ROLE OF VITAMIN D3 AND ITS METABOLITES ON CHICKEN OSTEOBLAST DIFFERENTIATION, LAYER BONE 3-DIMENSIONAL STRUCTURE DEVELOPMENT AND LAYING PERFORMANCE

by

CHONGXIAO CHEN

(Under the Direction of WOO-KYUN KIM)

ABSTRACT

The effects of vitamin D_3 on bone metabolism are complicated. There is limited study of vitamin D_3 on chicken osteoblasts differentiation and mineralization. Furthermore, the application of vitamin D_3 metabolites, such as 25-hydroxyvitamin D_3 on laying hens has shown various effects on egg production and bone quality. Seldom studies have focused on the long-term supplementation of 25-hydroxyvitamin D_3 on laying hen. At last, the unique characteristics of laying hen bone make it an attractive research model to understand further laying hen bone physiology as well as to develop a new strategy to alleviate osteoporosis. In this case, the serial studies were conducted to understand the vitamin D_3 molecular actions on chicken osteoblasts, the long-term supplementation of 25-hydroxyvitamin D_3 on layer bone development and laying performance, as well as modeling layer bone development pattern. In the first study, 1,25-hydroxyvitamin D_3 at various concentrations were subjected to treatment with

chicken osteoblasts to understand its role in osteoblast differentiation and mineralization. The results showed the role of vitamin D_3 on osteoblasts has a close relationship with cell differentiation. It mainly acted as an inhibitor on cell differentiation and mineralization during the early stage, but a promoter during the late stage. Furthermore, a study exploring the effects of long-term supplementation of 25-hydroxyvitamin D_3 in diets on layer bone development and egg laying performance were performed. The results showed early (0-60wk) supplementation of 25-hydroxyvitamin D_3 in the diets could increase bone structure size, subsequently, benefit the bone health. Moreover, 25-hydroxyvitamin D_3 did not have major effects on eggshell quality, but it increased the egg production up to 60wk. The research results highlight the importance of early and long-term supplementation of 25-hydroxyvitamin D_3 on bone development and egg production. At last, a bone modeling trial was conducted to understand the layer bone development pattern. The data showed there were dramatic changes in bone development pattern at the beginning of laying, evidenced by the significant increases in bone volume, bone mineral density, and bone mineral content. This indicates the layer nutrition at the point of producing the first egg may be critical for egg production and skeletal integrity during the later laying period.

INDEX WORDS: vitamin D_3, chicken osteoblasts, layer bone structure, laying performance, layer bone modeling

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DEDICATION

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CHAPTER 1

INTRODUCTION

Vitamin D_3 needs two biological conversions to become its bioactive form: 1,25-dihydroxyvitamin D_3. This process is strictly regulated (DeLuca, 2004; Haussler, et al., 2011; van Driel and van Leeuwen, 2014; Zierold, et al., 1995). The effects of vitamin D_3 on a living organism are intricated and involved in multiple systems, including the immune system, anti-oxidation, anti-cancer actions, cardiovascular benefits, mineral hemostasis and bone developments (Gil, et al., 2018; Haussler, et al., 2013). The general effects of vitamin D_3 on laying hens and broilers are promoting bone development, preventing bone fracture, maintenance the egg production and eggshell quality, and modulates immune responses (Rodriguez-Lecompte, et al., 2016; ŚWiĄTkiewicz, et al., 2017).

The understanding of vitamin D_3 molecular actions on chicken osteoblasts is limited. However, from other species, vitamin D_3 showed inconsistent results as either suppressive or provocative on osteoblasts differentiation and mineralization (St-Arnaud, 2008; Tarroni, et al., 2012; van Driel and van Leeuwen, 2014). There is still no completed explanation on the discrepancy results cross or within species. The chicken osteoblasts, with high calcifying activity, is an attractive research model which can give

supplementary data and contribute to the understanding of vitamin D_3 function in bone formation (Pande, et al., 2015).

Although the molecular actions of vitamin D_3 on bones are complicated, the applications of vitamin D_3 in the chicken diets are most beneficial to the avian bone formation (Atencio, et al., 2005b; Kim, et al., 2011). Especially the 25-hydroxyvitamin D_3 (25OHD) showed higher bioactivity compare to the standard vitamin D_3 in chicken diets (Atencio, et al., 2005a; Soares Jr, et al., 1995). However, the effects of 25OHD on layer bone quality and laying performance are inconstant (Käppeli, et al., 2011; Keshavarz, 2003; Koreleski and Świątkiewicz, 2005; Mattila, et al., 2011; Nascimento, et al., 2014; Roland and Harms, 1976; Silva, 2017; ŚWiĄTkiewicz, et al., 2017; Torres, et al., 2009). It may be related to the supplementation timing and duration. However, there is a lack of long-term study of evaluation of 25OHD on layer bone development and laying performance.

Laying hen osteoporosis is a serious animal welfare concern for the poultry industry (Webster, 2004). A significant number of researches focusing on osteoporosis and bone quality expanded our knowledge of the factors affecting laying hen bone quality (Webster, 2004; Whitehead, 2004; Whitehead and Fleming, 2000). In order to develop a strategy to alleviate osteoporosis in laying hens, a sound understanding of layer bone development is required. Using statistical models to describe the bone-related parameters can help us understanding the bone development pattern, which will provide a sound basis for developing a new strategy to enhance layer bone health. However, to our knowledge, there are some broiler bone modeling studies or periodically bone quality evaluations showed in the previous literature (Biewener, et al., 1986; Prisby, et al., 2014),

but no study is performed targeting on statistical modeling of modern layer bone development.

Under this research background, we conducted serial studies to explore the role of vitamin D_3 and its metabolites on chicken osteoblast differentiation, layer bone structure development and laying performance.

The main objectives of this study were:

1. To explore the role of 1,25-dihydroxyvitamin D_3 on chicken osteoblasts differentiation and mineralization

2. To evaluate the role of long-term supplementation of 25-hydroxyvitamin D_3 in layer diets on bone development and laying performance.

3. To understand the bone development pattern of modern layers.

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CHAPTER 2

LITERATURE REVIEW

1. The introduction of Vitamin D_3

Ergocalciferol (Vitamin D_2) and cholecalciferol (vitamin D_3) are two primary forms of vitamin D. Vitamin D_2 is plant origin, whereas vitamin D_3 is commonly presented in animal tissues. The bioactivity of Vitamin D_3 is much higher than vitamin D_2 in chicken. It is because the affinity of vitamin D binding proteins (DBP) in chicken plasma is much lower for circulating form of vitamin D_2. However, for mammalian, DBP affinity is similar for both (DeLuca, et al., 1988; Holick, 1989), but plasma concentration of DBP is much higher in laying hens compared with immature birds or adult males (Dacke, et al., 2015; Hurwitz, 1989). Regarding the function, the effects of vitamin D_3 on human and animals is intricated and involved in multiple systems, including the immune system, anti-oxidation, anti-cancer actions, cardiovascular benefits, mineral hemostasis and bone developments (Gil, et al., 2018; Haussler, et al., 2013).

2. Vitamin D_3 metabolism and regulation

2.1 vitamin D_3 metabolism and catabolism

Vitamin D_3 could be obtained from conversion of 7-dehydrocholesterol in the skin under the 290-315nm UV light (Deluca, 2014; Holick, et al., 1980) or directly from the diets. However, the modern chicken flocks are mostly kept entirely indoors (without direct sunlight). The primary source of vitamin D_3 is from diets as a form of vitamins premix (ŚWiĄTkiewicz, et al., 2017).

Vitamin D_3 is biologically inactive in animal organisms (DeLuca, 2004). Therefore, it requires two biological conversions to become an active form. First, vitamin D_3 is hydroxylated at the C-25 in the liver by microsomal CYP2R1 (25-hydroxylase) to become its major circulating form of 25-hydroxyvitaminD_3(25OHD). Then mainly in the kidney, it becomes a bioactive form of 1, 25-dihydroxyvitamin D_3 (1,25OHD) catalyzed by mitochondrial CYP27B1 (1α-hydroxylase). The 1,25OHD either binds on vitamin D receptor (VDR) to induce a number of vitamin responsive elements (VDREs) expression to exert genomic function or through membrane-associated receptor to induce nongenomic function (Haussler, et al., 2011; van Driel and van Leeuwen, 2014). The 1,25OHD and 25OHD metabolized by CYP24A (24-hydroxylase) in liver, to become 1,24,25OHD or 24,25OHD (Figure 2.1). However, these metabolites still showed some biological activities. The 24,25OHD is required for normal mineralization and homeostasis of calcium in chicken (Norman, et al., 1980). Kato, et al. (1998) showed 24,25OHD along with 1,25OHD improved bone mechanical strength in the vitamin D receptor knock-out chick model. Furthermore, the presence of 24,25OHD is essential for both normal bone integrity and the bone healing process in chicks with rickets (Ornoy, et al., 1978; Seo, et al., 1997). At last, the 24,25OHD3 was found to be involved in egg hatchability (Henry and Norman 1978), but it did not play a significant role in calcium transportation in laying hens (Grunder, et al. 1990).

2.2 vitamin D_3 metabolism regulation

The production of 1,25OHD is tightly regulated (Figure 1). 1,25OHD induce CYP24A synthesis to avoid access 1,25OHD formation (Zierold, et al., 1995). It is

mainly due to the 1,25OHD-VDR action on VDREs contains the promoters of gene-coding 24-hydroxylase (DeLuca, 2004). Besides this short negative feedback loop, the renal 1α-hydroxylation of 25OHD is tightly regulated by parathyroid hormone (PTH, produced by parathyroid), fibroblast growth factor 23 (FGF23, mainly produced by osteocytes), and estrogen.

CYP27B1 is principally regulated by PTH being the stimulator and FGF23 being the inhibitor. On the one hand, the parathyroid gland presents VDR (Brumbaugh, et al., 1975), while binding with 1,25OHD, suppresses the synthesis of PTH through a direct action on gene transcription (Demay, et al., 1992). This negative feedback loop neutralizes the stimulation of CYP27B1 activity by PTH when low-calcium circulation presented in the system, serves to limit the bone-resorbing effects of PTH. On the other hand, 1,25OHD induces the release of FGF23 from bone, specifically from osteocytes. The CYP24A1 gene is transcriptionally activated by 1,25OHD (Ohyama, et al., 1994) and FGF23. Meanwhile, the CYP27B1 gene is repressed by FGF23 and 1,25OHD, to achieve a net effect of reducing the amount of 1,25OHD in the system (Murayama, et al., 1999). Estrogen can increase 1α-hydroxylase activity and significantly suppress 24-hydroxylase activity. It is demonstrated by injecting estradiol-17β into a male bird, which completely suppressed the 24-hydroxylase and dramatically increased the 1α-hydroxylase activity (Tanaka, et al., 1976). Similar results are shown as the ovariectomy inhibited 1,25OHD synthesis. While treating with estradiol, 1,25OHD synthesis was stimulated (Baksi and Kenny, 1978). Different from the strict regulation of 1,25OHD, the 25-hydroxylation of vitamin D_3 in the liver is not. The production of 25OHD is mainly mediated by the available substrate. Therefore, 25OHD is the main circulation form and a

reliable indicator of how much vitamin D entered the system and the conversion efficiency (Holick, 2007).

2.3 Vitamin D_3 metabolism in the bones

It is well established that 25OHD mainly converts to 1,25OHD in the kidney, then exert its endocrine function (Haussler, et al., 2013; Holick, et al., 1980). However, CYP27B1 (1α-hydroxylase) expression and activity are not restricted to the kidney. 1α-hydroxylase has been reported to express in lymph nodes, skin colon, pancreases, dendritic cell, brain, pulmonary alveolar macrophages, pathological parathyroid glands, prostate cells, and bones in various animal models (Adams and Hewison, 2012; Atkins, et al., 2007; Panda, et al., 2001; van Driel, et al., 2006; Zehnder, et al., 2001). Local production of 1,25OHD in extra-renal tissues has been postulated to be essential for cell growth and differentiation (Huang, et al., 2002). As one of the most critical vitamin D targeted organs — bone, previous studies showed the regulation of CYP27B1 and CYP24 mRNA expression in the bone was different than in the kidney. There are no suppression effects of 1,25OHD on CYP27B1 and CYP24 function (Anderson, et al., 2005). The expression of CYP27B1 on osteoblasts has been found in the rat (Weber, et al., 2003), and human (van Driel, et al., 2006). However, there is no any evidence whether CYP27B1 expressed on chicken osteoblasts. The missing gene sequence of 1α-hydroxylase make the researches on this part difficult.

3. Vitamin D_3 function

The effects of vitamin D_3 on human and animals is intricated and involved in multiple systems, including the immune system, anti-oxidation, anti-cancer actions, cardiovascular benefits, mineral hemostasis and bone developments (Gil, et al., 2018; Haussler, et al., 2013). The general effects of vitamin D_3 on laying hens and broilers were promoting bone development, preventing bone fracture, maintaining the egg production and eggshell quality, modulating immune responses, etc.(Rodriguez-Lecompte, et al., 2016; ŚWiĄTkiewicz, et al., 2017).

3.1 Vitamin D_3 on calcium and phosphate metabolism

For calcium and phosphate absorption, reabsorption and transportation in the intestine and kidney, 1,25OHD showed multiple effects. The interaction between 1,25OHD and FGF23, mentioned above, is negative feedback for 1,25OHD metabolism. FGF23 also inhibited phosphate reabsorption in the proximal tubule of the kidney by inhibiting Npt2a expression. Furthermore, a coreceptor, Klotho is needed to exert this function. Klotho is also up-regulated by 1,25OHD. Together, 1,25OHD induced both FGF23 and Klotho expression to inhibit phosphate reabsorption in the kidney. In addition, 1,25OHD increased the expression of Npt2a and Npt2c expression, and consequently increased the phosphate reabsorption (Masuda, et al., 2010). Npt2a, Npt2c, FGF23, and Klotho together mediated the P balance. The calcium reabsorption was similarly regulated by the expression of CaBPs and TRPVs in both kidney and intestine. Both were up-regulated by 1,25OHD to increase Ca absorption and reabsorption (Chang, et al., 2005). However, the type of CaBPs and TRPV are different in kidney and intestine, which are $CaBP_{28k}$/TRPV5 in kidney and $CaBP_{9k}$/TRPV6 in the intestines. The effects of

1,25OHD on eggshell gland calcium balance seems not efficient based on previous studies. Eggshell gland Ca^{2+} transportation-related protein such as calbindin D_{28k} and carbonic anhydrases, are vitamin D_3 independent (Bar, 2008).

3.2 Vitamin D_3 on bones

3.2.1 Vitamin D_3 on bone resorption — osteoblasts mediated

For bone metabolism, it is well known that the primary effect of 1,25OHD is to promote osteoclast function. 1,25OHD regulates osteoclast mainly via osteoblasts (Baldock, et al., 2006). Among these factors showed in the scheme (Figure 2.2), M-CSF (Macrophage Colony-stimulating Factor) binds to cFMS (Colony Stimulating Factor) presented in osteoclast precursors, and RANKL binds to RANK in osteoclasts to exert their stimulatory effects on cell proliferation and the inhibitory effects on apoptosis. However, OPG binds to RANKL preventing the RANKL/RANK singling results in the inhibition of osteoclastogenesis. 1,25OHD enhance the expression of RANKL, M-SCF, while inhibiting OPG, showed a catabolic effect on bones (Haussler, et al., 2010).

This mechanism of RANKL system was well studied in mice model but limited in the avian model. *In vitro*, under the absence of exogenous M-CSF, treating with human RANKL increased the survival of TRAP-positive osteoclasts (Boissy, et al., 2001). Under the presence of M-CSF, treating with chicken RNAKL also induced osteoclast-like multinucleated cells formed in a dosage-depend manner. Moreover, same as in mice model, RANKL stimulatory effect could be curtailed by OPG treatment in chicken (Wang, et al., 2008). Furthermore, both *in vivo* and in chicken primary osteoclast culture,

RANKL could increase the osteoclast resorption (Wang, et al., 2008). In addition, the survival of bone marrow-derived dendritic cells, and bone marrow-derived macrophages was enhanced by treating chicken RANKL as well (Sutton, et al., 2015b).

Both mouse and avian studies showed osteocytes highly expressed RANKL. A study showed the possibility of osteocyte physically connect with osteoclast in chicken calvaria (Sugawara, et al., 2005), which made osteocyte original RANKL could directly exert its effect on osteoclast, even though osteocytes are usually buried in mineralized bone mineral matrix. In other models, RANKL expression is regulated by 1,25OHD, PTH, PGE, glucocorticoids and some cytokines (Nakashima, et al., 2012). In the avian model, it showed that BMP2 (bone morphogenic protein 2) upregulated chondrocytes RANKL expression, consequently increased osteoclast resorption (Usui, et al., 2008). The RANK-RANKL-OPG systems between mammal and avian are similar, because the RANKL, RANK, and OPG as a member of TNF superfamily, are highly conserved between mammals and birds (Bai, et al., 2011; Sutton, et al., 2015a).

The mature female birds formed unique medullary bones, a non-structural osseous tissue, served as a temporary storage site for calcium that is needed for eggshell production (Whitehead, 2004). Medullary bone mineral content changes rapidly during the oviposition cycle, while huge amount of calcium is mobilized from this site (Kerschnitzki, et al., 2014b). This process involves in estrogen change, Ca level regulated PTH change, and the other factors fluctuation (Dacke, et al., 2015). The unique biology properties of medullary bone caught researchers' interest, rendering it as a good model for studying RANK-RANKL-OPG.

3.2.2 Vitamin D_3 on osteoblasts

Osteoblasts are derived from mesenchymal stem cells (MSCs), which plays an important role in bone formation. The MSCs can differentiate into chondrocytes, adipocytes, and myocytes. For determining the MSC cell fate, 1,25OHD is found to favor the differentiation into osteoblast or adipocyte (Bellows, et al., 1994; Kelly and Gimble, 1998). During bone development, SOX9 and RUNX2 are two master transcription factors which are required for the determination of chondrocyte and osteoblast cell fate, respectively. 1,25OHD mainly showed a stimulatory effect on RUNX2 expression then lead to an osteoblast cell fate. Besides, the Wnt, Ihh, and BMP pathways are also involved in MSCs differentiation and osteoblast cell fate. Wnt singling pathway stimulated RUNX2 expression but inhibited Sox9 expression result in a positively affect osteoblast differentiation. In this pathway, 1,25OHD activates the Wnt/β-catenin signaling to favor the osteoblast cell fate (Xiong, et al., 2017). Similarly, the Ihh pathway induces osteoblast differentiation by regulating RUNX2 expression as well. Whereas, BMP and FGF signaling pathway increase both Sox9 and RUNX2 expression promoting the differentiation of both osteoblasts and chondrocytes from MSCs (Yang, 2009). The differentiation of osteoblasts also involved in growth factors: FGFs, TGFs, and hormones (PTH, PTHrP), which partly regulated by 1,25OHD-VDR signaling pathway. They will be described in detail below.

During the osteoblasts differentiation, there are three significant stages of osteogenesis: proliferation, matrix maturation, and mineralization (Capulli, et al., 2014; Soltanoff, et al., 2009). To investigate osteoblast differentiation, the key osteogenesis

marker mRNA was usually investigated, which includes RUNX2, ALP, COL1A2, SPP1, and BGLAP, whereas ALP and COL1A2 were regarded as early markers, BGLAP was a late marker (Chatakun, et al., 2014; Mörike, et al., 1995; Quarles, et al., 1992; Yamamoto, et al., 2010).

1,25OHD mainly via forming the 1,25OHD-VDR-RXR heterodimeric complex to induce the vitamin D response elements (VDREs) expression resulting in either activation or repression of transcription (DeLuca, 2004). Besides, 1,25OHD can bind on a membrane-localized receptor presents on osteoblasts (Boyan, et al., 2002), which has more rapid responses compare to the classical VDR pathway, which mainly related to calcium influxes (van Driel and van Leeuwen, 2014).

The role of 1,25OHD in osteoblast is complex. There are considerable disagreements on 1,25OHD as suppressive or provocative on osteoblasts differentiation and mineralization (St-Arnaud, 2008; Tarroni, et al., 2012; van Driel and van Leeuwen, 2014). Overall, 1,25OHD mostly showed stimulatory effects on osteoblast differentiation and mineralization in human osteoblasts regardless of the cell origin and treatment dosage (Chen, et al., 2002; Jorgensen, et al., 2004; Li, et al., 2018; Prince, et al., 2001; Tourkova, et al., 2017; Zhou, et al., 2006). Whereas there are still some human studies that show partial inhibitory effects of 1,25OHD (Viereck, et al., 2002). Mice osteoblasts showed the most inconstant responses to 1,25OHD with either no effects, inhibition or promotion (Chen, et al., 2016; Kim, et al., 2016; van Driel and van Leeuwen, 2014; Xiong, et al., 2017). Rat osteoblasts are not as popular as human and mice *in vitro* studies. Generally, the responses showed either inhibition or no changes to 1,25D treatment (HARRISON, et al., 1989; Kim and Chen, 1989). For avian species, there are

limited data available. *In vitro*, 1,25D mainly showed an inhibitory effect on osteoblasts differentiation and mineralization (Broess, et al., 1995; Pande, et al., 2015). The inconsistent results across the species are summarized by van Driel, 2004 (Table 2.1).

The inconstancy of results greatly related to the factors such as differentiation stage, cell origin, treatment time and dosage, and extracellular factors (Czekanska, et al., 2012; van Driel and van Leeuwen, 2014). Questions regarding whether vitamin D has an anabolic or catabolic effect on osteoblasts and the role of vitamin D during different stage remain unanswered.

Several hypotheses are made here to describe the discrepancy results. By observing the previous studies, first, the result from human & rats (stimulatory), and mouse (inhibitory) can be explained by the common cell lines they used in the research. The human (SaOs2, MG-63) and rat osteoblasts showed more mature osteoblast phenotypes compared to most commonly used mouse osteoblasts (MC3T3) (Czekanska, et al., 2014). Furthermore, the cell original related effects might be explained by that the calvarial isolated osteoblasts (inhibitory, or no effects) showed the more immature character compared with long bone isolated cells(stimulatory) (Yang, et al., 2013a). Moreover, for some hormones and cytokines that mediated by 1,25OHD, which involved in osteoblast differentiation stage, could explain the multiple effects of 1,25OHD during different cell stage (de Gorter and ten Dijke, 2013). This is also supported by the study showed that cell passaging, the age of donor of MSCs has a negative correlation with 1,25OHD stimulation of alkaline phosphatase activity and osteocalcin expression in human MSCs (Zhou, et al., 2012). Therefore, the stage of the cell may be one of the most

critical factors that affect the role of 1,25OHD in osteoblasts differentiation and mineralization.

1,25OHD is regarded as a regulator of bone metabolism and function by affecting the production of bone matrix proteins (Collagen type I, osteopontin, osteocalcin, matrix Gla protein, etc.), the activity of alkaline phosphatase, and the process of mineralization (Yamamoto, et al., 2013). A scheme showed the vitamin D_3 endocrine function which will be describe in detail below (Figure 2.2).

Osteopontin (mRNA: SPP1) is a highly-phosphorylated sialoprotein that has strong mineral binding capacities in the extracellular matrix. It is one of osteoblasts marker, but it disturbs the crystal formation and enhances osteoclast attachment and resorption during bone repairing and remodeling. Thus, showed an adverse effects on osteoblast mineralization. 1,25OHD mainly increase in osteopontin, which has been shown to inhibit mineralization (Staal, et al., 1996).

Osteocalcin (mRNA: BGLAP) is a late marker of osteoblasts differentiation. It the most abundant non-collagenous protein in the bones, produced by osteoblasts, but also released during degradation by osteoclasts (Chatakun, et al., 2014; Mörike, et al., 1995; Yamamoto, et al., 2010). The chicken osteoblasts express a higher level of BGLAP compared with other mammalian species (Pande, et al., 2015). The effect of 1,25OHD showed either increased or decreased the production of osteocalcin in different studies with various species (Chen, et al., 2002; Pols and van Leeuwen, 2004; Zhang, et al., 1997).

Collagen type I (mRNA: CO1A2) is an early-stage marker and codes the most abundant bone matrix protein in osteoblasts (Quarles, et al., 1992). The effect of 1,25OHD on collagen type I expression showed either inhibition, stimulatory or no effects (Franceschi, et al., 1988; Harrison, et al., 1989; Kim and Chen, 1989).

Alkaline phosphatase (mRNA: ALP) activity is essential for mineralization via enhancing the initial mineral crystal formation (Chatakun, et al., 2014). 1,25OHD positively interferers human osteoblasts mineralization by accelerating the production of ALP in the early period and accelerated the extracellular matrix maturation (Woeckel, et al., 2010). In the mouse study, 1,25OHD inhibit the ALP expression (Chen, et al., 2012; Kim, et al., 2016). However, for the human osteoblasts, 1,25OHD either showed no effects or promontory effects on ALP expression (Chen, et al., 2002; Matsumoto, et al., 1991; Siggelkow, et al., 1999)

RUNX2 as one of the most important osteoblast-specific transcription factors is essential for the activation of osteoblast differentiation and induce MSCs into mature osteoblasts (Komori, 2006; Lieben, et al., 2012; Mundlos, et al., 1997; Viereck, et al., 2002; Vimalraj, et al., 2015). The RUNX2 deficient mice is lack of osteoblast and fail to produce a mineralized bone (Otto, et al., 1997). RUNX2 can either positively or negatively regulate expression of a variety of osteoblast-specific genes including Col1, ALP, Spp1, etc. In addition, RUNX2 regulated expression of Osterix directly, which could also control the transcription of target genes related to bone differentiation (Vimalraj, et al., 2015).

Similarly, BMP2 is highly related to the induction of osteoblasts differentiation and enhancement of bone matrix production (Liu, et al., 2012; Zhou, Liu and Tan, 2006).

The effect of 1,25OHD on RUNX2 expression also depends on the species of osteoblast that was used in the study. The RUNX2 by the treatment of 1,25OHD in murine cells (Yang, et al., 2013b). In contrast, in other mouse studies, RUNX2 was inhibited by 1,25OHD (Ducy, et al., 1997; Kim, et al., 2016). For human osteoblasts, the responses are more complicated. A study showed the duration of 1,25OHD treatment affects human primary osteoblasts RUNX2 expression with a short 1h treatment that showed stimulatory effects; 48h treatment showed an inhibitory effect (Viereck, et al., 2002). Furthermore, for different types of human osteoblasts, RUNX2 is not affected by 1,25OHD in human osteoblasts cell lines (O4-T8, 03-CE6, and 03-CE10) (Prince, et al., 2001), but 1,25OHD upregulates RUNX2 in human marrow stromal cells (Geng, et al., 2011). Similarly to RUNX2, BMP2 is either increased or decreased by 1,25OHD across different species (Chatakun, et al., 2014).

In summary, 1,25OHD plays an important role in skeleton mineralization and development. Its effects show as either positive or negative on osteoblast development, which depends on the presence of other factors, such as development stage, species of cells, the presence of growth factors.

4. Vitamin D_3 toxicology

Animal has a higher resistance of 1,25OHD toxicity, because the production and degradation of 1,25OHD is tightly regulated in the organism (Figure 2.1), either through a directly negative impacted on 1α-hydroxylase or via 1,25OHD-VDR pathway to control PTH and FGF23 production, indirectly regulate the 1,25OHD production and degradation

(Haussler, et al., 2013). In chicken, previous study showed fed birds with vitamin D_3 up to 15,000 IU/kg had no negative effects on growth performance or egg production on 20-68wk Lohmann LSL white laying hens (Mattila, et al., 2004). Similarly, a short period (30d) dietary treatment up to 20,000IU on 87wks ISA brown molted laying hen has no effects on laying and growth performance (Park, et al., 2005). A more recent study brings up the non-toxic level of vitamin D_3 to 102, 200 IU/ kg on 19 to 58wk Hy-Line W36 laying hens (Persia, et al., 2013). However, while increasing vitamin D_3 dosage to 200,000 IU/kg, it starts showing a decrease of egg weight, eggshell quality and feed consumption (Ameenuddin, et al., 1986).

In general, the vitamin D_3 intoxication shows an elevation of the plasma concentrations of vitamin D_3 and its metabolites, such as 25OHD, 24,25OHD, 25,26OHD, but it is rarely seen that high concentration of 1,25OHD in plasma (Shepard and Deluca, 1980). Since high concentrations of 25OHD could bind the VDR and induce transcription (DeLuca, et al., 2011). The hypothesis of in toxicity of vitamin D_3 is due to the DBP saturation, and let free vitamin D_3 metabolites enter the cell, where directly effect on gene expression. However, this theory remains unproven (Jones, 2008). Furthermore, the excess 1,25OHD can trigger TNF-alpha mediated apoptosis (Pascher, et al., 1999), which is another mechanism for vitamin D_3 toxicity. The inhibitory effects of higher dosage 1,25OHD on osteoblast are also shown in the proliferation of osteoblast-like osteosarcoma cells, which is stimulated by the physiologic level of 1,25OHD (0.1nM), but retarded by higher dosage (10nM) (Yamaoka, et al., 1986). Furthermore, 1,25OHD suppressed NF-kB, and Smad activation induced by TFG-beta and BMP2 in a dose-dependent manner (Yamaguchi and Weitzmann, 2012). In these studies, 1,25OHD

showed a positive effect or no effect at a lower dosage but adverse effects at high dosage, which could potentially regard as a mild toxicity effect of 1,25OHD. 1,25OHD have varied effects on osteoblast differentiation and mineralization, how to distinguish whether the effects are inhibitory or because of toxicity is a question need to be answered.

5. The application of vitamin D_3 metabolite: 25-hydroxyvitamin D_3 in laying hens

Vitamin D_3 is commonly supplied in vitamin premix in poultry diets. However, the additional supplementation of vitamin D_3 than the requirement mostly has no beneficial effects (Keshavarz, 2003; Mattila, et al., 2004; Persia, et al., 2013). It may indicate that the conversion of vitamin D_3 to its bioactive form is insufficient (Koreleski and Świątkiewicz, 2005). Especially in old laying hens, the defection of liver and kidney results in the decreased production of active vitamin D_3 metabolites (Bar, et al., 1988; ŚWiĄTkiewicz, et al., 2017). 25OHD as the second metabolite of vitamin D_3 is commercially available in the market. It is two to four folds more active compared with vitamin D_3 in chicken diets (Atencio, et al., 2005a; Soares Jr, et al., 1995). It may due to the efficiency of biological conversion is not 100%, the same amount of 25OHD is more active than vitamin D_3 as it bypasses the first biotransformation.

5.1 25-hydroxyvitamin D_3 on bones

The application of 25OHD in poultry diets showed positive effects on chicken bones. 25OHD supplementation in the diets can attenuate outbreaks of lameness caused by BOC (bacterial chondronecrosis) in commercial broiler flocks (Wideman Jr, et al.,

2015), and significantly decreases the frequency and severity of tibial dyschondroplasia (Edwards Jr, 1989). Furthermore, 25OHD showed beneficial effects in breeder compare to standard vitamin D_3 (Atencio, et al., 2005b). These positive effects were explained as 25OHD eliminated the need for liver conversion of vitamin D_3 to 25OHD, consequently showed higher activity. The supplementation of 25OHD during the laying period mostly showed no effects on bones (Käppeli, et al., 2011; Koreleski and Świątkiewicz, 2005). However, some of the studies showed the addition of dietary 25OHD increased bone mineral density, bone ash, Ca, P, and serum vitamin D levels (Sahin, et al., 2009).

5.2 25-hydroxyvitamin D_3 on egg production

The effects of 25OHD on egg production and egg quality of laying hens or breeders are inconsistent. The varied 25OHD treatment duration, treatment timing, and laying stage contribute to the diverse results.

Silva (2017) showed that fed the laying hens with 69µg/kg of 25OHD on the top of 3000IU/kg of vitamin D_3 from 0-50 wks (remove 25OHD from the diets after 50wk), increased FCR during the period of 18-34 wk and increased egg production of 18-50wk, but there was no effect on cumulative egg production from 18-87wk. However, most of previous studies showed 25OHD had no beneficial effects on egg production or egg quality in laying hen or broiler breeders (Käppeli, et al., 2011; Keshavarz, 2003; Koreleski and Świątkiewicz, 2005; Mattila, et al., 2011; Nascimento, et al., 2014; Torres, et al., 2009). For the studies showing no effects, none of them included 25OHD in the diets during the rearing period or covered the entire laying period. The use of 25OHD

during the rearing period and early laying phases seems to promote bone development and protect bone loss during the peak production, which may benefit laying performance during later laying period (Silva, 2017). To our knowledge, limited studies have been performed on this approach to the use of 25OHD targeting rearing or early laying period.

The mechanism of vitamin D_3 on shell formation has not been fully understood. The missing effects on eggshell quality may be due to eggshell gland Ca^{2+} transportation-related protein such as calbindin D_{28k} and carbonic anhydrases, which are probably vitamin D_3 independent (Bar, 2008). The considerable expression of VDR at eggshell gland (Bar and Hurwitz, 1984) and the injecting 1,25OHD (100 nM) in eggshell gland which induced calbindin D_{28K} expression (OHIRA, et al., 1998), indicated 1,25OHD might have direct effects on calbindin regulated Ca^{2+} transportation, but the dietary supplementation may not be able to reach an active level at ESG or showed an undetectable effects on increasing shell formation. Most of the studies suggested the eggshell formation is more calcium mediate rather than the direct effects of Vitamin D_3 on ESG (Bar, 2009). The 25OHD could significantly increase the absorption of Ca^{+2} at intestine and resorption of bones may contribute the better shell quality (Spencer, et al., 1978).

