Dietary supplementation of 25-hydroxycholecalciferol increases tibial mass by suppression bone resorption in meat ducks

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Abstract

Leg problems often result from the rapid weight gain and poor bone quality in modern ducks, leading to a high risk of fractures and continuous pain. We hypothesized that improving bone quality in combination with delaying weight gain via a low nutrient density (LND) diet probably reverses these skeletal abnormalities. Studies indicated that 25-hydroxycholecalciferol (25-OH-D3), a vitamin D3 metabolite, is effective in treating bone-related disorders. Therefore, Exp. 1 evaluated the effects of 25-OH-D3 on tibial mass of meat ducks. Male meat ducklings were fed a standard nutrient density diet (containing a regular vitamin regimen) without or with 25-OH-D3 at 0.069 mg/kg for 35 d. The results showed that 25-OH-D3 supplementation improved the mineral content, microarchitecture and mechanical properties of tibias, and this accompanied by a decreased serum bone resorption marker and a concomitant decrement in osteoclast-specific marker genes expression. Subsequently, Exp. 2 was conducted to examine the impacts of 25-OH-D3 incorporating an LND diet on tibial quality of ducks under 2 different vitamin regimens (regular and high). Ducklings were allocated to a 2 × 2 factorial arrangement with 2 kinds of vitamin premixes and without or with 25-OH-D3 at 0.069 mg/kg in LND diets. The high premix had higher levels of all vitamins except biotin than the regular premix. The results demonstrated that high vitamin diets exhibited more significant effects than regular vitamin diets on inhibiting bone turnover and increasing minerals deposition. Tibial mineral content, microarchitecture, and strength of birds under the regular vitamin regimen were increased by 25-OH-D3 supplementation; however, these positive effects were not observed in ducks under the high vitamin regimen. To conclude, 25-OH-D3 supplementation improves tibial mass by suppressing osteoclast-mediated bone resorption in meat ducks, and this positive impact only was observed in regular but not high vitamin regimen when birds fed an LND diet.

1. Introduction

Increases in growth rate and breast muscle mass results from genetic progress and intensive nutrition had been an increased incidence of locomotion (gait) problems (Paxton et al., 2013). Estimates of the prevalence of gait problems of commercial meat ducks have been reported on 14% and 21% (Jones and Dawkins, 2010). Altered gait in livestock is an important welfare issue, causing a reduction in mobility, that may be associated with pain and a reduction in normal behaviors (Bradshaw et al., 2002).

The increased incidence of leg problems with recent years may be due in part to the change that has taken place in the rapid rate of growth and body weight (BW) in meat-type birds (Williams et al.,...
because heavier weight causes the cranial shift in the body's center of mass and increasing the risk of fractures (Corr et al., 2003; Paxton et al., 2013). Accelerating weight gain through increasing dietary nutrient density led to a higher incidence of gait abnormality, whereas feed withdrawal or reducing dietary nutrient density has been shown to alleviate the gait abnormality of broilers (Brickett et al., 2007). Our previous study also showed that a low nutrient density (LND) diet decreased the incidence of tibial dyschondroplasia (TD) and score by decelerating weight gain, improved the microarchitecture of bone trabecula, and promoted a down-regulation of bone turnover in meat ducks (Zhang et al., 2018).

Poor bone quality is certainly one of the main causes of skeletal disorders and increases the risk of fractures in domestic birds (Bradshaw et al., 2002). A lot of therapeutic strategies are used to improve bone mass and mechanical properties. According to some studies, the use of vitamin D and its metabolite fat soluble vitamins, regulating the absorption and metabolism of calcium (Ca) and phosphorus (P), has beneficial effects on bone mineralization and biomechanical properties (Jiang et al., 2015; Khan et al., 2010; Sun et al., 2013). Studies suggest supplementation of vitamin D3 improved the walking ability and bone quality characteristics, and consequently decreased the leg diseases (Jiang et al., 2015; Sun et al., 2013), and a deficiency can lead to a higher incidence of leg problems of birds (Khan et al., 2010). It is well-established that dietary vitamin D3 is absorbed in the small intestine and hydroxylated in the liver to form 25-hydroxycholecalciferol (25-OH-D3), the major circulating form of the vitamin D3 (Kulda, 2012). An evaluated trial of the relative bioavailability of 25-OH-D3 and vitamin D3 found that 25-OH-D3 was approximately twice as active as cholecalciferol (vitamin D3) in promoting bone strength in broilers (Han et al., 2016). Supplementation with 25-OH-D3 has been reported to significantly increase tibial mineralization of broilers (Ferket et al., 2009; Santiago et al., 2016; Wideman et al., 2015). All studies aforementioned focus on the therapeutic potential for 25-OH-D3 in treating leg problems and its related complications, the mechanisms underlying the beneficial effects of vitamin D or its metabolites and bone mass are still limited to poultry. A candidate mechanism for the increase of bone mass by vitamin D treatment as well-known is stimulating the intestinal absorption of Ca and P (Christakos et al., 2014). Another possible mechanism is to regulate bone remodeling by directly targeting to serval bone cell types including osteoblast, osteocyte and osteoclast (Nakamichi et al., 2018; van Driel and van Leeuwen, 2017).

Another important effect described for 25-OH-D3 was the dependence on the dietary basal vitamin level. Ren et al. (2017) reported that the positive impacts of maternal canthaxanthin and 25-OH-D3 supplementation on growth performance and serum 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 recommendations, different responses of dietary 25-OH-D3 to distinct vitamin regimen probably due to different doses of vitamin D or interaction between vitamins (Bonjour et al., 2018). There are various recommendations of vitamin premix in the duck industry, and the variations in each vitamin content of each other is very wide, especially National Nutrition Council (1994) and China Agricultural Industry Standards (NY/T 2122-2012). Accordingly, this is reasonable to assume that the biological effect of 25-OH-D3 on tibial mass may lie on the dietary basal vitamin regimen.

