Effects of dietary 25-hydroxycholecalciferol on growth, production performance, eggshell quality and bone traits of brown egg layers housed under commercial conditions

by

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ABSTRACT

The effects of dietary 25-hydroxycholecalciferol (25OHD) on growth, production performance, eggshell quality and bone characteristics of laying hens were evaluated in a field trial in Colombia. At day 1 of age, a total of 17,750 Hy-Line Brown pullets were placed on a commercial farm, in 3 houses and randomly distributed in 6 treatments with 4 replicates each; at 13 wk of age, 10,800 pullets were transferred to the layer farm, maintaining the same treatments, but divided into 9 replicates per treatment. Treatments were: a Positive Control (PC) with 25OHD plus vitamin D_3 at 2,760 and 3,000 IU/kg, respectively fed throughout the trial, a Negative Control (NC) with vitamin D_3 only (3,000 IU/kg) fed during the entire trial, and treatments with the PC diet fed from day 1 to either 15 (Early), 17 (Prelay), 34 (Peak), or 50 (Late) wk of age, and then switched to the NC diet until 90 wk of age. Feed intake (FI), mortality and egg production were recorded daily and analyzed along with BW and FCR at each diet change. To evaluate eggshell quality and bone traits, 45 eggs per treatment were randomly sampled every two to three weeks to measure eggshell strength (ESS) and thickness, whereas 1 to 2 birds per pen (n=54) was euthanized at 15, 34 and 90 wk of age, to assess bone mineral density by quantitative computed tomography, and breaking strength. Data were analyzed by ANOVA or analysis of covariance, and orthogonal contrasts were performed to compare treatments fed 25OHD versus the NC.

Dietary 25OHD decreased FCR at 3 wk, increased BW up to 8 wk, and FI up to 12 wk of age. At 26 and 84 wk of age, infectious coryza was observed, decreasing egg production and livability in all treatments. To 34 wk, NC hens had the lowest egg production, and highest FCR, PC and Peak hens had the highest egg production and lowest FCR; there were no differences within a dietary phase after this point. By 87 wk of age, Peak treatment resulted in the highest

cumulative egg production and lowest FCR. Overall, the PC treatment resulted in higher ESS than the Early treatment, while PC and Early groups had greater shell thickness compared to NC. At 34 wk of age, shank cortical density at 30% of the total length from the proximal epiphysis of NC hens was nearly significantly the lowest among treatments (P= 0.076), whereas at 90 wk of age, the NC hens had lower BBS than Early hens. Additionally, orthogonal contrast revealed a significant enhancement of cumulative egg production by the end of production, and greater shell quality from 53 to 87 wk of age when 25OHD was added throughout the cycle, compared to the NC group. Dietary 25OHD up to 34 wk of age had a positive impact on early development and egg production from the onset of lay to peak production, whereas 25OHD supplementation throughout the cycle promoted greater eggshell quality, especially at older ages without compromising bone integrity over time.

… "God delights in concealing things;
Scientists delight in discovering things."
Proverbs 25:2 (MSG)

DEDICATION

To my Lord, and my God. Every time I felt incapable, your written words, love, and faithfulness encouraged me.

To my dear wife, Sarita. I have no words to express how complete I feel beside you. Thanks for your patience, for your encouragement, for believing in me… in us.

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LIST OF ABBREVIATIONS AND SYMBOLS

µ - micro

µg – microgram

1,25OHD – 1,25 dihydroxycholecalciferol

24,25OHD – 24,25 hydroxycholecalciferol

25OHD – 25 hydroxycholecalciferol

7DHC – 7-dihydrocholecalciferol

BBS – Bone breaking strength (shank)

BW – Body weight

Ca – Calcium

DBP – vitamin D binding protein

Early – Treatment providing PC diet from day 1 to wk 15, and NC diet from 16 to 90 wk of age

ESS – Eggshell strength

FCR – Feed conversion ratio

FI – Feed intake

FSH – Follicle-stimulating hormone

g – gram

IgG – Immunoglobulin G

IU – International units

kDa – kilodalton

kg – kilogram

KgF – Kilograms of force

kN – kilonewton

kV - kilovoltage

L:D – Hours light:darkness hours in the photoperiod

Late – Treatment providing PC diet from day 1 to 50 wk, and NC diet from 51 to 90 wk of age

LED – Light-emitting diode

LPS - Lipopolysaccharide

m – meter

m^2 – Square meter

mg – milligram

Mid – middle of the shank bone (center of diaphysis)

mm - millimeter

mRNA – Messenger RNA

mTOR – Mechanistic target of rapamycin

n – Number of observations

NC – Negative Control; Treatment providing 3,000 IU/kg of vitamin D_3 from day 1 to 90 wk of age

NRC – National Research Council

ºC – Centigrade

PC – Positive Control; Treatment providing 3,000 IU/kg of vitamin D_3 plus 2,760 IU/kg of 25OHD from day 1 to 90 wk of age

Peak – Treatment providing PC diet from day 1 to 34 wk, and NC diet from 35 to 90 wk of age

Prelay – Treatment providing PC diet from day 1 to 17 wk, and NC diet from 18 to 90 wk of age

PTH – Parathyroid hormone

QCT – Quantitative computed tomography

VDR – Vitamin D_3 receptors

wk – week

1. LITERATURE REVIEW

1.1 INTRODUCTION

Between 2000 and 2015, egg production grew by 38.7% worldwide, with a population of around 7.3 billion hens and a production estimate of 1,340 billion eggs in 2015 (Conway, 2016). The intensification of the poultry industry through the years has led to a more efficient production of eggs, largely through genetic selection, management, and nutrition. However, these significant changes have also generated adverse effects on eggshell quality (al-Batshan et al., 1994), bone strength (Rath, 2000), and animal welfare (Yilmaz Dikmen, 2016). The poultry industry has responded to these effects in part through nutritional strategies, including the proper supplementation of vitamins, minerals, and other feed additives.

Studies regarding the importance of vitamin D_3 in calcium and phosphorus metabolism in birds are well documented (Abdulrahim et al., 1979; Norman and Hurwitz 1993; De Matos, 2008; Khan and Mukhtar, 2013). Although vitamin D_3 is naturally produced through the production of 7-dehydrocholesterol from cholesterol in the skin after UV light exposure (Khun et al., 2014), most commercial poultry have no direct exposure to sunlight. Therefore dietary vitamin D_3 and its metabolites are commonly supplied in poultry rations.

Compared to vitamin D_3, dietary supplementation of the vitamin D_3 metabolite, 25-hydroxycholecalciferol (25OHD), has resulted in greater performance and breast meat yield in broilers (Brito et al., 2010; Vignale et al. 2015), hatchability in breeders (Coto et al., 2010); eggshell quality (Al-Zahrani and Roberts, 2014) and production in laying hens (Ding et al., 2011; Rivera et al., 2014). Positive effects on bone traits have been reported both in broilers (Fritts and Waldroup, 2003) and laying hens (Rivera et al., 2014). Also, 25OHD supplementation promotes intestinal maturation and function (Chou et al., 2009; Ding et al., 2011), and modulates the

immune system (Saunders-Blades and Korver, 2015, Morris et al., 2015). Therefore, the aim of the current review is to explore the mechanisms and effects of vitamin D_3 sources, particularly 25OHD in poultry, on egg-laying chicken development, production performance, eggshell quality and bone traits.

1.2 VITAMIN D

Vitamin D is a secosteroid (a type of steroid with a broken ring) which shares a similar structure with steroid hormones, including estradiol and cortisol (Norman, 2008; Figure 1.1). The main effect of vitamin D_3 is to maintain plasma calcium and phosphorus homeostasis and therefore it is required for bone development and maintenance. (Soares et al., 1995; Khan et al., 2010). Additionally, other non-skeletal functions have been attributed to vitamin D_3 and its metabolites in domestic birds, including broiler performance (Fritts and Waldroup, 2003), eggshell formation (Keshavarz et al. 2003; Khan and Mukhtar, 2013), reproduction (Coto et al., 2010; Saunders-Blades and Korver, 2015), and immune response (Chou et al., 2009; Gomez-Verduzco et al., 2013). Therefore, vitamin D_3 is not only defined as a fat-soluble vitamin (Abawi and Sullivan, 1989), but also as a prohormone (Norman, 2008; DeLuca, 2008; Huff et al., 2000) and more recently, as an immunomodulatory nutrient (Correale et al., 2009; Shanmugasundaram and Selvaraj, 2012).

1.2.1 Vitamin D metabolism

Vitamin D_3 can be synthesized in the skin from 7-dihydrocholecalciferol (7DHC), a precursor of cholesterol, after ultraviolet radiation exposure (Edwards, 2003). Although birds do not produce vitamin D_3 in feather-covered skin, they produce high amounts of 7DHC in the legs and feet (or nonfeathered skin areas) to synthesize vitamin D_3, which is translocated into

circulation (Tian et al., 1994) bound to vitamin D binding protein (DBP), and carried to the liver (Norman, 1987; Elaroussi et al., 1994; Figure 1.1).

Hens managed in free-range systems and exposed to sunlight for up to 9 hours per day (plus 2,200 IU/kg of dietary vitamin D_3) had enhanced endogenous synthesis of vitamin D_3, increasing vitamin D_3 and 25OHD levels in the egg (by up to 400% and 45% more, respectively) compared to birds managed indoors and supplemented with same levels of vitamin D_3 (Kühn et al., 2014). Similarly, sunlight exposure was considered as a cause of the lack of differences in egg production and shell quality in layer breeders supplemented with 0 to 2,400 IU/kg of vitamin D_3, placed in an open sided house (Panda et al., 2006). The authors suggested that birds could synthesize sufficient vitamin D_3 through sunlight exposure. However, vitamin D_3 synthesis through sunlight in all vertebrates depends on weather, season and latitude (Sherman et al., 1990; Holick et al., 2007). Moreover, most commercial poultry facilities allow limited or no exposure to direct sunlight; thus, endogenous production of vitamin D_3 is low, and its dietary supplementation is required (Holick, 1995; Soares et al., 1995).

The plant form of vitamin D (ergosterol or vitamin D_2), lacks anti-rachitic effects, has low affinity for DBP and poor activity in birds (Norman, 1987; Soares et al., 1995; Bar, 2008); therefore, dietary supplementation of vitamin D in poultry diets is provided using synthetic vitamin D_3 or commercially available vitamin D_3 metabolites, such as 25OHD, 1,25-dyhydroxycholecalciferol (1,25OHD), and 1α-hydroxycholecalciferol (alpha-calcidiol; Han et al., 2012; Pesti and Shivaprasad, 2010). After consumption, vitamin D_3 is absorbed via diffusion by the enterocytes, mainly in the duodenum and upper jejunum (Borel, 2003), in the form of aggregates called micelles, along with other lipophilic food components, and transported to the liver as portomicrons (Elaroussi et al. 1994; Cooke and Haddad, 1989).

The metabolic pathway of vitamin D_3 is illustrated in Figure 1.1. To become the active form, vitamin D_3 must undergo two sequential hydroxylation steps. The first hydroxylation takes place at the 25-C position in the liver microsomes and mitochondria by 25-hydroxylase, to produce 25-hydroxycholecalciferol (25OHD). Conversion of vitamin D_3 to 25OHD also occurs in the intestine, kidneys (Norman, 1987), and the skin (Hansdottir et al., 2008) but at a lower rate. Then, 25OHD is transported via DBP to the kidneys, where it is transformed into 1,25-dyhydroxycholecalciferol (1,25OHD) by 1α-hydroxylase (Shanmugasundaram and Selvaraj, 2012). In addition, extrarenal expression of the 1α-hydroxylase has been reported in various tissues including white blood cells, the digestive tract, skeletal muscle fibers, pancreatic cells, parathyroid glands, and skin in mammals and birds (Zhang et al., 2002; Correale et al., 2009; Morris et al., 2014a; Shanmugasundaram and Selvaraj, 2012).

Although the hydroxylation of vitamin D_3 to 25OHD is not highly regulated, the action of 1α-hydroxylase and subsequent formation of 1,25OHD is tightly regulated by parathyroid hormone (PTH), plasma levels of calcium, phosphorus, estrogens, prolactin, 24,25 hydroxycholecalciferol (24,25OHD) and 1,25OHD itself, mainly to maintain calcium homeostasis, to avoid hyper or hypocalcemia (De Matos, 2008; DeLuca, 2008). In hypocalcemia, PTH is secreted to promote 1α-hydroxylase activation in the kidney and target tissues (Soares, 1984). 1,25OHD controls passive and active mechanisms of calcium absorption from the intestinal tract by increasing the synthesis of calcium-binding proteins (calbindins), mainly calbindin-D_{28k} in birds. The function of calbindin is primarily to transport calcium into epithelial cells of the intestine, kidney, eggshell gland and osteoblast (Wasserman and Taylor, 1966; Christakos et al., 2003). Other proteins involved in vitamin D_3 function include vitamin D receptor (VDR), a nuclear receptor that binds to 1,25OHD to promote the expression of specific

target genes (Christakos et al., 2003). Besides the kidney and intestine (Norman, 2006), VDR have been identified in bone (Dusso et al., 2005), eggshell gland (Takahashi et al., 1980), rooster epididymis (Dornas et al., 2006), and breast muscle (Vignale et al., 2015) among other tissues.

At normal or high plasma calcium concentrations, 24-hydroxylase activity is upregulated to convert 25OHD into 24,25OHD, and prevents the formation of new 1,25OHD, as 24,25OHD is the first stage of vitamin D_3 inactivation and elimination (Johnston and Ivey, 2002; Bar, 2008). Seo et al., (1997) suggested that circulating 24,25OHD could be an essential metabolite for proper bone mineralization in chickens (Seo et al., 1997); another study concluded that high levels of plasma 1,25OHD (in the absence of 24-hydroxylase), is a cause of impaired bone mineralization (St-Arnaud et al., 2000). 24,25OHD is also involved in normal hatchability (Henry and Norman, 1978), and fracture repair process (Seo et al., 1997; Seo and Norman, 1997)

In laying hens and broiler breeders, the increase in calcium demand following the onset of egg production is considered the most important factor affecting vitamin D_3 metabolism and subsequent 1,25OHD synthesis (Nys et al., 1992). On a daily basis, birds rely on the availability and utilization of dietary calcium and bone mineral, especially the medullary bone, to form the eggshell and maintain calcium plasma levels (Kerschnitzki et al., 2014). Thus, with every egg laid, there is an up-regulation in vitamin D_3 metabolism to counteract a temporary and physiological calcium deficiency (Bar, 2008). Among other factors that stimulate the synthesis of 1,25OHD in laying hens are the rise of plasma androgens and estrogens that comes with sexual maturity (Bar and Norman, 1981), the decrease in plasma calcium during eggshell calcification, and the consequent increase of plasma PTH (Sugiyama and Kusuhara, 2001; De Matos, 2008).

1.2.2 Dietary vitamin D and its metabolites in poultry

The poultry industry normally supplements vitamin D_3 at higher dietary concentrations than the current NRC (1994) requirements. It was concluded that NRC guidelines for poultry require a new edition that incorporates the changes in animal nutrition during the last 20 years (Applegate and Angel, 2014). Hence, the minimum requirement of vitamin D_3 in laying hens vary from 250 to 375 IU/kg (NRC, 1994), to 3,300 IU/kg as established by the Hy-Line Brown management guide (Hy-Line, 2014) or 3,000 to 4,000 IU/kg of vitamin D_3 plus 2,760 IU/kg of 25OHD (total of 5,760 to 6,760 IU/kg of vitamin D activity) during both the rearing and lay periods as recommended by the manufacturer of 25OHD (DSM Nutritional Products, 2016).

Depending on the response criteria, recommended dietary values for vitamin D activity well in excess of the NRC-recommended level for poultry have been suggested. For instance, at commercially-relevant levels of vitamin D_3 (4,000 to 6,000 IU/kg, with 3.2 to 4% of dietary calcium), eggshell quality increased compared to lower levels of vitamin D_3 (minimum of 2,000 IU/kg) in brown and white hens (El-Maksoud, 2010; Safamehr et al., 2013; Plaimast et al., 2015). In broiler breeders, 1,400 to 2,800 IU/kg are recommended levels to obtain proper egg production, hatchability and embryo viability (Atencio et al., 2006), while levels up to 4,000 IU/kg were suggested to increase BW gain in the offspring (Atencio et al., 2005a). Similarly, vitamin D_3.enriched eggs (with up to 34,800 IU/100 g of yolk) can be obtained by adding up to 102,200 IU of dietary vitamin D_3/ kg of feed in laying hens, without any adverse effect on egg quality (Yao et al., 2013) health or performance (Mattila et al., 2003; Persia et al., 2013). Moreover, there were no differences in egg production using different hen dietary levels of vitamin D_3 reported in several other studies (El-Maksoud, 2010; Safamehr et al., 2013; Yao et al., 2013), with low levels of 250 IU/kg (Keshavarz, 1996). Conversely, low levels of up to 500

IU/kg showed a negative effect on broiler breeder egg production compared to 1,000 IU/kg or higher levels of vitamin D_3 (Atencio et al., 2006).

In broilers, between 3,000 to 4,000 IU/kg of dietary vitamin D_3 enhanced mineral retention and bone mineralization, increased BW, and reduced tibial dyschondroplasia, compared to levels below 1,000 IU/kg of vitamin D_3 (Fritts and Waldroup, 2003; Atencio et al., 2005a; Rama-Rao et al., 2006; Khan et al., 2010), as well as decreasing the incidence of footpad lesions and hock dermatitis (Sun et al., 2013). In addition, up to 10,000 IU of vitamin D_3 per kg of feed was recommended to prevent tibial dyschondroplasia (Whitehead et al., 2004).

Several factors affect vitamin D_3 absorption and metabolism. Chicks showed a decrease in plasma 25OHD from one to 10 days of age (Saunders-Blades and Korver, 2014), which suggests a limited ability to hydroxylate vitamin D_3 in the liver. Furthermore, intestinal absorption of vitamin D_3 is limited because the gastrointestinal tract and its accessory organs are not fully developed after hatch (Noy and Sklan, 1995). Additionally, any challenge that reduces liver function might also reduce hydroxylation to 25OHD, including liver disease or malfunction (Yarger et al., 1995b; Edwards, 2000; Stokes et al., 2013), stress (Thaxton and Puvadolpirod, 2000; Wideman et al., 2015; Odihambo Mumma et al., 2006), bacterial infections (Peighambari et al., 2000), aging (McLoughlin and Soares, 1976; Soares, 1984), and mycotoxins (Yarru et al., 2009). For instance, a recent study showed a decrease in skeletal health, with a major development of osteoporosis, in birds with fatty liver (a common metabolic disorder in laying hens and breeders; Jiang et al., 2013). The authors suggested this was due to a decrease in enzyme activity, as fat is deposited in the damaged liver cells. An increase in serum osteocalcin, a marker of bone turnover, and dependent on vitamin D_3 (Bar, 2008; Sukumar et al., 2015) was also reported in hens with fatty liver. Consequently, when the natural production of 25OHD is

impaired, dietary 25OHD can enhance the vitamin D status of the bird. Commercially available 25OHD is marketed as HyD® (DSM Nutritional Products, 2016; Michalczuk et al., 2010; Käppeli et al., 2011a). The recommended dietary dose of 25OHD is 69 µg/kg (DSM Nutritional Products, 2016), which is equivalent to 2,760 IU/kg of vitamin D activity per kg of feed, based on the conversion of 0.025 µg/kg of cholecalciferol to 1 IU (NRC, 1994; Fritts and Waldroup, 2003), A list of studies that include various levels of vitamin D_3 and 25OHD are summarized in Table 1.1

Various positive characteristics have been attributed to 25OHD when comparing to vitamin D_3 and other metabolites. Dietary 25OHD is less dependent on bile salts for its absorption (Compston et al., 1981; Borel, 2003), and therefore has a higher absorption rate than vitamin D_3 (Bar et al., 1980). Secondly, 25OHD has lower daily excretion rate than other vitamin D_3 metabolites (Chou et al., 2009), higher retention (Bar et al., 1980), greater biopotency at low levels of supplementation (below 500 IU/kg; Fritts and Waldroup, 2003; Atencio et al., 2005b), and higher affinity to DBP than vitamin D_3 or 1,25OHD (Soares et al., 1995). Third, in vitro studies revealed a direct and rapid (within 1 minute) effect on calcium absorption by enterocytes (Phadnis and Nemere, 2003), and an increment of VDR expression, followed by the activation of mTOR (mechanistic target of rapamycin) which up-regulates cell growth, proliferation, and synthesis of protein when myoblast cells were treated with 25OHD (Vignale et al., 2015). Finally, 25OHD is the most abundant form of vitamin D_3 in blood circulation (Norman, 2008), making it the most appropriate indicator of vitamin D status in poultry (Bar et al., 2003; Bar, 2008; Soares, 1984). An increment of either dietary vitamin D_3 or 25OHD elevates serum levels of 25OHD; however, serum 25OHD levels increased more rapidly when 25OHD is added to the ration instead of comparable levels of vitamin D_3 (Yarger et al., 1995b). Additionally, dietary

25OHD significantly increases circulating 25OHD concentrations (by up to 126%), compared to similar levels of dietary vitamin D_3, both in broilers (Vignale et al., 2015) and laying hens (Käpelli et al., 2011a).

Lower weight of small intestine, longer villus length in the duodenum, shorter crypt depth and greater ratio villus length/crypt depth were reported when 2,760 IU/kg of 25OHD was added on top of 3,000 IU/kg in broiler diets up to day 35 of age (Chou et al. 2009). Similar effects were found in 7-day old chicks, from hens fed 25OHD at 4,000 IU/kg relative to 1,000 IU/kg of 25OHD (Ding et al., 2011). In both studies, the authors concluded that these changes might lead to a lower energy demand by the intestine because of reduced maintenance requirements and reduced enterocyte turnover, along with an enhanced rate of nutrient absorption because of the increased villus length. This could allow the bird to use the increased amount of available nutrients for development and other physiological functions (Moghaddam et al., 2012).

