RE-EVALUATION OF THE VITAMIN D_3 REQUIREMENT FOR GROWING CHICKENS

A Thesis

by

HECTOR ENRIQUE LEYVA-JIMENEZ

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Chair of Committee,	Cristopher A. Bailey
Committee Members,	Rosemary L. Walzem
	Delbert M. Gatlin III
Head of Department,	David J. Caldwell

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ABSTRACT

Three experiments were conducted with the purpose of re-evaluating the vitamin D_3 requirement of growing chickens. Due to intense genetic selection in the poultry industry, it is probable that modern strains have altered their nutritional requirements. Therefore, it is necessary to re-evaluate their ability to respond to modern feeding strategies. In the first experiment, broiler chickens were obtained from different commercial hatcheries and fed a basal vitamin D_3-deficient starter diet with the objective of evaluating the variability in maternal deposition of vitamin D_3 in the egg yolk. No significant ($p>0.05$) differences were found in the initial serum concentrations of 25-hydroxycalciferol (25-OH-D_3) within sources of broiler chickens. Maternal vitamin D reserves will deplete around day 10 regardless of the initial concentration in newly hatched chickens. In the second experiment, 500 Ross-308 broiler chickens were sampled after being fed with 3,850 IU/kg cholecalciferol (D_3) or 3,850 IU/kg D_3 plus one of two sources of 25-OH-D_3 for a 24-h period at different time points to evaluate the dynamics of serum 25-OH-D_3, and identify if there was any "circadian-type" trend in the broiler´s response to dietary 25-OH-D_3 over time, as well as identify important information about the effect of age in the metabolism of 25-OH-D_3 and bone ash mineralization. Both sources showed a significant linear increase in serum 25-OH-D_3 concentrations ($p<0.05$) over the 24-h exposure to treatment diets. Finally, for the third experiment, 96 Hy-line chickens were placed in a very controlled environment and a concentrated liquid form of vitamin D_3 (Pure Life® Labs) was directly gavaged in

precise increasing amounts to re-evaluate the requirement of this metabolite for growth performance and bone mineralization. The estimated requirement for growing leghorn chickens was 800 IU/kg D_3 for maximum growth and bone mineralization. Results of these studies show that in order to produce a vitamin D_3-deficient status in young broiler chickens, it is necessary to have strict control on the breeder's diet. The supplementation of 25-OH-D_3 increases the probability of maximum biological function with regard vitamin D. The re-evaluation of the vitamin D_3 requirement for leghorn chickens suggests that the NRC (1994) minimum vitamin D_3 requirement of 200 IU/kg might be inadequate for modern poultry leghorn strains.

DEDICATION

I dedicate this thesis work to my family.

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TABLE OF CONTENTS

Page

Page

LIST OF FIGURES

Page

Page

LIST OF TABLES

Page

Page

1. INTRODUCTION

Genetic selection in meat-type chickens over the past 20 years have led to increased productivity, thanks to the improvement in growth potential and feed efficiency. This was shown by Havenstein *et al.* (2003) when they compared two strains of broilers; Athens-Canadian Randombred Control (ACRBC) and the 2001 Ross-308 and fed representative 1957 and 2001 diets, respectively. They found that the Ross-308 broilers on the 2001 feed were estimated to have reached 1,815 g body weight (BW) at 32 days of age with a feed conversion (FC) of 1.47; whereas, the ACRBC on the 1957 feed would not have reached that BW until 101 days of age with a FC of 4.42. Because feed represents up to 70% of the total production costs, the opportunity to evaluate birds ability to respond to different extents of nutrient density and new alternative ingredients have become one of the main concerns in the poultry industry to reduce the cost of feed.

One consequence of the improvement in growth rate (35-50 g yearly increase in 42-49 day BW; Leeson and Summers, 2005) is leg health, which is an important component of broiler welfare, and therefore, in general broiler performance. Leg health encompasses a wide range of leg disorders leading to locomotion problems and high mortality (Kapell *et al.* 2012) which represents a major economic issue for the poultry industry. Sullivan (1994) estimated annual losses in the United States due to skeletal problems in broiler production around $80 to $120 million USD which adjusted to the annual inflation (U.S. Bureau of Labor Statistics) would represent $ 128,000,000 to 192,000,000 USD in 2014.

Common leg problems in rapid growth rate strains include tibial dyschondroplasia (TD), angular bone deformities, such as medial deviation of the distal tibia or proximal metatarsus, and rickets (Waldenstedt, 2006). Vitamin D is a term associated with a group of related steroid chemical compounds that possess antirachitic activity. There are two common forms, ergocalciferol (D_2) and cholecalciferol (D_3). Since D_2 has about 10% the bioactivity of D_3 for poultry (McDonald *et al.* 1995) D_3 is used to supplement poultry feeds. The main role of vitamin D is the control of bone development through its ability to strictly regulate Ca absorption (Aslam *et al*., 1998). Therefore, vitamin D has a strong relationship with calcium (Ca) and phosphorus (P) metabolism and ultimately bone health. The quantitative requirement of growing chickens for vitamin D is usually based on functional assessments using measurements of bone quality, such as bone ash content or incidence of rickets, which have been found to be more sensitive indicators of requirements than growth rate (Waldroup *et al*., 1963, Edwards *et al*., 1994). The vitamin D requirement reported by the NRC (1994) is 5 µg/kg (200 IU/kg) for both growing broilers and leghorn strains. Seventeen years ago, Baker *et al.* (1998) reported in agreement with the NRC (1994) that 5 µg/kg (200 IU/kg) appeared to be adequate for maximal weight gain and bone ash in broiler chickens from 8-20 days post hatching when fed adequate levels of available P. However, changes in available P can increase the requirement up to 37.5 µg/kg (1,500 IU/kg). More recent studies reported higher requirements for vitamin D; Kasim and Edwards (2000) estimated the vitamin D requirement for broiler chickens was 25 µg/kg (1,000 IU/kg). Fritts *et al*. (2003) reported that approximately 50 µg/kg (2,000 IU/kg) of vitamin D_3 was needed for

maximum bone ash in 21-day-old broilers. Whitehead *et al.* (2004) showed that vitamin D_3 requirements of broilers up to 14 days of age at sufficient dietary Ca and available P concentrations may be in the range 35-50 µg/kg (1,400-2,000 IU/kg) of feed for optimal cortical bone quality. Khan *et al*. (2010) concluded that performance, bone mineralization and immunity were significantly improved when supplementing high levels of vitamin D_3 [37.5-87.5 µg/kg (1,500-3500 IU/kg)] in broiler diets compared with the NRC requirement. Baird *et* al. (1935) estimated the vitamin D_3 requirement for growing brown egg layers to be 4.5 µg/kg (180 IU/kg). Thus, there is a big debate regarding the vitamin D requirement of growing chickens, as some authors (Baker *et al.*, 1998, Fowler *et al.* 2014) agree with the NRC (1994) requirements. However, a number of studies made post 1994 suggest higher doses are needed to support rapid growth of modern poultry strains and prevent the appearance of leg disorders such as TD (Kasim and Edwards, 2000, Ledwaba and Roberson, 2003, Whitehead et al. 2004, Khan *et al*., 2010).

The nutritional importance of vitamin D is not limited to Ca and P metabolism in bone mineralization. Other functions of vitamin D include stimulation of intestinal absorption of Ca and P, maintaining normal parathyroid hormone concentration, mediating kidney Ca reabsorption and influencing diverse immune functions (De Luca, 2004) such as cell-mediated and humoral antibody response. A deficiency in vitamin D can increase the susceptibility to infections due to impaired localized innate immunity and defects in antigen-specific cellular immune response (Wintergerst *et al*. 2007).

The aim of this thesis project is to re-evaluate the vitamin D_3 requirement in the growing chicken by tracking the maternal deposition of vitamin D_3 in the egg yolk and the dynamics of 25-hydroxycholecalciferol (25-OH-D_3) concentration in the blood.
This information will be used to establish an accurate procedure to test the requirement in growing chickens using an individual daily oral dose of a liquid form of pure cholecalciferol to have precise control in vitamin D_3 consumption.

2. LITERATURE REVIEW

2.1 HISTORY OF VITAMIN D

The history of vitamin D has been reviewed by Norman (1979), De Luca (2004) and Combs (2012). As with most of the known vitamins, their discovery is usually related or linked with common diseases or disorders that were observed in animals or humans at different stages of history. The first approach to vitamin D study was made in the times of ancient Greece when Hippocrates pointed out the importance of sunlight in human health and described conditions that resembled rickets (Mohr, 2009). The first descriptions of rickets were written by Daniel Whistler who wrote on the subject in his medical thesis at Oxford University in 1645 and Professor Francis Glisson of London who also wrote about the subject in 1650. The formal study of vitamin D started in the eighteenth century when rickets were accepted as a skeletal disorder and a health problem, especially during the industrial revolution in England where rickets were known as "The English Disease." Sbiadecki (1822) suggested that rickets were caused by lack of exposure to sunlight, when he compared the incidence between children that lived in urban and rural areas. The first description of the rachitic skeleton was made by Pommer in 1885. Palm (1980) pointed out the importance of sunlight to prevent rickets. Many factors were described as responsible for rickets: lack of fresh air, congenital factors, age, infectious agents and lack of physical activity were examples of some of these factors. At the same time vitamin A was recognized to prevent xeropthalmia and night blindness and was identified to be present in cod liver oil. In 1919, Edward

Mellanby undertook the studies of vitamin A and tried to develop a dietary model for rickets. He conducted several studies where he fed dogs a low-fat diet, and when he observed that the dogs developed rickets, he concluded that rickets were probably also due to a vitamin A deficiency and that cod liver oil was an excellent antirachitic agent; he also established the importance of dietary Ca and P in preventing rickets. However, McCollum (1922) who previously identified vitamin A, was convinced that this new antirachitic activity of cod liver oil was due to a different compound. After he bubbled oxygen through heated cod liver oil to destroy the vitamin A, he fed the oil to rats and chicks and found that the processed oil was still able to cure rickets. He concluded that a different compound was responsible for reducing the incidence of rickets, and he called it vitamin D. Goldblatt and Soames (1923) demonstrated that UV-irradiated rat livers that were fed to rachitic rats were able to cure rickets. Steenbock and Black (1924) also found that UV-irradiated food was also able to cure rickets in rachitic rats. Thus, the first relationship between light and vitamin D was formally established. The vitamin D_3 chemical structure was determined by Windaus´s group in 1936. It wasn´t until the 1970´s that Kodicek *et al*. (1970), DeLuca (1978) and Norman (1979) clearly defined the vitamin D metabolites and their controls.

2.2 PROPERTIES AND METABOLITES OF VITAMIN D_3

Vitamin D, also known as the "sunshine vitamin," is a fat-soluble vitamin that encompasses a group of steroid chemical compounds that possess antirachitic activity. Vitamin D_3 is soluble in organic non-polar solvents and oils; it has a melting point of 84-85°C and can be destroyed by excess irradiation of UV-light and peroxidation when

rancidifying polyunsaturated fatty acids are present. The use of some antioxidants such as vitamin E may prevent vitamin D destruction (Leeson and Summers, 2001). The most common forms of vitamin D are ergocalciferol (D_2) and cholecalciferol (D_3). Vitamins D_2 and D_3 have relatively the same potency for cattle, sheep and pigs, but D_2 has only 3% of the potency of D_3 for poultry (Leeson and Summers, 2001). Chen and Bosmann (1964) in a very controlled study using crystalline vitamins D_2 and D_3 reported an antirachitic activity ratio from 1:8 to 1:11 in poultry, respectively. One unit of vitamin D_3 [International Units (IU)] is defined as the activity of 0.025 µg of cholecalciferol (NRC, 1994).

Cholecalciferol is produced *in vivo* by irradiation of the provitamin D_3 7-dehydrocholesterol, in the epidermal layer of the skin where, by exposure to the ultraviolet light (290-310 nm) either from the sun or an artificial source, it is synthesized to cholecalciferol (Combs, 2012). The structures of vitamin D_3 and D_2 are shown in Figure 1. The precursors of vitamin D_3 have no effect in the body until the B-ring is opened between the 9 and 10 positions of the steroid. This occurs with ultraviolet irradiation forming a double bond between the carbon atoms at the 10 and 19 positions of the new compounds (Leeson and Summers, 2001). It has been found that ten times more provitamin D_3 is present in the featherless leg skin than on the back, indicating the importance of this area for vitamin D_3 metabolism (Tian *et al*. 1994). The synthesized cholecalciferol, formed in the skin, is absorbed and transported via the blood to the liver where it is converted to 25-hydroxycholecalciferol (25-HO-D_3), the major circulating form of vitamin D_3. The level of 25-OH-D_3 associated with adequate bone health is 30

ng/ml, and this is parameter is considered to be the best indicator of vitamin D status (Pettifor *et al*., 1977). The half-life of 25-OH-D_3 is 20 days (Combs, 2012). The 25-OH-D_3 must be transported to the kidneys for conversion into 1,25-dihydroxycholecalciferol (1,25$(OH)_2$ D_3) which is the active hormonal metabolite of the vitamin (Norman, 1979). The newly formed 1, 25$(OH)_2$ D_3 is transported via the blood to the intestine, bones, or anywhere else required to perform its metabolic function. Transport of cholecalciferol and metabolites is possible thanks to the vitamin D binding protein (DBP) which binds to the metabolites from the skin and dietary sources. The production of 1, 25$(OH)_2$ D_3 is regulated by parathyroid hormone (PTH) in response to Ca and P concentration in the blood. When the concentration of Ca in blood is low, PTH is secreted, and the kidneys react by releasing P into urine; this metabolic change stimulates the kidney to produce 1, 25$(OH)_2$ D_3. If Ca and P levels are normal, the kidney transforms 25-OH-D_3 into 1, 24, 25-trihydroxyvitamin D_3 an analog of vitamin D (Leeson and Summers, 2001). Holick *et al*. (1973) found that this analog 1, 24, 25-trihydroxyvitamin D_3 was less bioactive (around 60%) than 1, 25$(OH)_2$ D_3 in stimulating intestinal Ca absorption and bone Ca mobilization.

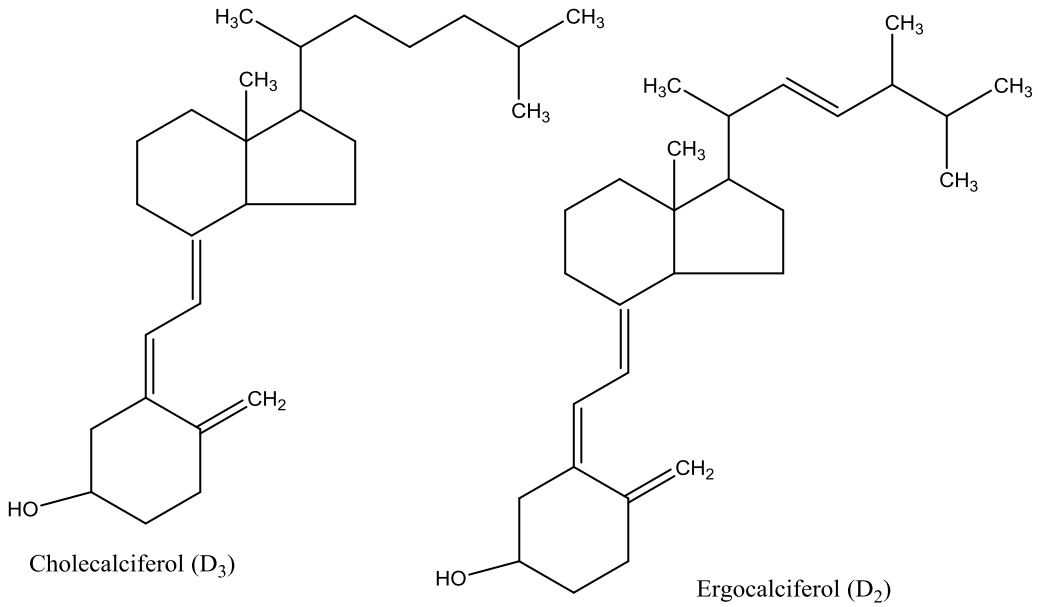


Figure 1. Chemical structures of cholecalciferol (D_3) and ergocalciferol (D_2). Structures drawn using ChemDraw® Professional 15.0 software.

2.3 ABSORPTION AND TRANSPORT

The absorption of vitamin D through the intestine is classified as micelle dependent passive diffusion and requires the presence of bile salts that help in the formation of micelle structures in the small intestine. The major absorption of vitamin D occurs in the distal part of the jejunum and ileum (Combs, 2012, Goncalves, 2015). Once it reaches the blood stream, vitamin D is rapidly distributed throughout the body, where the highest concentration will be found in the intestine, liver, kidneys, and spleen (Leeson and Summers, 2001). Vitamin D and its metabolites will be transported within the body with the help of the vitamin D binding protein (DBP) which binds the vitamin stoichiometrically. Also, endogenous vitamin D produced in the skin will be transported

into the blood stream with the help of DBP (Combs, 2012). Excretion of vitamin D is primarily through the feces with very little excretion in the urine (McDowell, 1989).

2.4 METABOLIC FUNCTIONS

Vitamin D supports bone growth and mineralization indirectly by elevating Ca and P levels in the plasma to a point necessary to support normal body functions (Underwood and DeLuca, 1984). Two hormones, thyrocalcitonin (calcitonin) and parathyroid hormone (PTH) have influence on the release of $1,25(OH)_2D_3$, and are needed to control levels of blood Ca and P. The role of calcitonin appears most important in controlling high serum Ca levels by depressing gut absorption, halting bone demineralization and depressing Ca absorption in the kidney. On the other hand, vitamin D_3 elevates plasma Ca and P by stimulating specific pump mechanisms in the intestine, kidney and bone, thus maintaining blood levels of Ca and P, from these body reserves (Leeson and Summers, 2001). The synthesis of $1,25(OH)_2D_3$ is tightly regulated by PTH, depending on the calcium status of the bird. Normal Ca concentration in blood is around 10 mg/dL. The D_3 metabolite $1,25(OH)_2D_3$ stimulates the expression of the calbindin-D_{28K}-mRNA gene in the intestinal wall (Hall and Norman, 1990) and induces the synthesis of calcium binding protein calbindin-D_{28K} . Theofan *et al*. (1986) measure the intestinal transcription level of calbindin-D_{28K}-mRNA in vitamin D_3 depleted white Leghorn cockerels treated with an intramuscular dose of $1,25(OH)_2D_3$. He concluded that the transcription of the calbindin-D_{28K}-mRNA gene is directly correlated to nuclear uptake and binding of $1,25(OH)_2D_3$ to its receptor. The presence of this protein reflects the ability of the intestine to absorb Ca by facilitating the movement of calcium through

the enterocytic cytosol while keeping the intracellular concentration of the free Ca ion below hazardous levels (Combs, 2012). Calcium binding protein calbindin-D_{28K} is virtually not expressed in the intestine during vitamin D deficiency, but is expressed in response to $1,25(OH)_2D_3$ supplementation (Theofan *et al*., 1986, Meyer *et al*., 1992). Bone formation is stimulated by $1,25(OH)_2D_3$, which induces osteoclastin production from osteoblasts (Standford, 2006). The ability to release Ca from the bones is vital for maintaining constant levels of Ca in the blood. Bone is a dynamic tissue that is constantly remodeled. In healthy individuals with adequate physiological functions bone resorption and formation are tightly balanced (Norman and Hurwitz, 1993). Intestinal P absorption is also favored by the $1,25(OH)_2D_3$ metabolite action which stimulates the Na^+/P co-transport in the intestine which is necessary to transport P through the intestinal wall (Combs, 2012). Vitamin D is also involved in Ca and P resorption in the renal tubes of the kidney to maintain general homeostasis in the body (Norman, 1979).

Evidence also suggests that vitamin D_3 plays a regulatory role in immune cell function (De Luca, 2004). This was also reported by Aslam *et al.* (1998) who found that broiler starter diets without supplemental vitamin D_3 depresses the cellular immune responses in young chicks by reducing the number of abdominal macrophages. Cantorna (2006) studied autoimmune disorders in mice and found that vitamin D had a positive effect in the regulation of T cell development and function which will decrease the susceptibility of autoimmune diseases such as multiple sclerosis and inflammatory bowel disease. Wintergerst *et al*. (2007) found that a deficiency in vitamin D can increase the

susceptibility to infections due to impaired localized innate immunity and defects in antigen-specific cellular immune response.

2.5 NUTRITIONAL REQUIREMENT FOR GROWING CHICKENS

Many factors influence the vitamin D_3 requirement of the chicken such as the source of P in the diet, the ratio of Ca to P and the extent of exposure of the animal to sunlight. There are a number of reports indicating that 11 to 45 minutes exposure to sunlight per day is sufficient to prevent rickets in growing chickens (Leeson and Summers, 2001). The vitamin D_3 requirement for starting leghorns and broiler strains reported by the NRC (1994) is 5 µg/kg (200 IU/kg). Baker *et al.* (1998) reported, in agreement with the NRC (1994), that 5 µg/kg (200 IU/kg) appeared to be adequate for maximal weight gain and bone ash in broiler chickens from 8-20 days post hatching when fed adequate levels of available P. However, changes in available P can increase the requirement up to 37.5 µg/kg (1,500 IU/kg). Baird *et al*. (1935) estimated the vitamin D_3 requirement for brown egg layers to be 4.5 µg/kg (180 IU/kg). Years later, several studies reported different requirements. Kasim and Edwards (2000) estimated the cholecalciferol requirement for growing broiler chickens ranged from 20.1 to 28.3 µg/kg (804-1,131 IU/kg). Fritts *et al*. (2003) reported that approximately 50 µg/kg (2,000 IU/kg) of vitamin D_3 was need for maximum bone ash in 21 day old broilers. Ledwaba and Roberson (2003) found that approximately 40-45 µg/kg (1,600-1800 IU/kg) D_3 was required to reduce rickets in male broiler chicks fed a vitamin D_3-deficient diet. Whitehead et al. (2004) showed that vitamin D_3 requirements of broilers up to 14 days of age at sufficient dietary Ca and available P concentrations may be in the range 35-50

µg/kg (1,400-2,000 IU/kg) of feed based on maximum cortical bone quality. Rao *et al.* (2006) concluded that performance and bone mineralization in broilers could be maintained with suboptimal concentration of Ca and P (0.5 and 0.25 % respectively) with high concentrations of vitamin D_3 [90 µg/kg (3,600 IU/kg)] in the diet. Khan *et al*. (2010) concluded that performance, bone mineralization and lymphoid organ weight/body weight ratio was significantly improved when supplementing high levels of vitamin D_3 at 37.5-87.5 µg/kg (1,500-3,500 IU/kg) in broiler diets when compared with the NRC (1994) requirement. As the ratio of Ca to available P becomes narrower, the requirement for vitamin D_3 increases (Leeson and Summers, 2001). Also, the requirement becomes higher if the source of P in the diet is of low availability. Mycotoxins may also significantly increase the requirement of vitamin D_3, possibly due to impaired absorption and liver functions (Leeson and Summers, 2001). Driver *et al.* (2006) studied the effect of maternal dietary vitamin D_3 and found that hens fed 50 µg/kg (2,000 IU/kg) vitamin D_3 of were able to deposit sufficient quantities of vitamin D_3 in the egg to maintain excellent 16-day BW of chickens and reduce the incidence and severity of TD when fed a TD-inducing diet. Recent literature suggesting higher requirements for vitamin D_3 presume this to be due to changes in metabolism of the rapid growth modern poultry strains.