Therefore, our goals for this study were to first evaluate if the 25-OH-D3 is efficient in increasing the tibial mass of meat ducks by affecting osteoblast and/or osteoclast activity. Second, considering that the beneficial effect of LND diet and the dependence of 25-OH-D3 on the dietary basal vitamin level, the present study also aims to evaluate the effect of 25-OH-D3 under different vitamin regimens on the tibial quality of meat ducks fed an LND diet.

2. Materials and methods

The experiments were carried out in accordance with the guidelines on the Sichuan Agricultural University Experimental Animal Committee.

2.1. Birds, diets and experimental design

One-day-old male Cherry Valley meat-type ducks were obtained from a commercial hatchery, and reared in individual cages (2.2 m × 1.2 m × 0.9 m) situated in a temperature- and humidity-controlled room. They were fed ad libitum pellet-type experimental diets, including the standard nutrient density positive control (PC) diets and LND diets. The PC diet was designed to satisfy the recommendations (NY/T 2122-2012) except vitamins. In the PC diet, the contents of Ca and non-phytate phosphorus (NPP) were 0.9%, 0.42% in starter diets (d 1 to 14) and 0.85%, 0.4% in grower diets (d 15 to 35), respectively. The LND diet contained 0.7% Ca, 0.35% NPP, 10.25 MJ/kg apparent metabolism energy (AME), and the grower diet of LND diets had a constant ratios of crude protein (CP) and essential nutrients, such as limiting amino acids relative to AME compared to PC diet (Table 1). The diets were designed to differ in their 25-OH-D3 and vitamin regimens. For this purpose, various amounts of 25-OH-D3 (Exp. 1) and/or vitamin regimens

| Item          | Starting diet (d 1 to14) | Grower diet (d 15 to 35) |
|---------------|-------------------------|--------------------------|
|               | PC diet                 | LND diet                 |
| **Ingredients, %** |                         |                          |
| Maize         | 58.05                   | 62.1                     |
| Soy oil       | 3                       | 0                        |
| Soybean meal  | 34.8                    | 23.48                    |
| DDGS          | 0                       | 3                        |
| Wheat bran    | 0                       | 2                        |
| Rapeseed meal | 0                       | 2.85                     |
| i-Lysine      | 0.08                    | 0.2                      |
| L-Methionine  | 0.145                   | 0.13                     |
| L-Threonine   | 0.03                    | 0.07                     |
| L-Tryptophan  | 0                       | 0.12                     |
| Choline chloride | 0.2                    | 0.2                      |
| Sodium chloride | 0.3                    | 0.3                      |
| Mineral premix | 0.2                    | 0.2                      |
| Vitamin premix | 0.03                   | 0.03                     |
| Limestone     | 1.06                    | 1.04                     |
| Dicalcium phosphate | 1.75           | 1.6                      |
| Bentonite     | 0.355                   | 0.07                     |
| Total         | 100                     | 100                      |
| **Chemical analysis, %** |               |
| AME (MJ/kg)   | 12.14                   | 12.14                    |
| CP            | 20.05                   | 17.50                    |
| Dig Lys       | 1.02                    | 0.76                     |
| Dig Met       | 0.42                    | 0.38                     |
| Calcium       | 0.90                    | 0.85                     |
| Total-phytate P | 0.65                  | 0.65                     |
| Non-phytic acid P | 0.42                | 0.40                     |

DDGS = distillers dried grains with soluble; AME = apparent metabolizable energy; CP = crude protein; Dig = digestibility.

1 PC diet, standard nutrient density positive control diet; LND diet, low nutrient density diet.
2 Provided per kilogram of diet: Cu (CuSO4·5H2O), 8 mg; Fe (FeSO4·7H2O), 80 mg; Zn (ZnSO4·7H2O), 90 mg; Mn (MnSO4·H2O), 70 mg; Se (NaSeO3), 0.3 mg; I (KI), 0.4 mg.
3 Provided per kilogram of diet: regular or high vitamin regimens as shown in Table 2.
(Exp. 2) were added to the basal ingredients. Vitamin regimens included regular and high, which was formulated based on the recommendations of NRC (1994) and China Agricultural Industry Standards (NY/T 2122–2012), respectively (Table 2). All vitamins used in this study were provided by DSM Ltd. (China).

During the experiments, BW and feed intake were recorded weekly, BW gain and feed conversion as the feed-to-BW gain ratio (F:G) was calculated on a per-cage basis. The mortality due to leg deformations, which is defined as the percentage of mortality and culled ducks due to leg deformations and fractures, also was recorded during feeding period on a pen basis.

2.1.1. Experiment 1
The aim of this trial was to compare the effects of 25-OH-D₃ on tibia mass of meat ducks fed the PC diet containing the regular vitamin regimen. With this aim, 256 male meat ducklings at 1 old were allocated to 2 dietary treatments with 8 replicates (16 birds each). They were fed PC diets containing either no (control group) or 25-OH-D₃ (0.069 mg/kg) for 35 d.

2.1.2. Experiment 2
The aim of this trial was to evaluate the effects of 25-OH-D₃ under different vitamin regimens on the tibial quality of meat ducks fed the LND diets. For this purpose, 512 ducks were assigned to one of 4 LND diets (8 replicate pens; 16 ducks/pen). The 4 experimental treatments consisted of a 2 × 2 factorial arrangement of 2 dietary inclusion vitamin regimens (regular and high) and 2 concentrations of 25-OH-D₃ (0 or 0.069 mg/kg diet). Ducklings were all fed the PC diets until d 14, subsequently, they were subjected to corresponding LND diets until d 35.

