ANIMAL WELL-BEING AND BEHAVIOR

Effect of dietary 25-hydroxycholecalciferol on the sternal mass of meat ducks under different vitamin regimens

H. Y. Zhang,* Q. F. Zeng,* S. P. Bai,* J. P. Wang,* X. M. Ding,* Y. Xuan,* Z. W. Su,* G. S. Fraley,† B. Yao,‡ and K. Y. Zhang*,1

*Institute of Animal Nutrition, Key Laboratory for Animal Disease-Resistance Nutrition of China, Ministry of Education, Sichuan Agricultural University, Chengdu, Sichuan 611130, China; †Department of Biology, Hope College, Holland, MI 49423, USA; and ‡DSM (China) Ltd., Shanghai 201203, China

ABSTRACT Genetic selection and intensive nutrition for increased growth rate in meat-type ducks has resulted in an imbalance between pectorales increment and sternal mass, which is detrimental to productivity and welfare. Reducing body weight and increasing sternal mass probably reverses these adverse effects. Therefore, 2 experiments (Expt.) were conducted to investigate the effects of 25-hydroxycholecalciferol (25-OH-D3), a vitamin D3 metabolite, on sternal mass. In Expt. 1, 512 1-day-old male ducks were randomly assigned to 4 low-nutrient density diets and received the following treatments in a 2 × 2 factorial arrangement: (i) NRC or China Agricultural industry standards (NY/T) vitamin premixes and (ii) 0.069 mg/kg 25-HyD in feed or not. At 49 D of age, regardless of 25-OH-D3, NY/T vitamin regimen inhibited bone turnover and consequently increased sternal trabecular bone volume and mineral deposition compared with NRC vitamin premix. Supplementing 25-OH-D3 to NRC but not NY/T vitamin regimen significantly improved sternal microarchitecture and mineral content, which accompanied by decreased serum bone resorption markers concentration, as well as downregulation of the gene expressions of osteoclast differentiation and activity. In Expt. 2, 256 1-day-old male ducks were fed a standard nutrient density diet contained NRC vitamin premix with 0 or 0.069 mg/kg of 25-OH-D3. Results also showed that 25-OH-D3 treatment significantly improved sternal mineral accumulation and microarchitecture, along with decreasing osteoblast and osteoclast numbers in bone surface, declining serum bone turnover markers levels, and increasing serum Ca concentration. Collectively, these findings indicated that the dietary administration of 25-OH-D3 increased sternal mass in NRC vitamin diet by suppressing bone resorption in 49-day-old meat duck.

Key words: 25-hydroxycholecalciferol, vitamin, sternal mass, meat duck

INTRODUCTION

Increases in growth rate and breast muscle mass through selective breeding and nutrient strategy in meat-type poultry has been accompanied by welfare problems. In addition to an increased incidence of poor gait (Duggan et al., 2015), respiratory problems were also noticed in birds, such as pulmonary diseases (Iyer and Rao, 1971) and ascites (Julian, 1988). In the avian respiratory system, the sternum is considered as primary ventilator (Claessens, 2009), which influences air sac volume, thereby is facilitating a unidirectional flow of air through the lung (Tickle et al., 2007). The rapid musculoskeletal development inadvertently puts stress on sternum and potentially outgrows pulmonary capacity and increases the occurrence rate of these diseases (Julian, 1998; Wideman, 2001). Based on the above phenomenon, slowing weight gain combined with promoting sternal mass might be able to reverse these adverse effects and improve bird’s health.

Vitamin D was originally discovered as an antirachitic agent capable of preventing a failure of bone mineralization. Vitamin D3 deficiency results a higher incidence of leg problems in birds (Khan et al., 2010). Supplementing vitamin D3 improved the walking ability and bone quality characteristics and consequently decreased the leg diseases in broilers (Sun et al., 2013; Jiang et al., 2015). A current view is that the beneficial effects of vitamin D3 on bone mineral density and reduction of
bone fracture incidence are caused by the suppression of bone resorption (Richy et al., 2005) and/or promoting bone formation (Turner et al., 2014; van der Meijden et al., 2014). Paradoxically, vitamin D3 has been also shown to enhance bone resorption in vitro and in vivo (Sato et al., 2007; Kogawa et al., 2010). Variations in vitamin D3 and is found to significantly promote tibia mineralization of broiler (Wideman et al., 2015; Santiago et al., 2016), and it was approximately twice as active as cholecalciferol in promoting bone strength in broilers (Han et al., 2016). However, Ren et al. (2017) found the positive impacts of maternal canthaxanthin, and 25-OH-D3 supplementation on growth performance and serum phosphorus (P) of ducklings only were observed in a low but not a high vitamin regimen, which has higher levels of all vitamins except nicotinic acid than the low vitamin regimen probably because of different doses of vitamin D or interaction among vitamins (Bonjour et al., 2018). There are various recommendations of vitamin premix in the duck industry, and the variations of each vitamin content between each other is wide, especially NRC (NRC, 1994) and China Agricultural Industry Standards (NY/T, 2012). Accordingly, this is reasonable to assume that the biological effect of 25-OH-D3 on sternal mass may lie on dietary vitamin regimen.

The rapid body gain in commercial domestic birds seems to be an important factor for the detrimental effects on bone. Accelerating weight gain via increasing dietary nutrient density resulted in a higher incidence of gait abnormality (Brickett et al., 2007), and the intensive nutrition in meat birds has been suggested as main cause for the inadequate bone quality (Williams et al., 2004). Feed withdrawal or reducing the nutrient density of diets played a critical role in alleviation of the gait abnormality of broiler (Brickett et al., 2007). Our previous study also showed that a low nutrient density (LND) diet decreased weight gain and promoted trabecular thickness (Tb.Th) via suppressing bone turnover in meat duck, both relieved the burden acted on the bone and improved the animal welfare (Zhang et al., 2018).

