# Role of long-term supplementation of 25-hydroxyvitamin D$_3$ on laying hen bone 3-dimensional structural development

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**ABSTRACT** Egg-laying hens have a unique bone development pattern due to the medullary bone formation and high bone turnover rate. The role of long-term supplementation of an intermediate form of vitamin D$_3$, 25-hydroxyvitamin D$_3$ (**25OHD**), on skeletal development of pullets and laying hens is not well established. Exploring its effects on layer bone development will help develop a strategy for preventing laying hen osteoporosis. The purpose of this study was to investigate the role of long-term supplementation of 25OHD in layer diets on bone 3-dimensional structural development. A total of 390 1-day-old Hy-Line W36 pullets were randomly allocated to 3 treatments with 10 replicate cages and 13 birds/cage. Dietary treatments were 1) vitamin D$_3$ at 2,760 IU/kg, 2) vitamin D$_3$ at 5,520 IU/kg, and 3) vitamin D$_3$ at 2,760 IU/kg plus 25OHD at 2,760 IU (69 μg)/kg. The level of 25OHD in the serum was tested throughout the whole experimental period (0–95 wk). Bone growth rate (**BGR**) was measured at 10 wk using a calcein injection technique. Femurs were scanned using Micro-CT for 3D structural analysis, and the whole-body composition analysis was performed using a dual-energy x-ray absorptiometry method at 17, 60, and 95 wk. Dietary supplementation of 25OHD significantly increased 25OHD level in the serum from 0 to 95 wk. During the rearing period (0–17 wk), 25OHD increased BGR, cortical tissue volume, and bone marrow area at 17 wk, 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**). This effect allowed more space for mineral deposition in bones during the later egg-laying period. At 60 wk, the 25OHD group had significantly greater BMD, which led to the highest total BMC, cortical volume, and trabecular bone connectivity. At 95 wk, the birds fed 25OHD had significantly higher cortical bone volume and lower porosity. The 25OHD group also had higher total BMD and medullary bone volume but a lower BMC and volume of trabecular bone than vitamin D$_3$ or double dosage vitamin D$_3$ treatment. This indicated that the bone resorption rate was lower in cortical bone than that in trabecular bone in the late laying period. In conclusion, supplementation with dietary 25OHD could stimulate bone growth and increase bone volume in pullets to provide more space for mineral deposition during the laying period with positive effects on laying hen bone quality.

Key words: 25-hydroxyvitamin D$_3$, 3-dimensional bone structure, pullet, laying hen

## INTRODUCTION

High egg-producing laying hens suffer from avian osteoporosis during the late laying period because of both medullary and structural bone being progressively resorbed for supplying calcium to eggshell formation (Whitehead, 2004; Kim et al., 2007). This issue has become a severe economic and welfare concern for the poultry industry. During the egg-producing period, medullary bones, a special calcium storage for eggshells, are formed inside the bone cavity of laying hens (Schraer and Hunter, 1985; Dacke et al., 1993; Whitehead, 2004; Kim et al., 2007; Werning, 2018). The high osteoclast (bone resorption cells) activity in medullary bone results in 10–15 times faster calcium mobilization than in cortical bone, considering medullary bone a reliable calcium source for eggshell formation in laying hens (Hurwitz and Bar, 1965; Simkiss, 1967; Van de Velde et al., 1984). However, the medullary bone has less structural strength than cortical and trabecular bones (Fleming et al., 2006) because of its low quantity and

**Table 1.** Diet formulation and calculated nutrient composition for rearing period (0–17 wk).[1]

| Ingredients, % (unit %) | Starter 1,[2] 1–3 wk | Starter 2, 4–6 wk | Grower, 49–60 wk | 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 |
| Defluorinated phosphate | 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[5] | | | | | |
| ME (kcal/kg) | 3,030 | 3,030 | 3,030 | 3,050 | 2,920 |
| 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 |

Abbreviations: 5 ME, metabolic enenrgy; CP%, crude protein percentage; Ca, calcium percentage; Available P, available phosphorus percentage.

[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 (69 μg)/kg.

[2]Starter 1 (0–3 wk), starter 2 (4–6 wk), grower (7–12 wk), developer (13–15 wk), prelay (15–17 wk).

[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.

randomly distributed collagen contents (Dacke et al., 1993). Furthermore, medullary bone formation always accompanies the cession of structural bone development (Whitehead, 2004). Thus, for laying hens, maintaining the balance of medullary bone and structural formation becomes critical in terms of bone health and egg production.

25-Hydroxyvitamin D3 (**25OHD**), an intermediate form of vitamin $D_3$, has become commercially available in the poultry industry. It has been exclusively studied

**Table 2.** Diet formulation and calculated nutrient composition for laying period (18–95 wk).[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 |
| Defluorinated phosphate | 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[5] | | | | | |
| ME (kcal/kg) | 2,840 | 2,900 | 2,820 | 2,840 | 2,820 |
| 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 |

Abbreviations: 5 ME, metabolic enenrgy; CP%, crude protein percentage; Ca, calcium percentage; Available P, available phosphorus percentage.

[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 (69 μg)/kg.

[2]Peaking (18–38 wk), layer 2 (39–48 wk), layer 3 (49–60 wk), layer 4 (61–75 wk), and layer 5 (76–95 wk).

[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 3.** Micro-CT scanning settings.

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

in broilers for improving bone health and Ca and P absorption (Edwards, 1989; Koreleski and Swiatkiewicz, 2005a; Wideman et al., 2015; Han et al., 2016; Świątkiewicz et al., 2017). However, most studies on laying hen suggested no significant positive effects on laying hen bone health (Koreleski and Świątkiewicz, 2005b; Käppeli et al., 2011; Nascimento et al., 2014; Silva, 2017; Świątkiewicz et al., 2017; Adhikari et al., 2020). It may be due to the fact that the studies in layers were mostly focusing on laying periods instead of pullets. The manipulation of nutrients only during a laying phase is less effective to increase bone quality or reverse the onset of osteoporosis. A meta-analysis on human clinical trials also showed there were no effect of supplementation of vitamin D or calcium on fracture incidence in older population (Zhao et al., 2017).

