

Article

Effect of 25-Hydroxycholecalciferol with Different Vitamin D₃ Levels in the Hens Diet in the Rearing Period on Growth Performance, Bone Quality, Egg Production, and Eggshell Quality

Dongdong Li ¹, Keying Zhang ¹ , Shiping Bai ¹ , Jianping Wang ¹, Qiufeng Zeng ¹, Huanwei Peng ¹, Zuowei Su ¹, Yue Xuan ¹, Sharina Qi ² and Xuemei Ding ^{1,*}

¹ Key Laboratory for Animal Disease-Resistance Nutrition of China, Ministry of Education, Institute of Animal Nutrition, Sichuan Agricultural University, Chengdu 611130, China; dongdong20172010@163.com (D.L.); zkeying@sicau.edu.cn (K.Z.); shipingbai@sicau.edu.cn (S.B.); wangjianping@sicau.edu.cn (J.W.); zqf@sicau.edu.cn (Q.Z.); phw@sicau.edu.cn (H.P.); wzs698@126.com (Z.S.); 71128@sicau.edu.cn (Y.X.)

² Sichuan Shengdile Village Ecological Food Co., Ltd., Zitong, Mianyang 622150, China; sharina_qi@126.com

* Correspondence: dingxuemei@sicau.edu.cn; Tel.: +86-28-8629-0922



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Abstract: A study was conducted to evaluate the effect of 25-hydroxyvitamin D₃ (25-OHD) with different levels of vitamin D₃ (VD₃) during the rearing period (1–20 weeks) on laying hen performance, bone quality, and eggshell quality. A total of four hundred 1-day-old Lohman pullets were randomly allotted into a 2 × 2 factorial design with 2 levels of dietary VD₃ (300 and 2800 IU/kg) and 2 levels of dietary 25-OHD (0 and 56 µg/kg). Each treatment had five replicates of 20 hens each. Increasing the dietary VD₃ level (2800 vs. 300 IU/kg) tended to increase body weight uniformity (0.05 < *p* < 0.1) at 8 weeks. Dietary VD₃ levels and supplementation of 25-OHD during the growing period (1–20 weeks) did not improve the laying performance and eggshell quality (eggshell strength, eggshell thickness, and eggshell relative weight) during the laying period (*p* > 0.05). Compared with the 300 IU/kg VD₃ group, the 2800 IU/kg VD₃ group showed higher serum calcium concentration and keel length at 10 weeks (*p* < 0.05). The addition of 25-OHD significantly increased serum calcium and 25-OHD concentration at 10 and 20 weeks and keel calcified rate at 20 weeks (*p* < 0.05). Increasing dietary VD₃ level (2800 vs. 300 IU/kg) increased tibia ash and phosphorus content at 10 weeks, tibia strength at 72 weeks (*p* < 0.05), and tended to increase tibia ash content at 72 weeks, tibia calcium content and tibia strength at 20 weeks (0.05 < *p* < 0.1). Dietary supplementation with 25-OHD increased tibia ash content at 10 weeks, tibia calcium content and tibia strength at 20 weeks (*p* < 0.05), and tended to increase tibia ash content at 72 weeks (0.05 < *p* < 0.1). Overall, the results gathered in this study indicate that dietary supplementation of high levels of VD₃ and 25-OHD during the growing period improved the tibia quality of laying hens during the early and later laying period, but had no effect on laying performance and eggshell quality during the laying period.

Keywords: laying hens; vitamin D₃; 25-hydroxyvitamin D₃; bone quality; eggshell quality

1. Introduction

For laying hens, bone quality is closely related with egg production and eggshell quality [1]. During egg formation, bone can be mobilized to provide up to 40 percent of the calcium (Ca) in the eggshell each day [2]. Previous studies have also shown that avian medullary bone material is rapidly changing during the oviposition cycle, and bone mineral content in medullary bone was significantly reduced during eggshell formation [3]. In addition, studies have shown that hen keel bone damage may be painful to birds and affect their production [4]. It is well known that bone growth of laying hens is mainly in the pullet period. For hens, at sexual maturity, estrogen levels in the body increase substantially

and lamellar cortical bone changes to non-structural medullary bone [2]. Thus, nutritional strategies aiming at improved egg production and eggshell quality should not be only focused on phase of lay cycle but in early stages of skeletal development [5]. A recent study showed that pullet fed low calcium and phosphorus diet had lower relative keel bone weight and ash content, and tibia ash content; and serum pyridinoline (a bone resorption marker) was significantly increased [6]. However, when bone resorption failed to maintain normal levels of calcium in the blood, birds developed hypocalcemia and secreted high levels of serum parathyroid hormone (PTH) [7]. Therefore, within the normal physiological range, high serum calcium levels are necessary to maintain good bone quality in pullets.

Vitamin D₃ (VD₃) is a fat-soluble vitamin, and it is a principal factor required for mineral and skeletal homeostasis. Earlier studies have shown that 300 IU/kg VD₃ in the diet is the minimum required level for hen [8]. However, in actual production, the feed of laying hens often contains a higher concentration of VD₃. Moreover, some studies have shown that high dietary levels of VD₃ can improve bone quality [5,9] and eggshell quality [1,5,10] in laying hens. However, for the young pullet, the functional defect of the liver and kidney resulted in the decrease of active VD₃ metabolites [11,12].

