628*
Source*Level			
Pvalue		*0.832*	*0.568*

[a-b]Means within the same column without a common superscript differ ($P<0.05$) Duncan's multiple range test; Means ± SEM.
[1]BW, Body weight (g/bird); WG, Weight gain (g/bird); FCR, Mortality corrected feed conversion ratio (g feed intake / g weight gain).
[2]Values for performance responses represent the mean average of n=5 replicate pens per treatment of 5 birds each at day 17.
Negative Control n=3 TBS and TBA were 17.3 ± 3.05 and 48.4 ± 0.72, respectively.

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3.3.3 Discussion

Supplementation of dietary 25-OH-D_3 as a complete or partial replacement for vitamin D_3 at the same level increased bone ash (Fritts and Waldroup, 2003; Świątkiewicz and Koreleski, 2005; 2006), bone calcification (Gómez-Verduzco et al., 2013), bone strength (Świątkiewicz and Koreleski, 2005; 2006), and reduced incidence and severity of tibial dyschondroplasia in broiler chickens (Rennie and Whithead, 1996). Goodgame et al. (2011) demonstrated the optimal level of 25-OH-D_3 was 10 μg/kg (400 IU/D_3) for promoting bone ash in broiler chicken diets. In contrast, research done by Fritts and Waldroup (2003) has shown the absence of differences in BWG, FCR, TBA, and TD severity in broiler fed diets with 12.5 to 100 μg/kg of 25-OH-D3. In agreement with that study, our results of dietary 25-OH-D_3 supplementation which ranged from 15 to 75 μg/kg (600-3,000 IU/D_3) showed no significant differences among treatment groups and when compared to the control. Therefore, the use of 25-OH-D_3 in poultry diets could provide more effective margin of safety in preventing performance reductions in the presence of low Ca and nPP diets.

In conclusion, relative bioavailability differences between D_3 sources could be due to differences in preparation methods. Differences in particle size and coating material could have caused segregation during the mixing process which could have reduced equal dispersion throughout the feed treatments. Although similar results were observed in dietary vitamin D treatments, it appears that, a vitamin D deficiency in our control treatment was not effectively achieved. This is partially due to high maternal stores of D_3 in the yolk which reduced the sensitivity of our experiments. Furthermore,

855 the maternal stores in the progeny increased the variability in the response and decreased
856 separation of the dietary 25-OH-D_3 treatments.
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4. ESTABLISHING A MARGINAL D_3-DEFICIENT BROILER BREEDER FLOCK TO PRODUCE MARGINAL D_3 PERFORMING D_3 PROGENY TO REASSESS THE D_3 REQUIREMENT IN MODERN MEAT-TYPE BROILER CHICKENS USING A NOVEL ORAL GAVAGE BIOASSAY

4.1. INTRODUCTION

The novel oral gavage bioassay established in the Bailey laboratory by Leyva-Jimenez, Hector, et al., (2019) aimed at depleting the maternal D_3 storage of commercial broiler chicks over a 10-day depletion period and then orally gavaging the birds with increasing levels of vitamin D_3. Previous work in the Bailey laboratory has shown that the control group does not always express D_3 deficiency symptoms within the 3-week time frame of the study. The hypothesis is that the maternal stores are extremely high in commercial broiler chicks because the commercial diets contain 20x more than the minimum requirement published in the most recent NRC. A study done by Atencio et al. (2005) looked at chicks hatched from eggs laid by broiler breeder hens fed various levels of vitamin D_3 (0-4,000 IU/kg of diet) and reported that the highest body weight gains and tibia ash were observed in chicks hatched from hens fed the highest levels of vitamin D_3. Most research that has been done looking at broiler leg abnormalities has focused on the manipulation of their diet or environment but there is limited research that has been done on the maternal diet of modern broiler chicks and the effects of maternal vitamin D_3 level in the diet on performance and tibia strength and ash of the progeny. This study utilized the progeny of a broiler breeder maternal flock that was marginally depleted of

vitamin D_3 in combination with the novel gavage bioassay as described by Leyva-Jimenez, Hector et al. (2019) to reassess the estimated D_3 requirement of modern meat-type broiler chickens.

4.2. MATERIALS AND METHODS

4.2.1. Birds, Diets and Management

All methods were approved by Texas A&M Institution Animal Care and Use Committee (IACUC 2017-0072). A pen containing 100 hens and 10 roosters (63-weeks old) Cobb X Hubbard breeders were fed a vitamin-D_3-deficient diet for a 2-week period to deplete any storages of vitamin D_3; eggs collected during this time were discarded. After the 2-week period, all birds were placed on a breeder diet set to vitamin D_3 NRC (1994) requirements of 200 IU/D_3. At the beginning of this 4-week period, 10 hens and 4 males were randomly selected and colored marked and blood was collected at the end of each week. Blood samples were collected within Eppendorf® 1.5-ml tubes and centrifuged at 3000 x *g* for 15 min to separate the serum which then was used to run a commercial ELISA Kit (25-OH-Vitamin D Kit Eagle Biosciences®). The assay procedure was done following the user's manual from the kit. After, the 2-week period on the vitamin D_3 NRC requirement diet, hatching eggs were collected three times a day for 7 days and stored in a cool room at 18°C. Eggs were then placed in a GQF® automatic incubator at an average temperature of 37°C and 50-60% humidity for 18 days and then transferred to a GQF® hatcher at an average temperature of 36°C and 60-70% humidity for the remaining 3 days.

A total of 209 hatched Hubbard chickens were wing banded and placed in two stainless steel battery brooders located in an environmentally controlled rearing room (#1215) at the Texas A&M Poultry Research Farm. Birds were fed a corn-soy vitamin-D_3-deficient broiler starter diet *ad libitum* for a 17-day rearing period. The first 9 days of the study served as a depletion period of the maternal D_3 stores followed by a 12-h fasting period. On day 10, birds were weighed in groups of 20 and an average BW was calculated. The average BW was used to create eight treatments groups with six replicates (n=26) with close to "identical" starting body weight and variance. 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 BlakRay, UVP, LLC. Upland, CA) to prevent the endogenous synthesis of D_3. From day 10 to the end of the trial, birds were orally gavage once a day with increasing levels of vitamin D_3. Oral D_3 treatments were offered for a 7-day period. Water was offered *ad libitum* during the whole trial using nipple drinkers. Birds were monitored daily with regards to general flock condition, temperature, lighting, water, feed, and any unanticipated events inside the rearing facility.

4.2.2. Dietary Treatments

A basal mash corn-soy broiler starter diet devoid of D_3 was formulated using a custom vitamin/mineral premix containing no D_3 and corn oil as the fat source (Table 4-3). Diets were formulated with a marginal concentration of Ca (0.75%) and (0.375%) non-phytate phosphorus (nPP) to increase the sensitivity of response variables to the experimental treatments. Daily oral gavage treatments were based on an estimated intake

of 0, 50, 100, 200, 400, 800, 1,600 and 3,200 IU D_3/kg of feed consumed over the last 7 days of the study. To create the experimental gavage 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). Conversion of D_3 to IU were based on a 1:1 where 1 IU of D_3= 0.025 µg of cholecalciferol. 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. Prepared D_3 solutions were separated in 7 daily doses (n=49) and then stored in a freezer at -20°C until needed. Birds in the control group containing 0 IU received 0.5 mL of corn oil without D_3 for 7 days. Birds were individually weighed prior to the first oral gavage and oral gavage treatments were performed from lowest to highest IU concentration using an 18-gauage stainless steel gavage needle and a 1-mL syringe graduated a 1/100 mL. Between treatments the gavage needle was flushed with plain corn oil to avoid any 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.

Table 4-1 Vitamin-D_3-Deficient Breeder Diet

Ingredient	Basal Diet[1] (%)
Yellow corn	67.50
Dehulled soybean meal	19.24
DDGS	2.00
DL-methionine	0.25
L-Lysine HCl	0.01
L-Threonine	0.01
Corn oil	0.75
Limestone	3.55
Oyster shell	5.33
Monocalcium phosphate	0.43
Sodium chloride (salt)	0.41
Vitamin-mineral premix[2]	0.50

[1]Calculated nutritional content was as follow: crude protein 17%, metabolizable energy 2736.94 kcal/kg, calcium 4.0 %, available phosphorus 0.38 %, methionine 0.43 %, methionine+cystine 0.73 %, lysine 0.86%, tryptophan 0.19 %, threonine 0.63% arginine 1.09%, crude fat 2.68% and crude fiber 2.33%
[2]Vitamin/mineral premix guaranteed analysis: Copper 2200ppm, Iodine 400 ppm, Iron 4,000 ppm, Manganese 2.5%, Zinc 2.5%, Selenium 40 ppm, vitamin A 1,596,650 IU/kg and vitamin E 7,964 IU/kg. Recommended inclusion level 5 kg/t to manufacture complete poultry feed.

