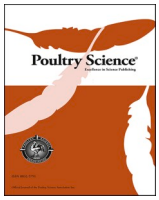
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Full-Length Article

Tibial adaptations to dietary 25-hydroxyvitamin D_3 supplementation under two distinct vitamin regimens in young ducks

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ABSTRACT

Tibia disorders in modern ducks are frequently associated with rapid weight gain and compromised bone quality, which is defined as the structural and material properties of bone tissue that determines its strength and fracture resistance. These factors significantly increase the risk of fractures and chronic pain. Studies suggest that 25-hydroxycholecalciferol (**25-OH-D_3**), a vitamin D_3 metabolite, effectively addresses bone disorders, potentially depending on dietary vitamin regimens, which are determined by the amount and ratio of vitamins in the diet. This study used a 2 × 2 factorial design to evaluate the effects of two vitamin regimens (regular and high) with or without exogenous 25-OH-D_3 (0.069 mg/kg) on leg health and tibia quality in meat ducks (1-14 d). The high-vitamin regimen contained greater amounts of all vitamins except biotin and significantly enhanced ash content, tibial microstructure, fracture load, and reduced tibial dyschondroplasia (**TD**) scores ($P < 0.05$) compared to the regular regimen, despite no impact on growth performance or tibia length, weight, and diameter ($P > 0.05$). Additionally, dietary 25-OH-D_3 supplementation increased weight gain ($P < 0.05$), improved bone quality, and strengthened bone formation and resorption processes. Notably, under the regular vitamin regimen, 25-OH-D_3 reduced TD scores and enhanced weight gain, tibia mechanical properties, and the serum content of procollagen type I N-terminal propeptide (**PN1P**) that a marker of bone formation (all $P < 0.05$). However, these effects were diminished in ducks fed the high-vitamin diet. There were some interactions that were noticed regarding serum 25-OH-D_3 content, trabecular area, tibia fracture load, and PN1P levels in the present study ($P < 0.05$). In conclusion, the biochemical effects of 25-OH-D_3 were influenced by the baseline levels of dietary vitamins, a high-vitamin diet or treatment with 25-OH-D_3 in a regular vitamin diet improved bone quality and reduced tibial dyschondroplasia by enhancing bone formation.

Introduction

The tibia of bird is primarily composed of hydroxyapatite, organic components like collagen and osteocalcin, and various cells, and plays a critical role in supporting posture and locomotion, regulating mineral metabolism, and contributing to endocrine functions (Florencio-Silva et al., 2015). Maintaining bone integrity is essential for tibia function, which involves the removal of aged or damaged bones by osteoclasts and its subsequent replacement with new bone synthesized by osteoblasts (Kenkre and Bassett, 2018). Disruption of this balance, which favors bone resorption and/or inhibits bone formation, likely increases the risk of compromised bone ash content and microstructure, resulting in a higher likelihood of fractures (Kitaura et al., 2020). In recent years, the breeding of meat-type birds has prioritized growth performance and carcass traits, such as growth rate, feed conversion rate, and pectorales yield. However, the neglect of skeletal traits and their imbalance with rapid body growth may lead to an increased incidence of leg problems. It has been reported that the prevalence of tibial dyschondroplasia (**TD**) in commercial meat duck is estimated to be above 40 % and lead to about 2 % mortality due to leg abnormalities (Zhang et al., 2018). Data from meat-type birds with different growth rate suggested that osteoblasts are not adequately filled in the bone canal in fast growth cohort, resulting in cortical bone porosity and reduced bone quality (Williams et al., 2004). In addition, comparing long bone development between historical and contemporary ducks found that the younger ages and smaller body weight (**BW**) birds exhibited the lower epiphyseal ash values in tibia

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(Van Wyhe et al., 2012). With the continuous breeding of meat ducks, the imbalance between weight gain and bone growth has led to leg diseases such as leg bone malformation and tibial chondrodysplasia in meat ducks (Robison et al., 2015). Study on the tibia characteristics of meat ducks showed a rapid bone growth and mineralization happened during 1 to 35 d and 1 to 42 d (Zhang et al., 2019a), respectively. This evidence suggests that it is critical to monitor those factors such as nutrients which affect bone mass in young ducks that can adapt the changed BW and reduce bone disorders.

Several nutritional strategies including the application of low nutrient density (**LND**) diet and dietary supplementation of calcium (**Ca**) (Zhang et al., 2018), amino acids (Castro and Kim, 2020), vitamins (Zhang et al., 2020a), zinc glycine (Wang et al., 2023), and resistant starch (Zhang et al., 2022) have been used to improve the bone quality of ducks. Vitamin D, a lipid-soluble vitamin, plays multiple roles in the regulation of bone metabolism through facilitating the absorption of Ca and phosphate (**P**) (Christakos et al., 2011), directly modifying bone formation and resorption (Nakamichi et al., 2018; van Driel and van Leeuwen, 2017), and exerting antioxidant and anti-inflammatory properties (Xu et al., 2024; Zhang et al., 2021). Low vitamin D status can result in a higher incidence of bone abnormality in birds (Landy et al., 2018). Over the past 100 years, although many breakthroughs and discoveries have been revealed in relation to vitamin D research (Gallagher and Rosen, 2023), there are still some unsolved mysteries, including the upper limit for safe supplementation (Rizzoli, 2021), the interaction with its metabolism, and the exact mechanisms underlying how vitamin D influences bone metabolism. Vitamin D_3 has been used as the prevention of osteoporosis (Demay et al., 2024), dietary supplementation with 2000 IU vitamin D and 0.069 mg/kg 25-hydroxycholecalciferol (25-OH-D_3) has also been reported to inhibit the proliferation of osteoclasts of ducks (Zhang et al., 2020a). However, it was also reported that the addition of near-physiological levels of 1, 25-$(OH)_2$-D_3 (10^{-10} M-5.0 $\times$ 10^{-10} M) to the human monocyte culture enhanced differentiation of small size osteoclasts and resorption activity (Zarei et al., 2016). The discrepancies could be partly explained by the dosage which has been used in these studies and test objects. As far as meat duck is concerned, the recommended levels of vitamin D_3 are very different among nutrient requirements standards for meat ducks. Taking National Research Council (NRC, 1994) and China Agricultural Industry Standards (NY/T, 2122-2012) as an example, the recommendations of vitamin D_3 is 400 and 2,000 IU, respectively. This would affect the exogenous supplementation of vitamin D in practice, which was evidenced by previous study in meat ducks (Zhang et al., 2020a). It is well-established that vitamin D_3 is first converted into 25-OH-D_3 in the liver and then reaches the kidneys where it is hydroxylated into the hormone metabolite 1,25-$(OH)_2$-D_3 to fulfill the biological functions of vitamin D. Several experiments found that 25-OH-D_3 was not affected by intestinal cholic acid secretion and fat, and is more easily absorbed than vitamin D_3 (Bouillon and Quesada Gomez., 2023). Moreover, the conversion loss of vitamin D_3 in the liver can be avoided. A study has shown that compared with vitamin D_3, 25-OH-D_3 can effectively increase poultry weight, breast weight, and feed conversion rate, and promote bone development (Bar et al., 2003). Combining the synergistic effects of 25-OH-D_3 and 1,25-$(OH)_2$-D_3 as osteogenic inducers in human dental pulp (Khanna-Jain et al., 2010), it is possible that the treatment of vitamin D_3 derived from 25-OH-D_3 is more effective regarding to bone development of young ducks.

Given the dependence of 25-OH-D_3 supplementation on dietary vitamin regimen and the alteration of rapid weight gain affecting bone metabolism, the present study was conducted to evaluate the influence of dietary 25-OH-D_3 inclusion under two different vitamin regimens on leg problems and tibia quality of meat ducks from 1 to 14 d. In addition, the bone formation and resorption were also assessed to reveal the mechanism highlighting how dietary 25-OH-D3 and vitamin premix regulate bone turnover. The findings of this study offer valuable insights into the role of vitamins in the diets of meat-type birds, emphasizing their contribution to animal welfare rather than solely focusing on production efficiency.