25-Hydroxyvitamin D₃ (25-OHD) is a metabolite of VD₃, which has higher activity and absorption efficiency than VD₃ [13,14]. Since 2006, 25-OHD has been approved as a source of vitamin D in the poultry industry [15]. Studies have shown that 25-OHD can significantly improve laying performance and egg quality of laying hens [16–18]. However, other studies have shown that 25-OHD is not beneficial to the laying performance and egg quality in laying hens [15,19–22]. The inconsistency of these results is related to the 25-OHD treatment duration and laying stage. Moreover, most of these studies focused on the laying stage. A scarcity of reports exists on the effects of 25-OHD on growth and development of pullet. Recent studies have shown that long-term supplementation of 25-OHD has positive effects on egg production and egg quality, and this beneficial effect occurs primarily in the early stages of egg production [12]. Previous results have also shown that 25-OHD promotes bone development of pullet and prevents bone loss during laying peak production [18]. However, dietary 25-OHD supplementation in these studies continued from the rearing stage to the laying stage; therefore, this improvement effect of laying performance may only be due to the addition of 25-OHD at the laying stage. Furthermore, the efficacy of 25-OHD may be related to the basal levels of dietary vitamin D₃.

Based on these, we hypothesized that dietary supplementation with high levels of VD₃ and 25-OHD in the early growth stage can improve the growth and bone quality of pullet, thus improving the laying performance, egg quality, and bone quality of laying hens. This study was performed to evaluate the effects of 25-OHD on the growth and development of 1–20 week-old laying hens under different dietary VD₃ levels, and by tracking the laying performance and egg quality in the laying stage, we studied whether dietary high levels of VD₃ and 25-OHD during the growing period can improve the laying performance and eggshell quality during the laying period.

2. Material and Methods

2.1. Experimental Birds, Diets, and Management

The present study was performed on Sichuan Shengdile Village Ecological Food Co. LTD (Mian'yang, China), and the experimental protocol used in the study was approved by the Animal Care and Use Committee of the Sichuan Agricultural University (SAUPN-19-02). This experiment included four treatment diets in a 2 × 2 factorial arrangement: two vitamin D₃ (VD₃) levels (300, 2800 IU/kg) and two 25-hydroxycholecalciferol (25-OHD) levels (0, 56 µg/kg). At the beginning of the experiment, according to the principle of no difference in body weight, a total of four hundred 1-day-old Lohman pullets were housed in wire cages and randomly allocated to 4 treatments with 5 replicates per treatment (20 pullets / replicate). At 13 weeks, the pullets were transferred to the laying hen house. Due to the bird loss of sampling during the pullet period, during the laying period, 18 birds were used for each replication.

Water and experimental diet were provided ad libitum from 0 to 72 weeks. From 1 to 20 weeks of age, 5 phases of diet were formulated for each treatment including starter 1 (week 0–4), starter 2 (week 5–8), grower (week 9–17), pre-laying (week 18–19), and laying (week 20). Basic dietary composition and nutrient levels are shown in Table 1. The basic diet was corn–soybean meal type, and the feed form was powder. After 20 weeks of age, birds were fed the same diet containing 2800 IU/kg vitamin D₃ without 25-OHD. The environment was controlled according to the Lohman Commercial Layer Management Guide (2018). The temperature was 35–36 °C at day of hatch and gradually decreased to 20 °C when the chicks reached 35 D of age. Light exposure was 24 h at day of hatch and decreased over time to 8 h at 8 weeks of age. At the age of 18 weeks, the lighting time was increased to stimulate egg production. Light duration was maintained at 14 h after 24 weeks of age. The light intensity was 20 Lux during 0–3 weeks, 6 Lux during 4–17 weeks, and 20 Lux during 18–72 weeks. The light color was warm white.

Table 1. Basal diet composition and nutrient levels.

Ingredient (%)	Week 0–3	Week 4–8	Week 9–17	Week 18–19	Week 20
Corn	59.92	63.98	68.34	62.42	57
Soybean meal	34.9	31.19	17.8	29.06	28.36
Wheat bran			9.22		
Soybean oil	0.44	0.52	0.75	1.5	3.24
DL-methionine	0.21	0.11	0.17	0.18	0.29
L-lysine HCl	0.17	0.03			
L-threonine			0.01	0.02	
Threonine	0.04				
NaCl	0.18	0.17	0.16	0.16	0.16
Choline chloride, 60%	0.05	0.05	0.05	0.07	0.07
NaHCO ₃					0.25
Calcium carbonate	1.27	1.26	1.36	3.9	8.67
Calcium hydrophosphate	2.08	1.95	1.4	1.95	1.57
Mineral premix ¹	0.5	0.5	0.5	0.5	0.15
Vitamin premix ²	0.23	0.23	0.23	0.23	0.23
Antioxidant (Ethoxyquin)	0.01	0.01	0.01	0.01	0.01
Total	100	100	100	100	100
Calculated nutrient content, %					
ME (kcal/kg)	2753	2789	2783	2775	2738
Crude protein	20	18.5	14.5	17.5	16.82
Calcium	1.05	1	0.9	2	3.73
Non-phytate P	0.48	0.45	0.37	0.45	0.38
Lysine	1.2	1	0.68	0.92	0.88
Methionine	0.51	0.4	0.39	0.45	0.55
Tryptophan	0.23	0.21	0.17	0.22	0.19
Threonine	0.8	0.71	0.54	0.67	0.65

¹ Provided per kilogram of diets (week 0–20): Cu (as copper sulfate) 5 mg, Fe (as ferrous sulfate) 25 mg, Mn (as manganese sulfate) 100 mg, Zn (as zinc sulfate) 60 mg, I (as potassium iodide) 0.5 mg, and Se (as sodium selenite) 0.2 mg. ² Provided per kilogram of diets (week 0–8, week 9–17, week 18–20): VA 10000, 10000, 10000 IU; VE 30, 30, 30 mg; VK₃ 3, 3, 3 mg; VB₁ 1, 1, 1 mg; VB₂ 6, 6, 4 mg; VB₆ 3, 3, 3 mg; VB₁₂ 20, 20, 25 µg; D-pantothenate 8, 8, 10 mg; niacin acid 30, 30, 30 mg; folic acid 1, 1, 0.5 mg; Biotin 50, 50, 50 µg.