2.5.1 Deficiency symptoms

The most evident signs of vitamin D_3 deficiency are rickets, TD, osteomalacia in adults and retarded growth. Also beaks and claws will become soft and pliable. Feathering is usually poor and other structural disorders may be noted such as a bent

spinal column and sternum deviated (Leeson and Summers, 2001). Lofton and Soares (1986) studied the effects of vitamin D_3 on leg abnormalities in broilers that were fed less than 5 µg/kg (200 IU/kg) of diet and found significantly lower body weights, feed efficiency values, serum ionic calcium, total serum calcium, tibial breaking strength, and percentage tibial ash values. In addition, Aslam *et al.* (1998) found in vitamin D-deficient chicks a reduction in the cellular immune response and a reduction in thymus weight. Also, Sunde *et al*. (1978) studied the effect of different D_3 metabolites in embryo development and found that deficient vitamin D diets led to defective upper mandibles and high mortality during the first two weeks of age.

2.5.2 Toxicity

Excessive intakes of vitamin D_3 also have negative effects, and most common signs of toxicity are related to abnormal high blood Ca concentration, known as hypercalcemia, and low levels of PTH that ultimately affect bone health (McDowell 1989). Cruickshank and Sim (1987) reported that excessive supplementation of vitamin D_3 100 µg/kg (4,000 IU/kg) combined with high stocking density may increase the incidence of twisted leg and negatively affect the feed conversion ratio. Leeson and Summers (2001) stated that high D_3 in poultry diets produces a syndrome characterized by resorption of bone salts and abnormal deposition of calcium in the viscera and soft tissues in growing chickens. Excessive D_3 can lead to renal damage, due to calcification of the kidney tubules while the aorta and other arteries may also become calcified. Nain *et al*. (2007) found that broilers fed excessive amounts of vitamin D_3 2,000 µg/kg (80,000 IU/kg) were more susceptible to sudden death syndrome associated with a higher

rate of cardiac arrhythmia with respect to broilers fed 125 µg/kg (5,000 IU/kg). Estimated safe upper intake levels of D_3 are presented in Table 1. Other toxicity signs may include anorexia, gastrointestinal distress, lameness and calcinosis (Combs, 2012).

Table 1. Estimated safe upper intake levels of vitamin D_3 (IU/kg)

Animal	Exposure time	
	< 60days	**> 60 days**
Chicken	40,000	2,800
Turkey	90,000	3,500
Japanese quail	120,000	4,700

Adapted from NRC (1987)

2.6 SOURCES OF VITAMIN D

Ergocalciferol is widely distributed in plants, fungi and molds while cholecalciferol is present in animals where vitamin concentration depends on the dietary vitamin D_3 and exposure of the tissue to sunlight (Combs, 2012). Sources of vitamin D are natural foods like milk, butter, veal, beef and vegetable oils; irradiated sebaceous material licked from skin or hair or directly absorbed products of irradiation formed on or in the skin. (McDowell, 1989). Some natural sources of vitamin D_2 or D_3 and estimated concentrations are presented in Table 2.

Table 2. Concentration of vitamin D in common foods and feedstuffs

Food /Feed Stuff	IU/100g
	Ergocalciferol (D_2)
Alfalfa, sun cured	142
Alfalfa silage	12
Corn grain	0
Corn oil	9
Corn silage	13
Molasses	58
Sorghum grain	2.6
Spinach	0.2
	Cholecalciferol (D_3)
Cod liver oil	10,000
Menhaden entire body oil	5,000
Blue fin tuna liver oil	4,000,000
Eggs	100
Beef Liver	8-40
Chicken Liver	50-65
Cow milk	4

Adapted from McDowell (1989) and Combs (2012)

3. MODELING MATERNAL VITAMIN D_3 IN BROILER CHICKENS

3.1 INTRODUCTION

Multiple factors influence the concentration of nutrients in the egg yolk. The age of the breeders, diet composition and nutrient density, housing conditions or general management can result in different maternal deposition of nutrients and specifically of vitamin D_3. Moran (2007) studied the effect of maternal deposition of nutrients in the egg yolk and found that any nutritional inadequacies in the parental flock will had a negative impact on the performance of the resulting chicks. An adequate nutrition of the breeder flock is vital to ensure good development and hatchability of the embryos; both excess and deficient nutrient density leads to poor performance and viability of the young chickens (Wilson, 1997). It is known that in the poultry industry high amounts of vitamin D_3 are fed to breeders, which leads to a high deposition of this fat-soluble vitamin in the egg yolk. All things being equal, once a chick is hatched, its viability will be closely related to the nutritional reserves from the yolk stores that were used as the primary nutrient source during the time spent adapting to a solid diet. Dietary vitamin D_3 in the breeder flock will affect egg shell thickness, egg production and egg mass that ultimately will affect performance of the progeny (Coto *et al*. 2010a)

Previous research done in our laboratory has found a high variability in response to dietary vitamin D_3. In some cases, it can be difficult to find a significant decrease in percentage bone ash as an indicator of the vitamin D activity in young broilers. The data collected from our laboratory suggest that the maternal vitamin D_3 in egg yolk may be

sufficient to sustain growth and the mineralization of bones, even when they were fed a vitamin D_3-deficient diet. The objective of this study was to model the maternal deposition of vitamin D in the egg yolk, comparing chickens from different commercial hatcheries and correlating this with the mineralization of bones and serum 25-OH-D_3 concentration during the first few weeks of age.

3.2 MATERIALS AND METHODS

3.2.1 Experimental design overview

Three experiments were conducted using newly hatch broiler chickens obtained at different dates from two commercial hatcheries. For the first experiment, broiler chickens were obtained from Sanderson Farms (*Bryan, TX*) and placed in a Petersime battery brooder. A basal vitamin D_3 deficient corn-soy broiler starter diet was formulated and served as a negative control (NC1) during the entire trial to control for residual maternal D_3 stores from the yolk. From the same basal diet a batch was separated and supplemented with 3,850 IU/kg cholecalciferol and served as the positive control (PC). For the second and third experiment, broiler chickens were obtained from the Cobb hatchery (*Timpson, TX*) and from Sanderson Farms (*Bryan, TX*), respectively, and placed in a Petersime battery brooder. Only, the vitamin D_3-deficient diet previously used in experiment 1, was fed to the broiler chickens for both trials (NC2 and NC3).

For the three experiments, minimal lighting was maintained in the rearing room to reduce endogenous synthesis of cholecalciferol. This was achieved by removing all lateral lamps in the room and by covering completely the roof lamps with brown paper. Blood samples were taken from at least 2 birds from each treatment every 2 days to be

analyzed for 25-OH-D_3 concentration using a commercial ELISA Kit (25-OH-Vitamin D Kit, Eagle Biosciences®). Tibia samples were also taken every 2 days from the same birds for bone ash determination to evaluate the vitamin D_3 activity.

3.2.2 Housing and daily observations

Birds were caged in one Petersime battery brooder located in an environmentally controlled rearing room (# 1215) at the Texas A&M Poultry Research Farm. Minimal lighting (as previously described) in the room was provided on a constant 24-h basis. The complete absence of UV light in the rearing rooms has been verified previously (Fowler *et al*. 2014). Birds were observed daily with regard to general flock condition, temperature, lighting, water, feed, and unanticipated events for the rearing facility.

3.2.3 Experimental subjects

Broiler chickens were obtained from two different commercial hatcheries; Ross-308 broilers from Sanderson Farms (*Bryan, TX*) or Cobb-500 from the Cobb hatchery (*Timpson, TX*).

3.2.4 Dietary treatments

A basal corn-soy vitamin D_3-deficient broiler starter diet was formulated; this was achieved by using a customized mineral/vitamin premix without vitamin D_3 and utilizing corn oil as the fat source (Table 3). This basal diet was subdivided into two batches. One batch was supplemented with 3,850 IU/kg cholecalciferol (PC) and the other was fed without any supplemental vitamin D_3 and served as a negative control (NC). Feed and water was offered *ad libitum.*

Table 3. Broiler starter experimental basal vitamin D deficient diet[1]

Ingredient	%
Corn	61.34
Dehulled soybean meal	32.19
DL-Methionine	0.23
L-Lysine HCl	0.18
Corn oil	2.31
Limestone	1.44
Monocalcium phosphate	1.55
Salt	0.55
Custom vitamin/mineral premix[2]	0.5*

[1]Calculated nutritional content was as follow: crude protein 22%, metabolizable energy 3050 kcal/kg, calcium 0.95 %, available phosphorus 0.45 %, methionine 0.56 %, methionine+cystine 0.92 %, lysine 1.31%, tryptophan 0.20 %, threonine 0.82% arginine 1.46%, crude fat 4.17% and crude fiber 2.15%

[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.*

3.2.5 Sera samples

Blood was drawn from at least 2 birds every 2 days and collected within Eppendorf® 1.5 ml tubes and centrifuged at 3000 xg for 10 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.

3.2.6 Bone ash determination

The left tibia was dissected from the same birds selected to draw blood for bone ash determination to evaluate vitamin D_3 activity. Muscle and cartilage were removed before being placed in 4 L of petroleum ether for 48 h at room temperature. Following this, tibias were dried for 24 h in a drying oven at 105°C. Finally tibias were ashed at 650°C for 23 h. Ash was evaluated as a percentage of the free-fat, dried bone weight**.**

3.2.7 Statistical analysis

The serum 25-OH-D_3 concentration data were subjected to repeated measures analysis using the GLM procedure of SPSS. Dietary treatments were used as fixed factor. Means were separated by Duncan´s multiple range test. Statistical significance was accepted at $p \leq 0.05$. Broken-line regression was used to analyze the percentage bone ash data using the "Nutritional Response Model Program" Version 1.01 from Gene Pesti and Dmitry Vedenov, University of Georgia. Individual birds served as the experimental unit for bone ash data analysis.

3.3 RESULTS

3.3.1 Maternal vitamin D_3 status

Experiment 1. After 21 days on the treatment diets a significant ($p<0.05$) difference between NC and PC was observed on days 7, 9, 11, 15 17 and 21 (Table 4), suggesting that on day 7 the maternal reserves of vitamin D_3 were considerably depleted. The lowest serum concentration of 25-OH-D_3 was reached at day 11 for the NC1. Even when there was no statistical difference on day 13 and 19 a clear numerical difference was found between treatments following the depletion trend observed on day 7.

Table 4. Experiment 1 ANOVA serum 25-OH-D_3 (ng/ml)

Day	Exp 1	
	NC1	PC
1	26.18±7.67	27.39±1.02
3	7.98±9.45	18.67±6.25
5	6.53±0.70	21.65±7.51
7	3.91±1.00[b]	20.97±0.01[a]
9	3.70±0.48[b]	17.16±4.49[a]
11	3.39±2.75[b]	23.06±1.42[a]
13	5.90±1.66	26.22±10.93
15	5.66±3.81[b]	31.19±2.02[a]
17	5.08±2.48[b]	35.04±0.35[a]
19	4.15±4.38	32.10±11.34
21	13.03±0.43[b]	38.93±7.44[a]

a,b Means within a row with no common superscript differ significantly (P<0.05).
± Standard deviation. n= 2 birds per day. Exp 1 Sanderson Farms (Bryan, TX).

The distribution of serum 25-OH-D_3 concentrations for experiment 1 are presented in Figure 2, where a clear difference is observed in the trends of NC1 and PC1 throughout time.

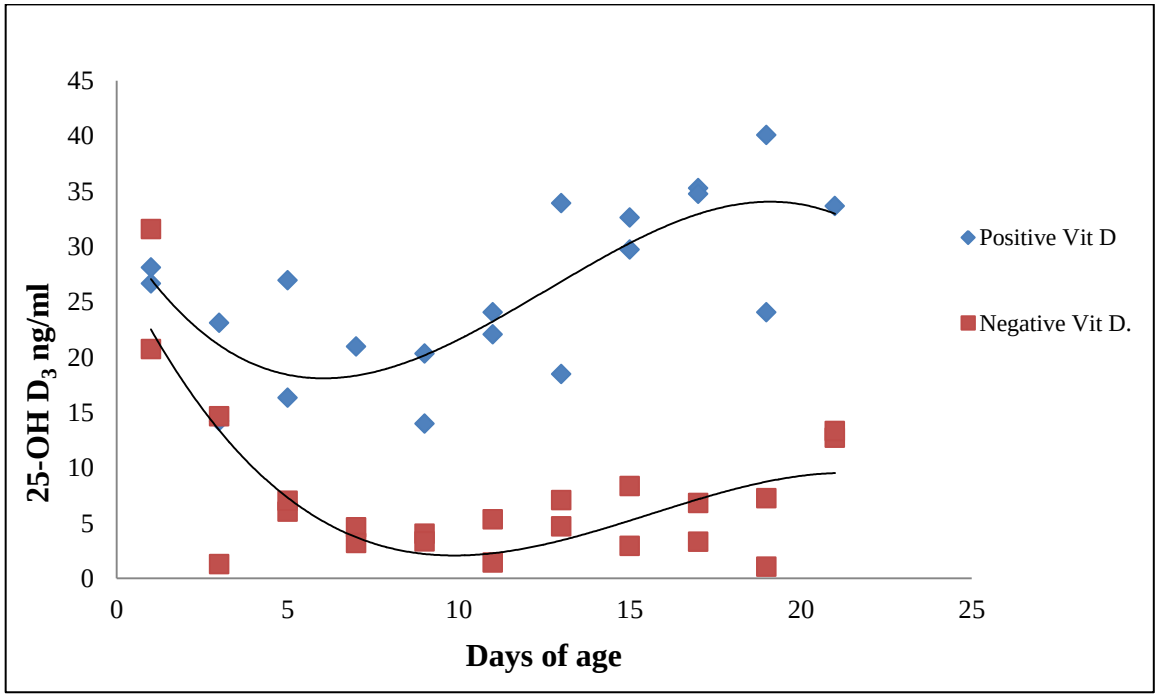


Figure 2. Maternal 25-OH-D_3 (mg/ml) trend NC1 vs PC1 Exp 1 Sanderson Farms (Bryan, TX).

Experiments 1-3. No difference ($p>0.05$) was found between flocks throughout 11 days on the vitamin D_3-deficient diet. Only on day 7 a significant difference was found, NC1 had higher serum 25-OH-D_3 values than NC2 and NC3 (Table 5). Data suggest that regardless of initial maternal reserves of vitamin D_3 the chickens will deplete at a similar rate (Figure 3).

Table 5. Flock variability ANOVA serum 25-OH-D_3 (ng/ml)

Day	Experiment		
	NC1[1*]	NC2[2**]	NC3[2*]
1	26.18±7.67	27.30±2.64	33.11±4.5
3	7.98±9.45	18.33±1.31	15.82±7.32
5	6.53±0.70	5.60±4.13	3.43±3.06
7	3.91±1.00[a]	1.36±1.26[b]	0.21±0.36[b]
9	3.70±0.48	4.62±3.75	8.76±1.66
11	3.39±2.75	3.53±2.63	9.57±4.35

a,b Means within a row with no common superscript differ significantly (P<0.05).
*± Standard deviation. [1] n= 2 birds per day [2] n= 3 birds per day. * Sanderson Farms (Bryan, TX) ** Cobb hatchery (Timpson, TX).*

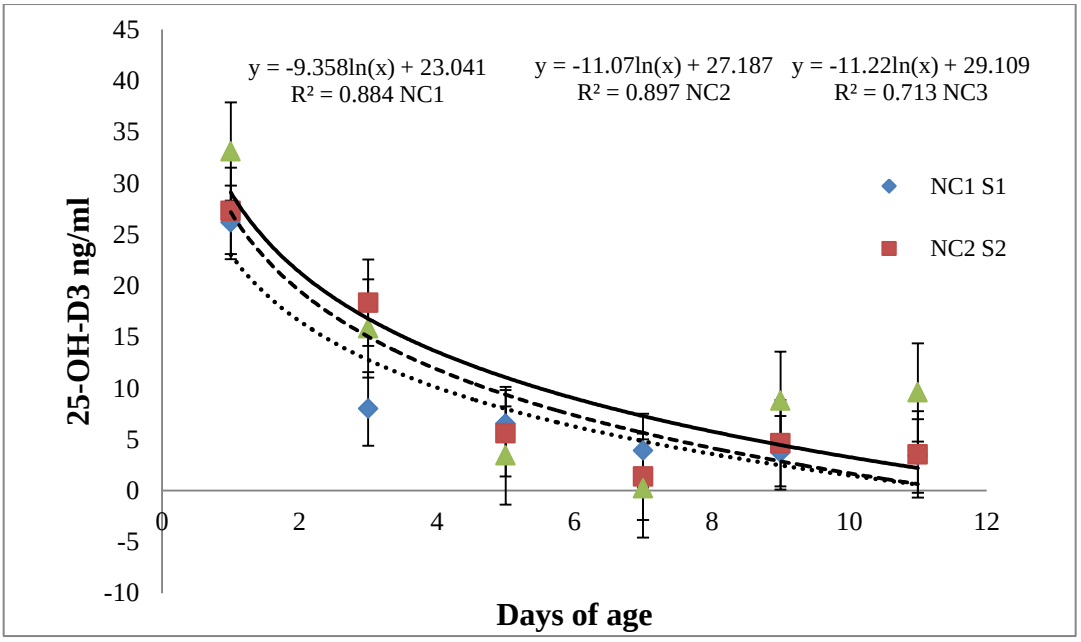


Figure 3. Maternal 25-OH-D_3 trend variability between broiler sources (ng/ml). S1=Sanderson Farms (Bryan, TX) S2=Cobb hatchery (Timpson, TX).

3.3.2 Bone ash

The percentage bone ash for each of the NC treatments and the PC from the first experiment is presented on Figures 4-7. The breaking point in the linear ascending line indicates the time to maximize bone mineralization. Similar percentage bone ash values were found for the PC and NC1 where both treatments maximize at 44%. However, NC1 maximized bone mineralization around 6 days while the PC took about 10 days. Low percentage bone ash was found for NC2 and NC3. Both NC flocks registered a maximum response of 34.82 and 31.95%, respectively.

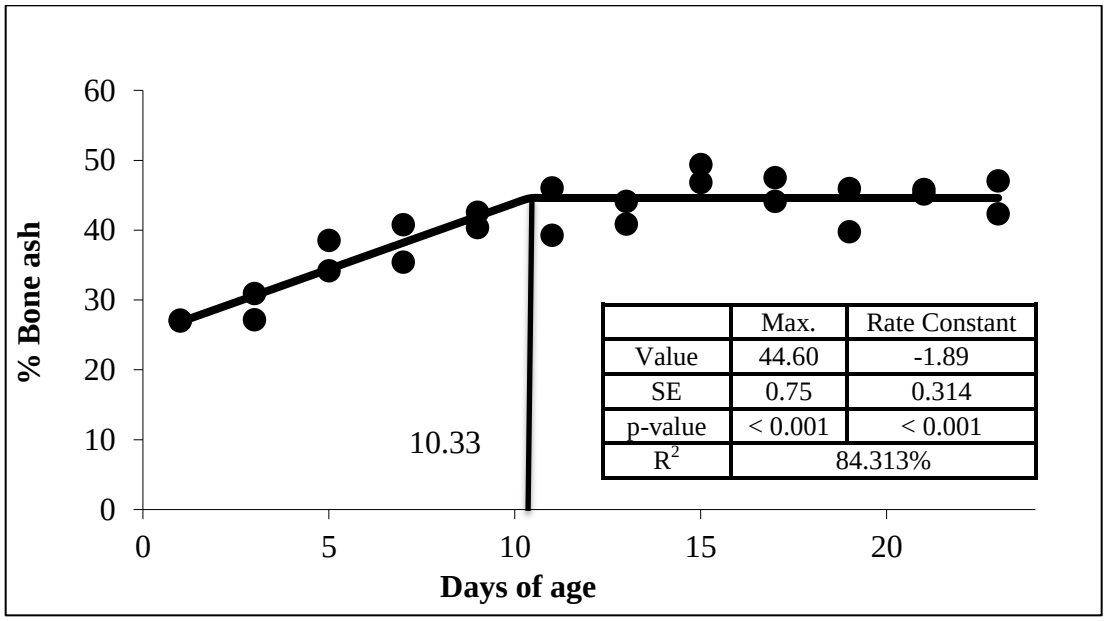


Figure 4. Positive control percentage bone ash (Exp 1) Source: Sanderson Farms (Bryan, TX). [y=Max + Rate Constant(Time to maximize –x)].*

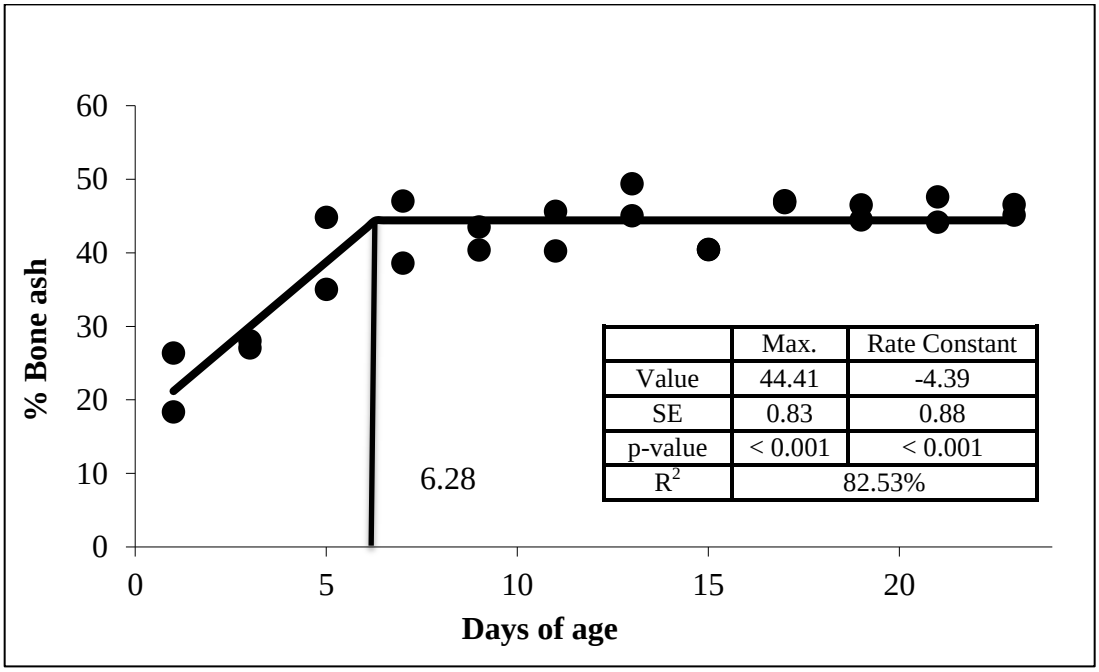


Figure 5. Negative control percentage bone ash (Exp 1) Source: Sanderson Farms (Bryan, TX). [y=Max + Rate Constant(Time to maximize –x)].*