Materials and methods

Birds and experimental design

All experiments were carried out following the guidelines of the Henan Agricultural University Experimental Animal Committee (approval No. 2022-021). A total of 384 1-day-old Cherry Valley meat-type male ducks were weighed and divided into 4 groups in a 2 $\times$ 2 factorial design with 2 vitamin regimens (Regular and High, Table 1) and 2 dietary 25-OH-D_3 concentrations (0 and 0.069 mg/kg). Each treatment included 7 replicates with 16 ducks per pen (2.2 $\times$ 1.2 $\times$ 0.9 m). The regular and high vitamin regimens referred to the recommendation of NRC (1994) and NY/T (2012), respectively. The dose of 25-OH-D_3 was based on our previous research in meat ducks (Zhang et al., 2020a). All vitamins used in this study were provided by DSM Ltd. (Shanghai, China). The experimental period was 14 d. All birds were fed the same basal diets to meet the nutrient requirements of Cherry Valley meat duck according to the recommendations of NY/T (2012, Table 2). Feed was supplied in pellet form, and the diameter of the pellets was 2 mm in the starter and 3 mm in the grower diet. Ducks were reared in a temperature- and humidity-controlled room and had free access to feed and water throughout the experimental period. The numbers of death and culling ducks, BW, and feed intake (**FI**) were recorded during the 14-d feeding period, and weight gain, the ratio of feed to gain, and survival proportion were calculated on a pen basis.

Evaluation of tibia abnormality

The mortality due to leg deformations, which is defined as the percentage of mortality or culled ducks due to tibia deformations and fractures, was recorded during the feeding period on a pen basis. Moreover, following the method described by Zhang et al. (2018), three birds were randomly selected from each pen, and the right tibia was evaluated for TD by longitudinally cutting the proximal end and assessing the proportion of cartilage, as following: 0 = normal bones with no cartilage lesions; 1 = cartilage developed in up to one-third of the cut area, 2 = cartilage developed in one third to one half of the cut area, 3 = cartilage developed in more than one half of the cut area. The incidence of TD was defined as a percentage with TD score > 0 based on this TD scoring system. One of the left tibiae from each pen was collected for dimension, weight, and materialization evaluation.

Table 1
Composition of the vitamin premixes for ducking (0-14 d)[1].

Item	Vitamin regimens		
	Regular	**High**	**Chang fold**
Fat soluble vitamin			
Vitamin D_3 (IU)	400	2000	5.00
Vitamin K_3 (mg)	0.5	2	4.00
Vitamin A (IU)	2500	4000	1.60
Vitamin E (IU)	10	20	2.00
Water-soluble vitamin			
Vitamin B_{12} (mg)	0.01	0.02	2.00
Pantothenic Acid (mg)	11	20	1.82
Folic Acid (mg)	0.55	1	1.82
Vitamin B_2 (mg)	4	10	2.50
Vitamin B_6 (mg)	2.5	4	1.60
Vitamin B_1 (mg)	1.8	2	1.11
Biotin(mg)	0.15	0.15	1.00
Niacin (mg)	55	50	0.91

Regular and high vitamin regimen referred to the vitamin levels recommended by the National Research Council (NRC, 1994) and the China Agricultural Industry Standards (NY/T, 2122-2012), respectively.
[1] Supplied in per kilogram of diet.

Table 2
Composition and nutrient levels in basal diets.

Ingredients, %	Day 1 to 15
Maize	58.05
Soya oil	3.00
Soybean meal	34.80
L-Lysine. HCl	0.08
DL-Methionine	0.145
L-Threonine	0.03
Choline chloride	0.20
Sodium chloride	0.30
Mineral premix [1]	0.20
Vitamin premix [2]	0.03
Limestone	1.06
Dicalcium phosphate	1.75
Bentonite	0.355
Total	100.00
Nutrient content, %	
Apparent metabolizable energy (kcal/kg)	2900
Crude protein	20.05
Digestible lysine	1.02
Digestible methionine	0.42
Calcium	0.90
Total P	0.65
Non-phytate P	0.42

[1] Provided per kilogram of diet: Cu ($CuSO_4 \bullet 5H_2O$), 8 mg; Fe ($FeSO_4 \bullet 7H_2O$), 80 mg; Zn ($ZnSO_4 \bullet 7H_2O$), 90 mg; Mn ($MnSO_4 \bullet H_2O$), 70 mg; Se ($NaSeO_3$), 0.3 mg; I (KI), 0.4 mg.
[2] Provided per kilogram of diet: Regular or High vitamin premixes as shown in Table 1.

Sampling

At d 15, one duck was selected from each pen based on its average BW, fasted for 12 h, and blood was collected via the jugular vein for serum Ca, P, total vitamin D_3, parathyroid hormone (**PTH**), and biomarkers of bony turnover determination. After euthanizing, the left tibial bones (the proximal end) were dissected and immediately flash-frozen in liquid N_2 for mRNA analyses. The proximal end of the right tibia was removed and fixed in 4 % paraformaldehyde for morphological analysis.

Biomechanical testing

Destructive biomechanical testing of tibia was performed by the texture analyzer (TA.XT. Plus, Stable Microsystems Ltd., Godalming, UK), employing a 3-point method. The tibia was rested on two points with a gap of 50 mm and pressure was perpendicularly applied at the center of the two points up to the breaking of the bone. Force-displacement data were recorded for bone stiffness, yield load, fracture load and the area under-the-curve (**AUC**) determination. The stiffness and yield load were defined as the slope of the linear portion of the load-displacement curve and the load at which the load-deformation relationship ceased to be linear, respectively (Turner, 2006).

Determination of bone mass

After undergoing biomechanical testing, the bone fragments were deposited in measuring cylinders to measure the volume (cm^3) based on the method of discharging volume. These tibias were then defatted by immersing petroleum ether for 48 h and oven-dried at 108°C for 24 h for the defatted weight (g) determination, thus the tibia density (g/cm^3) was calculated as the previously described (Zhang et al., 2019b).

$$\text{Density}\ \left(g/cm^3\right) = \text{Defatted weight}\ (g)/\text{Tibia volume}\ \left(cm^3\right)$$

Subsequently, the ash was determined by placing the tibia in a muffle furnace at 550°C for 24 h. Ca and P contents were also determined through Ethylenediaminetetraacetic acid (**EDTA**) titration and ammonium metavanadate colorimetry, respectively. The values of ash, Ca, and P were presented based on dry-defatted weight.

Bone microstructure measurement

The fixed tibias were decalcified with 10 % EDTA, embedded, sliced, and stained with toluidine blue. The micrographs of the bone sections were taken using a microscope (Nikon Eclipse TS100; Nikon Corporation, Tokyo, Japan). Total area (**T.Ar**), calcified bone area (**B.Ar**), and bone perimeter (**B.Pm**) were measured from at least 10 sections for each sample by a blinded examiner using Weibel Grid technique according to previously described procedure (Vidal et al., 2012). The trabecular bone area (**Tb.Ar**), trabecular number (**Tb.N**), thickness (**Tb.Th**) and spacing (**Tb.Sp**) are derived indirectly using the following equations:

$$\text{Tb.Ar} = B.\text{Ar}/\text{T.Ar} \times 100$$

$$\text{Tb.Th} = 2 \times B.\text{Ar}/\text{B.Pm}$$

$$\text{Tb.}N = (\text{B.Ar}/\text{T.Ar})\text{Tb.Th}$$

$$\text{Tb.Sp} = (1/\text{Tb.N}) - \text{Tb.Th}$$

Morphological analysis

Paraffin-embedded slice samples of the tibias were subjected to staining for alkaline phosphatase (**ALP**) and tartrate-resistant acid phosphatase (**TRAP**), according to the manufacturer's protocol (Sigma-Aldrich, Inc., St. Louis, MO). Images were obtained using a Nikon Eclipse imaging microscope (Nikon Corporation, Tokyo, Japan). Osteoblast and Osteoclast were counted on the external surfaces of the bone using the surgical defect as the field of view. A single operator at two separate time points performed the quantification using Image Pro-Plus (Media Cybernetics, Inc., Rockville, MD).