2.2. Data Collection and Sample Collection

During the whole experiment, the feed intake and mortality was frequently recorded. At 19 weeks of age, ten chickens were randomly selected from each replicate to measure shank length; shank length was measured with vernier caliper. At 8 and 19 weeks of age, each bird was weighed (SOVO TSC-E; Fly Pressure Horizontal Device Co., Ltd., Shenzhen, China) separately to calculate the body weight uniformity (BWU) of each repetition; the BWU of each repeat = the number of birds within the range of $\pm 10\%$ of the average weight of the replicate/the total number of birds in the replicate. In addition, the body weight gain (BWG) and average daily feed intake (ADFI) of 1–8 weeks, 9–19 weeks, and 1–19 weeks were calculated. From 21 to 72 weeks, egg number, total egg weight, unqualified eggs

(dirty egg, broken eggs, egg weight beyond the average egg weight ± 10 g, misshaped egg, and sand-shelled egg) were recorded daily for each replicate. Then, laying rate, average egg weight, total egg number, qualified egg number, and total egg weight of 21–72 weeks were calculated. At the end of 20, 40, 60, and 70 weeks of age, three eggs per experimental unit were collected for eggshell quality determination. At 10, 20, and 72 weeks, one bird from each experimental unit was euthanized to collect keel bone and right and left tibia and femur. Once collected, keel bones were stored in plastic bags and held at 4 °C until measured. Both tibia and femur bones were frozen at -20 °C until analysis.

2.3. Eggshell Quality

Eggshell quality measurements included eggshell thickness, eggshell strength, and eggshell relative weight. Eggshell strength was evaluated using an eggshell force gauge model II (Robotmation Co., Ltd., Tokyo, Japan). Eggshell thickness was measured on the blunt end, equatorial region, and sharp end, using an eggshell thickness gauge (Robotmation Co., Ltd., Tokyo, Japan).

2.4. Serum Parameters Analysis

At the end of 10 and 20 weeks, 20 hens (5 replicates for each treatment) were individually weighted, and the disposable blood collection needles were used to collect blood from the wing vein. Blood was collected into a vacuum tube without any anticoagulant. Samples were then centrifuged at $3000 \times g$ for 10 min, and then, serum was stored at 20 °C. Serum parathyroid hormone (PTH) and 25-hydroxyvitamin D (25-OHD) concentration were assayed using a commercial ELISA kit (ALPCO Diagnostics, NH) according to the manufacturers' recommendations. The concentration of calcium (Ca) and phosphorus (P) in blood was determined with biochemistry Analyzer (Yellow Springs Instrument Co.Inc., Yellow Springs, OH, USA).

2.5. Keel Development and Calcification

After stripping out the entire keel, the depth, length, and calcified length of the keel were measured. Keel depth is the distance between the endpoint of keel (near bird head) and the intersection of the first rib and the keel. Keel calcified rate (%) = calcified length/keel length $\times 100\%$.

2.6. Tibia Quality

The bones were cleaned of surrounding muscles and soft tissues and the strength was measured by the texture analyzer (TAXTPlus, Stable MicroSystems Corp., Godalming, UK). Then, bone samples were air-dried, degreased in petroleum ether, and then dried in an oven at 105 °C to a constant weight. Subsequently, fat-free bone index, bone ash, calcium, and phosphorus content were measured using the method described by Zhang et al. (2017) [23]. Fat-free bone index (g/kg) = fat-free bone weight/alive bird weight.

2.7. Statistical Analysis

The data were preliminarily sorted by Excel. Two-way ANOVA followed by Duncan's test (SAS 9.1) were used for analyses of dietary vitamin D₃ level, 25-OHD level, and their interaction. All data were expressed as the means and standard error of the mean (SEM). Significance was declared at $p < 0.05$.

3. Results

3.1. Growth Performance

Growth performance parameters were shown in Table 2. Dietary VD₃ and 25-OHD had no significant effect on pullet body weight (BW), body weight gain (BWG), average daily feed intake (ADFI), and shank length (SL) ($p > 0.05$). There was a significant interaction for BWU at 8 weeks between dietary VD₃ level and 25-OHD ($p < 0.05$), and increasing dietary VD₃ level from 300 to 2800 IU/kg tended to increase BWU at 8 weeks ($0.05 < p < 0.1$).

Table 2. Effects of dietary VD₃ level and 25-OHD on growth performance of laying hens.