Other vitamin D_3 metabolites such as the synthetic analog 1α-hydroxycholecalciferol (alpha-calcidiol), or 1,25OHD from plants as *Solanum glaucophyllum* and *Cestrum diurnum* (both from the *Solanaceae* family) have been studied as vitamin D sources for poultry. Alpha-calcidiol, for instance, does not require the kidney 1-hydroxylation and is transformed in the liver directly into 1,25OHD; thus, it was found to effectively provide vitamin D activity at 5 or 10 μg/kg in calcium- and phosphorus-deficient diets in the absence of vitamin D_3 (Han et al., 2012; Pesti and Shivaprasad, 2010). Nevertheless, since its activation is not dependent on calcium plasma levels, this product is more toxic than vitamin D_3 or 25OHD, showing signs of toxicity (kidney mineralization), at 15 μg/kg (600 IU/kg), three times its recommended dose (Soares et al., 1983; Pesti and Shivaprasad, 2010). Dietary 25OHD did not show any adverse effect on

health or performance at up to 6 times the recommended dose of 69 µg/kg of feed in laying hens (Terry et al., 1999) and up to 10 times in broilers (Yarger et al., 1995b). The presence of large amounts 1,25OHD glycosidic conjugate in the *Solanum* extract increases plasma levels of this metabolite (Napoli et al., 1977). Circulating 1,25OHD has a half-life of 6 to 8 hours, which is shorter compared to 25OHD (Rovegno et al., 2012). Similarly, plasma calcium increased in vitamin D-deficient chicks fed *Cestrum* leaf powder (Wasserman et al., 1976). Although some studies have shown positive effects of dietary 1,25OHD in laying hens, including on egg production (Frost et al., 1990), specific gravity (Carlos and Edwards 1998) shell thickness and FCR (Chennaiah et al., 2004), exceeding the recommended amount (5µg/kg) by just 2 µg/kg is enough to reach a toxic level, increasing mortality (Tsang et al.,1990) and decreasing egg production (Chennaiah et al., 2004). Therefore, positive effects of dietary 1,25OHD could be limited, since its safety margin is narrow (Newman and Leeson, 1999).

1.2.3 Vitamin D metabolites in the immune response

Immunomodulation through nutrition has become an important matter of investigation in poultry. Vitamin D_3 and its metabolites are part of a group of nutrients capable of playing a major role in immune function, including both innate and humoral immunity (Korver, 2012). Broilers fed 2,760 IU/kg of 25OHD, compared to 3,000 IU/kg of vitamin D_3 had higher level of serum antibodies (IgG) when challenged with *Salmonella typhimurium,* with a 10% increase in phagocytosis activity (Chou et al., 2009); and greater growth performance and reduced inflammation after a lipopolysaccharide challenge (LPS; Morris et al., 2014a; Morris et al. 2015). Dietary 25OHD (2,760 IU/kg) resulted in a decrease in interleukin1β (a proinflammatory cytokine) mRNA in broilers following an LPS injection, compared to 3,000 IU/kg of vitamin D_3 (Morris et al., 2014). Similarly, in laying chicks supplemented with 4,000 IU/kg (100 µg/kg) of

25OHD, a lower expression of interleukin 1β, and a 3.5-fold increase of the anti-inflammatory cytokine IL-10 was reported after a coccidiosis vaccine challenge, relative to lower levels of 25OHD (≤ 50 μg/kg; Morris et al., 2015). Other findings include a greater *in vitro* killing activity of leukocytes against *Escherichia coli* in the offspring of hens fed 2,760 IU/kg of 25OHD, compared to similar levels of vitamin D_3 (Saunders-Blades and Korver, 2015), and a greater nitric oxide production in chicken macrophages (Morris and Selvaraj, 2014). Finally, Shojadoost et al. (2015) concluded that vitamin D_3 modulates immune function in chicken macrophages, promoting nitric oxide production and reducing inflammation, by treating macrophages with 1,25OHD and following stimulation with LPS. These findings suggest that dietary 25OHD is a nutritional alternative to reduce the negative effects on performance associated with bacterial infections and inflammation processes while enhancing protective immune responses during an infection.

1.2.4 Effect of 25OHD on hen performance

Unlike broilers, laying hens and breeders have a high demand for calcium to support egg formation, especially during peak production, when a greater proportion of the total eggs are laid (Saunders-Blades et al., 2009), and subsequently, when the ability of the hen to produce eggs with strong and thick shells gradually decreases (al-Batshan et al., 1994). There are several explanations for the loss in eggshell quality over time. A reduction in calcium absorption efficiency (al-Batshan et al., 1994), a progressive decrease in the ability to hydroxylate vitamin D_3 (McLoughlin and Soares, 1976), and a low activity of 1-α-hydroxylase in the kidneys, with 44% less plasma 1,25OHD in 38 wk-old hens compared to 72 wk-old hens (Abe et al., 1982) are some of the findings as the hen ages. Conversely, a low adaptive response to an imbalance of calcium intake and hypocalcemia in old hens (80 and 120 wk of age) reduces shell calcification

rather than a decrease in the ability to produce the active form of vitamin D_3 (Bar and Hurwitz, 1987; Elaroussi et al., 1994). Considering that young birds (22 wk of age) increase 1-α-hydroxylase in response to calcium depletion (Elaroussi et al., 1994), 25OHD supplementation could increase 1,25OHD production during crucial periods such as at the beginning and peak of production.

Initial studies included low dietary supplementation of 25OHD (below the current manufacturer-recommended dose) compared to vitamin D_3. For instance, no differences in egg quality and production were found from 22 to 33 wk of age, comparing 120 or 360 IU/kg (3 or 9 μg/kg) of 25OHD to similar levels of vitamin D_3 (Abdulrahim et al., 1979). Supplementation of 600 IU/kg of 25OHD in place of the same level of vitamin D_3 decreased the incidence of soft-shelled eggs from 62 to 74 wk of age, but not at younger ages (McLoughlin and Soares, 1976). Keshavarz (1996; 2003) showed no effects of dietary 25OHD at 12.5 and 69 μg/kg compared to equivalent levels of vitamin D_3 in performance, bone traits or eggshell quality, in three experiments performed in mid-aged hens (from 45 to 66 wk of age). Furthermore, a high dose of 1.1 mg/kg of feed 25OHD (equivalent to 1,100 μg/kg or 44,000 IU/kg) relative to 2,200 IU/kg of vitamin D_3 did not enhance eggshell quality in either young or old hens with a record of thin-shelled and shell-less eggs (at 45, 70 or 83 wk of age), (Roland and Harms, 1976). The duration of the studies (from 4 to 20 wk of duration), the levels of 25OHD supplementation (from 3 up to 1,100 μg/kg), the variations in age and bird condition (birds laying shell-less or normal eggs) may explain the differences among these experiments.

Some recent publications, however, have reported enhancements in eggshell quality and performance using 25OHD or combinations of vitamin D_3 and 25OHD. An increase in shell thickness was found when brown hens were provided 138 μg 25OHD/kg of feed (5,520 IU/kg)

in addition to vitamin D_3 from 19 to 80 wk, compared to the recommended dose of 69 µg/kg (Alzahrani and Roberts, 2015). Similar results were reported when 69 µg/kg of 25OHD was added on top of 2,750 IU/kg of vitamin D_3 in 32 and 82 wk-old hens (Garcia-Hernandez et al. 2001), while Torres et al. (2009) did not find any significant differences in broiler breeder performance when 35 or 69 µg/kg of 25OHD plus 2,000 IU/kg of vitamin D_3 was added compared to 2,000 to 3,400 IU/kg of vitamin in D_3, although an increment in shell quality in older birds (40 and 60 wk old) was found.

In two studies, vitamin D_3 was substituted for 25OHD at 0, 25, 50, 75 or 100%, in laying hen diets, at 1,500 (Koreleski and Świątkiewicz, 2005) or 1,600 IU/kg of feed (Rivera et al., 2014) of total vitamin D activity. Greater eggshell quality was reported in both studies when at least 25% of the total vitamin D activity was provided as 25OHD. Rivera et al. (2014) also showed a lower feed intake and FCR with any combination of 25OHD and vitamin D_3 compared to either source used independently, at the same level of activity (1,600 IU/kg of feed). Despite no differences in egg production being reported, these results are in agreement with previous studies in broilers, which reported greater gain and lower FCR in broilers when 25OHD and vitamin D_3 were combined, compared to when either source was used individually at the same level (Papešová et al., 2008; Brito et al., 2010; Michalczuk et al., 2010).

A lower feed conversion ratio from 23 to 35 wk of age was obtained when 69 µg/kg of 25OHD was added instead of vitamin D_3 at the same level, along with thicker and stronger shells when 100 or 200 ppm of vitamin C was also included (Salvador et al., 2009). Conversely, FCR was not affected in laying breeders (from 45 to 51 wk) by 25OHD supplementation at 100 µg/kg compared to 25 µg/kg, but greater egg production and hatchability were reported (Ding et al., 2011). Atencio et al. (2005b) also found higher egg production in broiler breeders when 25OHD

was provided at a low level of dietary inclusion (3 µg/kg) compared with the same amount of vitamin D_3. Recently, the addition 69 µg/kg of 25OHD along with 6 mg/kg of canthaxanthin in broiler and duck breeders resulted in lower embryonic mortality, and higher number of viable chicks per hen, without differences in egg production from 53 to 61 wk (Duarte et al., 2015), plus greater shell strength from 39 to 77 wk of age (Ren et al., 2016).

1.2.5 Effect of 25OHD on bone traits

Hydroxyapatite $(Ca_3(PO_4)_2)_3$ $Ca(OH)_2$ represents 99% of the total calcium in a bird's skeleton (Johnston and Ivey, 2002); and calcium is the most important mineral in bone structure (Keshavarz, 2003). Overall, there is an agreement among various studies on the enhancement of bone traits and the prevention of various skeletal disorders in broilers and poults when 25OHD is included in the diet. A reduction in the incidence and severity of tibial dyschondroplasia (a disease of young chickens that affects bone growth and cartilage) was reported in broilers fed 40 to 100 µg/kg of 25OHD compared to similar levels of vitamin D_3 or lower levels of 25OHD (Rennie and Whitehead, 1996; Fritts and Waldroup, 2003; Ledwaba and Roberson, 2003). Additionally, greater calcium deposition in broiler tibias (Gomez-Verduzco et al., 2013), and a significantly lower incidence of lameness (Wideman et al., 2015) were reported when 25OHD was incorporated at its recommended dose in the feed (in the former), or the drinking water at 33.9 µg/L (in the latter), both experiments included industrial levels of vitamin D_3 (2,000 and 5,500 IU/kg, respectively). Similar results were reported in young poults with turkey osteomyelitis complex fed 99 µg/kg of dietary 25OHD compared to those fed 2,860 IU/kg vitamin D_3 (Huff et al., 2002).

One the other hand, the reported effects of 25OHD on bone traits in egg laying hens have not been as consistent as the results in broilers. Compared to the equivalent level of vitamin D_3,

25OHD at 69 µg/kg feed did not affect bone traits from 21 to 65 wk of age in laying hens (Salvador et al., 2009; Keshavarz, 2003), or broiler breeders (Saunders-Blades and Korver, 2015). However, when at least 50% of vitamin D activity was provided as 25OHD from wk 24 to 40 (with a total of 2,000 IU/kg of vitamin D activity), an increase in ash percentage and bone radiographic densitometry was reported at 40 wk, compared to lower proportions of 25OHD or vitamin D_3 alone (Rivera et al., 2014). Also, a 44 wk experiment in brown hens (using a total of 1,500 IU/kg of vitamin D activity), showed a positive tendency in bone breaking strength at 70 wk (*P*-value not shown), after a partial or total replacement of vitamin D_3 with 25OHD (Korelski and Świątkiewicz, 2005). No effects on the prevalence of keel bone deformities were found by Kapelli et al. (2011b), either during the rearing (18 wk of age) or laying periods (up to 65 wk of age) when 1,500 IU/kg of each 25OHD and vitamin D_3 were provided, compared to an equivalent level of vitamin D_3 alone (3,000 IU/kg). Accordingly, the effect of 25OHD was more evident at immature ages, when the bird's skeleton is being developed (Whitehead, 2004). Unfortunately, the effects of 25OHD on young pullets have not been studied in detail; however, most dietary interventions when hens are already in lay are unproductive in the enhancement of structural bone (Fleming, 2008). Alternatively, the combination of 25OHD and vitamin D_3 may enhance the use of dietary calcium for eggshell formation, by slowing down the rate of structural bone loss if supplemented before the beginning of egg production.

1.3 OBJECTIVES AND HYPOTHESES

Most of the reviewed studies do not cover a complete cycle in laying hens, from placement to depopulation. In addition, no studies of the effect of 25OHD have been performed in commercial laying hen facilities. Therefore, the aim of the current research was to determine

the effects of supplementing 25OHD for different lengths of time under commercial conditions, on pullet growth and development, production performance, livability, eggshell quality and bone traits. Additionally, due to two bacterial outbreaks that occurred during the trial, and considering the effect of 25OHD on immune responses reported in the literature, we also evaluated whether 25OHD minimized effects of the disease outbreaks on any of the measured parameters.

The objectives were achieved in this thesis by testing the following hypotheses:

1. **That dietary 25OHD, in addition to vitamin D_3 would enhance laying pullet growth and development, under field conditions in Colombia.**

These hypotheses were addressed in Chapter 2, by evaluating the performance of pullets and layers including, body weight, feed intake, feed conversion ratio, and livability. Body weight was measured at five time points during rearing, and three time points during production (with each dietary phase change).

2. **That dietary 25OHD in addition to vitamin D_3 would increase egg production and laying hen performance and livability under commercial conditions in Colombia.**

This hypothesis was addressed in Chapter 2 as well: body weight, egg production, feed intake, feed conversion ratio and livability were measured and analyzed. Egg production, feed intake and mortality were registered on a daily basis and analyzed with each diet change during the production cycle. Body weight was measured with every diet change as well. The effect of the bacterial infection was considered in this chapter.

3. **That dietary 25OHD in addition to vitamin D_3 would enhance bone traits, and eggshell quality of laying hens, under commercial conditions in Colombia.**

This hypothesis was addressed in Chapter 3, in which shank length was measured in selected birds during rearing. Shank breaking strength, mineral density and cross-sectional area (using quantitative computed tomography) were measured at three time points. In addition, eggs were sampled during the production period to measure and analyze eggshell strength and thickness.

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1.5 TABLES

Table 1.1 Summary of vitamin D_3 and 25OHD levels in various cited papers.

Citation	Experimental bird	Ages	Vitamin D_3 dose per kg feed[1]	25OHD dose per kg of feed[1]	Description
Atencio et al., 2005	Broiler Breeders	73 to 90 wk	124.8 IU (3.12 µg) 500 IU (12.5 µg)	124.8 IU (3.12 µg) 500 IU (12.5 µg)	Either vitamin D_3 or 25OHD
Chou et al., 2009	Broilers	0 to 39 days	3,000 IU (75 µg)	1,380 IU (34.5 µg) 2,760 IU (69 µg)	Vitamin D_3 combined with either two levels of 25OHD
Ding et al., 2011	Laying Breeders	45 to 51 wk	-	1,000 IU (25 µg) 4,000 (100 µg)	25OHD
El-Maksoud, 2010	Laying hens	24 to 40 wk	2,500 IU (62.5 µg) 3,000 IU (75 µg) 3,500 IU (87.5 µg) 4,000 IU (100 µg)	-	Vitamin D_3
Garcia-Hernandez et al., 2001	Laying hens	32 to 42 wk / 82 to 92 wk	2,750 IU (68.7 µg)	0 IU (0 µg) 2 ,760 IU (69 µg)	Vitamin D_3 alone or plus 25OHD
Gomez-Verduzco et al., 2013	Broilers	0 to 21 days	200 IU (5 µg) 0 IU (0 µg)	0 IU (0 µg) 2,760 IU (69 µg)	Either vitamin D_3 or 25OHD
			2,000 IU (50 µg)	0 IU (0 µg) 2,760 IU (69 µg)	Vitamin D_3 alone or plus 25OHD
Fritts and Waldroup, 2003	Broilers	0 to 42 days	125 IU (3.1 µg) 250 IU (6.2µg) 500 IU (12.5 µg) 1,000 IU (25 µg) 2,000 IU (50 µg) 4,000 IU (100 µg)	126 IU (3.1 µg) 250 IU (6.2µg) 500 IU (12.5 µg) 1,000 IU (25 µg) 2,000 IU (50 µg) 4,000 IU (100 µg)	Either vitamin D_3 or 25OHD
Khan et al., 2010	Broilers	0 to 42 days	200 IU (5 µg) 1,500 IU (37.5 µg) 2,500 IU (62.5 µg) 3,500 IU (87.5 µg)	-	Vitamin D_3
Käppeli et al., 2011a	Laying hens	0 to 8 wk	2,800 IU (70 µg) 1,400 IU (35 µg)	0 IU (0 µg) 1,400 IU (35 µg)	Vitamin D_3 alone, or 50% replacement with 25OHD
Käppeli et al., 2011b		9 to 18 wk	2,000 IU (50 µg) 1,000 IU (25 µg)	0 IU (0 µg) 1,000 IU (25 µg)	
		19 to 68 wk	3,000 IU (75 µg) 1,500 IU (37.5 µg)	0 IU (0 µg) 1,500 IU (37.5 µg)	
Koreleski and Świątkiewicz, 2005	Laying hens	26 to 70 wk	1,500 IU (37.5 µg) 1,125 IU (28.1 µg) 750 IU (18.7 µg) 375 IU (9.73 µg) 0 IU (0 µg)	0 IU (0 µg) 375 IU (9.73 µg) 750 IU (18.7 µg) 1,125 IU (28.1 µg) 1,500 IU (37.5 µg)	Either vitamin D_3 or 25OHD alone, or four levels of combination

Table 1.1 CONTINUED Summary of vitamin D_3 and 25OHD levels in various cited papers.

Citation	Experimental bird	Ages	Vitamin D_3 dose per kg feed[1]	25OHD dose per kg of feed[1]	Description
Michalczuk et al., 2010	Broilers	0 to 42 days	4,000 IU (100µg) 2,500 IU (62.5 µg) 1,240 IU (31 µg)	0 IU (0 µg) 1,500 IU (37.5 µg) 2 ,760 IU (69 µg)	Vitamin D_3 alone, and two levels of combination
Morris et al., 2014	Broilers	0 to 35 days	250 IU (6.25 µg)	250 IU (6.25 µg) 1,000 IU (25 µg) 2,000 IU (50 µg)	Either vitamin D_3 or 25OHD
			3,000 IU (75 µg)	2 ,760 IU (69 µg)	
Morris et al., 2015	Laying pullets	0 to 21 days	-	250 IU (6.25 µg) 1,000 IU (25 µg) 2,000 IU (50 µg) 4,000 IU (100 µg)	25OHD
Panda et al., 2006	Laying hens	72 to 88 wk	300 IU (7.5 µg) 600 IU (15 µg), 1,200 IU (30 µg) 2,400 IU (60 µg)	-	Vitamin D_3
Papešová et al., 2008	Broilers	0 to 37 days	5,000 IU (125 µg) 2,500 IU (62.5 µg)	0 IU (0 µg) 2,000 IU (50 µg)	Vitamin D_3 alone or 40% replacement with 25OHD
Plaimast et al., 2015	Laying hens	98 to 105 wk	2,000 (50 µg) 6,000 (150 µg)	-	Vitamin D_3
Rama-Rao et al., 2006	Broilers	2 to 42 days	200 IU (5 µg) 1,200 IU (30 µg) 2,400 IU (60 µg) 3600 IU (90 µg)		Vitamin D_3
Rivera et al., 2014	Laying hens	24 to 40 wk	1,600 IU (40 µg) 1,200 IU (30 µg) 800 IU (20 µg) 300 IU (7.5 µg) 0 IU (0 µg)	0 IU (0 µg) 300 IU (7.5 µg) 800 IU (20 µg) 1,200 IU (30 µg) 1,600 IU (40 µg)	Either vitamin D_3 or 25OHD, or four levels of combination
Safamehr et al., 2013	Laying hens	18 to 45 wk	3,300 IU (82.5 µg) 5,000 (125 µg)	-	Vitamin D_3
Salvador et al., 2009	Laying hens	23 to 35 wk	2,760 IU (69 µg)	2,760 IU (69 µg)	Either vitamin D_3 or 25OHD
Saunders-Blades and Korver, 2015	Broiler Breeders	29 to 63 wk	2,760 IU (69 µg)	2760 IU (69 µg)	Either vitamin D_3 or 25OHD
Torres et al., 2009	Broiler Breeders	32 to 67 wk	2,000 IU (50 µg) 3,400 IU (85 µg)	-	Vitamin D_3
			2,000 IU (50 µg)	1,400 IU (35 µg) 2,760 IU (69 µg)	Two levels of combination

Table 1.1 CONTINUED Summary of vitamin D_3 and 25OHD levels in various cited papers.