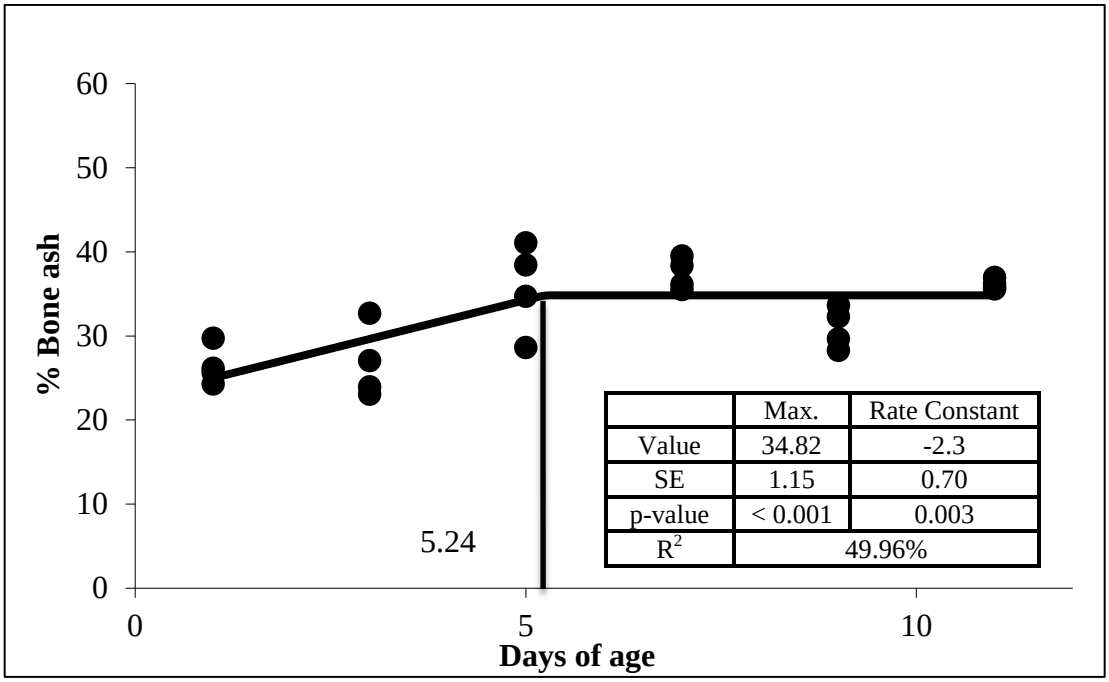


Figure 6. Negative control percentage bone ash (Exp 2) Source: Cobb hatchery (Timpson, TX). [y=Max + Rate Constant(Time to maximize –x)] .*

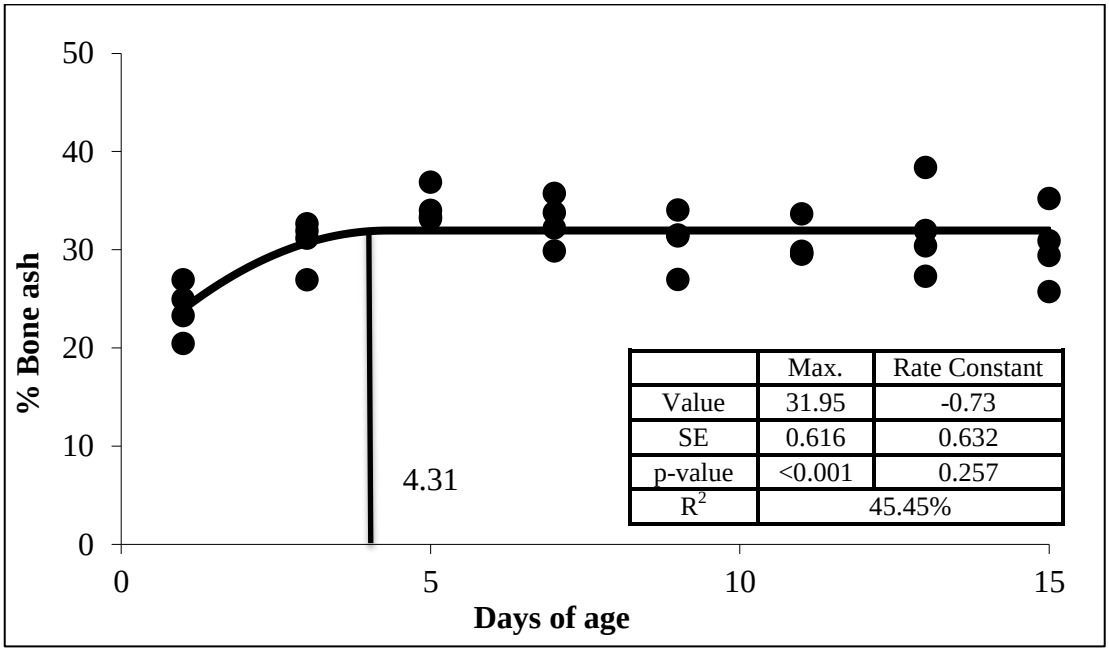


Figure 7. Negative control percentage bone ash (Exp 3) Source: Sanderson Farms (Bryan, TX). [y=Max + Rate Constant(Time to maximize –x)^2].*

3.4 DISCUSSION

The results of the present study show the high variability within flocks in response to a vitamin D_3-deficient diet when percentage bone ash is used to test the activity of vitamin D in bone mineralization of broiler chickens from different sources. The biggest effect of this variability is observed in the maximum percentage bone ash registered for each experiment. Maximum percentage bone ash of 44, 34.8 and 31.95% were registered for Exp. 1, Exp. 2 and Exp. 3, respectively. This is in agreement with Thorp and Waddington (1997) who studied the incidence of different bone pathologies in broiler chickens obtained from different European countries and found that the prevalence and type of pathologies observed varied with the source of the broilers. The effect of maternal nutrition may influence the response of the progeny to multiple assays in

research studies, especially in bone quality measurements that are highly correlated with the endogenous reserves of vitamin D_3 transferred in the egg yolk. Any nutritional deficiency or inadequacy transferred from the breeder to the egg has a negative impact on the performance of the progeny (Moran, 2007). Murphy *et al*. (1936) studied the effect of vitamin D intake of the breeder hen on bone calcification of the growing chick, and stated that the antirachitic potency of the egg yolk is directly related with the hens vitamin D intake level; these authors further state that a direct correlation results between the antirachitic intake of the hen and the degree of bone calcification in the resultant chick during the first few weeks of age. Mattila *et al*. (1999) showed that 25-OH-D_3 and cholecalciferol are transferred from the hen into the egg yolk and found a strong positive correlation between the hen's vitamin D dietary level and the deposition in the egg yolk. Other studies with broiler breeder hens also show a direct relationship between the source and level of dietary vitamin D on the carryover effect to young chicks for adequate development. Breeders that were supplemented with high concentrations of cholecalciferol were able to maintain an active carryover effect, and their chick's sustained growth and bone mineralization even 3 weeks after hatching when fed 5,500 IU/kg D_3. The supplementation with a dietary source of 25-OH-D_3 enhanced the effect by improving the general performance of the progeny (Coto *et al*. 2010b). This effect was also confirmed by Atencio *et al*. (2005b) who reported that chicks hatched from hens fed a dietary source of 25-OH-D_3 improved body weight gain, gain:feed, feed intake, tibia ash and lowered mortality when compared to chicks hatched from hens fed only cholecalciferol. Atencio *et al*. (2006) estimated that 2,000-4,000 IU/kg D_3 was required

in breeder diets to maximize body ash of the progeny. Atencio *et al*. (2005a) studied the relationship of dietary cholecalciferol in the breeder hen and Ca levels in the progeny diet. He found that chicks hatched from eggs laid by hens fed high levels of vitamin D_3 (50 µg/kg or 2,000 IU/kg D_3) and fed 0.9% Ca had the highest body weights and tibia ash and the lowest TD incidence versus those fed 0.63% Ca. Broiler breeder hens in the last phase of the laying cycle were able to transfer vitamin D more efficiently into the egg yolk than younger birds when fed high levels of cholecalciferol (50 µg/kg or 2,000 IU/kg D_3) during the entire production cycle (Driver *et al*. 2006). Other nutritional factors not related with the diet itself may also influence in the response and incidence of bone disorders. Bar *et al*. (1986) studied different outbreaks of leg disorders in turkeys and stated that the incidence of field rickets was related more to a defect in vitamin D metabolism or in its expression rather than dietary factors. The incubation process and first day post-hatch management is critical since they can play a key role with respect to reaching the genetic potential of modern broiler strains (Kidd, 2009). In agreement with my results, Coates (1962) described several disadvantages responsible of the variability in the response to dietary treatments when chickens are used as laboratory subjects. He stated that the social hierarchy in groups of birds kept together results in variable food consumption and uneven growth rates within experimental groups; also their feeding habits make difficult to obtain accurate records of food intake that are used to evaluate performance.

Bone ash values found for the first experiment in this study agree with those found by Lofton and Soares (1986) where percentage bone ash measured in 14-day-old

chicks was around 45% in birds fed a vitamin D_3 deficient diet. Lofton and Soares (1986) data suggest that maternal deposition of vitamin D_3 was enough to sustain growth and bone mineralization during the first 2 weeks of age. Contrary to Exp. 1, values registered in experiment 2 and 3 were below 45% in agreement with Edwards *et al*. (1994) who found similar low percentage bone ash (27%) in chicks that were excluded from ultraviolet light exposure for 16 days. Overall a high variability in the maximum percentage bone ash response to the vitamin D_3-deficient diet within flocks obtained from the same commercial hatchery and from different sources was observed; this variability may explain why in some cases, it can be difficult to find a consistent significant decrease in percentage bone ash as an indicator of the Vitamin D activity in young broilers.

3.5 CONCLUSION

In order to produce a vitamin D_3-deficient status in young broiler chickens it is necessary to have strict control on the breeder's diet. Regardless of the initial concentrations of vitamin D in newly hatched chickens obtained from different commercial hatcheries, the maternal reserves will deplete around day 10 of age but still a high variability in response to dietary vitamin D was observed when percentage bone ash was used to evaluate the vitamin D activity.

4. EVALUATION OF SERUM 25-OH-D_3 DYNAMICS IN BROILER CHICKENS

4.1 INTRODUCTION

By supplementing 25-OH-D_3 (the major circulating form of vitamin D in the blood), the activity of vitamin D is increased relative to feeding cholecalciferol alone. Yarger *et al.* (1995) compared the bio-potency of 25-OH-D_3 and cholecalciferol in broiler chickens. They found that serum 25-OH-D_3 concentration increased more rapidly in birds that were supplemented with the vitamin D metabolite and performance was improved by increasing body weight and feed efficiency. Fritts and Waldroup (2003) compared the performance and bone ash content of growing broiler chickens fed different levels of 25-OH-D_3 and cholecalciferol. Body weight and bone ash were significantly greater in birds fed the 25-OH-D_3 when compared to cholecalciferol alone. The incidence of TD was also improved when 25-OH-D_3 was supplemented to the diet. Ledwaba and Roberson (2003) studied the efficacy of 25OH-D_3 in low Ca diets; the inclusion of 70 µg/kg 25-OH-D3 had a positive effect in preventing TD in marginal Ca diets. Chou *et al*. (2009) reported that the supplementation of 25-OH-D_3 may improve the small intestine morphology and enhance the immune response of broiler chickens. In a previous study conducted by Huvepharma (*Peachtree city, GA*) and Tyson Foods Inc. (*Springdale, AR*), where 25-OH-D_3 serum concentrations were calculated from blood samples taken from different commercial production cycles at different times of the year during broiler development, there was high variability and no consistent trend with

respect to vitamin D status. This variability opened a wide range of possibilities to explain this phenomenon: genetic variability, maternal deposition of vitamin D in the broiler chick egg yolk, individual feed intake, growth rate, and housing conditions could all be factors affecting the vitamin D status within each study. Tibia bone ash was also measured and percent of ash decreased after the second week for all sample sites, which could also be attributed to high variability in the vitamin D status. As previously mentioned, in the modern poultry industry leg health is an important component of broiler welfare and therefore in general broiler performance which is a critical economic issue for any poultry business.

Little information exists about the dynamics of serum concentrations of 25-OH-D_3 when supplemented in broiler feeds to prevent rickets and other bone problems. The purpose of this study was to better understand the dynamics of 25-OH-D_3, identify if there was any "circadian-type" trend in the broiler´s response to dietary 25-OH-D_3 over time, as well as identify important information about the effect of age in the metabolism of 25-OH-D_3 and bone ash mineralization.

4.2 MATERIALS AND METHODS

4.2.1 Experimental design overview

Ross-308 newly hatched broiler chickens were purchased from a commercial hatchery and fed *ad libitum* a vitamin D_3-deficient corn-soy broiler mashed starter diet for a 21-day rearing period. To create three treatments, the basal diet was subdivided and supplemented with 3,850 IU/kg cholecalciferol or 3,850 IU/kg cholecalciferol plus one of two sources of 25-OH-D_3 at 0.5 kg/t (Hy-D™ and Bio-D™).

On day 1 of age, 112 birds were divided in four groups of 28 birds and received the treatment diets *ad libitum* for a 24 h period. Four birds were taken from each group at times 0, 1, 2, 4, 8 12 and 24 h after presentation to the treatment diets to draw blood. Sera were collected from the blood samples and used to run a commercial ELISA Kit (25-OH-Vitamin D Kit, Eagle Biosciences®). The left tibia of four birds sampled at 0-h of exposure to the treatment diets was removed to perform a bone ash determination as a percent of the fat-free, dried bone weight.

On days 4, 9, 14 and 19 of age, 88 birds were selected and divided into three groups and fasted overnight. The next day, after the fasting period, selected birds were offered the treatment diets *ad libitum* for 24 h. Four birds were taken from each group of the three treatments at times 0, 1, 2, 4, 8 12, and 24 h after presentation to the treatment diets to draw blood. Sera were collected from the blood samples and split into two sub-samples. One sub-sample was used to run a commercial ELISA (25-OH-Vitamin D Kit, Eagle Biosciences®) and the other one was sent to Heartland Assays (*Ames, IA*) for analysis of 25-OH-D_3 and calcium concentration. The left tibia of four birds sampled at 0-h of exposure to the treatment diets was removed to perform a bone ash determination as a percent of the fat-free, dried bone weight.

After the 24-h feeding period on days 2, 6, 11, 16 and 21, four remaining birds were individually marked with wing bands and placed back on the vitamin D_3-deficient diet for the rest of the trial. On day 21, all marked birds were euthanized then the left tibia was removed to perform a bone ash determination as a percent of the fat-free, dried bone weight.

4.2.2 Housing and daily observations

Birds were caged in two Petersime battery brooders located in an environmentally controlled rearing room (# 1215) at the Texas A&M Poultry Research Farm. Minimal lighting in the room was provided on a constant 24-h basis. The complete absence of UV light in the rearing rooms has been verified previously (Fowler *et al*. 2014). Birds were observed daily with regard to general flock condition, temperature, lighting, water, feed, and unanticipated events for the rearing facility.

4.2.3 Dietary treatments

A basal corn-soy vitamin D_3-deficient broiler starter diet was formulated using a customized mineral/vitamin premix without vitamin D_3 and utilizing corn oil as the fat source (Table 6). Treatment diets were supplemented with 3,850 IU/kg cholecalciferol, and with 0.5 kg/t Bio-D™ (*Peachtree, GA*) or Hy-D™ (*Naperville, IL*), which yields 34.8 ppb and 68.9 ppb of 25-OH-D_3 respectively. Feed samples were taken directly from a moving stream, and stored in a refrigerator at 11°C prior to being sent to Heartland Assays (*Ames, IA*) for vitamin D_3 and 25-OH-D_3 concentration analysis by Liquid Chromatography-Mass Spectrometry (LC/MS/MS).

4.2.4 Feed and watering method

Vitamin D_3-deficient diet was offered *ad libitum* for the 21-day rearing period except for the 24-h sample collection dates, when selected birds were offered the treatment diets after being fasted overnight. Water was offered *ad libitum* during the whole trial.

Table 6. Experimental basal vitamin D_3 deficient diet[1]

Ingredient	%
Corn	62.30
Dehulled soybean meal	32.04
DL-Methionine	0.23
L-Lysine HCl	0.18
Corn oil	1.97
Limestone	1.06
Monocalcium phosphate	1.19
Salt	0.46
Custom vitamin/mineral premix[2]	0.5*

[1]Calculated nutritional content was as follow: crude protein 22%, metabolizable energy 3050 kcal/kg, calcium 0.75 %, available phosphorus 0.37 %, methionine 0.56 %, methionine+cystine 0.92 %, lysine 1.31%, tryptophan 0.26 %, threonine 0.82% arginine 1.46%, crude fat 3.85% and crude fiber 2.16%

[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.*

4.2.5 Sera samples

Blood was drawn at specific times (0, 1, 2, 4, 8, 12 and 24 h) after exposure to treatment diets. The blood was collected within Eppendorf® 1.5 ml tubes and centrifuged at 3000 x g for 10 min to separate the serum which then was split into two sub-samples. One sub-sample was used to run a commercial ELISA (25-OH-Vitamin D Kit Eagle Biosciences®) and the other was sent to Heartland Assays (*Ames, IA*) for analysis of 25-

OH-D_3 and calcium concentration via liquid chromatography mass-spectrometry (LC/MS/MS) and colorimetric methods, respectively.

4.2.6 Bone ash determination

The left tibia was dissected from selected birds for bone ash determination to evaluate vitamin D_3 activity. Muscle and cartilage were removed before being placed in 4 L of petroleum ether for 48 h at room temperature. Following this, tibias were dried for 24 h in a drying oven at 105°C. Finally tibias were ashed at 650°C for 23 h. Ash was evaluated as a percentage of the free-fat, dried bone weight**.**

4.2.7 Statistical analysis

The serum 25-OH-D_3 concentration data were subjected to repeated measures analysis using the GLM procedure of SPSS. Dietary treatments were used as fixed factor. Means were separated by Duncan´s multiple range test. Statistical significance was accepted at $p \leq 0.05$. Assay type data were analyzed using the pair correlation function of the same software. Broken-line regression was used to analyze the serum Ca concentration and percentage bone ash using the "Nutritional Response Model Program" Version 1.01 from Gene Pesti and Dmitry Vedenov, University of Georgia. Individual birds served as the experimental unit for percentage bone ash, serum 25-OH-D_3 and Ca concentration data analysis.

4.3 RESULTS

4.3.1 Initial serum 25-OH-D_3

Table 7 shows the serum 25-OH-D_3 concentration (ng/ml) of day-old chicks, where time 0 values represents the initial maternal D_3 stores in egg yolk as birds were sampled prior to receiving any treatment feed.

Table 7. ANOVA day 1-ELISA serum 25-OH-D_3 (ng/ml)

Hour	Treatment			
	NC	Vit. D	Vit. D+ Bio-D	Vit. D+ Hy-D
0	40.36±8.11			
1	38.67±5.63	38.04±15.66	32.65±3.18	33.04±11.16
2	41.63±7.82	31.76±10.45	29.47±7.06	34.24±5.09
4	29.34±8.05	28.86±13.11	40.75±9.78	33.95±4.75
8	37.53±1.27^{b}	34.25±5.70^{b}	36.12±5.54^{b}	47.63±2.56^{a}
12	36.83±10.08	43.01±11.39	44.76±6.87	42.93±8.37
24	33.28±7.01	51.89±7.96	52.39±25.71	29.67±7.83

a,b Means within a row with no common superscript differ significantly (P<0.05).
± Standard deviation. n= 4 birds per treatment per hour (n= 3 birds for Vit. D 1 and 2 h, Vit.D+Bio-D for 8 and 24-h, NC for 12-h). Zero hour value represents the initial 25-OH-D_3 (ng/ml) average of 96 total birds.

Serum 25-OH-D_3 from 1-4, 12 and 24 h were not different ($p>0.05$) when compared to the negative control. A difference was found at 8-h exposure to the treatments diets ($p<0.05$), where Vit. D+Hy-D treatment had higher values than NC, Vit. D and Vit. D+Bio-D.

4.3.2 Serum 25-OH-D_3 assay type correlation

A correlation coefficient of 81% (r) was found between the assay type results (Table 8), ELISA Kit vs LC/MS/MS performed by Heartland Assays (Ames, IA), an increasing positive relationship was found for both assays; however, only 65% of the total variation (r^2) in the assay results can be explained by the linear relationship; therefore, a separate analysis of variance was done for each assay. Figures 10–17 and Tables 9-16 present the collected data for serum 25-OH-D_3 concentration (ng/ml).

Table 8. Assay type bivariate correlation

Assay Type	Coefficient of Correlation	p value
Heartland - ELISA Kit	0.81	<0.001

n= 335 for each assay

- *Serum 25-OH-D_3 concentration Day 5*

After 5 days of feeding the basal vitamin D_3-deficient diet to the chickens, initial serum 25-OH-D_3 concentration (0-h exposure to treatment diets) dropped from 40.4 of day-

old chicks to 3.46 and 7.05 ng/ml for the ELISA Kit and LC/MS/MS, respectively, as yolk derived D_3 started to deplete (Table 9).

ELISA Kit. - No significant ($p>0.05$) difference was found from 1-2 and 8-h exposure to treatment diets. At 4-h, Vit.D+Bio-D and Vit. D+ Hy-D registered higher values when compared to Vit. D alone ($p<0.05$). At 12 and 24 h Vit., D+Bio-D had intermediate values between Vit. D and Vit. D+Hy-D treatments ($p<0.05$) (Table 9).

LC/MS/MS.- There was no difference ($p>0.05$) between treatments on 1, 8 and 12 h. At 2 and 4-h of exposure to treatment diets, a significant difference was found; Vit. D alone had lower concentration values than Vit. D+Bio-D and Vit.D+Hy-D ($p<0.05$). After 24 h, Vit. D+Bio-D registered intermediate values between Vit. D and Vit.D + Hy-D ($p<0.05$) (Table 9).