Table 4-2 NRC Vitamin D_3 Requirement Breeder Diet

Ingredient	Basal Diet[1] (%)
Yellow corn	67.05
Dehulled soybean meal	19.32
Distillers Dried Grain	2.00
DL-methionine	0.25
L-Lysine HCl	0.01
L-Threonine	0.01
Corn oil	0.75
Limestone	8.29
Sodium bicarbonate	0.09
Sodium chloride (salt)	0.34
Customized vitamin-mineral premix[2]	0.25

[1]Calculated nutritional content was as follow: crude protein 17%, metabolizable energy 2736.94 kcal/kg, calcium 4.0 %, available phosphorus 0.38 %, methionine 0.43 %, methionine+cystine 0.73 %, lysine 0.86%, tryptophan 0.19 %, threonine 0.63% arginine 1.09%, crude fat 2.68% and crude fiber 2.33%
[2]Vitamin/mineral premix guaranteed analysis: Copper 2200ppm, Iodine 400 ppm, Iron 4,000 ppm, Manganese 2.5%, Zinc 2.5%, Selenium 40 ppm, vitamin A 1,596,650 IU/kg and vitamin E 7,964 IU/kg. Recommended inclusion level 5 kg/t to manufacture complete poultry feed.

Table 4-3 Broiler Starter Diet Devoid of Vitamin D_3

Ingredient	Basal Diet[1] (%)
Yellow corn	63.73
Dehulled soybean meal	32.05
DL-methionine	0.28
L-Lysine HCl	0.24
L-Threonine	0.04
Corn oil	0.52
Limestone	1.07
Monocalcium phosphate	1.21
Sodium chloride (salt)	0.33
Customized vitamin-mineral premix[2]	0.50

[1]Calculated nutritional content was as follow: 22% crude protein, 3,035 kcal/kg metabolizable energy, 0.75% calcium, 0.37% non-phytate phosphorus, 0.62% methionine, 0.98% methionine+ cystine, 1.33% lysine, 0.26% tryptophan, 0.86% threonine, 1.45% arginine, 3.30% crude fat, 2.16% crude fiber, 0.16% sodium, 0.92% potassium, 0.30% 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.

4.2.3. 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) the highest treatment (3,200 IU) was sent to a third-party commercial laboratory (Cornerstone Laboratories, LLC by AOAC-2002.05, 1775 Moriah Woods Blvd., Ste. 12 Memphis, TN 38117) for D_3 concentration analysis. Analyzed treatment concentration reported by the laboratory was 3,540 IU/mL (88.5 mcg/mL).

4.2.4. Performance Evaluation

Feed intake and BW per pen were recorded on day 10, after the fasting period, and on day 17 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 day 10 to 17 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 D3/kg of feed)

FI = Feed intake (g/bird)

4.2.5. Bone Mineralization

On day 17 of the trial, all birds per pen were euthanized via cervical dislocation and both tibiae were removed, labeled, and stored in a freezer (20°C) until further analysis. The right tibiae were defatted in petroleum ether for 48 h. Defatted bones were then dried in a force draft over (95°C) until a constant weight was reached. The dried bones where then used to assay (dry) breaking strength (TBS) using a texture analyzer (TA.XT Plus, Texture Technologies, Hamilton, MA.) charged with a 50-kg load cells, a crosshead speed of 100 nm/min with the tibia supported on a 3-point bending ring and a 2.5-cm constant span. Finally, the broken dried tibiae were ashed at 650°C for 23 h. Percent tibia bone ash (TBA) was calculated based on starting dry bone weight and remaining ash and expressed as a percent. The left tibiae were removed from the freezer in stored in a fridge for 12 h prior to breaking. The thawed bones were cleaned of any remaining tissue and used to assay (raw) breaking strength (TBS) using a texture

analyzer (TA.XT Plus, Texture Technologies, Hamilton, MA.) charged with a 50-kg load cells, a crosshead speed of 100 nm/min with the tibia supported on a 3-point bending ring and a 2.5-cm constant span. The constant span was reduced by 0.5-cm from previous chapter to decrease variance due to bending prior to breaking.

4.2.6. Statistics

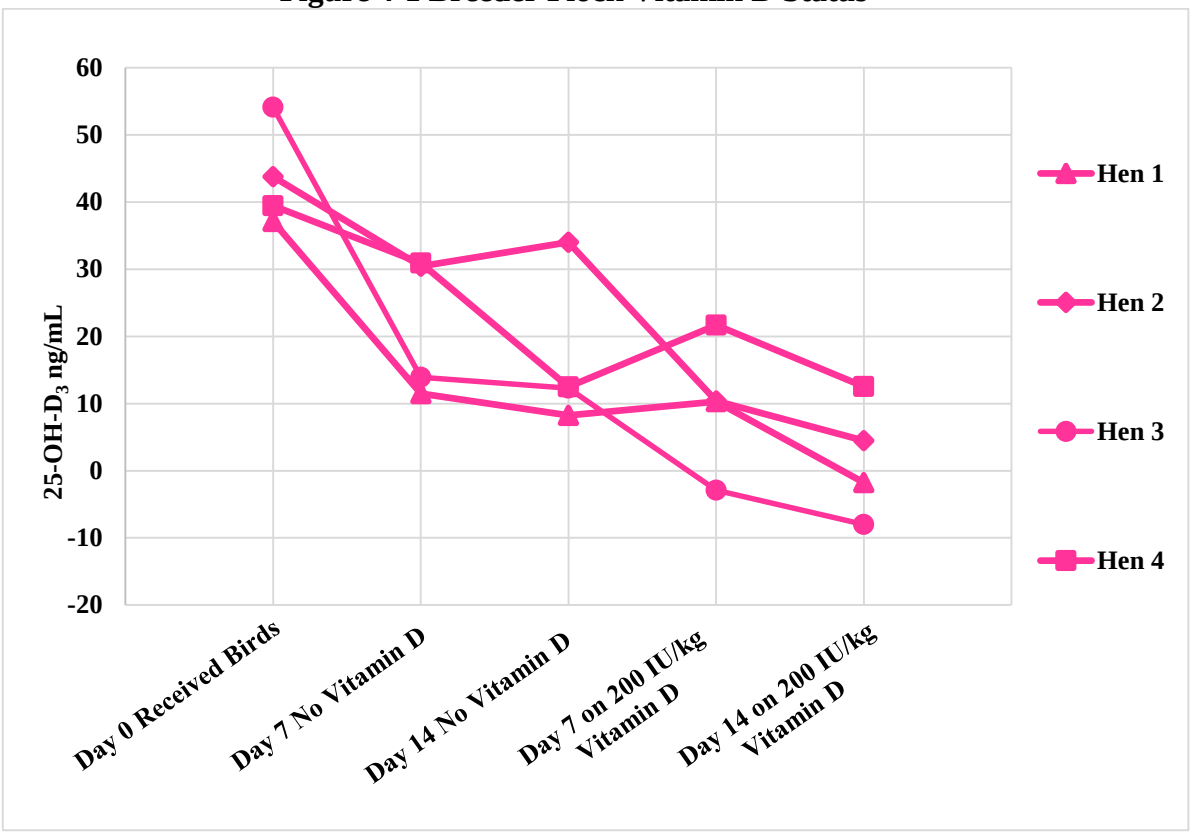
Collected data were analyzed as one way-ANOVA where treatment and block were used as fixed factors in the model. Battery level was used as the blocking factor. Means were separated by Duncan's multiple range test when appropriate. Linear and quadratic effects of graded levels of D_3 were investigated by regression analysis. The 0 IU group was included as common control to investigate linear and quadratic effects.

4.3. RESULTS AND DISCUSSION

4.3.1. Breeder flock

While no performance statistical analysis was done on the breeder flock, Figure 4-1 illustrates an observable decrease in the vitamin D status of the hens. This reduction in maternal vitamin D storages helped ensure the progeny birds used in the D_3 requirement oral gavage bioassay had homogenous and relatively low vitamin D status to increase the sensitivity of the protocol.

Figure 4-1 Breeder Flock Vitamin D Status



4.3.2. Performance Parameters

Performance results are presented in table 4-4. The D_3 treatments positively improved ($P<0.05$) BW and WG. Performance results were more consistent with increasing levels of D_3 when compare to the 0 IU D_3/kg, multiple range test was possible by looking at the Duncan's means separation. However, this demonstrated that 50-3200 IU/kg of feed were required to maximize BW and WG. Although, performance results differences were observed in this experiment, it appears high maternal stores of D_3 in the yolk still influenced the sensitivity of our experiment.

Table 4-4 Effect of Dietary Vitamin D_3 on the Performance of Broiler Chickens

IU D_3/kg feed[1,2]	n	17d BW	10-17d WG	10-17d FCR
T1 (0)	6	370 ± 24.5[b]	241 ± 25.9[b]	1.47 ± 0.15
T2 (50)	6	409 ± 16.9[a]	285 ± 12.5[a]	1.39 ± 0.13
T3 (100)	6	403 ± 16.0[a]	275 ± 15.5[a]	1.35 ± 0.06
T4 (200)	5	394 ± 12.9[a]	266 ± 14.4[a]	1.30 ± 0.14
T5 (400)	6	404 ± 13.7[a]	279 ± 12.7[a]	1.28 ± 0.22
T6 (800)	6	391 ± 16.5[a]	266 ± 17.8[a]	1.34 ± 0.19
T7 (1600)	6	400 ± 20.2[a]	270 ± 22.0[a]	1.25 ± 0.28
T8 (3200)	6	407 ± 9.8[a]	276 ± 11.7[a]	1.40 ± 0.08
Pvalue		*0.007*	*0.005*	*0.422*

[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.