Citation	Experimental bird	Ages	Vitamin D_3 dose per kg feed[1]	25OHD dose per kg of feed[1]	Description
Vignale et al., 2015	Broilers	0 to 42 days	2,760 IU (69 µg)	-	Vitamin D_3
			5,520 IU (138 µg)	5,520 IU (138 µg)	Either vitamin D_3 or 25OHD
Whitehead et al., 2004	Broilers	0 to 42 days	200 IU (5 µg) 800 IU (20 µg) 5,000 IU (125 µg) 10,000 IU (250 µg)	-	Vitamin D_3
Yao et al., 2013	Laying hens	19 to 59 wk	9,700 IU (242.5 µg) 17,200 IU (430 µg) 24,700 IU (617.5 µg) 102,200 IU (2,555 µg)	-	Vitamin D_3
Yarger et al., 1995a	Broilers	0 to 48 days	2,760 (69 µg) 8,280 IU (207 µg) 27,600 IU (690 µg)	2,760 (69 µg) 8,280 IU (207 µg) 27,600 IU (690 µg)	Either vitamin D_3 or 25OHD
Yarger et al., 1995b	Broilers	0 to 46 and up to 52 days	2,760 IU (69 µg) 3,440 IU (86 µg) 4,140 IU (103.5 µg)	1,380 IU (34.5 µg) 2,760 IU (69 µg) 4,140 IU (103.5 µg)	Either vitamin D_3 or 25OHD

[1] Conversion factor for IU of vitamin D_3: 1 IU vitamin D activity = 0.025 µg/kg of vitamin D_3 (NRC, 1994; Fritts and Waldroup, 2003).

1.6 FIGURES

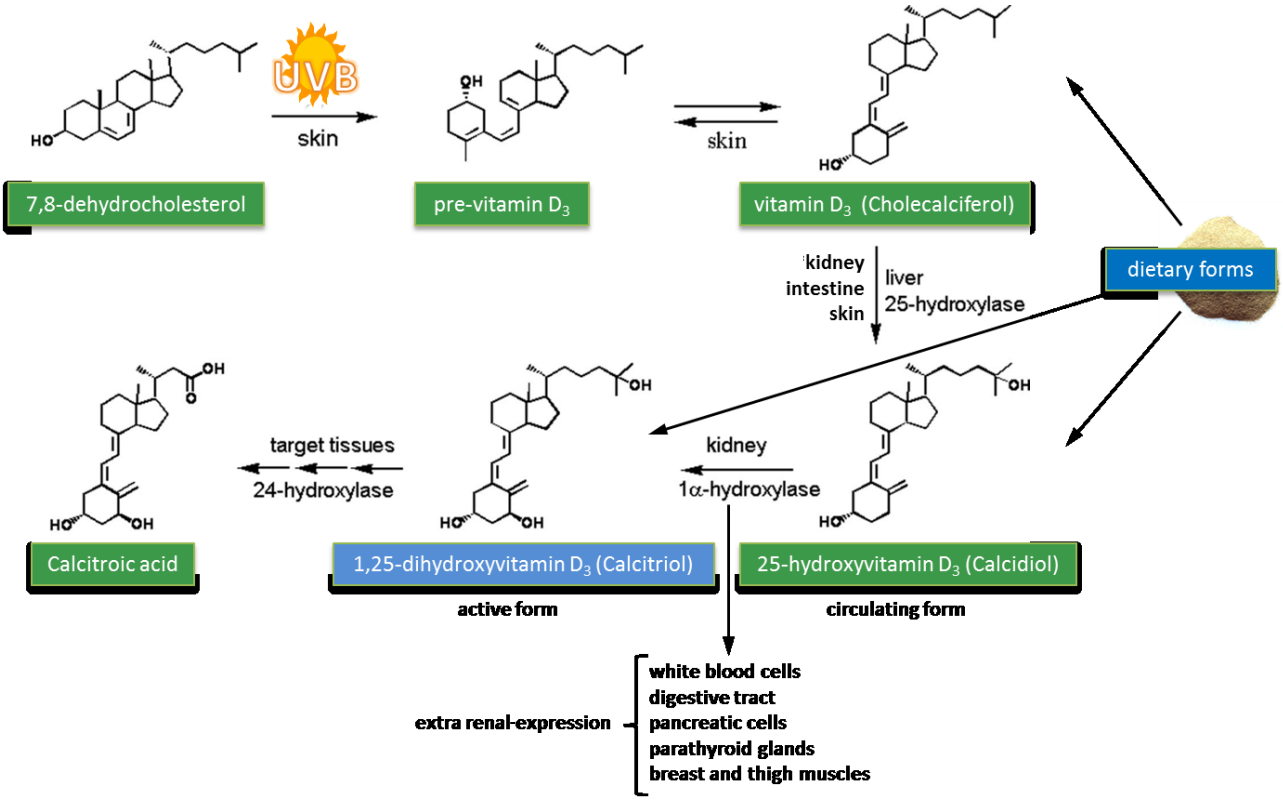

Figure 1.1 Vitamin D_3 synthesis, activation, and catabolism. Vitamin D_3 is produced in the skin by the photolytic cleavage of 7-dehydrocholesterol followed by thermal isomerization. Afterwards, it is transported to the liver via serum vitamin D binding protein, where it is converted to 25-hydroxyvitamin D_3, the major circulating metabolite of vitamin D_3. This conversion may take place at lower rates in the intestine, kidneys (Norman, 1987), and the skin (Hansdottir et al., 2008). The final activation step, 1-hydroxylation, occurs primarily, but not exclusively, in the kidney, forming 1,25-dihydroxyvitamin D_3, the hormonal form of the vitamin. Extra-renal expression of the 1α-hydroxylase has been reported various tissues including white blood cells, digestive tract, pancreatic cells, parathyroid glands and skin in mammals (Zhang et al., 2002; Correale et al., 2009; Morris et al., 2014) and, breast and thigh muscles in chickens (Shanmugasundaram and Selvaraj, 2012). Catabolism inactivation is carried out by 24-hydroxylase, which catalyzes a series of oxidation steps resulting in side chain cleavage. Adapted from Dusso et al., 2005.

2. EFFECT OF DIETARY HYDROXYCHOLECALCIFEROL ON GROWTH AND LAYING PERFORMANCE OF BROWN EGG LAYERS IN COMMERCIAL CONDITIONS IN COLOMBIA

ABSTRACT

A field trial was conducted to evaluate the effects of feeding 25-hydroxycholecalficerol (25OHD) plus vitamin D_3 on laying hen growth and performance over a whole production cycle of Hy-Line Brown laying hens. At day 1 to wk 12, a total of 17,750 Hy-Line Brown pullets were placed in 3 houses, with six treatments of 4 replicates each, and approximately 740 birds per pen. Treatments were: a Positive Control (PC) with 25OHD plus vitamin D_3 at 2,760 and 3,000 IU/kg, respectively throughout the trial, a Negative Control (NC) with vitamin D_3 only (3,000 IU/kg) during the entire trial, and treatments with the PC diet from day 1 to either 15 (Early), 17 (Prelay), 34 (Peak), or 50 (Late) wk of age, and then switched to the NC diet until 90 wk of age. At 13 wk, 10,800 pullets were transferred to the layer farm, maintaining the same treatments and randomly divided into 9 replicate pens per treatment with 200 birds per pen. Feed intake (FI), mortality and egg production were recorded daily and analyzed along with BW and FCR at each diet change. ANOVA and orthogonal contrasts to compare treatments fed dietary 25OHD versus the NC were performed. Dietary 25OHD enhanced BW, FI, and FCR at 3 wk, and BW at wk 8. Additionally, 25OHD increased FI up to 12 wk. At 26 wk and again at 84 wk, infectious coryza was observed, decreasing egg production and livability across treatments. To 34 wk, NC hens had the lowest egg production and highest FCR; PC and Peak hens had the highest egg production and lowest FCR; there were no differences after this point. By 87 wk, Peak treatment resulted in the highest cumulative egg production and lowest FCR. Dietary 25OHD addition up to 34 wk had a positive impact on early development and egg production from onset of lay to

peak production. Orthogonal contrast also revealed a significant enhancement of cumulative egg production when 25OHD was added throughout the cycle.

Keywords: 25-hydroxycholecalciferol, laying hen, performance, egg production, field trial.

2.1 INTRODUCTION

The main role of vitamin D_3 is the prevention of skeletal problems (antirachitic activity) through the metabolism of calcium and phosphorus; yet, additional functions in bird growth, development, and immune function of vitamin D_3 have been recently reported (Chou et al., 2009; Vignale et al., 2015; Morris et al., 2014). Since most birds in the poultry industry have no direct exposure to light, dietary supplementation of vitamin D_3 is necessary. Dietary vitamin D_3 is absorbed from the intestines, and transformed into 25-hydroxycholecalciferol (25OHD) in the liver by 25-hydroxylase. Then, 25OHD is converted to the active form, 1α,25 dihydroxycholecalciferol (1,25OHD) in the kidney by 1-α-hydroxylase (Bar et al., 1980; Soares et al., 1995). Currently, vitamin D_3 activity is provided in commercial poultry diets as vitamin D_3 or one of several vitamin D_3 metabolites. Vitamin D activity is expressed in IU per kg of feed (NRC, 1994); conventionally, 1 µg of vitamin D_3 is equivalent to 40 IU of vitamin D activity (NRC, 1994).

Compared to vitamin D_3, dietary 25OHD bypasses the first hydroxylation step in the liver (Kappelli, 2011), has a more efficient intestinal absorption, is less dependent on bile acids for absorption (Compston et al., 1981; Phadnis and Nemere, 2003), has a lower excretion and higher retention rates (Chou et al., 2009; Bar et al., 1980), and its safety margin is greater than other vitamin D_3 metabolites (Pesti and Shivaprasad, 2010; Newman and Leeson, 1999). Dietary 25OHD increased performance in replacement of vitamin D_3 at 69 or 40 to 100 µg/kg of feed in

broilers (Yarger at al., 1995) and turkey poults (Owens and Ledoux, 2000), respectively; and in broiler diets combining industrial levels of vitamin D_3 (2,500 to 2,760 IU/kg), plus 37.5 or 70 µg of 25OHD per kg of feed (Brito et al., 2010; Michalczuk et al., 2010). Moreover, 25OHD increases morphological maturation of the small intestine in broilers (Chou et al., 2009; Ding et al., 2011). Effects of 25OHD in pullets have not been previously reported.

During lay, recent studies have supported the enhancement of feed efficiency (Salvador et al., 2009) and egg production (Rivera et al., 2014) when dietary 25OHD is added, whereas previous experiments reported no effects on those traits (Roland and Harms, 1983; Garcia-Hernandez et al., 2001; Keshavarz, 2003). Because most published studies covered short periods of time and did not include rearing as part of the experiment, the relevance of these studies to commercial production may be limited.

Therefore, the aim of the present study was to investigate the effects of dietary 25OHD at its recommended dose in addition to vitamin D_3 over different periods of the hen's life under commercial conditions. It was hypothesized that dietary 25OHD plus vitamin D_3 would enhance pullet growth, egg production, and livability as compared to vitamin D_3 alone.

2.2 MATERIALS AND METHODS

2.2.1 Dietary treatments

Birds were distributed at day 1 of age into six dietary treatments, varying in the duration of dietary 25OHD supplementation. The dietary treatments were: a Negative Control (NC) with a basal level of vitamin D_3 (vitamin D_3) of 3,000 IU per kg of complete feed throughout life, a Positive Control (PC) which was the NC diet plus 69 µg of 25OHD throughout life (HyD®, DSM Nutritional Products, Parsippany, NJ; equivalent to 2,760 IU of vitamin D_3 activity), and four

treatments in which the birds were fed the PC diet from 0 to 15 (Early), 0 to 17 (Prelay), 0 to 34 (Peak) or 0 to 50 (Late) wk of age, and then changed to the NC diet until 90 wk of age (Figure 2.1).

2.2.2 Housing and management

2.2.2.1 Pullet farm

A field trial was conducted in its entirety on two farms owned by the Colombian table egg company Nutriavicola S.A. (Tulua, Colombia). A total of 17,750 Hy-Line Brown pullets were placed at one day of age on a rearing farm in the township of La Magdalena, Valle del Cauca, Colombia. The pullet farm was 1,000 meters above sea level. Chicks were distributed into 6 dietary treatments; each treatment group had 4 replicate pens of approximately 740 birds distributed in 3 houses, in a randomized block arrangement, with diet as the treatment and house as the block. During the first six days, houses had paper floor covers, plastic feeder trays, bell drinkers at floor level, supplemental drinkers, and propane-fired brooders. One-half of each pen was used for brooding and gradually expanded after the first week, allowing access to the full pen by d 18 of age, resulting in a final stocking density of 10.5 birds per m^2. House temperatures were maintained at approximately 34 ºC during the first 3 days and progressively decreased by 2 degrees every 3 days until reaching an ambient environmental temperature at d 19 of age. Chicks were exposed to an intermittent light program (4 hours of light followed by 2 hours of darkness; having 4 light-dark cycles per day) during the first week (Hy-Line, 2014), with a light intensity of approximately 40 lux. Following primary breeder management guide recommendations (Hy-Line, 2014), birds were exposed to 19L:5D from 1 to 2 wk, 18L:6D from 2 to 3 wk, and 16L:8D from 3 to 4 wk; then decreasing by 1 hour of light each wk until reaching 12L:12D at 8 wk, at approximately 25 lux during this 7 wk period. When the flock reached an average BW of 1,300

g, birds were exposed to natural daylight, adding 30 minutes of artificial light per week until reaching 14L:10D. Black curtains were used to control photoperiod and light intensity; LED lamps were used to supplement natural daylight as needed.

2.2.2.2 Layer farm

At 13 wk of age, 10,800 birds equally representing each treatment and pullet pen were transferred to a layer farm located in the municipality of San Pedro, Valle del Cauca, Colombia, at 220 meters above sea level. The birds were randomly divided into 9 replicate pens per treatment and 200 birds per pen; remaining birds were removed from the experiment. Birds were distributed into 3 houses with a stocking density of 15.8 birds per m^2; each pen also had one nesting space per 5 birds provided in two wood nest box units, with rice hull litter added periodically. The Valle del Cauca area has average rainfall rate of 1,000 to 1,500 mm per year (Bernal et.al., 1989), and an average temperature of 24ºC throughout the year.

2.2.2.3 Infrastructure and equipment

Both rearing and laying farms had similar infrastructure and equipment: open-sided houses with manual curtains, external metal mesh and reinforced plastic mesh between pens within a house, concrete floor with rice hull litter, bell drinkers (100 birds/drinker), and manual tube-type feeders (33 birds/feeder). Each pen was identified with a label on the door indicating the pen number, and a unique color for each treatment, and the number of birds in each pen.

2.2.2.4 Procedures

All experimental procedures were approved by the Animal Care and Use Committee-Livestock of the University of Alberta. Birds were reared and managed under the same conditions according to standard procedures established by Nutriavicola. Its protocols (which

included general observations such as feed and water consumption, beak trimming, vaccination programs, biosecurity procedures, management during brooding and production) are consistent with the recommendations of the primary breeder (Hy-Line, 2014).

2.2.2.5 Feed production

Corn-soy-based diets formulated to meet the nutrient requirements of typical commercial laying hens were provided throughout the experiment, as nine dietary phases. Diet changes occurred at 3, 8, 12, 15 and 17 wk in the rearing period (Table 2.1) and at 34, 50, and 75 wk, with the last lay diet fed up to 90 wk during production (Table 2.2). The feed was manufactured at Nutriavicola's feed mill (Tulua, Valle del Cauca, Colombia), and each of the two dietary treatments (with or without 25OHD) fed during each phase were packed in bags with a unique color; each bag of feed was distributed according to treatment in each pen.

2.2.3 Data recorded

2.2.3.1 Pullet phase

Feed consumption and mortality were recorded daily and summarized for each dietary period. Pullets had free access to feed throughout brooding and rearing. At each diet change, approximately 20% of the birds per pen were weighed. To take a random sample, birds from each pen were grouped, confining the approximate desired amount of birds on one corner of the pen, and individually weighed using a portable digital hanging scale (WeiHeng, WH-A17, Guangzhou WeiHeng Electronics Co. Ltd. CN). Average BW of the sampled birds was extrapolated to the number of birds per pen to calculate FCR (calculated for each pen as total feed intake divided by body weight gain in grams).

2.2.3.2 Laying phase

During production, feed allocation was established by company parameters to all the birds as a whole, and managed to maintain egg production and BW according to the management guide recommendations (Hy-Line, 2014). Birds were weighed following the same procedure stated for the pullet phase. Once the hens started to lay (18 wk of age), daily hen-day egg production was recorded by pen, compiled with each diet phase up to 87 wk of age and analyzed per period and for the duration of the laying phase. FCR during production was expressed as total feed consumed divided by the number of eggs produced (expressed in dozens) within each dietary phase up to 87 wk of age. In addition, a sample of the total population (around 3 to 5% of the flock) was individually weighed on a weekly or biweekly basis to monitor body weight; these data were not included in the study.

At each of 26 and 84 wk, the hens showed clinical signs of an acute respiratory disease compatible with infectious coryza, characterized by facial swelling under the eyes, swollen wattles, nasal discharge and sneezing (Rajurkar et al., 2010; Nabal-Muhammad and Sreedevi, 2015; Figure 2.2). Egg production and mortality were negatively impacted, especially during the second episode. Symptomatic birds were treated with injectable antibiotics, and the whole farm with antibiotics via the drinking water, as established by the veterinarian.

2.2.4 Statistical analysis

For all statistical analyses, the pen was the experimental unit. Data were analyzed using a one-way ANOVA, with dietary treatment as the fixed effect using the procedure for linear mixed models (PROC MIXED) of SAS 9.2© for Windows (SAS Institute, 1999). Variability due to the house was considered as a random term. The probability of differences was considered

significant at $P < 0.05$. Tukey's range test was used to compare treatment means. The analysis was in accordance with the following model:

$$Y_{ijk} = \mu + B_i + H_j + (BH)_{ik} + E_{ijk},$$

where μ was the population mean; B_i was the effect of house (block; i = 1 to 3); H_j was the effect of dietary 25OHD treatment (main effect; j = 1 to 6); $(B\text{-}H)_{ik}$ was the interaction of the house and treatment, and E_{ijk} was the residual error.

Orthogonal contrast analysis was performed to compare treatments fed dietary 25OHD up to a specific point versus the NC group. When a treatment group switched from 25OHD to vitamin D_3, that group was removed from the contrast analysis. For instance, at 34 wk, the PC, Peak, and Late groups were contrasted with the NC group, the Early and Prelay groups were not included in the analysis because they were no longer fed 25OHD to that point. Contrast differences were considered significant when $P < 0.05$.

2.3 RESULTS

2.3.1 Rearing performance

Dietary 25OHD increased BW at 3 wk of age; NC hens had lower BW, FI and the highest FCR compared to all other treatments (Table 2.3). As well, contrast analysis revealed that the NC diet resulted in lower BW from 4 to 8 wk of age, and the lowest FI up to 12 wk of age compared to the diets containing 25OHD. At 12 wk of age, NC birds had the lowest FCR among treatments, but there were no differences in FCR or other variables during the rest of the rearing period to 17 wk of age.

2.3.2 Production Performance

There were no significant differences in BW due to treatment during production (Table 2.4). At 34 wk, however, contrast analysis showed that birds fed 25OHD (PC, Peak and Late) had significantly higher BW than those fed vitamin D_3 only (NC; Table 2.4). Due to company logistics, BW was not measured at 87 wk. From 18 to 34 wk of age, hens fed 25OHD (PC and Peak by ANOVA; PC, Peak and Late by orthogonal contrast) had lower FCR than those fed NC (Table 2.4). There were no differences in FCR among treatments from 35 to 50 or from 51 to 75 wk of age, but a nearly significant trend was found in the latter period, indicating a lower FCR in the Peak treatment compared to the other treatments (P=0.070; Table 2.4).

Conversely, NC hens had lower FCR than Early hens from 76 to 87 wk of age; other treatment groups had intermediate FCR not different from either of these treatments. Cumulative FCR of Peak hens was lower than that of the Early and Prelay hens. FI was controlled during production to manage hen body weight; therefore, dietary treatment did not significantly affect hen FI at any age during this period (Table 2.4). Mortality did not show significant differences among treatments throughout the study (data not shown); but showed higher percentages during peak (18-34 wk; 1.53% overall mortality) and near the end of the production cycle (76 to 87 wk of age; 5.39% overall mortality) compared to the management guide parameters (1% and 1.7%, respectively; Hy-Line, 2014); these increases were associated with the infectious coryza outbreaks that took place at 26 and 84 wk of age. Effect of age on mortality was not analyzed statistically.

2.3.3 Egg production

During the first production period (18 to 34 wk of age), birds reached peak production at 25 wk of age (PC=95.8%, NC= 93.2%, Early=93.5%, Prelay=94.8%, Peak=95.7%, Late=94.3%),

followed by a drop in egg production during the next four wk when the flock experienced the first coryza outbreak, reaching the lowest value within this period at 29 wk (PC=85.2%, NC= 84.0%, Early=85.6%, Prelay=84.5%, Peak=86.8%, Late=85.1%). Egg production recovered by 32 wk of age (PC=94.4%, NC= 92.6%, Early=93.8%, Prelay=93.2%, Peak=94.1%, Late=94.3%). From 18 to 34 wk of age, PC and Peak had higher egg production than NC; similarly, orthogonal contrast showed greater egg production on birds being fed 25OHD at this point (PC, Peak, and Late) compared to those being fed the NC diet (Table 2.4). No other treatment effects were found after 34 wk of age. Cumulative egg production of PC hens remained higher than NC hens up to 50 wk of age. By 75 wk of age, egg production of the NC, Prelay and Late treatment hens was significantly lower than that of the Peak treatment hens, and by the end of the cycle, cumulative egg production of the Peak treatment hens was higher than that of the NC, Early and Prelay hens. Orthogonal contrast revealed that the 25OHD treatments (PC, Peak and Late to 34 wk; PC and Late to 50 wk, and PC to 75 and 87 wk of age) had higher cumulative egg production compared to the NC treatment (Table 2.5). At 84 wk, there was a second coryza outbreak, with decreasing egg production in all treatment groups, with the biggest drop between 85 and 86 wk of age (decreases in production: PC=23.7%, NC=20.2%, Early=21.4%, Prelay=20.1%, Peak=15.9%, Late=15.5%). Afterwards, all treatment groups appeared to show a gradual recovery in egg production.