2.2. Sampling
Eight ducks per treatment (1 bird per pen) were randomly selected and sacrificed by cervical dislocation on d 35. Left tibias (the proximal end) were dissected and fixed at 10% buffered formalin (pH 7.4) for tibial histology analysis. After fasting for 12 h, other 8 ducks per treatment, with BW close to the pen average, were selected and subjected to blood collection through the jugular vein. Then, the animals were sacrificed by cervical dislocation, and left tibias (the proximal end) were dissected and immediately flash-frozen in liquid N₂ for mRNA analyses. The right tibias were taken without soft tissues, and both the length and width (50% of length) were measured immediately. These tibias were kept frozen for later analysis of mineral content and biomechanical properties.

2.3. Evaluation of leg abnormalities
At the end of the experiments, 3 birds from each pen were randomly selected and sacrificed by cervical dislocation. Tibias were removed from both legs, and the proximal end was sectioned to grade for TD using the TD scoring system described by Edwards and Veltmann (1983). Tibial dyschondroplasia lesions were scored as 0, 1, 2 or 3 (0 = normal bone or no lesion, 1 = slight lesion, 2 = medium lesion and, 3 = most severe lesion).

2.4. Serum biochemical analysis
Serum Ca and P concentrations were determined with Biochemistry Analyzer (Yellow Springs Instrument Co. Inc., Yellow Springs, OH, USA). Serum parathyroid hormone (PTH) and 25-OH-D concentrations were assayed using commercial ELISA kit (ALPCO Diagnostics, NH, USA) according to the manufacturers’ recommendations. In addition, Serum procollagen type I N-terminal propeptide (PINP), alkaline phosphatase (ALP) (both bone formation markers), C-terminal cross-linked telopeptide of type I collagen (CTX) and tartrate-resistant acid phosphatase (TRAP) (both bone resorption markers) were measured using commercial assay kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China). All samples were tested in triplicate within each assay.

2.5. Tibia-breaking strength analysis
Breaking force test was performed to evaluate tibial strength using the texture analyzer (TA. XT Plus; Stable Microsystems), employing exponent lite express software (Stable Micro Systems). The tibia was rested on 2 points with a gap of 13 mm and pressure was applied with pressure sensitive load cell (50 kg) at the center of the 2 points, and the blade perpendicularly hit the tibia at its midpoint at a test speed of 5 mm/s for a distance of 30 mm.

2.6. Measurement of mineralization
Tibial fat-free weight and tibial density were determined according to our previous methods (Zhang et al., 2017). Specifically, bone volume (cm³) was measured based on quantum of water overflowing from a fully filled container, and subsequently these tibias were air-dried for 24 h at room temperature, extracted by refluxing diethyl ether in a Soxhlet apparatus for 16 h, oven-dried at 108 °C for 24 h for fat-free bone weight (g) determination, thus the tibial density (g/cm³) was calculated as tibial fat-free weight (g)/tibial volume (cm³). For ash, Ca and P analysis, dry-defatted tibia was ashed in a muffle furnace at 550 °C for 24 h, and the ash was measured on the basis of the percentage of dry-defatted weight. Tibial Ca and P were determined through ethylenediaminetetraacetic acid (EDTA) titration and ammonium metavanadate colorimetry, respectively, and values were also presented basing on dry fat-free weight.

2.7. Bone histological analysis
Bone histological analysis was performed as previously described (Vidal et al., 2012). Briefly, the fixed tibia samples were decalcified with 10% EDTA (pH 7.4) for d 21, dehydrated conventionally, and embedded in paraffin wax. The entire blocks containing the samples were cut into sagittal sections, after roughing the microtome approximately 120 times of 10-μm thickness. After that, slides of 4-μm thickness were obtained for the toluidine blue

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**Table 2**
Composition of the vitamin premixes for meat ducks.¹

| Item                  | d 1 to 14 | d 15 to 35 |
|-----------------------|-----------|------------|
|                       | Regular   | High       | Regular   | High       |
| Vitamin A, IU         | 2,500     | 4,000      | 2,500     | 3,000      |
| Vitamin D₃, IU        | 400       | 2,000      | 400       | 2,000      |
| Vitamin E, IU         | 10        | 20         | 10        | 20         |
| Vitamin K₃, mg        | 0.5       | 2          | 0.5       | 2          |
| Vitamin B₆, mg        | 1.8       | 1.8        | 1.8       | 1.5        |
| Vitamin B₁₂, mg       | 4         | 10         | 4         | 10         |
| Vitamin B₉, mg        | 2.5       | 3          | 2.5       | 3          |
| Vitamin B₁₃, mg       | 0.01      | 0.02       | 0.01      | 0.02       |
| Niacin, mg            | 0.5       | 0.5        | 0.5       | 0.5        |
| Pantothenic 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          |

¹ Supplied in per kilogram of diet.
² The vitamin levels recommended by the National Research Council (NRC, 1994).
³ The vitamin levels recommended by the China Agricultural Industry Standards (NY/T 2122–2012).
staining, in which the mineralized bone as violet blue and the osteoid as pale blue. The bone slides were visualized in a microscope equipped (Nikon Corporation, Tokyo, Japan). Histomorphometry were performed in 3 randomly selected non-overlapping microscopic fields in each section by Image-Pro Plus software (Media Cybernetics, Silver Spring, MD, USA). Undergoing gray scale transform, the manual grid was used to divide the samples in several fields with the same dimensions, allowing an easier quantification and analysis of the histomorphometry parameters. Some measurements such as total area (T.Ar), calcified bone area (B.Ar), bone perimeter (B.Pm) can be obtained directly from images, and several key parameters, including the trabecular bone area (Tb.Ar), trabecular number (Tb.N), thickness (Tb.Th) and spacing (Tb.Sp), are derived indirectly using the following equations:

\[
\text{Tb.Ar} = \frac{B.Ar \times T.Ar}{100},
\]

\[
\text{Tb.Th} = 2 \times \frac{B.Ar}{B.Pm},
\]

\[
\text{Tb.N} = \frac{\text{Tb.Ar} \times \text{Tb.Th}}{\text{Tb.Sp}},
\]

\[
\text{Tb.Sp} = \frac{1}{\text{Tb.N}} - \text{Tb.Th}.
\]

The selected area was similar in all samples and the same parameters were measured in order to standardize the analysis. At least 10 sections from each sample were analyzed.