### MATERIALS AND METHODS

Care, handling, and sampling procedures were approved by the Animal Care and Use Committee of Sichuan Agricultural University before initiation of the trial. Cherry Valley meat male ducks were reared in Table 1. Composition of the vitamin premixes for meat duck.

| Item                  | NRC (IU) | NY/T (IU) | NRC (IU) | NY/T (IU) |
|-----------------------|----------|-----------|----------|-----------|
| A (IU)                | 2,500    | 4,000     | 2,500    | 3,000     |
| D3 (IU)               | 400      | 2,000     | 400      | 2,000     |
| E (IU)                | 10       | 20        | 10       | 20        |
| K3 (mg)               | 0.5      | 2         | 0.5      | 2         |
| B1 (mg)               | 1.8      | 2         | 1.8      | 1.5       |
| B2 (mg)               | 4        | 10        | 4        | 10        |
| B6 (mg)               | 2.5      | 4         | 2.5      | 3         |
| B12 (mg)              | 0.01     | 0.02      | 0.01     | 0.02      |
| Niacin (mg)           | 55       | 50        | 55       |           |
| Pantothentic Acid (mg)| 11       | 20        | 11       | 10        |
| Biotin (mg)           | 0.15     | 0.15      | 0.15     | 0.15      |
| Folic acid (mg)       | 0.55     | 1         | 0.55     | 1         |

1Supplied in per kilogram of diet.
2The vitamin levels recommended by the NRC (NRC, 1994).
3The vitamin levels recommended by the China Agricultural Industry Standards (NY/T, 2012).

Table 2. Composition and nutrient levels in the basal diets (dry matter basis).

| Ingredients and analysis | 1–14 D | 15–35 D | 36–56 D | 56–66 D |
|-------------------------|--------|---------|---------|---------|
| Ingredients, %          |        |         |         |         |
| Maize                   | 58.15  | 62.15   | 62.71   | 56.61   |
| Soya oil                | 3      | 3       | 4.2     | 0       |
| Soybean meal            | 34.8   | 23.47   | 17.01   | 0       |
| DDGS                    | 0      | 3       | 4       | 4       |
| Wheat bran              | 0      | 2       | 4       | 26.6    |
| Rapeseed meal           | 2.5    | 4.5     | 8.61    |         |
| L-Lysine                | 0.08   | 0       | 0       | 0.2     |
| DL-Methionine           | 0.145  | 0.13    | 0.09    | 0.1     |
| L-Threonine             | 0.03   | 0       | 0       | 0.07    |
| L-Tryptophan            | 0      | 0       | 0.12    |         |
| Choline chloride        | 0.2    | 0.2     | 0.2     |         |
| Sodium chloride         | 0.3    | 0.3     | 0.3     | 0.3     |
| Mineral premix          | 0.2    | 0.2     | 0.2     | 0.2     |
| Vitamin premix          | 0.03   | 0.03    | 0.03    | 0.03    |
| Limestone               | 1.06   | 1.04    | 1.1     | 1       |
| Dicalcium phosphate     | 1.75   | 1.6     | 1.25    | 1       |
| Bentonite               | 0.55   | 0.07    | 0.11    | 0.96    |
| Total                   | 100    | 100     | 100     | 100     |
| Calculated nutrient analysis, % | | | | |
| AME (MJ kg⁻¹)           | 12.14  | 12.14   | 12.35   | 10.25   |
| CP                      | 20     | 17.5    | 16      | 13.28   |
| ME/CP                   | 145    | 166     | 184     | 184     |
| Dig Lys, P%             | 1.02   | 0.76    | 0.65    | 0.53    |
| Dig Met, P%             | 0.42   | 0.38    | 0.32    | 0.27    |
| Calcium                 | 0.9    | 0.85    | 0.8     | 0.71    |
| Total-phytate P         | 0.65   | 0.65    | 0.61    | 0.76    |
| Nonphytic acid P        | 0.42   | 0.4     | 0.35    | 0.35    |

Abbreviations: AME, apparent metabolizable energy; CP, crude protein; DDGS, distillers dried grains with solubles; Dig, digestibility; LND, low nutrient density; PC, standard nutrient density positive control.
1Provided per kilogram of diet: Cu (CuSO₄·5H₂O), 8 mg; Fe (FeSO₄·7H₂O), 80 mg; Mn (MnSO₄·H₂O), 70 mg; Se (NaSeO₃), 0.3 mg; I (KI), 0.4 mg.
2Provided per kilogram of diet: NRC or NY/T vitamin premixes as shown in Table 1.
cages (2.2 × 1.2 × 0.9 m) in a temperature-controlled and humidity-controlled room and had free access to feed and water throughout the experimental period. Diets were provided in pellet form. All vitamins used in this trial were provided by DSM Ltd. (Shanghai, China).

**Experimental Design and Animal Management**

There are 2 experiments (Expt.) in this study. In Expt. 1, a total of 512 one-day-old ducks were equally divided into 4 LND diets groups as follows (8 replicate pens; 16 ducks/pen) in a 2 × 2 factorial arrangement: 2 different vitamin regimens from NRC (1994) or NY/T (2012) with 0.069 mg/kg 25-OH-D3 in feed or not. The vitamin composition was displayed in Table 1. Ducklings were all fed a normal nutrient density starter diet until 14 D, and subsequently, they were subjected to an LND diet until 56 D. The starter diet was formulated based on NY/T (2012). The LND diet was designed according to our previous report (Zhang et al., 2018), which is with constant t ratios of CP and essential nutrients, such as limiting amino acids relative to metabolic energy compared with positive control (PC) diet (36–49 D) (Table 2). During the whole rearing period, BW by pen and feed intake (FI) were recorded weekly. Feed conversion was calculated as the feed to gain (F:G) ratio. At 42, 49, and 56 D of age, they were fasted for 12 h, and one bird in each pen was selected based on the average BW of each cage, and whole sternum was removed for fresh weight, morphometry, and mineralization property analysis. In addition, at 49 D of age, another bird in each pen was selected, and the blood was collected via jugular vein, also the serum was obtained. Sternal samples (0.5 × 0.5 cm) that located above the fontanelle and closest to the keel were dissected and put in liquid nitrogen and in phosphate-buffered formaldehyde immediately for gene expression and bone histomorphometry analysis, respectively.