2.4 DISCUSSION

Because this was a field trial conducted in the facilities of a commercial egg company, 25OHD supplementation was in addition to the regular levels of vitamin D_3 used by Nutriavicola. Therefore, experimental groups had a higher level of total vitamin D_3 activity

(5,760 IU/kg) than the NC group (3,000 IU/kg). In addition, this approach is recommended by the manufacturer of 25OHD (DSM Nutritional Products, 2016).

2.4.1 Rearing performance

Up to 15 wk of age, all treatments except the NC group had been fed 25OHD. Dietary 25OHD in addition to vitamin D_3 stimulated bird growth and development to 3 wk of age, promoting a greater FI, BW and reduced FCR (Table 2.3). By 3 wk, birds fed 25OHD consumed 2.8% more feed and were 8.7 to 11.2% heavier than NC birds. Published data on the effects of 25OHD on pullets during the rearing period are limited; however, compared to vitamin D_3 dietary 25OHD increased broiler BW (Yarger et al., 1995; Aburto et al., 1998), including during the first 10 days of life (Parkinson and Cransberg, 2004; Saunders-Blades and Korver, 2015) and under commercial conditions (Fritts and Waldroup, 2003). Furthermore, 14-d-old chicks absorbed a greater amount of 25OHD than vitamin D_3 when provided at similar dietary levels (Bar et al., 1980), whereas a combination of vitamin D_3 and 25OHD (2,500 and 2,000 IU/kg, respectively) resulted in higher broiler BW at d 21 and 37 relative to vitamin D_3 alone at 5,000 IU/kg (Papešová et al., 2008). In addition, broiler FCR was reduced and BW increased when 1,600 IU/kg of 25OHD was provided in addition to 1,100 IU/kg of vitamin D_3 in the diet up to 2 wk of age (Parkinson and Cransberg, 2004) or when 50% of dietary vitamin D_3 was replaced by 25OHD (a total of 3,500 IU/kg of vitamin D_3 activity) during the first 21 d of life (Świątkiewicz et al. (2006). However, other researchers did not find differences due to 25OHD on broiler performance (Angel et al., 2006; Fritts and Waldroup, 2005) using similar levels of vitamin D_3 or 25OHD. Despite the differences in the genetic lines (broiler vs. layer), the combination of 25OHD plus vitamin D_3 enhanced growth in young pullets, as in broilers. Although each diet in the present research met or exceeded the requirements for vitamin D_3 activity in laying hens

(NRC, 1994; Hy-Line, 2014), the differences in performance, particularly at 3 wk may be related to the limited ability of the birds to absorb and metabolize vitamin D_3 early in life. First, plasma 25OHD levels decrease during the first 10 d after hatch when broiler chicks are supplemented with vitamin D_3 alone (Saunders-Blades and Korver, 2014), probably due to a low hepatic production of 25OHD. These low plasma levels of 25OHD can be avoided when dietary 25OHD is supplemented (Mitchell et al. 1997). This could be particularly important in laying pullets, which have slower liver development than broiler chicks during the first days of life (Buzala et al., 2015). Secondly, the digestive tract of the newly-hatched chick is not completely developed, showing low secretion of lipase (Nitzan et al., 1991; Noy and Sklan, 1995) and low absorption of fats during the first days of life (Carew et al., 1972); as a fat soluble vitamin, both lipase and fats play a substantial role in the vitamin D_3 absorption (Norman, 1987). Third, 25OHD supplementation is linked to increased nutrient absorption in broilers by enhancing small intestine morphology (Chou et al., 2009; Ding et al., 2011; Bello et al., 2014), which may also promote the higher feed intake of birds fed 25OHD from 0 to 3 wk of age.

The immaturity of absorption and metabolism of dietary vitamin D_3, and lower nutrient absorption at early ages may explain, in part, why NC birds had lower BW and FCR at 3 wk of age compared to birds fed 25OHD. These physiological traits, observed in broiler chicks, could be similar in layer pullets; however, the mechanisms involved are not known. The BW differences at 8 wk of age of pullets fed 25OHD compared to the NC pullets could be a carry-over effect of the greater BW at 3 wk, as no differences in FCR were found from 4 to 8 wk of age, even though FI was higher in the 25OHD treatments (by contrast analysis). Furthermore, exceeding the target body weight during early pullet growth (9% higher BW on 25OHD treatments by 3 wk, compared to guide target BW) is desirable according to the management

guide (Hy-Line, 2014), since the digestive and immune systems undergo substantial development during this period (Dibner at el., 1998; Geyra et al., 2001; Yegani and Korver, 2008).

By 12 wk, when the pullet has developed most of its skeletal structure (Gjorgovska et al., 2014), and growth rate slows (Whitehead, 2004), the differences in BW among treatments disappeared, and the NC pullets had a lower FCR (compared to other treatments except PC) and FI at this point (compared to the other treatments). As the birds aged, the NC birds were able to overcome any early limits in nutrient absorption, reaching similar BW as the 25OHD treatment birds by this age. The lack of differences in BW, FCR and mortality from 12 to 17 wk are in agreement with a previous study that reported no differences in these parameters at 16 wk of age when dietary 25OHD was supplied along with vitamin D_3 (1,400 IU/kg of vitamin D_3 plus 1,400 IU/kg of 25OHD) relative to vitamin D_3 alone (2,800 IU/kg; Käppeli et al., 2011); however, the authors did not include any measurement of pullet performance at younger ages.

2.4.2 Production period

Similar to the results for FCR from 18 to 34 wk of age, laying hens fed 2,756 IU/kg of vitamin D_3 had greater FCR from 23 to 34 wk of age than hens fed an equivalent level of activity as 25OHD (Salvador et al., 2009). Similar findings were reported using a 50:50 mixture of vitamin D_3 and 25OHD at 1,600 IU of total vitamin D_3 activity per kg of feed from 24 to 40 wk of age (Rivera et al., 2014). In contrast, 25OHD added at 69 µg/kg of feed (2,760 IU/kg) on top of 2,750 IU/kg of dietary vitamin D_3 from 32 to 42 wk of age did not affect FCR in laying hens (Garcia-Hernandez et al., 2001), nor did as using either vitamin D_3 or 25OHD at 2,760 IU/kg diet (Keshavarz, 2003).

Increased egg production up to 34 wk of age in hens fed 25OHD (PC, Peak, Late; by contrast analysis) disagrees with previous reports in which dietary 25OHD had no effect on performance. Adding 69 μg/kg of 25OHD on top of an equivalent level of vitamin D_3 from 32 to 42 wk of age (Garcia-Hernandez et al., 2001) or using different proportions of 25OHD plus vitamin D_3 (from 25%, 50%, 75% or 100% of 25OHD of 2,000 IU/kg of total vitamin D_3 activity) from 24 to 40 wk (Rivera et al., 2014) resulted in no difference in productivity. Similarly, a longer study of laying hens from day 1 to 62 wk of age revealed no differences in FCR or egg production. Käppeli et al. (2010) added 2,800 IU/kg of vitamin D_3 in the starter diet, 2,000 IU/kg in the rearing diet, and 3,000 IU/kg vitamin D_3 during production compared to replacement of 50% of vitamin D_3 with 25OHD. The length of the studies, the 25OHD levels of inclusion and the exclusion of the rearing period in most of these studies could partially explain the lack of significance in the evaluated parameters. In our study, the decreased FCR, in addition to the lower BW at 34 wk (contrast *p*-value = 0.019), and the higher egg production to 34 wk, suggests that 25OHD promoted a more efficient use of nutrients for egg production rather than weight gain during this period. The responses to dietary 25OHD at 2,760 IU/kg, plus vitamin D_3 throughout rearing and up to 34 wk of age demonstrate the importance of pullet development on the long-term productivity of laying hens. Based on the different experimental designs of our study and that reported in the literature, supporting optimal pullet development during rearing may be a more effective strategy than supplementation beginning after the onset of egg production.

Our egg production results at 34 wk are in partial agreement with Saunders-Blades and Korver (2015) in which broiler breeders fed 69 μg/kg 25OHD from 23 to 64 wk reached peak production earlier than birds fed equivalent levels of vitamin D_3, although no differences in

weekly egg production were found thereafter. The mechanism by which egg production from onset of lay to peak was increased by 25OHD has not been completely explored. During the reproductive period of laying hens, estradiol raises kidney 1α-hydroxylase activity, and increases the production of 1,25OHD (Tanaka et al., 1976). Moreover, 1,25OHD is involved in the follicle selection process by decreasing antimullerian hormone in the granulosa cells in the ovary (Wojtusik and Johnson, 2012), which in turn up-regulates the follicle-stimulating hormone, and the recruitment of follicles (Wojtusik and Johnson, 2012; Johnson and Lee, 2016). Since dietary 25OHD increases 25OHD plasma levels in broilers and layers compared to similar levels of dietary vitamin D_3 (Vignale et al., 2015; Käppeli et al. 2010), a greater availability of plasma 25OHD, the precursor for active vitamin D_3 formation, may result in greater 1,25OHD production. The subsequent increase in VDR expression in the granulosa layer and the enhanced follicular recruitment reported by Wojtusik and Johnson (2012), may ultimately lead to higher egg production. VDR receptors have been identified in several tissues including kidney and intestine (Norman, 2006), bone (Dusso et al., 2005), eggshell gland (Takahashi et al., 1980) and rooster epididymis (Dornas et al., 2006). Up-regulation of VDR through 25OHD supplementation has been reported in broiler breast muscle (Vignale et al., 2015) and chicken macrophages (Morris and Selvaraj, 2014); however, additional research needs to identify the underlying process in layers egg production during peak.

The lack of dietary treatment effects on egg production and FCR after 34 wk, and the NC hens having lower FCR than the Early group from 76 to 87 wk of age are contrary to our hypotheses. However, no differences were reported in either of these parameters when 2,760 IU/kg of 25OHD were added compared to the same level of vitamin D_3 from 45 to 65 wk of age (Keshavarz, 2003), or on top of 2,750 IU/kg of vitamin D_3 from 82 to 92 wk of age relative to

vitamin D_3 alone in molted hens (Garcia-Hernandez et al., 2001). Substituting 25, 50, 75 or 100% of 1,500 IU/kg of vitamin D_3 with 25OHD resulted in no differences in egg production and FCR from 26 to 70 wk of age (Koreleski and Świątkiewicz, 2005). Still, none of these studies included 25OHD during rearing, or covered the entire life of the bird. Dietary 25OHD failed to increase hen productivity and performance after 34 wk. The higher egg production by hens provided 25OHD until 34 wk (Peak treatment) than hens fed 25OHD to 50 wk or throughout the cycle was unexpected. The use of 25OHD during the rearing period and early production seems to promote growth and skeletal development, which may allow the bird to avoid skeletal and shell quality problems during long production cycles. Coupled with good layer management, early feeding of 25OHD may be more effective than its use as a palliative measure during late production, after eggshell quality or bone health issues have occurred. To our knowledge, limited studies have evaluated this approach to the use of 25OHD in laying hens.

There might be a point where 25OHD supplementation has no further impact on egg production. As hens get older, activity of renal 1α-hydroxylase decreases with a subsequent lower biosynthesis of 1,25OHD (Abe et al., 1982). From that perspective, 25OHD addition would not be more effective than adding vitamin D_3, because the supply of precursor may no longer be the limiting factor to formation of active vitamin D_3. However; the higher cumulative egg production by Peak hens to 34 wk was sufficient to result in greater overall egg production in the subsequent periods until the end of the experiment. The effects early in production influenced overall production, since around 25% of the total eggs produced up to 87 wk of age are laid to 34 wk (Hy-Line, 2014).

One of the effects of 25OHD in this study may have been on the immune system. PC and Peak birds had greater egg production and lower FCR to 34 wk following the first coryza

outbreak at 26 wk of age. Maternal dietary 25OHD increased aspects of early broiler chick innate immune function (Saunders-Blades and Korver, 2015), while dietary 25OHD supplementation increased phagocytic activity of leukocytes against *Salmonella* and humoral immune response (Chou et al., 2009), decreased inflammation (Morris et al., 2014) and increased nitric oxide production in broilers (Morris and Selvaraj, 2014). Morris et al. (2015) also reported greater BW gain and an enhancement in host defense against coccidiosis by preventing excessive inflammation in Leghorn pullets when 25OHD was supplemented at 100 µg/kg, compared to 50 µg/kg or lower doses of 25OHD. Infectious coryza causes inflammation of the upper respiratory tract with an associated decrease in egg production of between 10 to 40% (Akter et al., 2013). Therefore, dietary 25OHD may have reduced the systemic impact of the bacterial infection, since total egg production and FCR to 34 wk of birds fed 25OHD were less affected than that of NC hens (Table 2.4). A reduction of inflammation during the coryza outbreak due to dietary 25OHD may also have reduced the negative impact of the infection on egg production to 34 wk of age. Birds of all ages are susceptible to coryza infection, but vulnerability increases with age (Rajurkar et al., 2009). This may explain the higher mortality and the lack of effect of dietary treatment on egg production from 76 to 87 wk of age (Table 2.3). Additional studies investigating the role of 25OHD on immune function during an infection in laying hens, both at the beginning and end of the production cycle are recommended.

25OHD early supplementation throughout rearing influenced growth on the early ages, and clearly impacted the productivity of the birds up to 34 wk of age. The development of a robust skeletal structure, a sound immune and digestive systems development during rearing may maintain productivity, health status during long production cycles. The reported effects of 25OHD on intestinal maturation (Chou et al., 2009; Ding et al., 2011), and immune system

modulation (Morris et al., 2015) along with the present results support the importance of including 25OHD during the rearing period.

In conclusion, supplementation of 2,760 IU/kg (69 μg/kg) of dietary 25OHD from 1 day of age in addition to a commercially-relevant level of vitamin D_3 enhanced early development and growth of the layer chick to 3 wk of age, possibly by increasing the absorption of nutrients. From the onset of lay to 34 wk, 25OHD promoted a higher and more efficient egg production compared to vitamin D_3 alone, even during an infectious disease challenge. Dietary 25OHD supplementation from brooding up to 34 wk may be an appropriate measure to promote proper development, higher egg production during peak production, with a higher cumulative egg production by the end of the cycle. Further research under controlled conditions, concerning the macro and microscopic changes in the small intestine and the molecular mechanisms that ultimately enhance body weight in the pullet and early egg production in the laying hen should be considered.

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2.6 TABLES

Table 2.1 Ingredient and calculated nutrient composition of experimental pullet diets fed during rearing (0 to 17 wk of age)[1]

Item	Starter 0-3 wk	Grower 4-8 wk	Pullet 1 9-12 wk	Pullet 2 13-15 wk	Prelay 16-17 wk
Ingredient %					
Corn, yellow				56.65	
Soybean meal				13.60	
Palm oil				2.50	
Corn gluten 60%				3.14	
Forage preparation				5.00	
Wheat bran				12.00	
Palm kernel cake				3.00	
Sea salt				0.18	
Sodium bicarbonate				0.28	
Dicalcium phosphate				0.77	
Calcium carbonate (fine)				1.31	
Methionine hydroxy analogue[2]				0.17	
Threonine				0.05	
L-Valine				0.05	
Lysine sulfate 55%				0.20	
Formic acid 17.1% + ammonium formate 9.4%[3]				0.10	
Premix and supplements%[4]				0.99	
Total				100	
Calculated nutrients					
CP (%)				16.0	
ME (kcal/kg)				2,820	
Digestible lysine %				0.70	
Digestible methionine + cysteine %				0.66	
Digestible threonine %				0.54	
Calcium %				0.92	
Digestible phosphorus %				0.36	
Sodium %				0.17	

[1]Missing diet ingredients have not been provided by the industry partner.

[2]Alimet, Novus International, St. Charles, MO, USA.

[3]Luctasalm, Lucta Animal Nutrition, Barcelona, Catalonia,Spain.

[4]Premix included vitamin D_3 at 3,000 IU/kg.

Table 2.2 Ingredient and calculated nutrient composition of experimental laying hen diets fed during production (from 18 to 87 wk of age)

Item	Lay 1 18-34 wk	Lay 2 35-50 wk	Lay 3 51-75 wk	Lay 4 76-87 wk
Ingredient %				
Corn, yellow	50.14	51.53	50.90	51.30
Soybean meal 48%	19.13	17.87	17.66	17.26
Palm oil	4.50	4.00	4.00	4.00
Corn gluten 60%	3.79	3.70	4.20	4.17
Forage preparation	4.50	3.50	3.50	3.50
Wheat bran	5.00	5.00	5.00	5.00
Palm kernel cake	1.50	3.00	3.00	3.00
Sea salt	0.22	0.20	0.20	0.20
Sodium bicarbonate	0.23	0.26	0.26	0.26
Dicalcium phosphate	0.74	0.55	0.48	0.48
Calcium carbonate (fine)	4.47	2.71	2.84	2.84
Calcium carbonate (coarse)	4.47	6.31	6.63	6.63
Methionine hydroxy analogue%[1]	0.13	0.12	0.11	0.10
Threonine	0.03	0.03	0.02	0.03
L-Valine	0.03	0.03	0.03	0.03
Lysine sulfate 55%	0.15	0.15	0.16	0.16
Formic acid 17.1% + ammonium formate 9.4%[2]	0.10	0.10	0.10	0.10
Premix and supplements %[3]	0.95	0.95	0.95	0.95
Total	100	100	100	100
Calculated Nutrients				
CP (%)	17.5	16.7	16.5	16.0
ME (kcal/kg)	2,830	2,820	2,820	2,810
Digestible lysine %	0.79	0.76	0.75	0.74
Digestible methionine + cysteine %	0.68	0.66	0.66	0.65
Digestible threonine %	0.58	0.56	0.56	0.56
Calcium %	3.80	3.85	4.00	4.00
Digestible Phosphorus %	0.34	0.33	0.32	0.32
Sodium %	0.17	0.17	0.17	0.17

[1]Alimet, 88% of methionine activity, Novus International, St. Charles, MO, USA.

[2]Luctasalm, Lucta Animal Nutrition, Barcelona, Catalonia, Spain.

[3]Premix included vitamin D_3 at 3,000 IU/kg.

Table 2.3 Effects of dietary 25-hydroxycholecalciferol on body weight, feed intake and feed conversion ratio of Hy-Line Brown pullets from 0 to 17 wk of age.[1]

	PC[2]	NC[3]	Early[4]	Prelay[5]	Peak[6]	Late[7]	SEM	Contrast P[8]	P-values
Age (weeks)	---------- BW (g) ----------								
3	211.6[a]	193.0[b]	213.2[a]	212.5[a]	215.6[a]	217.4[a]	1.96	< 0.001	< 0.001
8	614.0	604.7	627.8	624.7	622.8	614.0	7.83	0.032	0.172
12	1,042.9	1,035.2	1,040.5	1,037.4	1,049.4	1,033.9	7.05	0.470	0.656
15	1,301.5	1,274.8	1,267.6	1,276.7	1,289.1	1,284.7	9.59	0.396	0.191
17	1,393.4	1,375.1	1,383.7	1,366.7	1,382.9	1,369.5	8.12	0.742	0.197
	---------- FI (g/bird/day) ----------								
0 - 3	18.1[a]	17.5[b]	18.2[a]	18.1[a]	18.0[a]	18.0[a]	0.06	< 0.001	< 0.001
4 - 8	41.3[ab]	39.9[b]	41.3[ab]	41.4[a]	41.4[ab]	41.5[a]	0.39	0.008	0.025
9 - 12	60.1[a]	58.0[d]	59.9[bc]	60.1[abc]	59.8[c]	60.3[a]	0.18	< 0.001	< 0.001
13 - 15	67.7	67.1	67.3	67.5	67.7	67.2	0.86	0.206	0.394
16 - 17	70.0	69.7	69.2	69.9	70.1	69.4	2.66	0.838	0.634
	---------- FCR (g feed:g gain) ----------								
0 - 3	2.21[a]	2.40[b]	2.19[a]	2.20[a]	2.16[a]	2.14[a]	0.031	< 0.001	< 0.001
4 - 8	3.59	3.43	3.49	3.56	3.50	3.60	0.085	0.198	0.617
9 - 12	3.94[ab]	3.74[b]	4.06[a]	4.04[a]	4.01[a]	4.14[a]	0.078	0.003	< 0.001
13 - 15	5.83	6.23	6.79	6.42	6.26	5.95	0.302	0.943	0.289
16 - 17	11.26	10.34	9.41	10.92	10.98	13.64	1.319	0.361	0.350

[a-d] Treatment means within the same row with no common superscripts are significantly different ($P < 0.05$).

[1]From 0 to 12 wk of age, birds housed in 4 pens per treatment, in 3 houses, with approximately 740 birds per pen. At 13 wk of age, birds were transferred to a production facility, with 9 replicates per treatment in 3 houses with an initial number of 200 birds per pen.

[2]PC: 69 µg of 25-hydroxycholecalciferol + 3,000 IU of vitamin D_3/kg feed from 0 to 90 wk of age.

[3]NC: 3,000 IU of Vitamin D_3 /kg feed from 0 to 87 wk of age.

[4]Early: PC diet fed from 0 to 15 wk then switched to NC to 90 wk of age.

[5]Prelay: PC diet fed from 0 to 17 wk then switched to NC to 90 wk of age.

[6]Peak: PC diet fed from 0 to 34 wk then switched to NC to 90 wk of age.