To our knowledge, limited studies have been performed to evaluate the effect of early and long-term supplementation of 25OHD in pullet and laying hen diets on bone development. To bridge this gap, the current research project focused on developing a strategy to enhance laying hen bone health and laying performance by early and long-term supplementation of 25OHD in the diets. By conducting a 95-wk layer trial with 2 doses 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 a microcomputed tomography (**micro-CT**) with advanced 3-dimensional analysis techniques. The obtained data provided new aspects of how vitamin D$_3$ affects the layer bone 3-dimensional structures, as well as a strategy for the application of 25OHD to alleviate osteoporosis and welfare issues in laying hens.

## MATERIALS AND METHODS

### Housing, Birds, and Treatments

The study was approved by the Institutional Animal Care and Use Committee at the University of Georgia and conducted at the research facility of the Department of Poultry Science at the University of Georgia. A total of 390 one-day-old Hy-Line W36 pullets (3 trts × 10 reps × 13 birds per cage) were housed in wire colony 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$_3$ + 69 µg/kg 25OHD (**25D**; equivalent from DD; HyD, DSM Nutritional Products). The diets were formulated based on the Hy-Line W36 management guide (2016) (Tables 1 and 2). Birds were housed in colony cages until 17 wk and then transferred to individual cages. Water and experimental diets were offered *ad libitum* from 0 to 95 wk. The pullets received an intermittent lighting program during the first 7 d, with 4 h of light followed by 2 h of dark. The lighting management was customized by using the Hy-line North America lighting program throughout 2-17 wk (http://sales.hyline.com/NALighting/WebLighting.aspx). After 17 wk, hens received 15.5 h of light and 8.5 h of dark.

### Serum 25OHD Content Analysis

Blood samples (10 birds/treatment/time point) were collected from the wing vein at 0 (baseline), 6, 12, 17, 40, 60, 75, and 95 wk. After the blood was clotted, the blood samples were centrifuged at 1,500 × g in a refrigerated centrifuge (Eppendorf Centrifuge 5430R; Eppendorf, Hamburg, Germany) for 12 min. The serum was collected and transferred into a clean polypropylene tube. The samples were maintained at −80°C until analysis. The serum 25OHD level was determined using a mass spectrometry procedure (Heartland Assays, Ames, IA).

### Bone Growth Rate Using Calcein Labeling Technique

At 10 wk of age, one pullet/cage (10 birds/treatment) was injected with calcein solution (Millipore Sigma, St. Louis, MO) intraperitoneally at a dose of 20 mg/kg body weight. After a 10-d interval, pullets were injected
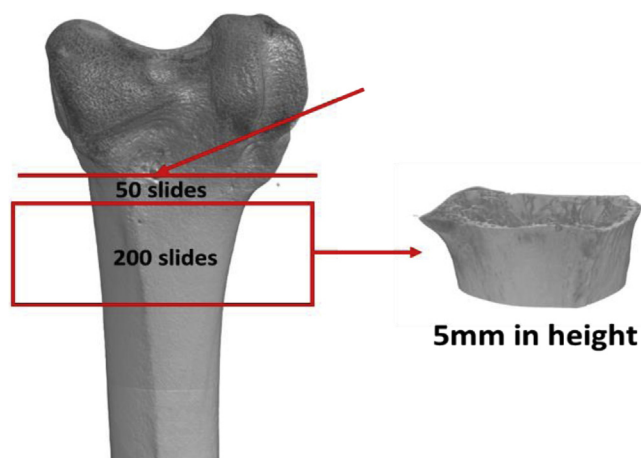


**Figure 1.** The volume-of-interests (VOI) selection is demonstrated in the pictures. The constant VOI was used throughout all the samples. The VOI starts from 50 slides (1.25 mm) down below the nutrient foramen in the distal femur. Two hundred slides (5 mm) were used for analysis. This part contains all 3 types of bones: cortical bone, trabecular bone, and medullary bone (after 17 wk), which is the optimal region to represent the bone quality.

**Table 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 |
| Bone mineral density (BMD) | 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 present study, BMD relates to the amount of bone within a mixed bone-soft tissue region |
| Bone mineral content (BMC) | 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 a 2D scale. In brief, a higher number means better connected trabecular structure. |

intraperitoneally again with calcein solution at the same dosage. Then, the pullets were euthanized 4 d after the second injection. Femurs were collected and preserved in 70% ethanol until analyzed. A thin slice of bone (0.5 mm) was taken from mid-diaphysis, and the bone slice was mounted on a glass slide. A fluorescence microscope with a blue filter (Olympus IX71; Olympus, Tokyo, Japan) was used to determine bone growth rate (**BGR**) by measuring the distance between the 2 calcein labels on the bones. Eight measurements were taken from each sample, and the average value was recorded for data analysis.

## *Micro-Computer Tomography (Micro-CT) Scanning*

The right femur was taken from one bird per cage (10 bones/treatment/time point) at 17, 60, and 95 wk. The soft tissue was removed completely. The samples were wrapped with cheesecloth soaked with phosphate buffered saline to keep the bones from drying. The bones were then stored at $-20°C$. Before the analysis, the samples were completed defrosted. A micro-CT scanner (Skyscan 1275; Bruker micro-CT, Belgium) was used for 3-dimensional image acquisition. The bone was held in a low-dense 50-mL tube; the extra cheesecloth was used for keeping the sample firmly inside the tube holder at a vertical orientation. The tube was then mounted on the scanning stage. Scanning settings are shown in Table 3. Before the scanning, the alignment test and flat field correction were performed according to the micro-CT manual (Bruker micro-CT, Belgium). The random movement and 180-degree scanning were applied. A 0.5-mm aluminum filter was used to reduce the beam hardening. After scanning, the pictures were
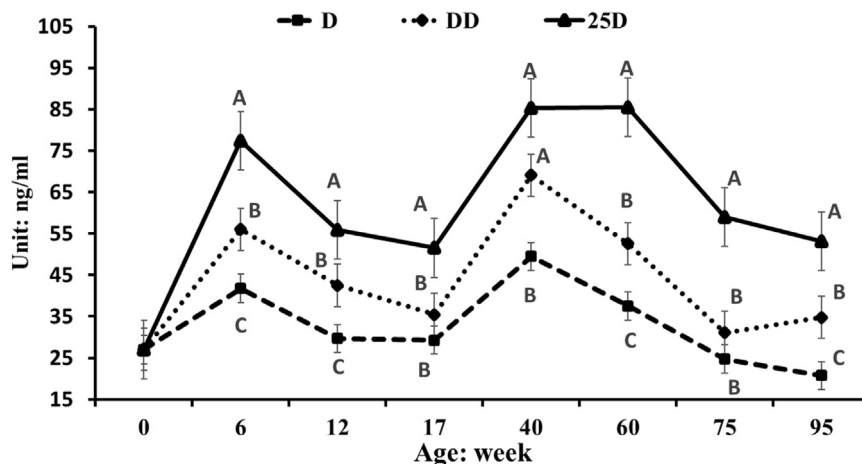