Some studies showed the beneficial effects of 25OHD on eggshell quality. The supplementation of 25OHD(35 or 69ug/kg, 32wk-67wks) resulted in better egg shells evaluated by specific gravity at 60 weeks of age (Torres, et al., 2009); replace 25% of vitamin D_3 with 25OHD (9.35ug/kg, 26-70wk) improved shell quality (Koreleski and Świątkiewicz, 2005); inclusion of 25OHD (69ug/kg, 0-50wks) for the rearing and early laying period increased the shell thickness (Silva, 2017). In these studies, the beneficial

effects mainly presented in the studies with long-term (at least 30wks feeding) or early (rearing or early laying stage) supplementation of 25OHD.

The calcium level and based vitamin D_3 level also affects the outcome of 25OHD in poultry diets. It is probably because the concentration of vitamin D_3 in diet and the calcium level in some studies are adequate to maintain egg quality. In this case, the additional vitamin D_3 will not improve laying hen egg quality (Bar, 2008; Plaimast, et al., 2015).

In older hens, the ability to produce the active metabolites — 25OHD, and 1,25OHD, is reduced (Bar and Hurwitz, 1987), and vitamin D_3 becomes more limited. Added 25OHD in the diets may be more effective at this stage. However, the effects of 25OHD is limited by the possibility of the insufficient 25OHD conversion to 1,25OHD in the kidney (Abe, et al., 1982), Furthermore, 25OHD may target on preventing bones loss and maintains the system Ca^{2+} homeostasis rather than increasing egg production during this period (Silva, 2017), which needs further investigation.

6. Laying hen bone physiology

6.1 Rearing period

Laying hen skeletal formation during the rearing period is similar to the mammal. It proceeds through two major mechanisms: intramembranous and endochondral ossification (Whitehead, 2004). In intramembranous ossification, osteochondral progenitors differentiate directly into osteoblasts to form membranous bone; during endochondral ossification, osteochondral progenitors differentiate into chondrocytes to

form a cartilage template of the future bone (Sturkie, 2012). The difference between mammalian bone and avian bone is shown as the avian bone did not have a secondary ossification center and the growth plate in avian bone was much well vascularized compare to mammalian (Whitehead, 2004).

A number of research in pullet's studies addressed the importance of early bone development, and its prolonged effects on the bone health at laying period (Casey-Trott, et al., 2017; Hester, et al., 2013; Regmi, et al., 2015). Human studies also emphasized the importance of bone health during the growing period and its subsequent benefits during adulthood (Bailey, et al., 1999). Previous nutritional studies seldom focused on the pullet period. However, when osteoporosis was observed during the laying period, it has already hard to reverse it by using nutritional strategy (Rennie, et al., 1997). A meta-analysis on human clinical trial also showed there are no effects of supplementation of vitamin D or calcium on decreasing older adults fracture incidence (Zhao, et al., 2017). In summary, early prevention of osteoporosis is suggested.

6.2 Laying period

During the sex maturation, the blood calcium levels are brought up by estrogen; carbonic anhydrase expression increased, which is involved in supplying the carbonate portion of the calcium carbonate in the shell; the skeletal weight of the pullets increases by 15-12 grams (4-5g calcium) (Sturkie, 2012). The most significant marker of sexual maturity is the medullary bone formation inside the bone cavity, which is throughout the whole avian species (Werning, 2018). Functionally, the medullary bone act as a constant

calcium source for eggshell formation (Dacke, et al., 1993), rather than a structural function (Fleming, et al., 2006).

The existence of medullary bone is more prominent in long bones, especially in leg bones, but the humerus remained mostly hollow (Dacke, et al., 1993; Whitehead, 2004). Medullary bone is a hormones-drive bone structure (Beck and Hansen, 2004; Wilson and Thorp, 1998). The injection of estradiol valerate to male Japanese quail induced medullary bone formation (Miller and Bowman, 1981; Yamamoto, et al., 2001). The content of medullary bone is similar to the trabecular and cortical bones, which mainly contains hydroxyapatite lattice (Ascenzi, et al., 1963), but with much lower collagen content and in a random distribution (Dacke, et al., 1993). The medullary bone can be metabolized at a rate at least 10–15 times faster than cortical due to the high osteoclast activity in this region (Hurwitz and Bar, 1965; Simkiss, 1967; Van de Velde, et al., 1984).

Because bone contributes calcium to eggshell formation, the eggshell quality is negatively correlated with bone quality (Kim et al., 2005). Eggshell formation increases calcium mobilization from bones, consequently reduces bone quality in laying hens (Kim, et al., 2012). A strong correlation between the incidence of osteoporosis and body weight and production was found in a previous study (Cransberg, et al., 2001). Bishop, et al., (2000) also showed an inverse relationship of bone quality with eggshell quality, osteoporosis.

7. The application of micro-CT in egg laying hen bone analysis

Micro-CT (Micro-computed tomography) is the "golden standard" for bone health assessment (Feldkamp, et al., 1989). The application of micro-CT in research begin in the early 1980s and dramatically increased in recent years (Schambach, et al., 2010). Compare to the clinic CT (voxel: around 1 mm^3), micro-CT has a much higher resolution (as low as voxel: 1μm^3) (Ritman, 2007), which lead to a higher sensitivity to detect small changes in the objects. Previous literature showed many examples of applications of Micro-CT in human, mice, rat and other species (Bouxsein, et al., 2010; Schambach, et al., 2010). However, micro-CT has not been widely used in poultry bone studies.

The common techniques that used in poultry bone research are well summarized by Kim, et al. (2012). Current evaluations of chicken bones are mostly by conducting bone ash analysis, breaking strength test or dual-energy x-ray absorptiometry (DEXA). These methods provide the results of planar morphology or bone mess. Although bone quantity and density are both important factors for bone strength (Hester, et al., 2004), these approaches did not consider the trabecular micro-architecture, which independently related to the bone strength (Siffert, et al., 1996; Webber, et al., 1998). An avian bone study demonstrated that more than 10% loss of trabecular bone could significantly impact the bone strain (Reich and Gefen, 2006), which suggested the integrity of trabecular bone is critical for fracture resistance.

Egg-laying hens have unique bone characters, especially the formation of medullary bone in the cavity (Whitehead, 2004), and the higher activity of bone resorption process compare to the mammal (Hurwitz and Bar, 1965; Simkiss, 1967; Van et al., 1984). These characters render egg-laying hen bone as an attractive research model. Furthermore, osteoporosis results from the progressive structural bones loss

which is a serious animal welfare concern in laying hen industry (Webster, 2004). In order to alleviate the osteoporosis in laying hens, a better understanding of laying hen bone is necessary. Micro-CT as a powerful bone assessment tool will become more and more popular in chicken bone studies.

The guideline for assessment of bone micro-architecture in rodents using micro-CT summarized the conventional methods, cautions, application, and limitations of micro-CT (Bouxsein, et al., 2010). However, while applying micro-CT to chicken bone structural analysis, some factors need to be adjusted carefully due to the unique chicken bone traits. In this review, the application of micro-CT on laying hen bone analysis, especially when medullary bone presents in the cavity is introduced; the recommended settings for scanning, reconstruction, and analysis are interpreted. At the end of the review, a customized automated bone separation algorithm is introduced. This algorithm allows us to separate laying hen bones into the cortical, trabecular and medullary region without any human bias. Then the segmented analysis can be applied to each part to obtain more defined data. The application of this method will greatly expand our understanding of layer bone physiology in future research.

7.1 The advantages and limitations of micro-CT in poultry bone research

The conventional optical or electron microscopy allows the researcher to visualize two-dimensional images of a bone biopsy surface or thin slices to get the inside structures, which assume the structures are all plate-like. Whereas, in most cases, a conclusion about three-dimensional object structures cannot be made on the base of two-

dimensional information. Micro-CT system enables us to visualize and measure the true 3-dimensional object structures. These measurements include micro-architecture, true density, bone/tissue volume, 3-dimensional structural arrangement, etc. Contrary to conventional approaches, all quantitative parameters were calculated from 3-dimensional data without any model assumption. Furthermore, the acquired images can be reconstructed to 3D model and analyzed non-destructively (Stauber and Müller, 2008), which allows the researcher to use the same sample for additional analysis. At last, by applying the bone separation, the different parts of bones can be isolated and analyzed individually to get more sophisticated data (Buie, et al., 2007). However, for laying hens, the separation of cortical bone, trabecular bone and medullary bone of the mature layer is difficult.

Due to the long scanning time and high resolution, lots of factors, for example, the temperature, sample movement, misalignment, beam hardening, dust on the sensory, etc., have significant impacts on outcomes, consequently, affect the accuracy of results (Ritman, 2004). Furthermore, the micro-CT usually has a small chamber for holding the samples. Thus, not all the samples can be fit inside. For example, the whole mature layer's tibia is too long for some scanners. In this case, they must be cut into parts before scanning.

7.2 Sample preparation and installation for the scanning

The adult layer bones are relatively larger than the mice or rat bones. The length of the modern layer femurs from day-old to 95wk were shown in Figure 2.3. This figure

shows the bone length reaches its peak at sexual maturity (around 18wks). During the laying period, the bone length keeps constant (Fleming, et al., 2006; Whitehead, 2004; Whitehead and Fleming, 2000). For a micro-CT scanner, a low-radio dense sample holder is used based on the length of bones and the chamber size. The relative larger layer bones result in longer scanning time than mice or rats, so the extra attention on avoiding dehydration of specimen during the scanning need to address. Several media can be used including ethanol, saline or water. However, the different medium affects x-ray attenuation (Nazarian, et al., 2008). The scanning medium must be constant throughout the samples. The soft tissues need to be carefully removed to avoid any effects on x-ray attenuation of bones. The samples should be firmly positioned in the holder with the same horizontal axis (Bouxsein, et al., 2010).

7.3 Image acquisition for laying hen bones

The attenuation of the x-ray photons results from either absorption or scattering depends on the energy (Grodzins, 1983). A rule for selecting energy is that the thicker and denser samples required higher energy (Bouxsein, et al., 2010). Mice have a smaller bone at a diameter less than 3mm, and a typical human bones diameter are around 10mm (Martín-Badosa, et al., 2003). However, the laying hen bone has a higher diameter compared to the mice, rats, or human. The layer femur bone width from day-old to 95wks is shown in Figure 2.4. The scanning energy should be relatively higher compared with the mice or human based on the longer travel distance of x-ray inside the laying hen bones (Grodzins, 1983), especially when the medullary bones were presented in the cavity, as the medullary bone is highly mineralized (Dacke, et al., 1993).

The intensity (number of photons during one projection) depends on current (μA), the exposure time (ms), and the frame averaging (number of pictures per rotation). The signal to noise ratio can be improved by increasing exposure time and average frame but will result in longer scanning time (Ritman, 2004). The scanning decision needs to be made by balancing the background noise and the optimal scanning time, as well as the outcome file size. Since the length of layer bones are usually longer than the mice and rats (Figure 2.3), accordingly, the scanning time is increased, which may result in a higher incident of misalignment and thermal drift during the scanning (Stock, et al., 2008). A mathematical post-alignment calibration needs to be applied for this scenario. An alternative way to reduce the scanning time is to shrink the scanning region and only focus on the interested area rather than the whole bone.

The voxel size can affect the accuracy of the analysis outcome. In general, the minimum ratio of voxels to the smallest structure size in an object is 2 (Bouxsein, et al., 2010). The finest structure (trabecular bone) in mice bones is around 20–30 μm (Martín-Badosa, et al., 2003). However, in modern laying hens, the trabecular thickness is between 60-220 μm (Figure 2.5). Therefore, the scanning resolution setting for laying hen bones can be lower than mice or rats. Furthermore, because of the slight changes in high-resolution scanning will not significantly affect the results on relatively high thickness structures (Müller, et al., 1996), the resolution setting in layer bone scanning may have even higher forgiveness. Nevertheless, for young pullets with underdevelopment trabecular bone as well as the aged laying hens with thin trabecular bone (Figure 2.5), the bone volume fraction to the total bone can be extremely low, which is similar to the record in a mouse study (Glatt, et al., 2007). In this case, the scanning

resolution needs to increase accordingly. In addition, if the research interests are on medullary bone structure, the pixel size needs to decrease further as the medullary bone has a much smaller structure size (Kerschnitzki, et al., 2014a).

In summary, a scanning setting should consider the contrast of the image, the potential artifacts as well as the balance of the scanning time and file size for laying hens bones. Additionally, the random movement can be applied to avoid ring effects, which results from dead pixels, dust on the detector or background noise. A flat field correction can be performed before the scanning to correct the inhomogeneity of the individual pixel of the detector (Salomé, et al., 1999).

7.4 The volume of interest (VOI) selection for laying hen bones

Based on the research questions, different parts of layer bones could be used. The humerus, tibia, femur and keel bone are the most common bones used in research. Keel bone and humerus become more and more popular in the free-range and aviary system studies (Guesdon, et al., 2004; Stratmann, et al., 2015; Whitehead and Fleming, 2000). However, to understand the metabolism of bones, the tibia and femur with the presence of all three types of bone structures are research of interest (Whitehead, 2004). While considering the chamber size of micro-CT, and a relatively large number of studies reporting the results from the femur (Bouxsein, et al., 2010), for laying hen bone study, the femur probably is one of the best choices. However, the shape of the femur is curved, the angle of bone axis need to be carefully adjusted before selecting a region of interests.

Whether the VOI represents the bone structure is the first question to ask while setting up a scanning. Usually, the diaphysis of long bones contained less trabecular bone compare to metaphysis (Rosen, et al., 2009). As go from the bonehead to the diaphysis, the trabecular bone structures become less and less. The extended volume of interests may dilute the trabecular bone volume fraction to the total bone, then mask the relevant difference between the treatments. Instead, the medullary bone is filled up throughout the whole bone inside the cavity. A 60wk old laying hen bone is scanned and analyzed to demonstrate the dilution of trabecular bone (Figure 6). Since the medullary bone, trabecular bone, and cortical bone are equally crucial for layer bone research. The metaphysis would be a more represented region for study, but the VOI need to carefully control in order to avoid the dilution of trabecular bones.

While considering the reproducible landmark for VOI, the nutrient foramina on distal of femur head can be used, as it constantly showed in this region (Figure 2.7A). However, in younger birds, the blood vessels have not invaded into the bones, and in the order birds, several nutrient foramina were shown in this region (Figure 2.8). Alternatively, another marker can be used which is described in Figure 2.7B. Both methods required to position the bone sample in a certain angle in 3D space to make sure the VOIs are constant.

7.5 Laying hen bone model reconstruction

Acquired images need to be reconstructed to a 3D model before analysis. During the reconstruction, beam hardening artifacts, ring artifacts, background noises, thermos

drift, which due to the high density of sample, dead pixies, air disturbing, and temperature change on x-ray source, respectively, need to be checked carefully. According to the inspection, the mathematical corrections are performed using software (Bouxsein, et al., 2010). The metal artifacts, are not typical for the laying hens. Instead, the motion artifact is the most frequent during the oversize scanning which results from sample movement or camera thermos drift.

7.6 Laying hen bone analysis

Most software for bone analysis only recognizes the binary pictures. By converting the grayscale picture into a binary picture, the same global threshold is applied. Threshold setting is more difficult in laying hen than the mammal due to the complex structural components in a bone. In some situations, a particular threshold is set for the porosity analysis in the cortical bone, or to show the fine structure of medullary bone.

8. The automated bone separation algorithms for laying hen bones

Bone separation could be achieved by manually drawing the ROI slice by slice or using automated algorithms (Ang, et al., 2018; Buie, et al., 2007; Kohler, et al., 2007). There are no automated algorithms for laying hens bone separation in previous reports. Even manual separation is extremely hard when the trabecular bones and medullary bones are presented at the same time.

Our automated separation algorithms are designed based on the morphology difference of medullary bone compared to trabecular bone and cortical bones. The medullary bone has two primary forms in the bones (Figure 2.9). During the early laying period, it showed as fine woven loose bones distributed throughout the cavity, which could be separated by applying the different thresholds. However, during the late laying period, most of the time, the medullary bone clustered together to form a calcium chunk, which is hard to separate by applying different thresholds. In this case, we defined the diameter of medullary chucks are higher than the trabecular bone thickness as a separation trait. The opening or closing procedure was used for the separation. It needs to be noticed that, if medullary bone chuck binds on the trabecular structure, this separation may result in artificial damages on trabecular bone structures. This automated bone separation process has combined both situations along with some additional processes to avoid errors such as holes on the cortical bones, foreign matters around the object, etc.

A diagram is presented to show the critical steps of the automated bone separation procedure (Figure 2.10). The image is converted to binary by applying (global) threshold followed by a despeckle to sweep all except the largest object in 3D space to exclude any potential foreign matters around the object to make sure a “clean” input for the separation process. The whole processed are summarized as below:

Step (1). Region of interest (ROI) shrink-wrap: ROI is shrunken wrap in 2D space with stretching over the holes to avoid any leaking of ROI inside the bone cavity due to the holes in the bones. After this step, the air around the object is excluded.

Step (2). Bitwise operations: Image = NOT image followed by image = image AND ROI. A small opening followed by despeckle is recommended at this step to

remove any pixels that potentially existed around the object due to the stretching over the holes functions.

Step (C-3). A closing procedure on the image at 2D space with a large radius is applied. The minimum radius setting should be larger than the thickest trabecular bone. An erosion of ROI is recommended to let ROI a few pixels away from the endocortical surface to make sure all the cortical bones have been selected.

Step (C-4). Bitwise operation: ROI=ROI SUB image. The final ROI is defined. After reloading the image, the final cortical bone separation outcome is shown in the picture.

Step (3). Bitwise operation: Image= NOT image. This process makes trabecular bone in the image artificially connect to the air around to avoid being removed by the next procedure.

Step (4). An opening is performed on the image at 2D space with a radius set between the diameter of medullary calcium chuck and thickness of the trabecular bone. Usually, the diameter of the chuck is much higher than trabecular bone thickness. This procedure is optimal to separate the calcium chuck and trabecular bone.

Step (5). Bitwise operation: ROI=ROI SUB Image. A small erosion of ROI is recommended after this step. The erosion procedure can shrink the ROI selection to make it a few pixels away from calcium chunk. This erosion avoids trabecular ROI including a “calcium shell” pealed from calcium chuck, which due to the calculation error of the opening procedure.

Step (6). The original binary image is reloaded in the process to further assisting ROI determination. Afterward, a bitwise operation is applied: image= image AND ROI

Step (M-7). A closing procedure was performed on ROI to fill the unselected calcium chuck region.

Step (M-8). Bitwise operation: ROI= ROI SUB image is applied to finalize the ROI for medullary bone selection

Step (T-7) ROI= image. The ROI of the trabecular bone region is determined.

This process may be problematic in the situation that medullary bone exclusively binds to the cortical bone area, which will result in an increasing volume of cortical bones. However, in this situation, the medullary bone may act as a structural bone as well since all the morphology traits are similar to cortical bones.

This process has not been statistically validated due to no similar methods or correlated parameters available for comparison. However, visually, it separated the major bones of each part (Figure 2.11), and the progress is logical and reasonable. There is no human bias while applying it to all the samples which makes it even more trustable. Nevertheless. A comparison or validation study need to perform to validate the method statistically in the future.

The size of hen bone and the exhibition of medullary bone inside the cavity are major factors distinguish the application of micro-CT on birds from the other mammals. An automated laying hen bone separation process is presented very first time in this review. Even though the statistical validation is missing due to the difficulties to perform

such an experiment, it provides the possibility and progress on advance chicken bone analysis.

9. Gap statements

Several gaps persist in the understanding of vitamin D_3 on chicken osteoblast differentiation and mineralization. Learning the specific effects of vitamin D_3 on chicken osteoblasts development can help to understand the vitamin D_3 function on early bone development in chicken. Furthermore, there is limited research on long-term and early supplementation of 25OHD in pullet layers and laying hens. Thus, no sound research data were available to develop an appropriate 25OHD supplementation strategy in laying hen. At last, there are no statistical models to describe the modern layer bone development. Understanding the layer bone model provides insight for creating new strategies to optimize bone development and alleviated laying hen osteoporosis.

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TABLES

Table 2.1 The effects of 1,25OHD on osteoblasts differentiation and mineralization in different species.

	Human	Rat	Mouse
Alkaline phosphatase	↑	↑/↓	↓/↑/=
Collagen type I	↑/=	↓/=	↓/↑/=
Osteocalcin	↑	↑	↓
Mineralization	↑	↑	↓/↑

FIGURES

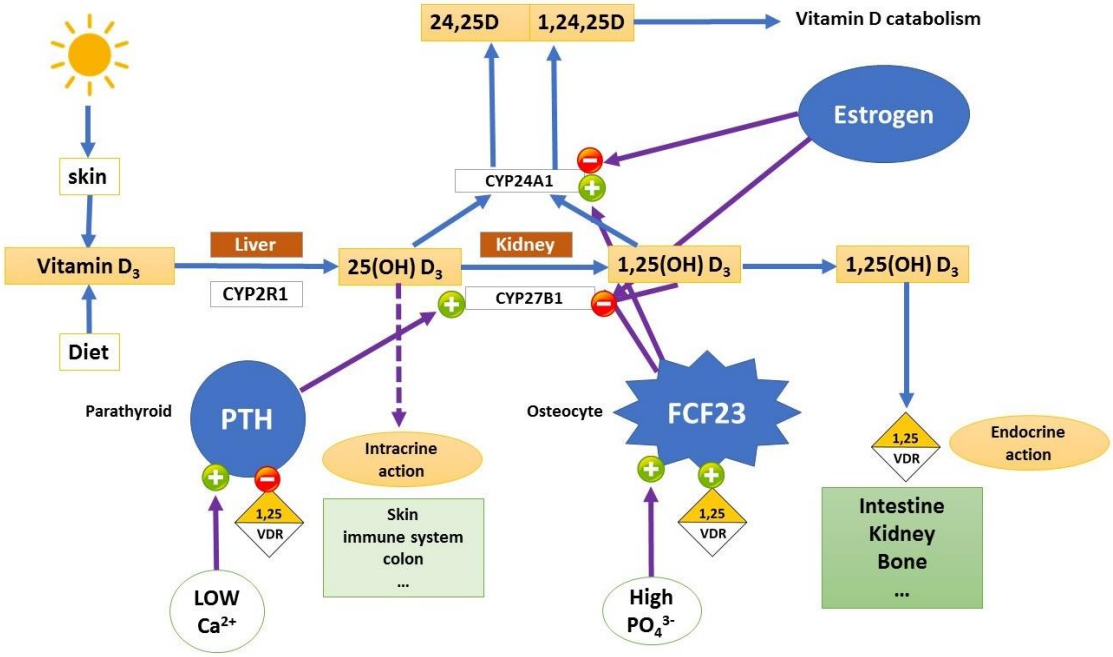

Figure 2.1 Vitamin D_3 metabolism and regulation.

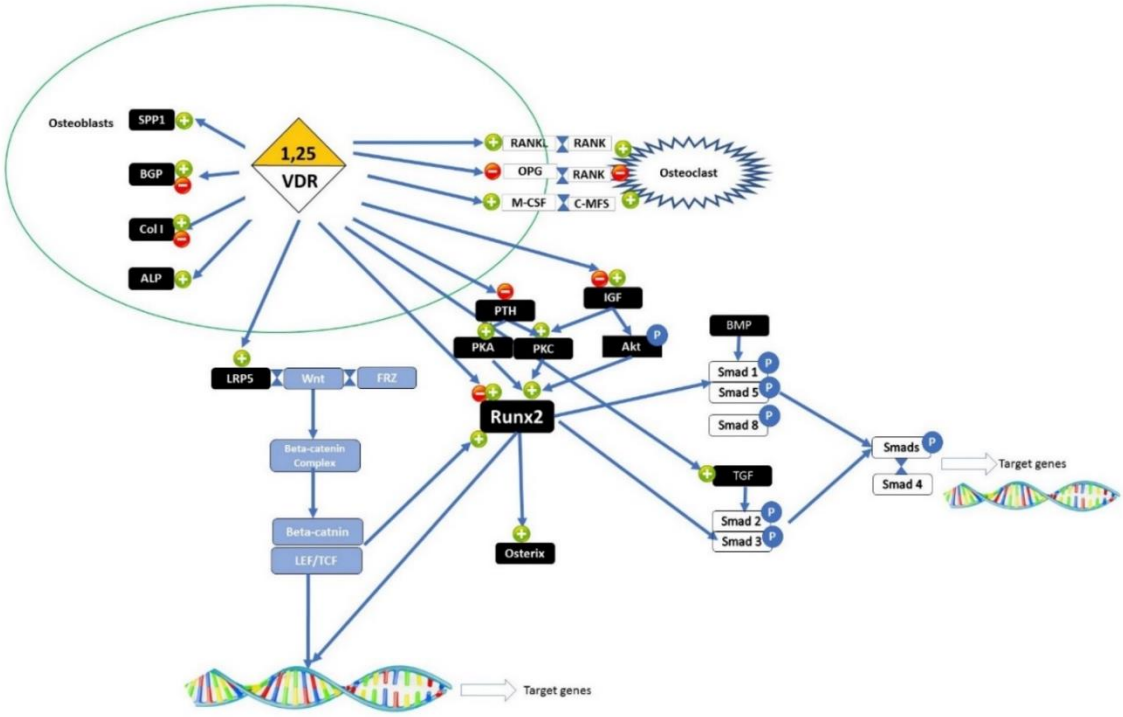

Figure 2.2 Vitamin D_3 endocrine function

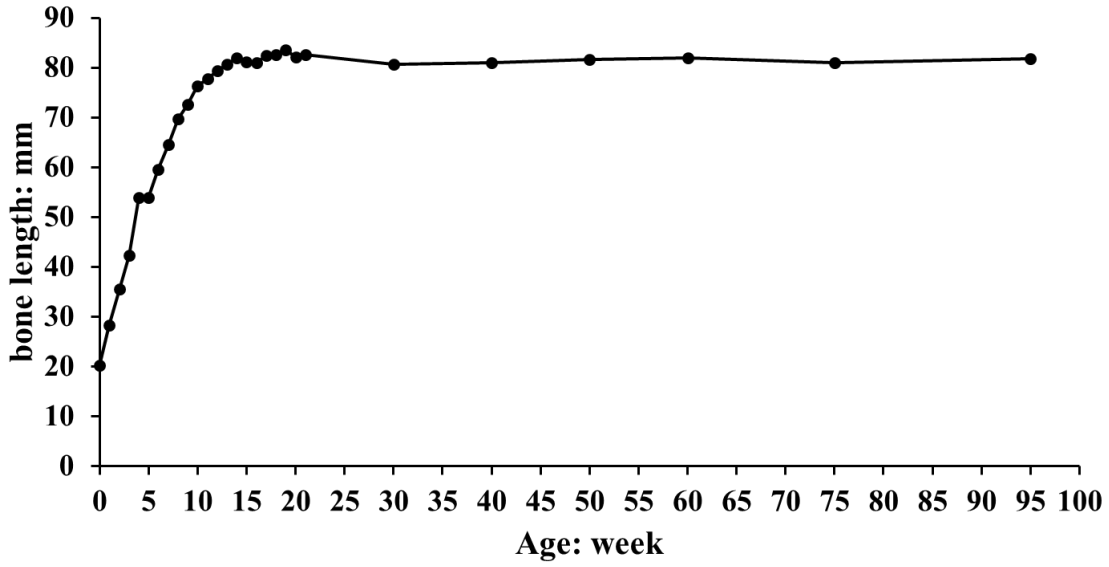

Figure 2.3 One-day-old Hy-line W36 layers were raised under the standard management and feeding program based on Hy-line W36 guide (2015) from 0-95wks. During the experimental period, ten birds femur/time points were collected. The bone length was measured using a caliper.

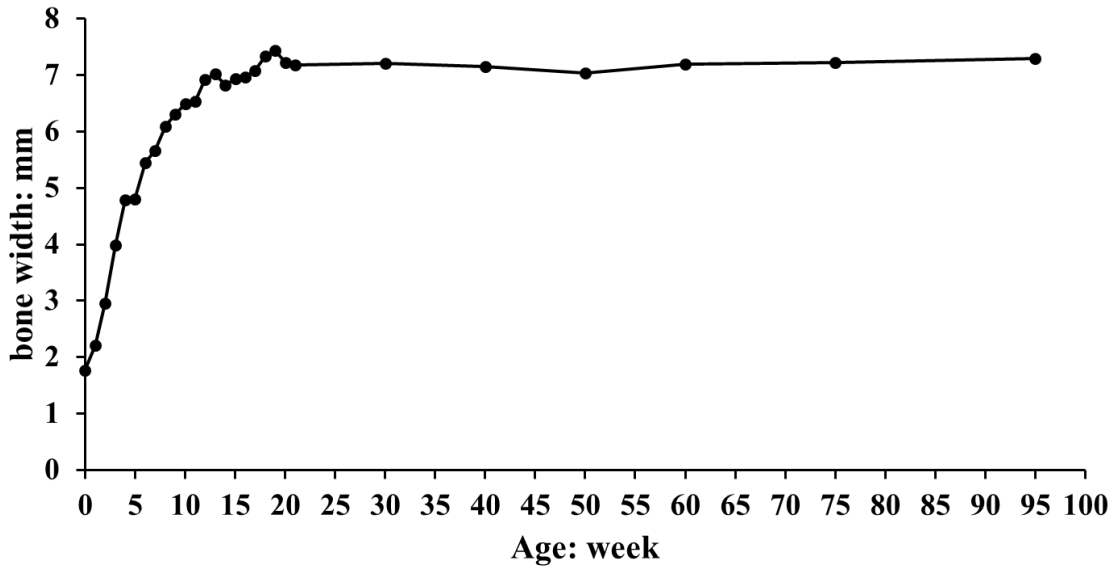

Figure 2.4 One-day-old Hy-line W36 layers were raised under the standard management and feeding program based on Hy-line W36 guide (2015) from 0-95wks. During the experimental period, ten birds femur/time points were collected. The bone width was measured using a caliper.

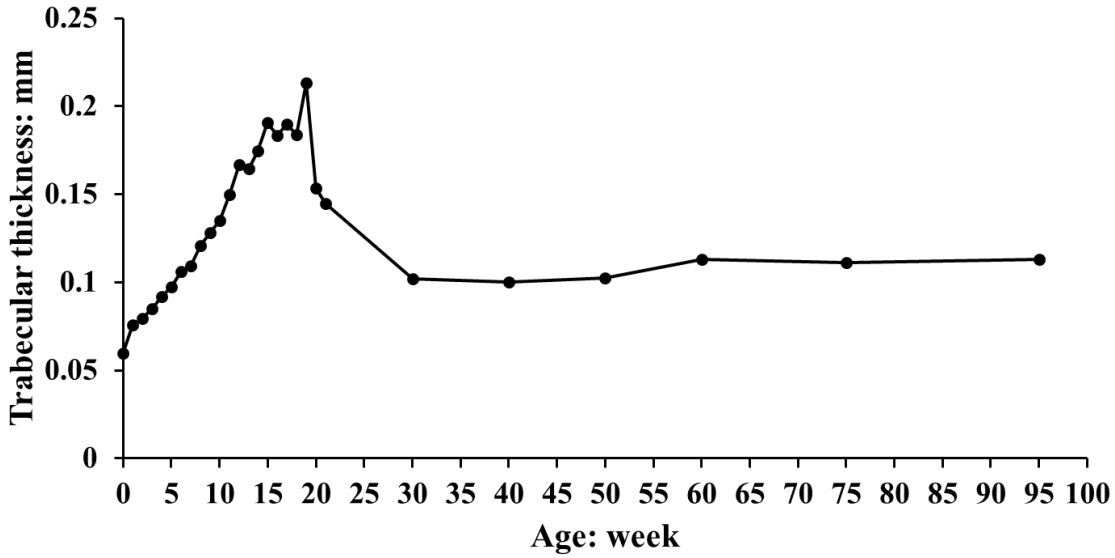

Figure 2.5 One-day-old Hy-line W36 layers were raised under the standard management and feeding program based on Hy-line W36 guide (2015) from 0-95wks. During the experimental period, ten birds femur/time points were collected. The bones were scanning and analysis using micro-CT (Skyscan 1275; Bruker microCT, Belgium), the trabecular bone thickness were measured by using CTan (Version: 1.16.4.1, Bruker microCT, Belgium).