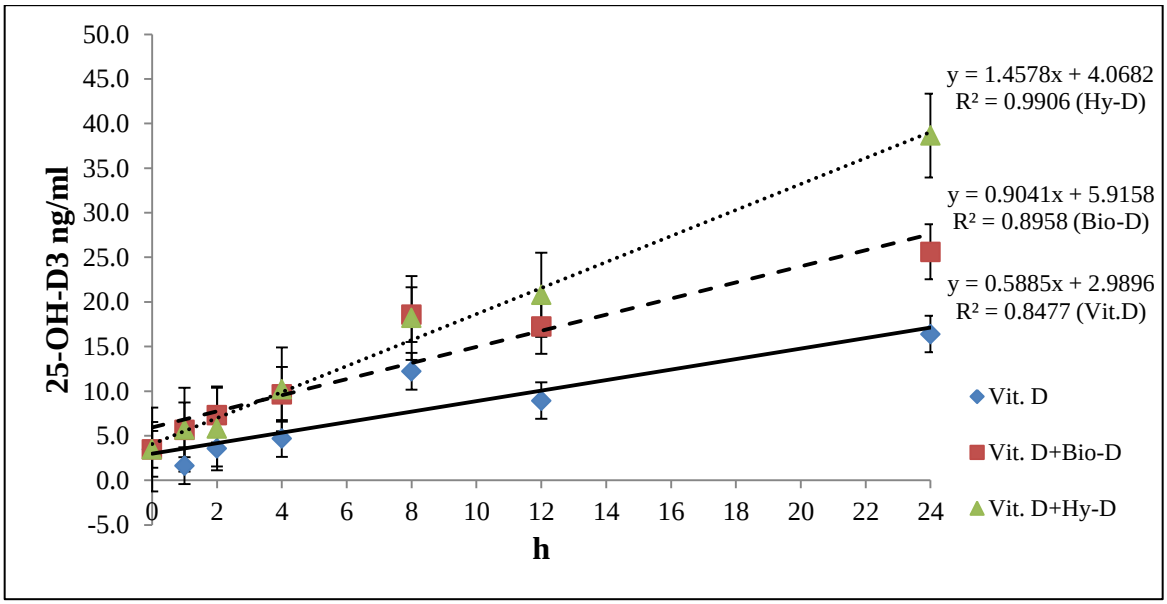
The comparative linear increase of the serum 25-OH-D_3 concentrations for both assays is presented in Figure 8.

Table 9. Day-5 ANOVA (ELISA Kit versus LC/MS/MS) serum 25-OH-D_3 (ng/ml)

Hour	Treatment		
	Vit. D	Vit. D+ Bio-D	Vit. D+ Hy-D
	ELISA Kit		
0		3.46±1.06	
1	1.63±1.52	5.65±4.70	5.68±0.83
2	3.58±2.08	7.29±2.99	5.81±2.88
4	4.69±2.42[b]	9.64±1.79[a]	10.20±1.32[a]
8	12.22±4.19	18.57±4.94	18.21±3.05
12	8.93±2.85[b]	17.25±7.01[ab]	20.79±6.82[a]
24	16.39±5.88[b]	25.62±16.10[ab]	38.65±4.86[a]
	LC/MS/MS		
0		7.05±1.53	
1	5.71±2.27	10.50±3.90	11.52±3.00
2	6.60±1.92[b]	12.35±2.95[a]	12.23±2.20[a]
4	11.02±4.04[b]	16.77±1.44[a]	19.47±1.31[a]
8	17.07±3.05	26.18±8.82	26.37±2.78
12	18.39±6.66	28.04±6.77	30.87±9.01
24	27.95±3.72[b]	39.96±16.37[ab]	52.31±5.90[a]

a,b Means within a row with no common superscript differ significantly (P<0.05).
± Standard deviation. n= 4 birds per treatment per hour. Zero hour value represents the initial 25-OH-D_3 (ng/ml) average of 76 total birds.

ELISA KIT



LC/MS/MS

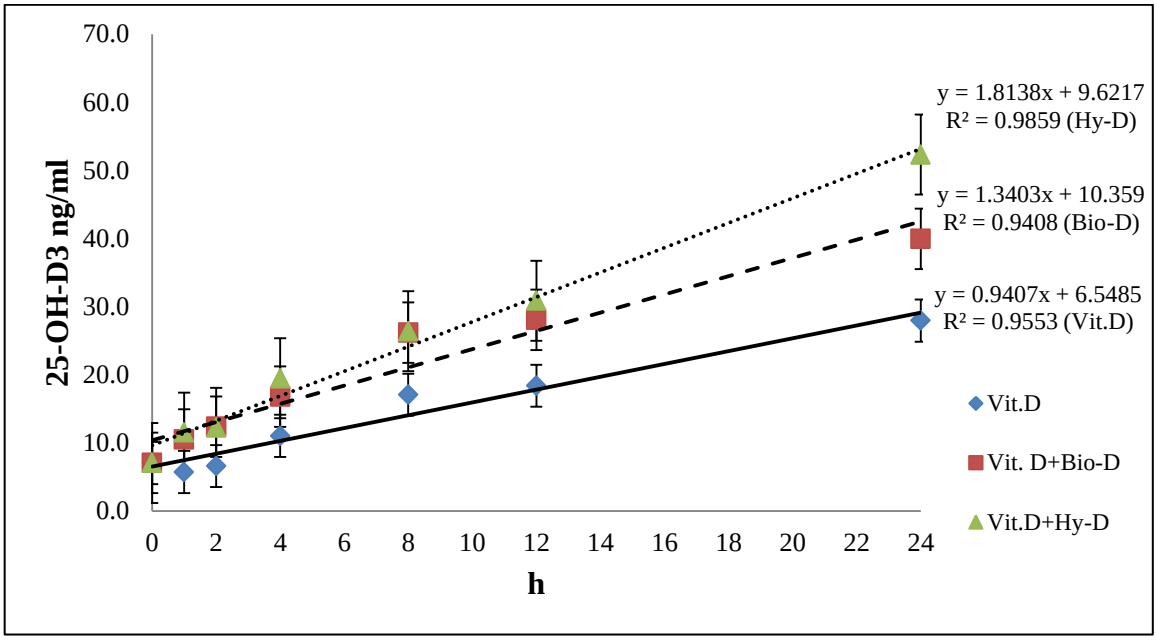


Figure 8.Comparative linear increase Day-5 ELISA vs LC/MS/MS serum 25-OH-D_3 values (ng/ml)

- *Serum 25-OH-D_3 concentration Day 10*

Initial serum 25-OH-D_3 concentration after 10 days on the vitamin D_3-deficient diet for ELISA Kit (4.26 ng/ml) and LC/MS/MS (6.28 ng/ml) was very similar to the 5-day value, a decrease was observed when compared to the day-old chick initial value 40.36 ng/ml that represents the initial maternal D_3 stores (Table 10). The linear increase trend for both assays is presented in Figure 9.

ELISA Kit.- At 8 h Vit. D+Bio-D had intermediate concentration when compared to Vit. D and Vit. D+Hy-D and at 24 h Vit.D+Bio-D and Hy-D had a significant increase in serum 25-OH-D_3 when compared to the Vit. D treatment. ($p<0.05$) (Table 10).

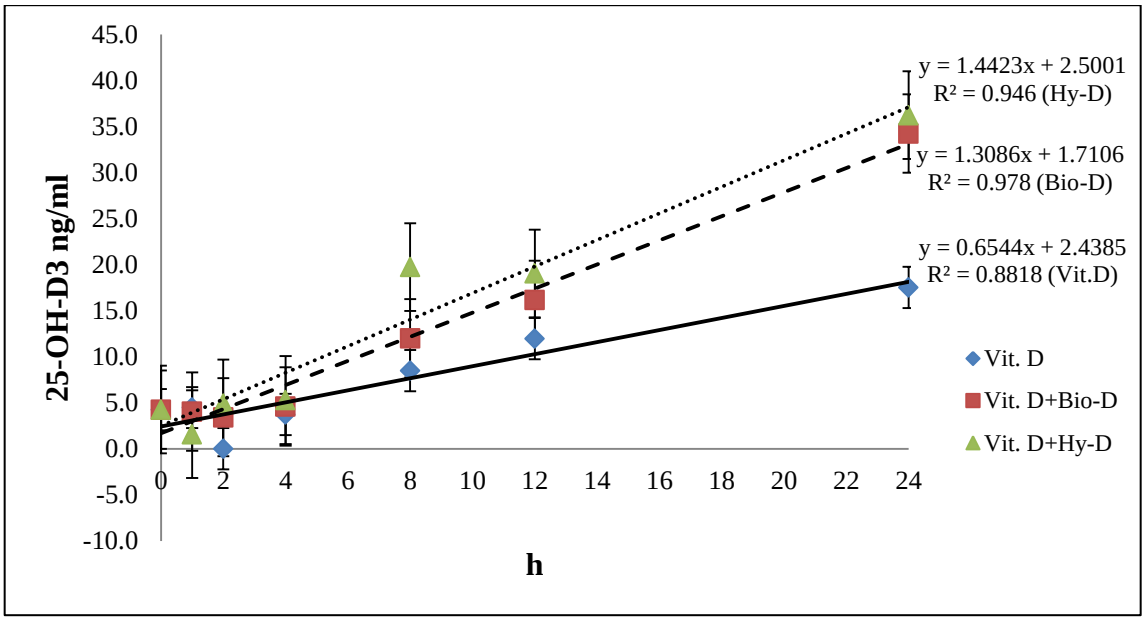
LC/MS/MS.- At 2 and 24 h, Vit. D+Bio-D and Hy-D had higher concentration values than Vit. D alone ($p<0.05$). After 8 and 12-h exposure to dietary treatments intermediate values for Vit.D+Bio-D were observed compared to Vit.D and Vit.D+Hy-D. (Table 10).

Table 10. Day-10 ANOVA (ELISA Kit versus LC/MS/MS) serum 25-OH-D_3 (ng/ml)

Hour	Treatment		
	Vit. D	Vit. D+ Bio-D	Vit. D+ Hy-D
	ELISA Kit		
0		4.26±3.04	
1	4.47±2.25	4.03±3.45	1.59±1.53
2	0.64±1.28	3.43±1.97	4.90±3.74
4	3.73±2.47	4.59±5.69	5.29±3.83
8	8.49±2.33^{b}	11.99±4.47^{ab}	19.72±5.73^{a}
12	11.96±3.02	16.17±6.08	19.04±2.31
24	17.50±3.32^{b}	34.22±5.82^{a}	36.23±10.52^{a}
	LC/MS/MS		
0		6.28±4.14	
1	10.02±3.92	9.07±5.07	6.58±2.47
2	5.38±4.11^{b}	14.15±2.21^{a}	12.88±4.84^{a}
4	9.95±1.40	19.02±6.52	16.78±5.94
8	19.10±3.88^{b}	27.64±5.80^{ab}	35.19±6.89^{a}
12	25.59±2.86^{b}	33.75±9.65^{ab}	40.22±5.27^{a}
24	33.36±6.18^{b}	60.08±8.89^{a}	56.46±15.28^{a}

a,b Means within a row with no common superscript differ significantly (P<0.05).
± Standard deviation. n= 4 birds per treatment per hour. Zero hour value represents the initial 25-OH-D_3 (ng/ml) average of 76 total birds.

ELISA Kit



LC/MS/MS

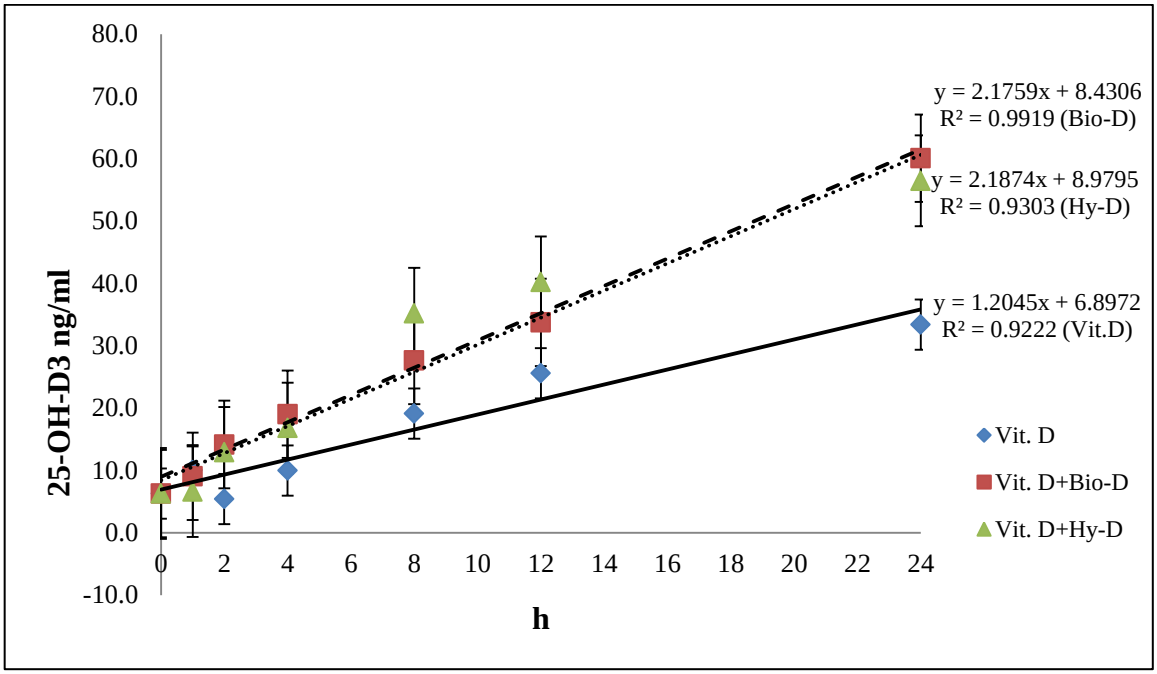


Figure 9. Comparative linear increase Day-10 ELISA vs LC/MS/MS serum 25-OH-D_3 values (ng/ml)

- *Serum 25-OH-D_3 concentration Day 15*

ELISA Kit. - At 2 h, Vit.D+Bio-D treatment registered higher concentration values than Vit.D and Vit.D+Hy-D. At 4-h Vit. D+Hy-D registered the highest values and after 8 h all treatments were significantly different. With Vit. D showing the lowest serum 25-OH-D_3 values. Intermediate values of Vit.D+Bio-D were found at 12 h and at 24 h. Both Vit.D+Bio-D and Hy-D had a significant increment in serum 25-OH-D_3 values compared to Vit.D treatment ($p<0.05$) (Table 11).

LC/MS/MS.- From 1 to 24 h, Vit. D+ Bio-D and Vit-D+Hy-D resulted in significant increment in 25-OH-D_3 concentration values when compared to Vit. D alone. Only at 4 h did Vit. D+Bio-D registered lower values than Vit.D+Hy-D ($p<0.05$) (Table 11).

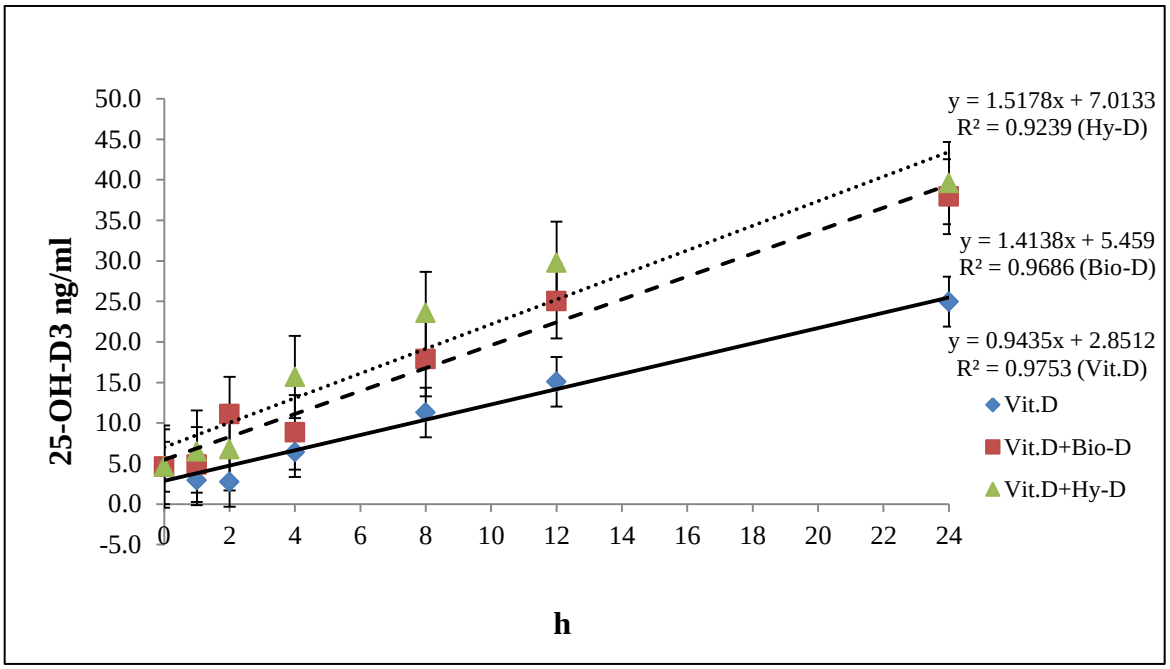
Figure 10 shows the linear trend of the serum 25-OH-D_3 values for both assays over time, maximum values were reached after 24 h exposure to treatment diets.

Table 11. Day-15 ANOVA (ELISA Kit versus LC/MS/MS) serum 25-OH-D_3 (ng/ml)

Hour	Treatment		
	Vit. D	Vit. D+ Bio-D	Vit. D+ Hy-D
	ELISA Kit		
0		4.61±4.06	
1*	2.94±2.31	4.86±0.30	6.47±2.56
2	2.74±1.25^{b}	11.10±0.76^{a}	6.76±4.44^{b}
4	6.40±2.68^{b}	8.85±1.14^{b}	15.67±3.91^{a}
8	11.30±0.86^{c}	17.90±4.85^{b}	23.58±1.55^{a}
12	15.08±5.11^{b}	25.03±4.48^{ab}	29.76±10.37^{a}
24	24.97±4.24^{b}	37.93±5.87^{a}	39.62±8.08^{a}
	LC/MS/MS		
0		7.71±3.15	
1*	7.29±1.86^{b}	11.06±1.45^{a}	13.97±2.76^{a}
2	8.72±0.93^{b}	18.00±1.45^{a}	17.47±3.98^{a}
4	13.15±2.53^{c}	20.56±1.86^{b}	26.84±3.64^{a}
8	19.07±2.73^{b}	33.54±6.11^{a}	33.37±4.12^{a}
12	22.95±5.47^{b}	34.69±5.36^{a}	39.89±8.71^{a}
24	29.29±4.77^{b}	49.79±6.73^{a}	52.68±7.68^{a}

a,b,c Means within a row with no common superscript differ significantly (P<0.05).
*± Standard deviation. n= 4 birds per treatment per hour. Zero hour value represents the initial 25-OH-D_3 (ng/ml) average of 76 total birds. * Vit.D+Bio-D (n= 3 brids).*

ELISA Kit



LC/MS/MS

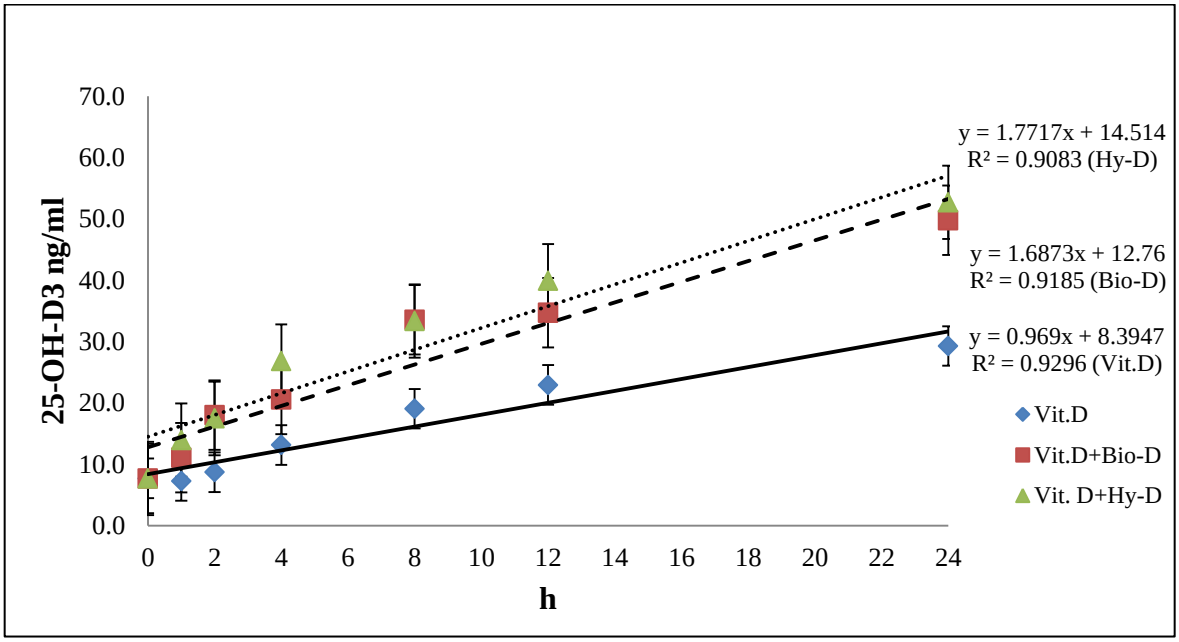


Figure 10. Comparative linear increase Day-15 ELISA vs LC/MS/MS serum 25-OH-D_3 values (ng/ml)

- *Serum 25-OH-D_3 concentration Day 20*

ELISA Kit. - Significant differences were found after 2-h exposure to treatments, with Vit. D+Hy-D having higher values than Vit.D and Vit. D+Bio-D. At 4 h, Vit. D had a significant lower values than the other two treatments. After 12 h, Vit. D+Bio-D had intermediate values between Vit. D and Vit.D+Hy-D ($p<0.05$) (Table 12).

LC/MS/MS.- At 1 and 2 h, Vit.D had an intermediate effect when compared to Vit.D and Vit.D+Hy-D. From 4 to 24 h Vit. D+Bio-D and Vit.D+Hy-D had significantly higher values than Vit.D. Only at 12 h was Vit. D+Bio-D significantly ($p<0.05$) lower than Vit.D+Hy-D (Table 12).