Expt. 2 was conducted under a standard nutrient density PC diet to verify the preferential effect of 25-HyD in NRC vitamin premix diet on the sternal mass of meat ducks. 256 one-day-old ducks were equally divided into 2 NRC vitamin diets with or without 0.069 mg/kg of 25-OH-D3 (8 replicate pens; 16 ducks/pen). The ducks were subjected to a 3-period feeding program consisting of starting (0–14 D), growing (15–35 D), and finishing (36–49 D) periods. The diets were formulated to meet all nutrient requirements of meat ducks to NY/T (2012) except vitamin (Table 2). At 49 D of age, BW was recorded per pen basis, then followed by 12 h fasting period, and 2 ducks with similar BW in each pen were selected, and whole sterna were removed from one bird for fresh weight, morphometry, and mineralization property analysis. Serum and sternal samples were also obtained as the same process as the Expt. 1 for bone metabolism analysis.

**Sternum Morphometry**

For estimating the morphometric change of sternum, 5 parameters including the distance between the 2 coracoids of the sternum, sternum central distance, posterior process distance, sternum length, and sternum depth were obtained from each sternum. Then, the sternum was cut open longitudinally along the keel, and the relative proportion of the sternum cartilage was measured using the method described by Zhang et al. (2019). All measurements were straight-line distances.

**Sternum Mineralization Analysis**

The fat-free weight and density of sternum were evaluated as previously described (Zhang et al., 2017), subsequently sternum was ashed in a muffle furnace at

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**Table 3. The primers for quantitative real-time PCR.**

| Gene   | Gene ID     | Primer       | Sequence (5'-3')   | Size (bp) |
|--------|-------------|--------------|--------------------|-----------|
| Phex   | XM_005010837.3 | Reverse      | tgcaactaatctggtgtgga | 99        |
|        |             | Forward      | ceggtagatcacecgagaaaa | 92       |
| Dmp1   | XM_005012780.3 | Reverse      | aaectctgaacttcatcagttc | 115      |
|        |             | Forward      | tggcaaatgtcgtgctgctta | 98       |
| Sclerostin | XM_005026106.3 | Reverse      | ggaagggtgggaaggtttta | 111      |
|        |             | Forward      | tgcctgtcatttggttggtagctgttc | 106      |
| Cathepsin K | XM_021277116.1 | Reverse     | ggaagggtgggaaggtttta | 100      |
|        |             | Forward      | tgcctgtcatttggttggtagctgttc | 105      |
| V-ATPase | XM_021267166.1 | Reverse     | ggaagggtgggaaggtttta | 94       |
|        |             | Forward      | tgcctgtcatttggttggtagctgttc | 105      |
| OPG    | XM_005017709.3 | Reverse      | ggaagggtgggaaggtttta | 100      |
|        |             | Forward      | gcaatcgtgacctcataatata | 105      |
| RANKL  | XM_021276016.1 | Reverse     | ggaagggtgggaaggtttta | 94       |
|        |             | Forward      | gcaatcgtgacctcataatata | 105      |
| β-actin | NM_001310408.1 | Reverse    | ggaagggtgggaaggtttta | 100      |
|        |             | Forward      | gcaatcgtgacctcataatata | 105      |
| GAPDH  | XM_005016745.3 | Reverse     | ggaagggtgggaaggtttta | 94       |
|        |             | Forward      | gcaatcgtgacctcataatata | 105      |

Abbreviations: Dmp1, dentin matrix protein 1; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; OPG, osteoprotegerin; Phex, phosphate regulating endopeptidase homolog x-linked; RANKL, receptor activator of nuclear factor-κB ligand; V-ATPase, Vacuolar-type H^+/ATPase.
Table 4. Effects of 25-OH-D3 and vitamin regimen on performance for meat duck1.

| Item               | 14 D  | 35 D  | 42 D  | 49 D  | 56 D  | 1 D  |
|--------------------|-------|-------|-------|-------|-------|------|
|                   | Body weight, g/duck | Gain, g/duck | Feed intake, g/duck | Feed: Gain, g:g |
| Vitamin regimen    | NRC   | NY/T  | NRC   | NY/T  | NRC   | NY/T |
|                    |       |       |       |       |       |      |
| 25-OH-D3           |       |       |       |       |       |      |
|                   | 641.99 | 669.95 | 661.56 | 672.73 | 639.30 | 672.73 |
|                   | 256.86 | 213.35 | 241.83 | 231.35 | 256.09 | 213.35 |
|                   | 194.90 | 194.90 | 194.90 | 194.90 | 194.90 | 194.90 |
|                   | 10.2  | 57.71  | 57.71  | 57.71  | 57.71  | 57.71 |
| SEM                | 10.2  | 10.2  | 10.2  | 10.2  | 10.2  | 10.2 |

Table 4. Continued...