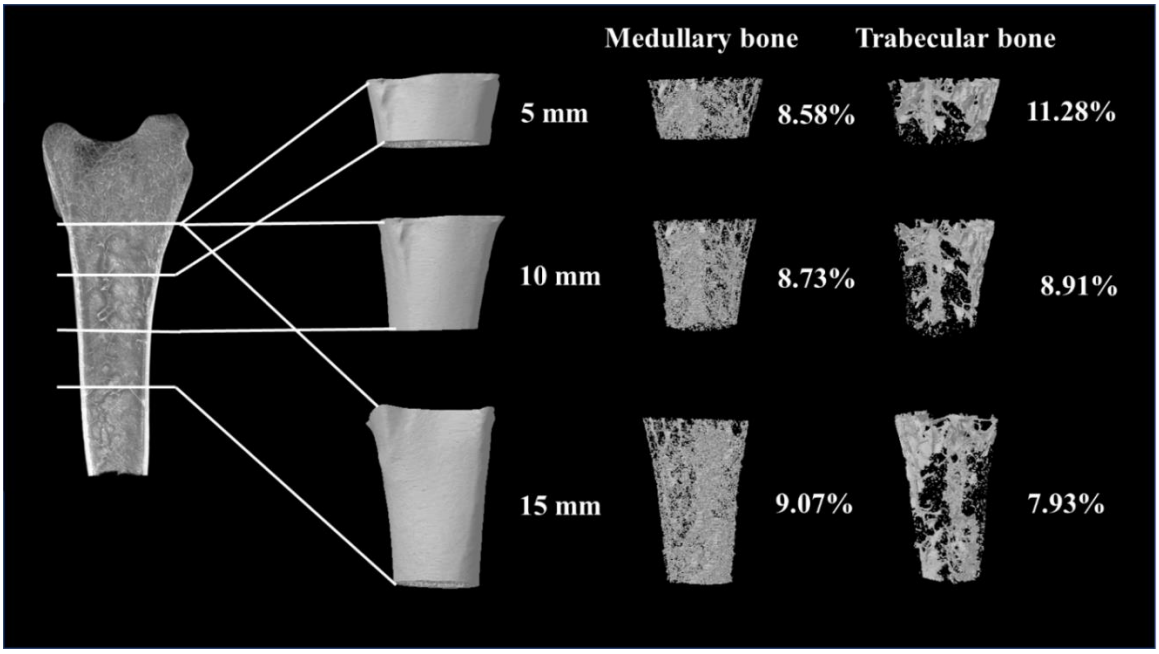

Figure 2.6 A represented 60wk Hy-line W36 laying hen femur was used to demonstrate the dilution effects showed in laying hen bone. The VOI was selected start from a nutrient foramen (see the introduction in Figure 5), 5mm, 10mm and 15mm was selected as VOI followed by a segmentation analysis. The numbers showed the ratio of parts bone volume (medullary bone or trabecular bone) vs. total bone volume. The analysis showed there is almost no changes on medullary bone percentage when expending the VOI but a trend of decrease of trabecular bone ratio was detected.

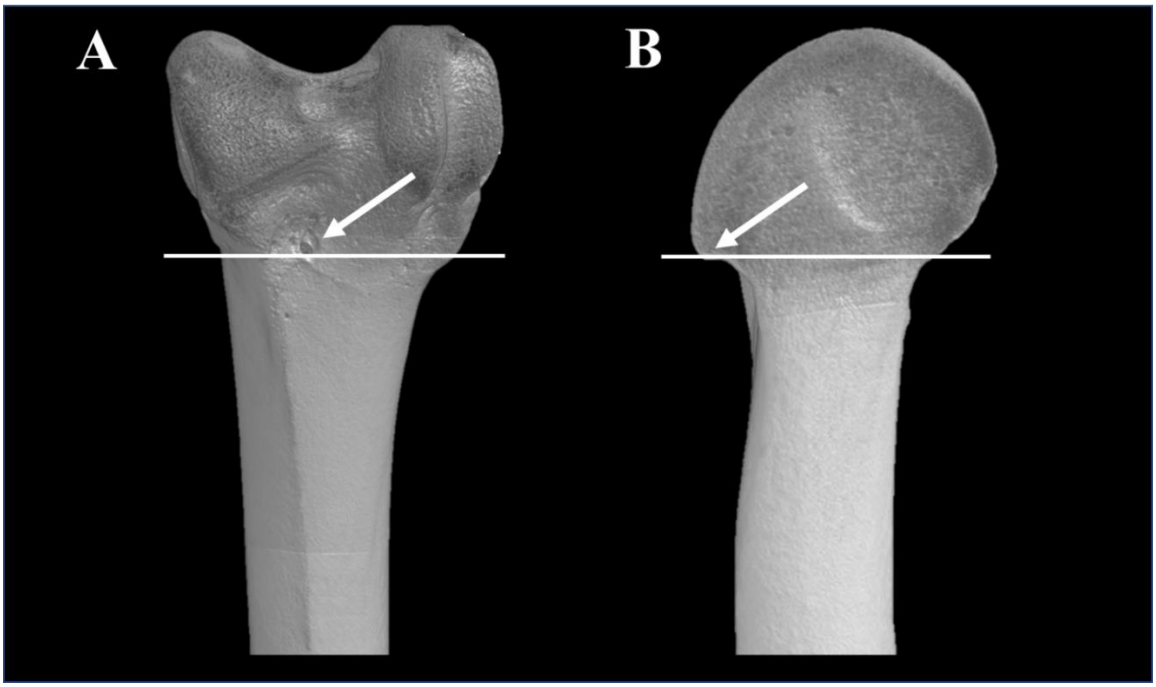

Figure 2.7 Two femur distal metaphysis image from a micro-CT scanning were showed to demonstrate the potential landmarks that can be used in VOI selection: (A) the nutrient foramen presented in the bonehead at similar position regularly. A VOI selection can be made from there, or a certain distance from there to avoid includes any holes inside the VOI; (B) for young and old birds, the widest part of bonehead showed in the pictures can be used as the landmark for the start of VOI selection.

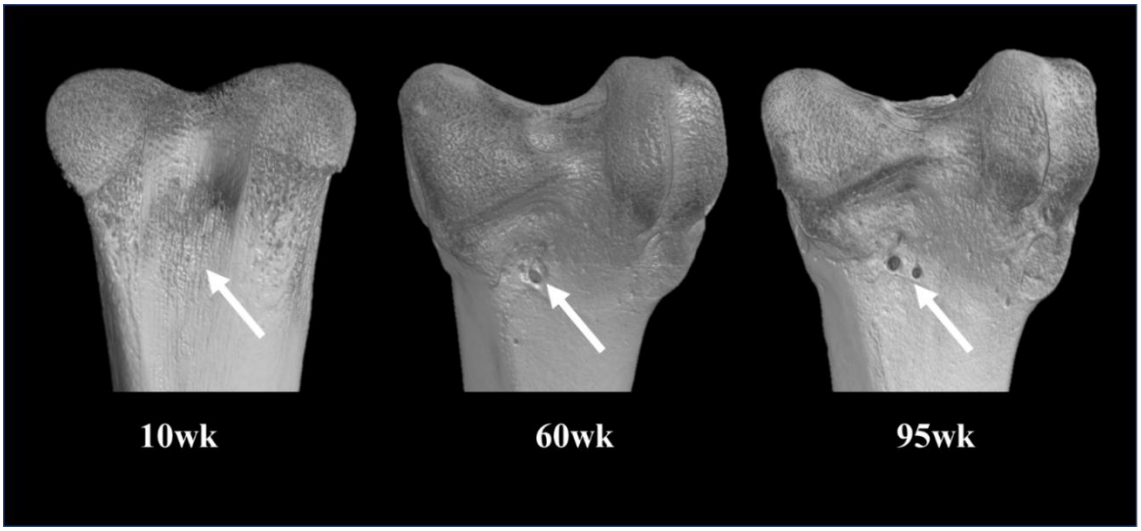

Figure 2.8 The femur from 10wk, 60wk, and 95wk of Hy-line W36 layers (under standard management and feeding program) were shown in the picture. The nutrient foramen (arrow) has not developed at the young age of birds, and multiple nutrient foramen usually showed at aged birds.

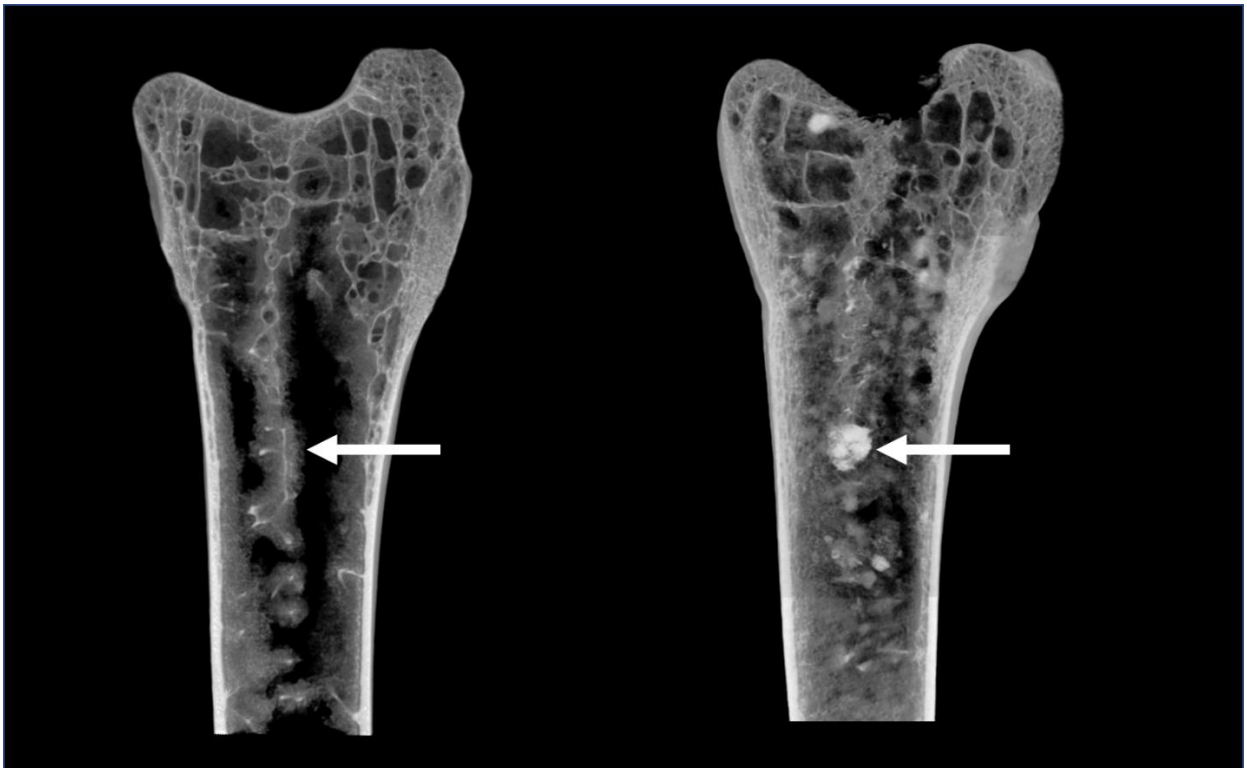

Figure 2.9 Two representative bones were presented to showed the type of structure of medullary bone (arrow).

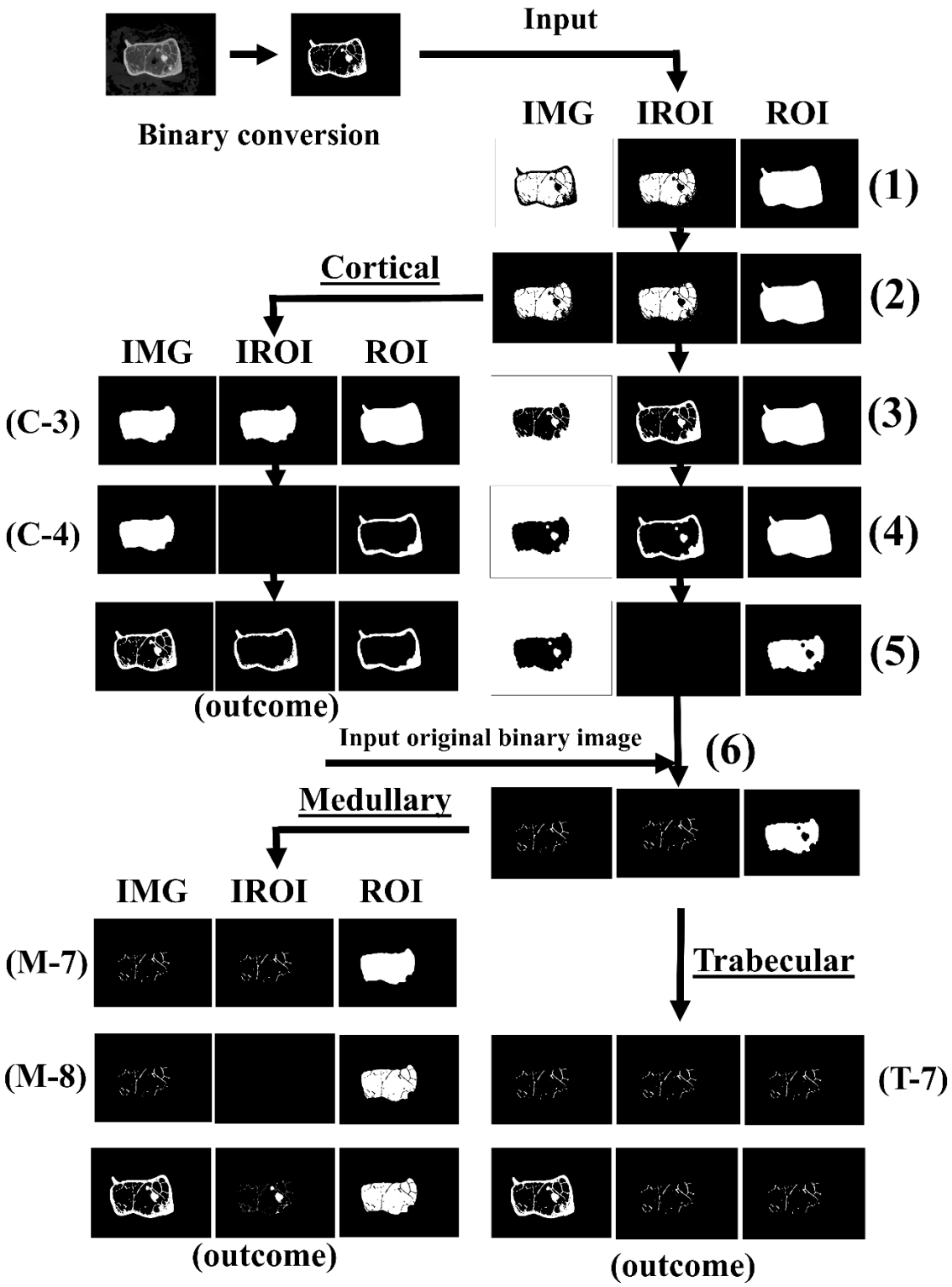
Input
Binary conversion
IMG
IROI
ROI
(1)
Cortical
(2)
IMG
IROI
ROI
(C-3)
(3)
(C-4)
(4)
(5)
(outcome)
Input original binary image
(6)
Medullary
IMG
IROI
ROI
(M-7)
Trabecular
(M-8)
(T-7)
(outcome)
(outcome)

Figure 2. 10 The diagram showed the critical step of automatic bone separation procedure. In order to better understand the process. Three types of views were presented in the diagram: IMG: view of the image; IROI: view of the image inside ROI (region of interest); ROI: view of ROI.

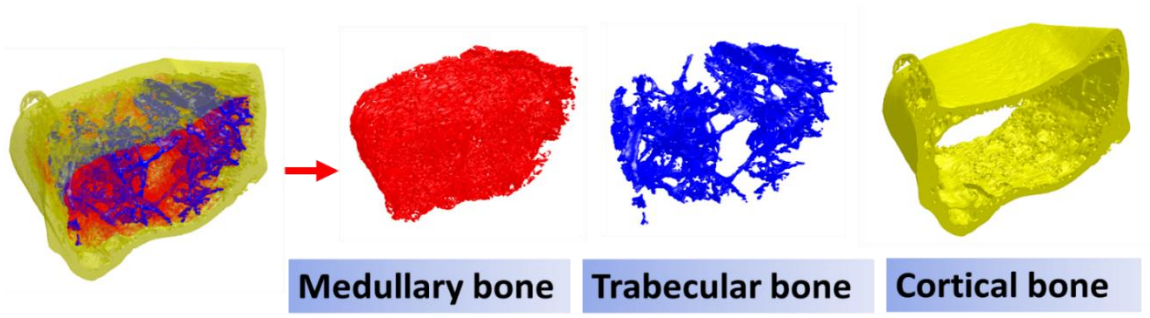

Figure 2.11 The diagram showed an example of laying hen bone separation outcome

CHAPTER 3

ROLE OF 1, 25-DIHYDROXYVITAMIN D3 ON OSTEOGENIC DIFFERENTIATION AND MINERALIZATION OF CHICKEN MESENCHYMAL STEM CELLS

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ABSTRACT

The direct role of 1,25-dihydroxyvitamin D_3 (1,25OHD) on osteogenic differentiation and mineralization in chicken mesenchymal stem cells (cMSC) derived from 2-day-old broiler bones was investigated. 1,25OHD has been suggested to play an important role in osteogenic differentiation and mineralization. However, limited data have been reported in avian species. In the present study, cMSCs were treated with control media (C), osteogenesis media (OM), osteogenesis media with 1, 5, 10 and 50nM 1,25OHD, respectively. The mRNA expression samples were obtained at 24h, 48h, 3d, and 7d to examine mRNA expression of key osteogenic genes (RUNX2, BMP2, COL1A2, BGLAP, BSP and ALP*)* at all time points. Cells were stained at 7, 14 and 21d using Von Kossa (mineralization), Alizarin Red (mineralization) and Alkaline Phosphatase (early marker) staining methods. From the mRNA expression results, we found a time-dependent manner of 1,25OHD on osteoblast differentiation and mineralization. In general, it showed an inhibitory effect on differentiation and mineralization during the early stage (24h and 48h), and a stimulatory effect during the late cell stage (3d and 7d). The staining showed, up to 14 days, 1,25OHD had an inhibitory effect on ALP expression and mineralization in a dosage-dependent manner. However, at 21d, there was no difference between the treatments. This study provides a novel understanding of the effects of 1,25OHD on osteogenic differentiation and mineralization of cMSCs depending on cell stage and maturity.

Keywords: 1,25-dihydroxyvitamin D_3, chicken osteoblast, gene expression, differentiation, mineralization

INTRODUCTION

Vitamin D_3 requires two steps of hydroxylation to become its active form, 1,25-dihydroxyvitamin D_3 (1,25OHD), to exert its biological functions (St-Arnaud, 2008). The role of 1,25OHD is intricate and involved in multiple systems, including the immune system, anti-oxidation, anti-cancer actions, and cardiovascular benefits (Gil, et al., 2018; Haussler, et al., 2013). Its most classical role is related to bone development and calcium homeostasis, which has been exhaustively studied (Driel van, et al., 2004). In general, it is well-established that 1,25OHD beneficially affects bone homeostasis either through its direct actions on bone cells or via regulating the minerals metabolism in the intestine, kidney, and parathyroid gland (Anderson, 2017; Gil, et al. 2018; Haussler, et al., 2013; van Driel and van Leeuwen, 2014). However, among all these properties, the direct effects of 1,25OHD on bone formation and mineralization remain inconclusive. There are considerable disagreements on 1,25OHD as suppressive or provocative on osteogenic differentiation and mineralization (St-Arnaud, 2008; Tarroni, et al., 2012; van Driel and van Leeuwen, 2014). Overall, 1,25OHD mostly showed stimulatory effects on osteogenic differentiation and mineralization in human osteoblasts regardless the cell original and treatment dosage (Chen, et al., 2002; Jorgensen, et al., 2004; Li, et al., 2018; Prince, et al., 2001; Tourkova, et al., 2017; Zhou, et al., 2006), whereas there are still some human studies that show partial inhibitory effects of 1,25OHD (Viereck, et al., 2002). Mice osteoblasts showed the most inconstant responses to 1,25OHD with either no effects, inhibition or promotion (Chen, et al., 2016; Kim, et al., 2016; van Driel and van

Leeuwen, 2014; Xiong, et al., 2017). Rat osteoblasts are not as popular as human and mice *in vitro* studies. Generally, the responses in rat osteoblasts showed either inhibition or no changes to 1,25OHD treatment (Harrison, et al., 1989; Kim and Chen, 1989). The inconsistent results across the species are greatly related to several factors such as differentiation stage, cell origin, treatment time and dosage, and extracellular factors (van Driel and van Leeuwen, 2014; Czekanska, et al., 2012). Even though the role of vitamin D on osteoblasts differentiation and mineralization has been comprehensively studied, questions regarding whether vitamin D has an anabolic or catabolic effect on osteoblasts and the role of vitamin D during different stages of cell growth and differentiation remain unanswered.

For avian species, there are limited data available. *In vitro*, 1,25OHD mainly showed an inhibitory effect on osteoblasts differentiation and mineralization (Broess, et al., 1995; Pande, et al., 2015). However, *in vivo* feeding trials, vitamin D_3 is mostly beneficial to avian bone formation (Atencio, et al., 2005; Kim, et al., 2011). The broiler and laying hen bone is an attractive bone model due to a robust calcifying bone model with higher rate of mineralization (Pande, et al., 2015), and unique bone turnover process (Whitehead, 2004). Chicken osteoblasts expressed higher osteocalcin and showed higher mineralization and ALP expression compared with Rat-MSCs, which may indicate that the rate of differentiation of chicken is higher compared with mammal (Pande, et al., 2015). Owen et al. (1991) points out that the effects of 1,25OHD on differentiation marks expression may be affected by the basal expression level. In this specific case, chicken osteoblasts model as high calcifying osteoblasts could give an important insight contributing to understand the vitamin D_3 function in bone formation.

In this study, we addressed the effects of various doses of 1,25OHD on osteogenic differentiation and mineralization of cMSCs during different cell stages. cMSCs isolated from day-old broiler compact bones were used in this study to investigate 1,25OHD on chicken osteogenic differentiation and mineralization. This study filled a research gap of 1,25OHD on chicken osteogenic differentiation and mineralization.

MATERIALS AND METHODS

Isolation of cMSCs, cell culture, and treatments

All practices regarding animal management were approved by the Institutional Animal Care and Use Committee of the University of Georgia. The isolation methods were adopted from R. Adhikari (Adhikari, et al., 2017). In brief, one-day-old broiler legs (3 birds) were removed from the hip joint and metacarpal after cervical dislocation and soaked in alcohol for two min. Then, dissected legs were kept in basal culture media (Dulbecco's Modified Eagle's medium (DMEM) (Mediatech Inc.,VA, USA) containing 10% Fetal Bovine Serum (FBS) (Mediatech Inc.,VA, USA), 100U/mL penicillin, 100µg/mL streptomycin and 0.292mg/mL L-glutamine (Thermo Fisher Scientific, MA, USA)). Muscles and connective tissues around tibia and femurs were removed. The cleaned bones were placed in the washing buffer containing phosphate-buffered saline (PBS) (Mediatech Inc., VA, USA) and 2% FBS. The epiphysis of the bone was removed, and bone marrow cavity was flushed with the washing buffer to remove the bone marrow and blood completely. The bones were then chopped to small fragments and suspended in

digestion media (DMEM containing 100 IU/ml penicillin and 100 ug/ml streptomycin, 0.25% collagenase (Sigma-Aldrich, MO, USA), and 20 % FBS). Then, the bone fragments were digested in an incubator shaker (Thermo Scientific SHKE4000, IA, USA) for 60 min at 37 °C and 180 rpm. Afterward, the digestion media containing bone fragments were mixed with DMEM media containing 10% FBS in 1:2 ratio, and then filtered with 40 µm sterile filter. Filtered contents were centrifuged at 1,200 rpm for 10 min to concentrate the cells. The cMSCs were plated in 100-mm cell culture dishes. Cultures were incubated at 37 °C in a humidified incubator containing 5% CO_2. Media was replaced every two days.

The cMSCs at P4 were seeded at a density of 20,000 cells /cm^2 in 24-well plates or 6 well-plates for further testing. On confluency, the cells were treated with osteogenic media (OM) containing DMEM with 10^{-7}M dexamethasone (DXA) (Sigma-Aldrich, MO, USA), 10 mM β-glycerophosphate (Sigma-Aldrich, MO, USA), 50 µg/ml ascorbate (Sigma-Aldrich, MO, USA), and 5% FBS for osteogenic induction. The cells cultured in DMEM basal media with 10% FBS were used as negative control. Fresh media was replaced every 2-3 days.

Alkaline Phosphate Assay

Alkaline phosphatase activity was tested following a protocol for analysis of osteogenic differentiation of MSCs (PromoCell, Heidelberg, Germany). The cells were rinsed with PBS twice and fixed in 10% neutral buffered formalin (Sigma-Aldrich, MO, USA) for 60 sec. Then cells were washed with washing buffer twice (0.05% Tween 20 (Sigma-Aldrich, MO, USA) in PBS), and 1mL substrate solution (10 ml distilled water contained one SigmaFast™ BCIP/NBT tablet (Sigma-Aldrich, MO, USA)) was added to

the wells. Cells were incubated at room temperature for 5-10 min based on color development. The reaction was stopped by rinsing wells with PBS.

Alizarin Red staining

Alizarin red staining test was conducted to stain for the mineral deposition following the protocol defined by Gregory, et al., (2004) to examine the mineralization level of cMSCs. In brief, Alizarin Red stain solution was prepared at the concentration of 20mg Alizarin Red S (Sigma-Aldrich, MO, USA)/ml distilled water at pH 4.1-4.3 (pH was adjusted by using 0.1% NH_4OH). The cells were rinsed twice with PBS (without Ca^{++}/Mg^{++}), then fixed using 10% buffered formalin for 30 min and washed twice with distilled water. The cells were stained with Alizarin Red solution for 45 min in the dark. The reaction was stopped by washing off the staining solution with distilled water.

Von Kossa Staining

Von Kossa stain was performed for detecting mineralization following the protocol previously described by Parhami, et al., (1997). In brief, cells were rinsed with PBS and fixed with 0.1% glutaraldehyde (Sigma-Aldrich, MO, USA) for 15 min. Then rinsed with distilled water and incubated with 5% silver nitrate (Sigma-Aldrich, MO, USA) in the dark for 30 min. The stained cells were rinsed again with distilled water. Then air dried under bright light to develop the color.

RNA extraction and real-time Quantitative Reverse Transcription Polymerase Chain Reaction

Total RNA of cells was extracted by using QIAzol Lysis reagent (Qiagen, MD, USA) according to the manufacturer's protocol. For each sample, 2ug of RNA was

reverse-transcribed to cDNA using a High-Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific, MA, USA) following manufacturer's protocol in a 96-well thermal cycler (Thermo Fisher Scientific, MA USA). cDNA samples were analyzed in duplicate by real-time PCR using iTaq^{TM} Universal SYBR Green Supermix (Bio-Rad, CA, USA). Primers (Table 1) and cDNA templates were subjected to qRT-PCR at 95° C for 10 min, followed by 40 cycles of 15 s denaturation at 95° C, annealing for 20 sec (annealing temperature was varied depending on the primers (Table 1)), and 15s extension at 72° C, followed by 95°C for 15 sec and a melt curve stage in a StepOneTM Real-Time PCR machine (Thermo Fisher Scientific, MA, USA). GAPDH was used as a housekeeping gene. Samples were normalized and analyzed by the ΔΔCT method.

Statistics

All experimental data were analyzed statistically by one-way ANOVA using SAS software Version 9.3 (SAS Institute, Cary, NC). Variability in the data was expressed as standard error mean (SEM). Differences between means were determined using Duncan's Multiple Range test. The level of significance was assessed at $P \leq 0.05$.

RESULTS

Key osteogenic differentiation marker gene expression

In order to reveal the role of 1,25OHD in chicken osteogenic differentiation of cMSCs, the mRNA samples were obtained at 24h, 48h, 3d, and 7d for the key osteogenic genes expression assessment (RUNX2, BMP2, COL1A2, BGLAP, BSP and ALP). These

results are summarized in Figures 1-3 OM induced mRNA expression of key osteogenic genes at all time points compared to C ($P<0.05$) (Figures 1-3). RUNX2 expression was inhibited by 1nM, 10nM and 50nM 1,25OHD treatments at 48h compared to OM treatment ($P<0.01$) (Figure 1B). In contrast, 1,25OHD upregulated RUNX2 expression at 3d with the 10 nM and 50 nM 1,25OHD compared with OM treatment ($P<0.0001$) (Figure 1C). No difference in RUNX2 expression was found at 24h and 7d among the treatments with 1,25OHD (Figure 1A and 1D). For BMP2 expression, at 48h, only the 1nM 1,25OHD inhibited its expression ($P<0.05$) (Figure 1F). However, the 50nM 1, 25D significantly stimulated BMP2 expression at 7 d ($P<0.001$) (Figure 1H). No difference was found at 24h and 3d compared with OM treatment (Figure 1E and 1G).

The effects of 1,25OHD on bone matrix protein mRNA expression followed a similar pattern as RUNX2 and BMP2. All concentrations of 1,25OHD decreased COL1A2 expression at 24 h and 48 h compared with OM treatment ($P<0.05$) (Fig 2A and 2B). On the contrary, at 3 d, 10 nM and 50 nM 1,25OHD increased COL1A2 expression ($P<0.0001$) (Figure 2C). BGLAP showed similar expression response to 1,25OHD as COL1A2. ($P<0.05$) (Figure 2E-H). Different than the other bone matrix proteins, SPP1 was increased by 50 nM 1,25OHD at 24h. ($P<0.001$) (Figure 3A). The stimulatory effects of 1,25OHD on SPP1 also showed at 3d and 7d ($P<0.001$) (Figure 3C and 3D). The ALP expression response to 1,25OHD, in the same pattern as COL1A2 at 24 h, 48h and 3d except 10 nM and 50 nM 1,25OHD at 24h, has no effects on ALP expression ($P<0.05$) (Figure 3E-G). During the late stage of differentiation, 1,25OHD showed a dual-function on ALP expression. At 7d, 5 and 10 nM 1,25OHD suppressed ALP expression, whereas

50nM increased ALP expression ($P<0.05$) (Figure 3H). The effects of 1,25OHD on osteogenic gene expression results are summarized in Figure 3.4.

ALP expression and mineralization

Three staining methods were performed at 7d, 14d and 21d to explore the ALP expression and mineralization process throughout the whole experimental period. The staining results were presented in two formats. The whole well staining picture was present by using a DSLR camera with a fixed exposure setting for all the treatments (Figure 4A, 5A, and 6A). At the same time, the staining pictures were taken by a microscope at 40x magnificent (Figure 4B, 5B, and 6B). As the cell continues to differentiate, the mineralization level became higher. While comparing the treatments at each time point, OM treatment dramatically increases the ALP expression and mineralization compared with C. The cells with 1,25OHD treatments showed an inhibitory effect on mineralization and ALP expression up to 14d compared with OM, but no difference between the treatment was detected at 21d. Furthermore, a consistent dose-dependent manner was observed. The higher concentration of 1,25OHD showed stronger inhibitory effects in the earlier stages.

DISCUSSION

1,25OHD is regarded as a regulator of bone metabolism and functions by affecting the production of bone matrix proteins (Collagen type I, osteopontin, osteocalcin, matrix Gla protein, etc.), the activity of alkaline phosphatase, and the process

of mineralization (Yamamoto, et al., 2013). However, its function on osteoblasts is pleiotropic due to a number of factors including osteogenic differentiation stage, cell origin, treatment timing, and extracellular factors. (St-Arnaud, 2008; van Driel and van Leeuwen, 2014; Yang, et al., 2013). It is challenging to compare or include all the factors in one study. Hence, this study mainly focused on the effects of various dosages of 1,25OHD on osteogenic differentiation at different differentiation stages.

The higher expression of osteogenesis key markers and mineralization in osteogenesis media (OM) was observed compared with cMSCs with control media, confirming that the experimental conditions were adequate for osteogenic differentiation. The results indicate a time-dependent manner of 1,25OHD on the expression of key osteogenic differentiation marker genes. We observed that an inhibitory effect during the early differentiation stages (24h and 48 h), and a stimulatory effect during the late differentiation stages (3d and 7d). Among these osteogenesis markers, RUNX2 as one of the most important osteoblast-specific transcription factors is essential for the activation of osteoblast differentiation and induce MSCs into mature osteoblasts. (Komori, 2006; Lieben, et al., 2012; Mundlos, et al., 1997; Viereck, et al., 2002; Vimalraj, et al., 2015). Similarly, BMP2 is highly related to the induction of osteogenic differentiation and enhancement of bone matrix production (Liu, et al., 2012; Zhou, Liu and Tan, 2006). Both are regarded as markers for osteogenic differentiation. In the current study, they followed the same pattern as other markers under 1,25OHD treatment, showed a dual-effect. However, 1,25OHD did not affect RUNX2 and BMP2 expression at 24h, which is similar to Yang's study that RUNX2 in primary murine osteoblasts was unaffected by 1,25OHD treatment. (Yang, et al., 2013). In contrast, in other mouse

studies, RUNX2 was inhibited by 1,25OHD (Ducy, et al., 1997; Kim, et al., 2016). For human osteoblasts, the responses are more complicated. The treatment duration of 1,25OHD affects its function on RUNX2 expression in human primary osteoblasts; a short 1h treatment showed stimulatory effects, whereas 48h treatment showed an inhibitory effect (Viereck, et al., 2002). Furthermore, the type of human osteoblasts is an important factor regulation RUNX2 expression; RUNX2 was not affected by 1,25OHD in human osteoblasts cell lines (O4-T8, 03-CE6, and 03-CE10) (Prince, et al., 2001), whereas 1,25OHD upregulated it in human marrow stromal cells (Geng, et al., 2011). Similarly to RUNX2, BMP2 is either increased or decreased by 1,25OHD across different species (Chatakun, et al., 2014). Due to many factors involved, it is not surprising how complicated the action of 1,25OHD on progenitor cells and osteoblasts. Our study carefully investigated 1,25OHD actions at various time points and provided a detail response related to cell differentiation stages, which reveals that cell differentiation stage and dosage are important for chicken osteogenic differentiation and mineralization.