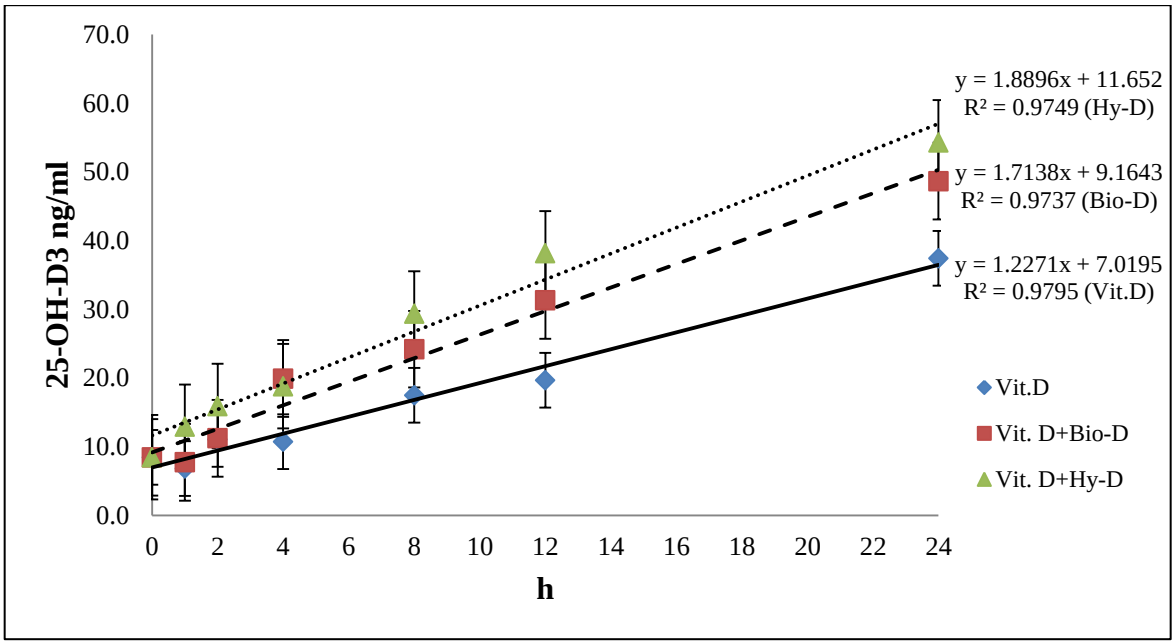
No "circadian" type trend was observed after the 24-h exposure to the treatment diets for any of the assays (Figure 11).

Table 12. Day-20 ANOVA (ELISA Kit versus LC/MS/MS) serum 25-OH-D_3 (ng/ml)

Hour	Treatment		
	Vit. D	Vit. D+ Bio-D	Vit. D+ Hy-D
	ELISA Kit		
0		8.47±2.32	
1	6.84±3.52	7.74±2.10	12.91±3.91
2	11.08±1.44^{b}	11.24±1.68^{b}	15.93±3.14^{a}
4	10.73±0.39^{b}	19.93±4.17^{a}	18.82±6.50^{a}
8	17.51±2.83	24.20±7.32	29.36±6.36
12	19.67±4.72^{b}	31.31±5.43^{ab}	38.15±11.52^{a}
24	37.40±7.85	48.65±8.83	54.27±13.79
	LC/MS/MS		
0		8.00±2.62	
1	8.25±0.93^{b}	12.47±4.89^{ab}	15.40±1.78^{a}
2	12.15±1.46^{b}	14.63±1.90^{ab}	16.53±1.99^{a}
4	12.20±1.56^{b}	22.74±2.81^{a}	24.30±4.74^{a}
8	17.75±1.97^{b}	26.81±5.84^{a}	30.05±2.24^{a}
12	21.74±2.13^{c}	30.47±3.32^{b}	38.12±6.38^{a}
24	30.38±4.38^{b}	47.57±7.94^{a}	47.92±9.20^{a}

a,b Means within a row with no common superscript differ significantly (P<0.05).
± Standard deviation. n= 4 birds per treatment per hour. Zero hour value represents the initial 25-OH-D_3 (ng/ml) average of 76 total birds.

ELISA Kit



LC/MS/MS

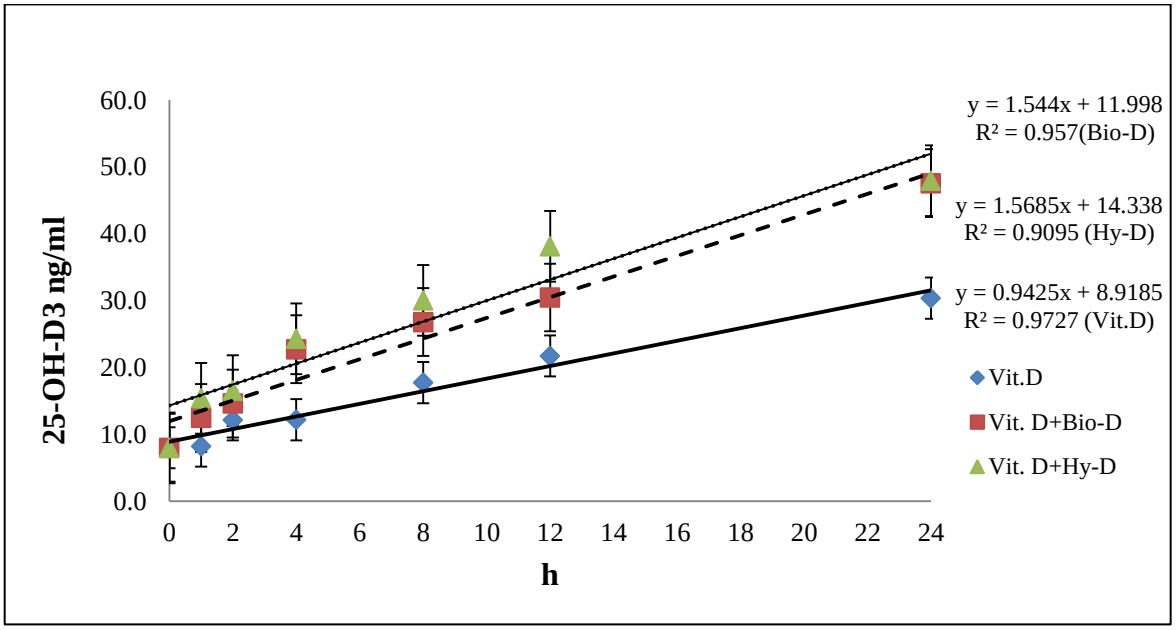


Figure 11. Comparative linear increase Day-20 ELISA vs LC/MS/MS serum 25-OH-D_3 values (ng/ml)

4.3.3 Serum Ca concentration

Calcium concentration was analyzed by Heartland Assays via colorimetric assay. Figures 12-23 show the Ca values at different samples times for the 24-h period exposure to the treatment diets. The breaking point of the ascending line indicates the approximate necessary time to reach stable levels in the blood.

- *Serum Ca concentration Day-5*

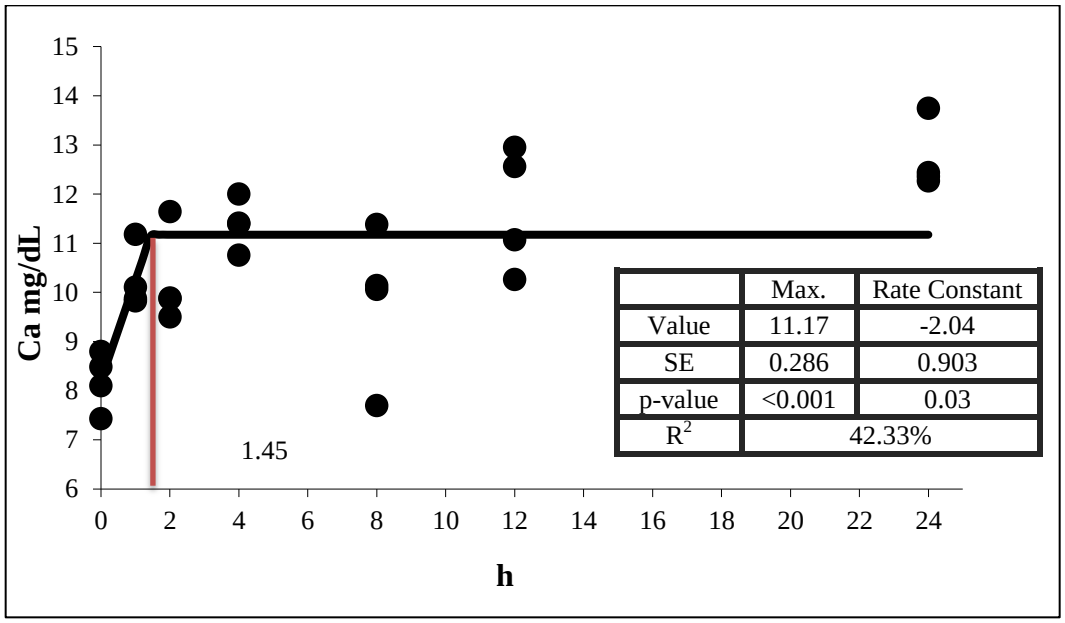


Figure 12. Vit. D treatment serum Ca concentration day-5 (mg/dL).
[y=Max +Rate constant(Time to maximize-x)].*

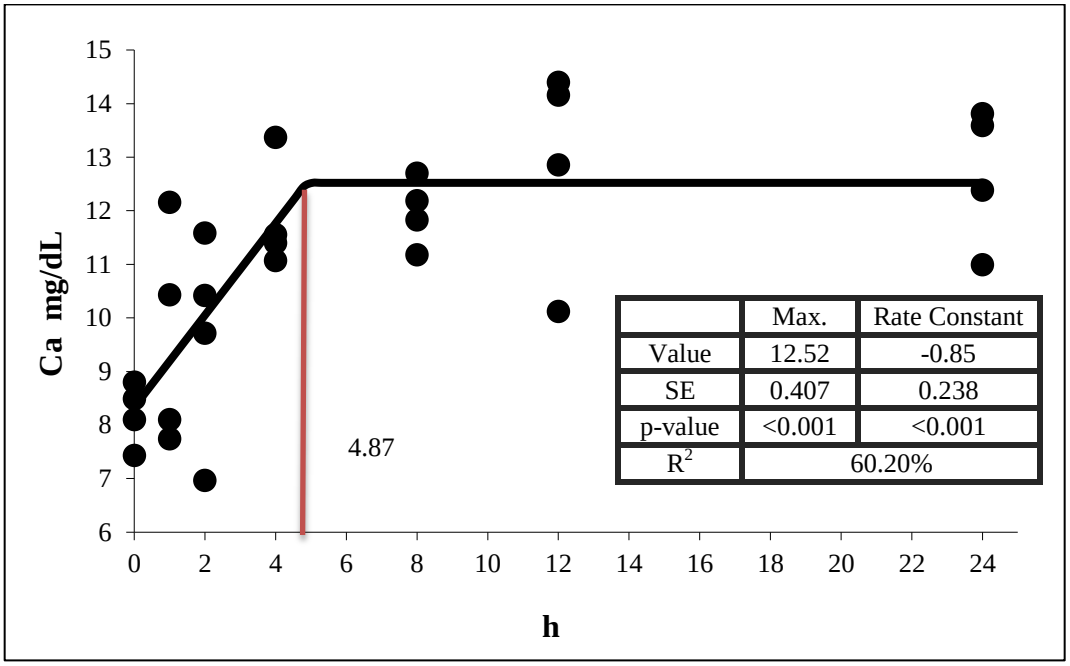


Figure 13. Vit. D + Bio-D treatment serum Ca concentration day-5(mg/dL).
[y=Max +Rate constant(Time to maximize-x)].*

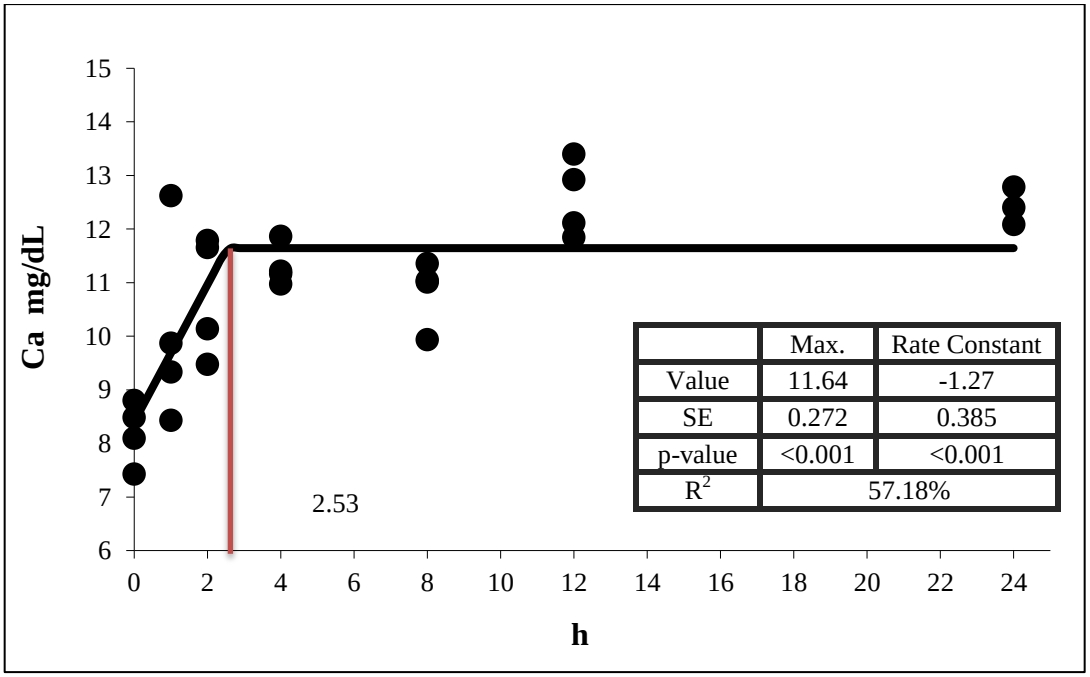


Figure 14. Vit. D + Hy-D treatment serum Ca concentration day-5(mg/dL).
[y=Max +Rate constant(Time to maximize-x)].*

- *Serum Ca concentration Day-10*

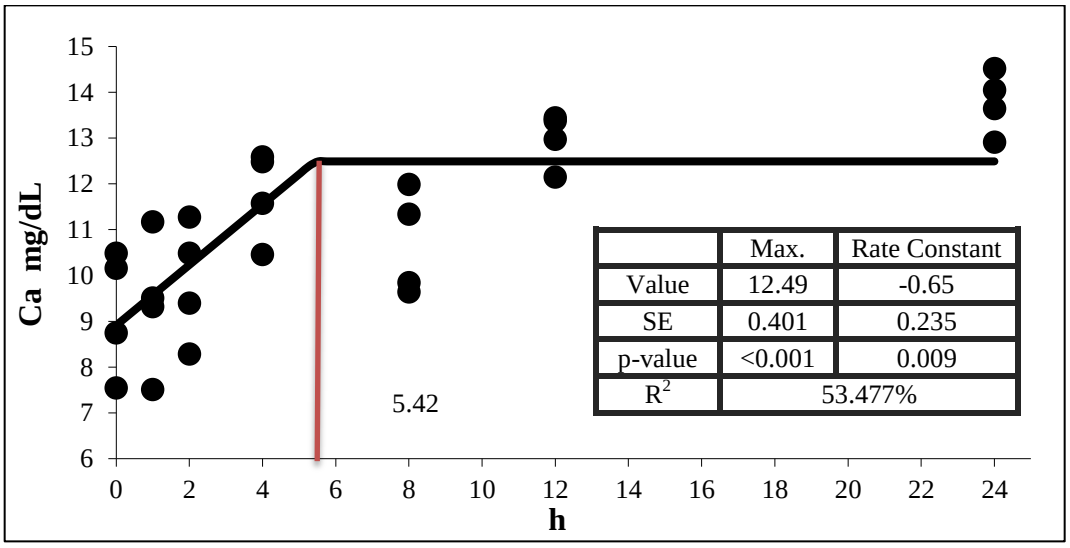


Figure 15. Vit. D treatment serum Ca concentration day-10 (mg/dL).
[y=Max +Rate constant(Time to maximize-x)].*

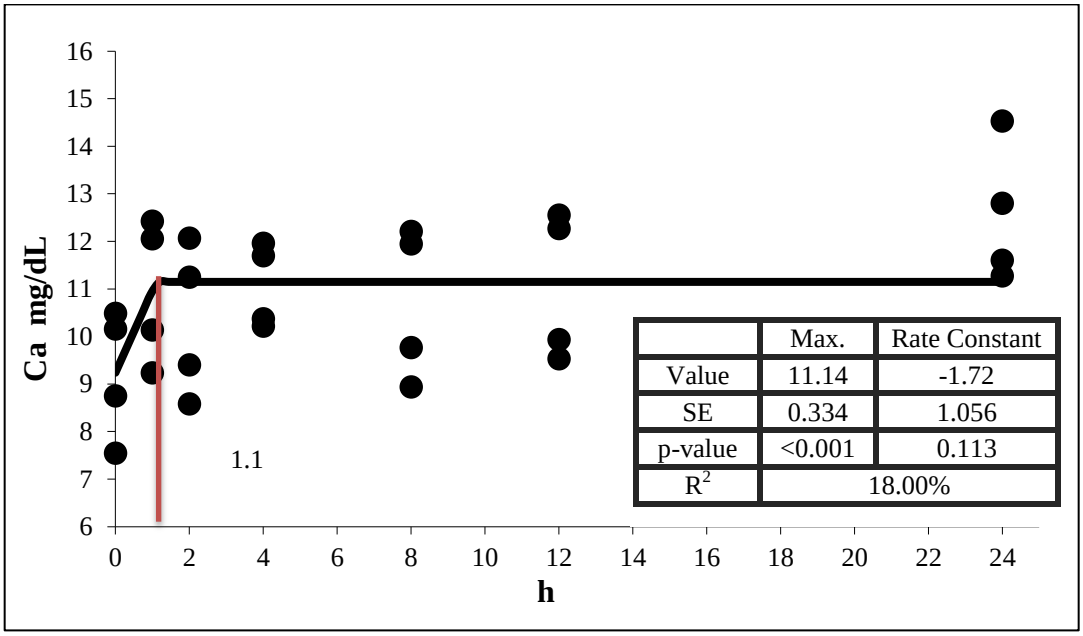


Figure 16. Vit. D + Bio-D treatment serum Ca concentration day-10 (mg/dL).
[y=Max +Rate constant(Time to maximize-x)].*

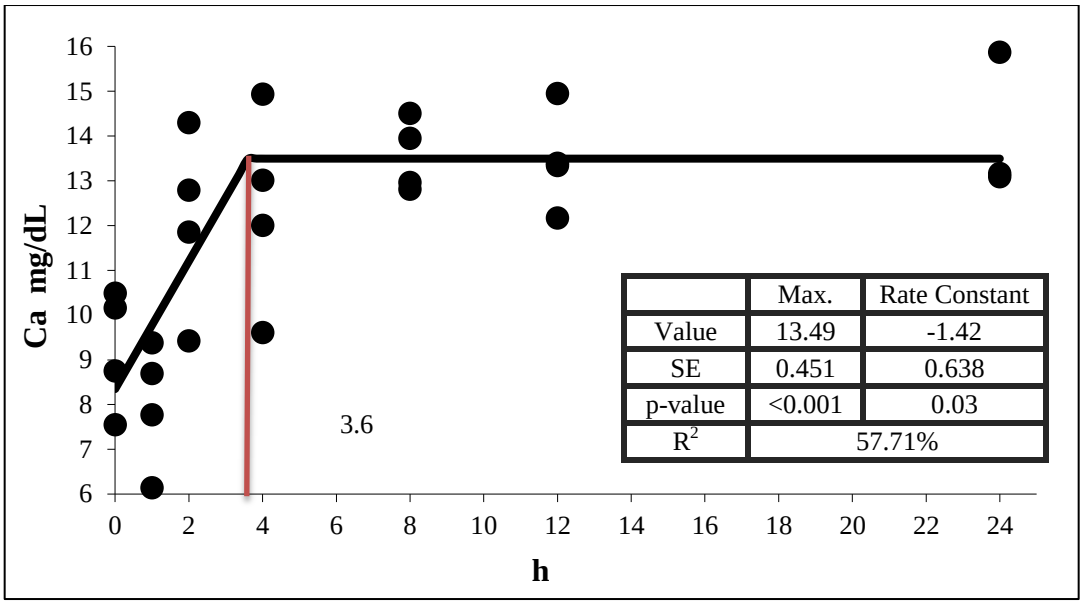


Figure 17. Vit. D + Hy-D treatment serum Ca concentration day-10 (mg/dL).
[y=Max +Rate constant(Time to maximize-x)].*

- *Serum Ca concentration Day-15*

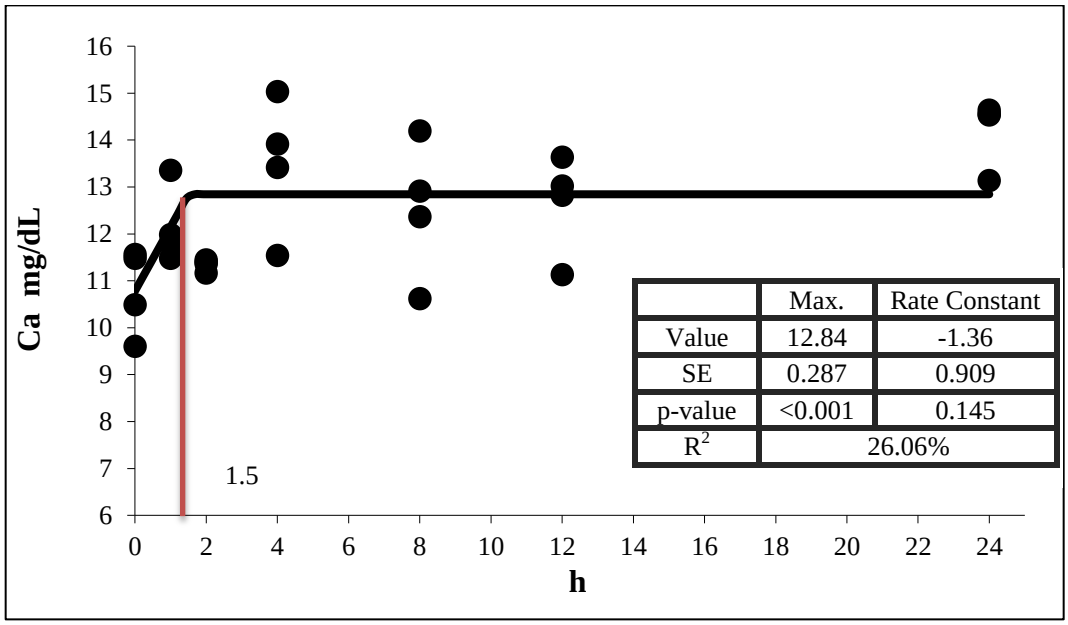


Figure 18. Vit. D treatment serum Ca concentration day-15 (mg/dL).
[y=Max +Rate constant(Time to maximize-x)].*