| Item               | 14 D  | 35 D  | 42 D  | 49 D  | 56 D  | 1 D  |
|--------------------|-------|-------|-------|-------|-------|------|
|                   |       |       |       |       |       |      |
| 25-OH-D3           |       |       |       |       |       |      |
|                   | 641.99 | 669.95 | 661.56 | 672.73 | 639.30 | 672.73 |
|                   | 256.86 | 213.35 | 241.83 | 231.35 | 256.09 | 213.35 |
|                   | 194.90 | 194.90 | 194.90 | 194.90 | 194.90 | 194.90 |
|                   | 10.2  | 57.71  | 57.71  | 57.71  | 57.71  | 57.71 |
| SEM                | 10.2  | 10.2  | 10.2  | 10.2  | 10.2  | 10.2 |

Vitamin regimen 25-OH-D3

NRC: 649.18, 2084.08, 2681.09, 3125.83, 3567.31, 600.67, 2032.79, 693.54, 738.35, 6022.49, 3855.90, 1.23, 2.97, 5.59

NY/T: 662.86, 2198.02, 2844.48, 3296.59, 3547.14, 614.36, 2172.44, 686.73, 747.81, 6019.26, 3751.50, 1.22, 2.89, 5.58

SEM: 7.17, 28.08, 38.56, 41.41, 46.78, 7.41, 38.89, 20.95, 11.96, 137.82, 110.49, 0.01, 0.05, 0.13

P-value

Vitamin regimen: 0.208, 0.008, 0.006, 0.007, 0.010, 0.207, 0.071, 0.811, 0.583, 0.078, 0.534, 0.598, 0.194, 0.907

25-OH-D3: 0.004, 0.289, 0.479, 0.401, 0.468, 0.004, 0.996, 0.832, 0.578, 0.048, 0.104, 0.203, 0.339, 0.258

Interaction: 0.425, 0.774, 0.908, 0.886, 0.906, 0.442, 0.897, 0.772, 0.951, 0.064, 0.287, 0.329, 0.038, 0.219

Harvested sternum samples were fixed, embedded, and sliced. For microarchitecture, the sections were stained with toluidine blue, the micrographs of the bone sections were taken using a microscope (Nikon Eclipse TS100; Nikon Corporation, Tokyo, Japan), and an image analyzer (Image Pro-Plus, Rockville, MD) at a magnification of 20X. Bone static histomorphometry parameters were performed by a blinded examiner using Weibel Grid technique, which includes the trabecular bone volume/tissue volume (BV/TV), trabecular number (Tb.N), Tb.Th, and spacing (Tb.Sp). For osteoblasts or osteoclasts detection, the sections were stained with alkaline phosphatase (ALP) or tartrate-resistant acid phosphatase (TRAP) detecting kit (Sigma-Aldrich, St. Louis, MO), respectively. The ALP positive staining represents osteoblasts, and TRAP-positive staining represents osteoclasts. Osteoblast and osteoclast number per bone surface (N.Ob/BS and N.Oc/BS) were counted on the external surfaces of the bone using the surgical defect as the field of view. A single operator at 2 separate time points performed the quantification using Image Pro-Plus.

Serum Biochemistry

Serum Ca and P concentrations were determined with Biochemistry Analyzer (Yellow Springs Instrument Co. Inc., Yellow Springs, OH). Serum parathyroid hormone (PTH) and 25-hydroxyvitamin D (25-OH-D) concentration were assayed using commercial ELISA kit (ALPCO Diagnostics, NH) according to the manufacturers’ recommendations. For 25-OH-D, samples and pretreatment reagent were combined to release the bound 25(OH)D from vitamin D binding protein. The mixtures are transferred to microplate wells and the ALP positive staining represents osteoblast, and TRAP-positive staining represents osteoclasts. Osteoblast and osteoclast number per bone surface (N.Ob/BS and N.Oc/BS) were counted on the external surfaces of the bone using the surgical defect as the field of view. A single operator at 2 separate time points performed the quantification using Image Pro-Plus.
Gene Expression Assays

mRNA levels of osteocyte-specific, osteoblast-specific, and osteoclast-specific marker genes were determined by quantitative RT-PCR. The frozen sternal samples were subjected to total RNA isolation, and the RNA quality (intact ribosomal RNA 28s/18s) was evaluated by agarose gel electrophoresis. The cDNA was synthesized from 200 ng of total RNA by using the PrimeScript RT Reagent Kit (Takara, Kusatsu, Japan). The RT-PCR analysis was performed on the ABI 7500 detection system (Applied Biosystems, Foster City, CA), and target cDNA was amplified by 40 cycles (1 cycle: 95°C for 5 s, 60°C for 34 s), and the melting curve analysis was performed at the end. The primers were designed using Primer 3 and are shown in Table 3. All reactions were run in duplicate, and a standard curve was generated to estimated reaction efficiency (slope) and genes expression. Glyceraldehyde-3-phosphate dehydrogenase and β-actin were selected as the reference genes, and a normalization factor was obtained by calculating the geometric mean of the values of the selected reference genes, which was subsequently used to normalize the relative amounts of RNAs of interest (Vandesompele et al., 2002).

Statistical Analysis

All data were expressed as the means and standard deviation (n = 8). Two-way ANOVA followed by Tukey’s test (SAS 9.2) were used for analyses of dietary vitamin regimen, 25-HYD level, and their interaction in LND diets. One-way ANOVA was used to compare the effect of 25-OH-D3 on sterna mass in the PC diets with NRC vitamin regimen. Statistical significance was detected at P < 0.05.

RESULTS

Effects on BW and Sternal Characteristics

As shown in Table 4, the addition of 25-OH-D3 increased (P < 0.05) the BW (14 D) and the weight gain (1–14 D). Dietary vitamin regimen significantly increased (P < 0.05) the BW of birds at 35, 42, 49, and 56 D of age, but did not change the gain, FI, and F:G in the whole period. The interaction of vitamin regimen and 25-OH-D3 did not notably affect the performance of ducks, except for F:G from 15 to 42 D of age.