Quantitative RT-PCR

Total RNA was extracted from frozen the proximal end of the tibia, and the RNA quality (intact ribosomal RNA 28s/18 s) was evaluated by agarose gel electrophoresis. The cDNA was synthesized from 200 ng of total RNA. The expression of interested mRNA in bone was detected using a Fast SYBR Green Master Mix protocol (Takara Biotechnology Co., Ltd., Dalian, China) in the ABI 7500 Real-Time PCR detection system (Applied Biosystems, Inc., Foster City, CA). Primers were designed using online Primer 3 and showed in Table 3. *β-actin* and glyceraldehyde-3-phosphate dehydrogenase (***GAPDH***) were selected as the reference genes and normalized the relative expressions of targeted genes, which included vitamin D receptor (***VDR***), osteoblastic and osteoclastic genes such as alpha-type I collagen (***Col1a***), *osteocalcin, ALP,* phosphate-regulating endopeptidase homolog X-linked (***Phex***), sclerostin (***Sost***), dentin matrix protein 1 (***Dmp1***), receptor activator of nuclear factor-κ B ligand (***RANKL***), osteoprotegerin (***OPG***), *cathepsin K,* and vacuolar-type H^+-ATPase (***V-ATPase***).

Serum biochemistry

The levels of Ca, total P, ALP, TRAP, C-terminal cross-linked telopeptide of type I collagen (**CTx**) concentration, and procollagen type I N-terminal propeptide (**P1NP**) were measured using commercial assay kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China). PTH concentration in serum was quantified by using an enzyme-linked immunosorbent assay (ELISA, LifeSpan BioSciences, Inc., Seattle, WA). Total vitamin D_3 was quantified by a commercial ELISA kit (ALPCO Diagnostics, NH) according to the manufacturer's suggested protocol.

Table 3
The primers for quantitative real-time PCR.

Gene	Gene ID	Primer	Sequence (5′−3′)	Size (bp)
VDR	XM_027445496.2	Reverse	GGAGCAGCAGAAAGTCATCG	88
		Forward	GGGCCGAAATTTGGTGAAGT	
Phex	XM_005010837.3	Reverse	TGCCAACTATCTGGTGTGGA	99
		Forward	CCGTAGATCACCCGAGAAAA	
Dmp1	XM_005012780.3	Reverse	AACCTTGGTCACCTTCATGC	92
		Forward	TCGGCAAAGTCCTGCTCTAT	
Sost	XM_005026106.3	Reverse	GGAAGGGTGGCAAGTGTTTA	115
		Forward	TGCCTGGTTCATTGTGTTGT	
Col1a	XM_021275738.1	Reverse	AAGAACAGCGTCGCCTACAT	89
		Forward	GCCCTGATCTCGATTTCGT	
ALP	XM_005031069.3	Reverse	GGCATCTCTCCATCCGAGTA	115
		Forward	AGGATGAAGGCAAGGCAGT	
Osteocalcin	XM_005024562.3	Reverse	GAAGTTGCCAATGCCTTTGT	85
		Forward	GCTCCATTGGGGTTTTGTAA	
Cathepsin K	XM_021277116.1	Reverse	ACTGCTGGTCCTGTTTGTCC	98
		Forward	GCTTGCGGTACGTTTTCTTC	
V-ATPase	XM_021267166.1	Reverse	TCCGTGTCTGGTTCATCAAA	111
		Forward	CAGGACACCAGACTTCAGCA	
OPG	XM_005017709.3	Reverse	GCCTAACTGGCTGAACTTGC	106
		Forward	GAAGGTCTGCTCTTGCGAAC	
RANKL	XM_021276016.1	Reverse	GCCTTTTGCCCATCTCATTA	100
		Forward	TAAGTTTGCCTGGCCTTTGT	
β-actin	NM_001310408.1	Reverse	CCAGCCATCTTTCTTGGGTA	105
		Forward	GTGTTGGCGTACAGGTCCTT	
GAPDH	XM_005016745.3	Reverse	TTTTTAACCGTGGCTCCTTG	94
		Forward	ACTGGGCATGGAAGAACATC	

VDR, Vitamin D receptor; Phex, Phosphate regulating endopeptidase homolog x linked; Dmp1, Dentin matrix protein 1; Sost, Sclerostin; Col1a, Alpha-type I collagen; ALP, Alkaline phosphatase; V-ATPase, Vacuolar-type H^+-ATPase; OPG, Osteoprotegerin; RANKL, Receptor activator of nuclear Factor-κ B ligand. GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

Statistical analysis

The statistical analysis was performed using JMP statistical software (SAS Institute). A statistical power of 0.80 (80 %) was obtained when the detectable effect size and significance level was set up at 1.0 and 0.05, respectively. Survival proportions were constructed using Kaplan-Meier method and analyzed by the log-rank test for trend. Data were analyzed by two-way analysis of variance (**ANOVA**) and Tukey's *post hoc* test after checking the normal distribution and homogeneity of variances. The statistical model included the main effects of dietary vitamin regimen, 25-OH-D_3 levels, and their interaction, as following:

$$Y_{ij} = \mu + \alpha_i + \beta_j + \gamma_{ij} + \varepsilon_{ij}$$

Here, Y_{ij} is the average of treatment *j*, μ is the overall average, α_i *and* β_j are the fixed effects of the dietary vitamin regimen and 25-OH-D_3, respectively, γ_{ij} is the interaction effect between the vitamin regimen and 25-OH-D_3, and ε_{ij} is the error term. $P \leq 0.05$ and $p < 0.1$ were defined as statistically significant and tendency, respectively.

Results

Growth performance and tibial dyschondroplasia accidence

As shown in Table 4, the vitamin regimens had no significant effect on growth performance ($P > 0.05$). However, dietary supplementation with 25-OH-D_3 significantly increased BW at 14 d and weight gain from d 1 to 14 (both $P < 0.05$), without affecting FI or the ratio of FI to weight gain during the study period. Additionally, there was no interaction between vitamin regimens and 25-OH-D_3 supplementation on growth performance in meat ducks ($P > 0.05$).

Regarding the incidence of TD, both the high-vitamin diet and 25-OH-D_3 supplementation notably reduced TD scores ($P < 0.05$). No interaction effect was observed between vitamin regimens and 25-OH-D_3 inclusion. Notably, 25-OH-D_3 supplementation significantly decreased TD scores in ducks fed the regular vitamin diet but not the high-vitamin diet (Fig. 1A, B). Meanwhile, neither the vitamin regimens nor 25-OH-D_3 supplementation had an obvious effect on mortality due to leg abnormalities or survival rates throughout the trial period (Fig. 1C, D).

Table 4
The performance of bird response to different vitamin regimen and 25-OH-D_3 treatments.