2.8. Tartrate-resistant acid phosphatase staining for osteoclast counts

To quantify the osteoclast, the tibial slides for staining with the TRAP detecting kit (Sigma–Aldrich, St. Louis, MO, USA). The numbers of osteoclast number per millimeter bone surface (N.Oc/mm) were determined in 5 consecutive microscopic fields (400× magnification). At least 5 serial vertical sections were evaluated for each animal per analysis. The slides were counted by 2 examiners, who was blinded to group status.

2.9. Gene expression assays

Real-time (RT) PCR was conducted to determine the mRNA levels of osteocyte-, osteoblast- and osteoclast-specific marker genes. Total RNA of the tibia proximal end was extracted with a standard Trizol protocol. The cDNA was synthesized from 200 ng of total RNA by a PrimeScript RT Reagent Kit (Takara). RT-PCR was performed on ABI 7500 RT-PCR detection system (Applied Biosystems), using Fast SYBR Green Master Mix (Thermo Fisher Scientific). Target cDNA was amplified by 40 cycles (one cycle: 95 °C for 5 s, 60 °C for 30 s) and a final melting curve analysis. All reactions were run in duplicate. Primers were designed using Primer 3 as shown in Table 3. The standard curve method was used to estimated reaction efficiency (slope) and genes expression. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH), 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 RNA of interests (Vandesompele et al., 2002).

2.10. Statistical analysis

Data represent the mean ± standard deviation. The statistical analysis was performed using SAS 9.2 (SAS Institute, Cary, NC, USA).

### Table 3

| Gene | Gene ID | Primer Sequence (5'→3') | Size, bp |
|------|---------|--------------------------|---------|
| Phex | XM_005010837.3 | Reverse: ggcaatctcctgttggga 99<br>Forward: cgtagataccgggagaa 99 | 0.56 |
| Dmp1 | XM_005012780.3 | Reverse: caaccttttgctctgctc 99<br>Forward: gcttggctgttctttgta 99 | 0.56 |
| Sclerostin | XM_005026106.3 | Reverse: gtagctgctagctgtgg 115<br>Forward: atcgctgctagctgtcg 115 | 0.56 |
| Cathepsin K | XM_021277116.1 | Reverse: tgcggctggtctttgctgc 111<br>Forward: acttgctgctagctgtcg 111 | 0.56 |
| V-ATPase | XM_021267716.1 | Reverse: tgcggctggtctttgctgc 111<br>Forward: acttgctgctagctgtcg 111 | 0.56 |
| OPG | XM_005017709.3 | Reverse: tgcggctggtctttgctgc 111<br>Forward: acttgctgctagctgtcg 111 | 0.56 |
| RANKL | XM_021276016.1 | Reverse: tgcggctggtctttgctgc 111<br>Forward: acttgctgctagctgtcg 111 | 0.56 |
| β-actin | NM_001312408.1 | Reverse: tgcggctggtctttgctgc 111<br>Forward: acttgctgctagctgtcg 111 | 0.56 |

Phex – phosphate regulating endopeptidase homolog x-linked; Dmp1 – dentin matrix protein 1; V-ATPase – vacuolar-type H+–ATPase; OPG – osteoprotegerin; RANKL – receptor activator of nuclear Factor kB ligand; GAPDH – glyceraldehyde-3-phosphate dehydrogenase.

Statistical power of 0.80 (80%) was obtained in this study when the minimally detectable effect size was 1.0 and the significance level was 0.05. Mortality and TD scores were χ²/n + 1 and arcsine transformed before analysis to satisfy the analysis of variance (ANOVA) requirements for normality and homogeneity of variance, respectively. Unpaired Student’s t-test was used to compare the effect of 25-OH-D3 on bone mass in the PC diets with regular vitamin regimen. Two-way ANOVA followed by Tukey’s test (SAS 9.2) were used to analyze the effect of dietary vitamin regimens, 25-OH-D3, and their interaction on bone mass in LND diets. Statistical significance was detected at P < 0.05.

3. Results

3.1. Experiment 1: tibial quality responses to 25-OH-D3 in PC diets contained regular vitamin regimen

3.1.1. Performance

The results of growth performance showed that supplementation of 25-OH-D3 (0.069 mg/kg diet) increased (P < 0.05) the BW (d 14) and BW gain (d 1 to 14), but did not notably change feed intake and F:G, as compared to the control group (without 25-OH-D3 supplementation) (Fig. 1).

3.1.2. Leg abnormalities and tibial growth

As shown in Fig. 2, the mortality due to leg deformations of the 25-OH-D3-fed birds was slightly lower than that of the control birds (Fig. 2A), and 25-OH-D3 treatment significantly decreased (P < 0.05) the TD score, an indicator for leg abnormalities, and increased (P < 0.05) the tibial fat-free weight when compared to the control group (Fig. 2B and E), but tibial length and width were not affected by the 25-OH-D3 diets (Fig. 2C and D).

3.1.3. Bone mass

The tibial mass was assessed through mineral content, bone histological analysis, and the osteocyte-specific marker genes expression in this study. As shown in Fig. 3, the birds fed the 25-OH-D3 diets exhibited markedly higher (P < 0.05) ash level, Ca content, density, and strength of tibia, as well as a slightly increase in tibial P content, when compared with the control ducks (Fig. 3A to E). The outcomes of bone histomorphometry analysis of the
Fig. 1. Effect of 25-hydroxycholecalciferol (25-OH-D₃) supplementation on growth performance in the standard nutrient density positive control (PC) diets with a regular vitamin regimen: (A) Body weight (BW), (B) BW gain, (C) feed intake, and (D) the ratio of feed intake to BW gain (F:G). Values are means ± standard deviation represented by vertical bars (n = 8). * Mean values are significantly different (Student’s t-test, P < 0.05).