**Figure 2.** Serum 25OHD concentration throughout the whole experiment period measured by using MS/LC. D: vitamin $D_3$ at 2,760 IU/kg; DD treatment: vitamin $D_3$ at 5,220 IU/kg; 25D treatment: vitamin $D_3$ at 2,760 IU/kg plus 25OHD at 2,760 IU (69 μg)/kg. Values of means represent 1 bird per 10 replicate cages (n = 10 birds) per treatment. MS/LC, mass spectrometry/liquid chromatography.
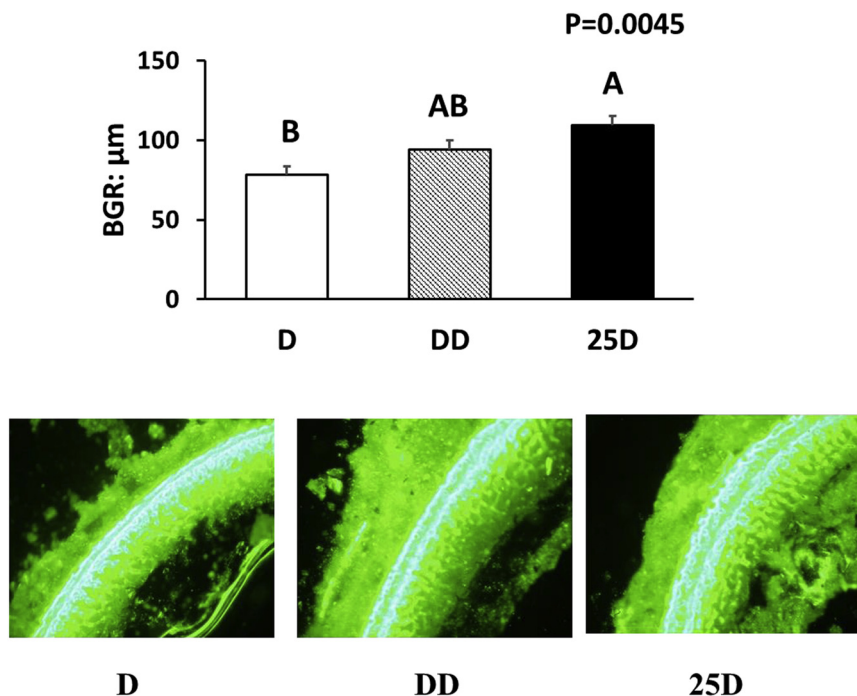
**Figure 3.** Effects of dietary supplementation of 25OHD on pullet bone growth rate (BGR) from 10–12 wk. Represented pictures were shown on the bottom. D: vitamin D$_3$ at 2,760 IU/kg; DD treatment: vitamin D$_3$ at 5,220 IU/kg; 25D treatment: vitamin D$_3$ at 2,760 IU/kg plus 25OHD at 2,760 IU (69 µg)/kg. Values of means represent 1 bird per 10 replicate cages (n = 10 birds) per treatment.

carefully screened, and the appropriate alignment and mathematical method 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 micro-CT, Belgium). The dynamic range for all the samples was set at 0–0.027. The volume of interest is shown in Figure 1. The 3D model underwent a custom process for bone separation. The separation process was designed based on the different density and morphology traits of each part of the bone. The 3D model was analyzed by using CTan (Version: 1.16.4.1; Bruker micro-CT, Belgium). The threshold was set at 90–255 (grayscale) for the bone samples at 17 wk and 100–255 (grayscale) for the bone samples at 60 and 95 wk samples; different threshold settings were used because of the difference in bone morphology between 17 vs. 60 and 95 wk. Two solid-state phantoms made by calcium hydroxyapatite were used for calibration. The outcome parameters in the present study are shown in Table 4.

### Dual-energy X-ray Absorptiometry

At 17, 60, and 95 wk, one bird per cage (10 birds/treatment/time point) was sacrificed for scanning by using a GE whole-body dual-energy X-ray absorptiometry (**DEXA**) scanner (GE Healthcare, Chicago, IL). The sample birds were euthanized by cervical dislocation and then positioned chest up on the scanner. The scanning mode was set for small animals. After scanning, the region of interest is set for individual bird. The data of whole-body bone density, mineral content, fat %, and lean muscle mass were collected.

### Statistical Analysis

All experimental data were analyzed statistically by one-way ANOVA with feed treatment as the main effect using SAS software version 9.4 (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

25OHD is the main circulation form of vitamin D$_3$ and a reliable indicator of vitamin D status in the body (Holick, 2007). The dietary supplementation of 25OHD could alter the 25OHD status in the bird because it skips the biological conversion of vitamin D3 to 25OHD in the liver. Thus, the 25OHD level in the serum was monitored in the present study. There was a fluctuation of 25OHD level in the serum throughout the experimental period. However, the treatment with 25D always had the highest 25OHD level in the serum compared to other treatments (Figure 2). At 40 wk, the serum 25OHD level reached a peak for all the treatments. After 40 wk, the serum 25OHD level gradually decreased, but the 25OHD treated birds maintained a high level of 25OHD in the serum throughout the whole experiment period.