[7]Late: PC diet fed from 0 to 50 wk then switched to NC of age.

[8]Orthogonal contrasts were performed to compare hens fed 25OHD in all dietary phases to that point with the NC treatment. When a treatment switched from 25OHD to vitamin D_3, it was removed from the analysis. For example, at 3, 8, 12 and 15 wk of age NC was contrasted against the rest of the treatments. At 17 wk, NC was contrasted against PC, Prelay, Peak and Late treatment, whereas Early treatment was removed from the analysis.

Table 2.4 Effects of dietary 25-hydroxycholecalciferol on body weight, feed intake, feed conversion ratio and egg production of Hy-Line Brown hens from 18 to 87 wk of age.

	PC[2]	NC[3]	Early[4]	Prelay[5]	Peak[6]	Late[7]	SEM	Contrast P[8]	P-values
Age (weeks)	---------- BW (g) ----------								
34	1,874.2	1,919.8	1,896.2	1,884.6	1,894.1	1,873.3	13.9	0.019	0.188
50	1,996.8	1,997.9	1,996.4	1,997.9	2,003.8	2,003.1	13.9	0.903	0.998
75	2,061.3	2,065.4	2,035.4	2,047.0	2,049.6	2,084.9	14.3	0.842	0.220
87[1]	-	-	-	-	-	-	-	-	-
	---------- Feed Intake (g/bird/day) ----------								
18 - 34	114.6	114.5	114.6	114.5	114.6	114.6	0.08	0.145	0.737
35 - 50	123.2	123.2	123.2	123.3	123.3	123.3	0.07	0.706	0.435
51 - 75	123.2	123.0	122.7	122.4	121.9	121.5	0.56	0.835	0.289
76 - 87	114.4	115.7	115.5	114.2	115.2	115.5	0.69	0.211	0.562
cumulative	119.8	119.9	119.8	119.6	119.7	119.8	0.15	0.762	0.933
	---------- FCR (kg feed: dozen eggs) ----------								
18 - 34	1.91[b]	1.99[a]	1.94[ab]	1.95[ab]	1.90[b]	1.93[ab]	0.016	<0.001	0.002
35 - 50	1.65	1.64	1.65	1.66	1.63	1.66	0.016	0.275	0.363
51 - 75	1.75	1.73	1.74	1.75	1.71	1.75	0.012	0.081	0.070
76 - 87	1.93[ab]	1.86[b]	1.99[a]	1.96[ab]	1.87[ab]	1.93[ab]	0.029	0.083	0.020
cumulative	1.78[ab]	1.77[ab]	1.79[a]	1.80[a]	1.75[b]	1.79[a]	0.011	0.654	0.012
	---------- % egg/hen/day ----------								
18 - 34	72.0[a]	68.9[b]	70.6[ab]	70.6[ab]	72.2[a]	71.0[ab]	0.59	<0.001	0.004
35 - 50	90.3	88.8	89.6	89.4	91.0	89.4	0.78	0.145	0.135
51 - 75	85.4	85.0	85.1	84.2	86.4	83.7	0.67	0.619	0.091
76 - 87	71.5	72.5	69.7	70.4	73.8	71.7	1.11	0.538	0.123

[a,b]Treatment means within the same row with no common superscript are significantly different ($P < 0.05$).

[1]BW at 87 wk of age was not measured.

[2]PC: 69 µg of 25-hydroxycholecalciferol + 3,000 IU of vitamin D_3/kg feed from 0 to 90 wk of age.

[3]NC: 3,000 IU of vitamin D_3/kg feed from 0 to 87 wk of age.

[4]Early: PC diet fed from 0 to 15 wk of age then switched to NC to 90 wk of age.

[5]Prelay: PC diet fed from 0 to 17 wk of age then switched to NC to 90 wk of age.

[6]Peak: PC diet fed from 0 to 34 wk of age then switched to NC to 90 wk of age.

[7]Late: PC diet fed from 0 to 50 wk of age then switched to NC to 90 wk of age.

[8]Orthogonal contrasts were performed to compare treatments fed 25OHD in all dietary phases to that point with the NC treatment. When a treatment switched from 25OHD to vitamin D_3 it was removed from the analysis. For example, at 34 wk, NC was contrasted against PC, Peak and Late, whereas Early and Prelay was removed from the analysis. At 50 wk, NC was contrasted against PC and Late. By 75 and 87 wk, NC was contrasted against PC.

Table 2.5 Effects of 25-hydroxycholecalciferol on cumulative egg production of Hy-Line Brown hens from 18 to 87 wk of age.

	PC[1]	NC[2]	Early[3]	Prelay[4]	Peak[5]	Late[6]	SEM	Contrast P[7]	P-values
	---------- %egg/hen/day ----------								
18 to 34 wk of age	72.0[a]	68.9[b]	70.6[ab]	70.6[ab]	72.2[a]	71.0[ab]	0.59	<0.001	0.004
18 to 50 wk of age	80.8[a]	78.4[b]	79.7[ab]	79.6[ab]	81.2[a]	79.7[ab]	0.55	0.001	0.001
18 to 75 wk of age	82.8[ab]	81.2[b]	82.0[ab]	81.5[b]	83.4[a]	81.4[b]	0.43	0.008	0.002
18 to 87 wk of age	81.1[ab]	79.3[b]	80.1[b]	79.6[b]	81.8[a]	80.0[ab]	0.47	0.006	0.001

[a,b]Treatment means within the same row with no common superscripts are significantly different ($P < 0.05$).

[1]PC: 69 µg of 25-hydroxycholecalciferol + 3,000 IU of vitamin D_3/kg of feed from 0 to 87 wk of age.

[2]NC: 3,000 IU of vitamin D_3/kg of feed from 0 to 87 wk of age.

[3]Early: PC diet fed from 0 to 15 wk of age then switched to NC to 90 wk of age.

[4]Prelay: PC diet fed from 0 to 17 wk of age then switched to NC to 90 wk of age.

[5]Peak: PC diet fed from 0 to 34 wk of age then switched to NC to 90 wk of age.

[6]Late: PC diet fed from 0 to 50 wk of age then switched to NC to 90 wk of age.

[7]Orthogonal contrasts were performed to compare treatments fed 25OHD in all dietary phases to that point with the NC treatment. When a treatment switched from 25OHD to vitamin D_3, it was removed from the analysis. For example, at 34 wk, NC was contrasted against PC, Peak and Late, whereas Early and Prelay were removed from the analysis. At 50 wk, NC was contrasted against PC and Late. By 75 and 87 wk, NC was contrasted against PC.

2.7 FIGURES

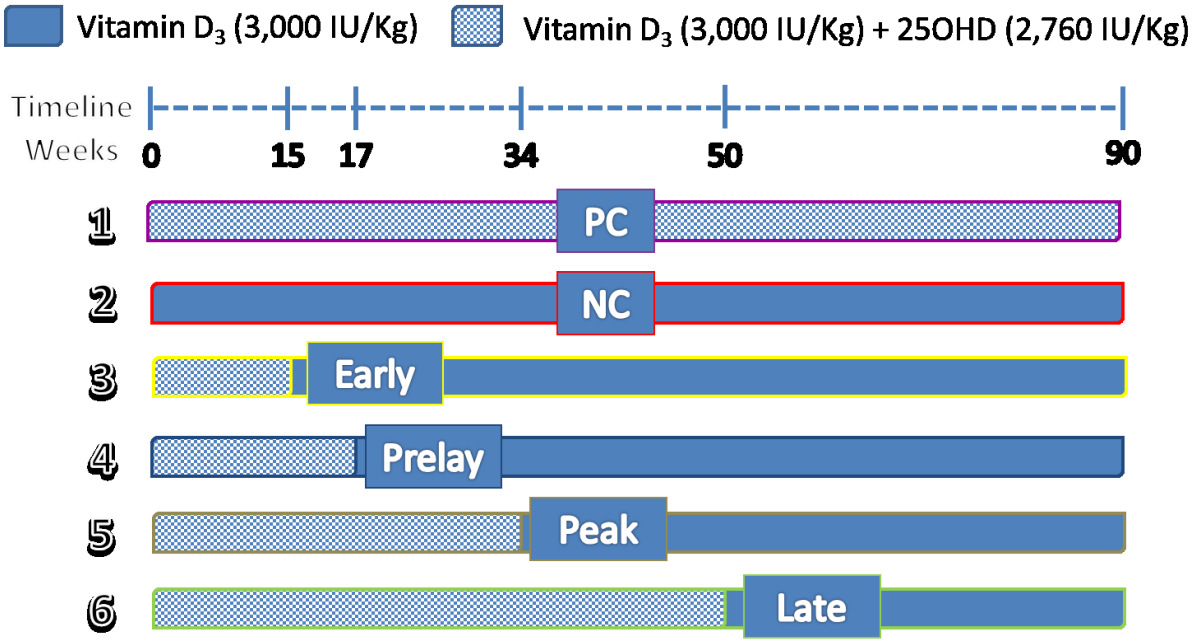

Figure 2.1. Experimental design. Treatments were: A positive control (PC) with 3,000 IU/kg of vitamin D_3 plus 2,760 IU/kg of 25OHD throughout life, a negative control (NC) with a basal level of 3,000 IU/kg of vitamin D_3 throughout life, and four diets in which the birds were fed the PC diet from 0 to 15 (Early), 0 to 17 (Prelay), 0 to 34 (Peak) or to 50 (Late) wk of age, and then changed to the NC diet until 90 wk of age.

Figure 2.2. A 34 wk old brown hen, showing facial swelling, a sign compatible with infectious coryza.

3. EFFECTS OF DIETARY 25 HYDROXYCHOLECALCIFEROL ON EGGSHELL QUALITY AND BONE TRAITS IN BROWN LAYING HENS IN COMMERCIAL CONDITIONS IN COLOMBIA.

ABSTRACT

A field trial was performed to evaluate the effect of dietary 25 hydroxycholecalciferol (25OHD) on eggshell and bone traits of Hy-Line brown laying hens. Treatments were: a Positive Control (PC) with 25OHD plus vitamin D_3 at 2,760 and 3,000 IU/kg, respectively throughout the trial, a Negative Control (NC) with vitamin D_3 only (3,000 IU/kg) during the entire trial, and four treatments with the PC diet from day 1 to either 15 (Early), 17 (Prelay), 34 (Peak), or 50 (Late) wk of age, and then switched to the NC diet until 90 wk of age. During rearing, 20 birds per treatment were randomly selected and wing-tagged to measure shank length every two wk. Throughout production, 45 eggs per treatment were randomly sampled every two to four weeks to measure eggshell strength (ESS) and thickness, whereas one or two birds per pen (9 to 18 per treatment) were euthanized at 15, 34 and 90 wk to assess bone mineral density (by quantitative computed tomography) and breaking strength. Data were analyzed using ANOVA or analysis of covariance, and orthogonal contrasts were used to compare treatments fed 25OHD to that point against the NC treatment. Across the experiment, the PC treatment resulted in higher ESS than the Early treatment, while PC and Early groups had greater shell thickness compared to NC. At 34 wk, shank cortical density at 30% of the total length from the proximal epiphysis of NC hens was nearly significantly the lowest among treatments (P= 0.076), whereas at 90 wk of age, the NC hens had lower BBS than Early hens. Orthogonal contrast showed that 25OHD increased average shank length, and maintained greater shell quality from 53 to 87 wk of age compared to the NC group. Dietary 25OHD supplementation up to 15 wk of age, and throughout the cycle

promoted greater egg-shell quality, especially at older ages without compromising bone integrity over time.

Keywords: laying hen, 25 hydroxycholecalciferol, shank length, eggshell quality, bone breaking strength.

3.1 INTRODUCTION

Eggshell quality can represent the difference between profits or losses for egg producers. Weak, abnormal, or cracked shells result in decreased economic returns (Dhawale, 2008) and are a major risk of internal egg contamination (Bain et al., 2006). Since eggshell quality decreases as birds get older (Abe et al., 1982; al-Batshan et al., 1994; Bar et al., 1999), maintaining shell quality over time is a crucial objective of the modern table-egg industry (Rossi et al., 2013). In the same way, bone tissue is the main reserve for eggshell formation and several metabolic processes. Its influence on eggshell formation is vital (Kim et al., 2012; Olgun and Aygun, 2016), especially in modern commercial lines which maintain eggshell quality at the expense of bone strength and density (Hocking et al., 2003; Kim et al., 2012), and could be exposed to environments that lead to osteoporosis and other skeletal disorders (Lay et al., 2011). Therefore, skeletal health is relevant to maintain productivity and welfare of laying hens (Whitehead and Fleming, 2000; Kim et al., 2012).

Besides genetics, management and age as significant factors (Hamilton et al., 1979; Abe et al., 1982) nutrition plays an important role in eggshell and bone quality (Nys, 1999; Edwards, Jr. 2000; Matilla et al., 2003). From a nutrition standpoint, calcium and vitamin D_3 supplementation are related to both eggshell and bone traits (Elaroussi et al., 1994; Edwards, Jr. 2000). Dietary vitamin D_3 is added to the diet to promote absorption, transport, use and

homeostasis of calcium and phosphorus (Frost et al., 1990; Soares et al., 1995; Khan et al., 2010). Compared to vitamin D_3, its metabolite 25OHD enhanced eggshell quality (Garcia Hernandez et al., 2001; Koreleski and Świątkiewicz, 2005; Rivera et al., 2014) and bone traits (Rivera et al., 2014) when added to laying hen diets; however, other studies showed no differences in these parameters compared to vitamin D_3 (Nascimento et al., 2014; Keshavarz, 2003; Käpelli et al., 2011a). The differences between studies may be explained by the short duration of the trials and the exclusion of the rearing period. It is at the end of the rearing period when structural bone reach maximum values prior to sexual maturity, with a subsequent switch in bone formation from structural to medullary bone (Fleming et al., 2008). Therefore, the present work comparing the effects of dietary 25OHD to vitamin D_3, had three objectives. First, to determine the effects on shank length during rearing; second, to determine the effects on eggshell quality throughout the production cycle, especially in late production; and third, to evaluate the effects on bone traits before onset of lay, at peak production and the end of the cycle. We hypothesized that 25OHD supplementation throughout the cycle would enhance eggshell quality and bone traits in brown egg-laying hens during the production cycle.

3.2 MATERIALS AND METHODS

3.2.1 Experimental design

Complete corn-soy-based diets were provided throughout the life of the birds (Chapter 2). Six dietary treatments were utilized as follows: a Negative Control (NC) with a basal level of vitamin D_3 of 3,000 IU/kg of complete feed throughout the life of the birds, a Positive Control (PC) with 69 μg of 25OHD (HyD®; DSM Nutritional Products, Parsippany, NJ; equivalent to 2,760 IU of vitamin D activity) plus vitamin D_3 (3,000 IU/kg of feed), throughout the life of the

birds, and four treatments in which birds were fed the PC diet from 0 to 15 (Early), 0 to 17 (Prelay), 0 to 34 (Peak) or 0 to 50 (Late) wk of age, then changed to the NC diet until 90 wk of age.

3.2.2 Housing and Management

3.2.2.1 Farms

Hy-Lyne Brown hens (n=17,750) were placed at day one of age on a rearing farm and randomly distributed among treatments. Each treatment group had four replicate pens of approximately 740 pullets, in a randomized block arrangement. At 13 wk of age, 10,800 birds were transferred to three houses at the layer facility, equally representing each pullet pen and remaining on their respective diets, including nine replicate pens per treatment. All birds were managed in the same way following company protocols and logistics (Chapter 2) which are in agreement with the recommendations of the primary breeder (Hy-Line, 2014).

3.2.2.2 Feed management

Following Nutriavicola S.A. standards, nine different dietary phases were formulated during the whole experiment. Diet changes took place at 3, 8, 15 and 17 wk of age during rearing and at 34, 50 and 75 wk, with the last diet fed up to 90 wk of age during production (Chapter 2). The feed was manufactured at Nutriavicola's feed mill (Tulua, Valle del Cauca, Colombia); the two diets fed during each phase were packed in uniquely colored bags.

3.2.3 Data recorded

3.2.3.1 Shank length

At wk 2 of age, five birds per pen (n= 20 birds per treatment) were randomly selected and wing-banded. Shank length (in mm) was measured every two wk from 2 to 18 wk of age as

described by Riczu et al. (2015), from the top of the flexed hock joint to the bottom of the footpad, using a vernier caliper, by one individual operator. Orthogonal contrast was performed to compare shank length between hens in the 25OHD treatments and the NC group from 0 to 18 wk of age.

3.2.3.2 Egg quality measures

From 24 to 36 wk of age, at two to four wk intervals, 5 eggs per pen (45 eggs per treatment) were randomly collected, and sent to an external laboratory (Nutrianalisis Ltda. Bogota, Colombia) for measurement of egg weight and eggshell strength (ESS), using a texture analyzer (model TA-XT2i plus, Stable Micro Systems Ltd, Vienna Court, Lammas Road, Godalming, Surrey, UK). From 40 to 87 wk of age, the parameters mentioned above, plus shell thickness were measured in-house by Nutriavicola using a Digital Egg Tester (DET6000, Nabel Co., Ltd, Kyoto, Japan). After collection, sampled eggs were delivered from the farm to Nutriavicola's laboratory (Tulua, Valle del Cauca, Colombia) for processing. Each egg was weighed, then placed horizontally on the holder to measure eggshell strength in KgF (force required to break the eggshell). Finally, a portion of shell from the middle of the egg was used to determine thickness using the DET6000 micrometer. All data were recorded by the tester and printed.

3.2.3.3 Bone traits measures

One bird per pen (54 in total) at each of 15 and 31 wk, and two birds per pen (108 in total) at 90 wk were randomly selected and euthanized by cervical dislocation, to excise both tarsometatarsi (shank) which were cleaned of adhering muscle tissue. Left shanks were packed in plastic bags, marked, frozen at -10ºC and sent to an external laboratory for bone breaking strength analysis (BBS; Lepton, S.A. Specialized Chemical Analysis and Support, Bogotá D.C.,

Colombia). Before processing, shanks were thawed at 4 to 6ºC for 24 hours, marked at the midpoint and broken using a Texture Analyzer (model TA-TX plus, Stable Micro Systems Ltd, Vienna Court, Lammas Road, Godalming, Surrey, UK). Using the compression method of "return to start" (Park et al., 2003); BBS was measured in KgF (force required to break the shank). Held by two brackets (36.4 mm apart), a physical force was applied to the midpoint of the shank by a static load cell (5 kN), with a trigger force of 20.0 g and a test speed of 2 mm/s.

The right shanks were fixed after collection in 10% buffered formalin for at least 42 hours, using ten times the volume of the specimen. After that, bones were rinsed with distilled water, and packed in labeled plastic bags containing a cotton ball soaked with distilled water, to keep the bone from drying out. All the shanks were shipped to the Poultry Research Centre of the University of Alberta. Bone mineral density and cross-sectional area were measured through quantitative computed tomography using a Stratec Norland XCT research scanner (Norland Medical Systems, Inc., Fort Atkinson, WI) having a 50 kV x-ray tube (Saunders-Blades et al., 2009). Measures were taken at 30% (Proximal) and 50% (Mid) of the total bone length from the proximal epiphysis. Bone mineral density and cross-sectional area were determined using the software Norland XMENU, version 5.40C (Norland Medical Systems, Inc., Fort Atkinson, WI) while bone mineral content (mg/mm; the amount of bone mineral in each 1 mm long cross-sectional scan) was calculated by multiplying density by cross-sectional area.

3.2.4 Statistical analysis

All data were analyzed using the MIXED procedure of SAS (SAS Institute, 1999), houses (n=3) were considered as blocks, with block as a random variable. Repeated measure analysis was performed, and the probability of differences was considered significant at $P < 0.05$. Tukey's range test was used to compare treatment means.

For shank length, ANCOVA was performed according to the following model:

$$Y_{ijklm} = \mu + BW + B_i + H_j + s(H)_{jk} + A_l + (HA_{jl}) + (BHA)_{iik} + E_{ijklm},$$

where μ = mean; BW = covariate used to account for the BW of individual pullets; B_i = house effect (block; i = 1 to 3); H_j = dietary 25OHD treatment effect (main effect; j = 1 to 6); $s(H)_{jk}$ = subject within treatment; A_l age period (l = from 2 to 18 wk) ; HP_{jk} interaction treatment x and age; BHP_{ijk} = interactions of house, treatment and time, and E_{ijklm} = residual error.

For egg weight, the ANOVA was performed with the statistical model being:

$$Y_{ijkl} = \mu + B_i + H_j + A_k + (HA_{jk}) + (BHA)_{iik} + E_{ijkl},$$

where μ = mean; B_i = house effect (block; i = 1 to 3); H_j = dietary 25OHD treatment effect (main effect; j = 1 to 6); A_k age effect (k = age in weeks, 24 to 87) ; HA_{jk} interaction treatment x age; BHA_{ijk} = interactions of house, treatment and age, and E_{ijkl} = residual error term.

For ESS and thickness, egg weight (EW) was included as covariate, and the ANCOVA was in accordance with the following model:

$$Y_{ijkl} = \mu + EW + B_i + H_j + A_k + (HA_{jk}) + (BHA)_{iik} + E_{ijkl},$$

where μ = mean; EW = covariate used to account for the egg weight of sampled eggs; B_i = house effect (block; i = 1 to 3); H_j = dietary 25OHD treatment effect (main effect; j = 1 to 6); A_k age effect (k = age in weeks, 24 to 87) ; HA_{jk} interaction treatment x age; BHA_{ijk} = interactions of house, treatment and age, and E_{ijkl} = residual error.