Fig. 2. Effect of 25-hydroxycholecalciferol (25-OH-D₃) supplementation on leg weakness and tibial growth in the standard nutrient density positive control (PC) diets with a regular vitamin regimen: (A) mortality due to leg abnormalities, (B) tibial dyschondroplasia (TD) score, and tibial growth including (C) length, (D) width, and (E) fat-free weight. Values are means ± standard deviation represented by vertical bars (n = 8). * Mean values are significantly different (Student’s t-test, P < 0.05).
tibia proximal end revealed that birds consuming the 25-OH-D₃ diets displayed a significant increase (P < 0.05) in Tb.Ar and Tb.N, and a pronounced decrease in Tb.Sp as compared to those fed the control diets (Fig. 3F to J). Furthermore, with regard to the osteocyte-specific marker genes expression, the 25-OH-D₃ diets increased (P < 0.05) the mRNA abundances of sclerostin compared to the control diets (Fig. 3K). The expression of phosphate regulating endopeptidase homolog x-linked (Phex) and dentin matrix protein 1 (Dmp1) appears to be similar in the control and the 25-OH-D₃-fed birds (Fig. 3L and M). Collectively, these results suggest that 25-OH-D₃ supplementation enhances tibial microstructure and bone mass.

Fig. 3. Effect of 25-hydroxycholecalciferol (25-OH-D₃) supplementation on tibial mineralization and microstructure in the standard nutrient density positive control (PC) diets with a regular vitamin regimen. Tibial mineralization including (A) ash, (B) calcium (Ca), (C) phosphorus (P) and (D) density, as well as (E) tibia-breaking strength; (F) toluidine blue stained and the morphometric analysis for (G) the trabecular bone area (Tb.Ar), (H) trabecular thickness (Tb.Th), (I) number (Tb.N), and (J) spacing (Tb.Sp) determined by histomorphometry. Real-time-PCR analysis of osteocyte marker genes including (K) phosphate regulating endopeptidase homolog x-linked (Phex), (L) sclerostin, and (M) dentin matrix protein 1 (Dmp1) in the tibia proximal end. Values are means ± standard deviation represented by vertical bars (n = 8). * Mean values are significantly different (Student’s t-test, P < 0.05).
3.1.4. Bone resorption

We explored the role of 25-OH-D3 in bone turnover by first investigating whether 25-OH-D3 altered the status of bone resorption. Thus, the osteoclast number and activity were evaluated histologically and biochemically in the present study, and these data showed that there was an apparent decrease in TRAP-positive cells from 25-OH-D3-fed birds (Fig. 4A), and supplementation of 25-OH-D3 reduced the N.Oc/BS by approximately 25% when compared to the control diets (Fig. 4B). The circulating concentrations of TRAP and CTx that reflect bone resorption were also decreased by the 25-OH-D3 diets as compared to the control diets (Fig. 4C and D). We then examined the effects of 25-OH-D3 on mRNA expression of osteoclastogenesis-related factors in bone. Here, the diets contained 25-OH-D3 increased (P < 0.05) the expression level of osteoprotegerin (OPG) mRNA, thereby decreasing (P < 0.05) the receptor activator of nuclear factor κB ligand (RANKL)-to-OPG ratio when compared to control diets (Fig. 4E to G). In addition, the ducks receiving 25-OH-D3 presented a significant decrease (P < 0.05) mRNA expression level of cathepsin K and a similar vacuolar H+-ATPases (V-ATPases) mRNA abundance than those fed the control diets (Fig. 4H and I). Together, these results suggest that 25-OH-D3 treatment suppressed osteoclast differentiation and activity.

3.1.5. Bone formation

Effects of 25-OH-D3 treatment on bone formation were assessed by serum biochemical parameters (Fig. 5A and B). No obvious differences in the serum ALP activity and P1NP level as the indicators of bone formation were observed in the presence and absence of 25-OH-D3 diets, suggesting that 25-OH-D3 is unlikely to be a positive regulator of bone formation.

3.1.6. Serum Ca, P, and calcitropic hormones

As shown in Fig. 5, 25-OH-D3 treatment significantly increased (P < 0.05) serum 25-OH-D and Ca levels when compared to the control group (Fig. 5C and D), whereas serum P levels were not altered by the 25-OH-D3 manipulation (Fig. 5E). Also, serum PTH concentrations were comparable between the control and 25-OH-D3-treated ducks (Fig. 5F), which indicates that the dosage of 25-OH-D3 employed in this study did not induce hypercalcemia.

3.2. Experiment 2: tibial quality responses to 25-OH-D3 and vitamin regimens in LND diets

3.2.1. Performance

Data on the growth performance of meat ducks are presented in Table 4. We found a significant increase (P < 0.05) in terms of BW on d 35 and BW gain from d 15 to 35 in the high vitamin diets as compared to regular vitamin diets. Likewise, supplementation of 25-OH-D3 markedly increased (P < 0.05) the BW on d 14 and BW gain from d 1 to 14, as well as decreased F:G (d 1 to 14) in regular but not high vitamin diets (Table 4).
3.2.2. Tibial characters

For leg abnormalities, there was no substantial effect \((P > 0.05)\) of dietary treatments on the mortality due to leg abnormalities of meat ducks during trial period (Fig. 6A); nevertheless, supplementation of 25-OH-D\(_3\) significantly decreased the TD score in regular but not high vitamin LND diets (Fig. 6B). With regard to tibial growth, no differences were found among all groups neither in length nor width (Fig. 6C and D). In addition, tibial fat-free weight was significantly increased \((P < 0.05)\) by 25-OH-D\(_3\) in regular vitamin diets (Fig. 6E).