The other key osteogenic markers, such as CO1A2 which is an early-stage marker and codes type I collagen in osteoblasts (Quarles, et al., 1992); early differentiation marker—ALP; and late-stage non-collagenous bone matrix proteins—BGLAP and SPP1 (Chatakun, et al., 2014; Mörike, et al., 1995; Yamamoto, et al., 2010), involved in osteoblast differentiation and mineralization. In the current study, COL1A expression was significantly inhibited by all the dosage of 1,25OHD at 24h. It may be due to the high expression level of COL21A at this time point because the higher basal expression most likely would be inhibited by 1,25OHD (Owen, et al., 1991). In line with this, the late differentiation marker BGLAP, which is supposed to have a lower expression at 24h,

was inhibited as well. It is probably because chicken MSCs express a higher level of BGLAP compared with other mammalian species (Pande, et al., 2015). In other species, the contradictory results were found. Either 1,25OHD upgraded BGLAP in human or down-regulated in mouse osteoblasts (Chen, et al., 2002; Zhang, et al., 1997). The SPP1 was stimulated at 24h with the highest treatment dosage, which is the only exception for all the inhibitory effects showed at this stage. Besides a marker of osteogenic differentiation, SPP1 has multiple functions with one of them showed as inhibiting mineralization (Chabas, 2005). The higher expression of SPP1 collaborated with other factors may contribute to the inhibitory effects on bone mineralization at early cell stage. ALP expression was tested in both the mRNA level and protein level. The ALP staining results indicated an inhibitory effect up to 14d by 1,25OHD, which agrees with the results in the mouse study (Chen, et al., 2012; Kim, et al., 2016), However, for the human osteoblasts, 1,25OHD either showed no effects or promoting effects on ALP expression (Chen, et al., 2002; Matsumoto, et al., 1991; Siggelkow, et al., 1999). It is hard to compare or summarize the effects of 1,25OHD on osteoblasts across all the species, not only because it involved in a number of factors, but also the multiple functions and expression level of each bone matrix proteins contribute to this complexity.

The staining results from this study showed the inhibitory effects on ALP expression and mineralization in a dosage-dependent manner up to 14d, which agrees with Chen's finding (Chen, et al., 2012). However, there no difference is observed at 21d, indicating that the promoting effects after 3d balanced off the earlier inhibitory effects. Contrary to our finding, Prince found the continuous treatment with 1,25OHD inhibited mineralization of the 04-T8 and CE6 cell lines but promoted mineralization in

the CE10 cell line where CE10 reflect an earlier stage osteoblasts phenotype than CE6 and O4-T8 (Prince, et al., 2001). Yang also pointed out the different mineralization response of calvaria and long bone osteoblasts to 1,25OHD is because of different maturation level of two cell origins (Yang, et al., 2013). Regarding the varied results in our current and previous other studies, they all pointed out that the effect of 1,25OHD on osteogenic differentiation and mineralization are related to the cell differentiation and mineralization stage and maturity. An alternative explanation for this phenomenon is the possible results of the high-level mineralization of cMSCs at the later stage, leading to an inconspicuous result among the treatments. Even though some of studies could detect the difference in staining among the treatments up to 24 d after the induction of osteogenic differentiation. (Yang, et al., 2013), for chicken MSCs, the optimal staining time may be less than 21d.

The response of 1,25OHD on chicken osteoblasts is seldom mentioned because there is limited information available, especially at the mRNA level. In general, 1,25OHD showed an inhibitory effect on osteoblast development (Broess, et al., 1995; Pande, et al., 2015). The detailed data from Broess's study also indicated a timing related function, which indicates that 10 nM of 1,25OHD slightly stimulated osteopontin and osteocalcin expression at the 17d but showed an inhibitory effect at 5 and 30 days. There is still no full explanation for these inconsistent results within or across species. However, the fact that cells in each study display a varied level of differentiation caught our attention (Czekanska, et al., 2012), which is one of the possible reasons that osteoblasts showed different responses to 1,25OHD across the species. Additional evidence was found from another study reporting that acute 1,25OHD treatment inhibited

alkaline phosphatase and collagen I expression which was at the highest basal expression levels in the early phase, but stimulated expression during the mineralization period of the cells when the basal expression levels were lowest (Owen, et al., 1991). In addition, (Woeckel, et al., 2010) reported that the pre-mineralization (before 10 days) and mineralization phase are two distinctive periods with only 0.6% (3 genes) overlap of genes regulated by 1,25OHD, which indicates that 1,25OHD may play different roles at various osteogenic differentiation stages .

In the current study, we found the effects of 1,25OHD on cMSCs has a close relationship with cell differentiation stage and 1,25OHD dosage. The results from the current study showed an inhibitory effect on osteoblasts differentiation at the early stage; however, it does not mean that 1,25OHD will inhibit the bone formation in a biological individual. Because 1,25OHD could also contribute to bone formation by affecting other organs such as intestine, kidney, and parathyroid (Haussler, et al., 2013). In addition, this study did not involve testing the effect of 1,25OHD on osteoclast. Consequently, the net effects of 1,25OHD on bones are indeterminate. However, the previous study has reached an agreement that 1,25OHD in favor of osteoclasts function for blood Ca homeostasis (Haussler, et al., 2010). In this case, 1,25OHD harms bone formation at early stage which is contradicting to its beneficial effects on bone health. In this case, we have a bold hypothesis.

Vitamin D is tightly regulated in the biological system (Anderson, 2017) and involved in a complex function to build a well-mineralized, efficiently remodeled and stronger bones rather than simply increase bone mass (Haussler, et al., 2013; Driel van, et al., 2004). It is a sophisticated regulator for bone remodeling. The bone remodeling is

initiated by osteoclastic resorption, which erodes a resorption lacuna, then followed by bone formation (Eriksen, 2010). Even though such a remodeling event may have significant overlaps during different remodeling stages (Schindeler, et al., 2008). However, the time interval between the activation of osteoblasts and osteoclast may benefit the efficiency of the bone remodeling process. The 1,25OHD inhibitory effects during the early differentiation stage of osteoblasts may be a sign of creating time interval during bone remodeling. However, due to the complexity of this process, it still required much more studies to confirm this theory.

In summary, this study emphasizes the various response to 1,25OHD during various differentiation stage of strong calcifying avian osteoblasts. It showed inhibitory effects on cMSC osteogenic differentiation and mineralization during early cell stage but promoting effects during the late cell stage. The staining results indicate a dosage-dependent manner that is the higher level of 1,25OHD show stronger inhibitory effects on mineralization of cMSC up to 14d. However, the detailed mechanism is still a complicated story. A number of factors contribute to the complexity of 1,25OHD effects on osteoblasts. Thus, the interpretation of data related to 1,25OHD on bone cells needs to address the factors such as the cell species, cell origin, differentiation stage, extracellular factor, treatment dosage, duration, etc. Exploring or systematic reviewing the interaction among these factors are important while discussing 1,25OHD on osteoblast differentiation and mineralization.

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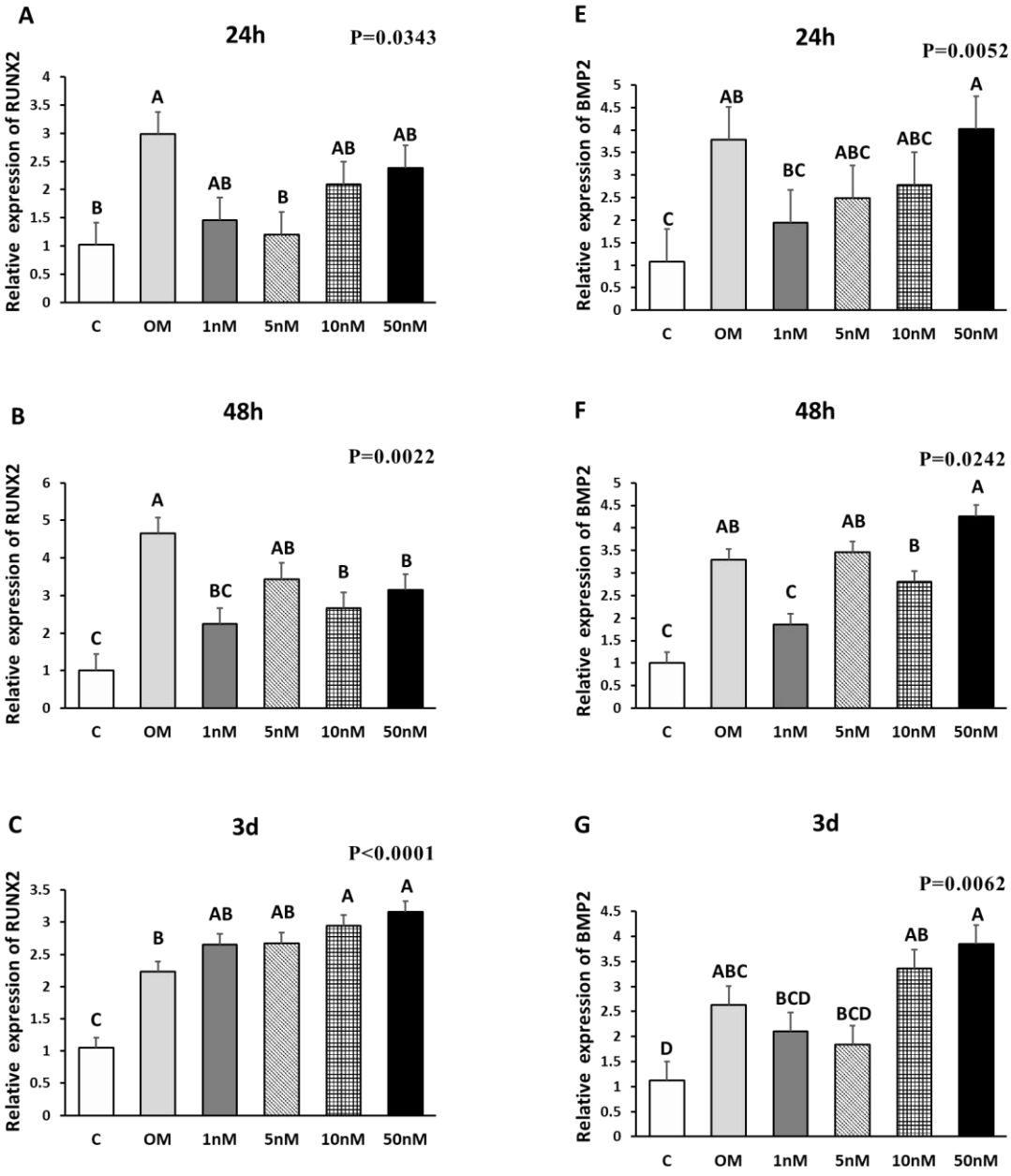
TABLES

Table 3.1 List of primers used in this study

Gene Name	Primer sequence (5'---3')	Product length (bp)	Annealing temperature (°C)
GAPDH	Fwd: GCTAAGGCTGTGGGGAAAGT	116	56
	Rev: TCAGCAGCAGCCTTCACTAC		
RunX2	Fwd: TCTCTGAACTCTGCACCAAGTC	229	58
	Rev: GCTCGGAAGCACCTGAGAGG		
Col1A2	Fwd: CTGGTGAAAGCGGTGCTGTT	222	57
	Rev: CACCAGTGTCACCTCTCAGAC		
SPP1	Fwd: GCCCAACATCAGAGCGTAGA	204	57
	Rev: ACGGGTGACCTCGTTGTTTT		
BMP2	Fwd: TCAGCTCAGGCCGTTGTTAG	163	57
	Rev: GTCATTCCACCCCACGTCAT		
BGLAP	Fwd: GGATGCTCGCAGTGCTAAAG	142	57
	Rev: CTCACACACCTCTCGTTGGG		
ALP	Fwd: CGACCACTCACACGTCTTCA	140	58
	Rev: CGATCTTATAGCCAGGGCCG		

FIGURES


A
24h
P=0.0343
Relative expression of RUNX2
B
A
AB
B
AB
AB
C
OM
1nM
5nM
10nM
50nM
E
24h
P=0.0052
Relative expression of BMP2
C
AB
BC
ABC
ABC
A
B
48h
P=0.0022
Relative expression of RUNX2
C
A
BC
AB
B
B
F
48h
P=0.0242
Relative expression of BMP2
C
AB
C
AB
B
A
C
3d
P<0.0001
Relative expression of RUNX2
C
B
AB
AB
A
A
G
3d
P=0.0062
Relative expression of BMP2
D
ABC
BCD
BCD
AB
A

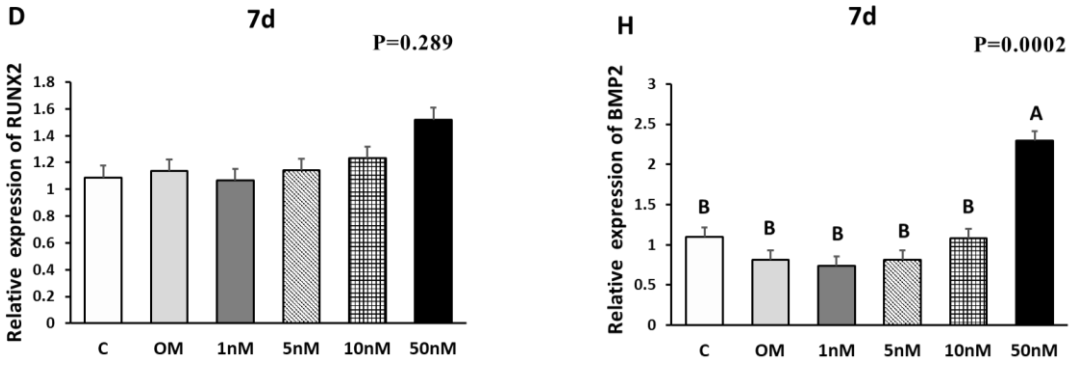


Figure 3.1. Effects of 1,25OHD on chicken osteoblasts RUNX2 and BMP2 gene expression by using qRT-PCR. Cell were treated with Control media, Osteogenesis media, or Osteogenesis media with 1nM, 5nM, 10nM or 50nM 1,25OHD. A-D showed the mRNA expression of RUNX2 at 24h, 48h, 3d and 7d respectively; E- H showed the mRNA expression of BMP2 at 24h, 48h, 3d and 7d, respectively. Mean separation was indicated by different letters on the top of bars (Value means ± SEM, 3 replicates/treatment).

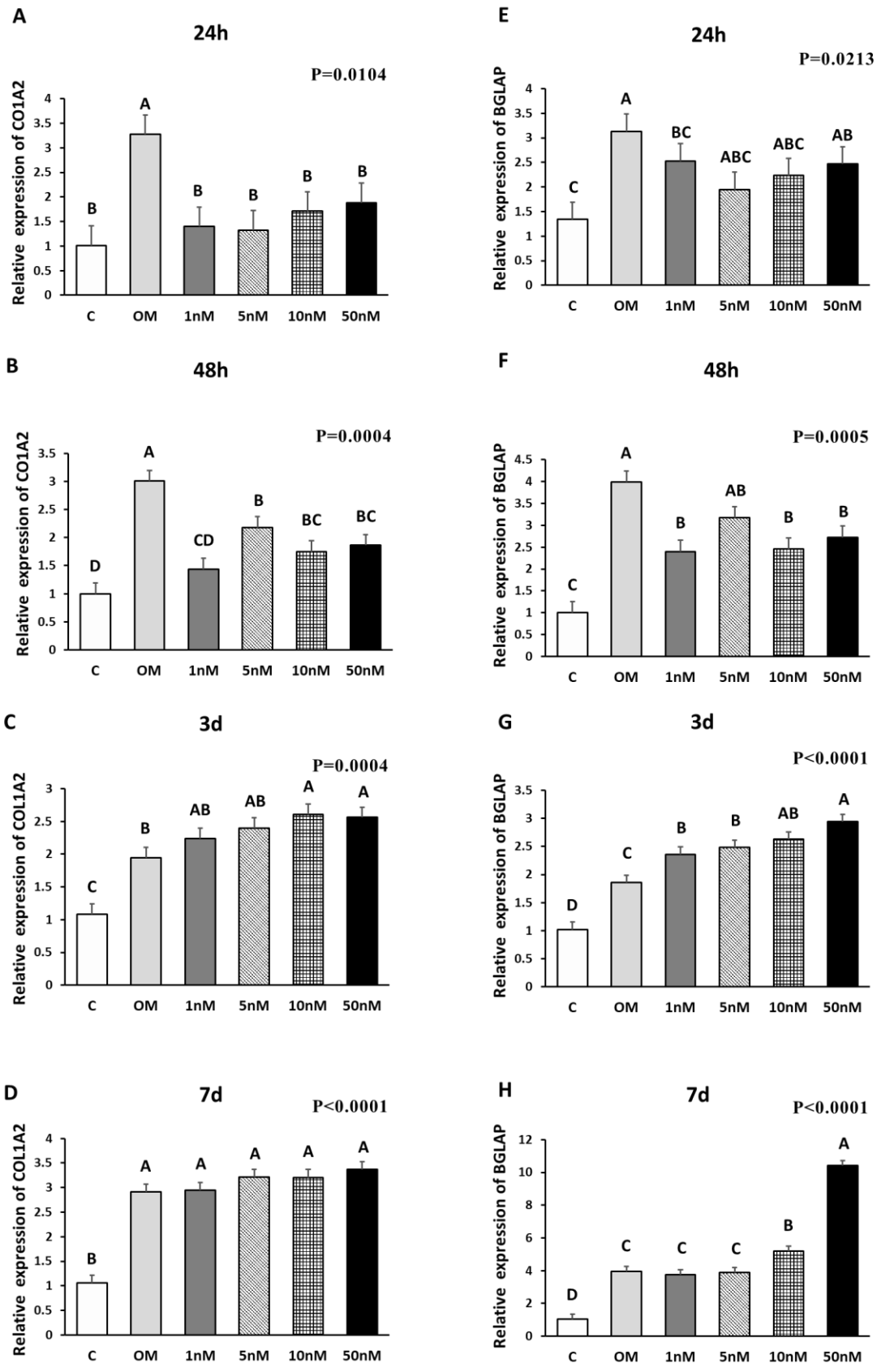

A
24h
P=0.0104
Relative expression of CO1A2
B
A
B
B
B
B
C
OM
1nM
5nM
10nM
50nM
E
24h
P=0.0213
Relative expression of BGLAP
C
A
BC
ABC
ABC
AB
B
48h
P=0.0004
Relative expression of CO1A2
D
A
CD
B
BC
BC
F
48h
P=0.0005
Relative expression of BGLAP
C
A
B
AB
B
B
C
3d
P=0.0004
Relative expression of COL1A2
C
B
AB
AB
A
A
G
3d
P<0.0001
Relative expression of BGLAP
D
C
B
B
AB
A
D
7d
P<0.0001
Relative expression of COL1A2
B
A
A
A
A
A
H
7d
P<0.0001
Relative expression of BGLAP
D
C
C
C
B
A

Figure 3.2. Effects of 1,25OHD on chicken osteoblasts COL1A2 and BGLAP gene expression by using qRT-PCR. Cell were treated with Control media, Osteogenesis media, or Osteogenesis media with 1nM, 5nM, 10nM or 50nM 1,25OHD. A-D showed the mRNA expression of COL1A2 at 24h, 48h, 3d and 7d respectively; E-H showed the mRNA expression of BGLAP at 24h, 48h, 3d and 7d, respectively. Mean separation was indicated by different letters on the top of bars (Value means ± SEM, 3 replicates/treatment).

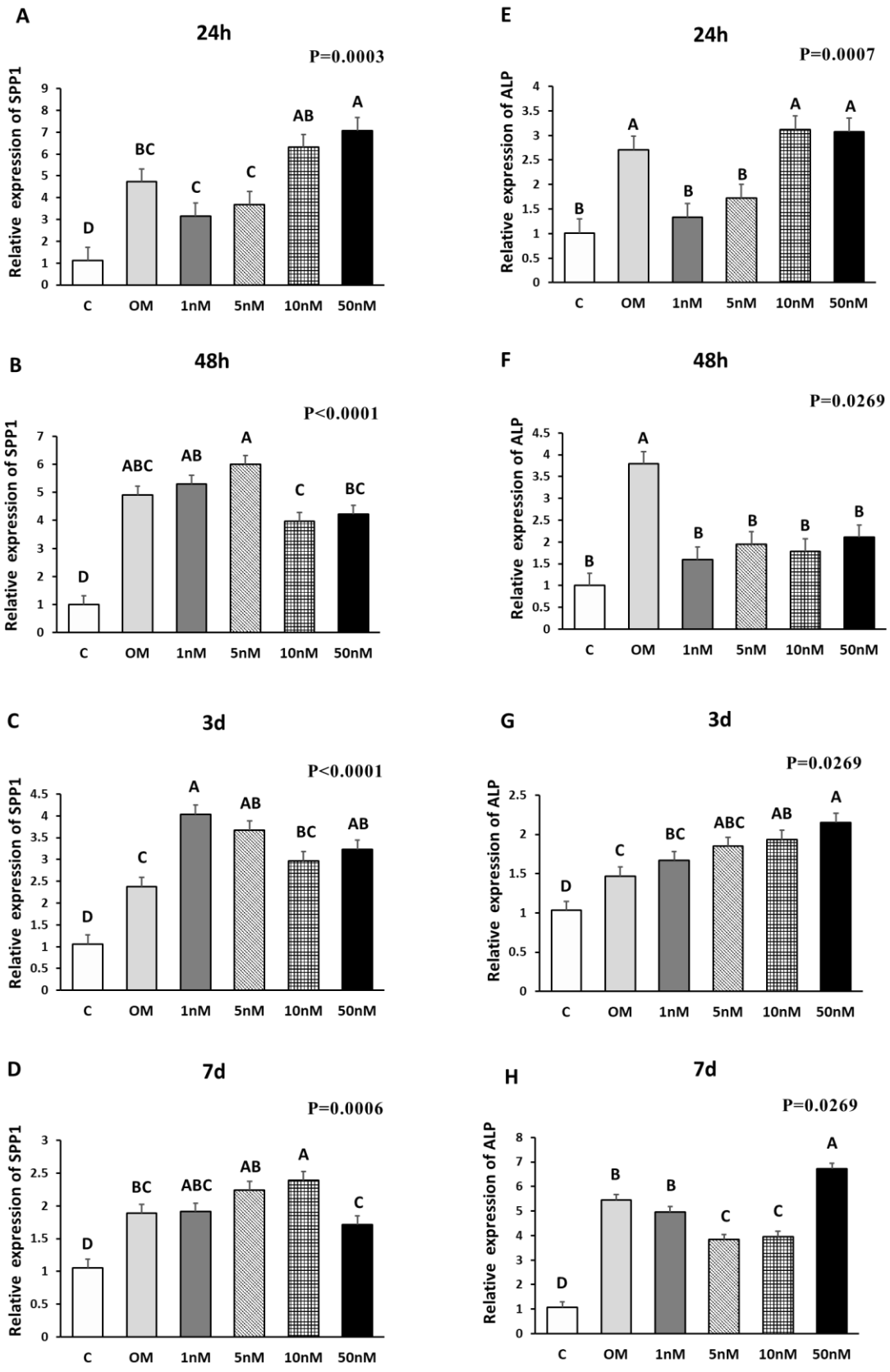

A
24h
P=0.0003
Relative expression of SPP1
D
BC
C
C
AB
A
C
OM
1nM
5nM
10nM
50nM
E
24h
P=0.0007
Relative expression of ALP
B
A
B
B
A
A
B
48h
P<0.0001
Relative expression of SPP1
D
ABC
AB
A
C
BC
F
48h
P=0.0269
Relative expression of ALP
B
A
B
B
B
B
C
3d
P<0.0001
Relative expression of SPP1
D
C
A
AB
BC
AB
G
3d
P=0.0269
Relative expression of ALP
D
C
BC
ABC
AB
A
D
7d
P=0.0006
Relative expression of SPP1
D
BC
ABC
AB
A
C
H
7d
P=0.0269
Relative expression of ALP
D
B
B
C
C
A

Figure 3.3. Effects of 1,25OHD on chicken osteoblasts SPP1 and ALP gene expression by using qRT-PCR. Cell were treated with Control media, Osteogenesis media, or Osteogenesis media with 1nM, 5nM, 10nM or 50nM 1,25OHD. A-D showed the mRNA expression of SPP1 at 24h, 48h, 3d and 7d respectively; E-H showed the mRNA expression of ALP at 24h, 48h, 3d and 7d, respectively. Mean separation was indicated by different letters on the top of bars (Value means ± SEM, 3 replicates/treatment).

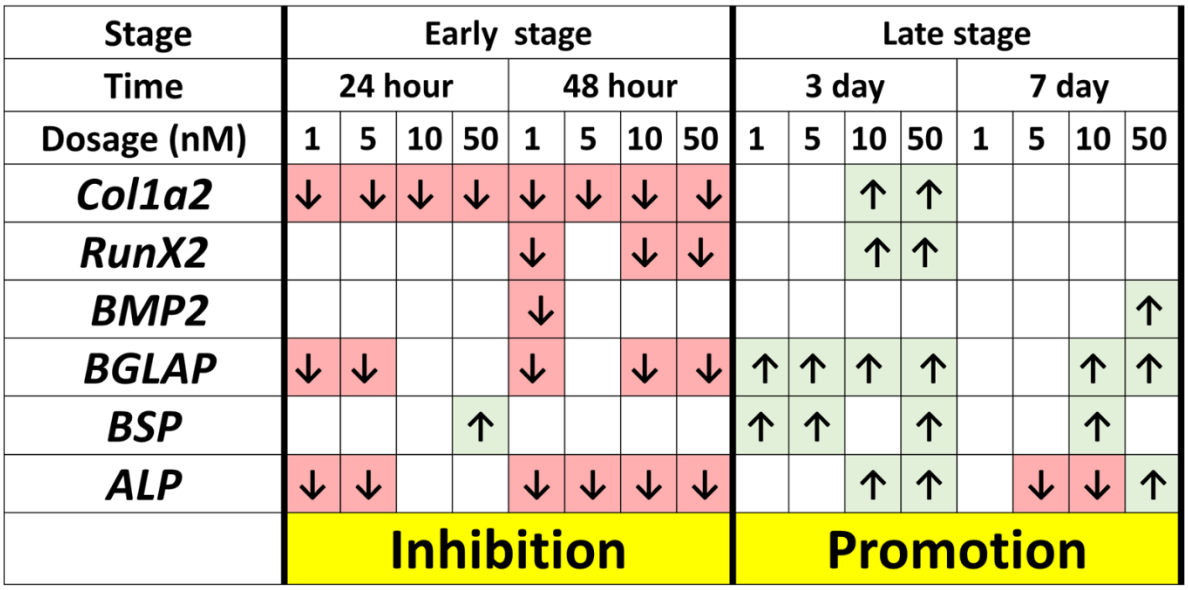


Figure 3.4 Effects of 1,25OHD on chicken osteoblasts key osteogenic gene expression by using qRT-PCR. Cell were treated with Control media, Osteogenesis media, or Osteogenesis media with 1nM, 5nM, 10nM or 50nM 1,25OHD. The results are summarized in the figure: the down-arrows (red) indicate the genes are down-regulated by 1,25OHD treatment compare to OM; the up-arrows (green) indicate the genes are up-regulated by 1,25OHD treatment compare to OM (Value means ± SEM, 3 replicates/treatment).

.

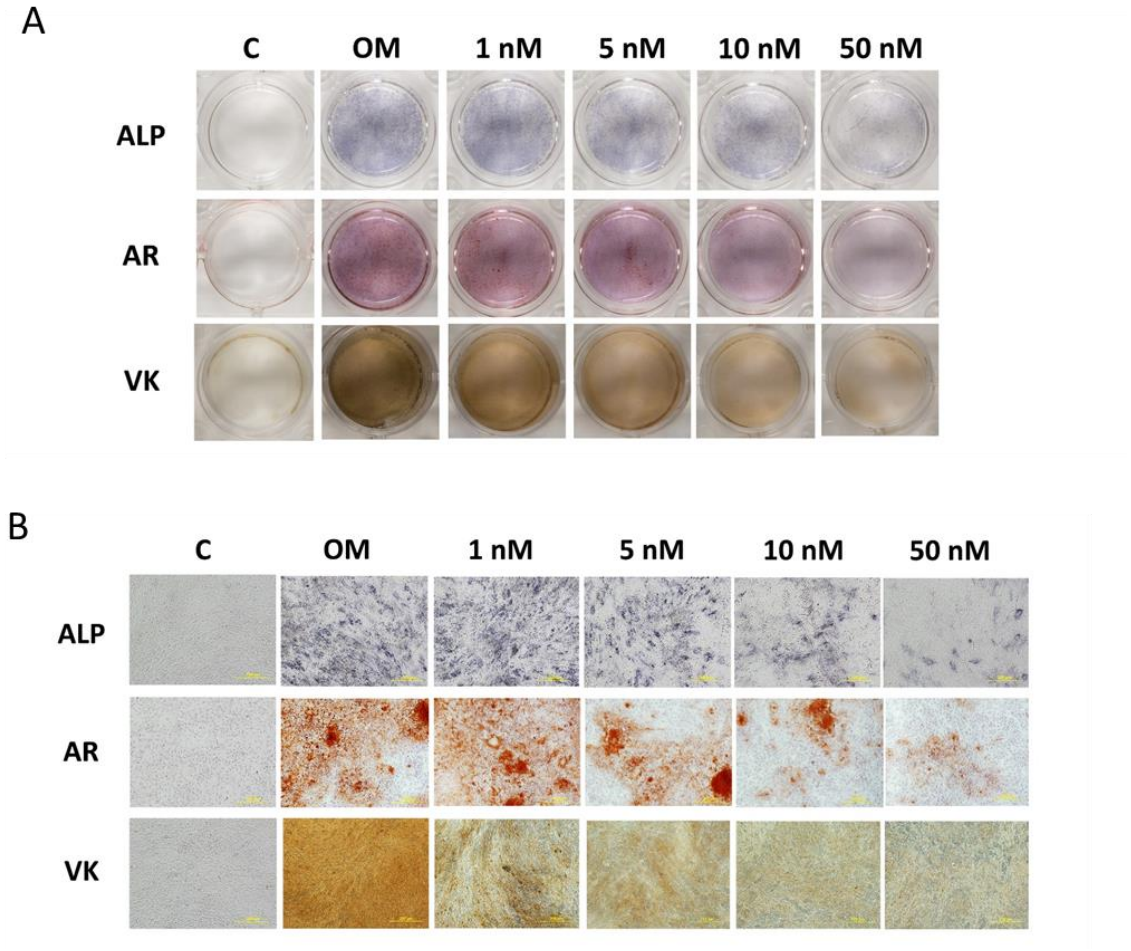


Figure 3.5 Effects of 1,25OHD on chicken osteoblasts ALP activity and mineralization at 7d. In each picture, the first row: ALP (Alkaline Phosphatase); the second row: AR (Alizarin Red stains the deposited minerals); the third row: VK (Von Kossa stains for the deposited minerals). A) Pictures were taken by DSRL camera, with same exposure setting across all the treatment. B) The picture was taken under the microscope at 40x. The bar indicated 200μm.