### Bone Quality Analysis

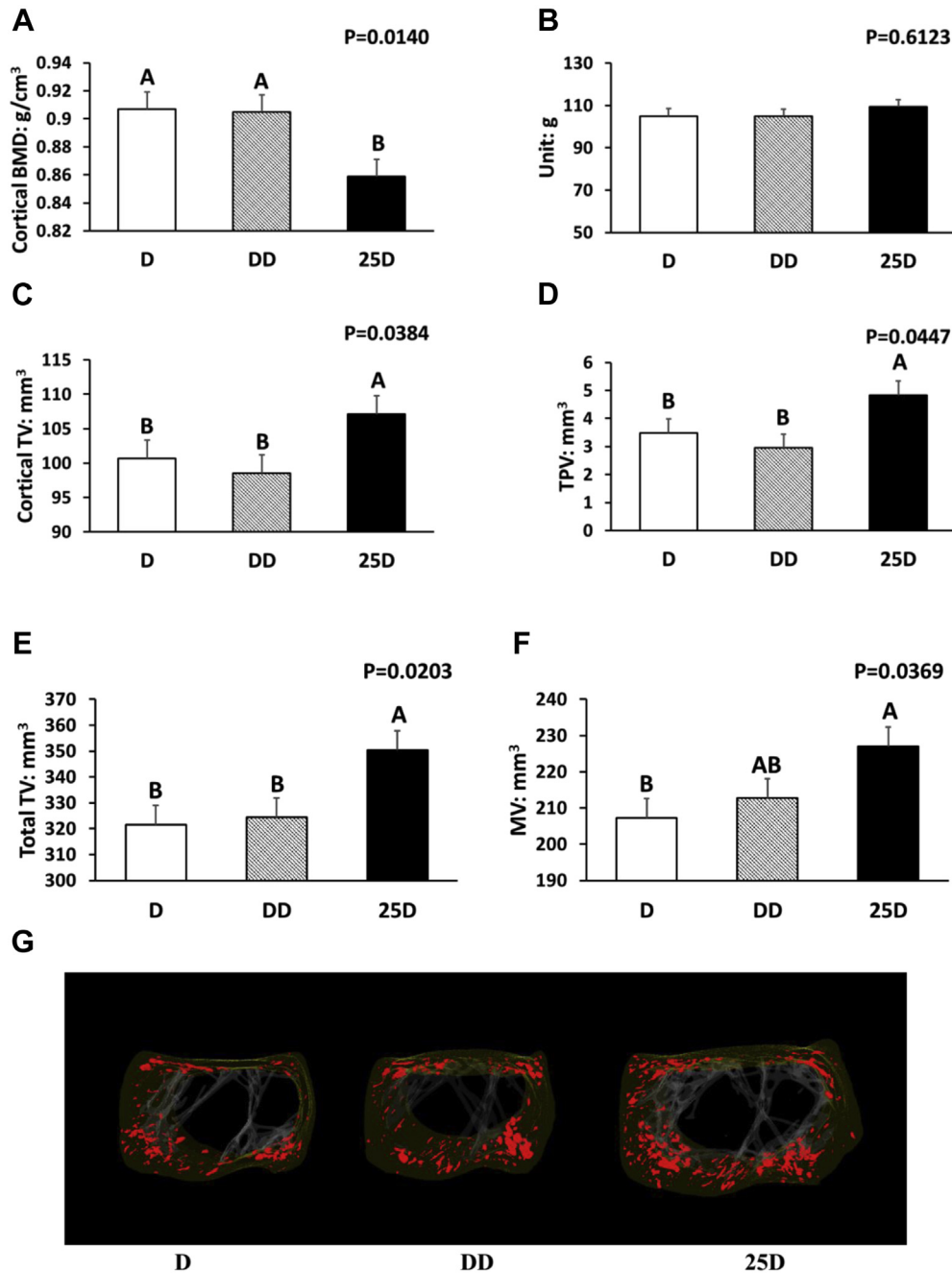During the pullet period, 25D treatment had a higher BGR than D treatment ($P < 0.05$) (Figure 3). However,

**Figure 4.** Effects of dietary supplementation of 25OHD on pullet bone development at 17 wk. 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 (69 μg)/kg. Values of means represent 1 bird per 10 replicate cages (n = 10 birds) per treatment. (A) Cortical bone bone mineral density (BMD); (B) cortical bone mineral content; (C) cortical bone volume; (D) cortical bone total volume of pore space; (E) total bone tissue volume (TV); (F) medullary volume; (G) pictures showed the cortical bone total volume of pore space from each treatment. The red color represents the pores. MV, medullary cavity volume; TPV, total pore volume.

no differences were detected from the whole body DEXA scanning by the end of the rearing period (17 wk, data not shown). From the femur micro-CT scanning results, at 17 wk, 25D treatment had a lower cortical bone mineral density (**BMD**; $P = 0.014$) than the others (Figure 4A), but no difference was observed in cortical bone mineral content (**BMC**; $P > 0.05$; Figure 4B). The segment analysis in each part of bones showed a lower BMD in cortical bone from the 25D treatment, which resulted from the increase of cortical bone volume ($P < 0.05$; Figure 4C) and cortical pores volume ($P < 0.05$; Figure 4D) in 25D treatment. Furthermore, 25D treatment had higher total bone tissue volume (TV; $P = 0.0203$) and medullary cavity volume ($P = 0.0369$) than D treatment (Figures 4E, 4F). The cortical bone image by micro-CT clearly showed that 25D made a larger bone with more pores inside of the cortical bone than the other treatments (Figure 4G). In summary, supplementation of 25OHD results in enlargement of the pullet bone structure, which may provide more space for mineral deposition and contribute to improve the bone quality during the laying period.