Item		Body weight, g/bird		Gain, g/bird	Feed intake, g/bird	F: G, g/g
		1 d	14 d	1-14 d	1-14 d	1-14 d
Regimen	25-OH-D_3					
Regular	-	48.48	630.06[b]	581.58[b]	728.69	1.25
	+	48.56	670.04[a]	621.48[a]	742.80	1.19
High	-	48.47	657.92[ab]	609.46[ab]	753.25	1.24
	+	48.55	674.93[a]	626.38[a]	753.08	1.20
SEM		0.04	10.64	10.64	17.56	0.02
Regimen						
Regular		48.52	650.05	601.53	735.75	1.22
High		48.51	666.43	617.92	753.17	1.22
SEM		0.03	7.52	7.52	12.42	0.02
25-OH-D3						
-		48.47	643.99[b]	595.52[b]	740.97	1.24
+		48.56	672.49[a]	623.93[a]	747.94	1.20
SEM		0.03	7.52	7.52	12.42	0.02
P-value						
Regimen		0.750	0.137	0.136	0.331	0.875
25-OH-D_3		0.173	0.013	0.011	0.695	0.052
Interaction		0.928	0.291	0.638	0.688	0.559

[a].
[b] Mean values with unlike letters were significantly different at $P < 0.05$.
Regular and high vitamin regimen referred to the vitamin levels recommended by the National Research Council (NRC, 1994) and the China Agricultural Industry Standards (NY/T, 2122-2012), respectively.
F: G, the ratio of feed intake to weight gain.

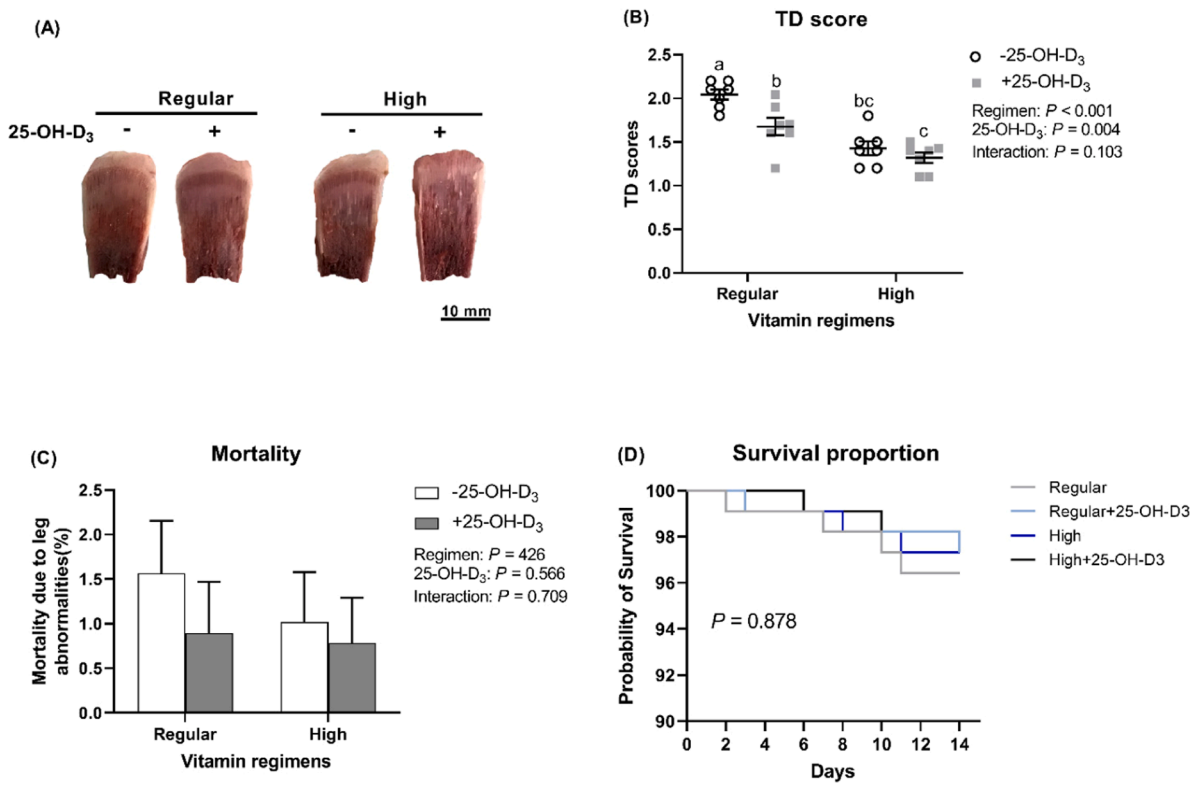


Figure 1. Effect of dietary vitamin regimen and 25-OH-D_3 on (A, B) tibial dyschondroplasia (TD) score, (C) the mortality due to leg abnormalities, and survival proportion of 14-d-old ducks. Data represent means with standard deviation represented by vertical bars ($n = 7$). [a-c] Different letters on bars means a significant difference at $P < 0.05$.

Bone growth, bone mass, and mechanical properties of the tibia

Dietary treatments, including different vitamin regimens and the addition of 25-OH-D_3, had no significant effects on tibial length, diameter, fresh weight, relative weight, or defatted weight ($P > 0.05$; Table 5). However, feeding a high-vitamin regimen diet notably increased ash, Ca, and P content in the whole tibia compared to the regular vitamin diet, contributing to higher bone density. Similarly, enhanced tibial contents of ash, Ca, and P, as well as fracture load and AUC were also observed in diets supplemented with 25-OH-D_3 ($P < 0.05$), although no interaction effects were detected regarding bone mass and mechanical properties of 14-d-old ducks (Table 6).

In terms of tibial mechanical properties, no apparent differences were observed in yield load or stiffness, as expressed by the slope of the linear portion of the load-displacement curve. Nevertheless, both the high-vitamin regimen and 25-OH-D_3 supplementation remarkably increased fracture load ($P < 0.01$) and reduced the AUC, indicating improved tibial mechanical strength. Notably, a significant interaction

Table 5
Effect of vitamin regimen and 25-OH-D_3 on tibia growth of 14-d-old ducks.

Item		Length, mm	Diameter, mm	Fresh weight, g	Relative weight, g/kg BW	Defatted weight, g
Regimen	25-OH-D_3					
Regular	-	72.59	4.55	4.35	0.72	1.55
	+	72.69	4.81	4.73	0.78	1.61
High	-	73.91	4.77	4.38	0.76	1.59
	+	74.44	4.76	4.91	0.81	1.68
SEM		0.91	0.08	0.12	0.02	0.05
Regimen						
Regular		72.64	4.68	4.69	0.75	1.55
High		74.17	4.77	5.00	0.79	1.64
SEM		0.64	0.06	0.08	0.01	0.03
25-OH-D_3						
-		73.25	4.66	4.58	0.74	1.55
+		73.56	4.79	5.12	0.80	1.64
SEM		0.64	0.06	0.08	0.01	0.03
P-value						
Regimen		0.104	0.309	0.815	0.753	0.071
25-OH-D_3		0.735	0.129	0.734	0.803	0.097
Interaction		0.813	0.813	0.260	0.868	0.902

[a,b]Mean values with unlike letters were significantly different at $P < 0.05$.
Regular and high vitamin regimen referred to the vitamin levels recommended by the National Research Council (NRC, 1994) and the China Agricultural Industry Standards (NY/T, 2122-2012), respectively.
BW, Body weight.

Table 6
Effect of vitamin regimen and 25-OH-D_3 on tibia mass and mechanical properties of 14-d-old ducks.