4.3.3. Bone Mineralization

Differences (P<0.05) in TBA, raw TBS, and dried TBD were observed between treatment groups and the 0 IU group. TBA showed significant improvement at 3200 IU D_3/kg from the remainder of the treatment groups. Between the lowest level (0 IU/kg) and the highest (3,200 IU/kg) a 4.6% reduction was achieved. Similarly, Leyva et al. (2019) observed a 2.4% reduction between the lowest level (0 IU/kg) and the highest (3,202 IU/kg) 48.9 and 51.3% respectively. TBA for the 0 IU/kg treatment in the current trial compared to the 0 IU/kg found in Hector et al. (2019) results with a 9.6% difference. Similar results were observed in TBA for the current trial and when compared to Hector et al. (2019) and when comparing with the 0 IU/kg treatment in the current study resulted in a 10.3% reduction in raw TBS. The reduced TBA and raw TBS in the present trial may be due to the marginally deficient breeder flock along with the 9 day depletion period and marginal Ca and nPP in the basal diet. It seems that the

marginal vitamin-D_3-deficient breeder flock increased the sensitivity of the progeny to graded levels of vitamin D_3. However, future studies should continue oral gavage vitamin D_3 treatments past the 7 days to increase separation between treatments.

Dried TBS was evaluated in this trial to determine if a more precise breaking point could be observed and reduce variability in bending and partial breaks. Multiple range tests in table 4-5 suggests that 50 IU/kg of feed were needed to maximize dried TBA. When looking at the linear broken line for raw and dried TBS, the raw broke at 252 IU/kg while the dried TBS broke at 400 IU/kg. Furthermore, R^2 values were much higher for the raw TBS compared to the dried bones (24.3 and 3.7, respectively). This demonstrates that not only is raw bone more sensitive to vitamin D_3 supplementation but could be a useful measurement for vitamin D requirements.

TBA was highly sensitive when looking at both the linear broken line and quadratic broke line models as shown in table 4-6. Overall, TBA resulted in the best R^2 values of 61.9 and 62.1 and was found to maximize at 73 and 121 IU/kg. The results of the present study are in agreement with post-NRC (1994) literature reports suggesting levels exceeding the standard 200 IU/kg of feed to maximize growth and bone mineralization (Kasim and Edwards, 2000; Fritts and Waldroup, 2003).

Table 4-5 Effect of Dietary Vitamin D_3 on Bone Mineralization of Broiler Chickens

IU D_3/kg feed[1]	n	TBA[2]	Raw TBS[2]	Dried TBS[2]
T1 (0)	5	39.3 ± 0.7^{c}	6.6 ± 0.9^{c}	8.7 ± 1.3^{c}
T2 (50)	5	42.2 ± 0.4^{b}	8.6 ± 0.5^{ab}	10.1 ± 0.9^{a}
T3 (100)	5	43.2 ± 0.4^{ab}	9.0 ± 0.6^{ab}	9.6 ± 0.9^{abc}
T4 (200)	5	43.3 ± 0.6^{ab}	8.1 ± 0.7^{b}	9.4 ± 1.2^{abc}

T5 (400)	5	43.6 ± 0.5^{ab}	8.8 ± 0.8^{ab}	8.4 ± 0.6^{c}
T6 (800)	5	43.4 ± 0.2^{ab}	8.6 ± 0.6^{ab}	8.8 ± 0.4^{bc}
T7 (1600)	5	43.5 ± 0.5^{ab}	8.8 ± 1.2^{ab}	8.5 ± 0.8^{c}
T8 (3200)	5	43.9 ± 0.5^{a}	9.6 ± 0.8^{a}	10.0 ± 1.4^{ab}
Pvalue		*0.000*	*0.000*	*0.018*

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

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1067 **Table 4-6 Vitamin D_3 requirement estimation and model comparison**

Response	Model	ER[1]	R^2(%)	P value
BA	Linear Broken Line	73	61.9	0.0000
	Quadratic	121	62.1	0.0001
RAW BS	Linear Broken Line	252	24.3	0.0116
	Quadratic	253	30.1	0.0252
DRY BS	Linear Broken Line	400	3.7	0.6135
	Quadratic	253	0.8	0.7542

[1]Estimated requirement, ER (IU D_3/kg of feed)

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Figure 4-2 Vitamin D_3 Raw Breaking Strength Linear Broken Line