Take account of tibial mass, under the circumstances of the absence of 25-OH-D\(_3\), birds receiving high vitamin diets had significantly higher \((P < 0.05)\) tibia strength, mineral depositions of Ca and P, and density than ducks receiving the regular vitamin diets. Dietary supplementation of 25-OH-D\(_3\) significantly increased \((P < 0.05)\) tibial mineral content, density and strength in regular but not high vitamin diets (Fig. 7). Taken together, these results suggest that a diet with high vitamin regimens probably is beneficial to tibial mass, and addition of 25-OH-D\(_3\) to regular vitamin diet could significantly increase the mineral content of the tibia.

3.2.3. Bone metabolism and serum biochemistry

Analyzing bone resorption by histomorphometry and serum biochemical parameters revealed that a significant increase \((P < 0.05)\) in TRAP-positive cells was observed in regular vitamin group when compared to high vitamin group (Fig. 8A). Dietary 25-OH-D\(_3\) decreased \((P < 0.05)\) TRAP-positive cells, serum TRAP activity and CTx level in regular but not high vitamin group (Fig. 8A to C). In addition, ducks consuming the high diets presented a significantly lower \((P < 0.05)\) TRAP activity and CTx level in serum than those fed the regular vitamin diets (Fig. 8B and C). As far as bone formation is concerned, the values of ALP were higher in regular diets when compared with those in high diets, but supplementation of 25-OH-D\(_3\) did not change the serum ALP activity within each dietary vitamin regimen (Fig. 8D). Similarly, the addition of 25-OH-D\(_3\) and vitamin regimens did not result in any detectable difference in serum P1NP among all groups (Fig. 8E).

Furthermore, the serum 25-OH-D was higher in the high vitamin diets than that in the regular vitamin diets (Fig. 8F). Supplementation of 25-OH-D\(_3\) notably increased \((P < 0.05)\) serum 25-OH-D in regular and high vitamin groups, Ca and P levels in the
For serum PTH, no influence of vitamin regimens or 25-OH-D3 in feed was observed among all groups (Fig. 8I). Overall, these results suggest that the actions of 25-OH-D3 on reducing bone resorption, and enhancing serum Ca and P levels depend on the dietary basal vitamin level of LND diets.

### Table 4

Growth performance of meat ducks in response to 25-hydroxycholecalciferol (25-OH-D3) administration in low nutrient density (LND) diets with 2 vitamin regimens.1

| Item                  | Vitamin regimen | 25-OH-D3 | 25-OH-D3 | Gain, g/duck | Feed intake, g/duck | Feed:Gain, g: g |
|-----------------------|----------------|----------|----------|--------------|---------------------|-----------------|
|                       | Regular        | -        | +        | d 1          | d 14                | d 35             | d 1 to 14      | d 15 to 35     | d 1 to 14      | d 15 to 35     |                   |
| Body weight, g/ducks  |                |          |          | 48.48        | 624.14             | 2,056.86         | 579.93         | 1,427.08       | 734.09         | 3,468.39       | 1.27a 2.46b      |
| Gain, g/duck          |                |          |          | 48.54        | 669.95             | 2,111.32         | 621.41         | 1,437.11       | 742.62         | 3,530.03       | 1.19b 2.46b      |
|SEM                    |                |          |          | 0.039        | 10.2               | 39.01            | 10.44          | 40.96          | 16.66          | 70.45          | 0.02 0.06       |
| High                  |                | -        | +        | 48.46        | 650.21             | 2,181.89         | 601.75         | 1,529.78       | 742.46         | 3,720.54       | 1.23b 2.43b      |
|                       |                | 48.55    | 674.93   | 2,213.35     | 626.38             | 1,535.68         | 753.08         | 3,569.74       | 1.20a 2.33b     | 1.20a 2.33b     |
|                       |                |          |          | 0.029        | 7.17               | 28.08            | 7.41           | 28.96          | 11.96          | 49.81          | 0.01 0.04       |
| SEM                   |                |          |          | 0.039        | 7.17               | 28.08            | 7.41           | 28.96          | 11.96          | 49.81          | 0.01 0.04       |
|                       |                |          |          | 0.028        | 7.41               | 27.13            | 7.17           | 29.98          | 11.57          | 51.57          | 0.02 0.04       |
|                       |                |          |          | 0.229        | 0.028              | 0.008            | 0.207          | 0.022          | 0.583          | 0.052          | 0.598 0.228     |
|                       |                |          |          | 0.177        | 0.004              | 0.289            | 0.004          | 0.850          | 0.578          | 0.539          | 0.023 0.397     |
|                       |                |          |          | 0.658        | 0.425              | 0.774            | 0.442          | 0.961          | 0.951          | 0.150          | 0.329 0.414     |

### Footnotes
1 Ducklings were all fed a normal nutrient density starter diet until d 14, subsequently, they were subjected to an LND diet until d 35.

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![Fig. 6](image-url)

**Fig. 6.** Leg weakness and tibial growth responses to 25-hydroxycholecalciferol (25-OH-D3) and vitamin regimen in a low nutrient density (LND) diet for meat ducks: (A) mortality due to leg abnormalities. (B) tibial dyschondroplasia (TD) score. Tibial growth was determined through (C) tibial length, (D) width, and (E) fat-free weight. Values are means ± standard deviation represented by vertical bars (n = 8). a, b Different letters on bars means a significant difference (two-way ANOVA, P < 0.05, Tukey’s post hoc test).
4. Discussion