Item		Bone mass				Mechanical properties			
		Ash, %	Ca, %	P, %	Density, g/cm^3	Slope, N/mm	Yield load, N	Fracture load, N	AUC, N*mm
Regimen	25-OH-D_3								
Regular	-	43.87^{b}	15.80^{b}	8.20^{b}	0.39^{b}	116.50	108.93	115.43^{c}	310.81^{a}
	+	46.00^{ab}	17.71^{ab}	9.09^{ab}	0.43^{ab}	136.70	138.81	166.08^{b}	257.46^{b}
High	-	46.56^{a}	18.40^{a}	9.16^{ab}	0.44^{ab}	128.64	125.50	176.34^{b}	251.62^{b}
	+	47.30^{a}	19.76^{a}	9.86^{a}	0.46^{a}	148.08	146.93	193.23^{a}	232.35^{b}
SEM		0.633	0.539	0.271	0.016	16.21	13.28	5.68	24.09
Regimen									
Regular		44.93^{b}	16.76^{b}	8.644^{b}	0.41^{b}	126.60	123.87	145.76^{b}	295.14
High		46.92^{a}	19.08^{a}	9.509^{a}	0.45^{a}	154.86	136.21	184.79^{a}	236.98
SEM		0.448	0.381	0.191	0.011	11.460	9.391	4.021	17.030
25-OH-D_3									
-		45.21^{b}	17.10^{b}	8.68^{b}	0.41	122.57	117.21	150.89^{b}	281.22^{a}
+		46.65^{a}	18.73^{a}	9.47^{a}	0.44	158.89	142.87	179.66^{a}	250.91^{b}
SEM		0.448	0.381	0.191	0.011	11.460	9.391	4.021	17.030
P-value									
Regimen		0.004	<0.001	0.004	0.049	0.494	0.382	<0.001	0.238
25-OH-D3		0.033	0.006	0.008	0.068	0.144	0.065	<0.001	0.021
Interaction		0.284	0.611	0.725	0.434	0.336	0.753	0.037	0.049

[a].
[b] Mean values with unlike letters were significantly different at $P < 0.05$.
Regular and high vitamin regimen referred to the vitamin levels recommended by the National Research Council (NRC, 1994) and the China Agricultural Industry Standards (NY/T, 2122-2012), respectively.
AUC, area under the load-displacement curve.

($P < 0.05$) was observed, indicating that the effect of 25-OH-D_3 supplementation was more pronounced in ducks feeding the regular vitamin diet compared to those on the high-vitamin diet (Table 6).

Tibial microstructure

The effects of 25-OH-D_3 supplementation and vitamin regimens on tibial microstructure are illustrated in Fig. 2. Toluidine blue staining revealed a visible reduction in bone marrow area and a significant increase in trabecular area following 25-OH-D_3 treatment in both vitamin regimens (Fig. 2A). Quantitative analysis demonstrated that 25-OH-D_3 supplementation in both vitamin regimens notably increased Tb.Ar and Tb.N, while reducing Tb.Sp in the proximal tibia (all $P < 0.05$). In addition, compared to the regular vitamin diet, the high-vitamin premix diet further elevated Tb.Ar, Tb.Th, and Tb.N, while significantly decreasing Tb.Sp ($P < 0.05$). Interaction effects between 25-OH-D_3 and vitamin regimens were observed for Tb.Ar and Tb.Sp, with the impact of 25-OH-D_3 being more obvious in ducks fed the regular vitamin diet than those on the high-vitamin premix diet.

Serum calciotropic hormones

The supplementation of 25-OH-D_3 significantly increased serum levels of 25-OH-D_3 and Ca in both regular and high vitamin diet groups (both $P < 0.05$). High vitamin diets also elevated these concentrations compared to regular vitamin diets ($P < 0.05$). No significant changes were observed in P concentrations across the groups ($P > 0.05$). Additionally, the comparable PTH levels suggest that the doses of 25-OH-D_3 and vitamin premixes used did not induce hypercalcemia, ensuring safety at the tested levels (Table 7).

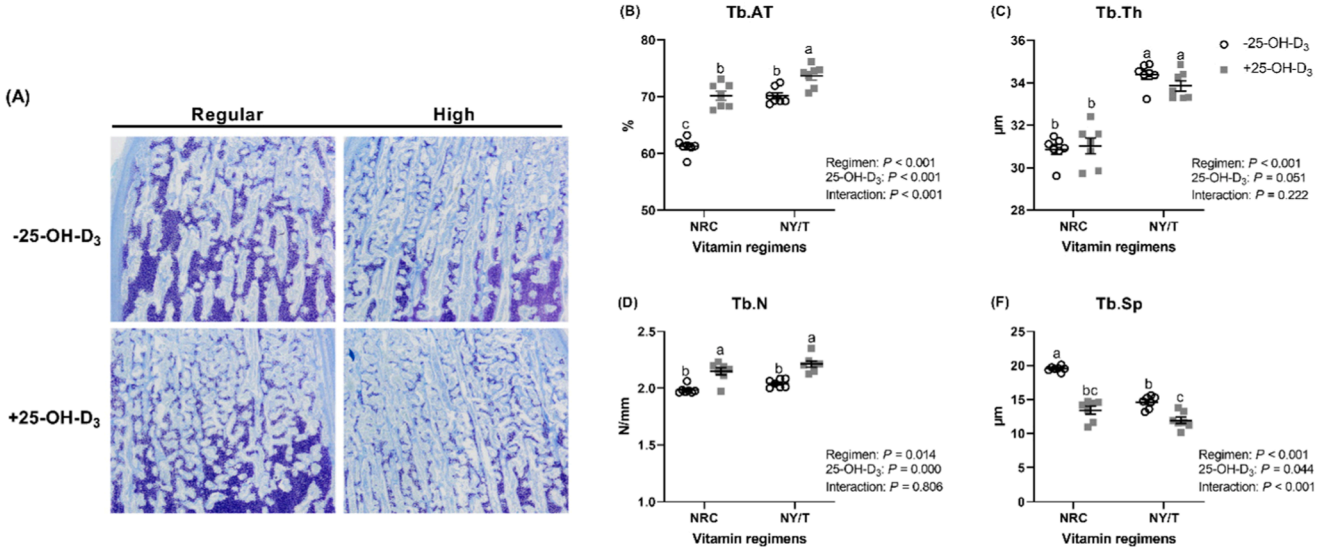


Figure 2. Bone microstructure response to dietary vitamin regimen and 25-OH-D_3 in 14-d-old ducks. (A) Toluidine blue stained and the morphometric analysis for (B) the trabecular bone area (Tb.Ar), (C) trabecular thickness (Tb.Th), (D) number (Tb.N), and (E) spacing (Tb.Sp) determined by histomorphometry. Data represent means with standard deviation represented by vertical bars ($n = 7$). [a-c] Different letters on bars means a significant difference at $P < 0.05$.

Table 7
Effect of vitamin regimen and 25-OH-D_3 on calciotropic hormones in serum.

Item		25-OH-D_3	Ca, mmol/L	P, mmol/L	PTH, ng/L
Regimen	25-OH-D_3				
Regular	-	216.17[d]	2.25[d]	5.95	33.60
	+	257.11[b]	2.53[b]	6.15	32.90
High	-	235.27[c]	2.43[c]	6.11	33.38
	+	293.86[a]	2.64[a]	6.20	30.70
SEM		3.93	0.03	0.09	1.33
Regimen					
Regular		236.64[b]	2.39[b]	6.05	33.25
High		264.57[a]	2.53[a]	6,15	32.04
SEM		2.78	0.02	0.06	0.94
25-OH-D_3					
-		225.72[b]	2.34[b]	6.03	33.49
+		275.48[a]	2.58[a]	6.18	31.80
SEM		2.78	0.02	0.06	0.94
P-value					
Regimen		<0.001	<0.001	0.262	0.371
25-OH-D_3		<0.001	<0.001	0.106	0.216
Interaction		0.034	0.327	0.553	0.461

[a].
[b] Mean values with unlike letters were significantly different at $P < 0.05$.

Regular and high vitamin regimen referred to the vitamin levels recommended by the National Research Council (NRC, 1994) and the China Agricultural Industry Standards (NY/T, 2122-2012), respectively.

Ca, calcium; P, phosphorus; PTH, parathyroid hormone.