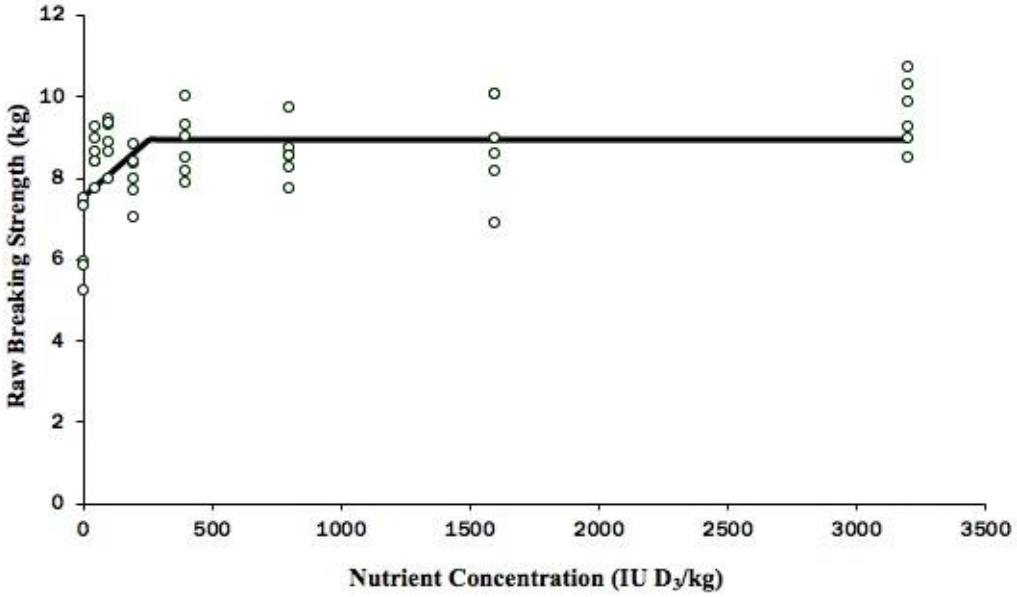


Figure 4-3 Vitamin D_3 Raw Breaking Strength Quadratic Broken Line

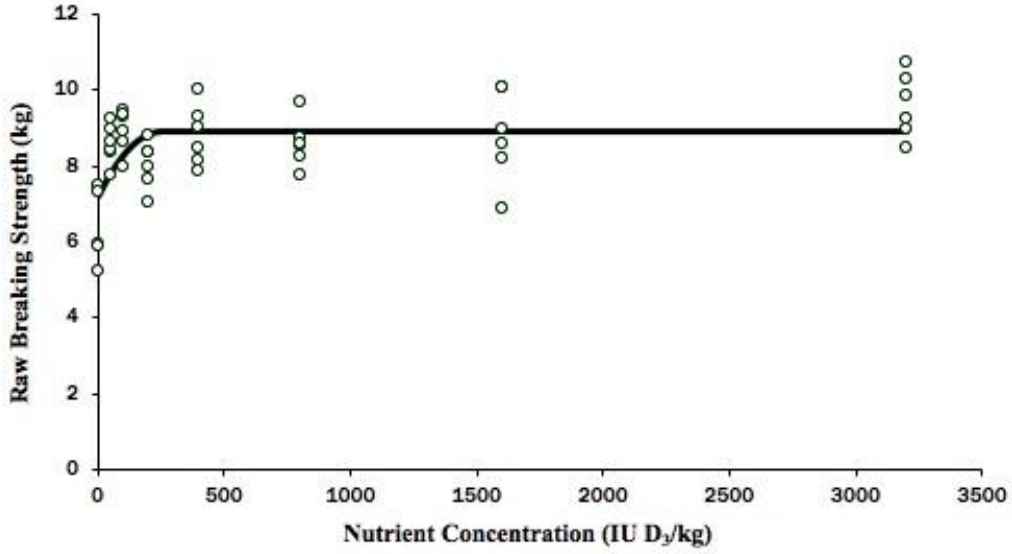


Figure 4-4 Vitamin D_3 Dry Breaking Strength Linear Broken Line

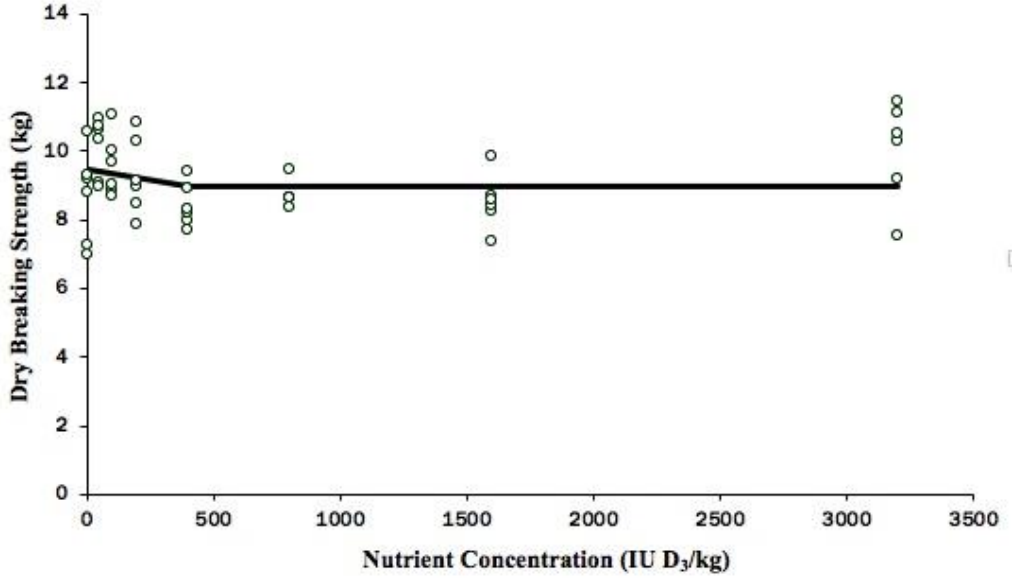


Figure 4-5 Vitamin D_3 Dry Breaking Strength Quadratic Broken Line

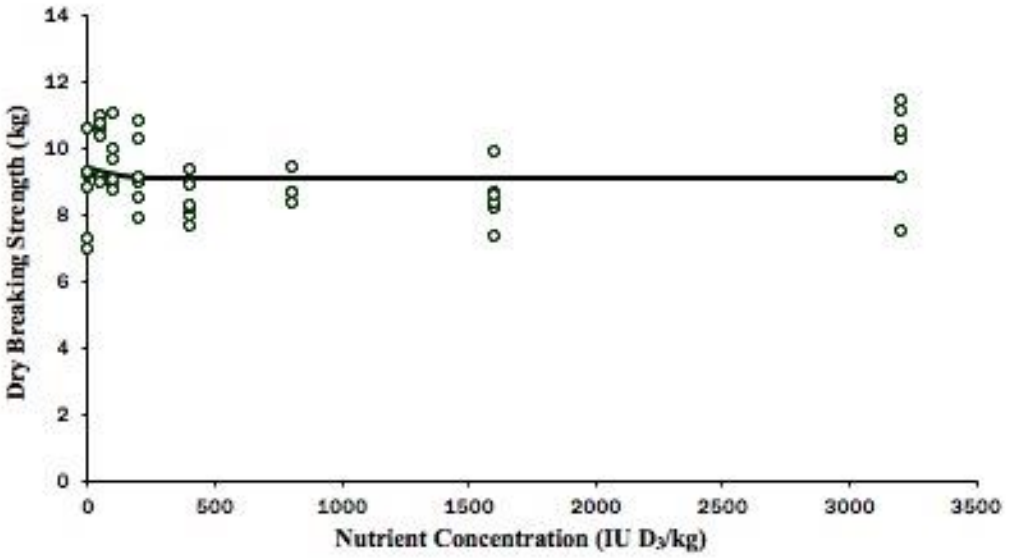


Figure 4-6 Vitamin D_3 Bone Ash Linear Broken Line

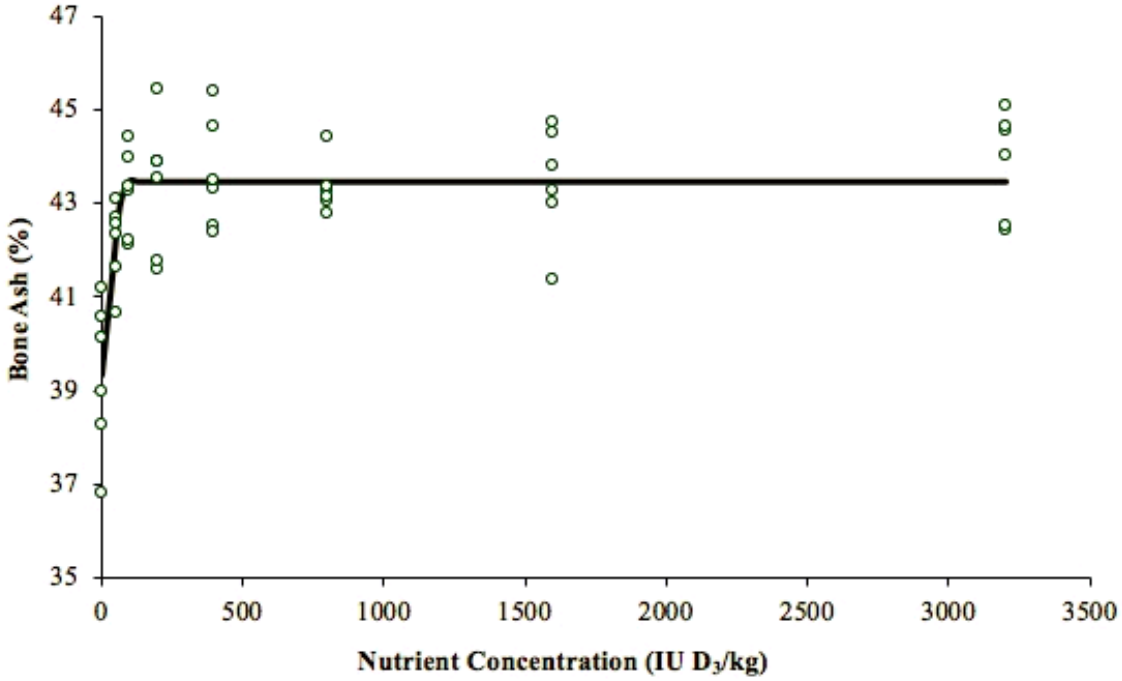
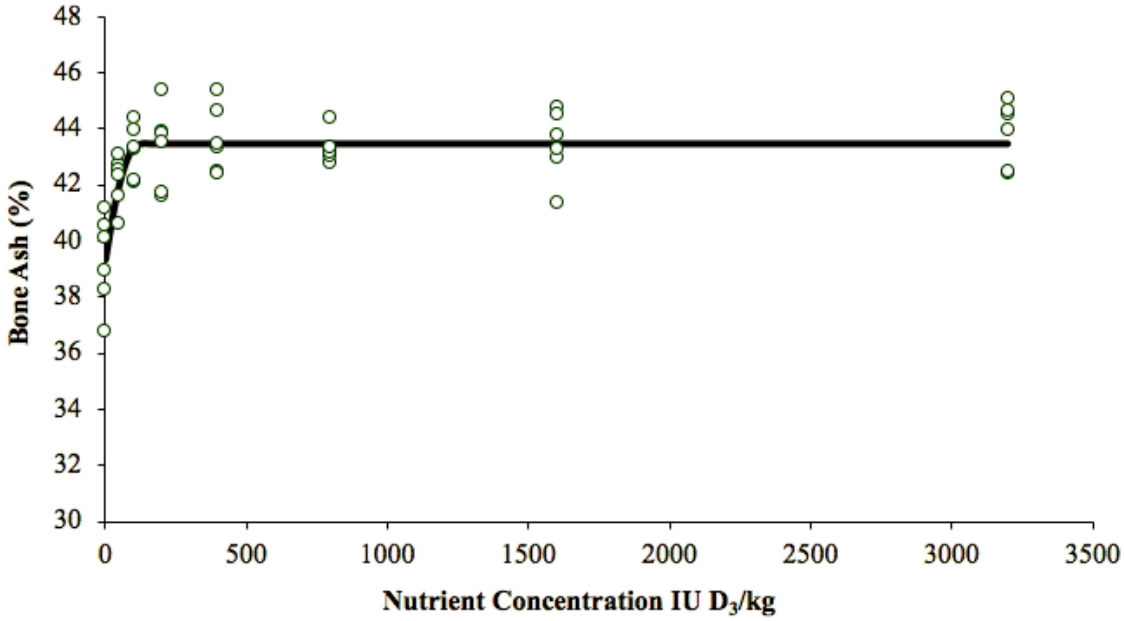


Figure 4-7 Vitamin D_3 Bone Ash Quadratic Broken Line



5. EVALUATION OF THE D_3 REQUIREMENT OF TWO COMMON COMMERCIAL BROILER STRAINS USING THE ORAL GAVAGE BIOASSAY

5.1. INTRODUCTION

This trial evaluated two broiler strains simultaneously to determine the significance of strain differences in response to oral gavage supplementation of vitamin D_3. Comparative studies between broiler strains have helped identify the morphological and physiological adaptation in response to distinct selective pressures. Additionally, this trial will help eliminate the variation due to the difference in cholecalciferol source preparation, feed mixing errors, selective feeding, and chemical nutrient analysis of the test treatments.

Commercial cholecalciferol is available in a variety of physical forms such as spray-dried or beadlet which play a crucial role in the bioavailability of these products. In the commercial industry, the potencies of cholecalciferol preparations or sources have a significant impact as the incidence of skeletal disorders is still common (Dinev, 2012). Numerous studies have been done that have observed differences in nutritional requirements between high-yield and multipurpose broiler strains (Corzo et al., 2005; Scheuermann et al., 2003; Smith and Pesti, 1998). Furthermore, dietary nutrient density and composition of finished feeds in commercial practices in crucial, not only because it has a significant effect on growth performance, carcass quality, and health of the broilers, but also because it in turn affects the economics of broiler productions (Scott, 2002; Sterling et al., 2005; Brickett et al., 2007). Whitehead et al. (2004) showed that broilers up to 14 days of age with sufficient dietary Ca and available phosphorus

concentrations required a range of 35-50 µg/kg (1,400-2,000 IU/kg) of vitamin D_3 based on maximum cortical bone quality. Rao et al. (2006) demonstrated that broiler performance and bone mineralization could be maintained with suboptimal concentrations of Ca and available phosphorus (0.5 and 0.25% respectively) when supplemented with high concentrations of vitamin D_3 [90 µg/kg (3,600 IU/kg)] in the diet. Fluctuation in feed and ingredient prices have promoted interest in lowering the dietary nutrient levels and studying the effects of diets formulated with suboptimal concentrations of nutrients in modern broilers (Waldroup et al., 2005; Kamran et al., 2008b).