Item ¹		300 IU/kg VD ₃		2800 IU/kg VD ₃		SEM	<i>p</i> -Value		
		25-OHD(−)	25-OHD(+)	25-OHD(−)	25-OHD(+)		VD ₃	25-OHD	VD ₃ × 25-OHD
BW (g)	1 d	41.8	41.7	41.8	41.6	0.2	0.793	0.371	0.930
	8 weeks	649.2	649.5	663.4	657.3	8.5	0.216	0.740	0.715
	19 weeks	1611.5	1590.6	1626.5	1627.6	18.4	0.177	0.596	0.559
BWG (g)	1–8 weeks	607.4	607.8	621.5	615.7	8.5	0.215	0.757	0.719
	9–19 weeks	962.3	941.1	963.1	970	16.2	0.369	0.669	0.396
	1–19 weeks	1569.7	1548.9	1584.7	1585.9	18.4	0.176	0.602	0.558
BWU (%)	8 weeks	64.6	73.8	79.4	72.8	4.0	0.079	0.728	0.047
	19 weeks	76.0	76.0	75.6	72.0	3.7	0.651	0.711	0.711
	1–8 weeks	30.7	30.3	30.3	29.6	0.3	0.480	0.246	0.829
ADFI (g)	9–19 weeks	73.8	72.8	72.8	72.5	0.8	0.430	0.428	0.664
	1–19 weeks	55.0	54.3	54.2	53.9	0.4	0.142	0.215	0.602
SL (mm)	19 weeks	100.8	101.6	101.8	102.1	0.6	0.184	0.260	0.667

¹ Within a row. BW, body weight; BWG, body weight gain; BWU, body weight uniformity; ADFI, average daily feed intake; SL, shank length; 25-OHD, 25-hydroxyvitamin D₃; −, 0 ug/kg 25-OHD; +, 56 ug/kg 25-OHD.

3.2. Laying Performance and Eggshell Quality

The flock reached the peak production (more than 90%) at 23 weeks; after the peak production, the HDLR (hen-day laying rate) and HHLR (hen-housed laying rate) gradually declined throughout the production period (Figure 1). Through further analysis of the HDLR, HHLR, AEW (average egg weight), ENHD (egg number per hen-day), ENHH (egg number per hen-housed), HDEW (hen-day total egg weight), HHEW (hen-housed total egg weight), QENHD (qualified egg number per hen-day), and QENHH (qualified egg number per hen-housed) of the egg-laying period (21–72 weeks), it was found that dietary VD₃ levels and 25-OHD had no significant effect on these parameters (Table 3; *p* > 0.05). Otherwise, dietary VD₃ levels and 25-OHD had no effect on eggshell quality (eggshell thickness, eggshell strength, and eggshell relative weight) at 20, 40, 60, and 70 weeks (Table 4; *p* > 0.05).

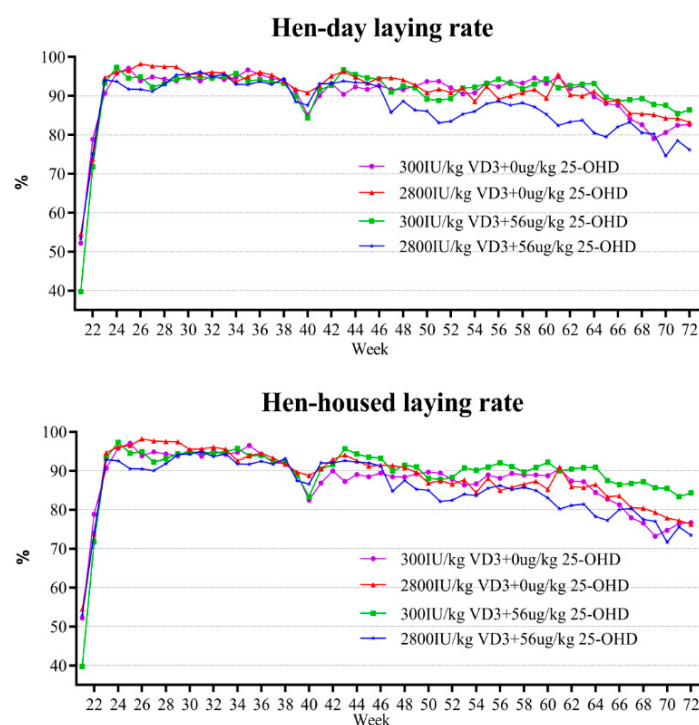


Figure 1. Effects of VD₃ level and 25-OHD in the diet of 1–20-week-old laying hens on laying rate from 21 to 72 weeks. 25-OHD, 25-hydroxyvitamin D₃. The number of hen-housed was calculated according to the actual number of birds at the age of 21 weeks.

Table 3. Effects of VD₃ level and 25-OHD in the diet of 1–20-week-old laying hens on laying performance (21–72 weeks) ¹.

Item ²	300 IU/kg VD ₃		2800 IU/kg VD ₃		SEM	<i>p</i> -Value		
	25-OHD(–)	25-OHD(+)	25-OHD(–)	25-OHD(+)		VD ₃	25-OHD	VD ₃ × 25-OHD
HDLR, %	91.0	91.4	91.7	87.7	1.5	0.320	0.234	0.151
HHLR, %	87.9	90.2	88.8	86.0	1.7	0.332	0.899	0.144
AEW, g	62.6	63.0	62.6	63.0	0.4	0.959	0.361	0.929
ENHD, No	327	329	330	316	5.2	0.319	0.233	0.148
ENHH, No	316	325	319	310	6.0	0.331	0.899	0.145
HDEW, kg	20.5	20.7	20.7	19.9	0.4	0.386	0.461	0.220
HHEW, kg	19.8	20.5	20	19.5	0.4	0.369	0.889	0.188
QENHD, No	323	325	325	310	5.6	0.274	0.254	0.139
QENHH, No	312	321	315	304	6.2	0.281	0.874	0.130

¹ The number of hen-housed was calculated according to the actual number of birds at the age of 21 week. ² Within a row, LS means with letter superscripts differs, $p < 0.05$ ($n = 5$). HDLR, hen-day laying rate; HHLR, hen-housed laying rate; AEW, The average egg weight; ENHD, egg number per hen-day; ENHH, egg number per hen-housed; HDEW, hen-day total egg weight; HHEW, hen-housed total egg weight; QENHD, qualified egg number per hen-day; QENHH, qualified egg number per hen-housed; 25-OHD, 25-hydroxyvitamin D₃; –, 0 ug/kg 25-OHD; +, 56 ug/kg 25-OHD.