Except for the sternum depth, the vitamin premix, 25-OH-D3, and their interaction did not affect the sternum dimension (Table 5). Supplementation of NY/T vitamin diet significantly increased the sternum depth of 42-day-old duck compared with NRC vitamin diet. Dietary 25-OH-D3 also increased the sternum depth of 49-day-old duck in this study. The bone weight, Ca, P, and density of sterna were comparable among all groups at 42 D and 56 D (Table 6). Vitamin premix and the interaction of vitamin regimen and 25-OH-D3 did not significantly change the sternal weight, ash, mineral...
Table 6. Effects of 25-OH-D3 and vitamin regimen on sternal weight and minerals content for meat duck.

| Item | Relative fresh weight | Fat-free weight, g | Ash, % fat-free weight | Ca, % fat-free weight | P, % fat-free weight | Vitamin level | 25-OH-D3 | SEM | P-value | VTM regimen | 25-OH-D3 |
|------|----------------------|-------------------|----------------------|---------------------|---------------------|---------------|-----------|-----|---------|-------------|----------|
|      | 42 D | 49 D | 56 D | 42 D | 49 D | 56 D | 42 D | 49 D | 56 D | 42 D | 49 D | 56 D | 42 D | 49 D | 56 D | 42 D | 49 D | 56 D | 42 D | 49 D | 56 D | 42 D | 49 D | 56 D | 42 D | 49 D | 56 D | 42 D | 49 D | 56 D | 42 D | 49 D | 56 D | 42 D | 49 D | 56 D | 42 D | 49 D | 56 D | 42 D | 49 D | 56 D | 42 D | 49 D | 56 D | 42 D | 49 D | 56 D | 42 D | 49 D | 56 D | 42 D | 49 D | 56 D | 42 D | 49 D | 56 D | 42 D | 49 D | 56 D | 42 D | 49 D | 56 D | 42 D | 49 D | 56 D | 42 D | 49 D | 56 D | 42 D | 49 D | 56 D | 42 D | 49 D | 56 D | 42 D | 49 D | 56 D | 42 D | 49 D | 56 D | 42 D | 49 D | 56 D | 42 D | 49 D | 56 D | 42 D | 49 D | 56 D | 42 D | 49 D | 56 D | 42 D | 49 D | 56 D | 42 D | 49 D | 56 D | 42 D | 49 D | 56 D | 42 D | 49 D | 56 D | 42 D | 49 D | 56 D | 42 D | 49 D | 56 D | 42 D | 49 D | 56 D | 42 D | 49 D | 56 D | 42 D | 49 D | 56 D | 42 D | 49 D | 56 D | 42 D | 49 D | 56 D | 42 D | 49 D | 56 D | 42 D | 49 D | 56 D | 42 D | 49 D | 56 D | 42 D | 49 D | 56 D | 42 D | 49 D | 56 D | 42 D | 49 D | 56 D | 42 D | 49 D | 56 D | 42 D | 49 D | 56 D | 42 D | 49 D | 56 D | 42 D | 49 D | 56 D | 42 D | 49 D | 56 D | 42 D | 49 D | 56 D | 42 D | 49 D | 56 D | 42 D | 49 D | 56 D | 42 D | 49 D | 56 D | 42 D | 49 D | 56 D | 42 D | 49 D | 56 D | 42 D | 49 D | 56 D | 42 D | 49 D | 56 D | 42 D | 49 D | 56 D | 42 D | 49 D | 56 D | 42 D | 49 D | 56 D | 42 D | 49 D | 56 D | 42 D | 49 D | 56 D | 42 D | 49 D | 56 D | 42 D | 49 D | 56 D | 42 D | 49 D | 56 D | 42 D | 49 D | 56 D | 42 D | 49 D | 56 D | 42 D | 49 D | 56 D | 42 D | 49 D | 56 D | 42 D | 49 D | 56 D | 42 D | 49 D | 56 D | 42 D | 49 D | 56 D | 42 D | 49 D | 56 D | 42 D | 49 D | 56 D | 42 D | 49 D | 56 D | 42 D | 49 D | 56 D | 42 D | 49 D | 56 D | 42 D | 49 D | 56 D | 42 D | 49 D | 56 D | 42 D | 49 D | 56 D | 42 D | 49 D | 56 D | 42 D | 49 D | 56 D | 42 D | 49 D | 56 D | 42 D | 49 D | 56 D | 42 D | 49 D | 56 D | 42 D | 49 D | 56 D | 42 D | 49 D | 56 D | 42 D | 49 D | 56 D | 42 D | 49 D | 56 D | 42 D | 49 D | 56 D | 42 D | 49 D | 56 D | 42 D | 49 D | 56 D | 42 D | 49 D | 56 D | 42 D | 49 D | 56 D | 42 D | 49 D | 56 D | 42 D | 49 D | 56 D | 42 D | 49 D | 56 D | 42 D | 49 D | 56 D | 42 D | 49 D | 56 D | 42 D | 49 D | 56 D | 42 D | 49 | content, and density at 49 D. Administration of 25-OH-D3 remarkably increased the sternal bone weight, mineral content, and density of 49-day-old ducks in NY/T but not NY/T vitamin regimens (Table 6).

Considered with the above sternal characterization results, therefore, more attention was given to the effect of 25-OH-D3 on bone phenotype between 42 and 49 D of age. Toluidine blue stain has shown that marrow area was intuitionally decreased, and the BV was obviously increased by 25-OH-D3 treatment in all vitamin regimen diets (Figure 1A). Outcomes of morphometric analysis confirmed that the diet contained NY/T vitamin, markedly increased the BV/TV and reduced the Tb.Sp compared with the NRC vitamin diet under the no 25-OH-D3-treated condition (Figures 1B, 1E). Dietary 25-OH-D3 manipulation significantly increased the BV/TV and the Tb.N and decreased the Tb.Sp among all groups (Figures 1B, 1D, 1E).