During the laying period, at 60 wk, 25D treatment still obtained the highest cortical TV ($P = 0.026$) and cortical bone volume (BV; $P = 0.0252$) (Figures 5A, 5B). At the
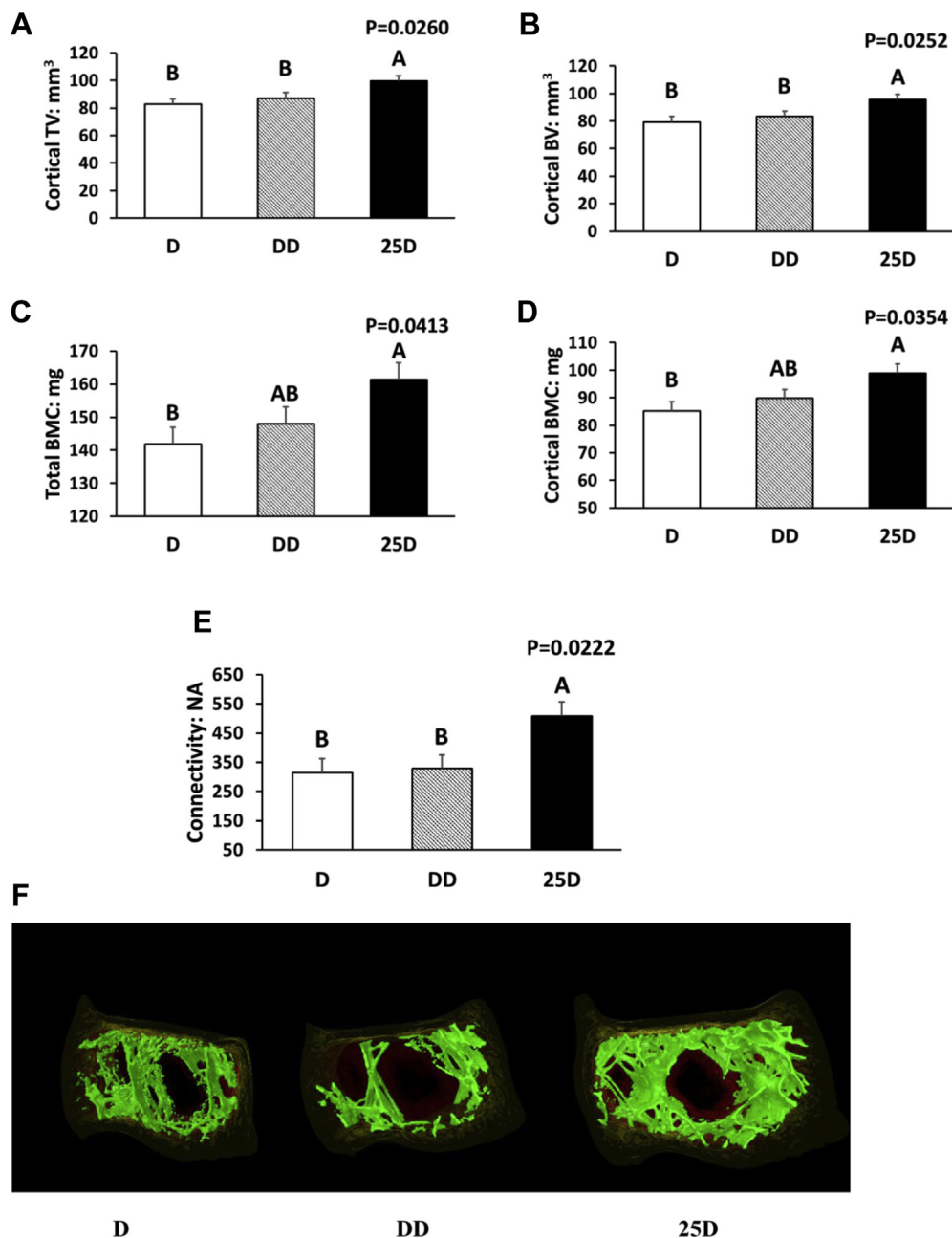
**Figure 5.** Effects of dietary supplementation of 25OHD on laying hen bone development at 60 wk. D: vitamin D$_3$ at 2,760 IU/kg; DD treatment: vitamin D$_3$ at 5,220 IU/kg; 25D treatment: vitamin D$_3$ at 2,760 IU/kg plus 25OHD at 2,760 IU (69 μg)/kg. Values of means represent 1 bird per 10 replicate cages (n = 10 birds) per treatment. (A) Cortical tissue volume (TV); (B) cortical bone volume (BV); (C) total bone mineral content (BMC), calculated by total bone mineral density (BMD)*total bone volume; (D) cortical BMC, calculated by cortical BMD*cortical tissue volume (TV); (E) trabecular bone connectivity; (F) the represented picture showed the trabecular bone structure from each treatment.

same time, there was no difference in cortical BMD and cortical porosity among the treatments at 60 wk ($P >$ 0.05; data were not shown). Instead, 25D treatment showed higher total BMC ($P = 0.0143$) and cortical BMC ($P = 0.0354$; Figures 5C, 5D). Furthermore, the trabecular bone structure analysis indicated higher trabecular connectivity in 25D ($P = 0.0222$) than in D or DD treatment (Figure 5E); moreover, the micro-CT image clearly showed that 25D had more connected trabecular bones inside of the bone marrow cavity (Figure 5F). The aforementioned data evidently indicated the larger bone structure from 25D treatment during the rearing

period (0–17 wk) allowed additional mineral deposition and consequently enhanced the bone quality during the later egg-laying period. The beneficiary effect of supplementation of 25OHD was also observed in improving trabecular structural quality.