Table 4. Effects of dietary VD₃ level and 25-OHD in the diet of 1–20-week-old laying hens on egg quality.

Item ¹	300 IU/kg VD ₃		2800 IU/kg VD ₃		SEM	<i>p</i> -Value		
	25-OHD(–)	25-OHD(+)	25-OHD(–)	25-OHD(+)		VD ₃	25-OHD	VD ₃ × 25-OHD
				Eggshell strength, kgf				
20 weeks	4.99	4.80	4.82	4.86	0.11	0.640	0.534	0.317
40 weeks	4.76	4.76	4.57	4.54	0.14	0.179	0.911	0.951
60 weeks	4.26	4.25	4.21	4.31	0.15	0.984	0.753	0.717
70 weeks	4.20	4.27	4.11	4.25	0.18	0.766	0.561	0.844
				Eggshell thickness, mm				
20 weeks	0.433	0.437	0.436	0.439	0.01	0.793	0.707	0.991
40 weeks	0.383	0.390	0.390	0.382	0.01	0.975	0.950	0.254
60 weeks	0.382	0.385	0.382	0.380	0.01	0.806	0.907	0.773
70 weeks	0.384	0.368	0.364	0.378	0.01	0.593	0.900	0.107
				Eggshell relative weight, %				
20 weeks	12.3	12.2	12.1	12.6	0.2	0.748	0.239	0.068
40 weeks	11.4	11.5	11.2	11.4	0.1	0.163	0.353	0.626
60 weeks	11.3	11.3	11.2	11.2	0.2	0.789	0.957	0.879
70 weeks	10.9	10.6	10.4	10.7	0.2	0.351	0.889	0.171

¹ Within a row, LS means with letter superscripts differs, $p < 0.05$ ($n = 5$). 25-OHD, 25-hydroxyvitamin D₃; –, 0 ug/kg 25-OHD; +, 56 ug/kg 25-OHD.

3.3. Serum Parameters

As shown in Table 5, the 2800 IU/kg VD₃ group showed a higher serum calcium concentration at 20 weeks compared with the 300 IU/kg VD₃ group ($p < 0.05$). The addition of 25-OHD also significantly increased the serum calcium and 25-OHD concentration at 20 weeks ($p < 0.05$). A significant interaction was observed between dietary VD₃ level and 25-OHD on serum PTH concentration at 20 weeks ($p < 0.05$). Dietary VD₃ levels and 25-OHD had no effect on serum phosphorus concentration ($p > 0.05$).

3.4. Keel Development and Calcification

As shown in Figure 2, the position of keel measurement is shown in Figure 2A, increasing dietary VD₃ levels (2800 vs. 300 IU/kg) significantly increased keel length at 10 weeks, and there was a significant interaction between VD₃ levels and 25-OHD for keel length at 10 weeks, (Figure 2B; $p < 0.05$). At 20 weeks, dietary supplementation with 25-OHD significantly increased the keel calcified rate (Figure 2D; $p < 0.05$). Dietary VD₃ levels and 25-OHD did not affect keel length at 20 weeks, keel depth at 10 and 20 weeks, and keel calcified rate at 10 weeks ($p > 0.05$).

Table 5. Effects of dietary VD₃ level and 25-OHD on serum parameters.

Item ¹	300 IU/kg VD ₃		2800 IU/kg VD ₃		SEM	<i>p</i> -Value		
	25-OHD(−)	25-OHD(+)	25-OHD(−)	25-OHD(+)		VD ₃	25-OHD	VD ₃ × 25-OHD
10 weeks								
Ca, mmol/L	0.67 ^a	1.06 ^{ab}	0.62 ^a	1.24 ^b	0.16	0.659	0.006	0.462
Pi, mmol/L	0.67	0.87	0.70	1.04	0.17	0.571	0.118	0.688
25-OHD, ng/mL	27.0 ^a	30.1 ^b	28.6 ^{ab}	30.2 ^b	0.89	0.280	0.009	0.351
PTH, pg/mL	112.4	108.6	107.5	107.3	6.7	0.652	0.770	0.796
20 weeks								
Ca, mmol/L	2.03 ^a	2.78 ^{ab}	2.8 ^{ab}	3.29 ^b	0.3	0.038	0.044	0.640
Pi, mmol/L	0.84	0.86	0.98	1.23	0.38	0.123	0.215	0.197
25-OHD, ng/mL	32.6 ^a	46.3 ^b	38.7 ^{ab}	50.4 ^b	4.2	0.249	0.011	0.823
PTH, pg/mL	105.3	107.4	115.2	98.1	9.5	0.827	0.341	0.042

¹ Within a row, ^{a,b} Mean values with unlike letters were significantly different, $p < 0.05$ ($n = 5$). PTH, parathyroid hormone; 25-OHD, 25-hydroxyvitamin D₃; −, 0 ug/kg 25-OHD; +, 56 ug/kg 25-OHD.