Bone formation

The effects of dietary vitamin supplementation on bone formation were assessed histologically and biochemically, as shown in Fig. 3. Ducks fed high-vitamin diets exhibited increased levels of P1NP ($P < 0.05$) and ALP ($P = 0.078$) compared to those on the regular vitamin diet (Fig. 3A, B). Similarly, supplementation with 25-OH-D_3 significantly elevated serum concentrations of P1NP and ALP (both $P < 0.05$). A notable interaction effect ($P < 0.05$) was observed for serum P1NP levels, indicating that the enhancement of P1NP by 25-OH-D_3 was more prominent in ducks fed the regular vitamin diet compared to those receiving the high-vitamin diet (Fig. 3A). Histological analysis revealed a notable increase in the number of osteoblasts (N.Ob/BS, ALP-positive cells) in response to both high-vitamin regimens and 25-OH-D_3 supplementation (Fig. 3C, D).

As far as osteoblastic gene expressions are concerned, dietary 25-OH-D3 supplementation upregulated the expression of *VDR, Col1a*, and *osteocalcin* in the proximal tibia. The high-vitamin regimen further enhanced the expression of these genes compared to the regular vitamin diet. However, dietary treatments did not obviously alter the transcription of ALP in any group. Additionally, osteocyte-related genes were examined and showed that diets containing 25-OH-D_3 or the high-vitamin regimen significantly upregulated (both $P < 0.05$) the expression of *Phex* and *Dmp1*, while no apparent changes were observed in the mRNA abundance of *Sost* in the tibia (Fig. 3E).

Bone resorption

To assess osteoclast frequency, the proximal tibia was stained using TRAP kit, revealing a significant reduction in TRAP-positive cells following supplementation with 25-OH-D_3 ($P = 0.026$, Fig. 4A, B). Similarly, serum biomarkers of bone resorption, including TRAP activity and CTx levels, were decreased in ducks fed 25-OH-D_3 diets (Fig. 4C, D). The manipulation of vitamin regimens and 25-OH-D_3 supplementation did not significantly alter the expression levels of *RANKL* or *OPG*, key factors related to osteoclastogenesis (Fig. 4E, F), whereas 25-OH-D_3 inclusion in the diet increased the RANKL-to-OPG ratio in bone tissue (Fig. 4G). Analysis of osteoclast-specific enzymes showed that the transcription of *cathepsin K* was reduced in ducks receiving 25-OH-D_3 diets (Fig. 4H), while no significant changes were observed in the mRNA levels of *V-ATPase* in the tibia (Fig. 4I). The dietary vitamin regimen

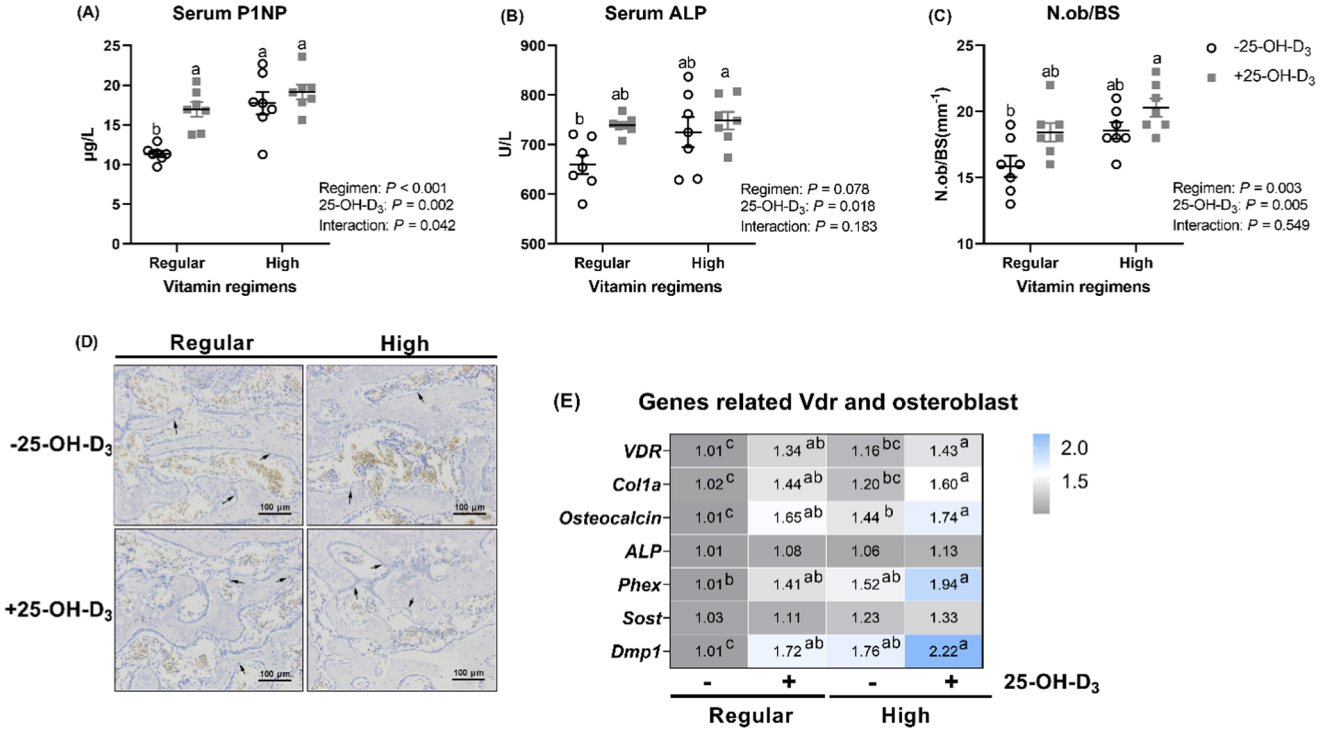


Figure 3. Impact of dietary vitamin regimen and 25-OH-D_3 on bone formation in 14-D-old ducks. Serum bone formation markers include (A) procollagen type I N-terminal propeptide (P1NP) and (B) alkaline phosphatase (ALP) activity. (C) osteoclast number per bone surface (N.Oc/BS) in tibia were determined through (D) ALP staining. (E) mRNA abundance of vitamin D receptor (*VDR*) and osteoblastic genes such as collagen Ia (*Col1a*), *osteocalcin, ALP*, phosphate regulating endopeptidase homolog x-linked (*Phex*), (f) sclerostin (*Sost*), and (g) dentin matrix protein 1 (*Dmp1*) in the tibia proximal end. Data represent means with standard deviation represented by vertical bars ($n = 7$). [a-c] Different letters on bars means a significant difference at $P < 0.05$.