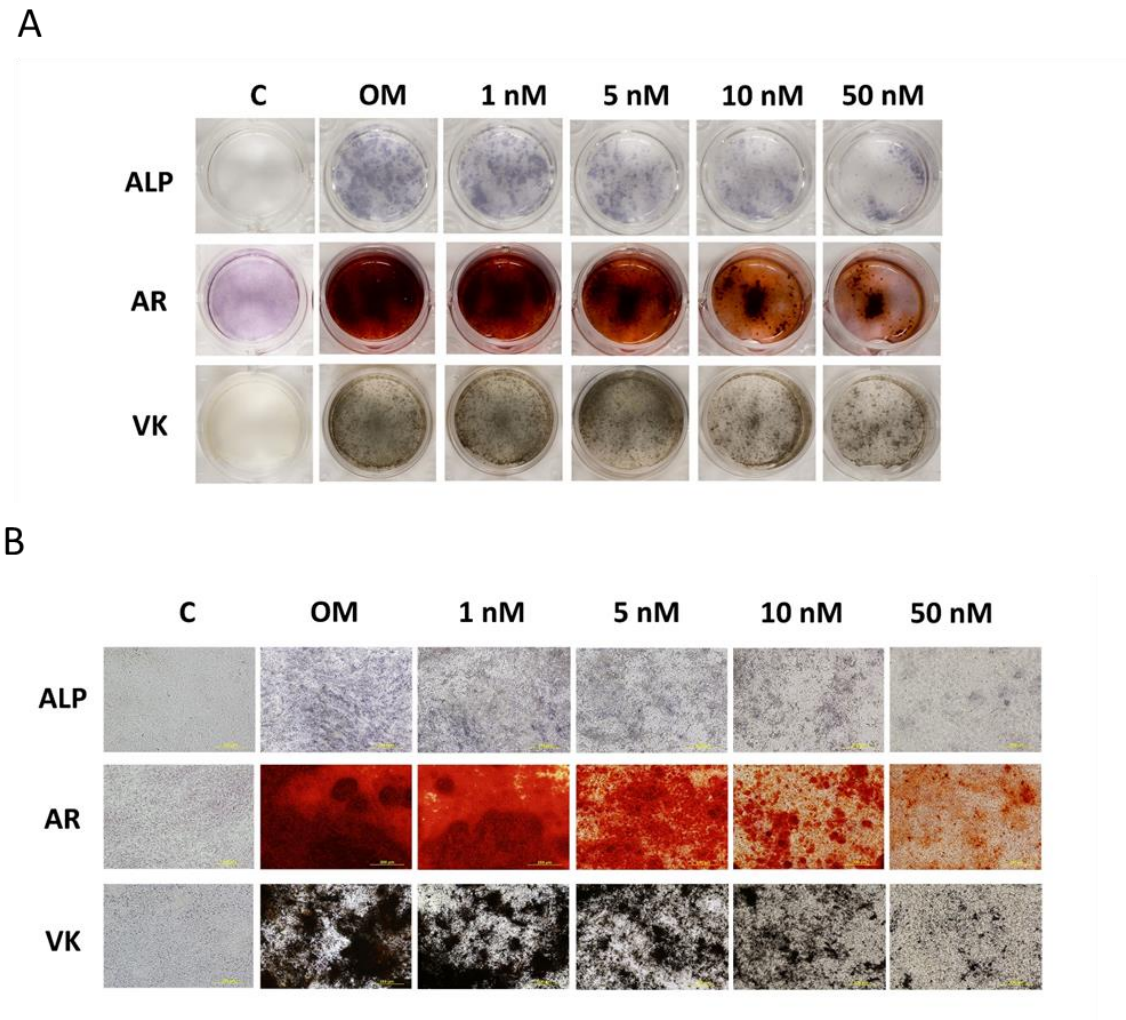


Figure 3.6 Effects of 1,25OHD on chicken osteoblasts ALP activity and mineralization at 14d. In each picture, the first row: ALP (Alkaline Phosphatase); the second row: AR (Alizarin Red stains the deposited minerals); the third row: VK (Von Kossa stains for the deposited minerals). A) Pictures were taken by DSRL camera, with same exposure setting across all the treatment. B) The picture was taken under the microscope at 40x. The bar indicated 200μm.

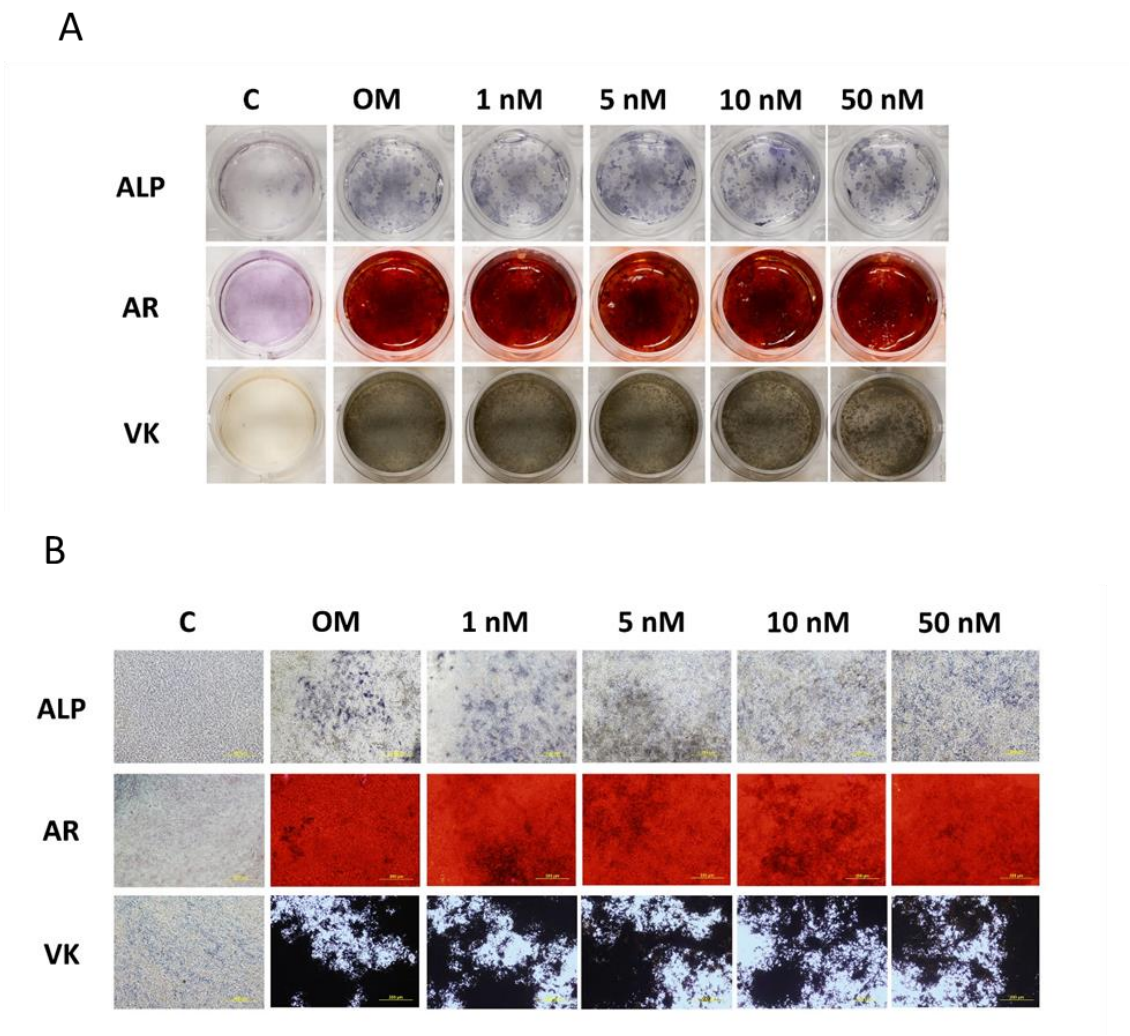


Figure 3.7 Effects of 1,25OHD on chicken osteoblasts ALP activity and mineralization at 21d. In each picture, the first row: ALP (Alkaline Phosphatase); the second row: AR (Alizarin Red stains the deposited minerals); the third row: VK (Von Kossa stains for the deposited minerals). A) Pictures were taken by DSRL camera, with same exposure setting across all the treatment. B) The picture was taken under the microscope at 40x. The bar indicated 200μm.

CHAPTER 4

ROLE OF LONG-TERM SUPPLEMENTATION OF 25-HYDROXYVITAMIN D_3 ON EGG PRODUCTION AND EGG QUALITY OF LAYING HENS

Chongxiao Chen, Bradley Turner, Todd Applegate, and Woo Kyun Kim. To be submitted to *Poultry Science*.

ABSTRACT

A study was conducted to evaluate the effect of dietary 25-hydroxyvitamin D_3 (25OHD) on laying hen growth performance, egg production, and egg quality from day-old to 95wk. A total of 390 1-day old Hy-Line W36 pullets were randomly allocated to 3 treatments with 10 replications and 13 birds per pen. Dietary treatments were: 1) vitamin D_3 at 2,760 IU/kg (D); 2) vitamin D_3 at 5,220 IU/kg (DD), and 3) vitamin D_3 at 2,760 IU/kg plus 25OHD at 2,760 IU (69µg)/kg (25D). Body weight, feed intake were recorded at the end of each stage: starter1 (0-3wk), starter2 (4-6wk), grower (7-12wk), developer (13-15wk), prelay (15-17wk), peaking (18-38wk), layer2 (39-48wk), layer3 (49-60wk), layer4 (61-75wk), and layer5 (76wk-95wk). Egg production was recorded daily, and egg quality was measured every 8 weeks from 18wk. There was no difference in growth performance during the rearing period (0-17wk). For laying period (18-95wk), DD showed lower feed intake at layer2, but higher at layer3 along with lower hen day production (HPD) from 18 to 48wk compared the others. During the same period, the DD group laid smaller eggs with higher specific gravity and shell thickness compared with the other treatments or D alone at wk40, which may be partly due to the lower body weight. In contrast, 25D had better FCR (feed intake/dozen of eggs) at layer2, and higher overall (18-60wk) HPD compared with DD. For the egg quality analysis, at 25 and 33wk, both DD and 25D had higher Haugh Unit compared with D. However, 25OHD has no effects on eggshell quality during the entire production period and no beneficial effects on egg production during later laying period (60wk-95wk). In summary, long-term and

early supplementation of 25OHD has positive effects on egg production and egg quality. The beneficial effects were mainly observed during the early laying stage (0-60wks).

Keyword: 25-hydroxyvitamin D_3, laying hen, growth performance, egg production, egg quality

INTRODUCTION

Vitamin D_3 can be obtained from the conversion of 7-dehydrocholesterol in the skin under the 290-315nm UV light (Holick, et al., 1980). However, the modern layer flocks are mostly kept entirely indoors (without direct sunlight). The primary source of vitamin D_3 is from the diet (ŚWiĄTkiewicz, et al., 2017). Vitamin D_3 is biologically inactive in animal body (DeLuca, 2004). Therefore, it requires two biological conversions to become an active form. First, vitamin D_3 is hydroxylated at the C-25 in the liver by microsomal CYP2R1 (25-hydroxylase) to become its major circulating form of 25-hydroxyvitaminD_3 (25OHD). Then mainly in the kidney, it becomes a bioactive form of 1,25-dihydroxyvitamin D_3 (1,25OHD) catalyzed by mitochondrial CYP27B1(1α-hydroxylase). The 1,25OHD either binds to vitamin D receptor (VDR) promoting a number of vitamin responsive elements (VDREs) expression to exert genomic function or binds to membrane-associated receptor inducing nongenomic function (Haussler, et al., 2011; van Driel and van Leeuwen, 2014). The general effects of vitamin D_3 on laying hens and broilers are stimulating on bone development, preventing bone fracture, maintaining the egg production and eggshell quality, and modulating immune responses (Rodriguez-Lecompte, et al., 2016; ŚWiĄTkiewicz, et al., 2017).

Vitamin D_3 is commonly supplied from vitamin premixes in poultry diets. However, the higher levels of inclusion than the requirement has no beneficial effects on laying performance and bone quality (Keshavarz, 2003; Mattila, et al., 2004; Persia, et

al., 2013). It may indicate that the conversion of vitamin D_3 to its bioactive form is limiting (Koreleski and Świątkiewicz, 2005). Especially in old laying hens, the defection of liver and kidney results in the decreased production of active vitamin D_3 metabolites (Bar, et al., 1988; ŚWiĄTkiewicz, et al., 2017). An alternative way to elevate the vitamin D_3 effects is by adding the active form of vitamin D_3 in the diets.

25-hydroxyvitamin D (25OHD) as the intermediate form of vitamin D_3 is commercially available in the market. It is two to four-fold more active compared with vitamin D_3 in chicken diets (Atencio, et al., 2005; Soares Jr, et al., 1995). The previous studies with 25OHD on laying hens showed inconsistent results, either no effects (Käppeli, et al., 2011; Mattila, et al., 2011; Keshavarz, 2003; Nascimento, et al., 2014; Roland and Harms, 1976) or beneficial effects (Koreleski and Świątkiewicz, 2005; Silva, 2017; Torres, et al., 2009) on eggshell quality and/or egg production. Among these studies, the inconsistent 25OHD treatment duration, treatment timing, and laying stage contribute to the varied results.

A hypothesis was proposed that the long-term supplementation of 25OHD in pullet layer diets could enhance egg production and egg quality. The past research was conducted mainly supplied 25OHD during the laying stage but seldom during the rearing period. Whereas, the accumulated effects, especially on bone development before sexual maturity which subsequently may affect laying performance, suggest the importance of pullet and early laying period nutrition (Silva, 2017). Thus, a study was performed to evaluate the effects of long-term supplication of 25OHD from day-old to 95wk on Hy-line W36 laying hen egg production and egg quality.

MATERIALS AND METHODS

Housing, birds, and treatments

The study was conducted at the research facility of the Department of Poultry Science at the University of Georgia. All experiments were performed following the guidelines for the use of animal in research as stated by the Institutional Animal Care and Use Committee at the University of Georgia. A total of 390 1-day old Hy-Line W36 pullets (3 treatments× 10 replicates × 13 birds per cage) were housed in wire cages and allocated to 3 treatment groups: control vitamin D_3 (D; 2,760 IU/kg); double dosage vitamin D_3 (DD; 5,520 IU/kg); and control vitamin D_3 + 69ug/kg 25OHD (25D; equivalent to DD; HyD®, DSM). The diets were formulated based on the Hy-Line W36 guide (2015) (Table 4.1 and 4.2). Birds were housed in colony cages until 17 weeks, then transferred to individual cages. Water and experimental diets were offered *ad libitum* from 0 to 95 weeks. The pullets received intermittent lighting program during the first 7 days with 4 hours of light followed by 2 hours of dark. The lighting management was followed the Hy-line North America lighting program throughout 2-17 weeks (http://sales.hyline.com/NALighting/WebLighting.aspx). After 17 weeks, layers received 15.5 hours of light and 8.5 hours of dark.

Performance data collection

Bird body weight and feed consumption were recorded in the conclusion of each feeding stage: starter1 (0-3wk), starter2 (4-6wk), grower (7-12wk), developer (13-15wk),

prelay (15-17wk), peaking (18-38wk), layer2 (39-48wk), layer3 (49-60wk), layer4 (61-75wk), and layer5 (76wk-95wk). Egg production was recorded daily. Hen-day production (HDP) was calculated at the end of each feeding stage after the layers reached the peak production (more than 90%, 22wk). Feed conversion ratio (feed intake/ body weight gain from 0-17 weeks, and feed intake/dozens of eggs from 22-95 week) was calculated by feeding phases.

Egg quality

Since wk17, egg quality was evaluated every eight weeks throughout the laying period. Thirty eggs were collected from each treatment each time (3 eggs/replication). Collected eggs were stored in 4℃ for overnight before analysis. At measurement, egg specific gravity was determined according to the method of Holder and Bradford (1979). Then the eggs were broken onto a flat surface where the height of the thick inner albumen was measured with Haugh unit tester (AMES, Model S8400) (Um and Paik, 1999). The yolk was separated from the albumen and weighed. The shells were washed and dried in a dryer at 50 ℃ for 2 days, then weighed. The shell thickness measurement was taken 3 times per sample near the middle part of shells; then average value were recorded. The weight of the albumen was calculated as the difference between the weight of the egg and the weight of the yolk plus shell.

Statistics analysis

All experimental data were analyzed statistically by one-way ANOVA with feed treatment as the main effect using SAS software Version 9.3 (SAS Institute, Cary, NC).

Differences between means were determined using Duncan's Multiple Range test. The level of significance was assessed at $P \leq 0.05$.

RESULTS

Growth performance

No difference in performance among the treatments was found during the rearing period at each feeding stage or overall (0-17wk; data were not shown). For the egg-laying period (18-95wk), DD treatment had the lowest body weight (BW) at wk40 ($P<0.0001$; Table 4.3), and the lowest feed consumption during layer2 (38-48wk; $P<0.0001$; Table 4.4) compared with the other treatments. At layer3 phase (49-60wk), with the DD treatment, the highest feed consumption was recorded ($P=0.0005$; Table 4.4), and the hens recovered from the low BW($P>0.05$; Table 4.3). For the later period, the D treatment resulted in the highest BW at wk75 ($P=0.0388$; Table 4.3).

Egg laying performance

The flock reached the peak production (More than 90%) at wk22, and after the peak, the hen day production (HDP) gradually declined throughout the production period (Figure 4.1). The treatment did not affect the time of egg peak production or the production before the peaking (data were not shown). When HDP was calculated based on feeding phases (start from wk22), 25D treatment showed higher egg production during the peak and layer2 periods compared with DD treatment ($P<0.0001$, Table 4.5), and the

highest overall production throughout the first 60wks of age (P=0.149; Table 4.5). The calculated FCR (Feed intake/dozens of eggs) showed 25D treatment has the lowest FCR compared with DD in layer2 (P=0.0405; Table 4.6). However, no difference in overall egg production, feed intake, and FCR were observed (wk18-95; P>0.05).

Egg quality

At wk25 and 33, both DD and 25D treatment had higher Haugh Unit compared with D (P<0.05; Figure 4.2A and 4.2B). At 41wk, DD group produced smaller eggs (P=0.009), with a lower yolk weight (P<0.0001) but a higher specific gravity (P=0.007) and shell thickness (P=0.042) compared with the other treatments or D alone (Figure 4.2C-4.2F). This was due to the lower body weight at 40wk (Summers and Leeson, 1983). There was no difference during the later laying period on egg quality (data were not shown).

DISCUSSION

The layer's body weight and feed consumption were affected by the treatments. The DD treatment had lower feed consumption during layer 2 (28-48wk) associated with a lower body weight at wk40. Consequently, lower egg production during this phase compared with the other two treatments was observed. As the results of the lower body weight, the DD treatment also had smaller eggs, parts weight, and denser eggshell. However, at layer 3 (49-60wk), the feed consumption was brought up by DD treatment. At the same time, the body weight was recovered. Subsequently, a trend of higher egg

production compared with the other two treatments was observed during this period ($P<0.1$ data were not shown). The results suggested the body weight, feed intake, and egg production are closely related (Bish, et al., 1985). However, the reason for the fluctuation of body weight, feed intake and egg production are unclear. The previous study showed fed birds with vitamin D_3 up to 15,000 IU/kg had no effects on growth performance or egg production on 20-68wk Lohmann LSL white laying hens (Mattila, et al., 2004). Similarly, a short period (30d) dietary treatment up to 20,000IU on 87wks ISA brown molted laying hen had no effects on laying and growth performance (Park, et al., 2005). A more recent study brought up the non-toxic level of vitamin D_3 to 102, 200 IU/ kg on 19 to 58wk Hy-Line W36 laying hens (Persia, et al., 2013). However, while increasing vitamin D_3 dosage to 200,000 IU/kg, it started showing a decrease of egg weight, eggshell quality and feed consumption (Ameenuddin, et al., 1986). Based on the current knowledge, the laying hen has considerable tolerance on a high level of dietary vitamin D_3, and the layer performance should not be affected by the dosage we applied in this study. Furthermore, D treatment has the highest body weight at wk75. The previous studies showed vitamin D influence the fat and muscle development (Ceglia, 2009; Ding, et al., 2012). However, we did not detect the birds' composition of lean or fat on having any difference at wk75 from dual-energy Dual-energy X-ray absorptiometry(DEXA) results (data was not shown). Nevertheless, if we overlook the entire laying period, the overall growth performance showed no difference between the treatments. These changes during each stage are more like a local fluctuation. It may result from the non-uniformity of layers, and the growth performances of layers are easily influenced by the egg production and other factors.

In respect to egg production, no difference was observed at the beginning of laying. 25OHD did not bring forward the onset of laying or the peak production. Moreover, 25OHD treatment did not affect the HDP before the peak production. However, supplementation of 25OHD benefited the HDP and FCR (feed intake/dozen of eggs) up to 60wks in the current study, which agrees with Silva (2017), when they fed the laying hens with 69μg/kg of 25OHD on the top of 3000IU/kg of vitamin D_3 from 0-50 wks (remove 25OHD from the diets after 50wk) and observed increased FCR during the period of 18-34 wk and increased egg production of 18-50wk, but had no effect on cumulative egg production from 18-87wk. However, most of previous studies showed 25OHD had no beneficial effects on egg production either on laying hen or broiler breeders (Koreleski and Świątkiewicz, 2005; Torres, et al., 2009; Mattila, et al., 2011; Käppeli, et al., 2011;). For the studies showing no effects, none of them included 25OHD in the diets during the rearing period or covered the entire laying period especially early laying period. The use of 25OHD during the rearing period and early laying phases seems to promote bone development and protect bone loss during the peak production, which may benefit the birds for egg production during later laying period (Silva, 2017). To our knowledge, limited studies have been performed on this approach to the use of 25OHD targeting rearing or early laying period.

For egg quality, like egg production on timewise, the beneficial effects were mainly observed during the early stage. It mainly exhibited as both DD and 25OHD increasing of the Haugh Unit at 25wk and 33wk compared with D treatment. The reason why the Haugh unit is increased by 25OHD is unclear. However, we did not detect any effects on eggshell quality for the entire production period (non-significant data were not

shown), which agrees with the previous studies (Keshavarz, 2003; Mattila, et al., 2011; Käppeli, et al., 2011; Nascimento, et al., 2014). The mechanism of vitamin D_3 on shell formation has not been fully understood. The missing effects on eggshell quality maybe due to eggshell gland Ca^{2+} transportation-related protein such as calbindin D_{28k} and carbonic anhydrases, which are probably not vitamin D_3 dependent (Bar, 2008). On the contrary, some studies are showing the beneficial effects of 25OHD on eggshell quality. The supplementation of 25OHD (35 or 69ug/kg, 32wk-67wk) resulted in better egg shells evaluated by specific gravity at 60 weeks of age (Torres, et al., 2009); replace 25% of vitamin D_3 with 25OHD (9.35ug/kg, 26-70wk) improved shell quality (Koreleski and Świątkiewicz, 2005); inclusion of 25OHD (69ug/kg, 0-50wk) for the rearing and early laying period increased the shell thickness(Silva, 2017). In these studies, the beneficial effects mainly presented in the studies with long-term (at least 30wks feeding) or early (rearing or early laying stage) supplementation of 25OHD. However, in our study, we failed to detect the effects of long-term supplementation of 25OHD on eggshell quality. It is probably because the single dosage vitamin D_3 diet and the calcium level in our study are adequate to maintain egg quality. In this case, the additional vitamin D_3 will not improve laying hen egg quality that is showed in previous literature (Bar, 2008; Plaimast, et al., 2015).

In older hens, the ability to produce the active metabolite, 25OHD, and 1,25OHD is reduced (Bar and Hurwitz, 1987), and vitamin D_3 become more limited. The current study indicated the additional 25OHD did not affect on egg production and egg quality during the late laying period. Besides the possibility of the insufficient 25OHD conversion to 1,25OHD in the kidney (Abe, et al., 1982), 25OHD may mainly target

preventing bones loss and maintains the system Ca^{2+} homeostasis rather than increasing egg production (Silva, 2017), which needs further investigation.

In summary, because the efficiency of biological conversion is not 100%, the same amount of 25OHD has greater physiological effects than vitamin D_3 as it bypasses the first biotransformation. In the current study, the long-term supplementation of 25OHD increased the egg production during the early stage (0-60wk). However, no beneficial effect was shown during late laying stage. It may due to the supplementation of calcium and the lowest vitamin D_3 concentration in this study is adequate for maintaining laying performance; or the kidney deficiency which is insufficient to convert 25OHD to 1,25OHD (Abe, et al., 1982); or perhaps 25OHD benefit effects mainly on maintaining bone loss at this stage. The calcium flow under 25OHD treatment in egg-laying hens needs further research. The body weight and feed consumption fluctuation maybe not the main effects of vitamin D_3 concentration in layer diets. Whereas, it showed an interesting relationship with egg production and quality. As the 25OHD become commercially available in the industry at a reasonable price, long-term or early supplementation of 25OHD on pullets and laying hen could be a potential strategy for increasing chicken laying performance.

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TABLES

Table 4.1 Diet formulation and calculated nutrient composition for rearing period (0-17wks).[1]

Ingredients, %	Starter 1[2]	Starter 2	Grower	Developer	Prelay
Corn	67.11	62.19	65.93	69.43	62.17
Soybean Meal	28.08	27.34	24.00	20.00	23.10
Soybean Oil	1.00	3.00	2.54	2.57	3.02
Limestone	0.68	0.71	0.8	1.95	4.68
Defluor. Phos.	2.03	2.01	1.92	1.85	2.01
NaCl	0.30	0.30	0.30	0.30	0.30
L-Lysine HCl	0.19	0.13	0.11	0.08	0.01
DL-Methionine	0.21	0.23	0.18	0.14	0.20
Threonine	0.23	0.08	0.06	0.05	0.03
Vitamin Premix[3]	0.05	0.05	0.05	0.05	0.05
Mineral Premix[4]	0.06	0.06	0.06	0.06	0.06
Amprolium	0.05	0.05	0.05	0.05	0.05
Sand	0	3.85	3.99	3.47	4.33
Calculated value					
ME(kcal/kg)	3030	3030	3030	3050	2920
CP%	20.00	18.25	17.50	16.00	16.50
Ca%	1.00	1.00	1.00	1.40	2.50
Available P (%)	0.50	0.49	0.47	0.45	0.48

[1] Treatments were added as the form of vitamin premix in the diet: D treatment: vitamin D_3 at 2,760 IU/kg; DD treatment: vitamin D_3 at 5,220 IU/kg; 25D treatment: vitamin D_3 at 2,760 IU/kg plus 25OHD at 2,760 IU (69µg)/kg.

[2] Starter1 (0-3wk), starter2 (4-6wk), grower (7-12wk), developer (13-15wk), prelay (15-17wk).

[3] Supplied per kilogram of diet: vitamin A, 9,900 IU; vitamin E, 22.10 IU; vitamin B12, 0.02 mg; biotin, 0.06 mg; menadione, 3.3 mg; thiamine, 2.20 mg; riboflavin, 6.60 mg; pantothenic acid, 11.00 mg; vitamin B6, 4.40 mg; niacin, 33.00 mg; folic acid, 0.90 mg; choline, 191.36 mg.

[4] Supplied per kilogram of diet: Mn, 80.4 mg; Zn, 64.2 mg; Mg, 16.08 mg; Fe, 15.78; Cu, 2.4 mg; I, 0.6 mg; Se, 0.24 mg

Table 4.2 Diet formulation and calculated nutrient composition for laying period (18-95wks).[1]

Ingredients, %	Peaking[2]	Layer 2	Layer 3	Layer 4	Layer 5
Corn	53.61	62.99	61.54	64.18	62.57
Soybean Meal	28.10	21.35	19.99	17.77	17.90
Soybean Oil	3.75	2.90	3.00	2.87	3.21
Limestone	7.44	6.89	6.87	7.13	7.33
Oyster shell	3.19	2.95	2.94	3.06	3.14
Defluor. Phos.	2.55	2.09	1.89	1.52	1.47
NaCl	0.30	0.30	0.30	0.30	0.30
L-Lysine HCl	0.46	0.09	0.04	0.05	0.04
DL-Methionine	0.33	0.22	0.17	0.14	0.14
Threonine	0.11	0.06	0.03	0.03	0.02
Vitamin Premix[3]	0.05	0.05	0.05	0.05	0.05
Mineral Premix[4]	0.06	0.06	0.06	0.06	0.06
Sand	0.05	0.05	3.11	2.84	3.78
Calculated value					
ME(kcal/kg)	2840	2900	2820	2840	2820
CP%	19.05	16.15	15.27	14.42	14.32
Ca%	4.94	4.48	4.40	4.42	4.51
Available P (%)	0.58	0.49	0.45	0.38	0.37

[1] Treatments were added as the form of vitamin premix in the diet: D treatment: vitamin D_3 at 2,760 IU/kg; DD treatment: vitamin D_3 at 5,220 IU/kg; 25D treatment: vitamin D_3 at 2,760 IU/kg plus 25OHD at 2,760 IU (69µg)/kg.

[2] Peaking (18-38wk), layer2 (39-48wk), layer3 (49-60wk), layer4 (61-75wk), and layer5 (76wk-95wk).

[3] Supplied per kilogram of diet: vitamin A, 9,900 IU; vitamin E, 22.10 IU; vitamin B12, 0.02 mg; biotin, 0.06 mg; menadione, 3.3 mg; thiamine, 2.20 mg; riboflavin, 6.60 mg; pantothenic acid, 11.00 mg; vitamin B6, 4.40 mg; niacin, 33.00 mg; folic acid, 0.90 mg; choline, 191.36 mg.

[4] Supplied per kilogram of diet: Mn, 80.4 mg; Zn, 64.2 mg; Mg, 16.08 mg; Fe, 15.78; Cu, 2.4 mg; I, 0.6 mg; Se, 0.24 mg

Table 4.3 Effects of different vitamin D_3 treatments on laying hen body weight during the laying period [3]

Weeks	D [1] g/bird	DD g/bird	25D g/bird	SEM[2]	P value
17	1262	1246	1236	10.1	0.218
40	1616^{A}	1419^{B}	1579^{A}	22.1	<.0001
60	1692	1637	1637	21.2	0.1285
75	1767^{A}	1703^{B}	1691^{B}	20.3	0.0388
95	1756	1700	1661	27.7	0.0829

[1.] D: vitamin D_3 at 2,760 IU/kg; DD: vitamin D_3 at 5,220 IU/kg; 25D: vitamin D_3 at 2,760 IU/kg plus 25OHD at 2,760 IU (69µg)/kg.

[2.] SEM: standard error means of treatments

[3.] Values of means represent 8 birds per 10 replicate pens (n = 80 birds) per treatment.

[A, B] means within a column with different superscripts are significantly different ($P<0.05$);

Table 4.4 Effects of different vitamin D_3 treatments on laying hen feed consumption during laying period [5]

Feeding stages	D[1] g/bird/day	DD g/bird/day	25D g/bird/day	SEM[2]	P value
22-38wks[4]	105.81	103.66	105.39	1.439	0.5419
39-48wks	105.97^{A}	95.86^{B}	104.91^{A}	1.358	<.0001
49-60wks	108.55^{B}	113.23^{A}	107.94^{B}	0.901	0.0005
61-75wks	108.74	110.47	108.32	0.949	0.3115
76-95wks	106.73	106.66	105.96	0.944	0.8427
22-60wks[3]	106.62	104.3	105.97	1.134	0.3462
61-90wks	107.55	108.53	107.1	0.858	0.5193
22-95wks	106.95	105.9	106.4	0.854	0.6955

[1.] D: vitamin D_3 at 2,760 IU/kg; DD: vitamin D_3 at 5,220 IU/kg; 25D: vitamin D_3 at 2,760 IU/kg plus 25OHD at 2,760 IU (69µg)/kg.

[2.] SEM: standard error mean of treatments

[3] The last three columns: accumulating feed consumption during the period indicated in the coloum.

[4] Peaking (22-38wk), layer2 (39-48wk), layer3 (49-60wk), layer4 (61-75wk), and layer5 (76wk-95wk).

[5.] Values of means represent 8 birds per 10 replicate pens (n = 80 birds) per treatment.

[A, B] means within a column with different superscripts are significantly different ($P<0.05$);

Table 4.5 effects of different vitamin D_3 treatments on laying hen egg production during the laying period[5]

Stages	D[1] %	DD %	25D %	SEM[2]	P value
22-38wks[4]	96.2^{A}	92.9^{B}	96.4^{A}	0.49	<.0001
39-48wks	89.5^{A}	82.4^{B}	90.1^{A}	0.79	<.0001
49-60wks	84.3	88.3	85.7	1.24	0.0985
61-75wks	78.0	82.0	77.6	1.67	0.156
76-95wks	64.6	70.8	66.8	2.76	0.3039
22-60wks[3]	90.7^{AB}	88.5^{B}	91.3^{A}	0.64	0.0149
61-90wks	70.3	75.6	71.4	2.22	0.2398
22-95wks	81.0	82.4	81.9	1.22	0.7402

[1.] D: vitamin D_3 at 2,760 IU/kg; DD: vitamin D_3 at 5,220 IU/kg; 25D: vitamin D_3 at 2,760 IU/kg plus 25OHD at 2,760 IU (69µg)/kg.

[2.] SEM: standard error mean of treatments

[3] the last three columns: accumulation egg production during the period indicated in the columns.

[4] Peaking (22-38wk), layer2 (39-48wk), layer3 (49-60wk), layer4 (61-75wk), and layer5 (76wk-95wk).

[5.] Values of means represent 8 birds per 10 replicate pens (n = 80 birds) per treatment.

[A, B] means within a column with different superscripts are significantly different ($P<0.05$);

Table 4.6 effects of different vitamin D_3 treatments on laying hen feed conversion ratio during the laying period [5]

Stages	D[1] g/dozen	DD g/dozen	25D g/dozen	SEM[2]	P value
22-38wks[4]	1.32	1.35	1.34	0.021	0.6763
39-48wks	1.34^{AB}	1.40^{A}	1.33^{B}	0.020	0.0405
49-60wks	1.60	1.61	1.56	0.025	0.3100
61-75wks	1.69	1.6	1.66	0.037	0.2762
76-95wks	2.01	1.79	1.82	0.119	0.4257
22-60wks[3]	1.41	1.43	1.39	0.021	0.3587
61-90wks	1.83	1.67	1.75	0.060	0.2255
22-95wks	1.54	1.51	1.51	0.026	0.6479

[1.] D: vitamin D_3 at 2,760 IU/kg; DD: vitamin D_3 at 5,220 IU/kg; 25D: vitamin D_3 at 2,760 IU/kg plus 25OHD at 2,760 IU (69µg)/kg.