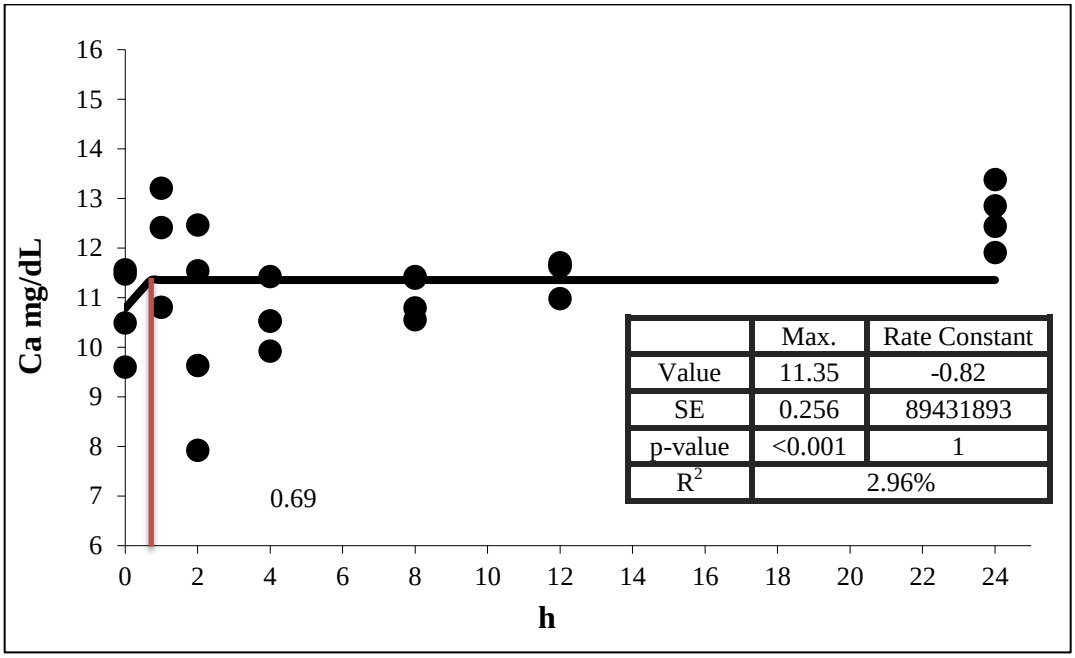


Figure 19. Vit. D + Bio-D treatment serum Ca concentration day-15(mg/dL).
[y=Max +Rate constant(Time to maximize-x)].*

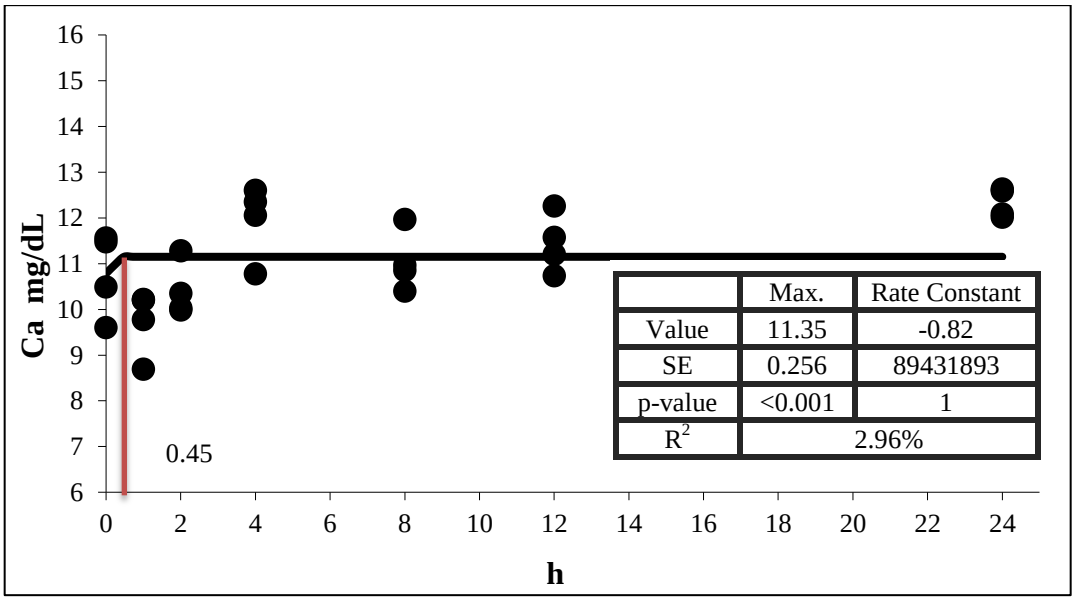


Figure 20. Vit. D + Hy-D treatment serum Ca concentration day-15 (mg/dL).
[y=Max +Rate constant(Time to maximize-x)].*

- *Serum Ca concentration Day-20*

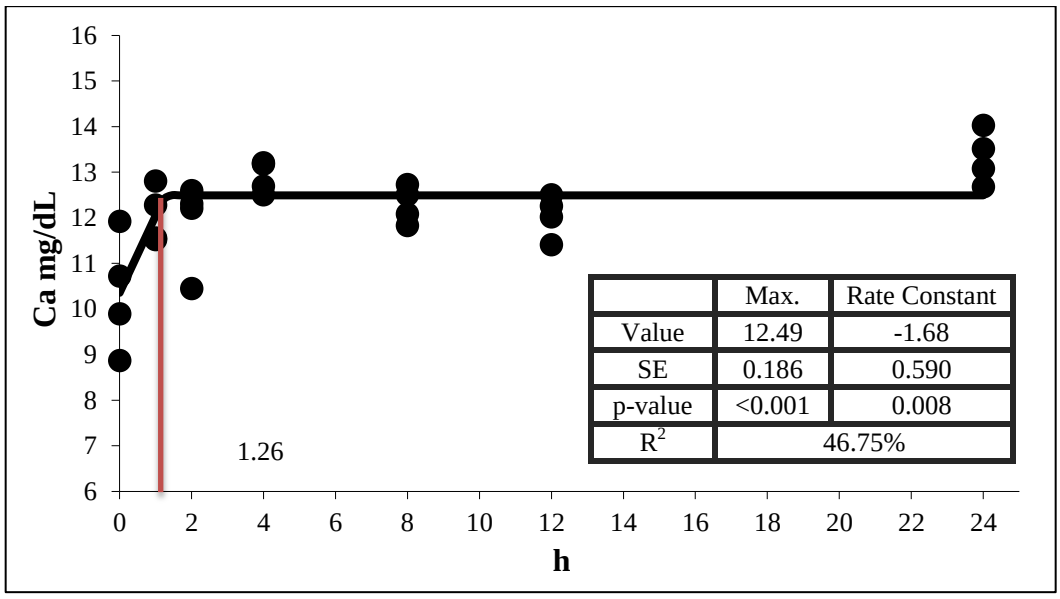


Figure 21. Vit. D treatment serum Ca concentration day-20 (mg/dL).
[y=Max +Rate constant(Time to maximize-x)].*

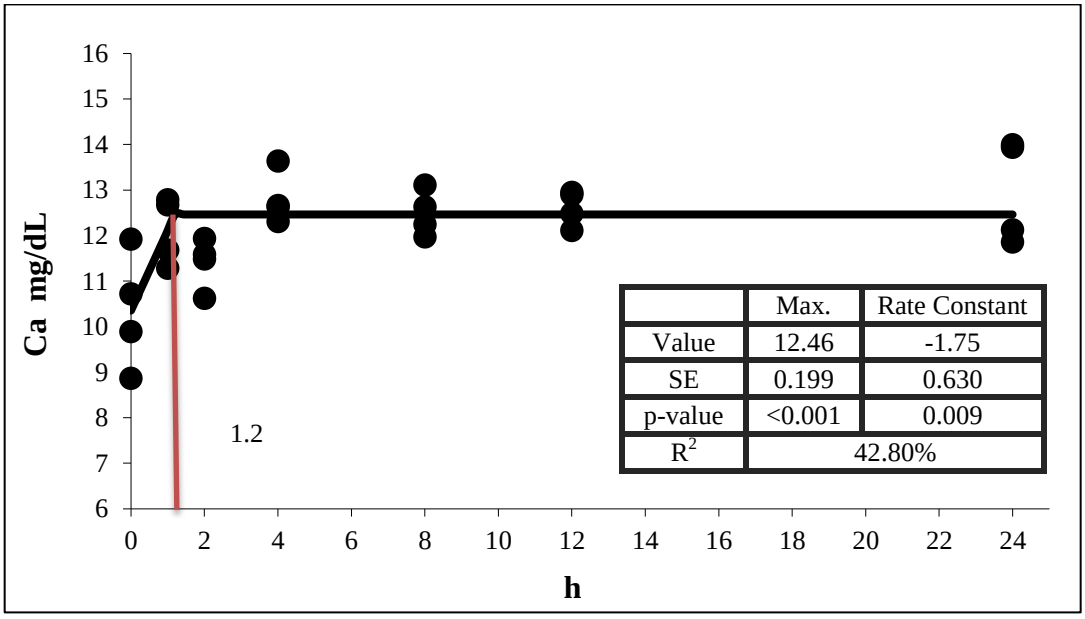


Figure 22. Vit. D + Bio-D treatment serum Ca concentration day-20 (mg/dL).
[y=Max +Rate constant(Time to maximize-x)].*

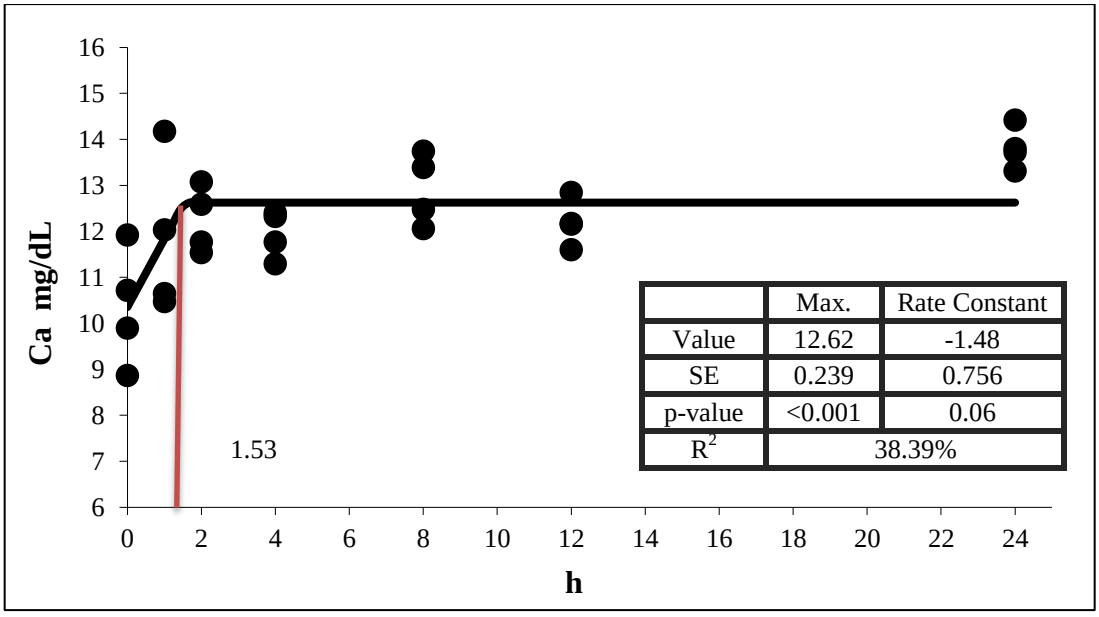


Figure 23. Vit. D + Hy-D treatment serum Ca concentration day-20 (mg/dL).
[y=Max +Rate constant(Time to maximize-x)].*

Figures 12-23 show the effect of the 25-OH-D_3 on the blood Ca levels. During the first 10 days the initial Ca concentration was around 9 mg/dL. For days 15 and 20 of age, Ca levels were already around 10 mg/dL which is the normal concentration in the blood and a lower response was observed. No consistent response was observed in Ca concentration among treatments.

The analysis of variance among treatments per sampled hour for the serum Ca values is presented in Tables 13-16.

At day-5 Vit.D+Bio-D had significantly higher Ca levels compared to Vit.D and Vit.D+Hy-D after 8-h exposure to treatment diets (Table 13).

Table 13. ANOVA day-5 serum Ca mg/dL

Hour	Product		
	Vit. D	Vit. D+ Bio-D	Vit. D+ Hy-D
0		8.20±0.59	
1	10.25±0.63	9.61±2.07	10.06±1.80
2	10.23±0.96	9.67±1.96	10.76±1.13
4	11.39±0.51	11.85±1.03	11.31±0.38
8	9.82±1.53^{b}	11.98±0.64^{a}	10.83±0.61^{ab}
12	11.71±1.26	12.88±1.95	12.57±0.71
24	12.70±0.70	12.69±1.29	11.85±1.17

a,b, Means within a row with no common superscript differ significantly (P<0.05).
± Standard deviation. n= 4 birds per treatment per hour . Zero hour value represents the initial Ca (mg/dL) average of 76 total birds.

Table 14. ANOVA day-10 serum Ca mg/dL

Hour	Product		
	Vit. D	Vit. D+ Bio-D	Vit. D+ Hy-D
0		9.23±1.35	
1	9.38±1.49[ab]	10.96±1.52[a]	7.99±1.40[b]
2	9.86±1.30	10.32±1.61	12.09±2.04
4	11.77±0.98	11.06±0.89	12.39±2.21
8	10.70±1.14[b]	10.71±1.61[b]	13.55±0.81[a]
12	12.98±0.59[a]	11.07±1.55[b]	13.46±1.14[a]
24	13.78±0.68	12.55±1.47	14.57±1.67

a,b, Means within a row with no common superscript differ significantly (P<0.05).
± Standard deviation. n= 4 birds per treatment per hour. Zero hour values represent the initial Ca (mg/dL) average of 76 total birds.

On day 10 of age a significant increment was observed for Vit.D+Bio-D when compared the other two treatments at 1-h exposure. At 8-h Vit.D+Hy-D was significantly higher than Vit.D and Vit.D+Bio-D. Finally at 12-h Vit.D+Bio-D had the lowest values of all (Table 14).

Table 15. ANOVA day-15 serum Ca mg/dL

Hour	Product		
	Vit. D	Vit. D+ Bio-D	Vit. D+ Hy-D
0		10.78±0.92	
1*	12.15±0.83[a]	12.14±1.22[a]	9.72±0.71[b]
2	11.34±0.12	10.39±2.02	10.41±0.60
4	13.47±1.45[a]	10.60±0.61[b]	11.94±0.80[ab]
8	12.52±1.47	11.04±0.43	11.04±0.66
12	12.65±1.07	11.49±0.33	11.44±0.64
24	14.22±0.72[a]	12.64±0.62[b]	12.33±0.32[b]

a,b, Means within a row with no common superscript differ significantly (P<0.05).
*± Standard deviation. n= 4 birds per treatment per hour * Vit.D+Bio-D (n= 3 brids). Zero hour values represent the initial Ca (mg/dL) average of 76 total birds.*

On day 15 Vit.D and Vit.D+Bio-D were significantly different from Vit.D+Hy-D during the first hour. At 4-h Vit.D+Hy-D had intermediate values between Vit.D and Vit.D+Bio-D. After 24-h Vit.D had significantly higher levels of Ca when compared to the other two treatments (Table 15).

Table 16. ANOVA day-20 serum Ca mg/dL

Hour	Product		
	Vit. D	Vit. D+ Bio-D	Vit. D+ Hy-D
0		10.35±1.29	
1	12.04±0.61	12.11±0.74	11.83±1.71
2	11.89±0.97	11.41±0.55	12.24±0.71
4	12.89±0.35[a]	12.81±0.57[a]	11.94±0.52[b]
8	12.28±0.40	12.49±0.49	12.91±0.78
12	12.04±0.46	12.61±0.39	12.19±0.51
24	13.32±0.58	12.98±1.14	13.81±0.45

a,b, Means within a row with no common superscript differ significantly (P<0.05).
± Standard deviation. n= 4 birds per treatment per hour. Zero hour values represent the initial Ca (mg/dL) average of 76 total birds.

At 20 days of age only one difference was found. At 4-h, Vit.D and Vit.D+Bio-D had a higher increment in Ca concentration than Vit.D+Hy-D. ($p<0.05$) (Table 16).

A correlation coefficient of 58% (r) between serum Ca and 25-OH-D_3 was found (Table 17). The weak relationship may be due to the stabilization of Ca levels that occurred during the first hours of exposure to the treatment diets, while the concentration of 25-OH-D_3 increased during the 24-h of exposure and stable levels were not reached during this time.

Table 17. Calcium vs 25-OH-D_3 bivariate correlation

Variables	Coefficient of Correlation	p value
Calcium – 25-OH-D_3	0.58	<0.001

n= 335 birds per variable

4.3.4 Percentage tibia bone ash

Figure 24 show the percentage bone ash of the birds sampled at 0-h exposure to the treatment diets, meaning that, they were always fed the deficient vitamin D_3 diet and served as a negative control. Bone ash data showed that chickens were able to support bone mineralization and growth with the maternal vitamin D_3 found in the egg yolk (40.36 ng/ml) up to 10.60 days when % tibia ash was maximized.

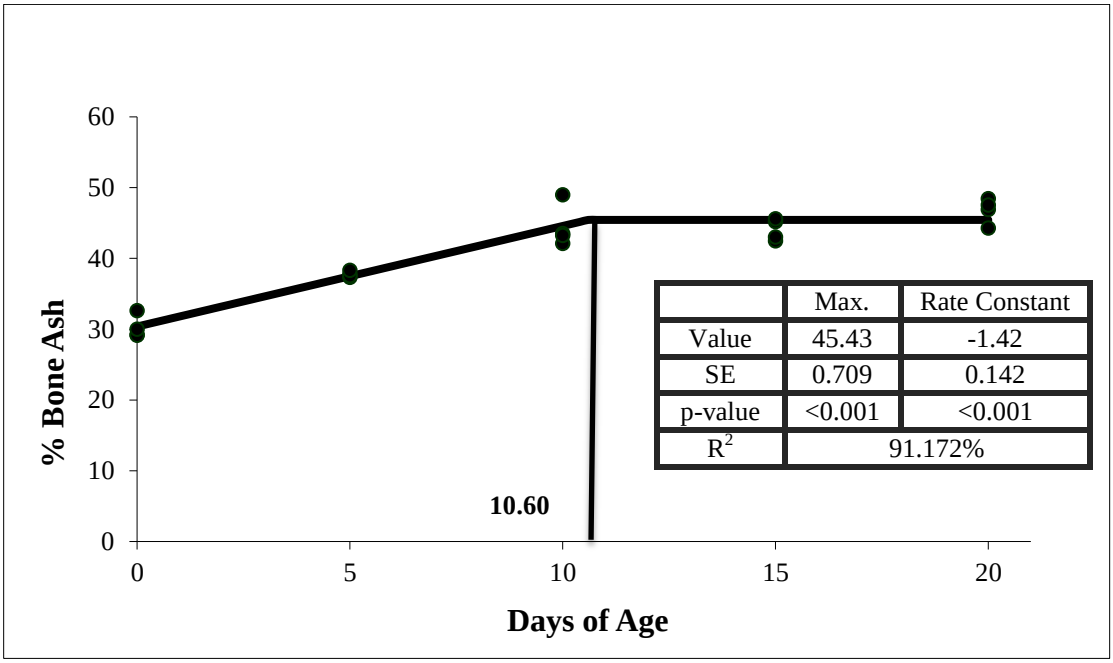


Figure 24. Percentage bone ash of chickens under vitamin D_3 deficient diet (NC).
The tibia was dissected on days 1, 5, 10, 15 and 20 days of age at 0 hours exposure to treatment diets. Four birds per day. [y=Max +Rate constant(Time to maximize-x)].*

Table 18 show the percentage tibia ash of chickens at 1, 5, 10, 15 or 20-days of age that were fed the treatment diets for 24-h and compared to the NC. The birds were placed back on the NC diet after the 24-h feeding period. All samples were taken at day-21 of age.

Table 18. Percentage bone ash evaluation one way ANOVA of chickens fed treatment diets one time for 24 h

% Bone Ash	NC	Vit. D	Vit. D +Bio-D	Vit. D +Hy-D
➢ **Day-old chicks fed one time treatment diets (NC)**				
	46.79±1.80	47.10±3.18	47.23±2.43	47.26±0.62
➢ **Fed treatment diets one time at Day-5**				
	46.79±1.80	47.49±1.67	47.01±0.58	48.49±2.17
➢ **Fed treatment diets one time at Day-10**				
	46.79±1.80	46.04±1.96	49.86±2.15	47.26±2.80
➢ **Fed treatment diets one time at Day-15**				
	46.79±1.80	48.87±0.98	46.83±2.02	49.01±1.54
➢ **Fed treatment diets one time at Day-20**				
	46.79±1.8^{b}	49.10±1.2^{a}	46.14± 0.7^{b}	49.37±1.3^{a}

a,b Means within a row with no common superscript differ significantly (P<0.05).
± Standard deviation. n= 4 birds per treatment.

No significant (p>0.05) difference was found in the percentage bone ash among treatments when compared to NC on days 1-15. On day 20, percentage bone ash values for Vit. D and Vit.D +Hy-D were significantly (p<0.05) higher than Bio-D and NC treatments (Table 18).

4.3.5 Feed analysis (Heartland assays analysis of 25-OH-D_3, 25-OH-D_2 and vitamin D_3 concentration via LC/MS/MS)

Samples were taken directly from a moving stream, and stored in a refrigerator at 11°C prior to being send to analysis. Tables 18-21 show the concentrations of vitamin D_3, 25-OH-D_3 and 25-OH-D_2 metabolites in the feed samples.