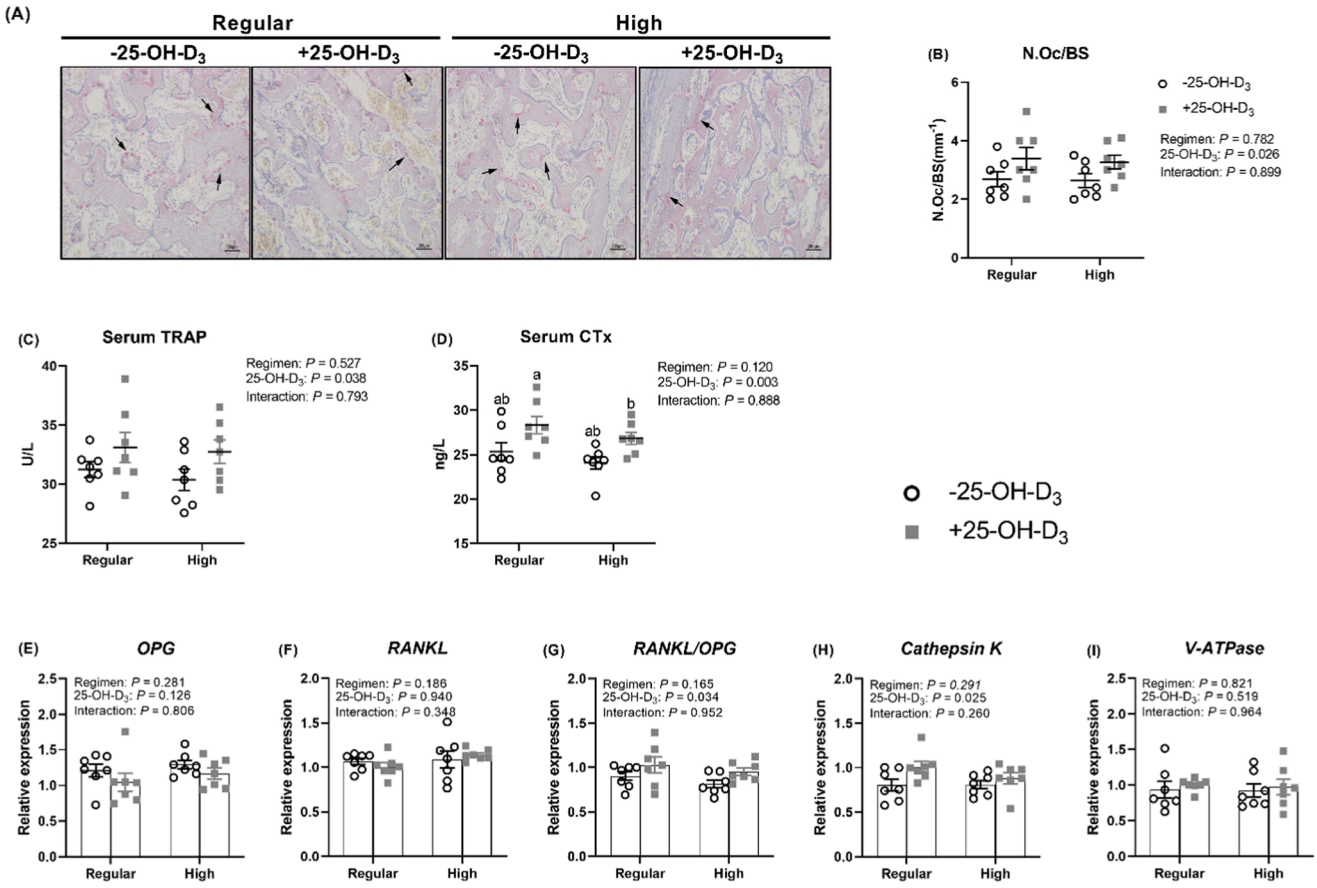


Figure 4. Effect of dietary vitamin regimen and 25-OH-D_3 on bone resorption in 14-d-old ducks. (A) Tartrate-resistant acid phosphatase (TRAP) staining of tibia sections. (B) Osteoclast number per bone surface (N.Oc/BS) in tibia determined by histomorphometry. Serum bone resorption markers (C) tartrate-resistant acid phosphatase (TRAP) and (D) C-terminal cross-linked telopeptide of type I collagen (CTx) content. (E-I) Osteoclastogenesis related genes including osteoprotegerin (*OPG*), receptor activator of nuclear factor kB ligand (*RANKL*), the ratio of *RANKL* to *OPG, cathepsin K*, and vacuolar-type H^+-ATPase (*V-ATPase*) in the tibia proximal end were qualified. Data represent means with standard deviation represented by vertical bars ($n = 7$). $^{a, b}$ Different letters on bars means a significant difference at $P < 0.05$.

itself did not affect bone resorption in 14-d-old ducks, as evidenced by similar osteoclast numbers, serum biomarkers of bone resorption, and osteoclast-specific gene expression across the groups (Fig. 4).

Discussion

With advancements in modern breeding techniques, the growth rate of meat ducks has accelerated significantly. However, this rapid growth has been linked to an increased incidence of leg diseases, which compromise animal welfare (Robison et al., 2015; Van Wyhe et al., 2012). There are many dietary factors influence bone characteristics, including nutrient density and vitamin content (Zhang et al., 2020a). Notably, significant differences exist between vitamin regimens used for meat ducks. For instance, compared to the NRC (1994) recommendations, the NY/T (2012) standards recommend higher levels of most vitamins, particularly vitamin D, while biotin levels remain lower. Given the rapid bone growth and mineralization occurring during 1-35 d and 1-42 d of age, respectively (Zhang et al., 2019b), this study investigated the tibial adaptations of young ducks to dietary 25-OH-D_3 supplementation under two vitamin regimens: the NRC (1994) and the NY/T (2012) guidelines. In addition to observing interactions related to serum 25-OH-D_3 content, trabecular area, tibia fracture load, and PN1P levels, the results demonstrate that a high-vitamin regimen, as well as 25-OH-D3 supplementation in a low-vitamin diet, improved bone quality and reduced the incidence of TD by promoting bone formation. Furthermore, the biochemical effects of 25-OH-D_3 were influenced by the baseline vitamin levels in the diet, particularly during the starter phase (1-14 d).

Evidence from broilers shows that supplementation with 25-OH-D_3 at a dose of 50 mg/liter through drinking water increased BW at 42 d of age and improved weight gain from 1 to 42 d, while maintaining comparable feed intake, and feed conversion ratio (**FCR**) throughout the trial (Santiago et al., 2016). The positive effects of dietary vitamin D_3 supplementation on BW gain are more pronounced in 14-d-old broilers (Aye Cho et al., 2020). Similarly, this study confirmed that a diet supplemented with 25-OH-D_3 significantly increased BW at 14 d and weight gain during the first 14 d, without notably affecting feed consumption, FCR, or survival rates in meat ducks. Notably, while the mortality rate due to leg abnormalities was not significantly altered by the vitamin regimen or 25-OH-D_3 supplementation in this study, the incidence of TD was reduced by either a high-vitamin diet or 25-OH-D_3 supplementation, as supported by recent research on meat ducks (Zhang et al., 2020a). Previous studies also reported that increased dietary vitamin D_3 significantly decreased the frequency of TD in broiler chickens (Driver et al., 2006; Rath et al., 2007). These findings suggest that a diet containing 0.069 mg/kg of 25-OH-D_3 can enhance weight gain and reduce leg abnormalities in ducks during the first 14 d. Additionally, supplementation with a high-vitamin diet further reduced TD scores in young ducks.

Compromised tibia quality is closely associated with leg disorders, which are linked to growth rate (Duggan et al., 2016; Paxton et al., 2013). Younger and smaller BW ducks have been observed to exhibit lower epiphyseal ash values during long bone development (Van Wyhe et al., 2012). An imbalance between weight gain and bone growth may

result in leg diseases such as leg bone malformations and chondrodysplasia in meat ducks (Robison et al., 2015). The rapid changes in BW and tibia properties in meat ducks from 1 to 14 d of age (Zhang et al., 2019b), including dimensions, weight, mineral deposition, and strength, prompted an investigation into tibia growth and mineralization. In this study, dietary interventions did not significantly alter tibia dimensions or weight, particularly relative weight to BW. This suggests that the observed improvements in leg abnormalities may not be related to enhanced bone growth. Bone ash, primarily composed of Ca and P, is directly correlated with bone mineral content and density. The increased ash, Ca, and P content observed with both the high-vitamin regimen and 25-OH-D_3 supplementation led to elevated tibial bone density, consistent with recent findings (Zhang et al., 2021, 2020a). Morphological analysis further revealed that, compared to the regular vitamin diet, the high-vitamin diet or 25-OH-D_3 supplementation increased Tb.Ar, Tb.Th, and Tb.N, while reducing Tb.Sp. These changes suggest that dietary inclusion of high vitamins or 25-OH-D_3 promotes the tibial microstructure in young ducks. Indeed, both fat-soluble and water-soluble vitamins are critical for bone mineralization. A high-vitamin diet has been shown to enhance sternum mineralization in meat ducks (Zhang et al., 2019a). In this study, except for niacin, the other vitamin levels, particularly vitamins D and K, were higher in the high-vitamin regimen compared to the regular vitamin premix. It is well-known that Vitamin D_3 is essential for Ca and P absorption and bone mass development, with a positive correlation to bone mineral density (**BMD**) (van Ballegooijen et al., 2015). The elevated serum Ca levels in the high-vitamin group likely reflect the higher vitamin D content, which enhanced mineral absorption into the tibia, accelerating calcification. It is worth noting that an interaction was observed between 25-OH-D_3 and the dietary vitamin regimen in terms of serum 25-OH-D_3 levels. This interaction may be attributed to the differing magnitudes of increase in serum 25-OH-D_3 when comparing high- and low-vitamin diets. In addition, Vitamin K, a key cofactor in synthesizing the Gla-protein family, plays a crucial role in regulating bone metabolism and preventing soft-tissue calcification (Shearer, 2000). Adequate levels of vitamin K have been shown to improve bone density (Yonemura et al., 2004), potentially contributing to the enhanced calcification observed with the high-vitamin regimen. Bone mass and trabecular structure are well known to be closely linked to biomechanical properties (Ito et al., 2002). In the present study, the enhanced tibial microstructure and increased mineral content resulting from a high-vitamin premix diet or 25-OH-D_3 supplementation contributed to greater fracture load and a reduced AUC, which measures the energy required to cause bone fracture (Turner, 2006). These findings suggest that the observed relief in leg abnormalities among young ducks is likely attributable to improved tibial quality.