[2.] SEM: standard error mean of treatments

[3] the last three columns: accumulation egg production during the period indicated in the columns.

[4] Peaking (22-38wk), layer2 (39-48wk), layer3 (49-60wk), layer4 (61-75wk), and layer5 (76wk-95wk).

[5.] Values of means represent 8 birds per 10 replicate pens (n = 80 birds) per treatment.

[A, B] means within a column with different superscripts are significantly different ($P<0.05$);

FIGURES

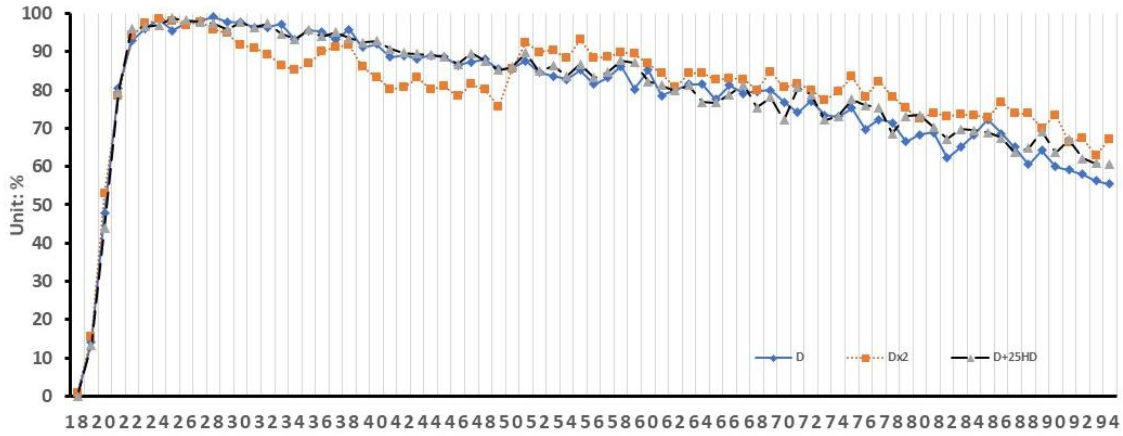

Figure 4.1 Effects of dietary supplementation of 25OHD on hen day production from wk17-wk95. Values of means represent 8 birds per 10 replicate pens (n = 80 birds) per treatment.

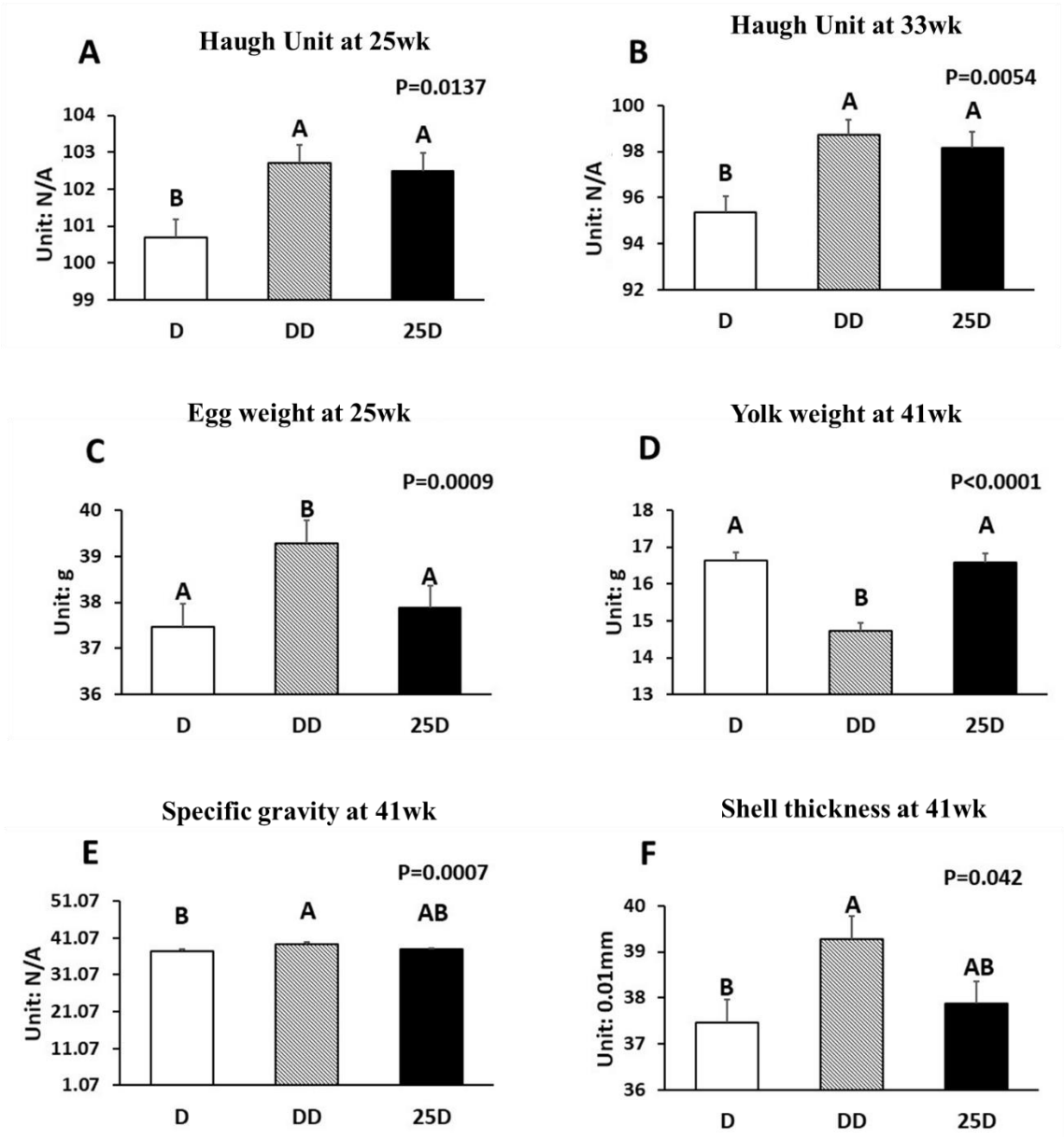

Figure 4.2 Effects of dietary supplementation of 25OHD on egg quality. D: vitamin D_3 at 2,760 IU/kg; DD: vitamin D_3 at 5,220 IU/kg; 25D: vitamin D_3 at 2,760 IU/kg plus 25OHD at 2,760 IU (69µg)/kg. (A) Haugh unit at wk25; (B) Haugh unit at wk33; (C) Egg weight at wk41; (D) Yolk weight at wk41 (E) Specific gravity at wk41; (F) Shell thickness at wk41. Mean separation was indicated by different letters on the top of bars

(Value means ± SEM). Values of means represent 3 eggs per 10 replicate pens (n = 30 birds) per treatment.

CHAPTER 5

ROLE OF LONG-TERM SUPPLEMENTATION OF 25-HYDROXYVITAMIN D_3 ON LAYING HEN BONE 3-DIMENSIONAL STRUCTURAL DEVELOPMENT

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ABSTRACT

Avian species have a unique bone metabolism pattern. The role of long-term supplementation of a higher bioactive form of vitamin D_3: 25-hydroxyvitamin D_3 (25OHD), on layer bone development is unclear. By exploring its effects on pullet and laying hen bone will help us better understand the role of 25OHD on bone development in avian model and help us to develop new strategies on preventing osteoporosis. In this study, micro-computed tomography (micro-CT) was used for bone quality assessment. Results showed the additional supplementation of 25OHD (2,760 IU/kg vitamin D3 + 69ug/kg 25OHD) in pullet and layer diets from 0-95wk showed diverse effects on pullets and laying hen bone 3-dimensional structural development. First, dietary supplementation of 25OHD significantly increased 25OHD level in the serum. During the rearing period, 25OHD increased bone growth rate, cortical tissue volume, and bone marrow area, simultaneously. 25OHD created more pores in cortical bone, which resulted in a lower cortical bone mineral density (BMD) but without alerting bone mineral content (BMC) in the pullet period. This effect allowed more space for mineral deposition in bones during the later egg-laying period. At 60wk, 25OHD group had significantly greater BMD, which led to the highest total BMC, and the highest cortical volume and trabecular bone connectivity. At 95wk, birds fed 25OHD had higher volume and lower porosity in cortical bone. 25OHD also had higher total BMD, medullary BV, but a lower BMC and volume of trabecular bone compared to vitamin D_3 or double dosage vitamin D_3 treatments. This indicated the trade-off between cortical bone and trabecular bone in the

late laying period. In conclusion, supplementation with dietary 25OHD could increase bone volume in pullets to provide more space for mineral deposition during the laying period with positive effects on laying hen bone quality. This research highlights the importance of early supplementation of 25OHD on bone development.

Keywords: 25-hydroxyvitamin D_3; 3-dimensional bone structure; pullet; laying hen

INTRODUCTION

The birds and reptiles have the ability to lay eggs with calcified eggshells, which is distinguished from the other vertebrate. When female bird reached sexual maturity, the structural bone development ceased. Meanwhile, the unique medullary bones were formed inside the bone cavity (Dacke, et al., 1993; Schraer and Hunter, 1985; Werning, 2018; Whitehead, 2004). Medullary bone is estrogen and androgen hormones-driven bones (Beck and Hansen, 2004; Miller and Bowman, 1981; Wilson and Thorp, 1998; Yamamoto, et al., 2001). The content of medullary bone is similar to the other part of bones, which mainly contained hydroxyapatite lattice (Ascenzi, et al., 1963) but with much lower collagen content in a random distribution (Dacke, et al., 1993). Therefore, medullary bone provided less structural strength compared to the other bone parts (Fleming, et al., 2006). The high osteoclast (bone resorption cells) activity in this region results in calcium metabolized at 10–15 times faster rate than cortical rendering it a reliable calcium source for eggshell formation (Hurwitz and Bar, 1965; Simkiss, 1967; Van de Velde, et al., 1984). Avian medullary bone represents a very active bone remodeling system due to the high number of osteoclasts compare to cortical bone (Van de Velde, et al., 1984). Furthermore, a quantitative trait locus (QTL) and expression QTL (eQTL) mapping study showed the chicken had implicated unexpected and large amount unknown genes in bone metabolism (Johnsson, et al., 2015), which indicates a different bone metabolism in avian species.

Besides the difference, human and layer femurs have lots of similarities in mechanical and structural properties, indicating that the chicken femurs with much lower cost is an optimal and adequate model for exploring human aspect researches (Passi and Gefen, 2005). Osteoporosis-related fractures and clinical costs are a severe socioeconomic issue in the US (Burge, et al., 2007). Likewise, in layers, the osteoporosis is also one of the most pressing animal welfare issues and frequently happened in aged laying hens (Webster, 2004). The typical way to prevent the osteoporosis in human is by supplementing vitamin D and calcium in the diets, which has been exclusively researched (Cranney, et al., 2008). However, the vitamin D action on bone metabolism involved in multi-factors especially the estrogen level in women, which is complicated (Anderson, et al., 2012). Furthermore, to track down the long-term effects of vitamin D on a human is hard and costly (Black, et al., 2006). Laying hen research will be an exciting research model for exploring the long-term effects of vitamin D_3 on bones due to the progressive bone loss in laying hens driven by the estrogen after sexual maturity, as well as a shorter lifespan compared to the human.

Higher bioactive vitamin D_3: 25OHD has become commercially available in the poultry industry, Its effects have been tested in broilers (Edwards Jr, 1989; Han, et al., 2016; Koreleski and Swiatkiewicz, 2005; ŚWiĄTkiewicz, et al., 2017; Wideman Jr, et al., 2015) with the effects of improving bone health, but mostly no effects were found in laying hens(Käppeli, et al., 2011; Koreleski and Świątkiewicz, 2005; Nascimento, et al., 2014; Silva, 2017; ŚWiĄTkiewicz, et al., 2017). However, there are missing studies of the long-term evaluation of 25OHD on layer bone developments.

In the current study, by conducting a 95wk layer trial with two dosages of vitamin D_3 and additional 25OHD, we were able to explore long-term effects of 25OHD on layer bone development before and after sexual maturity. The bone quality was mainly assessed using micro-computed tomography with advanced 3-dimensional analysis techniques. The obtained data provided a new aspect of how vitamin D_3 effects on the avian bone model, as well as the suggestion of the application of 25OHD in the poultry industry to alleviate osteoporosis welfare issues in laying hens.

MATERIALS AND METHODS

Housing, birds, and treatments

The study was conducted at the research facility of the Department of Poultry Science at the University of Georgia. The trial was conducted in accordance with the animal care guidelines of the research facility. A total of 390 1-day old Hy-Line W36 pullets (3 trt × 10reps × 13 birds per cage) were housed in wire cages and allocated to 3 treatment groups: control vitamin D_3 (D; 2,760 IU/kg); double dosage vitamin D_3 (DD; 5,520 IU/kg); and control vitamin D_3 + 69ug/kg 25OHD (25D; equivalent from DD; HyD®, DSM). The diets were designed based on the Hy-Line W36 guide (2015) (Table 5.1 and 5.2). Birds were housed in colony cages until 17 weeks, then transferred to individual cages. Water and experimental diets were offered *ad libitum* from 0 to 95 weeks. The pullets received intermittent lighting program during the first 7 days with 4 hours of light followed by 2 hours of dark circles. The lighting management was

customized by Hy-line North America lighting program throughout 2-17 weeks (http://sales.hyline.com/NALighting/WebLighting.aspx). After 17 weeks, layers received 15.5 hours of light and 8.5 hours of dark.

Serum 25-hydroxyvitamin D3 content analysis

Blood samples (10 birds/treatment/time point) were collected from the wing at 0 (baseline), 6wk, 12wk, 17wk, 40wk, 60wk, 75wk, and 95wk. After the blood clotted, serum was separated (3000rpm; 12mins) to determine serum 25OHD level using mass spectrometry (Heartland Assays, Ames, IA 50010).

Bone growth rate (BGR) using calcein labeling technique

At 10wk of age, one pullet/pen (10 birds/treatment) was injected with calcein solution intraperitoneally at a dose of 20 mg/kg body weight. After a 10-days interval, pullets were injected intraperitoneally again with calcein solution at same dosage. Then the pullets were euthanized 4 days after the second injection. Femurs were collected and preserved in 70% ethanol until analyzed. A thin slice of bone was taken from mid-diaphysis, the bone slice was mounted on a glass slide. A fluorescence microscope was used to determine the distance between the two calcein labels on the bones. Eight measurements were taken from each sample; the average value was recorded for data analysis.

Micro-Computer Tomography (Micro-CT) scanning

The right femur was taken from one bird per pen (10 bones/treatment/time point) at 17, 60 and 95wk. The soft tissue was removed completely. The samples were wrapped with cheesecloth contained PBS to keep the moister of bones. The bones were then stored

at -20 ℃. Before the analysis, the samples were completed deforest. Micro-CT scanner (Skyscan 1275; Bruker microCT, Belgium) was used for 3-dimensional image acquisition. The bone was held in a low-dense 50ml tube; the extra cheesecloth was used for keeping the sample in a vertical orientation and firmly inside the tube holder. The tube was then mounted on the scanning stage. Scanning settings are shown in Table 5.3. Before the scanning, the alignment test and flat field correction were performed according to Micro-CT manual. The random movement and 180-degree scanning were applied. The 0.5 mm aluminum filter was used to reduce the beam hardening. After scanning, the pictures were carefully screened, and the appropriate alignment and mathematical methods correction (beam-hardening correction: 35%; smoothing: NA; ring artifact reduction: NA) were applied for all samples during the reconstruction by using NRecon (version: 1.6.10.5, Bruker microCT, Belgium). Dynamic range for all the samples was set at 0-0.027. The volume of interested was shown in Figure 5.1, the 3D model undergoing a custom process for bone separation. The separation process was designed base on the different density and morphology traits of each part of the bone. The bone separation outcomes were demonstrated in Figure 2. The 3D model was analysis by using CTan (Version: 1.16.4.1, Bruker microCT, Belgium). The threshold was set at 90-255 for 17wk samples and 100-255 for 60wk and 95wk samples. Two calcium hydroxyapatite (HA) from a solid-state phantom (0.25g/cm3 and 0.75g/cm; diameter: 7mm) was used for calibration. The outcome parameters in the current study are introduced in Table 5.4.

Dual-energy x-ray absorptiometry (DEXA)

DEXA was used for whole bird's body composition analysis. At 17, 60, and 95wk, one bird per pen (10 birds/treatment/time point) was sacrificed for scanning. The whole bird was defined as a region of interest.

Statistics analysis

All experimental data were analyzed statistically by one-way ANOVA with feed treatment as the main effect using SAS software Version 9.3 (SAS Institute, Cary, NC). Differences between means were determined using Duncan's Multiple Range test. The level of significance was assessed at $P \leq 0.05$.

RESULTS

The 25OHD level in the serum

There was a fluctuation of 25OHD level in the serum throughout the experimental period. However, the treatment with 25OHD always had the highest 25OHD level in the serum compared to the other treatments (Figure 5.3).

Bone quality analysis

During the pullet period, 25D treatment had a higher BGR compared with the others (Figure 5.4). However, no differences were detected from the whole body DEXA scanning by the end of the rearing period (17wk, data were not shown). From the femur Micro-CT scanning results at 17wk, 25D treatment had a lower cortical bone mineral

density (BMD; P=0.014) in comparison with the others (Figure 5.5A), but no difference was observed in cortical bone mineral content (BMC; P>0.05; Figure 5.5B). The segments analysis in each part of bones showed the lower BMD resulted from the increase of cortical bone volume (P<0.05) and pores volume (P<0.05; Figure 5.5C and 5.5D). Furthermore, 25D treatment had a higher total bone tissue volume (TV; P=0.0203), medullary cavity volume (MV; P=0.0369) compared with D treatment (Figure 5.5E and 5.5F), which indicates that 25OHD stimulated the bone structural size increase during this period rather than build a denser bone. In summary, supplementation of 25OHD results in enlargement of the pullet bone structure, which provided more space for mineral deposition and contributed to the bone quality in the laying period.

During the laying period, at 60wk, 25D treatment still obtained the highest cortical TV (P=0.026) and cortical BV (P=0.0252) (Figure 5.6A and 5.6B). With the mineral deposition, there was no difference in cortical BMD and Porosity among the treatment by 60wk (P>0.05; data were not shown). Instead, 25D treatment showed a higher total BMC (P=0.0143) and cortical BMC (P=0.0354; Figure 5.6C and 5.6D). The trabecular bone structure analysis showed higher trabecular connectivity in 25D (P=0.0222) compared with D treatment (Figure 5.6E). The data above clearly indicated the larger bone structure from 25HD treatment during the rearing period (0-17wk) allowed additional mineral deposition and consequently enhanced the bone quality during the later egg-laying period.

During the late laying period, at 95wk, 25D treatment had the higher total BMD (P=0.0131), Medullary BMD (P=0.0418), total BMC (P=0.0040) and cortical BMC (P=0.0051) along with a higher total BV(P=0.0168), cortical TV (P=0.0120), cortical BV

(P=0.0101), and Medullary BV (P=0.0459) compared with DD treatment (Figure 5.7A-5.7H). 25D treatment had lowest close pore volume (CPV; P=0.0429), closed porosity (CPP; P=0.0298), but highest pore number (PN; P=0.0392) in the cortical bone (Figure 5.7I-5.7K), which indicated the mineral deposition processing in this area. However, a lower trabecular BMC (P=0.0009), trabecular BV (P=0.0010) and connectivity (P=0.0003) were detected compared with D treatment (Fig 5.7L-5.7N). The data above showed the expending of volume and mineral deposition in 25HD treatment mainly occurred in the cortical bone region. Moreover, an increase of trabecular bone resorption was found at this period.

The DEXA had a similar trend as Micro-CT results at 60 and 95wk. The 25D group had higher BMD and BMC compared with D or Dx2 treatment ($P<0.05$; Figure 5.8A-5.8D).

DISCUSSION

25OHD is the main circulation form and a reliable indicator of the amount of vitamin D entering the system (Holick, 2007). The serum 25OHD test demonstrated that dietary supplementation of 25OHD could significantly increase the circulating 25OHD in the birds, reconfirm the experimental condition. The similar results were found previously in laying hen (Käppeli, Fröhlich, Gebhardt-Henrich, Pfulg, Schäublin, Zweifel, Wiedmer and Stoffel, 2011). In the current study, we were able to observe the

long-term supplementation of 25OHD on layer bone 3-dimensional structural changes owe to the Micro-CT 3D scanning and automatic bone separation process.

The bone development during the rearing period is similar to mammals. It proceeds through two primary mechanisms: intramembranous and endochondral ossification but with a faster rate compared to mammals (Whitehead, 2004). In the current study, the principal function of 25OHD in bone development was as increasing bone structural size. However, the expending of bone size with the same amount of bone mineral content results in a low-density bone. While taking into account the bone structural development ceased at the onset of sexual maturity (Whitehead, 2004), probably creating a larger bone before laying is more meaningful. The benefits of exercise in laying hen also showed as a lower bone mineral density during the rearing period with a greater cross-sectional area on the total bone (bone width) (Casey-Trott, et al., 2017). The beneficial effects of larger bone size (width) affect the bone health during the laying period by increasing breaking strength as the broader bones correlated with higher bending force (Rauch, 2007). A number of research in pullet's studies also addressed the importance of early bone development, and its prolonged effects on the bone health at laying period (Casey-Trott, Korver, Guerin, Sandilands, Torrey and Widowski, 2017; Hester, et al., 2013; Regmi, et al., 2015). Human studies also emphasized the importance of bone healthy during the growing period and its subsequent benefits during adulthood (Bailey, et al., 1999). However, this low BMD probably could be avoided by further increasing calcium and phosphate content in the diets. But this hypothesis needs further researches.

Previous nutritional studies seldom focused on the 25OHD on pullet period. The supplementation of 25OHD during the laying period mostly showed no effects on bones (Käppeli, et al., 2011; Koreleski and Świątkiewicz, 2005; Nascimento, et al., 2014; Silva, 2017; ŚWiĄTkiewicz, et al., 2017). Which may due to the manipulation of nutrient during laying period is hard to increase bone quality or reverse the onset of osteoporosis. A meta-analysis on human clinical trial also showed there are no effects of supplementation of vitamin D or calcium on decreasing older adults fracture incidence (Zhao, et al., 2017). In summary, early supplementation of 25OHD to prevent the osteoporosis is suggested.

During the laying period, 25OHD still possessed the higher bone volume. As the mineral deposition processing, the pores at cortical bones were filled up and showed no difference in pores volume. The larger volume of bone allowed more space for mineral deposition. With the effects of 25OHD increasing calcium absorption and reabsorption (Bar, 2008; Zhao and Nemere, 2002), bone BMC and BMD were elevated.

In the current study, higher connectivity of trabecular bone was found. The past research on evaluation of chicken bone mostly based on bone ash, breaking strength or dual-energy x-ray absorptiometry. These methods are mainly based on the results of planar morphology or bone mess. Although bone quantity and density are both important factors for bone strength (Hester, et al., 2004), these approaches did not consider the trabecular architectural changes, which independently related to the bone strength (Siffert, et al., 1996; Webber, et al., 1998). An *in vitro* avian model study demonstrated that more than 10% loss of trabecular bone could significantly impact the bone strain (Reich and Gefen, 2006). It suggested the integrity of trabecular bone is critical for bone

resisting to the force. Unfortunately, we did not test bone strength directly in this study, but the increase of trabecular bone connectivity indicated the higher fracture resistance elevated by dietary supplementation of 25OHD.

The structural bone (trabecular bone and cortical bone) continuously loss during the laying period associated with medullary bone modeling and remodeling and the pressure of eggshell formation (Cransberg, et al., 2001; Fleming, et al., 2006). Meanwhile, a trade-off between bone quality and laying performance showed in many previous studies (Bishop, et al., 2000; Cransberg, et al., 2001; Kim, et al., 2012; Rennie, et al., 1997). Maintaining structure bone during late laying period without affecting egg production is critical and challenging. The role of vitamin D_3 on balancing mineral between the bone and egg is sophisticated in egg laying birds (Bar, 2008). In the current study, interesting changes were observed at late laying stage (95wk). The mineral deposition seems to have its preference under the 25OHD treatment, which showed an increase of deposition in cortical bone and medullary bone with a trade-off at the trabecular bone. In a rat study, vitamin D did show its effects on bone resorption depend on bone site. Vitamin D exerts its inhibitory effects on bone resorption through controlling osteoblasts osteoprotegerin secretion. However, Baldock, et al., (2006) showed the cancellous bone do not undergo this inhibition process. It may partly explain the reason why more trabecular bones were resorbed under 25OHD treatment. Furthermore, the trabecular bone became very thin due to the continuously bone resorption process throughout the laying period (Whitehead, 2004). At the same time, the medullary bone cluster together to form numbers of calcium chuck with larger diameter compare to trabecular bone (Figure 5.9). Bone resorption has a preference for resorbing

smaller mineral particles during shell formation (Kerschnitzki, et al., 2014), which rendering trabecular bone more sensitive to the bone resorption. The loss of trabecular bone may diminish the bone strength. However, the increase of cortical bone at this period may balance this adverse effect.

Histology evidence showed the trabecular bone volume declined by approximately 50% between 16-30wk. Meanwhile, the medullary bone increased more than 2-fold. It indicated the cost of medullary bone formation is most likely partly from trabecular bone (Cransberg, et al., 2001). In this case, the resorption of trabecular bone in 25D treatment may contribute to the higher medullary bone showed in this study. It agrees with a previous study that 25OHD increase medullary bone ash (Akbari Moghaddam Kakhki, et al., 2018).

In summary, long-term supplementation of 25OHD increased the circulating 25OHD concentration. However, 25OHD showed diverse effects throughout the rearing and laying period. 25OHD increased bone growth rate and bone size during the pullet period, which provided more space for minerals deposition during the later period. For the laying period, feeding 25OHD had positive effects on bone mineral deposition and improvement of structural bone quality. During the late stage of laying (95wk), 25OHD had a two-sided effect. It enhanced the mineral deposition in the cortical bone region while increasing the resorption of trabecular bones. Our result suggested that early supplementation of 25OHD has prolonged beneficial effects on the layer bone healthy. The higher Ca and P content in the diet during the rearing period is suggested to meet the requirement of increasing bone size. This study demonstrated that 25OHD stimulate bone growth, improves structural integrity in laying hens to prevent avian osteoporosis.

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TABLES

Table 5.1 Diet formulation and calculated nutrient composition for rearing period (0-17wks).[1]

Ingredients, %	Starter 1[2]	Starter 2	Grower	Developer	Prelay
Corn	67.11	62.19	65.93	69.43	62.17
Soybean Meal	28.08	27.34	24.00	20.00	23.10
Soybean Oil	1.00	3.00	2.54	2.57	3.02
Limestone	0.68	0.71	0.8	1.95	4.68
Defluor. Phos.	2.03	2.01	1.92	1.85	2.01
NaCl	0.30	0.30	0.30	0.30	0.30
L-Lysine HCl	0.19	0.13	0.11	0.08	0.01
DL-Methionine	0.21	0.23	0.18	0.14	0.20
Threonine	0.23	0.08	0.06	0.05	0.03
Vitamin Premix[3]	0.05	0.05	0.05	0.05	0.05
Mineral Premix[4]	0.06	0.06	0.06	0.06	0.06
Amprolium	0.05	0.05	0.05	0.05	0.05
Sand	0	3.85	3.99	3.47	4.33
Calculated value					
ME(kcal/kg)	3030	3030	3030	3050	2920
CP%	20.00	18.25	17.50	16.00	16.50
Ca%	1.00	1.00	1.00	1.40	2.50
Available P (%)	0.50	0.49	0.47	0.45	0.48

[1] Treatments were added as the form of vitamin premix in the diet: D treatment: vitamin D_3 at 2,760 IU/kg; DD treatment: vitamin D_3 at 5,220 IU/kg; 25D treatment: vitamin D_3 at 2,760 IU/kg plus 25OHD at 2,760 IU (69µg)/kg.

[2] Starter1 (0-3wk), starter2 (4-6wk), grower (7-12wk), developer (13-15wk), prelay (15-17wk).

[3] Supplied per kilogram of diet: vitamin A, 9,900 IU; vitamin E, 22.10 IU; vitamin B12, 0.02 mg; biotin, 0.06 mg; menadione, 3.3 mg; thiamine, 2.20 mg; riboflavin, 6.60 mg; pantothenic Acid, 11.00 mg; vitamin B6, 4.40 mg; niacin, 33.00 mg; folic acid, 0.90 mg; choline, 191.36 mg.

[4] Supplied per kilogram of diet: Mn, 80.4 mg; Zn, 64.2 mg; Mg, 16.08 mg; Fe, 15.78; Cu, 2.4 mg; I, 0.6 mg; Se, 0.24 mg

Table 5.2 Diet formulation and calculated nutrient composition for laying period (18-95wks).[1]

Ingredients, %	Peaking[2]	Layer 2	Layer 3	Layer 4	Layer 5
Corn	53.61	62.99	61.54	64.18	62.57
Soybean Meal	28.10	21.35	19.99	17.77	17.90
Soybean Oil	3.75	2.90	3.00	2.87	3.21
Limestone	7.44	6.89	6.87	7.13	7.33
Oyster shell	3.19	2.95	2.94	3.06	3.14
Defluor. Phos.	2.55	2.09	1.89	1.52	1.47
NaCl	0.30	0.30	0.30	0.30	0.30
L-Lysine HCl	0.46	0.09	0.04	0.05	0.04
DL-Methionine	0.33	0.22	0.17	0.14	0.14
Threonine	0.11	0.06	0.03	0.03	0.02
Vitamin Premix[3]	0.05	0.05	0.05	0.05	0.05
Mineral Premix[4]	0.06	0.06	0.06	0.06	0.06
Sand	0.05	0.05	3.11	2.84	3.78
Calculated value					
ME(kcal/kg)	2840	2900	2820	2840	2820
CP%	19.05	16.15	15.27	14.42	14.32
Ca%	4.94	4.48	4.40	4.42	4.51
Available P (%)	0.58	0.49	0.45	0.38	0.37

[1] Treatments were added as the form of vitamin premix in the diet: D treatment: vitamin D_3 at 2,760 IU/kg; DD treatment: vitamin D_3 at 5,220 IU/kg; 25D treatment: vitamin D_3 at 2,760 IU/kg plus 25OHD at 2,760 IU (69µg)/kg.

[2] Peaking (18-38wk), layer2 (39-48wk), layer3 (49-60wk), layer4 (61-75wk), and layer5 (76wk-95wk).

[3] Supplied per kilogram of diet: vitamin A, 9,900 IU; vitamin E, 22.10 IU; vitamin B12, 0.02 mg; biotin, 0.06 mg; menadione, 3.3 mg; thiamine, 2.20 mg; riboflavin, 6.60 mg; pantothenic Acid, 11.00 mg; vitamin B6, 4.40 mg; niacin, 33.00 mg; folic acid, 0.90 mg; choline, 191.36 mg.

[4] Supplied per kilogram of diet: Mn, 80.4 mg; Zn, 64.2 mg; Mg, 16.08 mg; Fe, 15.78; Cu, 2.4 mg; I, 0.6 mg; Se, 0.24 mg

Table 5.3 Micro-CT scanning settings

	Voltage (kv)	Current (mA)	Exposure time (ms)	Rotation (degree)	Average frame	Pixel size Um/pixel
17wk	80	125	55	0.4	6	25
60wk	85	117	55	0.4	6	25
95wk	85	117	55	0.4	6	25

Table 5.4 Micro-CT outcome parameters interpretation

Parameters	Introduction
Tissue volume (TV)	The volume of the volume-of-interest (VOI), including pores and cavity inside the bone
Bone volume(BV)	The volume of binarized objects (mineralized bones) within the VOI
BMD (Bone mineral density)	BMD is defined as the volumetric density of calcium hydroxyapatite (CaHA) in biological tissue regarding g.cm-3. It is calibrated using phantoms with a known density of CaHA. In the current study, BMD relates to the amount of bone within a mixed bone-soft tissue region
BMC (Bone mineral content)	BMC is calculated by using BMD*TV
Pore number (PN)	The total number of closed pores within cortical bone
Closed pore volume(CPV)	Closed pores in 3D is a connected assemblage of space and surrounded on all slides in 3D by solid voxels. CPV is the volume of closed pores.
Closed porosity (CPP)	The volume of pores as a percentage of total cortical tissue volume
Total Pore volume (TPV)	The total volume of pores within VOI of cortical bone
Trabecular connectivity	Developed and defined by Hahn et al. (1992). It is a 3D calculation based on 2D scale. In brief, a higher number means better connected trabecular structure.