For many years, selection for growth rate has been associated with an increase in leg disorders due to this weight redistribution, which results in considerable attention being given to the morphological characteristics and composition of leg bones. Many factors can affect bone properties, including genetics, sex, growth rate, bone deformities, nutrition, infection, and stocking density, etc. (Bradshaw et al., 2002). Specifically, differences between sexes have been reported in bone growth and composition of broilers by Yalçın et al. (2001). For this reason, the male ducks only were selected as research subjects to exclude gender implications. Herein we investigated the repercussions of 25-OH-D3 supplementation in the tibial mass of meat ducks. Our data showed that 25-OH-D3 improved the tibial mass of meat ducks by suppressing osteoclast-mediated bone resorption that corroborates the lower number of osteoclasts, decreased serum TRAP activity and CTx level, as well as the downregulation osteoclast-specific marker genes expression in trabecular bone. Furthermore, 25-OH-D3 supplementation increased tibial mass of meat ducks by suppressing osteoclast-mediated bone resorption that corroborates the lower number of osteoclasts, decreased serum TRAP activity and CTx level, as well as the downregulation osteoclast-specific marker genes expression in trabecular bone. Furthermore, 25-OH-D3 supplementation increased tibial mass which was characteristic of higher minerals deposition and strength in regular but not high vitamin regimen when birds fed the LND diets, showing that the effects of 25-OH-D3 on the tibial mass of meat ducks depended on the dietary basal vitamin level of the LND diets.

Findings from previous experiments indicated that supplementation of vitamin D3 at 2,000 IU/kg increased the BW of broilers (Gomezverduzco et al., 2013). The positive effect of supplementing 25-OH-D3 was also reflected in an increased BW gain and feed conversion ratio (Santiago et al., 2016). The present study similarly demonstrated that 25-OH-D3 administration significantly increased BW and BW gain on d 14. Moreover, a beneficial impact of 25-OH-D3 on leg problems of meat ducks was also noticed in the present study, which was confirmed by the lower mortality due to leg abnormalities and TD score in the 25-OH-D3 group. These results were in agreement with the study conducted by Khan et al. (2010) who reported that a feeding level of 1,500 or 3,500 IU/kg of vitamin D3 notably decreased mortality and TD score in broilers. Vitamin D3 exerts a variety of actions on bone health of broilers. These actions were presented by the study of Światkiewicz et al. (2017), which showed that the supplementation of vitamin D at about 3,000 IU/kg, much higher than National Nutrition Council (1994) recommendations, is optimal for mineral digestibility and bone quality of broilers. In the present study, supplementing 25-OH-D3 at 0.069 mg/kg (the equivalent of vitamin D3 at 2,760 IU/kg) to PC diets that containing regular vitamin regimen was found to significantly increase tibial minerals deposition and density for the 35-d-old meat ducks. These findings were in accordance with previous studies conducted in broilers (Santiago et al., 2016; Sun et al., 2013; Wideman et al., 2015). Morphometric analyses further demonstrated that dietary 25-OH-D3 administration significantly increased the Tb.Ar and Tb.N, and decreased the Tb.Sp of tibias in our current trial. Reports have also consistently showed that treated with eldecalcitol, an active vitamin D analog, increased bone volume by increasing Tb.N and Tb.Th, as well as decreasing Tb.Sp in tibias of mice (Nakamichi et al., 2017). It is well established that bone density and trabecular architecture are strongly related with yield strength of bone (Ito et al., 2002). Thus, enhancing trabecular microstructure and increasing the mineral content due to supplementation of 25-OH-D3 contributed to an increase in bone biomechanical strength in this study. Taken together, these findings suggested that supplementation with 25-OH-D3 improved tibial mass and strength. These conclusions were further bolstered by the upregulation of mRNA expression of Phex, sclerostin, and Dmp1 in 25-OH-D3-treated ducks. In general, osteocytes are the most cells in bone matrix that account for 90% to 95% of all bone associated cells. In addition to

![Fig. 7. Tibial mineralization responses to 25-hydroxycholecalciferol (25-OH-D3) and vitamin regimen in a low nutrient density (LND) diet for meat ducks: (A) tibial ash, (B) calcium (Ca), (C) phosphorus (P) and (D) density, as well as (E) tibia-breaking strength. Values are means ± standard deviation represented by vertical bars (n = 8). a, b Different letters on bars means a significant difference (two-way ANOVA, P < 0.05, Tukey’s post hoc test).](image-url)
regulating bone mass, osteocytes may determine bone quality (Milovanovic et al., 2013). An upregulation of osteocyte-specific genes implied that the increase of osteocyte number is probably associated with an enhanced bone mass.

In spite of the anti-rachitic activity of vitamin D in vivo, the impacts of vitamin D on osteoblast are still less uniform. Some studies showed a stimulation of osteoblast differentiation and mineralization after vitamin D treatment (Turner et al., 2014; van der Meijden et al., 2014), but also an inhibition of osteoblast differentiation in mice (Nakamichi et al., 2017) and in cultures of osteoblast lineage cells (Chen et al., 2012). This discrepancy could be partly explained by the stage differentiation of osteoblast, Ca condition, or the dose of vitamin D used in these studies (Lieben and Carmeliet, 2013; van der Meijden et al., 2014). In the present study, however, circulating bone formation markers, P1NP and ALP, were not discernibly altered by supplementation of 25-OH-D3, which suggested that the positive effect of 25-OH-D3 on bone mass of meat ducks is unlikely mediated by increasing bone formation.