During the late laying period, at 95 wk, 25D treatment still had 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 ($P = 0.0459$) than DD treatment (Figures 6A–6H). 25D treatment
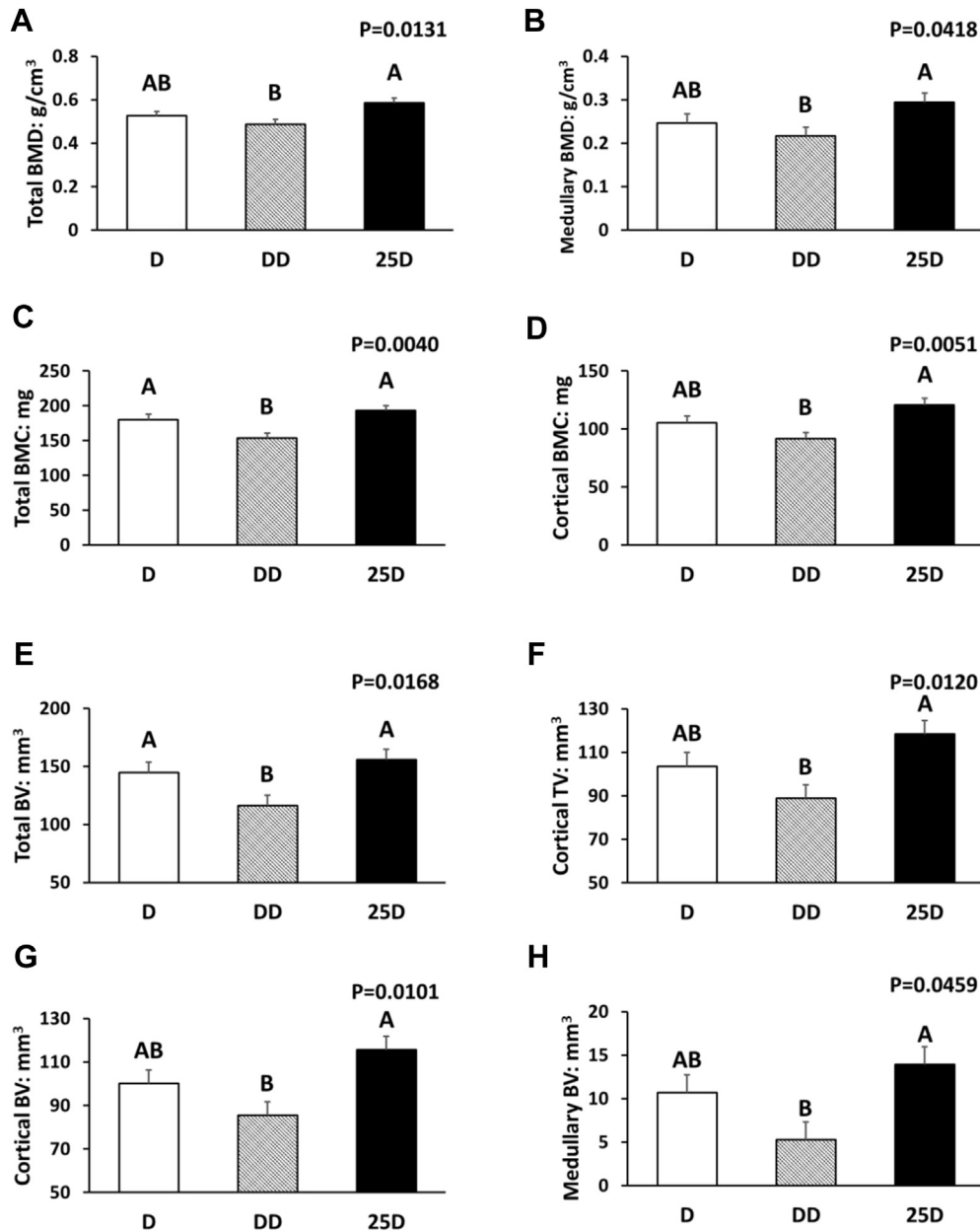
**Figure 6.** Effects of dietary supplementation of 25OHD on laying hen bone development at 95 wk. D: vitamin $D_3$ at 2,760 IU/kg; DD treatment: vitamin $D_3$ at 5,220 IU/kg; 25D treatment: vitamin $D_3$ at 2,760 IU/kg plus 25OHD at 2,760 IU (69 µg)/kg. Values of means represent 1 bird per 10 replicate cages (n = 10 birds) per treatment. (A) Total bone mineral density (BMD); (B) medullary BMD; (C) total bone mineral content (BMC), calculated by total BMD*total tissue volume (TV); (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 (CPV); (J) cortical bone closed porosity (CPP; 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 as there are no pores inside trabecular bones; (N) trabecular bone connectivity.

had the lowest closed pore volume ($P = 0.0429$) and closed porosity ($P = 0.0298$) but the highest pore number ($P = 0.0392$) in the cortical bone (Figures 6I–6K), which indicated the mineral deposition process continuously happened in the cortical bone area and filled up the space that were created during the rearing period. However, a lower trabecular BMC ($P = 0.0009$), trabecular BV ($P = 0.0010$), and connectivity ($P = 0.0003$) were detected than D treatment (Figures 6L–6N). The aforementioned data showed the expansion of volume and mineral deposition in 25OHD

treatment mainly occurred in the cortical bone region at 95 wk. However, an increase of trabecular bone resorption was found during this period in the 25D treatment.