Finally, the model used for bone traits was:

$$Y_{ijk} = \mu + B_i + H_j + (B\text{-}H)_{ij} + E_{ijk},$$

where μ was the population mean; B_i was the effect of house (block; i = 1 to 3); H_j was the effect of dietary 25OHD treatments (main effect; j = 1 to 6); $(B\text{-}H)_{ik}$ were the interactions of

the house and treatment, and E_{ijk} was the residual error. For this analysis, different variances were estimated for each sample using the statement: repeated / group=treatment.

Orthogonal contrast analysis was performed to compare groups fed 25OHD up to specific time points versus the NC. When a treatment group was switched from 25OHD to vitamin D_3, it was removed from the contrast analysis. For instance, at 34 wk, PC, Peak and Late were contrasted to NC, and Early and Prelay were not included in the analysis. For egg quality orthogonal contrast was performed between NC and PC alone in the overall result. For contrast analyses, differences were considered significant when $P < 0.05$.

3.3 RESULTS AND DISCUSSION

3.3.1 Shank length

No differences were detected among treatments at any time point up to 18 wk (ANOVA, Table 3.1). However, treatments including 25OHD up to 18 wk (PC, Peak, Late) resulted in longer shanks when compared with the NC treatment (contrast from 2 to 18 wk; Table 3.1). Birds that reach the target BW and proper skeletal development during rearing are less prone to prolapse during production compared to birds achieving target BW but with shorter shank lengths, indicating poor skeletal growth (Lesson and Caston, 1991). Longer shanks are also related with an enhanced adaptability of laying hens to high environmental temperatures (30 to 32ºC), by increasing heat dissipation (Lesson and Caston, 1993; Chen et al., 2004).

Despite the lack of treatment effects on BW at 18 wk (Chapter 2), our results in BW up to 8 wk of age (Chapter 2) and overall shank length indicate that 25OHD enhanced skeletal development during rearing. To our knowledge, studies on the effects of 25OHD on growth of layer pullets have not been published. Dietary 25OHD during the rearing period did not affect

the prevalence of keel bone deformities in white pullets (Lohmann LSL; Käppeli et al., 2011b); however, several studies indicate that 25OHD stimulates growth rate in broilers (Soares et al., 1995; Yarger et al., 1995; Fritts and Waldroup, 2003).

Through the years, several studies have considered shank length as a parameter of skeletal development and frame size in white pullets and broiler breeder chicks (Lerner, 1936; Leeson and Caston, 1991; Anderson et al., 2012; Zuidhof et al., 2015), our results confirmed a similar use for brown pullets. As expected, there was an age effect on shank length, showing a considerable growth up to 14 wk of age, when pullets had an average shank length of 100 mm, followed by slower growth afterward (Table 3.1). A similar result was reported in White Leghorn pullets, in which shank and other long bones reached their maximum size at around 13 to 14 wk of age, when fusion of epiphyseal plates occurs (Matsuzawa, 1981; Leeson and Caston, 1993; Kwakkel et al. 1998). Gjorgovska et al. (2014) also reported that skeletal growth rate of brown layer pullets was greatest at 13 wk of age. This slowdown of growth after 13 to 14 wk coincides with the beginning of sexual development (Bordas and Minvielle, 1999; Hy-Line, 2014), and the subsequent development of medullary bone (Whitehead, 2004). In agreement, Fleming et al. (2008) found early signs of ovarian development and initial formation of medullary bone on 15 wk-old hens.

3.3.2 Egg quality measures

No differences were found in egg weight through the trial at any time point by ANOVA, or by contrast (Table 3.2). Overall, the PC hens produced eggs with stronger shells the entire study than those from Prelay hens (Table 3.3). Compared to the NC diet, orthogonal contrast also revealed that the PC diet resulted in stronger shells from 77 to 87 wk of age. There was a significant treatment by age interaction for eggshell thickness, even though no significant

differences were revealed at any time point (Table 3.4). This apparent interaction could be the result of small differences among the weeks that together, showed a significant difference in the overall result; PC and Early treatments had thicker shells on average than NC treatment. Orthogonal contrast showed greater shell thickness in the eggs from PC group from 53 to 75 wk, and from 77 to 87 wk of age compared to the NC group; similarly, the PC treatment resulted in stronger and thicker shells than the NC treatment over the entire trial (Tables 3.3 and 3.4).

In a literature review, Soares et al. (1995) concluded that around half of the evaluated studies showed an increase in shell quality when 25OHD was fed to hens compared to vitamin D_3, whereas no effect was reported in the remaining studies; most of those experiments only included late stages of production. Interestingly, effects in newer studies remained diverse. As in our study, these reports indicated that dietary 25OHD, compared to similar levels of vitamin D_3 increased shell quality indices when fed with vitamin C during peak production (Salvador et al., 2009), doubling the recommended 25OHD dose (138 μg/kg; Al-Zahrani and Roberts, 2015), or along with higher levels of calcium (from 3.3 to 4.5% vs. lower levels; Kim et al., 2009). Moreover, enhanced shell quality was found when 25OHD was combined with vitamin D_3 either in young birds (32 to 42 and 24 to 40 wk of age, respectively; Garcia-Hernandez et al., 2011; Rivera et al., 2014), or old hens (82 to 92 wk of age, after molt at 60 wk of age; Garcia-Hernandez et al., 2001); and during longer trials (from 26 to 70 wk; Koreleski and Świątkiewicz, 2005). This outcome can be explained by the greater absorption and transport of dietary calcium at the intestinal level when 25OHD is supplemented in laying hens (Jassen et al., 1981) and the greater biological activity when vitamin D_3 and 25OHD are combined compared to either source supplemented individually at the same basal level, which was also concluded in studies in broilers (Brito et al., 2010; Michalczuk et al., 2010). In addition, since 25OHD upregulates

vitamin D_3 receptors (VDR) in breast muscle in broilers (Vignale et al., 2015) it might have a similar effect in the VDR receptors located in the shell gland, which increase progressively in number after hens reach sexual maturity, as well as greater VDR mRNA expression as the follicles increase in size (Yoshimura et al., 1997; Wojtusik and Johnson 2012). The exact mechanisms by which 25OHD supplementation increased shell quality remain to be identified. Conversely, other studies have revealed no effects on shell quality through dietary 25OHD, either in young or old hens with a record of poor shell quality (Roland and Harms, 1976), molted hens (Nascimento et al., 2014) or by combining vitamin D_3 and 25OHD from rearing up to 68 wk (Käppeli et al., 2011a). A mixture of factors such as duration of the trials, vitamin D_3 and 25OHD levels, the type and age of birds, and the environmental conditions might have contributed to the different outcomes of these studies.

Dietary 25OHD at 2,760 IU/kg during the first 15 wk of life (Early treatment) may cause an enhancement in dietary calcium utilization, possibly by augmenting intestinal calcium absorption during and beyond the rearing period, developing a sound skeleton structure, and ultimately impacting shell thickness throughout production. Dietary 25OHD promoted intestinal development and nutrient absorption in broilers (Chou et al., 2009; Ding et al., 2011). However, 25OHD supplementation throughout the study also increased eggshell quality after 53 wk of age and final bone quality compared to vitamin D_3 alone (NC treatment; by orthogonal contrast). Given the lack of effect of dietary 25OHD on shell quality in some studies using older hens, it is interesting to note the increase in shell thickness for both the PC and Early treatments relative to the NC treatment. Optimal skeletal development during the pullet phase may reduce the risk of poor quality eggshells, particularly in long laying cycles.

The effects of 25OHD on overall ESS and thickness may be related to differences in the ultrastructure of the shell, and the uterine fluid composition (Nys et al. 1999; Roberts, 2004; Dominguez-Vera et al., 2000). In addition to the amount and thickness of the shell, the structural characteristics of the shell influence its strength (Nys et al. 1999). Proteins in the uterine fluid (e.g. ovotransferrin and lysozyme) influence the morphology and growth phases of calcite crystals of the eggshell and ultimately impact the shape and strength of the eggshell (Gautron et al., 2001; Dominguez-Vera et al., 2000). Specifically, a uterine protein with a molecular weight of 45kDa, also identified as ovalbumin (Hincke, 1995), was reduced in 59 wk-old hens fed vitamin D_3-deficient diets, suggesting that could be a vitamin D_3 dependent protein (Kaur et al., 2013). Therefore, 25OHD may positively influence ovalbumin and other uterine proteins involved in shell deposition. Kaur et al. (2013) also concluded that low molecular weight proteins in the uterine fluid that decline with age could be related to deterioration of eggshell quality. Nevertheless, the effect of 25OHD on the eggshell at the microscopic level, and the uterine fluid composition would require further studies.

Shifts in ESS and thickness throughout the production cycle (Table 3.3 and 3.4) may be in response to higher environmental temperatures during or close to the sampling days (Mashaly et al., 2004; Lin et al., 2004; Dhawale, 2008). Birds exposed to high temperatures had reduced feed intake, plasma calcium levels (Mashaly et al., 2004) and calcium absorption (Mahmoud et al., 1996) which eventually decreased shell quality. Additionally, lower levels of calbindin-D28k were found in the intestine and shell gland of laying hens exposed to heat stress (Ebeid et al., 2012). Calbindin-D28k plays a role in calcium transport in the intestine and eggshell gland, and its synthesis is promoted by the active form of vitamin D_3 (Wasserman and Taylor, 1966; Christakos et al., 2003). Since the functions of vitamin D_3 and calbindin-D28k are closely

related, 25OHD supplementation could help to maintain shell quality during high-temperature periods. Valle del Cauca area has an average temperature of 24ºC during the year (Bernal et al. 1989), with maximum temperatures from 29.5 to 30.6ºC throughout the year (Hurtado, 2012); temperature was not measured during the trial to confirm or eliminate heat stress as a possible cause.

The decreasing values in ESS and shell thickness with age in laying hens are widely supported by several studies (al-Bashtan et al., 1994; Albano Jr. et al., 2000; Kaur et al., 2013). Among the explanations for this decline are a progressive reduction in the ability of the liver to hydroxylate vitamin D_3 to 25OHD (McLoughlin and Soares, 1976) and a decreased activity of 1α-hydroxylase in the kidney, with subsequently lower circulating 1,25OHD levels (Abe et al., 1982). Decreased intestinal calcium absorption, coupled with an increase in egg size (al-Bashtan et al., 1994; Grobas et al., 1999). These physiological changes may cause a progressive decrease in the formation of the active form of vitamin D_3, with a subsequent drop in eggshell quality and bone strength with age. Considering that 25OHD bypasses liver hydroxylation (Käppeli et al., 2011a), its supplementation could have ameliorated the low hepatic hydroxylation of vitamin D_3 as the hen gets older, providing additional substrate for the synthesis of the active form. This ultimately may have maintained shell quality as reported over the duration of the study, and especially manifest through the orthogonal contrast between PC and NC.

3.3.3 Bone traits

Bone mineralization measurements at Proximal (30% of the length of the shank from the proximal epiphysis) and Mid points (50% of the length of the shank from the epiphysis) were taken to obtain a more complete indication of the status of the sampled bone. Measures performed on the shank bone are assumed to reflect the status of other portions of the skeleton

(Korver et al., 2004). No differences were reported at 15 wk in BBS (Table 3.5) or bone mineralization (Tables 3.6 to 3.8). At 90 wk, BBS of the Early hens was significantly higher than the NC treatment; nevertheless, orthogonal contrast also revealed that PC hens had stronger bones than NC hens (Table 3.5). By 34 wk of age, no significant differences in cortical bone density were found except by orthogonal contrast (Table 3.6). Treatments that included 25OHD up to 34 wk (PC, Peak and Late) had greater density than the NC treatment; whereas the analysis of variance showed a trend (P=0.076; the NC group had the lowest value). Compared to vitamin D_3, 25OHD enhanced bone traits in broilers including bone ash, (McNutt and Haussler, 1973, Fritts and Waldroup; 2003), bone breaking strength (Rennie and Whitehead, 1996), and reduced the incidence of lameness (Wideman et al., 2015). Limited studies on bone traits of young pullets have been documented, with no reported effect of 25OHD compared to vitamin D_3 (Käppeli et al., 2011b). From 18 to 34 wk of age, hens reached peak production (Chapter 2), a period when the medullary bone volume has been built up more than two-fold compared to previous ages, and the structural bone has started to diminish (Fleming et al., 1998; Cransberg et al., 2001; Whitehead, 2004). Since structural bone is not formed as long as the hens are actively laying eggs (Hudson et al., 1993; Fleming et al., 2008), the difference in cortical density by orthogonal contrast at this point could mean that the normal cortical bone loss was alleviated by the 25OHD supplementation, this may be as a result of 25OHD promoting a more efficient dietary calcium utilization (enhancing calcium absorption in the intestine) to form the eggshells (Bar et al., 1977) and reduced reliance on bone calcium . This effect could be vital considering that hens were at peak production. However, this was not consistent as it was not seen in the Mid scan of the shank bones at this age, nor at other times.

At the end of the production cycle, BBS of NC hens was the lowest among treatments, but was significantly lower than only the Early treatment (Table 3.5). The reason why 25OHD fed to 15 wk of age resulted in stronger bones than any of the other treatments at the end of the cycle is unknown, especially because no differences were found in BBS at 15 and 34 wk of age (Table 3.6). One possibility is that 25OHD may not affect BBS compared to vitamin D_3 early in production but an increased dietary calcium absorption and transport from the intestine to the eggshell could reduce the reliance on calcium mobilization from the bone, since no differences in medullary bone mineral density and cross-sectional area were found, yet the Early treatment resulted in the greatest eggshell thickness. Another possibility is that there could be a point where continued 25OHD supplementation does not result in additional protection from bone mineral loss. This could be the reason why some research has shown significant differences in these variables, whereas others studies have not. Along with the enhancement of pullet BW up to 8 wk of age (Chapter 2) and greater overall shank length, the response of the Early treatment in BBS at 90 wk of age underscores the potential significance of 25OHD supplementation during the rearing period on bone status in late stages of production. Proper pullet management and nutrition, including strategies to optimize skeletal development before the onset of production, may be a more effective approach to support long egg production cycles than corrective measures applied after problems with skeletal health or shell quality are observed at later stages of egg production.

The orthogonal contrast, alternatively, indicated that 25OHD addition during the whole cycle maintained greater bone strength compared to the NC. In agreement with our study, Koreleski and Świątkiewicz (2005) found a positive trend (*P* value not reported) in BBS of 70 wk old brown hens when vitamin D_3 was partially replaced with 25OHD from 26 to 70 wk of

age. Correspondingly, a parallel study demonstrated that 25OHD supplementation increased yielding load and stiffness of bone compared to vitamin D_3 (Świątkiewicz and Koreleski, 2005). Conversely, no differences in bone traits were reported in 65-wk-old broiler breeder hens (Saunders-Blades and Korver, 2015), nor in molted hens at 80 wk of age (Nascimento et al., 2014) when supplementing either vitamin D_3 or 25OHD. Furthermore, bone traits in laying hens from 50 to 65 wk of age were not affected by 25OHD compared to vitamin D_3 (Keshavarz, 1996; 2003).

Type of birds, housing, and diet may play a role in the lack of effect of 25OHD in bone traits. Brown laying hens produce stronger eggshells at early and mid-ages of production (Kaur, et al., 2013), with a higher shell weight relative to egg weight as the age of the hen increased (Silversides and Scott, 2001). Brown hens also showed stronger bones by 65 wk of age compared to White Leghorns, making these birds less vulnerable to caged layer fatigue (osteoporosis, Riczu et al., 2003), or shell quality issues. Brown birds may mobilize more efficiently calcium from medullary bone preserving the cortical bone. Housing systems that promote movement and exercise enhanced bone strength (Riczu et al., 2003; Jendral et al., 2008). Additionally, birds placed in floor pens had greater cortical bone density and cross-sectional area in the wing and leg bones (Silversides et al., 2012). Finally, the addition of coarse calcium particles in addition to fine particles in production diets (Table 2.2; Chapter 2), may help to maintain bone mineralization over time. Large calcium particles remain in the gizzard for a longer time, and have a slower dissolving rate compared to ground particles, providing higher availability of dietary calcium for shell formation, especially during the dark period (Saunders-Blades et al., 2009; Oliveira et al., 2013; Xavier et al., 2015). Therefore, the use of cage-free

brown hens in the current study, fed a nutritionally complete diet, could have limited the effects of 25OHD on eggshell and bone traits.

25OHD supplementation from day 1 up to 15 wk of age, or during the entire cycle increased overall shank length at 18 wk of age and eggshell thickness throughout the study. Hens fed 25OHD may utilize dietary calcium more effectively to maintain eggshell quality, without compromising bone health, and in fact, maintaining it at later ages. The mechanisms by which dietary 25OHD addition increased eggshell strength and thickness should be studied further. A greater effect of 25OHD on the evaluated parameters may not have been seen because even the NC hens were fed diets that met the requirement for vitamin D_3. This, coupled with the lack of major problems during the trial resulted in little room for effects on skeletal health or shell quality. Further studies focused on the impact of 25OHD supplementation on the pullet development are recommended to clarify the effects observed in overall shank length, overall shell strength and thickness, and BBS at the end of the production cycle. Although no differences in eggshell quality traits were found at any time point, orthogonal contrasts showed that 25OHD maintained greater shell thickness after 53 wk of age. Long field trials like the present study are recommended to test 25OHD in White Leghorns, and in different housing types, considering the higher susceptibility to osteoporosis of these birds in cage-type houses.

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3.5 TABLES

Table 3.1 Effects of dietary 25-hydroxycholecalciferol on shank length of Hy-Line Brown pullets from 2 to 18 wk of age[1].

	PC[2]	NC[3]	Early[4]	Prelay[5]	Peak[6]	Late[7]	SEM
week	----------	----------	----------	mm ----------	----------	----------	----------
2	43.4	42.1	42.8	43.4	43.0	44.1	0.38
4	57.5	56.1	56.6	57.9	57.2	57.4	0.47
6	69.7	64.4	68.3	69.1	69.2	68.6	1.40
8	79.4	77.2	78.0	78.0	79.1	79.0	0.74
10	89.0	87.5	85.9	88.2	88.1	88.1	0.85
12	96.5	95.7	95.0	95.0	95.6	95.8	0.65
14	100.6	99.8	99.3	99.4	99.7	100.7	0.62
16	100.5	100.0	100.0	99.8	100.0	100.5	0.60
18	103.1	102.4	102.0	102.7	102.0	103.3	0.55
Overall	82.2	80.6	80.9	81.5	81.6	82.0	0.48
P-values for main effects and interaction							
Diet							0.162
Age							<0.001
Diet*Age							0.107
BW(Covariate)							0.104
P-values for contrast (25OHD vs. NC)[8]							
2 - 18 wk							0.024

[1]BW of sampled birds was used as a covariate. From wk 0 to 12, birds were placed in 4 pens per treatment, in three houses, with approximately 740 birds per pen. At 13 wk of age, birds were transferred to a production facility, with 9 replicates per treatment in 3 houses with an initial number of 200 birds per pen.

[2]PC: 69 µg of 25-hydroxycholecalciferol + 3,000 IU of vitamin D_3/ kg of feed from 0 to 90 wk of age.

[3]NC: 3,000 IU of vitamin D_3/kg of feed from 0 to 90 wk of age.

[4]Early: PC diet from 0 to 15 wk of age then switched to NC to 90 wk of age.

[5]Prelay: PC diet from 0 to 17 wk of age then switched to NC to 90 wk of age.

[6]Peak: PC diet from 0 to 34 wk of age then switched to NC to 90 wk of age.

[7]Late: PC diet from 0 to 50 wk of age then switched to NC to 90 wk of age.

[8]Orthogonal contrast was performed to compare treatments being fed 25 hydroxycholecalciferol in specific dietary phases with the NC treatment. When a treatment switched from 25 hydroxycholecalciferol to vitamin D_3, it was removed from the analysis. For example, from 2 to 18 wk of age PC, Peak and Late were contrasted against the NC treatment.

Table 3.2 Effects of dietary 25-hydroxycholecalciferol on egg weight of Hy-Line Brown hens from wk 24 to 87 of age.

	PC[1]	NC[2]	Early[3]	Prelay[4]	Peak[5]	Late[6]	SEM
Week	----------	----------	----------	g ----------	----------	----------	----------
24	56.6	56.7	57.8	56.8	57.6	57.5	0.801
28	60.8	61.6	61.0	60.9	61.2	60.8	0.839
34	62.1	61.4	62.1	62.7	61.8	62.5	0.808
36	60.4	61.1	60.6	62.3	61.5	61.5	0.814
40	63.8	64.7	65.9	66.6	65.6	63.8	0.808
42	62.1	62.1	62.5	63.4	62.6	61.9	0.809
44	63.5	63.9	63.3	62.8	63.4	63.0	0.808
46	64.1	63.8	65.1	64.8	63.4	65.1	0.814
48	64.2	63.0	64.4	64.1	64.4	63.7	0.801
53	62.3	63.5	63.3	63.2	62.7	64.0	0.808
56	62.2	63.1	62.1	61.7	61.7	62.6	0.814
59	63.2	62.8	64.6	63.9	63.6	64.0	0.822
61	61.9	61.8	62.0	62.1	62.0	63.8	0.814
64	62.8	63.4	63.6	62.8	62.8	64.0	0.835
66	62.2	62.3	62.8	62.6	62.4	63.5	0.825
68	64.6	63.3	63.7	61.6	63.9	63.8	0.821
71	64.4	66.7	67.4	67.3	67.9	66.1	0.828
73	65.3	66.1	66.2	67.9	68.2	67.1	0.836
75	67.5	64.4	67.4	65.5	66.1	66.9	0.828
77	66.5	67.5	66.6	66.9	66.2	66.5	0.835
79	67.3	66.6	65.6	64.3	67.7	67.1	0.836
81	68.1	66.8	67.7	67.9	68.1	67.4	0.828
83	67.0	66.6	66.8	67.0	67.2	67.7	0.821
85	67.1	67.3	67.8	67.9	67.5	66.1	0.814
87	63.9	64.8	64.8	64.5	64.6	65.7	0.808
Overall	63.7	63.8	64.2	64.1	64.2	64.2	0.163
P-values for main effects and interaction							
Diet							0.161
Age							<0.001
Diet*Age							0.926
P-values contrast (25OHD vs. NC)[7]							
24 - 34 wk							0.976
36 - 48 wk							0.707
53 - 75 wk							0.821
77 - 87 wk							0.934
Overall							0.799

[1]PC: 69 µg of 25-hydroxycholecalciferol + 3,000 IU/kg of vitamin D_3 to 90 wk of age.