Thus, the vitamin D requirement may change depending on many factors such as dietary Ca and P, availability of P sources, housing conditions, stocking density, and strain growth differences. Baker et al. (1998) found that Ca an P utilization can be improved when fed at sub-optimal concentration by supplementing high concentrations of cholecalciferol in the diet. Similarly, Edwards (2002) showed significant improvement of feed efficiency (FE) by feeding 220 µg/kg of D_3 with 0.30% dietary nPP to chicks from day 1-16. Due to the cost of synthetic cholecalciferol being lower than that or inorganic phosphorus sources, reducing the dietary calcium and phosphorus and supplementing high concentrations of cholecalciferol may be beneficial for reducing feed cost without reducing broiler performance.

Using the protocol previously developed by our research team, the present study corresponds to results found in previous chapters to further investigate differences in vitamin D_3 requirements of modern broiler strains.

5.2. MATERIALS AND METHODS

5.2.1. Birds, Diet and Management

All methods were approved by Texas A&M Institution Animal Care and Use Committee (IACUC 2017-0072). A total of three hundred Cobb-500 and three hundred Cobb-700 newly-hatched off sex male broiler chicks were purchased from a commercial hatchery, individually wing banded, and allocated in 2 stainless steel battery brooders (~10 birds per cage). A basal D_3-deficient corn-soy broiler starter diet was fed *ad libitum* throughout the 17-day trial period. The first 9 days of the study served to deplete the maternal stores of D_3 followed by a 12-h fasting period. On day 10 of the trial birds were weighed in groups of 20 per strain and an average body weight was calculated. The average body weight per strain were used to create 96 groups (48 per strain) with close to "identical" body weight and variance. 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 a 7-day period. Water was offered *ad libitum* during the whole trial using nipple drinkers. Birds were monitored daily with regards to general flock condition, temperature, lighting, water, feed, and any unanticipated events inside the rearing facility.

5.2.2. Dietary Treatments

A basal mash corn-soy broiler starter diet devoid of D_3 was formulated using a custom vitamin/mineral premix containing no D_3 and corn oil as the fat source (Table 4-3). Diets were formulated with a marginal concentration of Ca (0.75%) and (0.375%) non-phytate phosphorus (nPP) to increase the sensitivity of response variables to the

experimental treatments. Daily oral gavage treatments were based on an estimated intake of 0, 50, 100, 200, 400, 800, 1,600 and 3,200 IU D_3/kg of feed consumed over the last 7 days of the study. To create the experimental gavage 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). Conversion of D_3 to IU were based on a 1:1 where 1 IU of D_3= 0.025µg of cholecalciferol. 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. Prepared D_3 solutions were separated in 7 daily doses (n=49) and then stored in a freezer at -20°C until needed. Birds in the control group containing 0 IU received 0.5 mL of corn oil without D_3 for 7 days. Birds were individually weighed prior to the first oral gavage and oral gavage treatments were performed from lowest to highest IU concentration using an 18-gauage stainless steel gavage needle and a 1-mL syringe graduated a 1/100 mL. Between treatments the gavage needle was flushed with plain corn oil to avoid any 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.

Table 5-1 Basal Broiler Starter Diet Devoid of Vitamin D_3

Ingredient	Basal Diet[1] (%)
Yellow corn	63.73
Dehulled soybean meal	31.05
DL-methionine	0.28
L-Lysine HCl	0.24
L-Threonine	0.05
Corn oil	0.52
Limestone	1.08
Monocalcium phosphate	1.22
Sodium chloride (salt)	0.34
Customized vitamin-mineral premix[2]	0.50

[1]Calculated nutritional content was as follow: 21.5% crude protein, 3,000 kcal/kg metabolizable energy, 0.75% calcium, 0.37% non-phytate phosphorus, 0.61% methionine, 0.96% methionine+ cystine, 1.32% lysine, 0.27% tryptophan, 0.84% threonine, 1.38% arginine, 3.07% crude fat, 2.21% crude fiber, 0.16% sodium, 0.80% potassium, 0.31% 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.

Table 5-2 Experimental Treatments

	Cobb-500	Cobb-700
Basal (NC)	IU/D_3 kg of feed	IU/D_3 kg of feed
0 IU D_3/kg of feed	50	50
	100	100
	200	200
	400	400
	800	800
	1,600	1,600
	3,200	3,200

5.2.3. 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) the highest treatment (3,200 IU) was sent to a third-party commercial laboratory (Cornerstone Laboratories, LLC by AOAC-2002.05, 1775 Moriah Woods Blvd., Ste. 12 Memphis, TN 38117) for D_3 concentration

analysis for D_3 concentration analysis. Analyzed treatment concentration reported by the laboratory was 3,177.92 IU/mL (79.4 mcg/mL).

5.2.4. Performance Evaluation

Feed intake and BW per pen were recorded on day 10, after the fasting period, and on day 17 of the trial to calculate weigh gain (WG) and feed efficiency (FE). The BW of dead birds was recorded daily and used to adjust FE. Feed intake data collected from day 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)

5.2.5. Bone Mineralization

On day 17 of the trial, all birds per pen were euthanized via cervical dislocation and both tibiae were removed, labeled, and stored in a freezer (20°C) until further analysis. The right tibiae were defatted in petroleum ether for 48-h. Defatted bones were then dried in a force draft over (95°C) until a constant weight was reached. Finally, the dried tibiae were ashed at 650°C for 23-h. Percent tibia bone ash (TBA) was calculated based on starting dry bone weight and remaining ash and expressed as a percent. The left

tibiae were removed from the freezer in stored in a fridge for 12-h prior to breaking. The thawed bones were cleaned of any remaining tissue and used to assay (raw) breaking strength (TBS) using a texture analyzer (TA.XT Plus, Texture Technologies, Hamilton, MA.) charged with a 50-kg load cells, a crosshead speed of 100 nm/min with the tibia supported on a 3-point bending ring and a 2.5-cm constant span.

5.2.6. Statistical Analysis

Collected data were analyzed by ANOVA as a 2 x 8 factorial arrangement of 2 strains, Cobb-500 and 700, and 8 intubation concentrations of vitamin D_3 calculated to be from 0 to 3,200 IU/kg diet. 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 a common control to investigate linear and quadratic effects.

5.3. RESULTS AND DISCUSSION

Performance Parameters

Performance results are presented in table 5-3. When reviewing day 10 BW, day 17 BW, WTG, and FCR, no differences were observed between level of orally gavage D_3 or strain. Additionally, when reviewing the main effects, no differences were observed for level of orally gavage D_3 treatments. However, significant strain differences were observed for all performance variables, which showed higher response from the Cobb-500 strain to orally gavage D_3. Sakkas et al. (2018) observed similar strain differences when comparing Ross-308 and Ross-708 broilers fed 1 of 4 diets varying in vitamin D (1,000 IU D_3/kg, 4,000 IU D_3/kg, 7,000 IU D_3/kg, and 1,000 D_3

+3,000 25-OH-D_3 IU/kg). Performance results from this study concluded that diet and interaction with strain did not affect any of the performance variables. However, main effects of strain as seen in the present study significantly affected BW and FCR. The Ross-308 achieved a higher BW at the end of each phase measured and a significantly lowered FCR.

In reviewing the strains independently, as shown in table 5-4, no significant differences between oral gavage treatments for each strain were observed. However, numerical differences can be observed for a reduced BW and WG when looking at the Cobb-700. This is to be expected with slow-growing or slow-yielding strains, as these birds develop more slowly going into the grower and finishing feed phases.