The whole-body bone quality in each treatment during various periods was similar to the 3D analysis on femurs. No difference was observed in whole-bird BMD and BMC measured by DEXA at 17 wk (data not shown). However, the 25D group had higher whole-body BMD and BMC than D or DD treatment at 60 and 95 wk, respectively ($P < 0.05$; Figures 7A–7D).
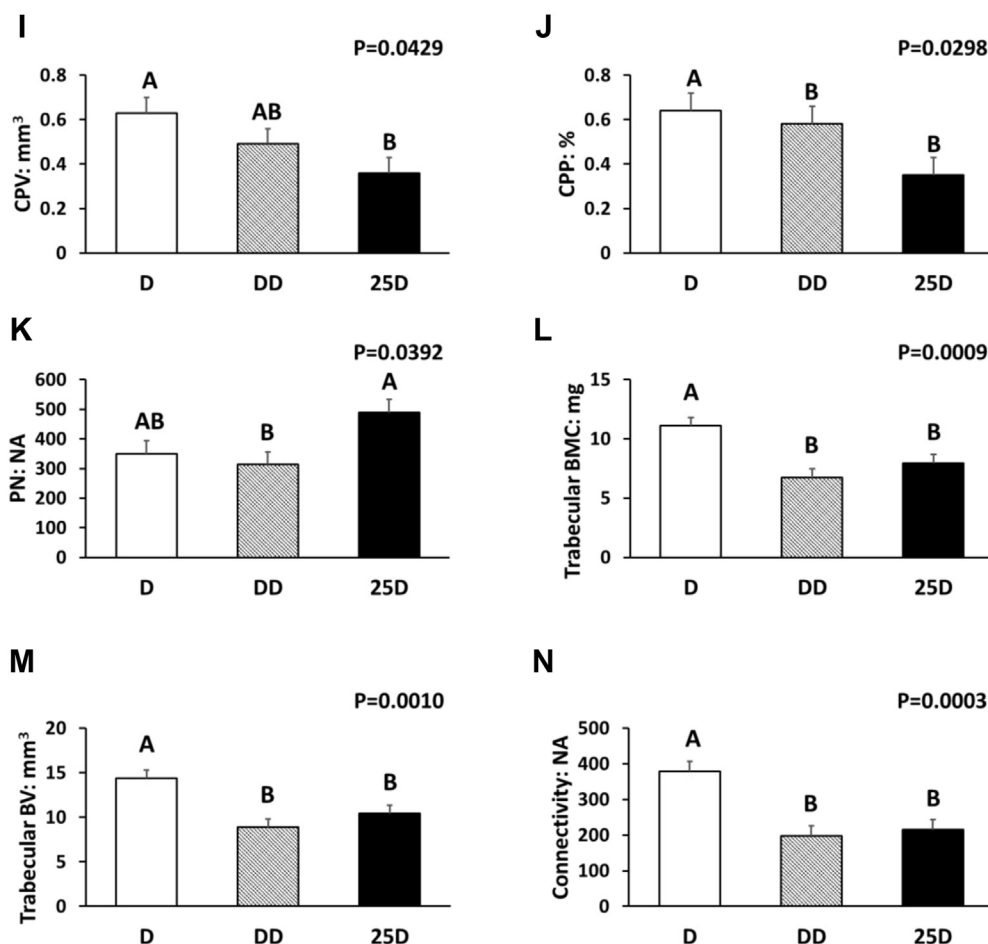
**Figure 6.** Continued

## DISCUSSION

Vitamin D$_3$ is converted in the liver to become its primary circulating form of 25OHD. However, the efficiency of biological conversion is limited, especially in old laying hens (Bar and Hurwitz, 1987). 25OHD is more active than vitamin D$_3$ as it bypasses the first biotransformation. The serum 25OHD results demonstrated that dietary supplementation of 25OHD could significantly increase the circulating 25OHD in the birds, suggesting that dietary supplementation of 25OHD is more efficient to provide 25OHD in the circulation than the same or even higher amount of regular vitamin D supplementation. Similar results were found previously in laying hen (Käppeli et al., 2011; Wang et al., 2020). A higher serum 25-OHD is advantageous for optimal calcium and skeletal homeostasis, immune system modulation, muscle cell differentiation, and growth performance (Wang et al.,2020).

In the present study, we were able to observe the long-term supplementation of 25OHD on layer bone 3-dimensional structural changes using the micro-CT 3D scanning and automatic bone separation process. Several studies addressed the importance of early bone development before sexual maturity and its prolonged effects on bone health during laying periods (Hester et al., 2013; Regmi et al., 2015; Casey-Trott et al.,

2017). Human studies also emphasized the importance of bone healthy during the growing period and its subsequent benefits during adulthood (Bailey et al., 1999). The laying hen bone development during the rearing period is similar to mammals' bone development. It proceeds through 2 primary mechanisms—intramembranous and endochondral ossifications—but with a faster rate than mammals (Whitehead, 2004). In the present study, the principal function of 25OHD in early bone development was increasing bone structural size. However, the expansion of bone size with the same amount of BMC results in a low-density bone. A low BMD is usually associated with a high risk of bone fracture (Ammann and Rizzoli, 2003). However, this trade-off between BMD and structure size is probably more favorable, while taking into account the bone structural development ceased at the onset of sexual maturity (Whitehead, 2004). The impact of the larger bone size (width) on bone health during the laying period has been shown as increasing breaking strength because of the broader bones correlated with higher bending force (Rauch, 2007). The benefits of exercise in laying hen also showed a similar pattern. During the rearing period, exercise increased the tibia cross-sectional area but reduced cortical BMD in tibia (Casey-Trott et al., 2017). However, this low BMD could be minimized potentially by manipulating calcium and phosphate
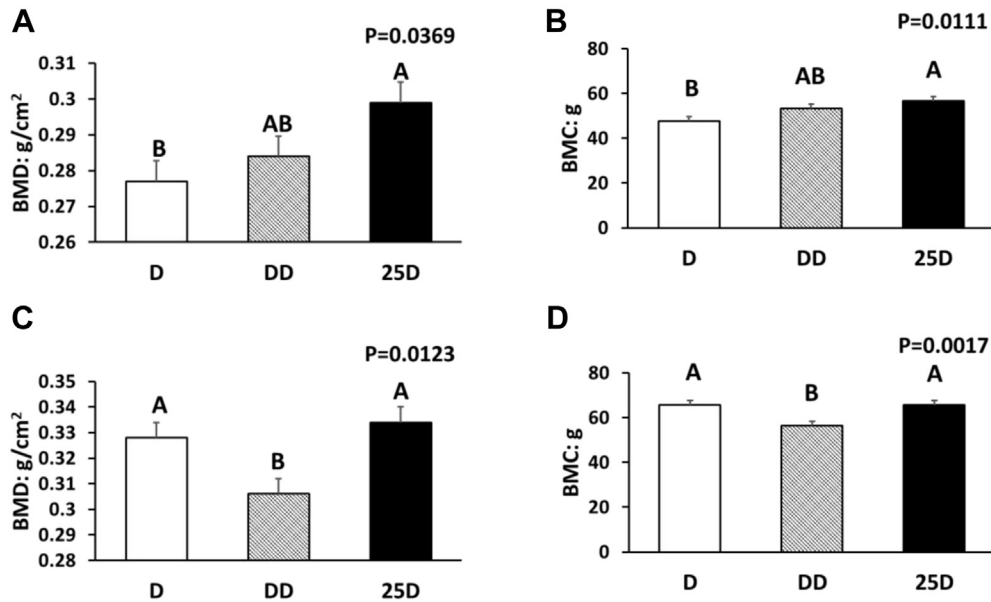
**Figure 7.** Effects of dietary supplementation of 25OHD on layer bone development by using dual-energy x-ray absorptiometry method (DEXA). D: vitamin $D_3$ at 2,760 IU/kg; DD treatment: vitamin $D_3$ at 5,220 IU/kg; 25D treatment: vitamin $D_3$ at 2,760 IU/kg plus 25OHD at 2,760 IU (69 μg)/kg. Values of means represent 1 bird per 10 replicate cages (n = 10 birds) per treatment. (A) Whole-body bone mineral density (BMD) at 60 wk; (B) whole-body BMD at 60 wk; (C) whole-body BMD at 95 wk; (D) total body bone mineral content (BMC) at 95 wk.