[2]NC: 3,000 IU/kg of vitamin D_3 from 0 to 90 wk of age.

[3]Early: PC diet from 0 to 15 wk of age then switched to NC to 90 wk of age.

[4]Prelay: PC diet from 0 to 17 wk of age then switched to NC to 90 wk of age.

[5]Peak: PC diet from 0 to 34 wk of age then switched to NC to 90 wk of age.

[6]Late: PC diet from 0 to 50 wk of age then switched to NC to 90 wk of age.

[7]Orthogonal contrasts were performed to compare treatments fed 25OHD in all dietary phases to that point with the NC treatment. When a treatment switched from 25OHD to vitamin D_3 it was removed from the analysis. For example, from 24 to 34 wk of age, NC was contrasted against PC, Peak and Late, whereas Early and Prelay was removed from the analysis. From 36 to 48 wk of age, NC was contrasted against PC and Late. From 53 to 75 wk and 75 and 87 wk of age, NC was contrasted against PC.

Table 3.3 Effects of dietary 25-hydroxycholecalciferol on eggshell strength of Hy-Line Brown hens from 24 to 87 wk of age[1].

	PC[2]	NC[3]	Early[4]	Prelay[5]	Peak[6]	Late[7]	SEM
week	grams of force						
24	4,207.3	4,122.9	4,316.9	4,159.6	4,207.0	4,282.6	109.04
28	4,189.4	4,218.7	4,393.4	4,370.0	4,318.1	4,237.7	113.22
34	4,269.7	4,275.6	4,131.4	4,409.3	4,386.3	4,400.3	108.35
36	4,421.6	4,373.6	4,298.0	4,385.4	4,364.9	4,270.6	110.82
40[8]	4,426.4	4,215.0	4,404.7	4,155.0	4,094.0	4,282.4	111.69
42	4,298.5	4,348.8	4,158.5	4,321.7	4,003.6	4,464.2	109.41
44	4,153.2	4,139.7	4,278.9	4,240.0	3,963.6	4,048.6	108.18
46	4,164.9	4,160.5	4,389.2	4,098.8	4,154.2	4,157.4	110.43
48	4,044.5	4,049.6	4,167.2	4,163.4	4,196.4	4,155.7	110.43
53	3,815.8	3,683.4	3,728.0	3,452.8	3,594.1	3,758.5	111.68
56	3,782.2	4,059.6	3,832.1	3,726.3	3,921.3	4,147.2	112.94
59	3,929.9	3,964.7	3,727.3	3,649.8	3,908.2	3,791.5	110.49
61	4,176.3	4,057.3	4,013.3	4,139.6	4,110.7	3,869.9	109.35
64	3,673.3	3,662.5	3,545.0	3,478.3	3,562.0	3,510.2	112.87
66	3,849.8	3,753.9	3,873.7	3,581.6	3,823.0	3,685.3	114.37
68	3,705.5	3,760.2	3,847.3	3,810.3	3,660.7	3,663.2	115.57
71	3,894.0	3,614.6	3,616.6	3,632.3	3,596.6	3,633.8	114.44
73	3,581.1	3,710.1	3,524.1	3,454.8	3,591.4	3,493.1	113.06
75	3,573.0	3,559.6	3,523.1	3,684.3	3,516.6	3,579.6	115.64
77	3,401.3	3,415.0	3,538.3	3,461.8	3,456.1	3,406.3	114.35
79	3,605.7	3,444.3	3,707.5	3,453.3	3,367.2	3,571.7	112.95
81	3,622.4	3,469.0	3,570.5	3,410.2	3,574.9	3,531.6	111.89
83	3,689.1	3,393.6	3,397.0	3,498.1	3,533.1	3,583.9	113.05
85	3,685.4	3,358.8	3,350.9	3,336.3	3,365.3	3,728.4	109.52
87	3,672.3	3,480.5	3,487.7	3,555.5	3,660.2	3,652.4	110.43
Overall	3,917.2[a]	3,852.8[ab]	3,872.4[ab]	3,825.1[b]	3,836.7[ab]	3,876.2[ab]	22.0
P-values for main effects and interaction							
Diet							0.048
Age							<.0.01
Diet*Age							0.595
Covariate							0.295
P-values for contrast (25OHD vs. NC)[9]							
24 - 34 wk							0.337
36 - 48 wk							0.670
53 - 75 wk							0.769
77 - 87 wk							<0.001
Overall							0.038

[a,b]Treatment means within the same row with no common superscripts are significantly different (P < 0.05).

[1]Weight of sampled eggs was used as a covariate.

[2]PC: 69 µg of 25-hydroxycholecalciferol + 3,000 IU of vitamin D_3/ kg of feed from 0 to 90 wk of age.

[3]NC: 3,000 IU of vitamin D_3/kg of feed from 0 to 90 wk of age.

[4]Early: PC diet from 0 to 15 wk of age then switched to NC to 90 wk of age.

[5]Prelay: PC diet from 0 to 17 wk of age then switched to NC to 90 wk of age.

[6]Peak: PC diet from 0 to 34 wk of age then switched to NC to 90 wk of age.
[7]Late: PC diet from 0 to 50 wk of age then switched to NC to 90 wk of age.
[8]From 24 to 36 wk of age eggshell strength was measured sing a texture analyzer (model TA-XT2i plus, Stable Micro Systems Ltd, Vienna Court, Lammas Road, Godalming, Surrey, UK). From 40 to 87 wk of age, the parameters eggshell strength was measured in-house by Nutriavicola using a Digital Egg Tester (DET6000, Nabel Co., Ltd, Kyoto, Japan).
[9]Orthogonal contrasts were performed to compare treatments fed 25 hydroxycholecalciferol in all dietary phases to that point with the NC treatment. When a treatment switched from 25 hydroxycholecalciferol to vitamin D_3 it was removed from the analysis. For example, from 24 to 34 wk of age, NC was contrasted against PC, Peak and Late, whereas Early and Prelay was removed from the analysis. From 36 to 48 wk of age, NC was contrasted against PC and Late. From 53 to 75 wk and 75 and 87 wk of age, NC was contrasted against PC. Similarly, overall contrast was performed comparing NC against PC.

Table 3.4 Effects of 25-hydroxycholecalciferol on eggshell thickness of Hy-Line Brown hens from 40 to 87 wk of age[1].

	PC[2]	NC[3]	Early[4]	Prelay[5]	Peak[6]	Late[7]	SEM
week	---------- mm ----------						
40	0.427	0.436	0.463	0.445	0.422	0.429	0.0074
42	0.419	0.415	0.445	0.428	0.419	0.416	0.0073
44	0.403	0.408	0.413	0.396	0.407	0.417	0.0073
46	0.373	0.386	0.383	0.388	0.389	0.385	0.0073
48	0.379	0.377	0.383	0.392	0.387	0.381	0.0072
53	0.398	0.371	0.371	0.368	0.371	0.369	0.0072
56	0.375	0.374	0.361	0.363	0.371	0.381	0.0073
59	0.372	0.375	0.373	0.376	0.372	0.375	0.0073
61	0.389	0.392	0.392	0.397	0.401	0.371	0.0073
64	0.375	0.387	0.377	0.379	0.370	0.381	0.0074
66	0.392	0.374	0.412	0.392	0.391	0.385	0.0075
68	0.376	0.375	0.388	0.376	0.367	0.374	0.0074
71	0.392	0.386	0.395	0.389	0.381	0.391	0.0074
73	0.395	0.359	0.375	0.375	0.368	0.366	0.0076
75	0.372	0.360	0.364	0.384	0.358	0.382	0.0075
77	0.396	0.357	0.373	0.378	0.364	0.360	0.0075
79	0.366	0.351	0.353	0.378	0.377	0.361	0.0076
81	0.376	0.362	0.382	0.361	0.371	0.373	0.0075
83	0.356	0.361	0.368	0.354	0.345	0.352	0.0075
85	0.390	0.348	0.354	0.364	0.358	0.378	0.0073
87	0.366	0.363	0.362	0.363	0.365	0.366	0.0073
Overall	0.385[a]	0.377[b]	0.385[a]	0.383[ab]	0.379[ab]	0.381[ab]	0.0016
P-values for main effects and interaction							
Diet							<0.001
Age							0.001
Diet*Age							<0.001
Covariate							<0.001
P-values for contrast[8]							
40 - 48 wk							0.719
53 - 75 wk							0.033
77 - 87 wk							<0.001
Overall							<0.001

[a,b]Treatment means within the same row with no common superscripts are significantly different ($P < 0.05$).

[1]Egg weight was included as a covariate.

[2]PC: 69 μg of 25-hydroxycholecalciferol + 3,000 IU of vitamin D_3/ kg of feed from 0 to 90 wk of age.

[3]NC: 3,000 IU of vitamin D_3/kg of feed from 0 to 90 wk of age.

[4]Early: PC diet from 0 to 15 wk of age then switched to NC to 90 wk of age.

[5]Prelay: PC diet from 0 to 17 wk of age then switched to NC to 90 wk of age.

[6]Peak: PC diet from 0 to 34 wk of age then switched to NC to 90 wk of age.

[7]Late: PC diet from 0 to 50 wk of age then switched to NC to 90 wk of age.

[8]Orthogonal contrasts were performed to compare treatments fed 25 hydroxycholecalciferol in all dietary phases to that point with the NC treatment. When a treatment switched from 25 hydroxycholecalciferol to vitamin D_3 it was removed from the analysis. For example, from 40 to 48 wk of age, NC was contrasted against PC and Late.

Table 3.5 Effects of 25-hydroxycholecalciferol on shank breaking strength of Hy-Line Brown hens at 15, 34 and 90 wk of age.

	PC[1]	NC[2]	Early[3]	Prelay[4]	Peak[5]	Late[6]	Contrast P[7]	P-values
week	grams of force + SEM							
15	11,003 ± 302	10,411 ± 370	11,155 ± 401	10,474 ± 476	10,477 ± 453	10,815 ± 390	0.364	0.643
34	21,198 ± 877	21,598 ± 1,025	21,428 ± 1,111	22,295 ± 1,076	21,333 ± 1,118	21,543 ± 664	0.835	0.982
90	21,819^{ab} ± 948	19,082^{b} ± 782	22,309^{a} ± 585	20,669^{ab} ± 827	21,120^{ab} ± 1,041	20329^{ab} ± 688	0.029	0.035

^{a,b}Treatment means within the same row within effect with no common superscripts are significantly different ($P < 0.05$).

[1]PC: 69 µg of 25-hydroxycholecalciferol + 3,000 IU of vitamin D_3/kg of feed from 0 to 90 wk of age.

[2]NC: 3,000 IU of vitamin D_3/kg of feed from 0 to 90 wk of age.

[3]Early: PC diet from 0 to 15 wk of age then switched to NC to 90 wk of age.

[4]Prelay: PC diet from 0 to 17 wk of age then switched to NC to 90 wk of age.

[5]Peak: PC diet from 0 to 34 wk of age then switched to NC to 90 wk of age.

[6]Late: PC diet from 0 to 50 wk of age then switched to NC to 90 wk of age.

[7]Orthogonal contrasts were performed to compare treatments fed 25 hydroxycholecalciferol in all dietary phases to that point with the NC treatment. When a treatment switched from 25 hydroxycholecalciferol to vitamin D_3 it was removed from the analysis. For example, at wk 15 of age NC was contrasted against the rest of the treatments. At wk 34, NC was contrasted with PC, Peak and Late, whereas Prelay was removed from the analysis. At wk 90, NC was contrasted with PC.

Table 3.6 Effects of 25-hydroxycholecalciferol on bone mineral density of shank bones of Hy-Line Brown hens at wk 15, 34 and 90 of age.

Proximal[1]	Total			Cortical			Trabecular + Medullary		
	15 wk	34 wk	90 wk	15 wk	34 wk	90 wk	15 wk	34 wk	90 wk
	mg/cm^3								
PC[3]	307.68 ± 13.25	409.21 ± 8.39	415.94 ± 13.26	924.13 ± 4.36	964.73 ± 13.17	957.45 ± 10.38	29.23 ± 10.3	47.02 ± 6.70	99.17 ± 8.42
NC[4]	315.92 ± 14.68	392.71 ± 14.85	429.67 ± 12.92	907.7 ± 7.27	939.39 ± 7.98	948.07 ± 10.59	40.07 ± 10.4	54.96 ± 8.67	102.23 ± 8.79
Early[5]	324.48 ± 18.92	392.64 ± 9.06	460.74 ± 11.66	928.86 ± 12.43	952.16 ± 12.28	969.34 ± 11.26	30.51 ± 10.92	50.56 ± 7.65	125.38 ± 14.11
Prelay[6]	302.60 ± 15.31	397.77 ± 13.13	425.00 ± 10.01	909.13 ± 5.98	976.01 ± 11.32	962.69 ± 11.02	36.50 ± 9.36	44.22 ± 8.25	106.37 ± 9.73
Peak[7]	315.46 ± 14.68	401.04 ± 14.89	429.82 ± 14.67	910.24 ± 4.07	944.90 ± 10.15	956.97 ± 11.21	41.75 ± 8.46	55.57 ± 3.14	104.67 ± 10.48
Late[8]	303.84 ± 19.17	400.17 ± 6.89	430.89 ± 7.66	931.5 ± 8.65	957.38 ± 10.16	956.87 ± 8.21	33.82 ± 10.09	57.76 ± 9.78	116.82 ± 6.80
Contrast P[9]	0.743	0.517	0.463	0.131	0.048	0.530	0.468	0.877	0.803
P-values	0.935	0.858	0.197	0.130	0.076	0.837	0.742	0.755	0.481
Middle[2]									
PC[3]	348.27 ± 7.40	441.07 ± 9.51	472.99 ± 15.50	958.31 ± 8.01	1025.54 ± 14.72	1052.86 ± 6.56	20.37 ± 7.63	37.31 ± 8.01	92.15 ± 10.52
NC[4]	364.18 ± 10.71	436.90 ± 15.75	483.49 ± 11.93	955.49 ± 4.49	1023.83 ± 7.62	1051.33 ± 6.61	33.62 ± 5.79	48.51 ± 9.04	106.91 ± 11.52
Early[5]	362.39 ± 17.48	446.98 ± 8.99	519.19 ± 11.97	950.00 ± 18.09	1025.81 ± 6.81	1070.34 ± 6.39	30.32 ± 6.39	42.41 ± 3.35	124.06 ± 11.91
Prelay[6]	363.36 ± 10.21	449.87 ± 11.36	485.89 ± 9.03	953.19 ± 7.22	1040.13 ± 6.53	1065.89 ± 6.90	29.67 ± 6.24	44.25 ± 5.29	99.81 ± 9.06
Peak[7]	368.08 ± 9.38	457.14 ± 14.51	498.05 ± 13.78	959.52 ± 9.91	1034.18 ± 4.20	1059.66 ± 9.07	31.55 ± 5.79	48.13 ± 4.47	96.21 ± 10.94
Late[8]	345.60 ± 12.90	427.46 ± 6.81	480.21 ± 7.31	967.67 ± 5.74	1031.07 ± 7.28	1061.74 ± 6.70	20.70 ± 6.48	50.42 ± 8.38	100.86 ± 7.32
Contrast P[9]	0.585	0.774	0.594	0.730	0.507	0.324	0.155	0.740	0.351
P-values	0.557	0.397	0.150	0.818	0.626	0.870	0.412	0.679	0.482

[a,b]Treatment means within the same column within effect with no common superscripts are significantly different ($P < 0.05$).

[1]Quantitative computed tomography scan at 30% of the length of the shank from the proximal epiphysis.

[2]Quantitative computed tomography scan at 50% of the length of the shank from the proximal epiphysis.

[3]PC: 69 µg of 25-hydroxycholecalciferol + 3,000 IU/kg of vitamin D_3 to 90 wk of age.

[4]NC: 3,000 IU/kg of vitamin D3 from 0 to 90 wk of age.

[5]Early: PC diet from 0 to 15 wk of age then switched to NC to 90 wk of age.

[6]Prelay: PC diet from 0 to 17 wk of age then switched to NC to 90 wk of age.

[7]Peak: PC diet to 34 wk of age then switched to NC to 90 wk of age.

[8]Late: PC diet to 50 wk of age then switched to NC to 90 wk of age.

[9]Orthogonal contrasts were performed to compare treatments fed 25 hydroxycholecalciferol in all dietary phases to that point with the NC treatment. When a treatment switched from 25 hydroxycholecalciferol to vitamin D_3 it was removed from the analysis. For example, at wk 15 of age NC was contrasted against the rest of the treatments. At wk 34, NC was contrasted with PC, Peak and Late, whereas Prelay treatment was removed from the analysis. At wk 90, NC was contrasted with PC.

Table 3.7 Effects of 25-hydroxycholecalciferol on cross sectional areas of shank bones of Hy-Line Brown hens at 15, 34 and 90 wk of age.

	Total			Cortical			Trabecular + Medullary		
Proximal[1]	15 wk	34 wk	90 wk	15 wk	34 wk	90 wk	15 wk	34 wk	90 wk
	---------- mm^2 ----------								
PC[3]	40.40 ± 1.10	42.06 ± 1.33	46.59 ± 1.21	12.65 ± 0.38	16.41 ± 0.38	16.85 ± 0.39	27.55 ± 0.96	25.15 ± 1.03	28.39 ± 0.94
NC[4]	40.21 ± 1.15	43.63 ± 1.26	45.38 ± 1.13	12.45 ± 0.35	16.56 ± 0.37	17.32 ± 0.44	27.65 ± 0.92	26.60 ± 1.18	27.03 ± 0.85
Early[5]	39.42 ± 1.47	42.86 ± 1.48	44.30 ± 0.66	13.00 ± 0.34	16.24 ± 0.44	17.63 ± 0.32	26.34 ± 1.42	26.25 ± 1.01	26.37 ± 0.50
Prelay[6]	39.49 ± 1.21	44.00 ± 0.96	44.75 ± 0.82	12.17 ± 0.20	16.63 ± 0.47	16.68 ± 0.34	27.15 ± 1.14	27.04 ± 0.75	27.71 ± 0.71
Peak[7]	39.53 ± 0.97	42.32 ± 0.86	44.64 ± 1.13	12.46 ± 0.34	16.32 ± 0.42	17.01 ± 0.36	26.85 ± 0.75	25.58 ± 1.05	27.23 ± 0.98
Late[8]	40.07 ± 1.14	44.47 ± 1.30	44.30 ± 1.00	12.33 ± 0.30	16.80 ± 0.22	16.43 ± 0.32	27.56 ± 1.16	27.15 ± 0.93	27.37 ± 0.79
Contrast P[9]	0.678	0.623	0.434	0.852	0.916	0.916	0.549	0.634	0.294
P-values	0.950	0.602	0.661	0.540	0.866	0.866	0.941	0.690	0.494
Middle[2]									
PC[3]	31.06 ± 0.78	32.91 ± 0.67	36.48 ± 0.90	11.12 ± 0.19	13.63 ± 0.25	14.51 ± 0.33	20.17 ± 0.72	19.57 ± 0.71	21.88 ± 0.87
NC[4]	30.66 ± 0.96	34.22 ± 1.20	36.26 ± 0.71	11.17 ± 0.34	14.03 ± 0.26	14.53 ± 0.33	19.60 ± 0.70	21.01 ± 0.94	21.63 ± 0.63
Early[5]	31.01 ± 0.94	34.25 ± 1.04	35.22 ± 0.59	11.38 ± 0.35	14.05 ± 0.53	14.76 ± 0.29	19.60 ± 0.75	19.93 ± 0.64	20.35 ± 0.54
Prelay[6]	29.55 ± 0.75	34.44 ± 0.79	34.97 ± 0.72	11.08 ± 0.24	14.07 ± 0.29	14.07 ± 0.24	19.00 ± 0.40	20.30 ± 0.68	21.13 ± 0.66
Peak[7]	30.43 ± 0.68	33.25 ± 0.49	35.20 ± 0.84	11.25 ± 0.24	13.86 ± 0.29	14.49 ± 0.24	19.33 ± 0.48	19.39 ± 0.64	20.61 ± 0.77
Late[8]	30.98 ± 0.62	35.72 ± 0.98	35.06 ± 0.71	10.95 ± 0.29	13.85 ± 0.29	13.93 ± 0.24	20.17 ± 0.67	21.78 ± 0.73	21.15 ± 0.59
Contrast P[9]	0.953	0.844	0.844	0.946	0.436	0.968	0.942	0.471	0.814
P-values	0.650	0.283	0.641	0.948	0.896	0.256	0.649	0.273	0.605

[a,b]Treatment means within the same column within effect with no common superscripts are significantly different ($P < 0.05$).

[1]Quantitative computed tomography scan at 30% of the length of the shank from the proximal epiphysis.

[2]Quantitative computed tomography scan at 50% of the length of the shank from the proximal epiphysis.

[3]PC: 69 µg of 25-hydroxycholecalciferol + 3,000 IU/kg of vitamin D_3 to 90 wk of age.