Table 5-3 ANOVA Day 10-17 Performance

Treatment		n	10d BW[3]	10-17d BW[3]	10-17d WTG[3]	10-17d FCR[3]
0	Cobb-500	6	211 ± 0.9	540 ± 10.8	328 ± 10.7	1.36 ± 0.2
50	Cobb-500	6	213 ± 0.9	529 ± 10.8	315 ± 10.7	1.32 ± 0.2
100	Cobb-500	6	214 ± 0.9	557 ± 10.8	343 ± 10.7	1.28 ± 0.2
200	Cobb-500	6	213 ± 0.9	529 ± 10.8	317 ± 10.7	1.31 ± 0.2
400	Cobb-500	6	213 ± 0.9	558 ± 10.8	345 ± 10.7	1.28 ± 0.2
800	Cobb-500	6	213 ± 0.9	549 ± 10.8	336 ± 10.7	1.29 ± 0.2
1600	Cobb-500	6	213 ± 0.9	553 ± 10.8	340 ± 10.7	1.29 ± 0.2
3200	Cobb-500	6	214 ± 0.9	555 ± 10.8	341 ± 10.7	1.28 ± 0.2
0	Cobb-700	6	180 ± 0.9	484 ± 10.8	305 ± 10.7	1.35 ± 0.2
50	Cobb-700	6	180 ± 0.9	473 ± 10.8	294 ± 10.7	1.37 ± 0.2
100	Cobb-700	6	179 ± 0.9	485 ± 10.8	307 ± 10.7	1.35 ± 0.2
200	Cobb-700	6	177 ± 0.9	482 ± 10.8	305 ± 10.7	1.34 ± 0.2
400	Cobb-700	6	180 ± 0.9	474 ± 10.8	294 ± 10.7	1.35 ± 0.2
800	Cobb-700	5	179 ± 0.9	479 ± 10.8	300 ± 10.7	1.34 ± 0.2
1600	Cobb-700	6	179 ± 0.9	472 ± 10.8	293 ± 10.7	1.36 ± 0.2
3200	Cobb-700	6	178 ± 0.9	483 ± 10.8	306 ± 10.7	1.35 ± 0.2
Pvalue			*0.409*	*0.648*	*0.632*	*0.679*
Main Effect						
Level						
0		12	194 ± 16.9	508 ± 34.6	314 ± 22.2	1.33 ± 0.04
50		12	196 ± 17.5	501 ± 40.1	305 ± 30.0	1.34 ± 0.06
100		12	196 ± 18.8	521 ± 52.5	325 ± 41.0	1.32 ± 0.06
200		12	195 ± 18.7	506 ± 33.0	311 ± 22.7	1.32 ± 0.07
400		12	196 ± 17.7	516 ± 53.1	319 ± 39.1	1.32 ± 0.06
800		11	198 ± 18.0	517 ± 42.5	319 ± 28.3	1.31 ± 0.05
1600		12	196 ± 18.0	513 ± 47.9	316 ± 33.9	1.32 ± 0.05
3200		12	196 ± 18.6	520 ± 40.7	323 ± 24.1	1.31 ± 0.06
Pvalue			*0.720*	*0.604*	*0.618*	*0.587*
Strain						
Cobb-500		48	213 ± 0.33a	546 ± 3.8a	333 ± 3.7a	1.30 ± 0.01a
Cobb-700		47	179 ± 0.33b	479 ± 3.8b	300 ± 3.7b	1.35 ± 0.01b
Pvalue			*<.0001*	*<.0001*	*<.0001*	*0.0001*

[a-b]Means within the same column without a common superscript differ (P<0.05) Duncan's multiple range test; Means ± SEM. N= 6 reps per treatment

[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); FCR, Mortality corrected FCR (g weight gain/g feed intake).

Table 5-4 Effect of Dietary Vitamin D_3 on Performance of Modern Broiler Strains

		Cobb-500			Cobb-700		
IU D_3/kg feed[1,2]	**n**	**Response[3,4]**					
		17d	**10-17d**	**10-17d**	**17d**	**10-17d**	**10-17d**
		BW	WG	FCR	BW	WG	FCR
0	6	537 ± 29.0	326 ± 27.9	1.31 ± 0.04	484 ± 12.5	305 ± 10.5	1.35 ± 0.03
50	6	529 ± 37.6	315 ± 37.9	1.32 ± 0.07	473 ± 16.7	294 ± 16.2	1.37 ± 0.05
100	6	557 ± 47.0	343 ± 46.4	1.28 ± 0.06	485 ± 28.1	307 ± 28.1	1.35 ± 0.04
200	6	529 ± 27.9	317 ± 27.5	1.31 ± 0.06	482 ± 17.2	305 ± 17.2	1.34 ± 0.08
400	6	558 ± 28.5	345 ± 28.0	1.28 ± 0.06	474 ± 34.3	294 ± 32.8	1.35 ± 0.05
800	6	549 ± 29.4	336 ± 29.0	1.29 ± 0.06	479 ± 7.1	300 ± 8.7	1.34 ± 0.04
1600	6	553 ± 22.3	340 ± 22.7	1.29 ± 0.04	472 ± 25.0	293 ± 26.0	1.36 ± 0.03
3200	6	555 ± 10.6	341 ± 9.6	1.28 ± 0.05	484 ± 21.8	306 ± 20.3	1.35 ± 0.05
Pvalue		*0.477*	*0.519*	*0.811*	*0.924*	*0.858*	*0.966*

Means ± SEM. N=6 reps per treatments
[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); FCR, Mortality corrected FCR (g weight gain/g feed intake).
[4]Values for performance responses represent the mean average of n=6 replicate pens per treatment of 6 birds each at respective age.

5.3.2. Bone Mineralization

Results for bone mineralization are shown in table 5-5. Differences in TBS were observed between level and strain main effects. However, multiple range test suggest that 100 IU/kg of feed were needed to maximize TBS and that the 200 IU/kg treatment group had the lowest TBS overall. In contrast, Kasim and Edwards (2000) estimated the D_3 requirements for growing broiler chickens ranging from 100-1,100 IU/kg of feed to

maximize performance parameters. Furthermore, this present protocol allows an estimate of nutritional D_3 requirements, however, it is still highly influenced by the maternal reserves of D_3 found in the egg yolk. Results in table 5-6 show strain comparison for the eight oral gavage D_3 treatments. Multiple range tests suggest that the Cobb-500 responded quicker to the oral gavage treatments as shown by TBS.

Table 5-7 illustrated the linear broken line and quadratic broken line for TBA and TBS for the two strains. The Cobb-500 was able to fit both broken lines for TBA and TBS, however, the TBA resulted in closer estimates (219 and 292) to that of the 1994 NRC. When looking at the Cobb-770 response, TBA required approximately 1290 and 504 IU/kg of feed for the linear broken line and quadratic broken line, respectively. TBS for the Cobb-700 resulted in 808 and 293 for the two broken line models as well. This suggest that the Cobb-700 vitamin D_3 requirement is much higher than that of the Cobb-500 to maximize TBA and TBS.

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Table 5-5 ANOVA Day 17 Broiler Bone Mineralization

Treatment		N	TBA	TBS
0	Cobb-500	6	46.4 ± 0.6	11.8 ± 0.4
50	Cobb-500	6	45.8 ± 0.6	10.8 ± 0.4
100	Cobb-500	6	46.2 ± 0.6	12.7 ± 0.4
200	Cobb-500	6	46.7 ± 0.6	10.6 ± 0.4
400	Cobb-500	6	46.3 ± 0.6	12.1 ± 0.4
800	Cobb-500	6	47.0 ± 0.6	11.8 ± 0.4
1600	Cobb-500	6	47.0 ± 0.6	12.2 ± 0.4
3200	Cobb-500	6	46.3 ± 0.6	11.6 ± 0.4
0	Cobb-700	6	46.4 ± 0.6	10.1 ± 0.4
50	Cobb-700	6	45.6 ± 0.6	10.1 ± 0.4
100	Cobb-700	6	46.4 ± 0.6	10.6 ± 0.4
200	Cobb-700	6	45.5 ± 0.6	10.2 ± 0.4
400	Cobb-700	6	46.8 ± 0.6	10.1 ± 0.4
800	Cobb-700	5	47.0 ± 0.6	10.7 ± 0.4
1600	Cobb-700	6	47.6 ± 0.6	10.5 ± 0.4
3200	Cobb-700	6	47.4 ± 0.6	10.6 ± 0.4
Pvalue			*0.648*	*0.173*
Main Effect				
Level				
0		12	46.4 ± 0.4	11.0 ± 0.3^{abc}
50		12	45.7 ± 0.4	10.4 ± 0.3^{bc}
100		12	46.3 ± 0.4	11.6 ± 0.3^{a}
200		12	46.1 ± 0.4	10.4 ± 0.3^{c}
400		12	46.5 ± 0.4	11.1 ± 0.3^{ab}
800		11	47.0 ± 0.4	11.2 ± 0.3^{a}
1600		12	47.3 ± 0.4	11.3 ± 0.3^{a}
3200		12	46.9 ± 0.4	11.1 ± 0.3^{ab}
Pvalue			*0.137*	*0.007*
Strain				
Cobb-500		48	46.4±0.2	11.7±0.1^{a}
Cobb-700		47	46.6±0.2	10.3±0.1^{b}
Pvalue			*0.646*	*<.0001*

[a-d]Means within the same column without a common superscript differ (P<0.05) Duncan's multiple range test; Means ± SEM. N=6 reps per treatment.
[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.

Table 5-6 Effect of Vitamin D_3 on Modern Broiler Strains Bone Mineralization

		Cobb-500		Cobb-700	
IU D_3/kg feed[1,2]	**n**	**Response[3,4] 0-17 day**			
		TBA	TBS	TBA	TBS
0	6	46.1 ± 1.2	11.8 ± 1.4^{abc}	46.4 ± 1.0	10.1 ± 0.9
50	6	45.8 ± 0.9	10.8 ± 0.8^{bc}	45.6 ± 1.7	10.1 ± 0.7
100	6	46.2 ± 1.1	12.7 ± 1.3^{a}	46.3 ± 1.6	10.6 ± 0.9
200	6	46.7 ± 1.6	10.6 ± 0.5^{c}	45.5 ± 1.0	10.1 ± 0.3
400	6	46.3 ± 1.2	12.1 ± 1.1^{a}	46.8 ± 1.0	10.1 ± 0.8
800	6	47.0 ± 1.8	11.8 ± 0.5^{ab}	46.9 ± 1.4	10.4 ± 0.3
1600	6	47.0 ± 1.5	12.2 ± 1.1^{a}	47.6 ± 1.5	10.5 ± 1.1
3200	6	46.3 ± 1.7	11.6 ± 0.5^{abc}	47.4 ± 1.5	10.6 ± 0.8
Pvalue		*0.811*	*0.006*	*0.105*	*0.815*