content and calcium particle size in the diets. But this hypothesis needs to be further studied.

During the laying period, 25D treatment still possessed a higher bone volume than the other treatments in the present study. Along with mineral deposition, the pores at cortical bones were filling up and led to no difference in pores volume between the treatments. The larger bone volume allowed more space for mineral deposition. BMC and BMD were also elevated in 25D treatment, which may be associated with the positive effects of 25OHD on calcium absorption and reabsorption in the intestine and kidneys (Zhao and Nemere, 2002; Bar, 2008).

In the present study, higher connectivity of trabecular bone in 25D treatment was found at 60 wk. The past research on evaluation of chicken bone was mostly based on bone ash, breaking strength, or DEXA (Hester et al., 2004; Castro et al., 2018; Adhikari et al., 2020). These

methods are mainly based on the results of planar morphology or bone mass. Although the bone quantity and density are important factors for bone strength (Hester et al., 2004), these parameters do not consider the trabecular architectural changes that are 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 on the bone strain (Reich and Gefen, 2006), suggesting that the integrity of trabecular bone is critical for bone resistance to the force. The increase of trabecular bone connectivity in 25D treatment indicated the higher fracture resistance elevated by dietary supplementation of 25OHD.

The loss of the structural bones (trabecular and cortical bones) during the laying period is associated with medullary bone modeling and remodeling and the pressure of daily eggshell formation (Cransberg et al.,
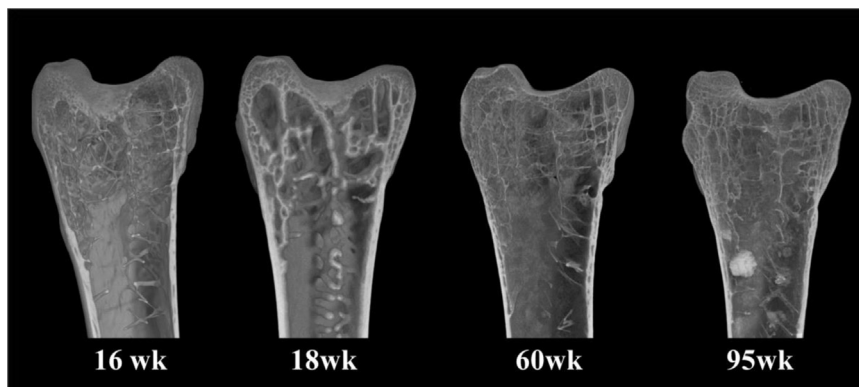


**Figure 8.** The picture showed the structure of cortical, trabecular, and medullary bones at 16, 18, 60, and 95 wk. Compared to 17 wk, the structure bone progressively lost, especially the trabecular bone became very thin during the late laying period. At the same time, the medullary bone formed calcium chucks inside the bone cavity.

2001; Fleming et al., 2006; Kim et al., 2007). Meanwhile, a trade-off between bone quality and laying performance has been reported in many previous studies (Rennie et al., 1997; Bishop et al., 2000; Cransberg et al., 2001; Kim et al., 2012). Maintaining structure bone during a 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 present study, interesting changes were observed at the late laying stage (95 wk). The mineral deposition continuously increased under 25D treatment, which showed an increase of mineral deposition in cortical bone and medullary bone with a trade-off at the trabecular bone. In a rat study, vitamin D regulated bone resorption through controlling osteoprotegerin secretion by osteoblasts (Baldock et al., 2006); however, the trabecular bone did not undergo this inhibition process. It may partly explain the reason why more trabecular bones were resorbed under 25D treatment. Furthermore, previous research had shown that the bone resorption has a preference for resorbing smaller mineral particles (Kerschnitzki et al., 2014). The trabecular bone became very thin resulting from the continuous bone resorption process throughout the laying period (Whitehead, 2004). Meanwhile, the medullary bone clustered together to form a number of larger calcium chucks compared to the trabecular bone (Figure 8). These morphological characteristics may render trabecular bone more sensitive to the bone resorption, and the loss of trabecular bone may diminish bone strength. However, the increase in cortical bone at this period could balance off this adverse effect.

In summary, long-term supplementation of 25OHD increased the circulating 25OHD concentration. The dietary 25OHD in pullets and laying hens showed beneficial effects on bone development and integrity throughout the rearing and laying periods. 25OHD increased BGR and bone size during the pullet period, which provided more space for mineral deposition during the later period. During the early laying period (18–60 wk), 25OHD supplementation had positive effects on bone mineral deposition and improvement of structural bone quality. During the late stage of laying (95 wk), 25OHD enhanced the mineral deposition in the cortical bone region while increasing the resorption of trabecular bones. This study suggests that early supplementation of 25OHD has prolonged beneficial effects on the layer bone health, and 25OHD is a promising feed additive to stimulate bone growth and improve structural integrity in laying hens to prevent avian osteoporosis and contribute to the practice of extending the laying periods without molting.

## ACKNOWLEDGMENTS

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