[4]NC: 3,000 IU/kg of vitamin D_3 from 0 to 90 wk of age.

[5]Early: PC diet from 0 to 15 wk of age then switched to NC to 90 wk of age.

[6]Prelay: PC diet from 0 to 17 wk of age then switched to NC to 90 wk of age.

[7]Peak: PC diet to 34 wk of age then switched to NC to 90 wk of age.

[8]Late: PC diet to 50 wk of age then switched to NC to 90 wk of age.

[9]Orthogonal contrasts were performed to compare treatments fed 25 hydroxycholecalciferol in all dietary phases to that point with the NC treatment. When a treatment switched from 25 hydroxycholecalciferol to vitamin D_3 it was removed from the analysis. For example, at wk 15 of age NC was contrasted against the rest of the treatments. At wk 34, NC was contrasted with PC, Peak and Late, whereas Prelay treatment was removed from the analysis. At wk 90, NC was contrasted with PC.

Table 3.8 Effects of 25-hydroxycholecalciferol on bone mineral content of shank bones of Hy-Line Brown hens at wk 15, 34 and 90 of age.

Proximal[1]	Total			Cortical			Trabecular + Medullary		
	15 wk	34 wk	90 wk	15 wk	34 wk	90 wk	15 wk	34 wk	90 wk
	mm/cm								
PC[3]	12.37 ± 0.42	17.16 ± 0.43	19.24 ± 0.51	11.70 ± 0.40	15.82 ± 0.34	16.15 ± 0.40	0.77 ± 0.27	1.23 ± 0.21	2.73 ± 0.18
NC[4]	12.72 ± 0.70	17.03 ± 0.48	19.46 ± 0.62	11.31 ± 0.39	15.54 ± 0.29	16.41 ± 0.39	1.10 ± 0.27	1.40 ± 0.19	2.72 ± 0.22
Early[5]	12.69 ± 0.58	16.81 ± 0.66	20.39 ± 0.48	12.09 ± 0.40	15.48 ± 0.54	17.08 ± 0.30	0.80 ± 0.28	1.33 ± 0.21	3.27 ± 0.34
Prelay[6]	11.82 ± 0.41	17.47 ± 0.59	18.99 ± 0.45	11.07 ± 0.22	16.23 ± 0.49	16.05 ± 0.34	0.95 ± 0.25	1.16 ± 0.21	2.94 ± 0.28
Peak[7]	12.46 ± 0.39	16.90 ± 0.44	19.07 ± 0.53	11.34 ± 0.33	15.43 ± 0.45	16.28 ± 0.38	1.11 ± 0.22	1.41 ± 0.07	2.76 ± 0.23
Late[8]	12.07 ± 0.57	17.78 ± 0.52	19.02 ± 0.35	11.49 ± 0.32	16.07 ± 0.22	15.71 ± 0.26	0.91 ± 0.275	1.60 ± 0.28	3.23 ± 0.22
Contrast P[9]	0.565	0.665	0.788	0.611	0.629	0.078	0.353	0.975	0.974
P-values	0.816	0.833	0.335	0.450	0.454	0.646	0.552	0.840	0.453
Middle[2]									
PC[3]	10.79 ± 0.24	14.50 ± 0.36	17.13 ± 0.45	10.67 ± 0.23	13.96 ± 0.26	15.28 ± 0.34	0.42 ± 0.15	0.75 ± 0.17	1.93 ± 0.19
NC[4]	11.15 ± 0.38	14.86 ± 0.50	17.51 ± 0.52	10.67 ± 0.31	14.36 ± 0.25	15.27 ± 0.31	0.65 ± 0.10	0.97 ± 0.17	2.32 ± 0.28
Early[5]	11.20 ± 0.52	15.30 ± 0.54	18.26 ± 0.45	10.82 ± 0.41	14.41 ± 0.54	15.80 ± 0.30	0.59 ± 0.12	0.85 ± 0.08	2.49 ± 0.23
Prelay[6]	10.72 ± 0.36	15.45 ± 0.35	16.95 ± 0.31	10.51 ± 0.24	14.64 ± 0.34	14.99 ± 0.25	0.56 ± 0.12	0.89 ± 0.11	2.14 ± 0.22
Peak[7]	11.19 ± 0.29	15.16 ± 0.37	17.21 ± 0.39	10.79 ± 0.25	14.34 ± 0.32	15.36 ± 0.28	0.61 ± 0.11	0.92 ± 0.08	1.94 ± 0.20
Late[8]	10.67 ± 0.32	15.27 ± 0.49	16.79 ± 0.35	10.60 ± 0.29	14.28 ± 0.28	14.78 ± 0.21	0.40 ± 0.12	1.13 ± 0.21	2.15 ± 0.18
Contrast P[9]	0.581	0.840	0.220	0.981	0.981	0.980	0.182	0.867	0.262
P-values	0.798	0.607	0.581	0.977	0.977	0.185	0.506	0.738	0.490

[a,b]Treatment means within the same column within effect with no common superscripts are significantly different ($P < 0.05$).

[1]Quantitative computed tomography scan at 30% of the length of the shank from the proximal epiphysis.

[2]Quantitative computed tomography scan at 50% of the length of the shank from the proximal epiphysis.

[3]PC: 69 µg of 25-hydroxycholecalciferol + 3,000 IU/kg of vitamin D_3 to 90 wk of age.

[4]NC: 3,000 IU/kg of vitamin D_3 from 0 to 90 wk of age.

[5]Early: PC diet from 0 to 15 wk of age then switched to NC to 90 wk of age.

[6]Prelay: PC diet from 0 to 17 wk of age then switched to NC to 90 wk of age.

[7]Peak: PC diet to 34 wk of age then switched to NC to 90 wk of age.

[8]Late: PC diet to 50 wk of age then switched to NC to 90 wk of age.

[9]Orthogonal contrasts were performed to compare treatments fed 25 hydroxycholecalciferol in all dietary phases to that point with the NC treatment. When a treatment switched from 25 hydroxycholecalciferol to vitamin D_3 it was removed from the analysis. For example, at wk 15 of age NC was contrasted against the rest of the treatments. At wk 34, NC was contrasted with PC, Peak and Late, whereas Prelay treatment was removed from the analysis. At wk 90, NC was contrasted with PC.

4. RESEARCH SYNTHESIS

4.1 INTRODUCTION

The total number of eggs produced worldwide increased by 39% from 1995 to 2005 (Scanes, 2007) and by another 39% from 2005 to 2015, with a production of 1,340 billion eggs in 2015 (Conway, 2016). This steady growth has been achieved through continuous advances in technology, genetic selection, management, and nutrition. Nevertheless, these significant changes have also caused adverse traits in eggshell quality (al-Batshan et al., 1994), bone strength (Rath, 2000), and animal welfare (Yilmaz Dikmen, 2016). Vitamin D_3 plays a major role in allowing birds to lay eggs with strong and thick shells (El-Maksoud, 2010; Plaimast et al., 2015), along with healthy bones (Fritts and Waldroup, 2003) and promoting well-being (Sun et al., 2013). Compared to vitamin D_3, dietary supplementation of the vitamin D_3 metabolite, 25-hydroxycholecalciferol (25OHD), has resulted in greater egg production (Ding et al., 2011), eggshell quality (Al-Zahrani and Roberts, 2014) and bone density (Rivera et al., 2014). In addition, 25OHD supplementation promotes intestinal maturation in the chick (Chou et al., 2009), and modulation of the immune system (Morris et al., 2015). The aim of the study was to evaluate the effects of feeding 25OHD plus vitamin D_3 on laying hen growth, productivity, eggshell quality and bone traits, from day 1 to the end of the production cycle under commercial conditions. Additionally, due to two bacterial outbreaks that occurred during the trial, we were able to evaluate the influence of dietary 25OHD on egg production, feed efficiency, and mortality in light of the effects of 25OHD immune responses reported in the literature.

A field trial was conducted in Colombia. Treatments were: a Positive Control (PC) with 25OHD plus vitamin D_3 at 2,760 and 3,000 IU/kg, respectively during the entire trial, a Negative Control (NC) with vitamin D_3 only (3,000 IU/kg) through the entire study, and four treatments

with the PC diet from day 1 to either 15 (Early), 17 (Prelay), 34 (Peak), or 50 (Late) wk of age, and then switched to the NC diet until 90 wk of age. Egg production, efficiency, and shell quality were analyzed up to 87 wk, and bone traits up to wk 90 of age.

4.2 SUMMARY

The objectives were achieved in the thesis research by testing the following hypotheses:

1. **That dietary 25OHD in addition to vitamin D_3 would enhance layer pullet growth and development, under field conditions in Colombia.**

This hypothesis was addressed in Chapter 2, by evaluating the performance of pullets including body weight, feed intake, feed conversion ratio, and livability. Body weight was measured at five time points during rearing and three time points during production (with each dietary phase change). The hypothesis was partially supported by the results, especially at early ages. Dietary 25OHD decreased FCR and enhanced BW at 3 and 8 wk of age. Dietary 25OHD also promoted FI up to 12 wk of age compared to vitamin D_3 alone. Conversely, FCR was lower in birds fed only vitamin D_3 from 9 to 12 wk of age.

2. **That dietary 25OHD in addition to vitamin D_3 would increase egg production, laying hen performance and livability under commercial conditions in Colombia.**

This hypothesis was addressed in Chapter 2 as well: body weight, egg production, feed intake, feed conversion ratio and livability were measured and analyzed. Egg production, feed intake and mortality were recorded on a daily basis and analyzed at each diet change during the production cycle. Body weight was measured at each diet change, and the effect of the bacterial infections was also considered in this chapter. This hypothesis was partly supported by the

results. Compared to vitamin D_3, birds fed 25OHD to 34 wk of age had lower BW than NC at that point, whereas the PC and Peak groups had lower FCR (kg of feed per dozen of eggs) and greater egg production compared to the NC treatment group. During this period, birds experienced the first bacterial outbreak which resulted in decreased egg production. Birds fed 25OHD (PC, Peak, and Late) started laying eggs sooner, reached a higher peak of production and had a more rapid recovery after the bacterial infection. From 76 to 87 wk of age, NC hens had lower FCR compared to the Early group, whereas no differences in egg production were found among treatments during the second bacterial outbreak. At the end of the trial, cumulative results revealed that Peak hens had lower FCR compared to Prelay and Late groups, and higher egg production compared to Early, Prelay and NC hens. Moreover, orthogonal contrast revealed that PC hens had greater cumulative egg production when compared to the NC hens. No differences in livability were found among treatments during the whole trial.

3. **That dietary 25OHD in addition to vitamin D_3 would enhance bone traits and eggshell quality of laying hens under commercial conditions in Colombia.**

This hypothesis was addressed in Chapter 3, in which shank length was measured in selected birds during rearing every two wk. Shank breaking strength (BBS), bone mineral density and cross-sectional area (using quantitative computed tomography) were measured at three time points (15, 34 and 90 wk of age). In addition, eggs were sampled every two to three wk during the production period to measure eggshell strength (ESS) and thickness. This hypothesis was only partially supported. Orthogonal contrast showed that birds fed 25OHD up to 18 wk (PC, Peak, and Late treatments) had longer shanks than NC birds, but no consistent differences were found from 2 to 18 wk. Overall results from 24 to 87 wk of age revealed that the PC treatment resulted in higher ESS than the Early treatment, whereas the PC and Early

groups had greater shell thickness compared to the NC group. Orthogonal contrast showed that birds fed 25OHD throughout the trial had greater eggshell quality in older ages (after 53 wk of age) compared to NC. At 34 wk, cortical density at 30% of the total length from proximal epiphysis of NC hens was the lowest among all treatments, but the difference was not statistically significant (P= 0.076). At the end of the trial, NC hens had lower BBS than Early hens. Orthogonal contrast showed that the PC treatment resulted in greater ESS and thickness in the overall results and higher BBS at 90 wk than the NC treatment.

4.3 ANALYSIS AND IMPLICATIONS

The achievement of body weight targets, the development of sound immune and digestive systems, and a strong skeletal frame are important in proper pullet rearing. Despite the importance of the rearing period in the hen's future productivity, published reports on the effects of dietary 25OHD on pullet growth and development, and laying hen productivity are limited. In the present study, the supplementation of 25OHD in addition to vitamin D_3 (2,760 IU/kg and 3,000 IU/kg, respectively) reduced FCR during the first three wk of age and increased body weight up to 8 wk of age compared to vitamin D_3 alone. These results could be due to the enhanced rate of nutrient absorption caused by the longer villus length, and shorter crypt depth reported when 25OHD was added on top of vitamin D_3 in broilers (Chou et al. 2009; Ding et al., 2011). Additionally, 25OHD supplementation resulted in longer shanks compared to the NC treatment, indicating an enhancement of skeletal development (Chapter 3). Even though no further differences in BW were reported during rearing, or in bone strength or density at 15 wk of age, continuous supplementation of 25OHD up to 34 wk of age resulted in a more productive and efficient laying hen, as seen from onset of lay to 34 wk of age (Chapter 2). The effect of 25OHD on growth and intestinal development on young pullets should be further studied.

Considering the importance of the rearing period on production performance, our results indicate that 25OHD supplementation plus industrial levels of vitamin D_3 could be an effective to enhance development and growth of young pullets. This result leads to new questions regarding the specific effects of 25OHD on laying pullet growth, intestinal development, and the potential triggered mechanisms (such as mTOR in broilers; Vignale et al., 21015); which may be studied in depth in future research.

The current study was conducted on two farms of the second-largest egg production company in Colombia (Nutriavicola S.A.; WATT, 2014), which produces its own feed, and has well-standardized processes. Through field trials, researchers and producers can evaluate the effect of certain products and the animal response under commercial conditions, where many factors are uncontrolled and commonly interrelate (weather, proximity to other farms, endemic diseases, particular vaccination programs). This proximity to "reality", along with studies under controlled environmental conditions, brings tools to both scientists and producers in the generation of reliable and useful information. For instance, the sudden bacterial outbreak (indicative of infectious coryza) allowed us to evaluate the effect of dietary 25OHD supplementation on egg production, performance, and mortality as birds experienced the infection. Previous studies have shown an effect of dietary 25OHD on immune responses during experimental bacterial challenges including higher levels of serum antibodies (IgG) and greater phagocytosis (Chou et al., 2009), higher growth performance and nitric oxide production, and reduced inflammation (Morris and Selvaraj., 2014; Morris et al., 2014b). Infectious coryza is a bacterial disease that decreases egg production from 10 to 40% (Akter et al., 2013), and is considered a relatively common infectious disease in Colombia (ICA, 2013). Although no specific measures of immune response were performed during the study, birds fed 25OHD had

higher egg production and lower FCR compared to NC from 18 to 34 wk of age (the first outbreak occurred at 26 wk of age), which may imply these hens were less affected by the infection during this period. From this perspective, a producer who identifies the vulnerability of his facilities to certain bacterial diseases may consider 25OHD supplementation as part of a program to protect birds from the effects of potential infections, in crucial times such as peak of production.

Since 25OHD bypasses liver metabolism, its effects compared to vitamin D_3, are more evident when the hepatic function and hydroxylation capacity are limited. Bacterial infections (Peighambari et al., 2000), stress (Wideman et al., 2015; Odihambo Mumma et al., 2006) or liver disease (Yarger et al., 1995; Stokes et al., 2013) are scenarios where 25OHD may show greater effects than vitamin D_3. By the beginning of egg production, hens start to use available nutrients to form the eggs in addition to meeting maintenance requirements; in our study, the bacterial infection may have influenced the effects of 25OHD on egg production and FCR.

Birds from the NC treatment had higher FCR and body weight at 34 wk of age compared to 25OHD-fed groups; this suggests that dietary 25OHD stimulated the partitioning of more nutrients toward egg formation rather than body weight relative to vitamin D_3. PC and Peak hens produced 4.3 to 4.6% more eggs than NC from 18 to 34 wk of age; whereas Peak hens laid 3% more eggs than NC during the whole production cycle. These results highlight the importance of reaching a high peak of production, as no difference in egg production were seen in any other period of the production cycle. Still, Peak treatment resulted in significantly higher cumulative egg production than NC until the end of the trial. Based on our results and those reported in the literature, 25OHD supplementation beginning at rearing may promote the development of a

sound skeletal frame, a robust immune and digestive system, which ultimately allow the hen to maintain better productivity and skeletal health during long production cycles.

As observed in Chapter 3, dietary 25OHD did not affect eggshell strength nor thickness at any of the analyzed time points, but showed greater thickness (after 53 wk of age) and strength (after 75 wk of age) when PC and NC were compared through orthogonal contrast. Therefore, 25OHD supplementation during the whole cycle has the potential to maintain eggshell quality compared to vitamin D_3 at older ages, where hens normally produce larger eggs with thinner and weaker shells (Abe et al., 1982; al-Batshan et al., 1994). This outcome was also reported in previous studies combining dietary vitamin D_3 and 25OHD in old hens from 70 to 80 wk of age (Garcia-Hernandez et al., 2001; Korelski and Świątkiewicz. 2005). From the producer standpoint, eggs with greater shell quality reduce losses due to fissured or broken eggs during collecting, processing and transporting the product to its final destination. Besides, groups fed 25OHD from day 1 showed higher cortical density at 34 wk compared to birds fed vitamin D_3 alone, which means that birds may use dietary calcium more efficiently from the beginning of production to 34 wk to form the eggshells, since no differences were reported in either medullary bone traits or eggshell strength and thickness during this specific period. Interestingly, NC birds had lower BBS than Early hens at 90 wk of age, whereas other treatment groups had intermediate BBS, not different from either of these treatments; therefore, there might be a point were 25OHD supplementation has no further effect on bone strength. This particular result could explain why some studies evaluating the effect of 25OHD relative to vitamin D_3 showed enhancements in bone traits, while other research did not. The enhancement of pullet BW and shank length during rearing, plus the response of the Early treatment in overall shell thickness and BBS at 90 wk of age, mark the potential importance of 25OHD addition during rearing and

its effect on bone status in old hens. However, orthogonal contrast revealed that hens supplemented with 25OHD throughout life (PC) had stronger bones at 90 wk of age than NC hens. A study focused on bone development, and maintenance during production, with more frequent sampling over time, is recommended for future investigations.

Overall, faster growth in the early stages of life and earlier attainment of peak production, with greater cumulative egg production and higher efficiency up to 34 wk of age are the main findings of this study. These important effects took place in critical stages of the birds's life, when the immune and digestive systems develop in the immature bird, and when birds reach their physical maturity and lay eggs to maximum yield. In addition, the effects of 25OHD on eggshell quality showed a positive effect after 53 wk of age without compromising bone integrity over time. A reasonable recommendation to a producer would be the supplementation of 25OHD throughout rearing, instead of its addition after the onset of egg production. Enhancement of pullet growth along with advanced skeletal development may reduce the occurrence of poor eggshell quality, especially in long laying cycles. Finally, studies on vitamin D_3, its metabolites and vitamin D analogs continue to generate interest in human and animal research (DeLuca, 2008). The comprehension of all its functions remains to be known.

4.4 RECOMMENDATION FOR FUTURE RESEARCH

The present study offers a broad perspective for future experiments on the effects of 25OHD plus vitamin D_3 at commercially-relevant levels on laying hen development and overall productivity, and under commercial conditions. To our knowledge, no studies have been performed covering a complete cycle from placement, from day one to depopulation. It will be significant to identify the molecular mechanisms influenced by 25OHD supplementation that ultimately enhanced early BW, and egg production from the onset of lay to peak, with or without

bacterial challenges. Some of these effects have been studied in broilers but to a limited extent in egg-type pullets. A similar trial comparing brown and white hens, under different housing systems, could help to understand how 25OHD effects in shell quality and bone health may differ in conventional cage systems (Silversides et al., 2012), or in birds that are more prone to have skeletal problems (Riczu et al., 2004). It would be interesting to assess the progression of bone traits (including using technologies as micro-computed tomography) and its relationship with eggshell quality in more age points over time.

4.5 STUDY LIMITATIONS

Field trials offer the possibility to test the effects of certain products or conditions, beyond the experimental laboratory, under conditions that are closer to the reality of the average producer environment and could give them greater confidence in the results if those are in agreement with trials made under experimental settings. Nevertheless, factors such as the company logistics, housing conditions, or potential changes in health status, may also bring limitations. In this study, the well-standardized procedures of the company, the proper management of each experimental unit, plus the sample size and distribution of the birds helped to overcome some of these initial limitations.

An alternative experimental design would have included control diets with the same levels of vitamin D activity as the Positive Control (5,760 IU/kg total vitamin D) provided as either vitamin D_3 or 25OHD alone, and data collection of more time points to evaluate the effect of 25OHD in the hen's life cycle. However, the inclusion of those levels of vitamin D_3 or 25OHD, and the inclusion of more treatment groups would have required a greater logistical effort by the company. The number of egg samples for eggshell quality and birds to evaluate bone traits was limited in quantity and frequency since a greater number of sampled eggs or birds

would have negatively affected the profitability of the flock. However, the number of replicates per treatment during production, and the proper management of all the pens as a whole population partially overcome these limitations. The study brought the opportunity to discriminate egg grade, the percentage of broken or cracked eggs; unfortunately, due to the logistics of the company a proper collection of this data was not possible. On the other hand, the bacterial infections that took place at 26 and 84 wk of age may have influenced the interpretation of the results; still, these unexpected events allowed evaluating other reported effects of 25OHD in the literature related to the immune function, as the birds' productivity and efficiency under a bacterial challenge (Morris et al., 2015).

Having a 90 wk field trial may bring challenges in the supervision and control of research conditions. Even though the lack of permanent presence of a research team member was a limitation, frequent visits to the farms on the main periods of the trial, regular contact with the professionals in charge of the farms, and constant flow of information favored an adequate control of the experiment.

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