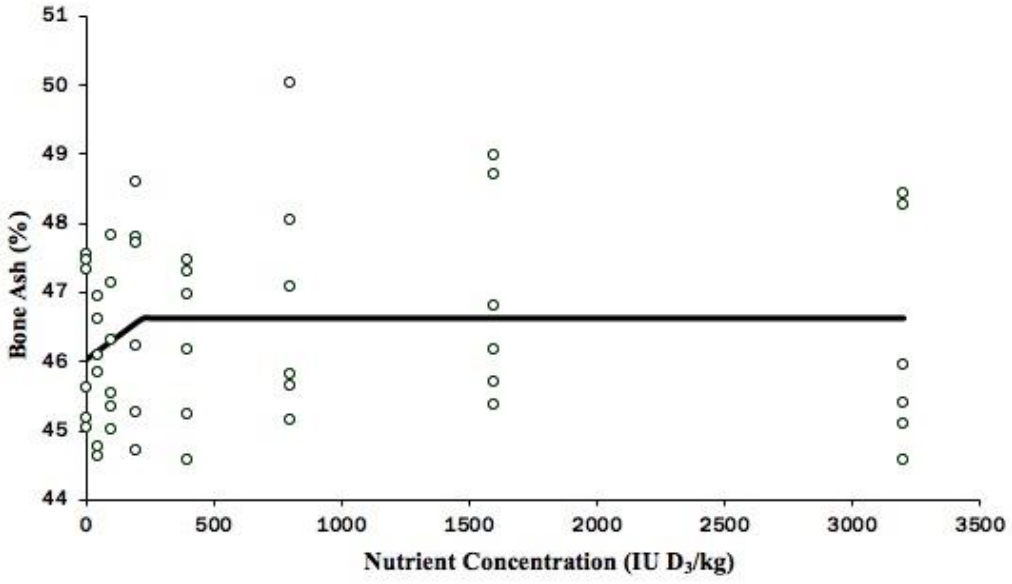
[a-c]Means within the same column without a common superscript differ (P<0.05) Duncan's multiple range test. Means ± SEM.
[1]Calculated IU D_3 /kg feed (Adjusted IU D_3/kg feed based on feed intake data).
[2]Serial dilutions using the stock solution were performed to create dietary D_3 treatments so 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)
[4]Values for bone mineralization responses represent the mean average of n=6 replicate pens per treatment of 6 birds each at respective age.

Table 5-7 Vitamin D_3 requirements and model comparison

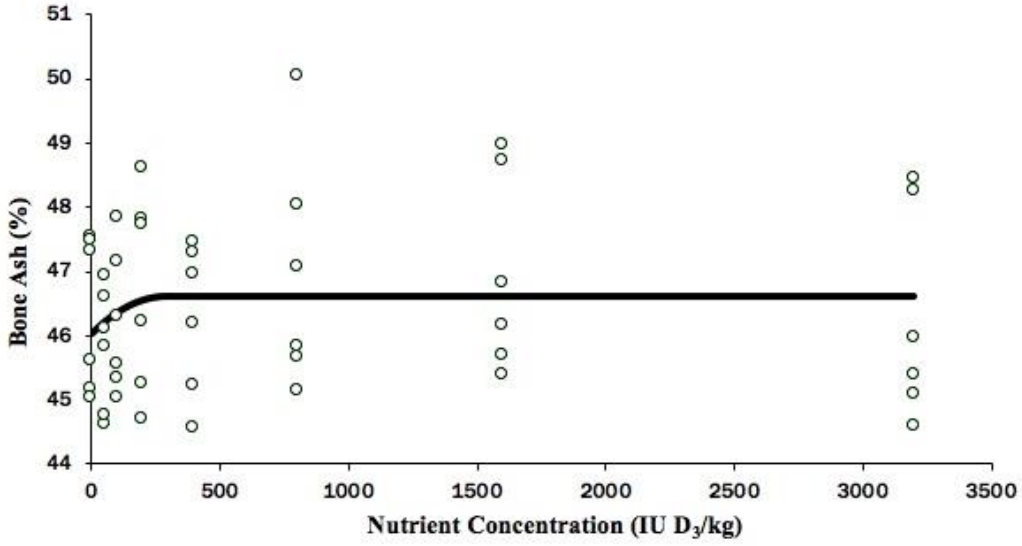
	Response	Model	ER[1]	R^2(%)	Pvalue
Cobb-500	BA	Linear Broken Line	219	2.796	0.3614
		Quadratic	292	2.400	0.5860
	BS	Linear Broken Line	100	0.991	0.7656
		Quadratic	122	0.204	0.8698
Cobb-700	BA	Linear Broken Line	1290	10.035	0.1339
		Quadratic	504	10.855	0.2821
	BS	Linear Broken Line	808	4.514	0.2883
		Quadratic	293	2.304	0.5624

[1]Estimated requirement, ER (IU D_3/kg of feed)

Figure 5-1 Cobb-500 Bone Ash Linear Broken Line



1319 **Figure 5-2 Cobb-500 Bone Ash Quadratic Broken Line**



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1324 **Figure 5-3 Cobb-700 Bone Ash Linear Broken Line**
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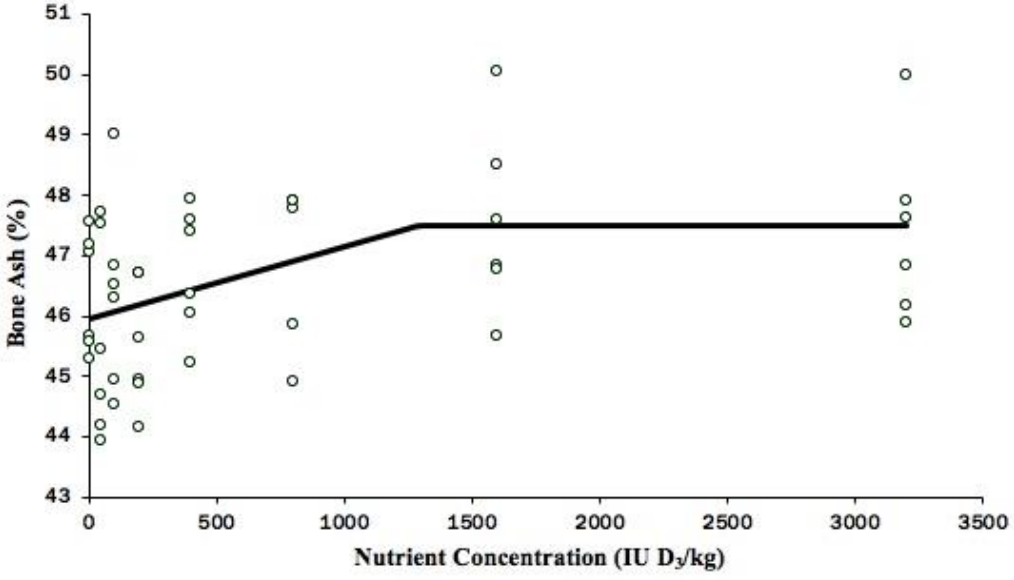

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Figure 5-4 Cobb-700 Bone Ash Quadratic Broken Line

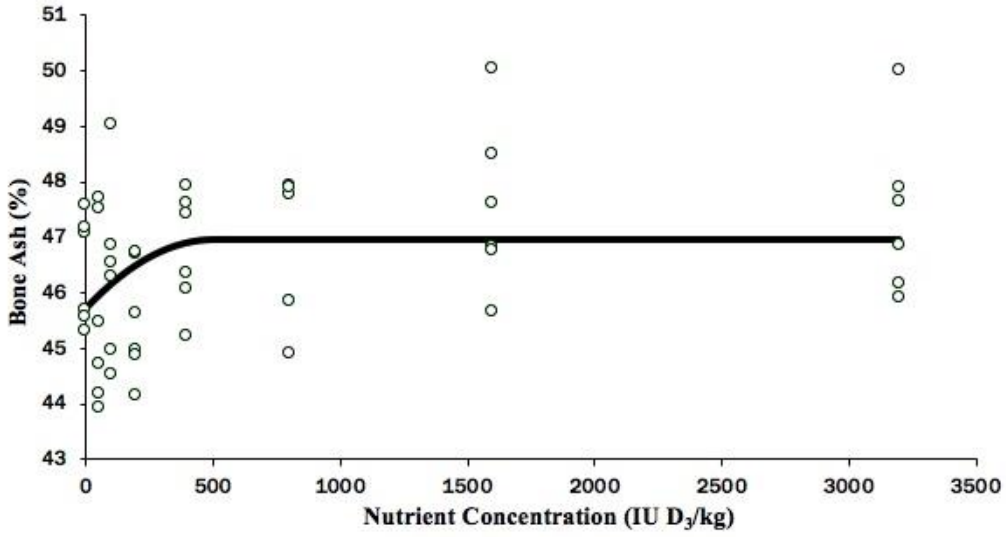
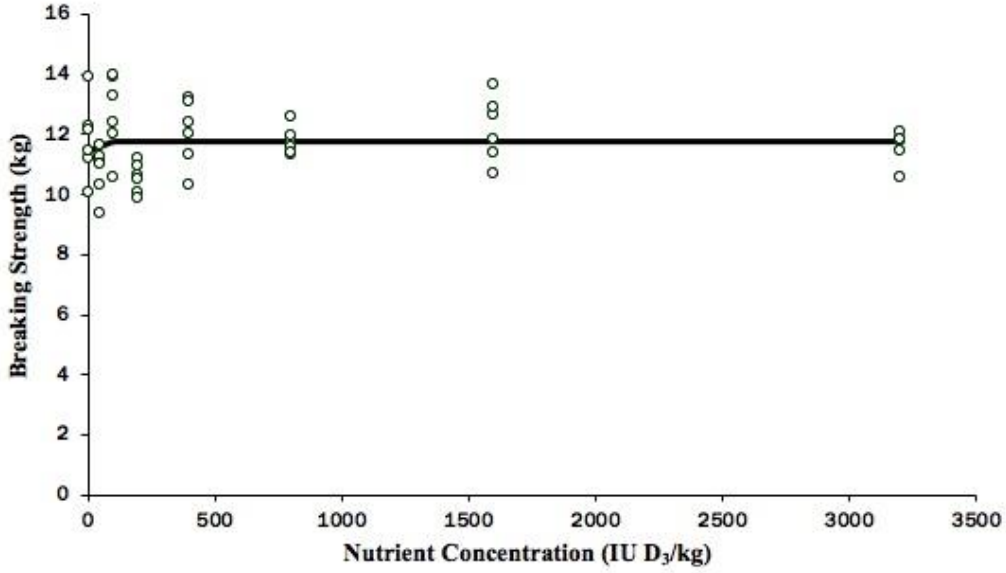