FIGURES

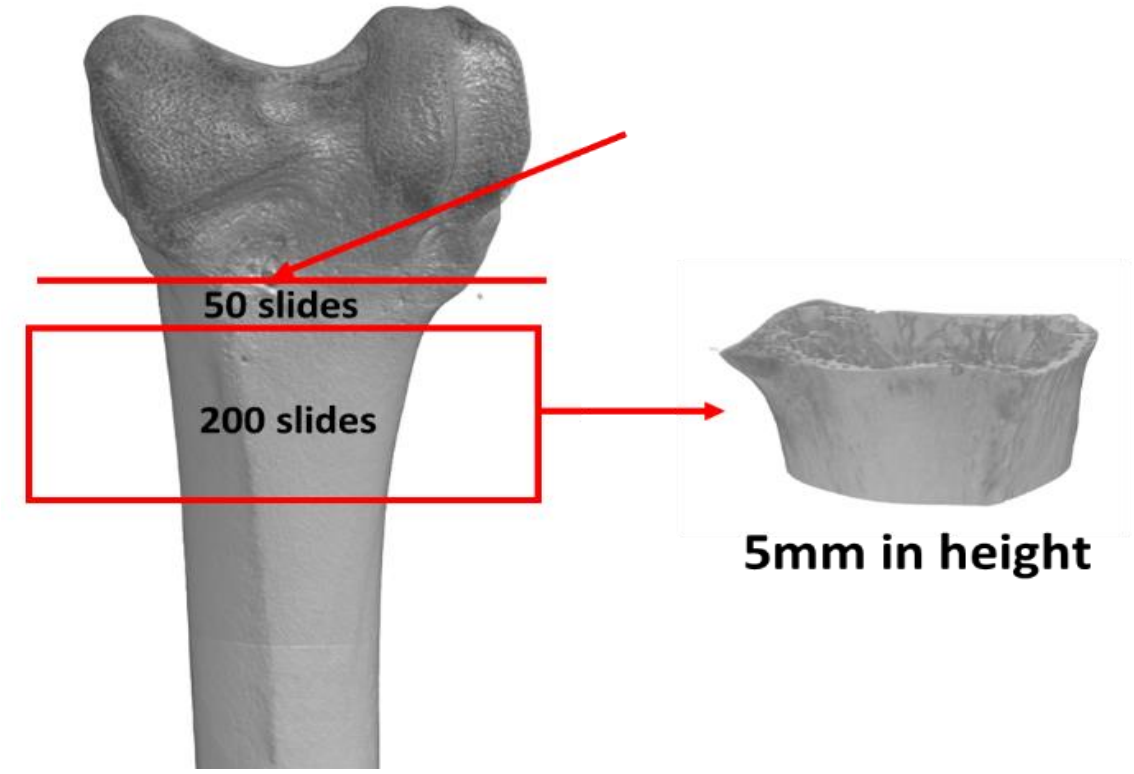


Figure 5.1 The volume of interests (VOI) selection is demonstrated in the pictures. The constant VOI was used throughout all the samples. The volume of interest starts from 50 slides (1.25mm) down below the Nutrient foramen in the distal femur. 200 slides (5mm) was used for analysis. This part contains all three types of bones: cortical bone, trabecular bone and medullary bone (after 17wks), which is the optimal region to represent the bone quality.

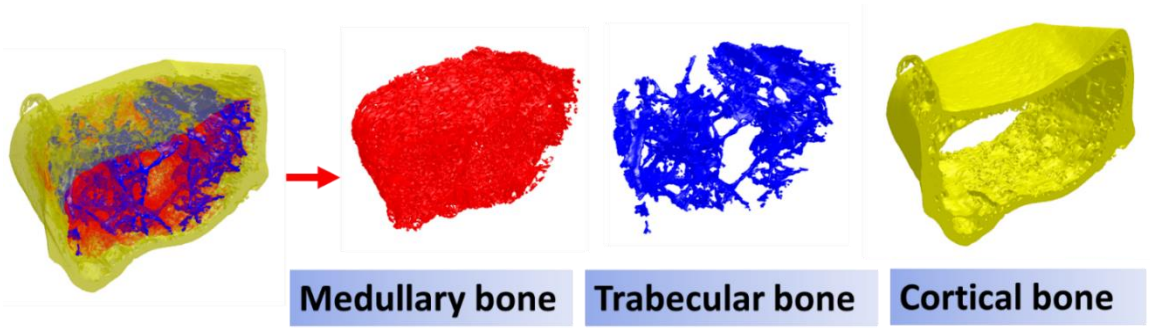


Figure 5.2 A customized automatic bone separation program was applied in the current study. the detail steps were introduced in chapter 1

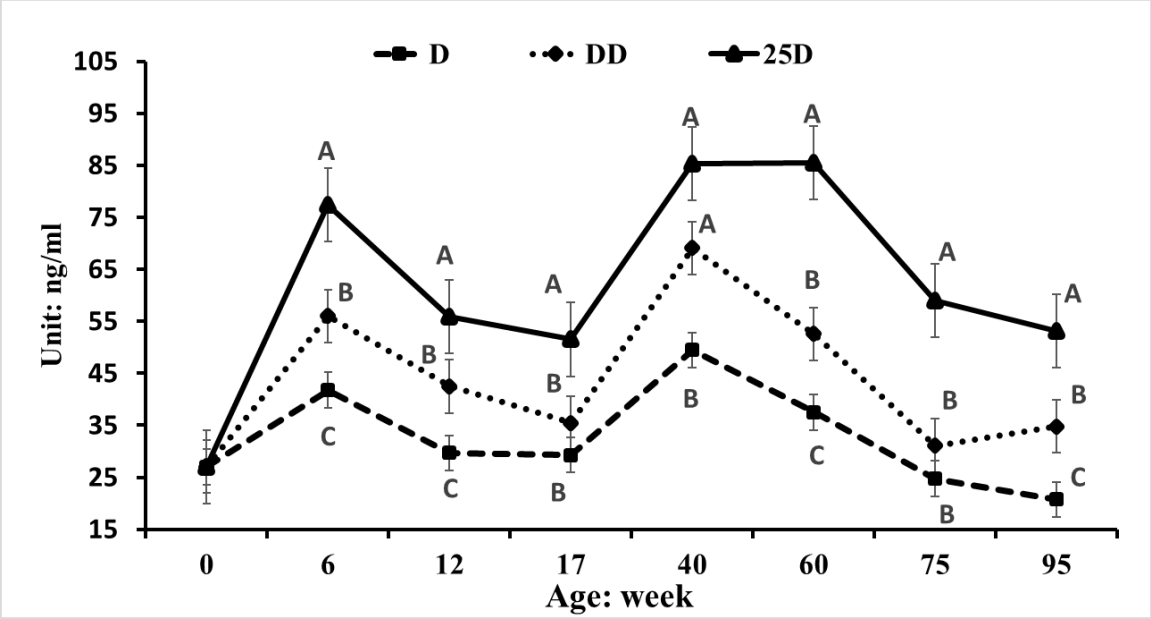


Figure 5.3 Serum 25OHD concentration throughout the whole experiment period measured by using MS/LC. D: vitamin D_3 at 2,760 IU/kg; DD treatment: vitamin D_3 at 5,220 IU/kg; 25D treatment: vitamin D_3 at 2,760 IU/kg plus 25OHD at 2,760 IU (69µg)/kg. Values of means represent 1 bird per 10 replicate pens (n = 10 birds) per treatment.

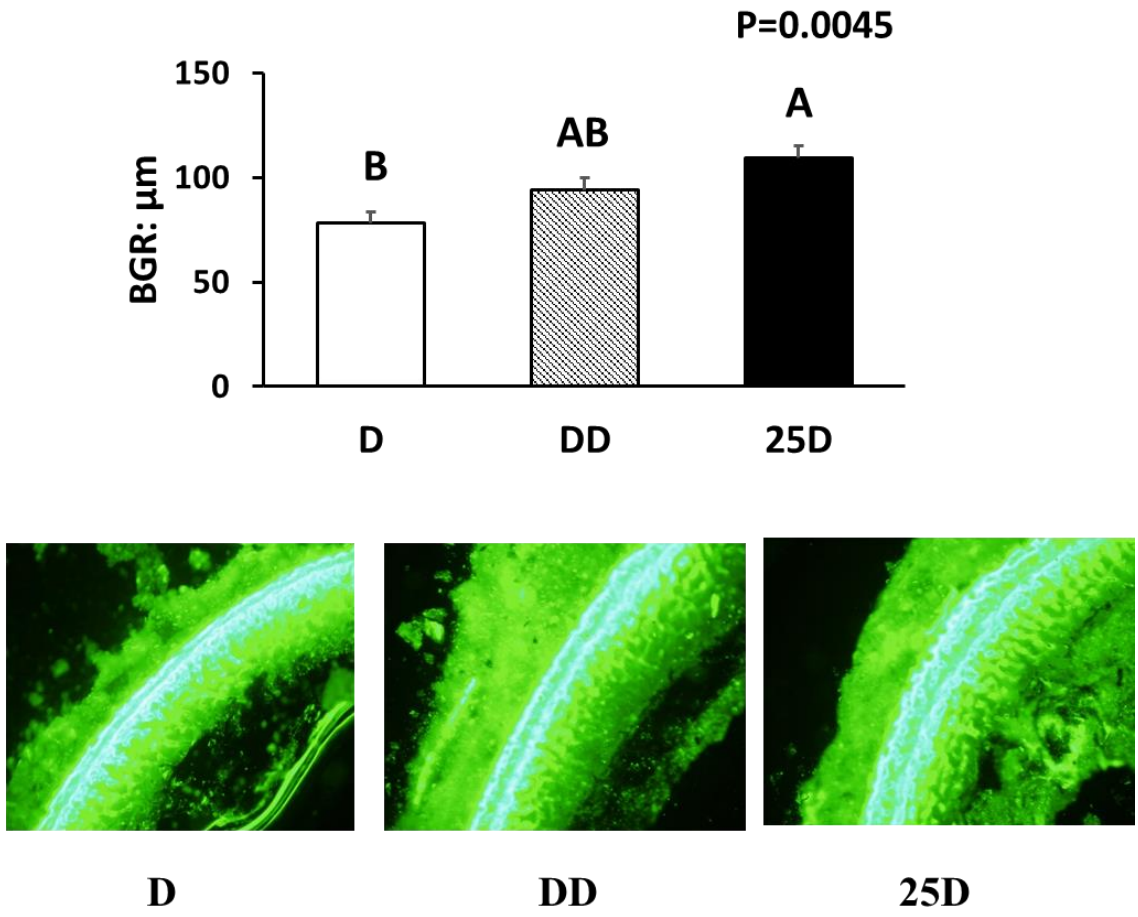


Figure 5.4 Effects of dietary supplementation of 25OHD on pullet bone growth rate from wk10-wk12. Represented pictures were shown on the bottom. D: vitamin D_3 at 2,760 IU/kg; DD treatment: vitamin D_3 at 5,220 IU/kg; 25D treatment: vitamin D_3 at 2,760 IU/kg plus 25OHD at 2,760 IU (69µg)/kg. Values of means represent 1 bird per 10 replicate pens (n = 10 birds) per treatment.

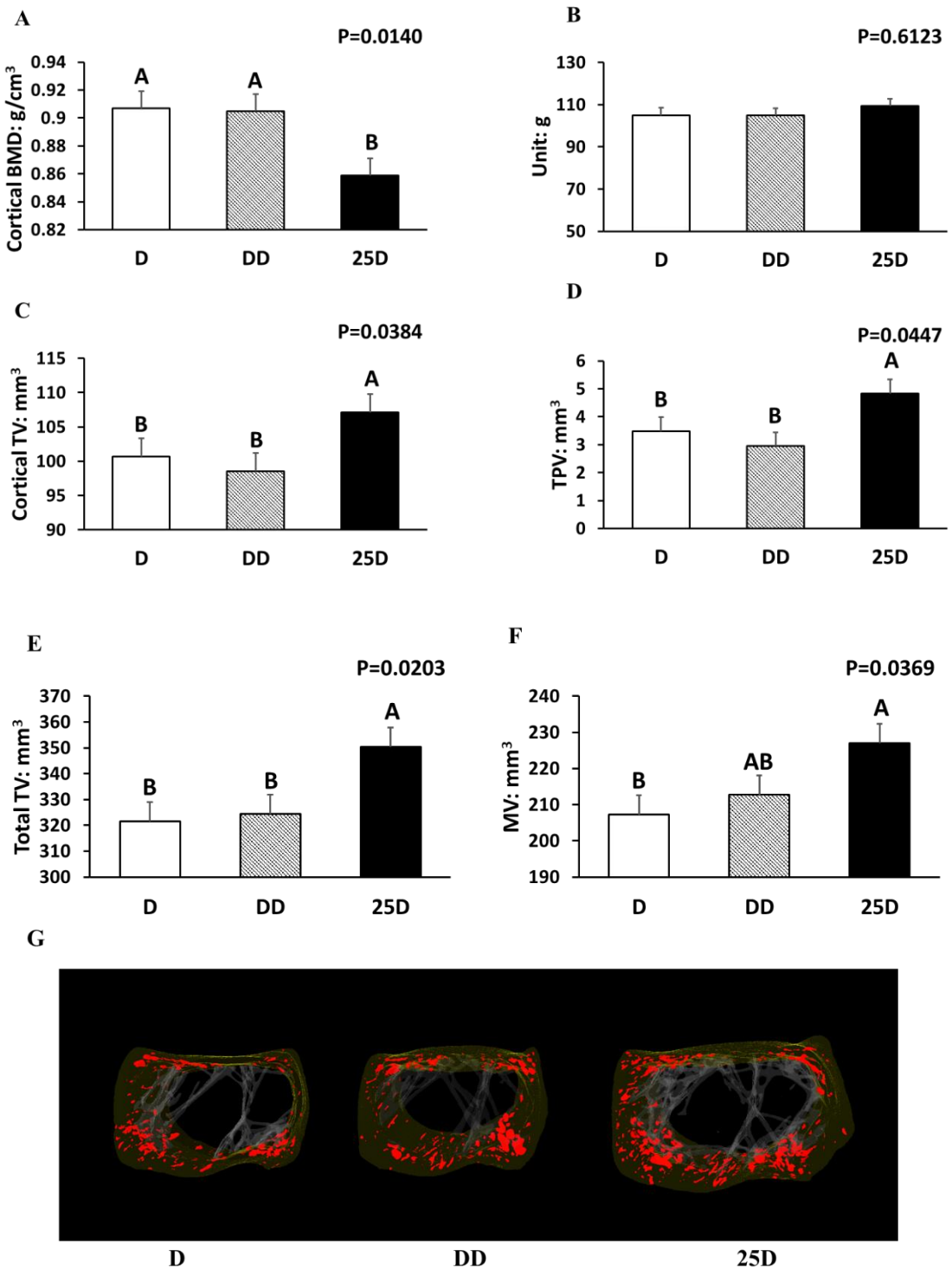


Figure 5.5 Effects of dietary supplementation of 25oHD on pullet bone development at 17wks. D: vitamin D_3 at 2,760 IU/kg; DD treatment: vitamin D_3 at 5,220 IU/kg; 25D

treatment: vitamin D_3 at 2,760 IU/kg plus 25OHD at 2,760 IU (69µg)/kg. Values of means represent 1 bird per 10 replicate pens (n = 10 birds) per treatment. (A) Cortical bone bone mineral density (BMD); (B) Cortical bone mineral content (BMC); (C) Cortical bone volume; (D) Cortical bone total volume of pore space at; (E) Total bone tissue volume at; (F) Medullary volume; (G) pictures showed the cortical bone total volume of pore space from each treatment at. The red color represents the pores.

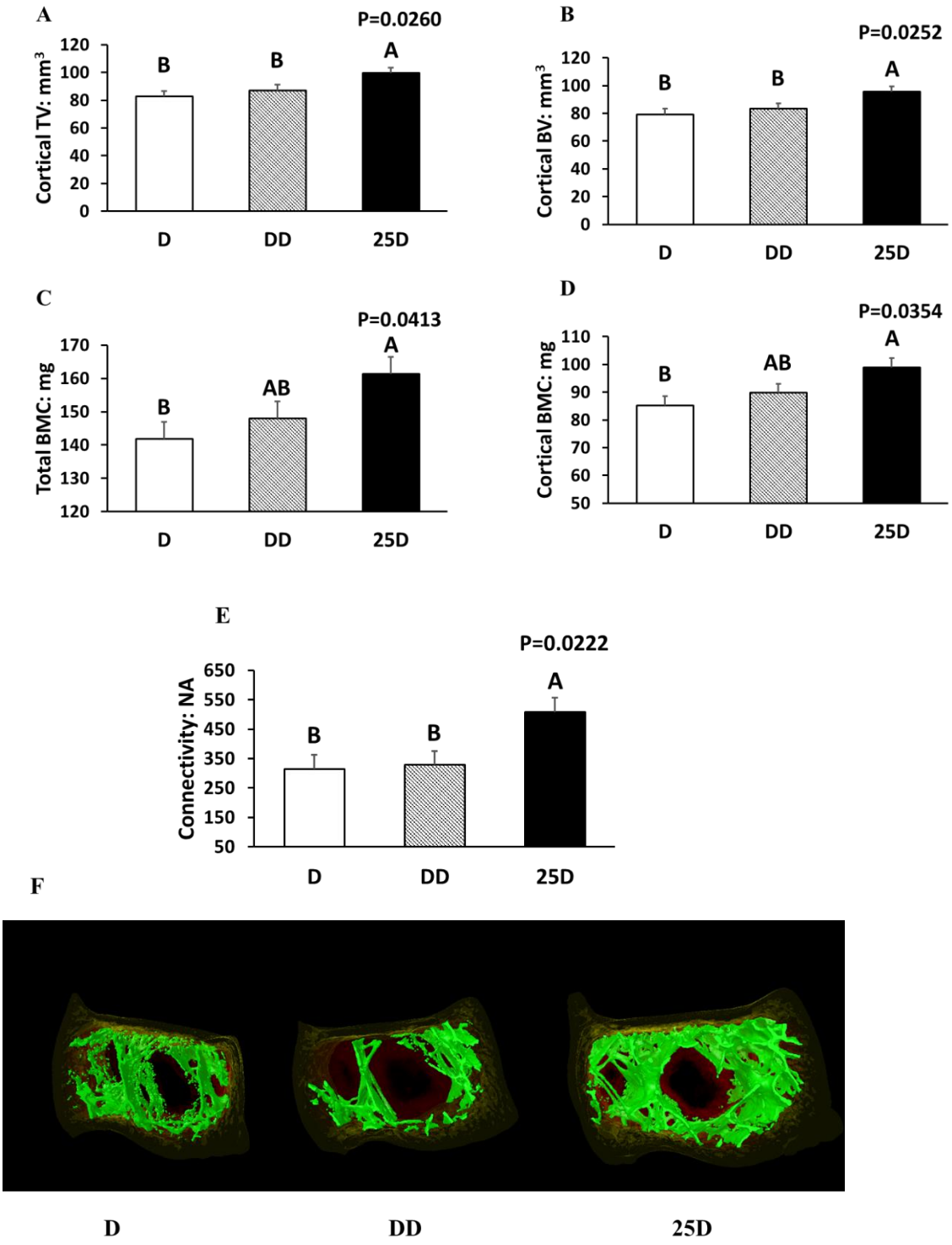
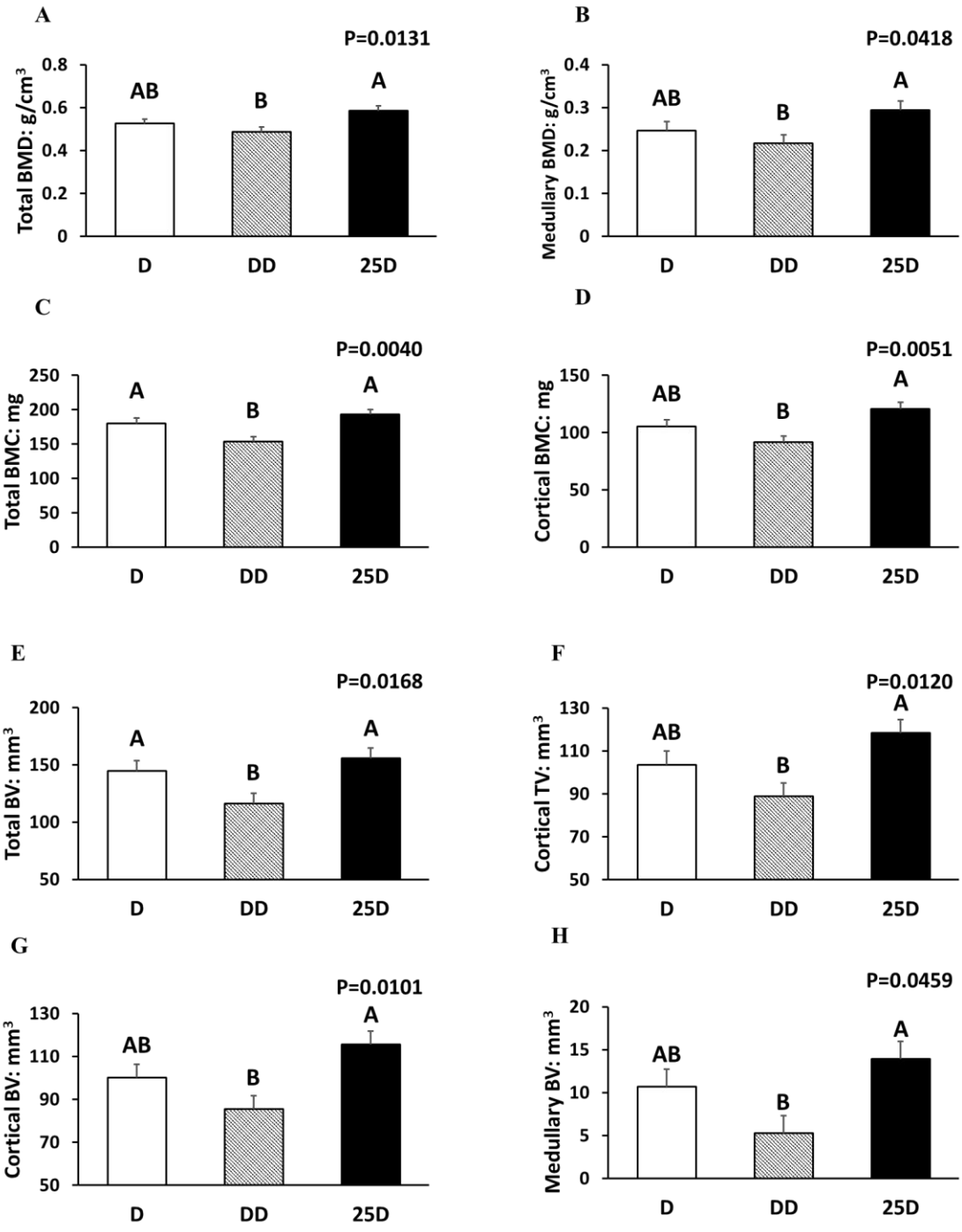


Figure 5.6 Effects of dietary supplementation of 25OHD on laying hen bone development at wk60. D: vitamin D_3 at 2,760 IU/kg; DD treatment: vitamin D_3 at 5,220 IU/kg; 25D

treatment: vitamin D_3 at 2,760 IU/kg plus 25OHD at 2,760 IU (69µg)/kg. Values of means represent 1 bird per 10 replicate pens (n = 10 birds) per treatment. (A) Cortical bone volume; (B) Cortical bone volume (C) Total BMC, calculated by total BMD*total bone volume; (D) Cortical BMC, calculated by cortical BMD*cortical tissue volume; (E) trabecular bone connectivity; (F) the represented picture showed the trabecular bone structure from each treatment.


A
P=0.0131
Total BMD: g/cm³
0.8
0.6
0.4
0.2
0
AB
B
A
D
DD
25D
B
P=0.0418
Medullary BMD: g/cm³
0.4
0.3
0.2
0.1
0
AB
B
A
D
DD
25D
C
P=0.0040
Total BMC: mg
250
200
150
100
50
0
A
B
A
D
DD
25D
D
P=0.0051
Cortical BMC: mg
150
100
50
0
AB
B
A
D
DD
25D
E
P=0.0168
Total BV: mm³
200
150
100
50
A
B
A
D
DD
25D
F
P=0.0120
Cortical TV: mm³
130
110
90
70
50
AB
B
A
D
DD
25D
G
P=0.0101
Cortical BV: mm³
130
110
90
70
50
AB
B
A
D
DD
25D
H
P=0.0459
Medullary BV: mm³
20
15
10
5
0
AB
B
A
D
DD
25D

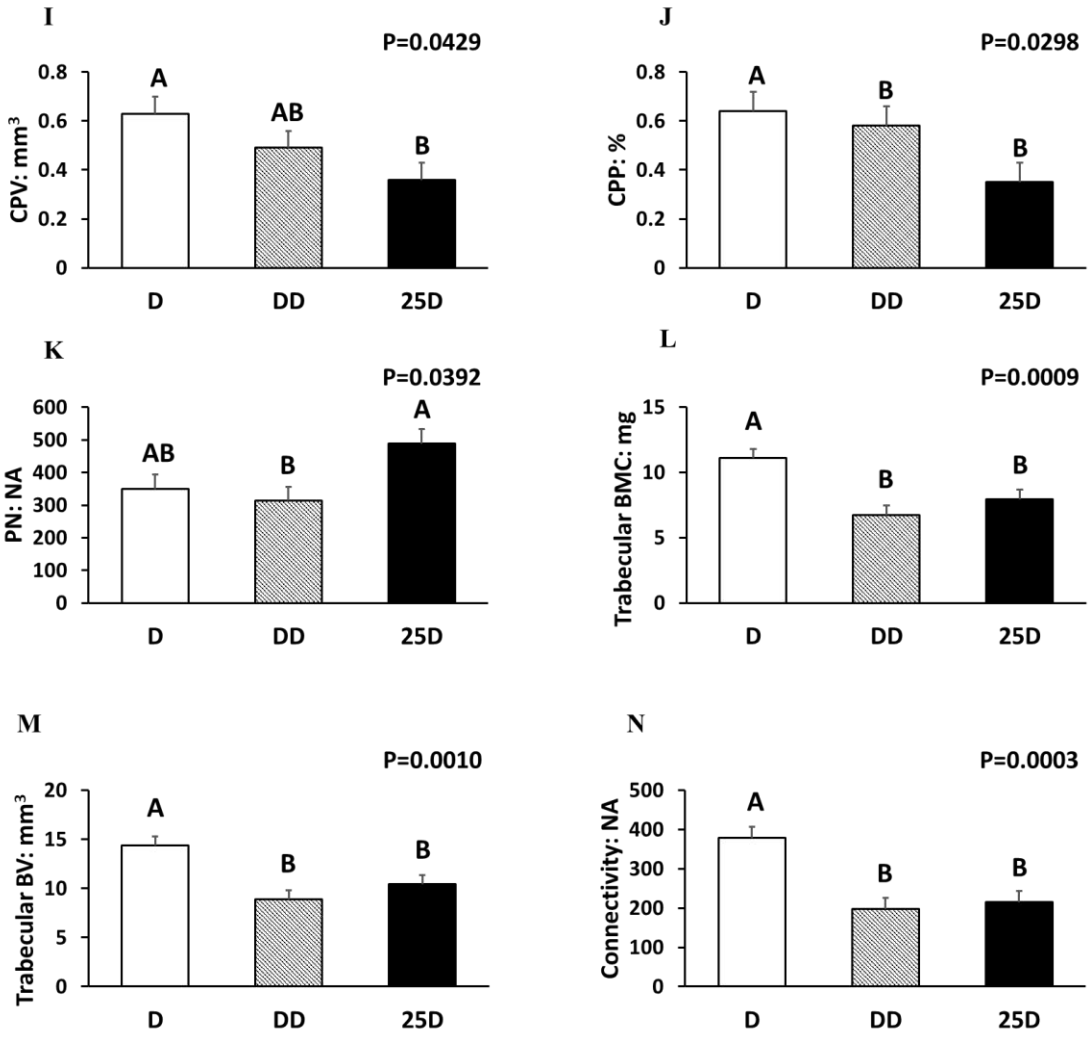


Figure 5.7 Effects of dietary supplementation of 25OHD on laying hen bone development at wk95. D: vitamin D_3 at 2,760 IU/kg; DD treatment: vitamin D_3 at 5,220 IU/kg; 25D treatment: vitamin D_3 at 2,760 IU/kg plus 25OHD at 2,760 IU (69µg)/kg. Values of means represent 1 bird per 10 replicate pens (n = 10 birds) per treatment. (A) Total BMD; (B) Medullary BMD (C)Total BMC, calculated by total BMD*total tissue volume; (D) cortical BMC, calculated by cortical BMD*cortical tissue volume (E) Total bone volume (F) Cortical tissue volume; (G) cortical bone volume (H) Medullary bone volume (I) cortical bone closed pore volume (J) Cortical bone closed porosity(close pore

volume/tissue volume; (K) cortical bone pores number (L) Trabecular BMC, calculated by trabecular BMD*trabecular tissue volume (M) trabecular bone volume which is equal with tissue volume since there are no pores inside trabecular bones; (N) trabecular bone connectivity

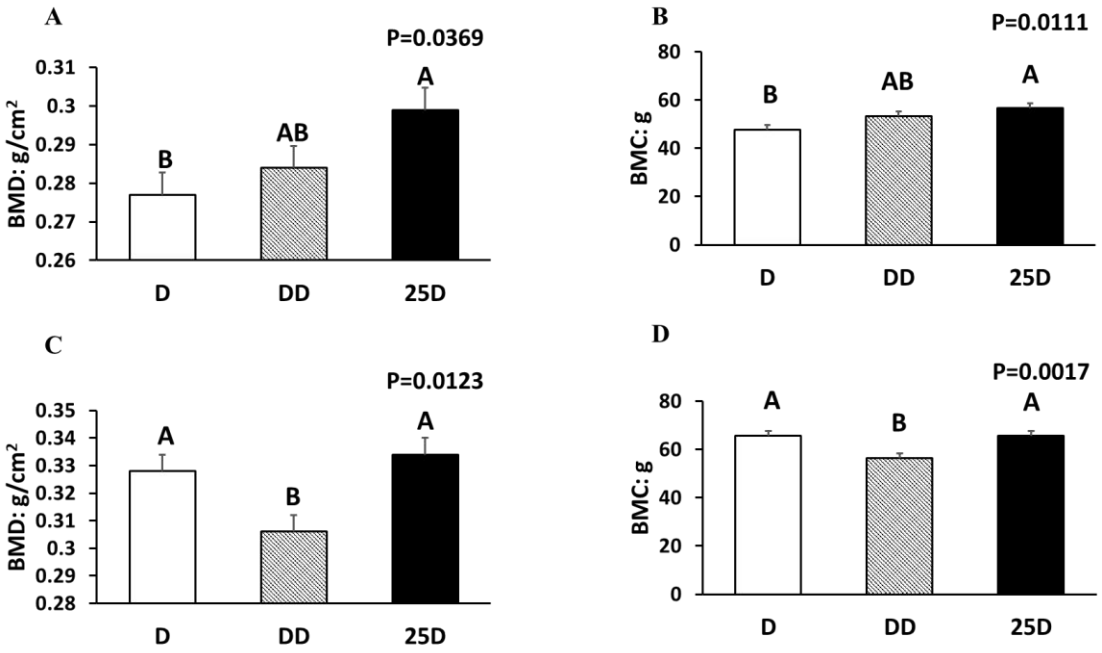


Figure 5.8 Effects of dietary supplementation of 25OHD on layer bone development by using DEXA. D: vitamin D_3 at 2,760 IU/kg; DD treatment: vitamin D_3 at 5,220 IU/kg; 25D treatment: vitamin D_3 at 2,760 IU/kg plus 25OHD at 2,760 IU (69µg)/kg. Values of means represent 1 bird per 10 replicate pens (n = 10 birds) per treatment. (A) whole body BMD at 60wk; (B) whole body BMD at 60wk; (C) whole body BMD at 95wk; (D) total body BMC at 95wk.

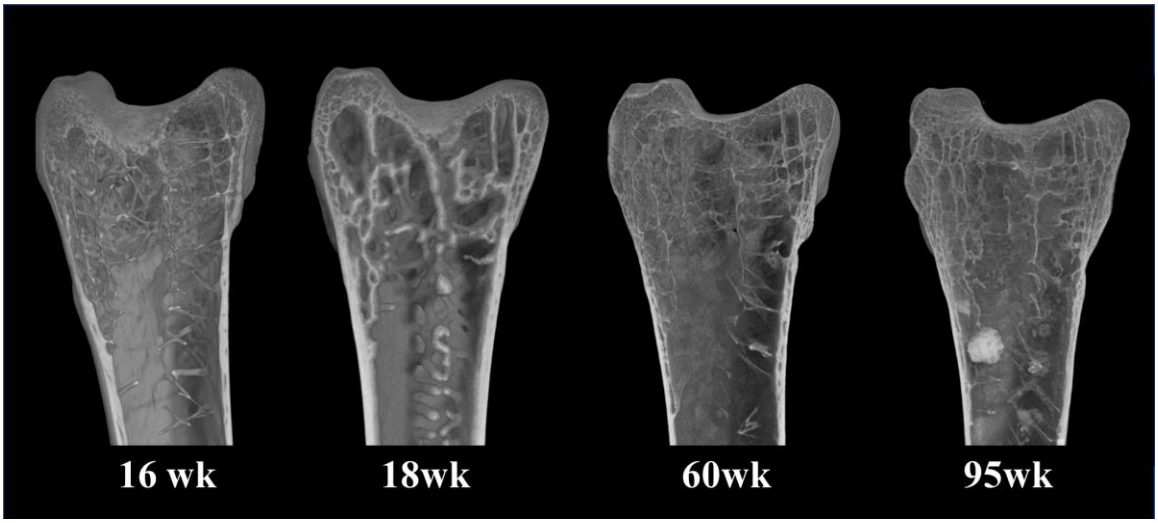


Figure 5.9 The picture showed the structure of cortical, trabecular and medullary bones at 16wk, 18wk, 60wk, and 95wk. Compare to 17wks, the structure bone progressively lost, especially the trabecular bone becomes very thin during the late laying period. At the same time, the medullary bone formed calcium chucks inside the bone cavity.