Table 19. Basal vitamin D_3-deficient diet (NC) 25-OH-D_3, 25-OH-D_2 and vitamin D_3 concentration (ppb)

Test	Name	Result	Unit
25-OH-D_3 (LC/MS/MS)	25-OH-D_2	< 0.5	ppb
25-OH-D_3 (LC/MS/MS)	25-0H-D_3	3.6	ppb
Vitamin D_3 (LC/MS/MS)	Vitamin D_3	18.2	ppb

Table 20. Vitamin D_3 diet (Vit. D) 25-OH-D_3, 25-OH-D_2 and vitamin D_3 concentration (ppb)

Test	Name	Result	Unit
25-OH-D_3 (LC/MS/MS)	25-OH-D_2	< 0.5	ppb
25-OH-D_3 (LC/MS/MS)	25-OH-D_3	23.8	ppb
Vitamin D_3 (LC/MS/MS)	Vitamin D_3	335.1	ppb

Table 21. Vitamin D_3 + Bio-D diet (Vit.D+Bio-D) 25-OH-D_3, 25-OH-D_2 and vitamin D_3 concentration (ppb)

Test	Name	Result	Unit
25-OH-D_3 (LC/MS/MS)	25-OH-D_2	< 0.5	ppb
25-OH-D_3 (LC/MS/MS)	25-OH-D_3	89.7	ppb
Vitamin D_3 (LC/MS/MS)	Vitamin D_3	398.9	ppb

Table 22. Vitamin D_3+ Hy-D diet (Vit.D+Hy-D) 25-OH-D_3, 25-OH-D_2 and vitamin D_3 concentration (ppb)

Test	Name	Result	Unit
25-OH-D_3 (LC/MS/MS)	25-OH-D_2	< 0.5	ppb
25-OH-D_3 (LC/MS/MS)	25-OH-D_3	117.5	ppb
Vitamin D_3 (LC/MS/MS)	Vitamin D_3	304.8	ppb

4.4 DISCUSSION

A good understanding of the 25-OH-D_3 dynamics in the broiler chicken is fundamental to assess the metabolism and status of vitamin D (Bouillon, 1976), which ultimately will allow us to have a precise knowledge of its effects when different dietary levels and sources of vitamin D are studied. In this study, no "circadian type" trend was found between experimental treatments but rather a linear positive increase throughout time was observed. A significant difference between feeding cholecalciferol alone or

cholecalciferol plus one of the two sources of 25-OH-D_3 was also shown. In agreement with the results obtained in this study, Clark and Potts (1977) found that there is a linear relationship between the intake of vitamin D_3 and the serum levels of 25-OH-D_3. Also, Yarger *et al*. (1995) compared the effect of feeding cholecalciferol alone or with 25-OH-D_3 at different concentration levels and found a significant dose response relationship with serum 25-OH-D_3 concentrations increasing more rapidly in birds fed 25-OH-D_3 than in birds fed cholecalciferol alone.

Our results showed that the reduction in the serum 25-OH-D_3, when compared to the initial concentration sampled in the day-old chickens at 0-h exposure to treatment diets (40.36 ng/ml), is a good indicative of the depletion status of the maternal vitamin D stores in the egg yolk.

A significant difference was found in percentage bone ash in chickens fed the treatment diets for 24 h at day 20 of the trial. Feeding cholecalciferol plus 25-OH-D_3 as Hy-D™ resulted in an improvement on bone ash of 5% when compared to the negative control, while feeding cholecalciferol plus Bio-D™ was not able to significantly improve the percentage bone ash. This is contrary to the previous results, form research done in our laboratory which support the hypothesis that supplementation of 25-OH-D_3 as Bio-D™ outperforms equivalent amounts of cholecalciferol (62.5, 125, 250, 500, and 1000 IU/kg) and improves percentage bone ash values when compared to those of vitamin D_3-deficient chickens (Fowler *et al*., 2014). Boris *et al*. (1982) supported the positive effect of supplemental 25-OH-D_3 in broiler diets. He compared the activity of cholecalciferol and 25-OH-D_3 in bone ash and found the vitamin D_3 metabolite promote 50-100%

increase in percentage bone ash content. Positive effects of feeding supplemental 25-OH-D_3 in performance and mineralization of the bones have been suggested in recent studies. Fritts and Waldroup (2003) compared performance and bone ash content of growing broiler chickens fed different levels of 25-OH-D_3 and cholecalciferol. Body weight and bone ash were significantly greater in birds fed 25-OH-D_3 when compared to cholecalciferol alone. Bar *et al*. (2003) stated that 5-10 µg/kg 25-OH-D_3 were sufficient to ensure normal body weight and bone ash in chickens under continuous lighting. The inclusion of 40-70 µg/kg 25-OH-D3 improved phytate P retention and decreased the incidence of TD. A total of 70 µg/kg 25-OH-D3 was needed to decrease TD in diets with marginal concentration of Ca (Ledwaba and Roberson, 2003). Chou et al. (2009) studied the effect of supplemental 25-OH-D_3 in broiler diets and found an improvement in the small intestine morphology, villus length of the duodenum and jejunum were longer. Also, a higher ratio of villus length to crypt depth was observed in duodenum and jejunum and a better humoral immunity response to *Salmonella* challenge was also observed. In broiler breeder hens the supplementation of 25-OH-D_3 had positive effects when compared to cholecalciferol with respect to increasing body ash of the progeny to 1.08 and 1.02 times higher at 3.125 µg/kg and 12.5 µg/kg 25-OH-D_3 respectively. Hatchability was also increased 1.67 times at 3.125 µg/kg 25-OH-D_3 (Atencio *et al*., 2005b). Contrary to the previous studies, Cantor and Bacon (1978) fed between 1.25 and 5 µg/kg of vitamin D_3 or 25-OH-D_3 to cage-reared, male broiler chicks and observed no differences in frequency of broken bones when comparing the two vitamin D_3 forms; although no negative effects of 25-OH-D_3 supplementation were observed either.

In the present study, the supplementation of 25-OH-D_3 resulted in an increase in plasma Ca during the first few hours after the chickens were exposed to the treatments diets. This effect was more evident during the first weeks of age until Ca levels stabilized at around 10 mg/dL, but no consistent trend was found in Ca levels throughout time in response to the supplemental sources of 25-OH-D_3. This is in agreement with Knowles *et al*. (1935) who described the variation of Ca levels in laying hens using blood samples drawn at different time points. Experimental hens responded to subcutaneous injections of parathormone by increasing the blood Ca concentration. The response was described as rapid and sudden after the subcutaneous injection. This rapid increase of Ca levels in the blood is also supported by Koch *et al*. (1984) who measured the responsiveness of parathyroid function in chickens with induced hypoglycemia, birds responded within minutes to an ethyleneglycol-bis-(B-aminoethylether)-N,N'-tetraacetic acid (EGTA) challenge used to induce depression of the blood ionic Ca concentration. McNutt and Haussler (1973) evaluated the nutritional effect of 25-OH-D_3 and l,25$(OH)_2$ D_3 in preventing rickets, weight gain and maintenance of plasma calcium in white Leghorn chickens. He found that l,25$(OH)_2$ D_3 and 25-OH-D_3 were 1.5 to 2.2 times as effective as cholecalciferol with respect to stimulation of weight gain and maintenance of plasma calcium levels.

In the present study, dietary Ca and available P were lowered from 1.0 and 0.45 % (NRC, 1994) to 0.75 and 0.37 %, respectively, with the purpose of increasing the pressure of the treatment diets to stabilize plasma Ca levels, and identify any effect in preventing vitamin D-deficiency signs in the broiler chickens. However, the three

treatment diets were able to increase the plasma Ca within the first hours of exposure to the treatment diets at a similar rate and no deficiency signs were observed either. Contrary to our results, The importance of adequate dietary calcium was studied by Williams *et al*. (2000) where chickens being fed a low-Ca diet had higher incidence of tibial dyschondroplasia and hypocalcaemia rickets, suggesting that diets for rapid growth modern strains may be low in available P but not in Ca.

4.5 CONCLUSION

No "circadian" trend line was found in serum 25-OH-D_3 concentrations over time but a positive linear increase was observed, supporting the theory that 25-OH-D_3 is in fact a partially active storage form of vitamin D_3 and is a good marker to assess the status of vitamin D in the broiler chicken. A significant difference was found between 25-OH-D_3 supplemental sources when compared to feeding cholecalciferol as the only source of vitamin D_3. In general feeding Bio-D™ or Hy-D™ as a supplemental source of 25-OH-D_3 had a significant positive effect in increasing the serum levels the 25-OH-D_3 and therefore increase the availability of this metabolite for further biological functions, especially after 10 days of age. No consistent trend was found in the response of blood Ca concentrations to supplemental 25-OH-D_3.

5. EVALUATION OF INCREASING DOSES OF VITAMIN D_3 ON THE REQUIREMENT, GROWTH PERFORMANCE AND BONE MINERALIZATION OF GROWING CHICKENS

5.1 INTRODUCTION

Intensive genetic selection in modern poultry strains has led to increase productivity, thanks to the improvement of growth rate and feed efficiency. Along with this intensive process, changes in the nutrient requirements for growing chickens are probable as suggested by different authors whose research indicates that higher requirements than those established in the NRC (1994) are needed to maximize performance.

Vitamin D_3 is known to have an important function in the regulation of Ca and P levels in plasma to support good mineralization of bones, which is very important when raising broiler chickens under intensive industry conditions where mortality due to bone problems represents a major economic issue. In order for vitamin D_3 to have biological function, it must undergo a hydroxylation in the liver to form 25-hydroxycholecalciferol (25-OH-D_3) which is the major circulating form of Vitamin D_3 in the blood, and then a second hydroxylation takes place in the kidneys to form 1,25-dihydroxycholecalciferol (l,25-$(OH)_2$-D_3) the most active metabolite in bone. The NRC (1994) vitamin D_3 requirement for growing Leghorn and broiler chickens is 200 IU/kg, some authors still consider this requirement as adequate to promote bone mineralization, growth and immune functions in modern strains of chickens (Baker *et al.*, 1998, Fowler *at al*., 2014),

while others suggest that higher amounts of vitamin D_3 (1,000-3,500 IU/kg) are needed to support the growth rate of modern strains (Kasim and Edwards, 2000, Ledwaba and Roberson, 2003, Whitehead *et al*., 2004, Khan *et al*., 2010). The quantitative requirement of growing chickens for vitamin D is usually based on measurements of bone quality, such as bone ash content or incidence of rickets, which have been found to be more sensitive indicators than growth rate (Edwards *et al*., 1994). The vitamin D requirement may change depending on many factors such as dietary Ca and P, housing conditions, or metabolic disorders like impaired liver activity due to stress conditions such as bird density, heat stress, and diseases; mycotoxicosis, enteritis, malabsorption syndrome, and certain immune disorders (Yarger *et al*., 1995).

The use of the GE Dual X-ray absorptiometry (DEXA) scan offers a good alternative assay to analyze bone quality and body composition. The DXA´s ability to assess bone in a precise and fast way using a low radiation dose expands the research boundaries to evaluate the activity of nutrients such as vitamin D_3 in bone mineralization and health.

Recent research done in our laboratory supports the lower requirement suggested by the NRC (1994) and Baker *et al*. (1998). The objective of this study was to re-evaluate the vitamin D_3 requirement using a precise and controlled protocol where leghorn chickens received a daily oral gavage of pure cholecalciferol and bone mineralization and performance response to the treatments were evaluated using a broken-line regression analysis.

5.2 MATERIALS AND METHODS

5.2.1 Experimental design overview

A pen containing 50 hens and 7 roosters (64 week old) Hy-Line breeders was fed a vitamin D_3-deficient breeder diet for 1 month for maternal vitamin D_3 depletion purposes; eggs collected during this period were discarded. After the 4-week depletion period, hatching eggs were collected twice a day for 7 days and stored in a cool room at 18.3° C. These eggs were placed in a GQF® automatic incubator at 37.5 °C and 50-60 % humidity for 18 days and then transferred to a GQF® hatcher at 36.9 °C and 60-70% humidity. A total of 96 newborn Hy-line chickens (6 birds per pen, 2 pen reps per treatment) were placed in one Petersime battery brooder unit located in an environmentally controlled rearing room (# 1215) at the Texas A&M Poultry Research Farm, and fed *ad libitum* a corn-soy vitamin D_3-deficient broiler starter diet for a 21-day rearing period. Birds were kept away from any source of UV light that may influence calcification from endogenous synthesis of cholecalciferol.

An external liquid concentrated source of Vitamin D_3 (Pure Life® Labs) containing 50,000 IU/ml was diluted to achieve concentrations yielding 0, 40, 80, 160, 200, 400, 800, 1,600 IU D_3/kg. Chickens received a daily gavage during the 21-day rearing period. Weekly feed consumption and daily body weight per pen were recorded to evaluate performance. Birds were monitored twice a day with regard to general flock condition, mortality, temperature, lighting, water, feed, and unanticipated events for the rearing period. At day 21 of age the left tibia was removed to perform a bone ash

determination as a percent of the fat-free, dried bone weight to evaluate vitamin D_3 activity.

5.2.2 Housing and daily observations

Breeders: A total of 50 hens and 7 roosters (64 week old) Hy-Line breeders were placed in a pen located at building #1210 at the Texas A&M Poultry Research Farm. During the depletion and collecting periods daily observations were done with regard general flock condition, mortality, water etc.

Chickens: Birds were caged in one Petersime battery brooder located in an environmentally controlled rearing room (# 1215) at the Texas A&M Poultry Research Farm. Minimal lighting in the room was provided on a constant 24-h basis. The complete absence of UV light in the rearing rooms has been verified previously (Fowler *et al*. 2014) Birds were observed twice a day with regard to general flock condition, temperature, lighting, water, feed, and unanticipated events for the rearing facility. Weekly feed consumption and daily body weight per pen were recorded to evaluate performance.

5.2.3 Egg management and incubation

Eggs were collected and discarded during the whole depletion period (4 weeks). After the depletion period eggs were collected for 7 days twice a day and stored in a cool room at 18.3°C. Dirty eggs or with any shell anomaly were discarded. Collected eggs were placed in a GQF® automatic incubator at 37.5 °C and 50-60 % humidity for 18 days and then transferred to a GQF® hatcher at 36.9 °F and 60-70% humidity. Eggs were monitored once a day with regard temperature and humidity.

5.2.4 Liquid vitamin D_3 source

A concentrated liquid vitamin D_3 (Pure Life® Labs) containing 50,000 IU D_3/ ml was used to supplement the increasing doses of cholecalciferol.

5.2.5 Dietary treatments

Breeder and pullet-starter basal corn-soy vitamin D_3-deficient diets were formulated using a customized mineral/vitamin premix without vitamin D_3 and utilizing corn oil as the fat source (Table 23). NRC (1994) body weight (225 g) and feed consumption (485 g) data for leghorn-type chickens was used to estimate the increasing doses of vitamin D_3 per kg of feed consumed over a 20-day period. Daily gavage treatments were based on estimated consumption of 0, 40, 80, 160, 200, 400, 800, 1,600 IU D_3/kg of feed consumed over the 20 day rearing period. The liquid source of Vitamin D_3 was diluted in corn oil to yield a concentration of 77.6 IU D_3/ml corresponding to the highest treatment dose (1,600 IU/kg) and then diluted again to yield the other treatments so that a daily constant dose was contained in 0.5 ml. The vitamin D_3 treatments were separated in 20 daily doses and stored in a freezer at -28.88 °C until required.

5.2.6 Feed and watering method

Both basal vitamin D_3-deficient diets and water were offered *ad libitum*.

5.2.7 Bone ash determination

The left tibia was dissected from selected birds at 21 day of age for bone ash determination to evaluate vitamin D_3 activity. Muscle and cartilage were removed before being placed in 4 L of petroleum ether for 48-h at room temperature. Following this, tibias were dried for 24-h in a drying oven at 105°C. Finally tibias were ashed at 650°C for 23-h. Ash was evaluated as a percentage of the free-fat, dried bone weight**.**

Table 23. Breeder and pullet starter experimental basal vitamin D-deficient diets

Ingredient	Breeder[1] %	Pullet starter[2] %
Corn	64.35	66.35
Dehulled soybean meal	23.55	29.29
DL-Methionine	0.17	0.13
L-Lysine HCl	0.00	0.08
Corn oil	0.00	0.00
Limestone	10.00	1.72
Monocalcium phosphate	1.31	1.58
Salt	0.30	0.331
Custom vitamin/mineral premix[3]	0.5*	0.5*

[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]Calculated nutritional content was as follow: crude protein 20%, metabolizable energy 2900 kcal/kg, calcium 1 %, available phosphorus 0.45 %, methionine 0.45 %, methionine+cystine 0.78 %, lysine 1.10%, tryptophan 0.26 %, threonine 0.82% arginine 1.46%, crude fat 2.81% and crude fiber 2.6%

[3] 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.*

5.2.8 Dual X-ray absorptiometry (DEXA)

The 96 Hy-line chicken carcasses were separated by treatment and identified by pen, and then stored in a freezer at -28.88°C. Two days before the DEXA scan the chicken carcasses were thawed and the feathers were removed by hand, and then stored in a

refrigerator at 5°C overnight.

Chickens were placed in supine position with their wings and legs at the sides of the body. The analysis was made using the GE Dual X-ray absorptiometry (DXA) scan located in the Applied Exercise Science Laboratory at Texas A&M University, and the GE small animal software which is specifically designed for animals <20 kg. Total bone mineral content (g) was obtained.

5.2.9 Statistical analysis

Data were subjected to repeated measures analysis using the GLM procedure of SPSS. Dietary treatments were used as a fixed factor. Means were separated by Duncan´s multiple range test. Statistical significance was accepted at $p \leq 0.05$. Bone ash assays data were analyzed using the pair correlation function of the same software. Broken-line regression was used to analyze the percentage bone ash using the "Nutritional Response Model Program" Version 1.01 from Gene Pesti and Dmitry Vedenov, University of Georgia. Each pen served as the experimental unit for performance and bone ash data analysis.

5.3 RESULTS

5.3.1 Performance

After 1 week on their respective treatments no significant ($p>0.05$) differences in weight gain, feed gain ratio, productivity index and mortality were observed in Hy-line chickens consuming increasing doses of cholecalciferol (Table 24). However, significant ($p<0.05$) differences were detected in body weight and feed weight ratio. Body weight for chickens consuming 300 IU D_3/kg registered the lowest values while those consuming 2,009 IU D_3/kg had the highest values when compared to the NC treatment.

For the second week of the study, a significant ($p<0.05$) difference was found in body weight and weight gain between Hy-line chickens consuming the increasing doses of cholecalciferol (Table 25). Birds consuming 145.6 IU D_3/kg registered the worst weight gain and body weight while birds consuming 2,009 IU D_3/kg registered the highest values for both parameters.

No significant ($p>0.05$) differences in feed gain ratio, productivity index and mortality were observed in Hy-line chickens after 2 weeks consuming different concentrations of cholecalciferol.

After 3 weeks being orally gavaged with increasing doses of cholecalciferol no significant ($p>0.05$) difference was found in feed gain ratio, feed weight ratio, productivity index and mortality among treatments in Hy-line chickens (Table 26). However, significant ($p<0.05$) differences were detected for body weight and weight gain with respect 62.3 and 145.6 IU D_3/ kg. But, no significant difference was found when compared to the NC treatment.

Table 24. ANOVA Body weight, weight gain, feed gain ratio, feed weight ratio, productivity index and mortality in Hy-line chickens after 1 wk of consuming 0, 62.3, 145.6, 242.2, 300, 525, 1,043 and 2,009 IU D_3/kg.

	IU D_3/kg							
	0	**62.3**	**145.6**	**242.2**	**300**	**525.5**	**1043**	**2009**
BW	77.34±1.81^{cbd}	73.50±3.06^{cbd}	72.92±0.35^{cd}	76.16±1.88^{cbd}	70.92±1.06^{d}	79.16±3.30^{abc}	80.41±1.53^{ab}	80.92±6.01^{a}
FI	0.062	0.056	0.062	0.060	0.054	0.067	0.068	0.069
C-FI	0.062	0.056	0.062	0.060	0.054	0.067	0.068	0.069
WG	32.34±2.35	28.34±1.88	28.09±0.12	31.67±1.64	25.42±0.82	34.09±2.00	34.92±3.65	35.58±8.13
P-F:G	1.93±0.06	1.97±0.63	2.20±0.16	1.92±0.13	2.13±0.49	1.98±0.13	1.97±0.15	1.99±0.31
F:W	0.81±0.1^{ab}	0.76±0.07^{a}	0.85±0.05^{ab}	0.79±0.03^{ab}	0.76±0.01^{a}	0.85±0.04^{b}	0.85±0.01^{ab}	0.86±0.01^{b}
PI	57.06±2.77	53.13±3.96	47.36±3.58	56.81±5.35	47.54±1.83	57.21±6.33	58.55±5.83	59.59±13.68
Mort	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00

a,b,c,d Means within a row with no common superscript differ significantly (P<0.05). ± Standard deviation. n= 2.
Body weight (BW),weight gain , kilograms of feed consumed per bird (FI), cumulative kilograms of feed consumed per bird (C-FI), (WG), phase-feed gain ratio (P-F:G), feed weight ratio (F:W), productivity index (PI), Mortality (Mort)

Table 25. ANOVA Body weight, weight gain, feed gain ratio, feed weight ratio, productivity index and mortality in Hy-line chickens after 2 wk of consuming 0, 62.3, 145.6, 242.2, 300, 525, 1,043 and 2,009 IU D_3/kg.

	IU D_3/ kg							
	0	**62.3**	**145.6**	**242.2**	**300**	**525.5**	**1043**	**2009**
BW	135.50±1.41^{abc}	125.75±10.25^{cd}	118.00±1.41^{d}	136.83±2.12a^{bc}	132.83±1.64^{bcd}	145.08±10.72^{ab}	149.42±3.41b^{ab}	151.58±11.43^{a}
FI	0.106	0.095	0.086	0.104	0.106	0.124	0.124	0.134
C-FI	0.169	0.151	0.148	0.164	0.160	0.192	0.192	0.203
WG	58.17±0.23^{bc}	52.25±7.^{18cd}	45.08±1.06^{d}	60.67±0.23^{abc}	61.92±2.70^{abc}	65.92±7.42^{ab}	69.00±1.81^{a}	70.66±5.42^{a}
P-F:G	1.83±0.01	1.82±0.02	1.93±0.07	1.71±0.02	1.72±0.06	1.9±0.16	1.8±0.07	1.90±0.23
F:W	0.79±0.01	0.75±0.03	0.73±0.02	0.76±0.01	0.80±0.05	0.86±0.04	0.83±0.01	0.88±0.10
PI	52.86±0.29	49.18±4.64	43.74±2.08	56.93±1.48	55.19±1.35	54.81±8.91	59.19±1.23	57.47±11.15
Mort	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00

a,b,c,d Means within a row with no common superscript differ significantly (P<0.05). ± Standard deviation. n= 2.
Body weight (BW),weight gain , kilograms of feed consumed per bird (FI), cumulative kilograms of feed consumed per bird (C-FI), (WG), phase-feed gain ratio (P-F:G), feed weight ratio (F:W), productivity index (PI), Mortality (Mort)

Table 26. ANOVA Body weight, weight gain, feed gain ratio, feed weight ratio, productivity index and mortality in Hy-line chickens after 3 wk of consuming 0, 62.3, 145.6, 242.2, 300, 525, 1,043 and 2,009 IU D_3/kg.