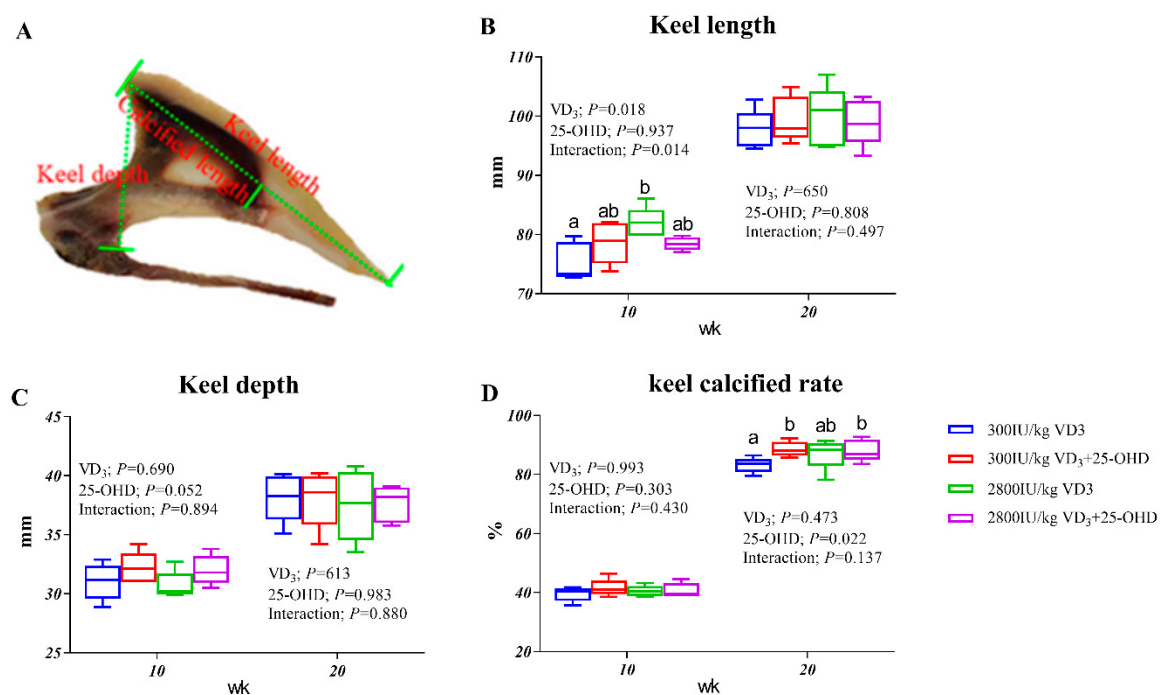


Figure 2. Effects of dietary VD₃ level and 25-OHD on the keel development and calcification of laying hens. (A) Picture showing the measure location of keel depth and calcified rate; (B) keel length; (C) keel depth; (D) keel calcified rate. a,b Mean values with different letters are significantly different.

3.5. Tibia Quality

At 10 weeks, compared with the 300 IU/kg VD₃ group, the 2800 IU/kg VD₃ group showed a higher content of ash and p phosphorus in tibia (Figure 3B,D; $p < 0.05$). Dietary supplementation with 25-OHD increased the content of ash in tibia (Figure 3B; $p < 0.05$). Dietary VD₃ levels and 25-OHD had no effect on tibia strength, fat-free tibia index, and calcium content in tibia at 10 weeks. (Figure 3A,C,E; $p > 0.05$)