Figure 5-5 Cobb-500 Breaking Strength Linear Broken Line



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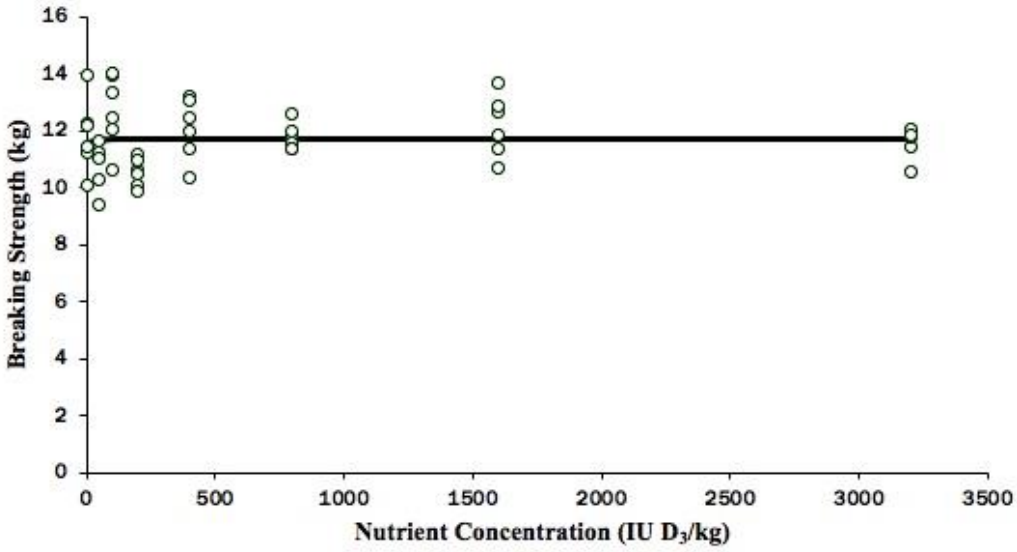
1335 **Figure 5-6 Cobb-500 Breaking Strength Quadratic Broken Line**



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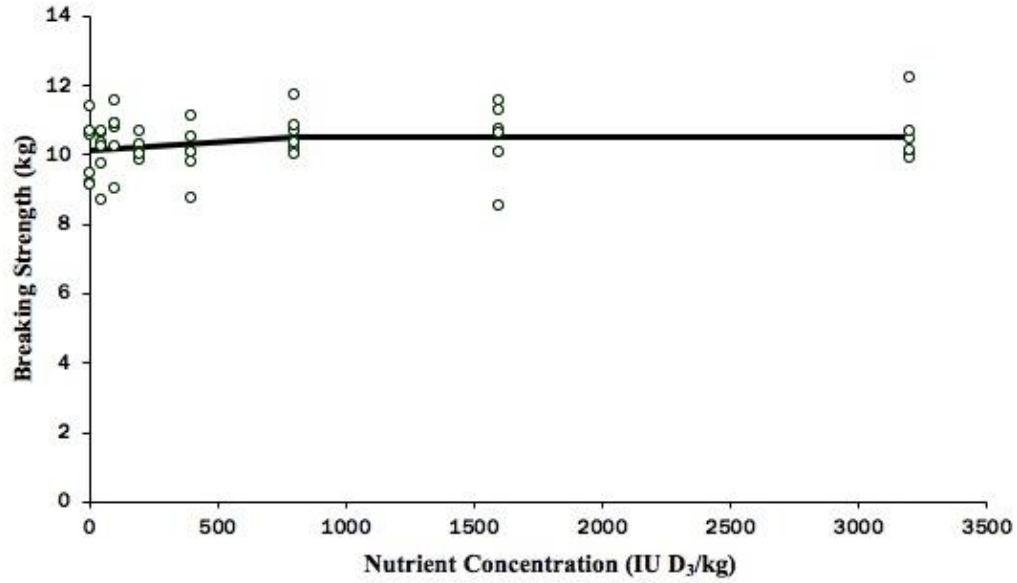
1338 **Figure 5-7 Cobb-700 Breaking Strength Linear Broken Line**



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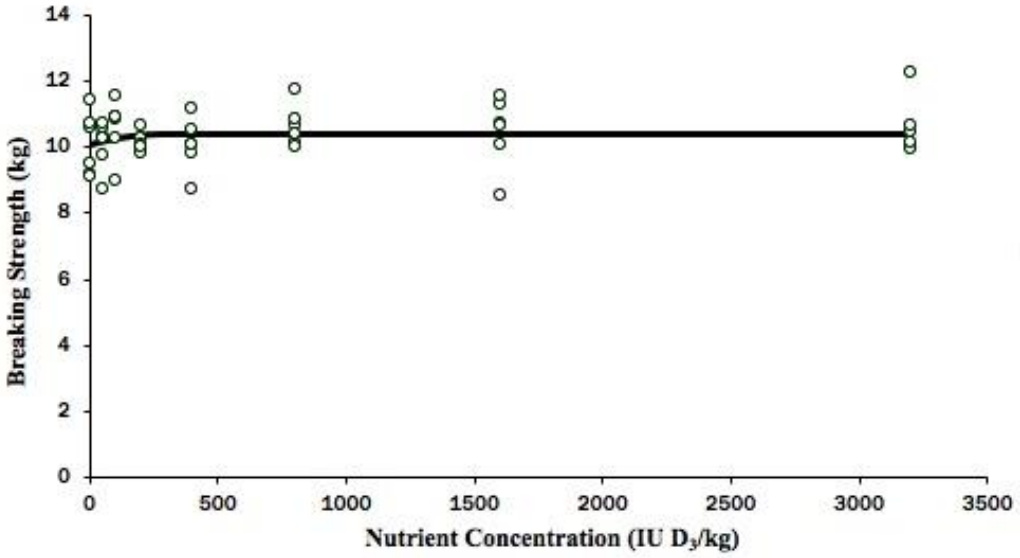
1341 **Figure 5-8 Cobb-700 Breaking Strength Quadratic Broken Line**



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6. CONCLUSIONS

Nutritionist rely on literature references such as the National Research Committee (NRC) to formulate diets that meet the animal's nutritional requirements. However, today's modern broiler breeds are much different genetically and have been selected for fast growth and yield parameters. Therefore, it is possible that their dietary requirements have changed to meet their needs for optimal growth.

The poultry NRC was established with nutritional recommendations for growing poultry, however, most of the requirements trials date back to early 1960s. This supports the need for developing new protocols to evaluate nutrient requirements in modern broiler strains. Post NRC literature suggest that dietary supplementation of D_3 (up to 20x the 1994 NRC) is required for optimal growth of broilers, however, the NRC was estimated at 200 IU/kg of feed.

To further evaluate the vitamin D_3 requirement in modern broiler strain, this dissertation adapted methodology from a previous protocol develop in our laboratory that would precisely estimate the vitamin D_3 requirement. Additionally, supplementation of D_3 metabolites such as 25-hydroxycholecalciferol (25-OH-D_3) has become a routine supplement via the feed or water administration. However, the NRC has limited information regarding the use of feed additive including vitamin metabolites such as 25-OH-D_3. Which lead to the second objective of this dissertation which was to compare the effects of dietary levels of D_3 and 25-OH-D_3 on performance, bone mineralization, and vitamin D status. It can be concluded that the supplementation of 25-OH-D_3 is more

effective than D_3 in promoting bone mineralization and increasing vitamin D status in broilers.

Due to high supplementation of D_3 to the broiler breeders in commercial practices, high maternal stores in the egg yolk is carried over into the progeny of these birds. This "carry-over" highly influences the vitamin D_3 requirement of growing broilers and reduces sensitivity of this protocol. Future research should focus on establishing a vitamin D depleted breeder flock, perhaps in the pullet phase, to have a homogenous and low vitamin D status.

Accelerated growth of commercial broilers over the last decade has led to the development of modern broiler strains with improved feed efficiency and higher meat yields. It can be concluded from the last part of this dissertation that modern broiler strains respond significantly different to supplementation of vitamin D_3. Additionally, that TBS was seen to be more sensitive to estimating vitamin D_3 requirements as compared to the AOAC recommended TBA method.

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