In addition to facilitating the absorption of Ca and P, vitamin D plays a critical role in bone remodeling, a vital physiological process involving osteoblast-initiated bone formation and osteoclast-mediated bone resorption (Kenkre and Bassett, 2018). Disruption of bone remodeling increases the risk of compromised bone mass and heightened fracture susceptibility. Osteoblasts, which differentiate from mesenchymal stem cells, are responsible for producing bone matrix proteins. Among these, Col1a is the most abundant extracellular protein in bone and plays a key role in tissue mineralization (Zainal Ariffin et al., 2022). The synthesis of Col1a is accompanied by an increased production of P1NP, which reflects the activity of osteoblasts and is used to measure the rate of bone formation (Song, 2017). Additionally, bone-related extracellular matrix proteins, such as ALP and osteocalcin, are essential for tissue mineralization, with elevated ALP activity being a major contributor to the initiation of bone calcification. In this study, diets containing high-vitamin regimens or 25-OH-D_3 supplementation facilitated bone formation, as evidenced by increased serum levels of P1NP and ALP in 14-d-old ducks. The upregulated expression of *VDR, Col1a*, and *Osteocalcin* in ducks supplemented with 25-OH-D_3 in both regular and high-vitamin regimens further indicated enhanced bone formation due to dietary 25-OH-D_3 inclusion. Consistent with these findings, Rong etc. (2022) demonstrated that treatment with eldecalcitol, an active vitamin D analog, promoted new bone formation and osteoblast activity by increasing *ALP, Col1a*, and *Osteocalcin* levels in mouse femur (Rong et al., 2022). A plausible explanation for these effects in the current study is the increased frequency of osteoblasts in the proximal tibia, resulting from supplementation with 25-OH-D_3 and the high-vitamin premix. Furthermore, the increased transcription of osteocyte-specific genes, such as *Phex* and *Dmp1*, suggests that the dietary treatments with 25-OH-D_3 or the high-vitamin regimen promoted the differentiation of osteocytes from osteoblasts in young ducks. These findings align closely with recent observations in the tibia and keel of meat ducks (Zhang et al., 2020a and 2020b).

Alterations in serum TRAP and CTx levels are considered key markers of bone resorption, reflecting osteoclast activity (Song et al., 2019). In this study, both TRAP and CTx levels were significantly increased by dietary supplementation with 25-OH-D_3 but not by the high-vitamin regimen, suggesting that 25-OH-D_3 may induce bone resorption. To explore this further, the expression of genes related to osteoclastogenesis and activity, including *OPG, RANKL, cathepsin K*, and *V-ATPase*, was analyzed. Both RANKL and OPG are secreted by osteoblasts and osteocytes, where RANKL promotes osteoclast differentiation and activity by binding to the RANK receptor on pro-osteoclasts, while OPG inhibits this process by acting as a decoy receptor for RANKL (Yamamoto et al., 2006). The observed increase in osteoclast frequency in the proximal tibia, along with a higher RANKL-to-OPG ratio, underscores the role of 25-OH-D_3 as a stimulator of osteoclast proliferation. Furthermore, mature osteoclasts release enzymes such as cathepsin K and V-ATPase to degrade collagen and other components of the bone matrix during resorption (Nakayama et al., 2011). The elevated transcription of cathepsin K in ducks receiving 25-OH-D_3 diets further supports the association between 25-OH-D_3 supplementation and increased osteoclast activity. Interestingly, previous studies in rats reported that the active form of vitamin D, 1α, 25-$(OH)_2$-D_3, inhibits bone resorption by depressing osteoclast formation and activity (Gu et al., 2021). While vitamin D_3 is commonly used as a therapeutic agent for bone loss, it has been reported to both stimulate and inhibit bone resorption in vivo, depending on specific conditions (Rong et al., 2022). These discrepancies could be partially attributed to differences in dosage across studies (Zarei et al., 2016). Taken together, these findings indicate that dietary supplementation with 25-OH-D_3 enhances both bone formation and resorption, where the regulation of dietary 25-OH-D_3 primarily favors bone formation, contributing to a net increase in bone mass in 14-day-old ducks.

Moreover, we found that the biochemical effects of 25-OH-D_3 on BW and bone metabolism in young ducks are highly dependent on dietary vitamin levels in this study, in which the high-vitamin premix contained higher levels of all vitamins except biotin when compared to the regular premix. This is consistent with our recent research (Zhang et al., 2020a, 2020b). Dietary supplementation with 25-OH-D_3 increased weight gain and reduced the TD score in the regular vitamin diet but not in the high-vitamin diet. Furthermore, the improvements in the mechanical properties of the tibia, particularly the AUC, and the bone formation response to 25-OH-D_3 supplementation were more pronounced in the regular vitamin diet than in the high-vitamin diet. This differential response suggests a dose-dependent effect or an interaction between dietary vitamins. In this study, the high-vitamin premix contained higher levels of all vitamins except biotin compared to the regular premix. While extensive evidence has shown that high-vitamin diets positively influence bone mass (Zhang et al., 2019a), the specific mechanisms and interactions between vitamins remain unclear. In addition to the well-known beneficial roles of vitamins D, K, and E in bone metabolism, the improved tibia quality induced by the high-vitamin diet may also involve water-soluble vitamins, particularly vitamin B_{12} and folic acid, both of which have been reported to improve bone microarchitecture and reduce bone fragility (Fratoni and Brandi,

2015). Conversely, vitamin A has been recognized as a negative regulator of osteoblast mineralization (Lind et al., 2013). Thus, the positive impact of a high-vitamin diet on tibial mass in meat ducks is the result of complex interactions between various vitamins. Further studies are needed to explore these interactions in more detail.

In conclusion, the obtained data revealed that a high-vitamin diet positively impacts tibial quality and reduces the incidence of TD by promoting bone formation. This implies that the vitamin composition and proportion in the high vitamin regimen (NY/T, 2012) may be sufficient to support tibia quality in 14-d-old ducks. Furthermore, dietary supplementation with 0.069 mg/kg of 25-OH-D_3 promoted weight gain, bone mass, and tibial strength, accompanied by active bone turnover, leading to net bone mass deposition. Additionally, the biochemical effects of 25-OH-D_3 on BW and bone metabolism were found to be dependent on the dietary vitamin levels. Specifically, supplementation with 25-OH-D_3 significantly enhanced tibial mechanical properties and bone formation in the diet with the regular vitamin levels (NRC, 1994) for 14-d-old meat ducks.

Disclosures

No potential conflict of interest was reported by the authors.

Declaration of competing interest

We declare that we have no financial and personal relationships with other people or organizations that can inappropriately influence our work, and 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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