CHAPTER 6

MODELING BONE DEVELOPMENT CHARACTERISTICS OF LAYERS BY USING MICRO-COMPUTED TOMOGRAPHY

Chongxiao Chen, Samuel E. Aggrey, and Woo Kyun Kim. To be submitted to *Frontier in Physiology*

ABSTRACT

Understanding the layer bone model parameters and their relationships provides insight into creating new strategies to optimize bone development and alleviate osteoporosis during the laying period. A study was conducted to explore the bone development pattern of modern high egg producing laying hens. A total of 480 1-day-old Hy-line W36 pullets were raised until 21wk based on Hy-line W36 guide. From 0 to 21wk, 10 birds per week were weighed, and femurs were taken for Micro-computed tomography (micro-CT) analysis. Segmented linear and exponential models were fitted to the body weight (BW), total bone tissue volume (TV), calcified bone volume (BV), bone mineral density (BMD), and bone mineral content (BMC) data using NLIN procedure. From results, The BW and TV fitted to the logistic exponential model ($P<0.001$, $R^2>0.979$). BW and TV increased exponentially by 0.256 g/wk and 0.320 mm^3/wk, respectively. BV and BMC fitted to linear model from 0-16wk and exponential model from 17-21wk ($P<0.001$, $R^2>0.970$). BV had a growth rate of 105.1 mm^3/wk up to 17wk and followed by an exponential growth by 1.608 mm^3/wk. Similarly, BMC increase by 0.100g/wk and 1.608g/wk respectively in two periods. Unlike the others, BMD remained constant from 8-17wk at 0.329 g/cm^3, then increased exponentially at 17wk by 1.559 g/cm^3/wk ($P<0.001$, $R^2=0.884$). The data were also grouped according to the developmental age period: period 1 (0-4wk), period 2 (6-16wk), and period 3 (17-21wk). The correlation among the parameters was evaluated by using CORR procedure at each period. The BW had a strong correlation with TV, BV, and BMC up to 17wk ($P<.001$;

R^2>0.975), and a moderate correlation after 17wk (P<.05, R^2=0.449 to 0.607). However, BW has a moderate relationship with BMD up 4wk (P<0.001, R^2=0.603), and no correlation (P>0.05) with BMD during 6-17wk. In conclusion, there were dramatic changes in bone developing pattern at the beginning of laying (18wk), evidenced by the significant increases in BV, BMD, and BMC. This indicates that calcium and other minerals are mobilized considerably from bones before laying for eggshell formation, and layer nutrition at the point of producing the first egg may be critical for egg production and skeletal integrity during the later laying period.

Keywords: modeling, Layer, bone development pattern, linear, logistic

INTRODUCTION

Osteoporosis is a serious animal welfare issue for commercial laying hens (Webster, 2004). A great number of research focusing on osteoporosis and bone quality expanded our knowledge of the factors affecting laying hen bone quality. (Webster, 2004; Whitehead, 2004; Whitehead and Fleming, 2000). These factors include dietary nutrition, housing system, genetic, the chance of exercise and age (Fleming, 2008; Jendral, et al., 2008; Rodriguez-Navarro, et al., 2018). The laying hen is an attractive model for studying bone pathologies such as osteoporosis (Kerschnitzki, et al., 2014), because of its unique medullary bone formation during sexual maturity, which acts as a constant calcium source for eggshell formation (Dacke, et al., 1993). Due to the high egg production, the laying hen experiences a progressive loss of bone strength and mass during the laying period (Dale, et al., 2015). How to improve bone health and laying performance is a challenge for the poultry industry.

There is a poor understanding of modern layer bone development. To our knowledge, there are some broiler bone modeling studies or periodically bone quality evaluations showed in the previous literature (Biewener, et al., 1986; Prisby, et al., 2014). However, there is no study on modeling the modern layer bone development so far. The objective of the current research is using curve fitting to describe the bone-related parameters and to understand the biological model meaning and relationship. It will provide a sound basis for developing a new strategy targeting on layer bone health. The

principal tool applied in the current study is the micro-computed tomography (Micro-CT), which is the "golden standard" for bone health assessment (Bouxsein, et al., 2010). It allows us to visualize and measure the true three-dimensional object structures without destroying the bones. All quantitative parameters were computed directly from 3-dimensional data without any model assumption. Due to the high resolution and 3-dimensional calculation, the accuracy of data was significantly improved compared to DEX and conventional CT (Regmi, et al., 2017).

MATERIALS AND METHODS

Housing, birds, and treatments

The study was conducted at the research facility of the Department of Poultry Science at the University of Georgia. The trial was conducted in accordance with the animal care guidelines of the research facility. A total of 480 1-day old Hy-Line W36 pullets were raised by feeding standard diets to meet the nutrition requirement based on Hy-Line W36 guide (2015) (Table 1). Birds were housed in colony cages until 17 weeks, then transferred to individual cages. Water and experimental diets were offered *ad libitum* from 0 to 21 weeks. The lighting management was customized by Hy-line North America lighting program throughout 0-17 weeks (http://sales.hyline.com/NALighting/WebLighting.aspx). After 17 weeks, layers received 15.5 hours of light and 8.5 hours of dark.

Performance data collection and sampling

Ten birds were taken randomly from the flock every week throughout the whole experimental period. The body weight was recorded. The right femur was carefully dissected out at the hip joint. The soft tissue was removed completely. The samples were wrapped with cheesecloth with PBS to keep the moister of bones. The bones were then stored at -20 ℃. Before the analysis, the samples were completed deforest.

Micro-CT scanning:

Skyscan 1275 (Bruker microCT, Belgium) was used for 3D image acquisition. The bone was held in a low-dense 50ml tube; the extra cheesecloth was used for keeping the sample in a vertical orientation and firmly inside the tube holder. Then the tube was mounted on the scanning stage. Scanning settings were determined based on the contrast of images considering all the ages, which is shown in Table 2. Before the scanning, the alignment test and flat field correction were performed according to Micro-CT manual. The random movement and 180-degree scanning were applied for the scanning. The 0.5 mm aluminum filter was used to reduce the beam hardening. After scanning, the pictures were carefully screened, and the appropriate alignment and mathematical methods correction (beam-hardening correction: 35%; smoothing: NA; ring artifact reduction: NA) were applied for all samples during the reconstruction by using NRecon (version: 1.6.10.5, Bruker microCT, Belgium). Dynamic range for all the samples was set at 0-0.035. The 3D model was analysis by using CTan (Version: 1.16.4.1, Bruker microCT, Belgium). The threshold was set at 80-255. Two calcium hydroxyapatite (HA) from a solid-state phantom (0.25g/cm3 and 0.75g/cm; diameter: 7mm) was used for calibration. The outcome parameters in the current study are introduced in Table 3.

Data analysis

By observing, the data followed two patterns, non-linear or a mix of linear and non-linear. Therefore, segmented linear and exponential models were fitted to the body weight(BW), total bone tissue volume(TV), calcified bone volume (BV), bone mineral density(BMD) and bone mineral content (BMC) data by using NLIN procedure.

Linear Model

The following equation describes the linear model:

$$W_t = d + (b \times t)$$

Where Wt is the parameter value at age t. d is the cross section on Y axis, and t is the linear growth rate.

Logistic Model

The following equation describes the logistic (Aggrey, 2002) growth model:

$$W_t = W_A/[1 + \exp -K(t - tc)]$$

Where Wt is the parameter value at age t, W_A is the asymptotic parameter value, K is the exponential growth rate, and tc is the age at the inflection point.

The data were also grouped according to the developmental age period: period 1 (wk0-4), period 2 (wk6-16), and period 3 (wk17-21). The correlation among the parameters at each period was evaluated using the CORR procedure. All the data were analyzed using SAS software Version 9.3 (SAS Institute, Cary, NC). The level of significance was assessed at $P \leq 0.05$.

RESULTS

The BW fitted to the logistic exponential model ($P<0.001$, $R^2=0.9800$), with an exponential increase rate at 0.256g/wk (Figure 1). Similarly, the TV fitted to the logistic exponential model ($P<0.001$, $R^2=0.9793$) with an exponential increase rate at 0.320 mm^3/wk (Figure 2). However, a mixed model must be applied to interpret BV with a linear model from wk0-16 and logistic model from 17-21wk ($P<0.001$, $R^2>0.970$) (Figure 3). BV had a growth rate of 105.1 mm^3/wk up to 17wk and followed by an exponential growth by 1.608 mm^3/wk. Similarly, BMC showed the same pattern as BV, it increases by 0.100g/wk and 1.608g/wk respectively in two periods (Figure 4). A unique pattern was showed on BMD; it kept constant from 8-17wk at 0.239 g/cm^3, then increased exponentially at 17wk by 1.559 g/cm^3/wk ($P<0.001$, $R^2=0.884$) (Figure 5).

The correlation is summarized in Table 3. Three periods were manually grouped based on the development stage: period 1 (0-4wk), period 2 (6-16wk), and period 3 (17-21wk). Three levels of correlation were summarized based on P value and R^2 value. In summary, BW had a strong correlation with TV, BV, and BMC up to 17wk ($P<.001$; $R^2>0.975$), and a moderate correlation after 17wk ($P<.05$, $R^2=0.449$ to 0.607). However, BW has a moderate correlation with BMD up 4wk ($P<0.001$, $R^2=0.603$), but no correlation ($P>0.05$) with BMD during 6-16wk.

DISCUSSION

BW and bone tissue volume in layers followed a similar development pattern throughout the experimental period. Furthermore, the bone tissue volume strongly correlated with BW. It is well recognized that bone size is closely related to body weight in living terrestrial vertebrates (Anderson, et al., 1985; Martin-Silverstone, et al., 2015). Whereas, BV and BMC have a different development pattern compare to BW and TV. TV represents the structure size of a bone as well as the holding capacity of minerals, while BV and BMC indicate the mineral amount in the bone. The study's results suggested the development of bone structure and mineral deposition are two different processes.

The current study showed a constant BMD value at 0.239 g/cm^3 before the sexual maturity. The constant value may result from the increase of bone mineral content along with increasing volume of bones (TV). Similarly, by comparing the BMD of broiler tibia at 2, 4, and 6wk, the results showed no difference between these time points at proximal metaphysis but decrease at mid-diaphysis as age increases (Charuta, et al., 2013). However, in a turkey study, the BMD increases along with bone breaking strength as the age increase (Krupski and Tatara, 2007). Nevertheless, the BMD changes were not affected by the age and did not correlate with BW after 7wk in the current study. Although the inconsistent results were shown in the previous study regarding the age, bone mineral density (BMD) is still an essential and common measure for bone quality

assessment in chicken (Almeida Paz and Bruno, 2006; Whitehead and Fleming, 2000). Nevertheless, it is more logical to consider the evaluation of bone strength as the major endpoint in animal models (Ammann and Rizzoli, 2003), because the primary concern for laying hen is the bone fracture.

In most of the studies, the higher BMD frequently showed a higher bone strength (Biewener, 1993; Jendral, Korver, Church and Feddes, 2008; Kim, et al., 2012). However, the human study showed increased BMD account for less than 25% if the overall reduction in fracture risks (Small, 2005) rendering BMD an imperfect indicator for fracture risks. On the one hand, it may be because the testing points of bones result in a different response (Biewener, 1993). On the other hand, bone strength is influenced by multi-factors such as bone size, shape, turnover, and architecture (Ammann and Rizzoli, 2003). Zhang and Coon (1997) highlight that the bone force is important with bone volume. When birds reached the sexual maturity, the BMD becomes more complicated due to the medullary bone formation. Medullary bone is radiodense materials and significantly contributes to BMD and BMC, but it does not contribute bone strength as much as the structure bones do (Dacke, et al., 1993). The relationship of BMD to bone strength needs to be further studied systematically. While interpreting the BMD data on bone quality especially during different development stages, more factors including the bone size, micro-architecture, etc. need to be taken account when possible.

Other studies suggest an increased mineral deposition as age increases (Charuta, et al., 2013; Krupski and Tatara, 2007). However, it needs to be noticed at 17wk, when birds are preparing for the first egg, BMD, BMC, and BV dramatically increased. It has been reported previously that the entirely skeletal weight of the pullets increases by 15-12

grams (4-5g calcium) at the sexual maturity (Sturkie, 2012). Our results also indicated that a serve mineral deposition happened during this period. In the diet, higher calcium level was provided to the birds to meet the demand for bone mineralization. However, it is not the primary reason for this phenomenon. The medullary bone is hormones-driven bones (Dacke, et al., 1993; Rath, et al., 2000; Whitehead, 2004); The injection of estradiol valerate to male Japanese quail induced medullary bone formation (Miller and Bowman, 1981). The content of medullary bone is similar to the other part of the bones, which mainly contain hydroxyapatite lattice (Ascenzi, et al., 1963), but with much lower collagen content and exhibit a random distribution pattern. (Dacke, et al., 1993). The medullary bone can be metabolized at a rate of at least 10–15 times faster than cortical due to the high osteoclast activity in this region (Hurwitz and Bar, 1965; Simkiss, 1967; Van de Velde, et al., 1984), which is an essential source for egg formation (Bishop, et al., 2000; Cransberg, et al., 2001; Kim, et al., 2012). It is important to have the appropriate nutritional supplementation and optimal management during this period in order to meet the requirement of bone development to achieve a healthier bone and potentially better egg production and quality later.

In conclusion, bone mineral deposition and bone structural development is two independent process. They follow different development patterns. The mineral deposition during the sex maturity is critical and shows a high flux of minerals to bone formation. The dietary requirement and management must be optimal. TV increases steadily and seems to reach a plateau at the onset of sexual maturity. It suggests that an increase in bird's bone structural size probably is the primary task to achieve better bone quality during the rearing period. At last, BMD is not an optimal predictor of layer bone

development during the growing period, the interpretation of BMD in poultry bone research needs to accompany with the other factors as much as possible.

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TABLES

Table 6.1 Diet formulation

Ingredients, %	Starter 1	Starter 2	Grower	Develope	Prelay	Peaking
Corn	67.11	62.19	65.93	69.43	62.17	53.61
Soybean Meal -48%	28.08	27.34	24.00	20.00	23.10	28.10
Soybean Oil	1.00	3.00	2.54	2.57	3.02	3.75
Limestone	0.68	0.71	0.8	1.95	4.68	7.44
Defluor. Phos.	2.03	2.01	1.92	1.85	2.01	3.19
Common Salt	0.30	0.30	0.30	0.30	0.30	2.55
L-Lysine HCl	0.19	0.13	0.11	0.08	0.01	0.30
DL-Methionine	0.21	0.23	0.18	0.14	0.20	0.46
Threonine	0.23	0.08	0.06	0.05	0.03	0.33
Vitamin Premix	0.05	0.05	0.05	0.05	0.05	0.11
Mineral Premix	0.06	0.06	0.06	0.06	0.06	0.05
Amprolium	0.05	0.05	0.05	0.05	0.05	0.06
Sand	0	3.85	3.99	3.47	4.33	0.05
ME(kcal/kg)	3030	3030	3030	3050	2920	2840
CP%	20.00	18.25	17.50	16.00	16.50	19.05
Ca%	1.00	1.00	1.00	1.40	2.50	4.94
Available P (%)	0.50	0.49	0.47	0.45	0.48	0.58

[1] Starter1 (0-3wk), starter2 (4-6wk), grower (7-12wk), developer (13-15wk), prelay (15-17wk), preaking (18-21wk)

[2] Supplied per kilogram of diet: vitamin A, 9,900 IU; Vitamin E, 22.10 IU; vitamin B12, 0.02 mg; Biotin, 0.06 mg; Menadione, 3.3 mg; Thiamine, 2.20 mg; Riboflavin, 6.60 mg; Pantothenic Acid, 11.00 mg; Vitamin B6, 4.40 mg; Niacin, 33.00 mg; Folic Acid, 0.90 mg; Choline, 191.36 mg.

[3] Supplied per kilogram of diet: Mn, 80.4 mg; Zn, 64.2 mg; Mg, 16.08 mg; Fe, 15.78; Cu, 2.4 mg; I, 0.6 mg; Se, 0.24 mg

Table 6.2 Micro-CT scanning settings

Voltage (kv)	Current (mA)	Exposure time (ms)	Rotation (degree)	Average frame	Pixel size Um/pixel
80	125	55	0.4	6	25

Table 6.3 Micro-CT outcome interpretation

Parameters	Introduction
Tissue volume(TV)	The volume of the volume-of-interest (VOI), including pores and cavity inside the bone
Bone volume(BV)	The volume of binarized objects (mineralized bones) within the VOI
BMD (Bone mineral density)	BMD is defined as the volumetric density of calcium hydroxyapatite (CaHA) in biological tissue regarding g.cm-3. It is calibrated using phantoms with a known density of CaHA. In the current study, BMD relates to the amount of bone within a mixed bone-soft tissue region
BMC (Bone mineral content)	BMC is calculated by using BMD*TV

Table 6.4 The correlation of bone parameters with body weight

	Correlation	P and R^2 value	0-4wk	5-17wks	18-21wks
BW	Strong correlation	P<.001; R^2>0.975	TV; BV; BMC	TV; BV; BMC	
	Moderate correlation	P<0.05; R^2=0.449-0.607	BMD		TV; BV; BMC
	No correlation	P>0.05		BMD	BMD

FIGURES

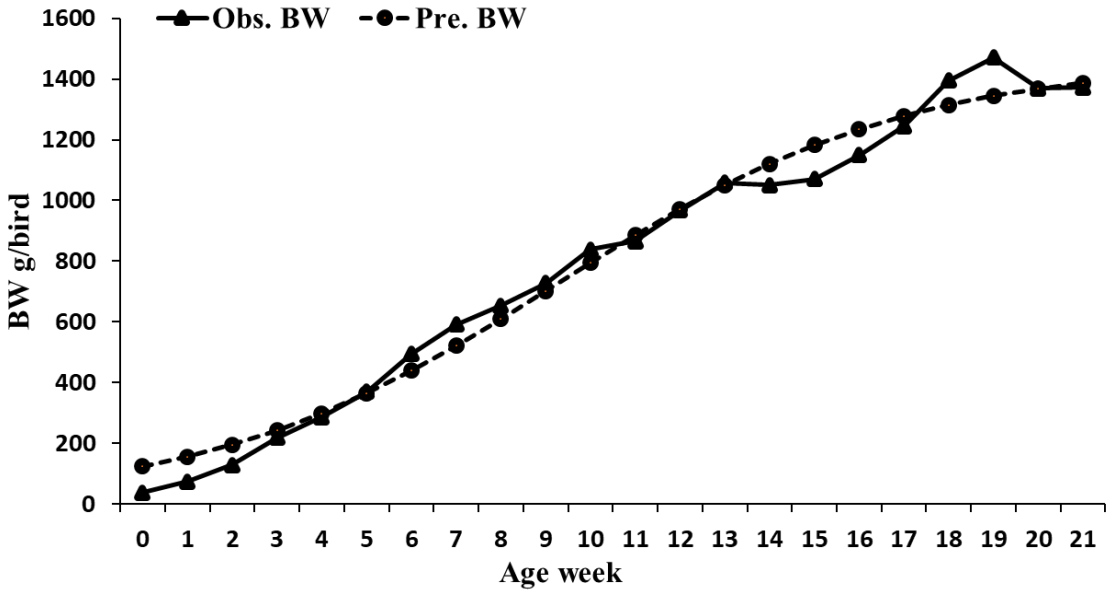

Figure 6.1 Growth curve is predicted by logistic model. P< 0.0001; R2=0.9800;

BW=1456.2/[1+exp -0.2561(age-9.2853)]

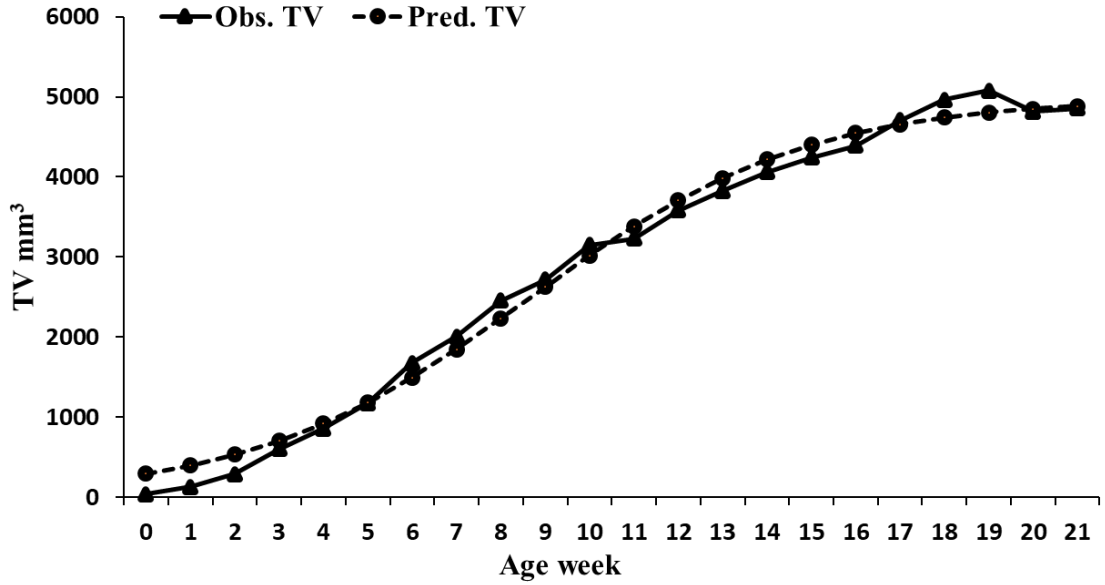

Figure 6.2 Bone tissue volume (TV) is predicted by logistic model: P= <.0001; R^2 =0.9793; TV=4980.4/[1+exp-0.3197(age-8.6522)]

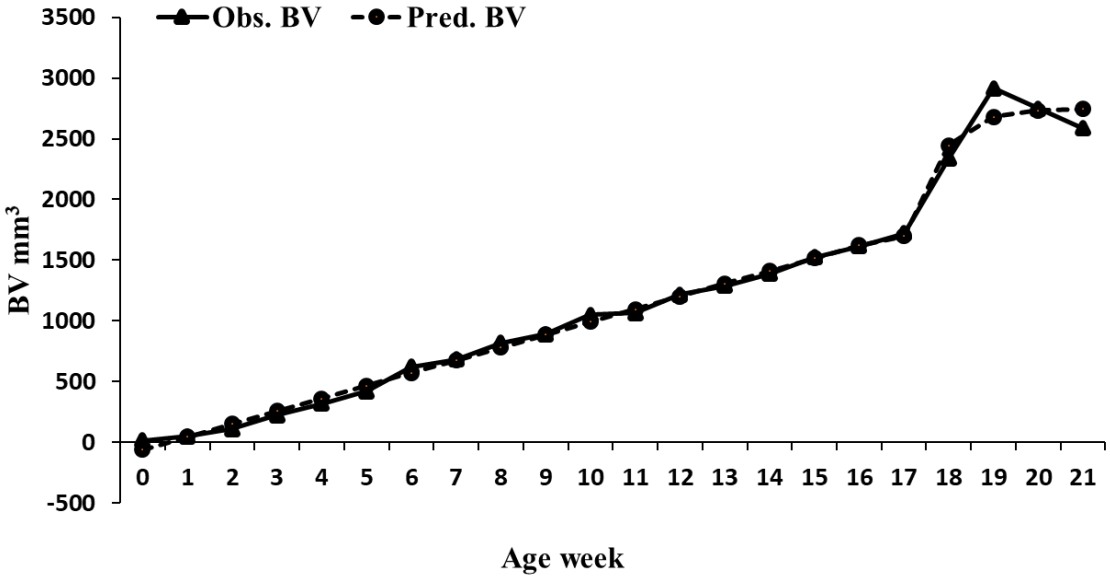

Figure 6.3 Bone volume (BV) is predicted by combining linear and logistic models: P< 0.0001; R^2 = 0.9704; 0-17wk: BV=-60.058+(105.1*age); 18-21wk: BV=2747.0/[1+exp-1.6080(age-16.7013)]

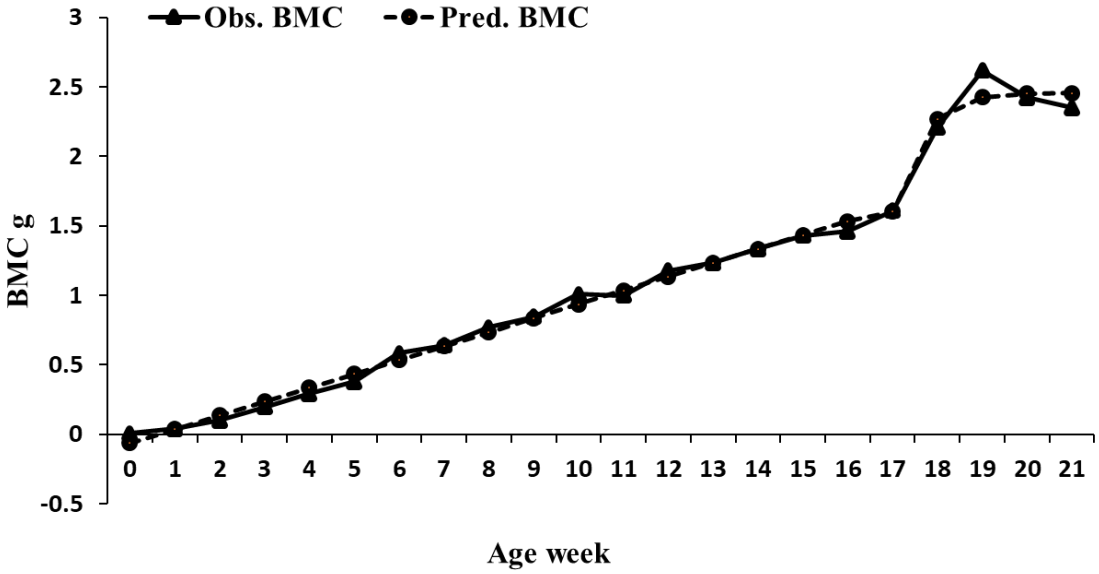

Figure 6.4 Bone mineral content (BMC) is predicted by combining linear and logistic models: $P<0.0001$; $R^2 = 0.9794$; 0-17wks: BMC=-0.0634+(0.0998*age); 18-21wks: BMC=2.4572/[1+exp-1.8684(age-16.6648)]

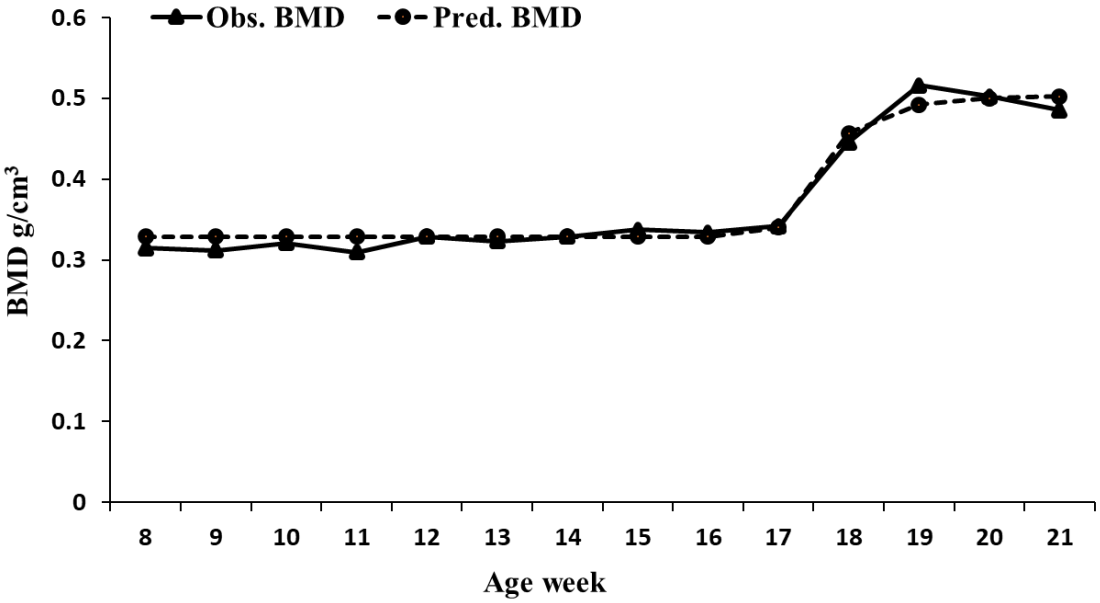

Figure 6.5 Bone mineral density (BMD) has a constant value at 0.329g/cm3 from 8-17wks; BMD is predicted by logistic model from 18-21wks: BMD=0.5025/[1+exp-1.5586(age-16.5261)]. The overall P= <0.0001; R^2 = 0.8841.

CHAPTER 7

CONCLUSIONS

These studies reveal the molecular actions on chicken osteoblasts differentiation and mineralization, the higher active form of vitamin D_3 on layer bone development and laying performance, and the pattern of layer bone development. The results significantly expanded our understanding of vitamin D_3 function, its interaction with bone development and egg production as well as the layer bone physiology.

A number of factors contribute to the complexity of 1,25-dihydroxyvitmain D_3 (1,25OHD) effects on osteoblasts. The *in vitro* study of 1,25OHD on chicken osteoblasts differentiation and mineralization indicated 1,25OHD's time-dependent and dosage-dependent manners. In general, it showed an inhibitory effect during the early differentiation stage (24h and 48 h), and a promontory effect during the late differentiation stage (3d and 7d). For the staining results, the cells with 1,25OHD treatments showed an inhibitory effect on mineralization and ALP expression up to 14d, but no difference between the treatments at 21d. Furthermore, a consistently dose-dependent manner was observed, which is the higher level of 1,25OHD showed stronger inhibitory effects. These results provide a novel understanding of the effects of 1,25D on osteoblast differentiation and mineralization depending on the cell stage and maturity.

The in-vivo farm trial for evaluating long-term dietary supplementation of 25-hydroxyvitamin D_3 (25OHD) on pullets and laying hen showed long-term, and early supplementation of 25OHD has positive effects on egg production and egg quality. The beneficial effects were mainly observed during the early laying stage (60wks). Furthermore, as the application of micro-CT on bone quality analysis, we can get more sophisticated data on bone structure changes. 25OHD showed diverse effects throughout the rearing and laying period. It increased bone growth rate and bone size during the pullet period, which provided more space for minerals deposition during the later period. For the laying period, 25OHD has positive effects on bone mineral deposition and improvement of structural bone quality. During the late stage of laying (95wk), 25OHD had a two-sided effect. It enhanced the mineral deposition in the cortical bone region while increasing the resorption of trabecular bones. Our result suggested that early supplementation of 25OHD has prolonged beneficial effects on the layer bone healthy. The higher Ca and P content in the diet during the rearing period is suggested to meet the requirement of increasing bone size. This study demonstrated that 25OHD stimulates bone growth, improves structural integrity in laying hens to prevent avian osteoporosis and has beneficial effects on egg production up to 60wks.

The bone modeling research enables us using statistical models to describe the bone development pattern. It showed bone mineral deposition and bone structural development is two independent process. A dramatical increase of mineral deposition occurred during the onset of sexual maturity, which suggested the dietary requirement and management must be optimal at this period. Bone structure size increased steadily and seemed to reach a plateau at the onset of sexual maturity. It suggested increase birds

structural size probably is the primary task to achieve a healthy bone. At last, BMD has a weak correlation with birds body weight which is also not an optimal predictor of layer bone development during the growing period. The interpretation of BMD in poultry bone research needs more attention.

Due to the limitation of chicken osteoblast culture system, we are unable to explore the interaction of osteoblast with other cells or organs. Subsequently, the net effects of 1,25OHD on bones are indeterminate. Further research could be conducted to investigate the specific effects of 1,25OHD during each stage by applying intermittent treatments. The farm trial for evaluating 25OHD suggested a higher calcium and phosphate content in the diet to meet the requirement of increasing bone size by 25OHD during the rearing period. The experiments of exploring the interaction of Ca/P concentration with 25OHD supplementation in pullet layer diets could be conducted in the future. At last, the modeling of bone development during the laying period would be interesting due to the progressive loss of structure bones, which could help us to find the osteoporosis traits. The modeling of humerus and keel bone development are important for aviary system study because of increased movement of wings and high incidence of keel bone fracture. The trials on the effects of the lighting program, cage system and dietary factors on laying hen bone development pattern could also be performed in the future to expand our knowledge of layer bones, consequently, help us to design a better strategy to increase layer bone health.