**Effects on the Formation and Bone Resorption**

Effects of 25-OH-D3 on bone resorption under 2 different vitamin regimens was assessed histologically and biochemically. TRAP-positive cells are intuitionally higher in NRC vitamin regimens in the absence of 25-OH-D3 (Figure 2A). In NRC but not NY/T vitamin diet, supplementation of 25-OH-D3 markedly reduced the N.Oc/BS (Figure 2B), and circulating TRAP and CTx, both were resorption markers (Figures 2C, 2D). The osteoclast-related factors including OPG, RANKL, and H+ATPase were examined using RT-PCR and shown that the expression of Phex and Sclerostin mRNA were pronouncedly upregulated by 25-OH-D3 treatment in NRC but not in NY/T vitamin regimen (Figures 1F, 1G). The interaction of vitamin regimen and 25-OH-D3 did not significantly affect the bone phenotypes of 49-day-old duck. These results suggest that the LND diet with NY/T vitamin premix produced positive role on sterna mass of 49-day-old duck compared with NRC vitamin diet and supplementation 25-OH-D3 to NRC, but not NY/T vitamin regimens could significantly promote the mineral deposition of sternum, even though sternal microarchitecture was improved in NY/T vitamin diet.
Effects of 25-OH-D3 on bone formation under 2 different vitamin regimens was also assessed biochemically. The serum indicators for bone formation, ALP and P1NP, were reduced significantly by the 25-OH-D3 treatment in NRC but not in NY/T vitamin regimen (Table 7). Responded to 25-OH-D3 supplementation, serum 25-OH-D and Ca concentrations were notably higher in 25-OH-D3-treated duck than that in untreated ducks (Table 7), but serum P and PTH concentrations were not altered by dietary 25-OH-D3 in any of the groups (Table 7). Thus, the administration of 25-OH-D3 promoted the sternal mass not through enhancing bone formation, perhaps as the result of indirect increase in Ca absorption from tissues.

**Effect on Sternal Characteristics and Bone Metabolism in PC Diet**

To verify the preferential effect of 25-OH-D3 in NRC vitamin premix diet on the sternal mass of meat ducks, another animal trial was conducted in a standard nutrient density diet contained NRC vitamin premix with 0 or 0.069 mg/kg of 25-OH-D3, and the results showed that 25-OH-D3 administered to birds for 49 D significantly increased the final BW but has no apparent change the sternal relative weight and dimension (Figure 3). Dietary 25-OH-D3 addition increased the mineralization area (darker colors) and sternal minerals deposition in 49-day-old ducks (Figures 4B–4F). Toluidine blue stained and morphometric analysis revealed that parameters of the trabecular bone structure, such as BV/TV and Tb.Th were significantly increased, and Tb.Sp was significantly decreased by 25-OH-D3 treatment (Figures 4G–4K). The influence of 25-OH-D3 on bone turnover also was determined histologically and biochemically and showed that both osteoblast and osteoclast number were remarkably decreased by 25-OH-D3 (Figures 5B, 5G). Serum levels of CTx, TRAP, and ALP activity were significantly suppressed by dietary 25-OH-D3 supplementation (Figures 5C, 5E, 5F). Meanwhile, 25-OH-D3 treatment markedly increased serum 25-OH-D and Ca concentrations (Figures 5I, 5J) but not significantly altered serum P and PTH level in 49-day-old ducks (Figures 5K, 5L). Overall, supplementation 25-OH-D3 to PC diet with NRC vitamin regimen improved the sternal mass through decreasing bone turnover and inducing Ca resorption from intestine and/or kidney.

**DISCUSSION**

In the current study, we examined the effect on sternal mass and bone metabolism of the daily administration of 25-OH-D3 in LND diet with different vitamin regimen...
and PC diet. NY/T vitamin regimen increased sternal mass compared with NRC vitamin premix in LND diet. Supplementing 25-OH-D3 to NRC vitamin regimen improved the bone mass of the sterna with the suppression of bone resorption and bone formation in LND and PC diet. It was clear that the increase in sternal mass was mainly because of the suppression of bone resorption. The decrease in bone formation caused by 25-OH-D3 may be related to the coupling reaction induced by the suppression of bone resorption.

Recent studies have indicated that diets supplemented with 2,000 IU/kg vitamin D3 improved broiler’s BW in comparison with birds fed according to NRC (1994) recommendations (Gomezverduzco et al., 2013). The positive effect of supplement 25-OH-D3 was also reflected in an increased BW gain and feed conversion ratio (Santiago et al., 2016). Coincidentally, the present study revealed 25-OH-D3 administered significantly increased the final BW of 49-day-old ducks in PC diet, as well as increased BW (14 D), gain (1–14 D) and decreased FI (15–42 D) and F: G in LND diet with any given vitamin premix. A positive impact of NY/T vitamin diet on BW also noticed in the present study. The birds fed NY/T vitamin diet had a significantly higher BW at 35, 42, 49, and 35 D of age, which was consistent with our previous observation that feeding high-vitamin level diet increases the BW of meat duck than low-vitamin level diet (Ren et al., 2017; Zhang et al., 2019), and it is partly because NY/T had higher levels of all vitamins except nicotinic acid than NRC recommendations (Table 1).