In addition, vitamin D has also been reported to be a moderator of osteoclastic activity. TRAP, a histochemical and biochemical marker for osteoclast, allows for the examination of osteoclastic differentiation and function (Halleen et al., 2000). Our current results revealed that 25-OH-D3 decreased the number of TRAP-positive cells, consistent with previous studies, which reported that calcitriol, the active form of vitamin D, strongly inhibited the proliferation of osteoclasts (Li et al., 2017). In another study, vitamin D strongly stimulated the formation of osteoclasts and induced bone resorption (Sato et al., 2007). These controversial data may be due to the different concentrations and methods of vitamin D administration (Zarei et al., 2016). The effect of vitamin D on osteoclastic activity has largely been established to mediated via osteoblast, which mainly regulates the secretion of the RANKL and OPG (Baldock et al., 2006). Receptor activator of nuclear factor κB ligand binds to receptor activator of nuclear factor kappa-B (RANK) that expressed on the surface of osteoclast to induce osteoclast differentiation, whereas OPG acts as a decoy receptor by blocking the interaction of RANKL with its functional receptor RANK (Yamamoto et al., 2006). The ration of RANKL to OPG is therefore an important index of osteoclastogenic stimulation and plays a key role in the regulation of bone metabolism. In this study, dietary 25-OH-D3 increased the mRNA expression of OPG but did not significantly change the RANKL mRNA level, thereby decreasing the ratio of RANKL to OPG, and subsequently reduced the osteoclast numbers in trabecular bone. Such metabolic effects of active 25-OH-D3 were in line with a previous...
report that vitamin D decreased the osteoclast numbers by inducing the expression of OPG mRNA and decreasing the RANKL-to-OPG ratio in mice (Nakamichi et al., 2017). Besides, that some important enzymes such as V-ATPases and cathepsin K are secreted by mature osteoclasts to dissolve the organic and inorganic components of bone in the process of bone resorption (Fujisaki et al., 2007; Rihonen et al., 2007). Downregulation of cathepsin K mRNA expression by the 25-OH-D3 administration in our study indicated that 25-OH-D3 inhibited bone resorption in meat ducks. Decreased circulating bone resorption markers, TRAP and CTx, also further confirmed the inhibitory effect of 25-OH-D3 on tibia of meat ducks. Collectively, these results demonstrate that treating with 25-OH-D3 suppressed bone resorption by decreasing osteoclastic numbers and activities in meat ducks.

Moreover, studies indicated that the biochemical actions of 25-OH-D3 are highly dependent on the dietary vitamin regimen in ducks (Ren et al., 2016; 2017). In agreement with our results, 25-OH-D3 treatment significantly increased tibial mineral content, density and strength in the regular but not high vitamin diets. These observations suggest that the actions of 25-OH-D3 on improving bone mass depend on dietary basal vitamin level in LND diets. Also, the increase of tibial mass due to supplying 25-OH-D3 in the regular vitamin diets was accompanied by the decreased concentrations in serum TRAP and CTx, indicating that the positive effects of 25-OH-D3 on tibial mass were mainly because of the suppression of bone resorption in the LND diets with regular vitamin regimen. This differential response of dietary 25-OH-D3 to distinct vitamin regimens on tibial mass appears to be a dosage dependent or interaction between dietary vitamins. Specifically, the high premix had higher levels of all vitamins except biotin than regular premix in the present study.

Regarding to the effect of vitamin regimens on tibial mass. Our previous study found that supplementation of 25-OH-D3 in a high vitamin diet significantly promoted the minerals deposition of sterna compared to a low vitamin diet for 49-d-old meat ducks (Zhang et al., 2019). Consistent with this finding, feeding the high vitamin diets in this study improved the tibial fat-free weight, mineral deposition and strength compared to the regular vitamin diets, suggesting that the high vitamin diets had a positive effect on tibial mass. The increase of bone mass probably is a result of decreased bone turnover rate (Takeda et al., 2015), evidenced by the lower levels of both bone formation markers (ALP and P1NP) and bone resorption markers (TRAP and CTx) on the high diets than those on the regular diets.

There are 2 limitations in our study. The first is the bone histological assessment. Although the skills for the histomorphometry can be applied in other reports (Vidal et al., 2012), however, the bias from sampling, sectioning, acquisition images and data are inevitability. Therefore, we admit the possibility that some of our conclusions may include overestimation or underestimation of roles of 25-OH-D3 in tibial mass. The second is experiments using the LND diet. Due to birds possess the ability to regulate feed intakes based on the dilution of nutrient density, it was expected that birds receiving the LND diets could consume more foods than birds fed the PC diets (Kamran et al., 2008). The current study is failing to examine the effect of the nutrient density on feed intake of meat ducks. A possible explanation for this discrepancy might be there is a certain range of the nutrient density of duck to regulate feed intake based on the dilution of dietary nutrient density. Further studies would be essential to elucidate these possibilities.

5. Conclusions

This study provides further evidence proving the beneficial functions of 25-OH-D3 on the tibial mass of meat ducks. The supplementation of 25-OH-D3 to regular vitamin regimen increased the tibial mass of meat ducks in both PC and LND diets by suppression of bone resorption, and this positive impact only was observed in regular but not high vitamin regimen when birds fed LND diets. In addition, the LND diets with high vitamin regimen made a favorable effect on the tibial mass of meat duck via decreasing bone turnover rate.

Author contributions

Huaiyong Zhang, Qifeng Zeng, Bing Yao and Keying Zhang: conceptualization; Huaiyong Zhang, Qifeng Zeng, Shiping Bai and Bing Yao: validation; Keying Zhang, Jianping Wang and Gregory S. Fraley: formal analysis; Shiping Bai, Jianping Wang and Xuemei Ding: investigation; Huaiyong Zhang, Qifeng Zeng, Xuemei Ding Yue Xuan and Zhoutuwei Su: data curation; Huaiyong Zhang, Gregory S. Fraley, Shiping Bai and Keying Zhang: writing — original draft preparation; Huaiyong Zhang, Qifeng Zeng and Shiping Bai: writing — writing and editing; Gregory S. Fraley, Jianping Wang and Keying Zhang: visualization; Huaiyong Zhang, Xuemei Ding and Qifeng Zeng: supervision; Keying Zhang: project administration and funding acquisition.

Conflict of interest

We declare that we have no financial and personal relationships with other people or organizations that can inappropriately influence our work, there is no professional or other personal interest of any nature or kind in any product, service and/or company that could be construed as influencing the content of this paper.

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