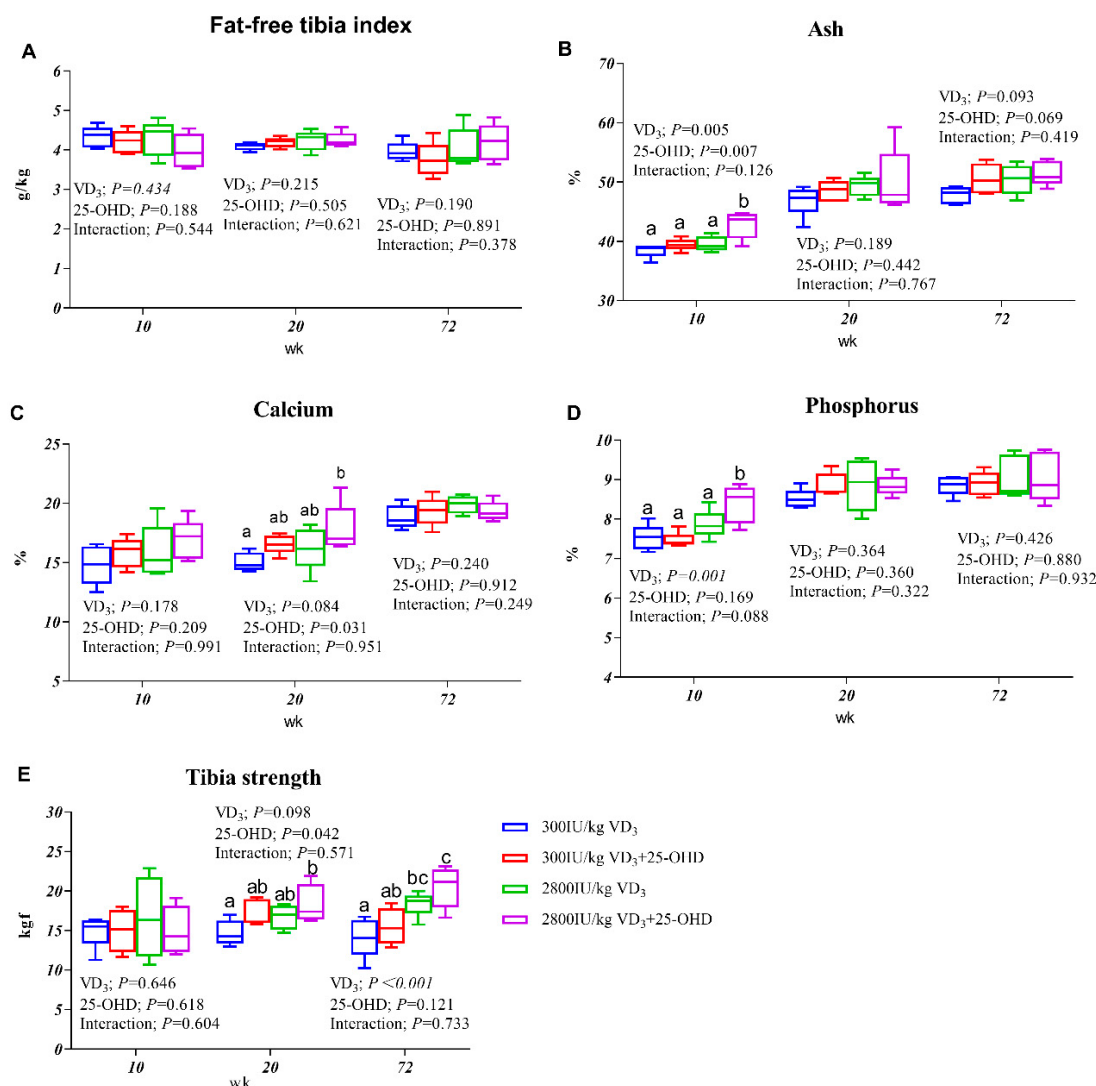


Figure 3. Effects of dietary VD₃ level and 25-OHD on tibia quality. (A) Fat-free tibia index; (B) Ash content in tibia; (C) Calcium content in tibia; (D) Phosphorus content in tibia; (E) Tibia strength. a–c Mean values with different letters are significantly different.

At 20 weeks, compared with the 300 IU/kg VD₃ group, the 2800 IU/kg VD₃ group had a higher tibia calcium content and tibial strength (Figure 3C,E; $0.05 < p < 0.1$). The addition of 25-OHD significantly increased the tibia calcium content and tibial strength (Figure 3C,E; $p < 0.05$). Dietary VD₃ levels and 25-OHD had no effect on fat-free tibia index, tibia ash content, and tibia phosphorus content (Figure 3A,B,D; $p > 0.05$).

At 72 weeks, increasing dietary VD₃ levels (2800 vs. 300 IU/kg) significantly increased tibia strength (Figure 3E; $p < 0.05$), and tended to increase the tibia ash content (Figure 3B; $0.05 < p < 0.1$). Dietary supplementation with 25-OHD also tended to increase the tibia ash content (Figure 3B; $0.05 < p < 0.1$). Dietary VD₃ levels and 25-OHD had no effect on the fat-free tibia index and calcium and phosphorus content in tibia (Figure 3A,C,D; $p > 0.05$).

4. Discussion

As we all know, in actual production, the shank length, body weight, and body weight uniformity of the pullets are important indicators to evaluate the growth and development of the hens in the rearing period. A previous study has shown that dietary supplementation with 69 µg/kg 25-OHD significantly increased the pullet body weight and feed intake at 3 weeks of age compared to the negative control (3000IU VD₃/kg of feed) [18]. However,

in the current study, we found that dietary supplementation with 25-OHD had no effect on body weight, ADFI, and shank length of pullets; this is in agreement with a recent research indicating that dietary supplementation of 69 µg/kg 25-OHD did not affect the growth performance of pullets compared with 2760 IU/kg VD₃ treatment [12]. Otherwise, in our study, dietary VD₃ levels also had no effect on body weight and shank length of pullets. However, increasing dietary VD₃ levels increased BWU at 8 weeks, but had no effect on BWU at 19 weeks. The results suggest that a 300 IU/kg VD₃ diet cannot adequately satisfy the growth and development of young hens from day of hatch to 8 weeks. This may be caused by liver and kidney dysfunction in young hens [11]. Birds that reach the target body weight and proper bone development during rearing are less prone to prolapse during production compared to birds achieving target BW, but with shorter shank lengths [24]. Previous studies have shown that treatments including 25-OHD up to 18 weeks resulted in longer shanks when compared with the 3000 IU/kg VD₃ treatment [18]. However, in our study, dietary VD₃ levels and 25-OHD had no effect on shank length at 19 weeks. This may be due to differences in basic diet nutrition levels, experimental environment, and breed of laying hens.