	IU D_3/kg							
	0	**62.3**	**145.6**	**242.2**	**300**	**525.5**	**1043**	**2009**
BW	219.33±2.12^{ab}	202.42±20.62^{bc}	178.92±2.24^{c}	226.00±7.77^{ab}	217.00±4.70^{ab}	234.58±21.09^{a}	240.83±5.89^{a}	246.42±16.85^{a}
FI	0.129	0.159	0.117	0.155	0.162	0.177	0.179	0.182
C-FI	0.298	0.311	0.266	0.320	0.323	0.369	0.372	0.386
WG	83.83±0.70^{ab}	76.66±10.37^{b}	60.92±0.82^{c}	89.17±5.65^{ab}	84.16±3.06^{ab}	89.50±10.36^{ab}	91.42±2.47^{ab}	94.84±5.42^{a}
P-F:G	1.54±0.50	2.11±0.45	1.93±0.01	1.74±0.06	1.93±0.07	1.98±0.02	1.96±0.14	1.93±0.18
F:W	0.59±0.19	0.79±0.14	0.66±0.01	0.68±0.01	0.75±0.04	0.75±0.01	0.74±0.05	0.74±0.07
PI	71.69±24.24	47.19±14.82	44.11±0.62	61.72±4.35	53.58±0.93	56.48±5.83	58.70±2.81	61.30±10.10
Mort	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00

a,b,c, Means within a row with no common superscript differ significantly (P<0.05). ± Standard deviation. n= 2.
Body weight (BW),weight gain , kilograms of feed consumed per bird (FI), cumulative kilograms of feed consumed per bird (C-FI), (WG), phase-feed gain ratio (P-F:G), feed weight ratio (F:W), productivity index (PI), Mortality (Mort)

A clear difference in body weight was observed in the Hy-line chickens after 20 days consuming their respective concentrations of cholecalciferol (Figure 25). Poor growth and feathering was observed in those birds consuming low concentrations of cholecalciferol (0-242.2 IU D_3/kg) with respect higher doses (1,043-2,009 IU D_3/kg).

Figure 25. Selected experimental subjects at 20 days of age.
From left to right: 0 IU/kg, 300IU/kg and 2,009 IU/kg cholecalciferol.

Average final body weight per pen (6 birds) of Hy-line chickens consuming increasing doses of cholecalciferol was used in the broken-line regression analysis (Figure 26). The breaking point in the ascending line of the graph corresponds to the estimated vitamin D_3 requirement for growing chickens when performance is used to test the maximum response to different dietary levels of cholecalciferol.

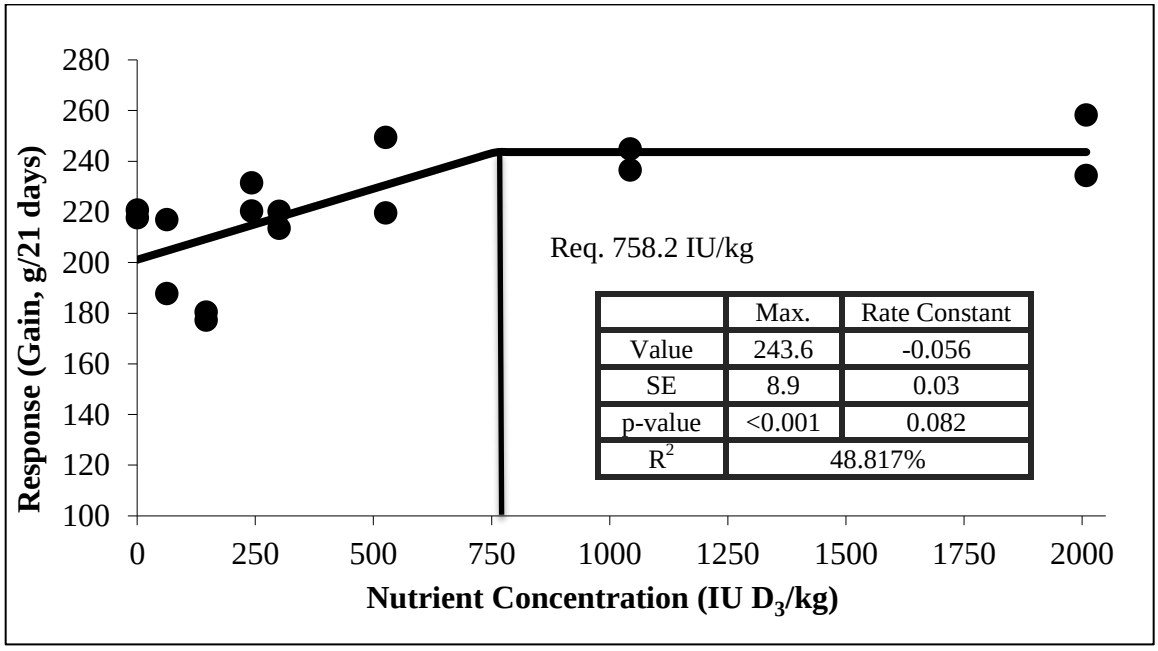


Figure 26. Average final body weight (g) per pen (6 birds) broken-line response to increasing concentrations of cholecalciferol (0, 62.3, 145.6, 242.2, 300, 525, 1,043 and 2,009 IU D_3/kg). [y=Max +Rate constant(Time to maximize-x)].*

5.3.2 Bone ash

The results of the percentage bone ash and total ash weight are presented in Figures 27 and 28. Again, the breaking point of the ascending line of the graph corresponds to the calculated vitamin D_3 requirement of growing chickens.

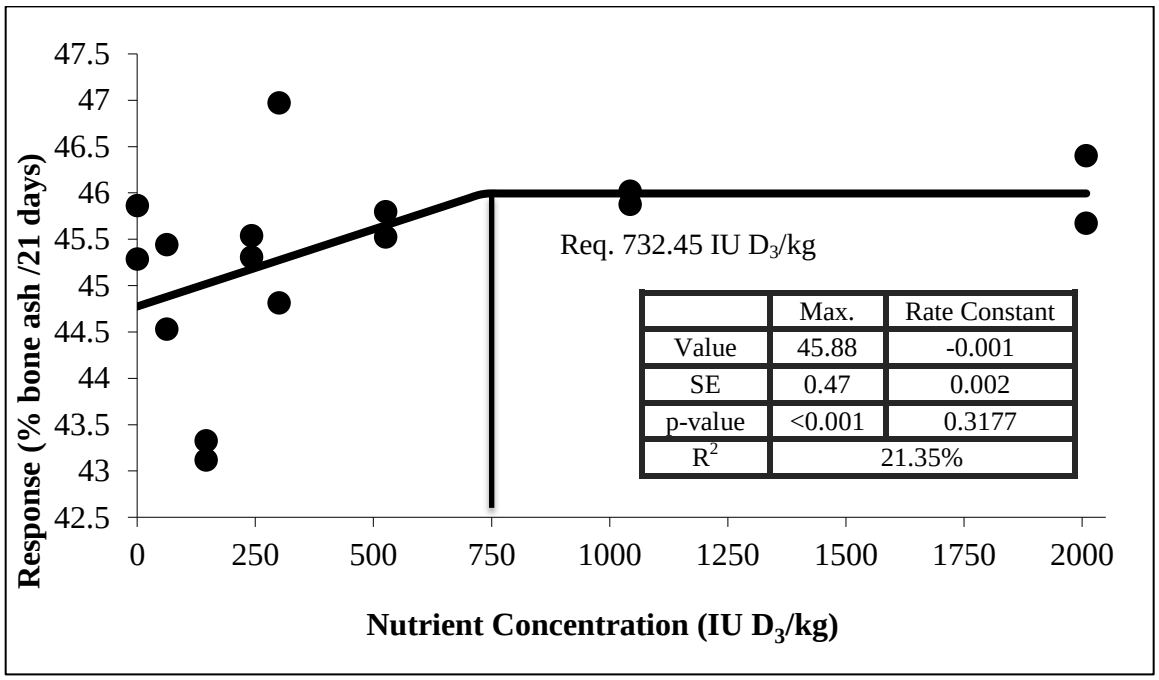


Figure 27. Percentage bone ash broken-line response to increasing concentrations of cholecalciferol (0, 62.3, 145.6, 242.2, 300, 525, 1,043 and 2,009 IU D_3/kg).
[y=Max +Rate constant(Time to maximize-x)].*

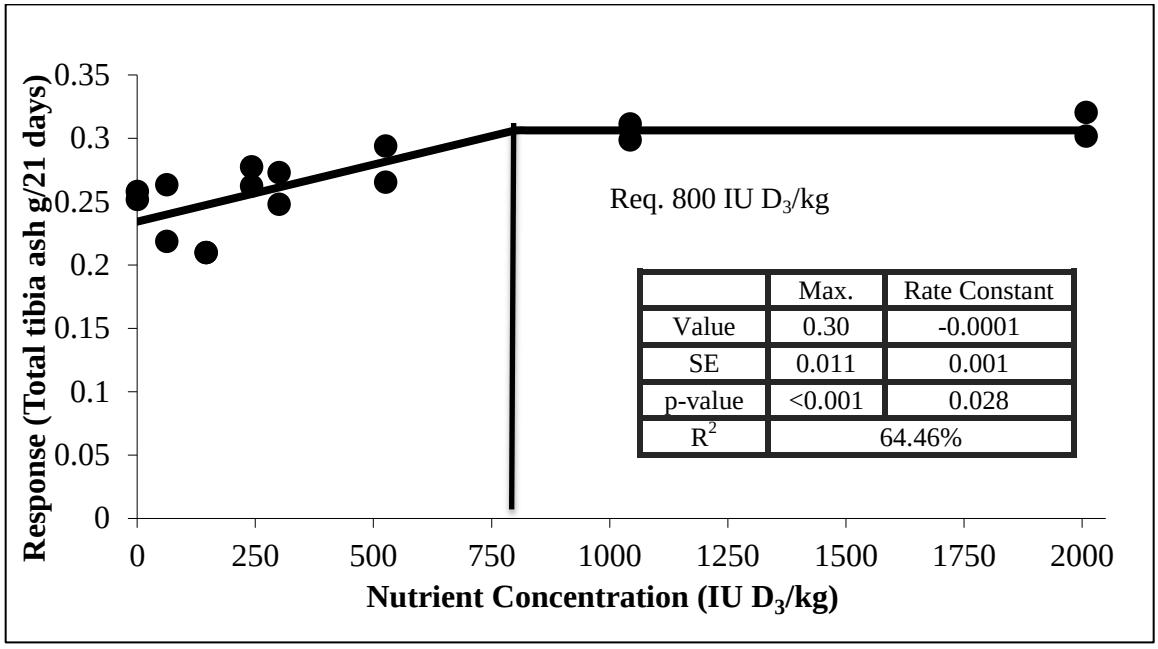


Figure 28. Total tibia ash broken-line response to increasing concentrations of cholecalciferol (0, 62.3, 145.6, 242.2, 300, 525, 1,043 and 2,009 IU D_3/kg).
[y=Max +Rate constant(Time to maximize-x)].*

5.3.3 Total body bone mineral content (BMC) DEXA scan

The total body mineral content (g) of the scanned chickens is presented in Figure 29. Chickens were scanned individually and the average BMC was calculated for each pen. The breaking point of the ascending line indicates the estimated vitamin D_3 requirement for growing Hy-line chickens.

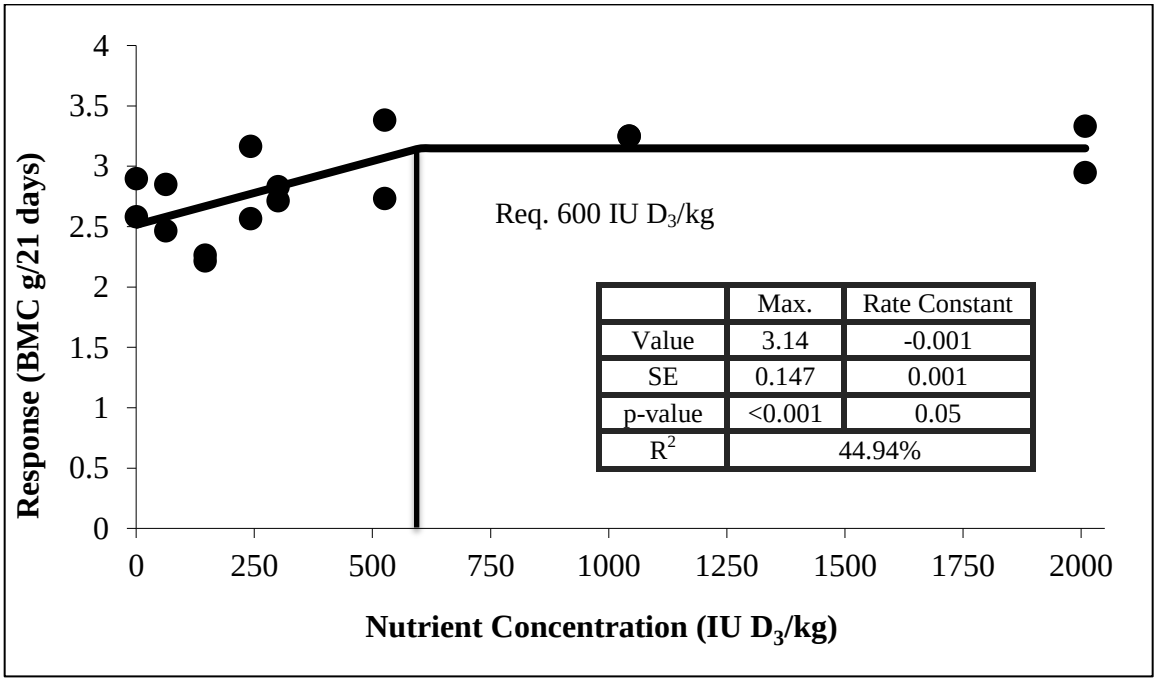


Figure 29. Total body bone mineral content (g) broken-line response to increasing concentrations of cholecalciferol (0, 62.3, 145.6, 242.2, 300, 525, 1,043 and 2,009 IU D_3/kg). [y=Max +Rate constant(Time to maximize-x)].*

Bivariate correlations between DEXA (BMC) versus final body weight (BW), percentage bone ash (PBA) and total tibia ash (TTA) were calculated to study the statistical relationships between criteria used to evaluate the vitamin D_3 response. Calculated coefficients of correlations were 93% (BMC vs BW), 71% (BMC vs PBA), 92% (BMC vs TTA) (Table 27).

Table 27. Bivariate correlations between the DEXA versus final body weight, total tibia ash and percentage bone ash.

Variables	Coefficient of Correlation	p value
➢ DEXA vs final body weight response		
BMC- BW	93%	<0.001
➢ DEXA vs percentage bone ash		
BMC-PBA	71%	0.002
➢ DEXA vs total ash content		
BMC-TTA	92%	<0.001

n= 16 per assay. Bone mineral content (BMC), body weight (BW), percentage bone ash (PBA), total tibia ash (TTA)

5.4 DISCUSSION

The results of the present study indicate that percentage bone ash was not very useful to precisely determine the vitamin D_3 requirement for growing leghorn chickens. The coefficients of determination for percentage bone ash and final body weight were 21.35 % and 48.8 % respectively. Contrary to our results, it has been stablished that the quantitative requirement of growing chickens for vitamin D is usually based on measurements of bone quality, such as bone ash content or incidence of rickets, which have been found to be more sensitive indicators than growth rate (Waldroup *et al*., 1963 and Edwards *et al*., 1994). However, in agreement with the previous authors, when the percentage bone ash data from my study was expressed as total tibia ash, the coefficient

of determination improved to 64.46% providing the best model to predict the vitamin D_3 requirement.

In this study, the data obtained from the four criteria (final body weight, percentage bone ash, total tibia ash and BMC) used to evaluate the chickens response to increasing doses of cholecalciferol was subjected to broken-line regression analysis and the breaking points were estimated to be 758.2 IU/kg for 21-day body weight, 732.45 IU/kg for percentage bone ash, 800 IU/kg for total tibia ash weight and 600 IU/kg for BMC. This results support the hypothesis that the NRC (1994) vitamin D_3 requirement of 200 IU/kg might be inadequate for modern poultry industry leghorn strains and higher levels may be needed to ensure maximum performance. In agreement with this, Heiman and Tighe (1942) reported that the vitamin D_3 requirement for white-leghorn chickens was 500 IU/kg. Also, in more recent studies several authors suggest that higher amounts of vitamin D_3 1000-3500 IU/kg are needed to support the growth rate of modern poultry strains (Kasim and Edwards, 2000, Ledwaba and Roberson, 2003, Whitehead et al. 2004, Khan *et al*., 2010). Contrary to our results, Baird *et al*. (1935) estimated the vitamin D_3 requirement for brown egg layers to be 180 IU/kg. McNaughton *et al*. (1977) found that 198 IU/kg of cholecalciferol were enough for 3-week-old growing chickens when adequate (1%) Ca is contained in the diet. Baker *et al.* (1998) reported, in agreement with the NRC (1994) that 200 IU/kg D_3 appeared to be adequate for maximal weight gain and bone ash in broiler chickens from 8-20 days post hatching when fed adequate levels of available P. Additionally, recent research in our laboratory estimated the vitamin D_3

requirement to be 54.8 IU/kg to support growth and bone mineralization in young growing chickens in agreement with the NRC (1994) (Fowler *et al*., 2014).

High positive correlations were obtained for all parameters when compared with the bone mineral content (BMC) reported by the DEXA scan. Coefficients of correlation were 93%, 71% and 92% (r) for BMC versus body weight, percentage bone ash and total tibia ash, respectively. In agreement with these results, Swennen *et al*. (2004) compared the accuracy of the DEXA scan in Cobb broilers, using different measurements of body composition and bone, with the chemical carcass analysis, and found a strong correlation between BMC and total ash weight of 95% (r). Schreiweis *et al*. (2005) evaluated bone integrity in Leghorn hens using the DEXA scan, and found a coefficient of correlation of 85% (r) between BMC and excised bones. Contrary to our results, where percentage bone ash registered the lowest coefficient of correlation, Onyango *et al*. (2003) found a correlation between BMC and percentage bone ash of 92% (r) in 3-week-old Ross-308 broiler chickens.

5.5 CONCLUSION

The results of this study indicate that the effect of intensive genetic selection in the modern poultry industry have altered birds nutritional requirement for vitamin D_3.

My results suggest that higher amounts of vitamin D_3 are required to promote growth and bone mineralization in leghorn-type chickens.

Feeding 4 times the recommended NRC (1994) vitamin D_3 requirement seems to be needed to ensure maximum performance and bone quality.

My results indicate that percentage bone ash was not the best parameter to predict the vitamin D_3 requirement in leghorn chickens.

The Dual X-Ray Absorptiometry proved to be a useful tool for the evaluation of bone quality. However, further research has to be done in order to standardize protocols that will allow the accurate prediction of nutritional requirements.

6. SUMMARY

Modern industry poultry strains are selected for rapid growth, high production and maximum feed efficiency. It is probable that the intensive genetic selection over the past 20 years have altered the nutritional requirements of modern poultry strains.(Havenstein *et al*., 2003, Schmidt *et al*., 2009) Therefore, it is necessary to re-evaluate their ability to respond to modern diets and feeding strategies.

One consequence of the improvement in growth rate and feed efficiency is the persistent incidence of bone abnormalities, which are an important component of the birds welfare, and therefore, in general performance. Several leg disorders such as tibial dyschondroplasia (TD), rickets and angular bone deformities represent a major economic issue for the poultry industry. Sullivan (1994) estimated annual losses in the United States due to skeletal problems in broiler production around $80 to $120 million USD which adjusted to the annual inflation (U.S. Bureau of Labor Statistics) would represent $ 128,000,000 to 192,000,000 USD in 2014.

Vitamin D is a term associated with a group of related steroid chemical compounds that possess antirachitic activity. The main role of vitamin D is the control of bone development by strictly regulating mineral absorption (Aslam *et al*., 1998). There are two common forms, ergocalciferol (D_2) and cholecalciferol (D_3). Since D_2 has about 10% the bioactivity of D_3 for poultry (McDonald *et al.* 1995) D_3 is used to supplement poultry feeds. The quantitative requirement of growing chickens for vitamin D_3 is usually based on functional assessments using measurements of bone quality, such as bone ash content

or incidence of rickets, which have been found to be more sensitive indicators of requirements than growth rate (Waldroup *et al*., 1963, Edwards *et al*., 1994). The vitamin D_3 requirement reported by the NRC (1994) is 200 IU/kg of feed for both growing broilers and leghorn strains. There is a big debate regarding the vitamin D_3 requirement. Some authors, in agreement with the NRC (1994) support the 200 IU/kg of feed to be valid (Baker *et al*., 1998, Fowler *et al*., 2014). Other authors suggest that higher amounts of vitamin D_3 (1,000-3,500 IU/kg) are needed to support the growth rate of modern strains (Kasim and Edwards, 2000, Ledwaba and Roberson, 2003, Whitehead *et al*., 2004, Khan *et al*., 2010). Recent literature suggesting higher requirements for vitamin D_3 presume this to be due to changes in metabolism of the rapid growth modern poultry strains.

Three experiments were conducted to better understand the effect of the intensive genetic selection in the nutritional requirements of modern poultry strains. In experiment one, broiler chickens where obtained from two commercial hatcheries**,** Sanderson Farms (*Bryan, TX*) or Cobb hatchery (*Timpson, TX*), and caged in a Petersime battery brooder. Broiler chickens were fed *ad libitum* a basal vitamin D_3-deficient broiler starter diet with the objective of evaluating the variability in maternal deposition of vitamin D_3 in the egg yolk. No significant ($p>0.05$) differences were found in the initial serum concentration of 25-hydroxycholecalciferol (25-OH-D_3) within sources of broiler chickens. Initial serum 25-OH-D_3 concentration was around 30 ng/ml which decreased linearly to 5 ng/ml over a 10 day period. Maternal vitamin D reserves will deplete around day 10 regardless of the initial concentration in newly hatched chickens. In the second experiment, newly-

hatched broiler chickens were placed in a Petersime battery brooder, and blood samples were taken at different time points during a 24 h period to evaluate the dynamics of serum 25-OH-D_3. A significant ($p<0.05$) linear increase in serum 25-OH-D3 was found over the 24 h exposure to the treatment diets. Finally, in experiment three, a total of 96 Hy-line chickens were placed in a very controlled environment and a concentrated liquid form of cholecalciferol was directly gavaged in precise increasing amounts to re-evaluate the requirement of vitamin D_3 of growing leghorn chickens. The estimated requirement was 800 IU D_3/kg of feed for maximum growth and bone mineralization.

These experiments demonstrate the importance of having a strict control on the breeder´s diet to produce a vitamin D_3-deficient chick. Additionally, the depletion rate of vitamin D_3 is similar in broiler chickens obtained from different commercial hatcheries. This study also corroborates that the concentration of serum 25-OH-D_3 is highly correlated with the antirachitic dietary source and level, supporting the hypothesis (Pettifor *et al*., 1977) that 25-OH-D_3 is in fact a good marker to assess the status of vitamin D in poultry species. It also suggests the NRC (1994) estimated vitamin D_3 requirement of growing leghorn chickens is inadequate to support growth and bone mineralization of modern leghorn strains.

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