Vitamin D3 also exerts a variety of actions on maintaining a healthy mineralized skeleton (Goltzman, 2018). In an excellent review published by Świątkiewicz et al. (2017), it was shown that the supplementation about 3,000 IU/kg vitamin D3, which much higher than NRC (1994) recommendations, is optimal for mineral digestibility and bone quality of broilers. Meat-type duck is no exception, and the present study confirmed that supplementing 25-OH-D3 (the equivalent of 2,760 IU/kg vitamin D3) to NRC vitamin regimen significantly increased the mineral deposition.
and density of sternum in LND diet in the 49-day-old duck. These findings were in accordance with previous studies conducted in broilers (Wideman et al., 2015; Santiago et al., 2016). However, an apparent increment of sternal mass induced by 25-OH-D3 was not observed in NY/T vitamin diets, suggesting the effect of 25-OH-D3 on sternum quality of meat duck probably depended on dietary vitamin regimen (Ren et al., 2017). Also, it was noticed that these positive effects of 25-OH-D3 on sternal mass were not prominently stood out in 42-day-old and 56-day-old duck in the LND diet. It is likely related to the sternum mineralization kinetic. Our laboratory has recently proposed the rapid mineralization of sternum occurs in between 42 and 49 D of age and achieve the plateau phase after 49 D for meat duck (Zhang et al., 2017). Thus, more attention was given to the impact of 25-OH-D3 on sternal mass at 49-day-old duck. As previously reported (Nakamichi et al., 2017), morphometric analysis has further demonstrated that dietary 25-OH-D3 administration significantly promoted sternal mineralization at 49 D, evidenced by the increase of BV/TV and Tb.N, and the decrease of Tb.Sp among all diets in a similar fashion. Overall, LND diet with NY/T vitamin premix is beneficial to sterna mass of 49-day-old duck compared with NRC vitamin diet and supplementation 25-OH-D3 to NRC but not NY/T vitamin regimens could significantly promote mineral accumulation of sternum in spite of perfect sternal microarchitecture in NY/T vitamin diet.

It is well established that the main function of vitamin D3 is the maintenance of Ca homeostasis, although it is also involved in multiple other biological effects (Dittmer and Thompson, 2011). During hypocalcemia, vitamin D3 will activate the Ca absorption in intestines and kidney and mobilize the Ca from bone to maintain normal Ca concentration in blood, which also depends on the PTH concentration (Lips and van Schoor, 2011). Hence, the effect of vitamin D3 on the bone (stimulation or inhibition of bone resorption and mineralization) also depends on the PTH level in blood.

Figure 3. Body weight and sternal characters of 49-day-old meat duck responses to 25-OH-D3 in PC diet with NRC vitamin regimen. (A) Body weight, (B) Sternum relative weight (per 100 g body weight), (C) fat-free weight, (D) sternum length, (E) depth, (F) coracoid distance, (G) sternum central distance, and (H) posterior process distance. Values are means and standard deviation represented by vertical bars (n = 8). a, bMean values with different letters are significantly different (one-way ANOVA, P < 0.05, Tukey’s post hoc test). Abbreviations: 25-OH-D3, 25-hydroxycholecalciferol; PC, positive control.
In the present study, serum Ca concentration was increased because of the dietary 25-OH-D3 supplementation LND diets at 49 D, but the PTH concentration was did not significantly change by the dietary 25-OH-D3, which indicates that the blood Ca level was still within the normal range, and the Ca content in LND diets is sufficient to meet the requirements of meat duck. Alternatively, the increased serum Ca concentration might suggest that the positive effect of 25-OH-D3 on sternal mass in vivo at least partly is through inducing the Ca absorption in intestine or kidney.

In addition to the well-known vitamin D3 stimulation of the intestinal absorption of Ca, this positive effect on sternum was also identified to directly regulate the several bone cell types including osteoblasts, osteocytes, and osteoclasts (Goltzman, 2018). Vitamin D3 was reported to act on osteoclasts by stimulating or inhibiting bone resorption in vivo (Sato et al., 2007; Harada et al., 2012), although vitamin D3 has been used as therapeutic agents for osteoporosis (Richy et al., 2005). The discrepancies could be partly explained by the dose used in these studies (Zarei et al., 2016). In terms of circulating bone
Table 7. Effects of 25-OH-D3 and vitamin regimen on serum biochemistry for meat duck at 49 D of age.

| Item        | Ca, mmol/L | P, mmol/L | 25-OH-D, ng/L | PTH, ng/L | ALP, U/L | P1NP, μg/L |
|-------------|------------|-----------|---------------|-----------|----------|------------|
| Vitamin level | 25-OH-D3   |           |               |           |          |            |
| NRC         |             |           |               |           |          |            |
| –           | 2.33c       | 3.61      | 435.78c       | 39.84     | 773.50c  | 14.49c     |
| +           | 2.44abc     | 3.72      | 475.62abc     | 37.79     | 741.83bc | 12.35bc    |
| NY/T        |             |           |               |           |          |            |
| –           | 2.37bcd     | 3.66      | 453.65bc      | 38.40     | 755.83bc | 13.40bc    |
| +           | 2.46abc     | 3.76      | 488.78abc     | 37.96     | 734.00bc | 11.89bc    |
| SEM         | 0.03        | 0.04      | 8.36          | 1.64      | 20.53    | 0.42       |
| Vitamin regimen |        |           |               |           |          |            |
| NRC         |             |           |               |           |          |            |
| 2           | 2.39        | 3.66      | 455.70b       | 38.81     | 757.67   | 13.42      |
| NY/T        |             |           |               |           |          |            |
| 2           | 2.41        | 3.71      | 471.21ab      | 38.18     | 744.92   | 12.64      |
| SEM         | 0.02        | 0.03      | 5.91          | 1.16      | 14.52    | 0.31       |
| 25-OH-D3    |             |           |               |           |          |            |
| –           | 2.34bc      | 3.63      | 444.72bc      | 39.12     | 764.67b  | 13.94b     |
| +           | 2.45ab      | 3.74      | 482.19ab      | 37.87     | 737.92b  | 12.11b     |
| SEM         | 0.02        | 0.03      | 5.91          | 1.16      | 14.52    | 0.31       |
| P-value     |             |           |               |           |          |            |
| Vitamin regimen |        |           |               |           |          |            |
| 2           | 0.433       | 0.259     | 0.041         | 0.705     | 0.398    | 0.083      |
| 25-OH-D3    |             |           |               |           |          |            |
| 2           | 0.004       | 0.058     | 0.003         | 0.456     | 0.032    | 0.004      |
| Interaction | 0.833       | 0.929     | 0.755         | 0.639     | 0.068    | 0.471      |

abc Mean values with unlike letters were significantly different (two-way ANOVA, \( P < 0.05 \), Tukey’s post hoc test).