In this study, we hypothesized that dietary supplementation of high levels of VD₃ and 25-OHD in the early growth stage could improve laying performance and egg quality at laying stage. However, this assumption has not been achieved. Similar to our results, when the laying hens were fed with 69 µg/kg of 25-OHD + 3000 IU/kg of VD₃ from 0 to 17 weeks, compared with 3000 IU/kg VD₃ treatment, no differences in cumulative egg production and egg weight was observed overall (18–87 weeks) [18]. A recent research found that the long-term supplementation of 25-OHD had no effect on overall (22–95 weeks) egg production [12]. In addition, a number of previous studies failed to find any beneficial effects on egg production in laying hens or broiler breeders [15,16,19,20,22,25]. Unlike our study, these studies focused on the laying period of laying hens. In our study, we found that dietary VD₃ levels and 25-OHD at growth period had no effect on eggshell quality. This may be because the calcium level in the diet is sufficient for eggshell formation; another reason may be that the calcium released by bone absorption under the action of osteoclasts maintains the normal quality of the eggshell. Similarly, a recent article also showed that long-term addition of 25-OHD to diets containing 2760 IU/kg VD₃ did not improve eggshell quality [12]. Otherwise, many studies have shown that addition of 25-OHD had no effect on egg shell quality [19,20,22]. On the contrary, a previous study has shown that the use of 25-OHD during the rearing period (0–15 weeks) and whole phases (0–87 weeks) could increase the thickness of the eggshell, which is not consistent with our results [18]. Therefore, the application of 25-OHD at the rearing period for improving the eggshell quality needs further research.

Serum 25-OHD concentration is an important index for vitamin D status in the body. In our study, the addition of 25-OHD increased the concentrations of 25-OHD. This is consistent with the results of most studies, which maintained high serum 25-OHD levels by supplementing the diet with 25-OHD [21,26,27]. Vitamin D can facilitate the absorption of dietary calcium in the small intestine [28]. In our study, high level of dietary VD₃ and the addition of 25-OHD increased the concentrations of serum calcium. Parathyroid hormone is important for regulating serum calcium ion concentration. When hypocalcemia occurs, elevated PTH can stimulate the activity of renal 1α hydroxylase and increase the production of 1,25(OH)₂VD₃, while the increase of extracellular calcium mediated by 1,25(OH)₂VD₃ will decrease the secretion of PTH [29]. In our study, PTH concentration was not significantly changed by the dietary VD₃ level and 25-OHD, which indicates that the blood Ca level was within the normal range.

For laying hens, with the increase of the laying rate and the prolongation of the laying cycle, the number of mineralized structural bones of hens will decrease gradually; this leads to an increase in the incidence of osteoporosis [30,31]. Therefore, it is necessary to improve the bone quality as much as possible during the growth and development stage of bone. In our study, dietary high levels of VD₃ or dietary supplementation with 25-OHD improved

tibia strength at 20 and 72 weeks, tibia ash content at 10 and 72 weeks, tibia ca content at 20 weeks, and tibia phosphorus content at 10 weeks. In agreement with our findings, one study showed that the use of 25-OHD during the rearing period (0–15 week) increased shank breaking strength of Hy-Line Brown hens at 90 weeks of age [18]. There are few studies on the effect of VD on the bone quality of young pullets. A positive trend in bone strength of 70-week-old brown hens had been found, when VD_3 was partially replaced with 25-OHD from 26 to 70 weeks of age [16]. Increasing dietary VD_3 levels increased Lohman laying hens bone strength compared to the 2500 IU/kg VD_3 treatment [9]. In broilers, studies have also shown that 25-OHD enhanced bone traits including bone ash [32] and reduced the incidence of lameness [33]. In our study, high levels of dietary VD_3 and 25-OHD increased the concentrations of calcium ions and 25-OHD in serum; this allowed calcium ions to deposit into the bone, thus improving the bone quality. Recent studies have reported that 25-OHD increased bone growth rate and bone size during the pullet period, which provided more space for mineral deposition during the later period [27]. This may be the reason why dietary supplementation with high levels of VD_3 and 25-OHD during the pullet period increased tibia strength and tibia ash content of laying hens at 72 weeks.

For laying hens, in addition to long bones, the development of the keel is also noteworthy. Keel bone damage is an important welfare problem in laying hens and may cause economic losses [34]. A previous study on laying hens found that 25-OHD did not affect the prevalence of keel bone deformities [35]. However, in our study, it was shown that high levels of VD_3 in diets increased the keel length at 10 weeks, and dietary supplementation of 25-OHD promoted the calcification of the keel at 20 weeks. This may be due to increased serum calcium and 25-OHD concentrations in laying hens. Similar to our results, dietary supplementation with 69 $\mu\text{g/kg}$ 25-OHD significantly improved sternal mineral accumulation of meat ducks [26]. It is worthy of further study to determine whether supplementation with 25-OHD during the pullet period can improve the quality of birds keels and reduce the incidence of keel fractures.

5. Conclusions

This study showed that increasing dietary VD_3 levels (2800 vs. 300 IU/kg) during the pullet period improved pullet BWU and tibia quality during the early and later stages. Moreover, the addition of 25-OHD during the pullet period could promote keel calcification and improve tibial quality during the early and later stages. Dietary VD_3 level and 25-OHD during the pullet period had no effect on laying performance and eggshell quality. This study suggests that dietary supplementation of high levels of VD_3 and 25-OHD during rearing is necessary to maintain good bone quality in laying hens.

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Institutional Review Board Statement: All animals were kept in a pathogen-free environment and fed ad lib. The procedures for care and use of animals were approved by the Ethics Committee of the Sichuan Agricultural University (Ethic approval number: SICAUAC201710-7) and all applicable institutional and governmental regulations concerning the ethical use of animals were followed.

Informed Consent Statement: This study did not involve humans.

Data Availability Statement: Some or all data, models, or code that support the findings of this study are available from the corresponding author upon reasonable request.

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