Abbreviations: 25-OH-D, 25-hydroxycholecalciferol; ALP, alkaline phosphatase; Ca, calcium; NY/T, China Agricultural industry standards; P, phosphorus; P1NP, procollagen type I N-terminal propeptide; PTH, parathyroid hormone.

Figure 5. Bone metabolism of 49-day-old meat duck responses to 25-OH-D3 in PC diet with NRC vitamin regimen. To reflect bone formation, sternum was subjected to a (A) alkaline phosphatase (ALP) staining for (B) osteoblast number (N.Ob/BS) calculation, and serum was obtain for (C) ALP and (D) procollagen type I N-terminal propeptide (P1NP) analyses. Serum levels of (I) 25-hydroxyvitamin D (25-OH-D), (J) calcium (Ca), (K) phosphorus (P), and (L) parathyroid hormone (PTH) were also further measured. Values are means and standard deviation represented by vertical bars (\( n = 8 \)). abc Mean values with different letters are significantly different (one-way ANOVA, \( P < 0.05 \), Tukey’s post hoc test). Abbreviations: 25-OH-D3, 25-hydroxycholecalciferol; PC, positive control.
resorption markers (TRAP and CTx) which were examined in this study, both TRAP activity and CTx concentration were significantly decreased by the 25-OH-D3 treatment in the LND diet with NRC but not with NY/T vitamin premix, which indicates that the supplementation of 25-OH-D3 to NRC vitamin premix could suppress bone resorption of sternum in 49-day-old meat ducks. This inhibitory effect of 25-OH-D3 probably was because of the decreasing osteoclast differentiation in this study, which was evidenced in the treatment of 25-OH-D3, in which RANKL/OPG ratio, an important regulator of osteoclastogenesis markedly declined, and consequently the osteoclast number decreased in NRC vitamin regimen. In agreement with our results, previous findings also showed administration of vitamin D3 decreased the ratio of RANKL to OPG in vivo and in vitro (Tang and Meng, 2009; Nakamichi et al., 2017). In addition, some important enzymes, such as vacuolar H^+ -ATPases (V-ATPases) and cathepsin K, identified in numerous studies could also associated with the function of osteoclast through dissolving the organic and inorganic components of bone in the process of bone resorption (Fujisaki et al., 2007; Riihonen et al., 2007). Downregulated expression of cathepsin K because of 25-OH-D3-treated in NRC but not NY/T vitamin diets implied the suppression of osteoclast activity induced by 25-OH-D3, and this may be another a contributor to the decreased bone resorption. Taken together, these results may indicate that the addition of 25-OH-D3 to the LND diet with NRC vitamin regimen acted on osteoclast to inhibit bone resorption of sternum in 49-day-old ducks.

Vitamin D3 was reported to enhance osteoblast differentiation and mineralization (Turner et al., 2014; van der Meijden et al., 2014). In contrast, consistent with the previous findings on eldecalcitol, an active vitamin D analog (Harada et al., 2012; Nakamichi et al., 2017), 25-OH-D3 supplementation to NRC vitamin regimen significantly decreased the serum ALP activity and P1NP levels, which suggests that 25-HyD is unlikely to be a positive regulator for bone formation. This suppressive effect on bone formation appears to be an indirect action of 25-OH-D3 and is a consequence of coupling of bone resorption to bone formation (Nakamura et al., 2003). Sclerostin was recently proposed to be a key mediator for the coupling process (Masuki et al., 2010). In the current study, 25-OH-D3 treatment upregulated osteocyte-specific genes transcription including sclerostin, suggesting that excessive bone mass induces a higher expression of sclerostin and subsequently sclerostin in turn regulated bone formation to return to the normal level. Further studies would be essential to exclude this possibility.

Under a standard nutrient density diet, we further showed that dietary 25-OH-D3 treatment remarkably decreased osteoblast and osteoclast number, as well as serum bone turnover markers concentration. Meanwhile, dietary 25-OH-D3 also significantly increased the serum total vitamin D3 and Ca concentrations. As a result, supplementation 25-OH-D3 to the PC diet with NRC vitamin regimen has a positive role in promoting sternal microstructure and bone mass in 49-day-old meat duck.

NY/T vitamin regimen increased the minerals accumulation of sternum and declined these indicators that reflect both the bone formation and resorption compared with NRC vitamin premix in LND diet. More importantly, the outcome of histomorphometry showed that dietary 25-OH-D3 administration significantly improved the trabecular microstructure of sternum, but it did not significantly alter the mineral content of the whole sternum and bone metabolism of sternum in NY/T vitamin diet. It is possible that the vitamin composition and proportion of NY/T vitamin regimen are adequate to sternal mass. Additionally, the higher bone mass in the NY/T diet compared with the NRC diet could be the result of a lower bone turnover ratio (Kolp et al., 2017). Further experiments would be essential for understanding the overall mechanisms of the NY/T vitamin regimen actions on bone metabolism.

Taken together, the present findings demonstrated that the LND diet with NY/T vitamin regimen had a favorable effect on the sternal mass of duck through decreasing bone turnover, and the 25-OH-D3 administration notably induced an increase in bone mass in NRC vitamin regimen, which is mediated by suppressing bone resorption in 49-day-old meat duck.

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