Effect of dietary 25-hydroxycholecalciferol supplementation and high stocking density on performance, egg quality, and tibia quality in laying hens

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ABSTRACT This study was conducted to determine the effects of 25-hydroxycholecalciferol (**25-OH-D$_3$**) on performance, egg quality, tibia quality, and serum hormones concentration in laying hens reared under high stocking density. A total of 800 45-week-old Lohmann laying hens were randomly allotted into a 2 × 2 factorial design with 2 levels of dietary 25-OH-D$_3$ levels (0 and 69 μg/kg) and 2 rates of stocking densities [506 (low density) and 338 (high density) cm^2/hen]. Laying hens were monitored for 16 wk. High stocking density decreased laying rate, egg weight, and feed intake compared with low stocking density ($P < 0.01$) during 1 to 8 wk and 1 to 16 wk. Overall, high stocking density increased eggshell lightness value and decreased shell redness and yellowness value, strength, thickness, and relative weight compared with low stocking density ($P < 0.05$). Dietary supplementation with 25-OH-D$_3$ reduced the value of the eggshell lightness and increased its yellowness and eggshells weight ($P \leq 0.05$). The increase in eggshell thickness was more pronounced when 25-OH-D$_3$ was supplemented to layers under high stocking density (interaction, $P < 0.05$). Layers under high stocking density had lower ash content and calcium content in the tibia than layers under low stocking density ($P = 0.04$); dietary 25-OH-D$_3$ increased tibia strength compared with no addition ($P = 0.05$). Layers under high stocking density had higher serum concentrations of 25-OH-D$_3$, corticosterone (**CORT**), lipopolysaccharide (**LPS**), and osteocalcin (**OC**; $P < 0.05$), lower content of parathyroid hormone (**PTH**) compared with layers under low stocking density ($P < 0.01$). Dietary 25-OH-D$_3$ increased serum concentration of 25-OH-D$_3$, carbonic anhydrase (**CA**), and calcitonin (**CT**) ($P < 0.01$) and reduced corticosterone, lipopolysaccharide and osteocalcin concentration ($P \leq 0.05$). The increase effect in PTH was more pronounced when 25-OH-D$_3$ was supplemented to layers under high stocking density (interaction, $P = 0.05$). Overall, the results gathered in this study indicate that high stocking density result in reducing production performance, shell color and quality, and tibia health, whereas dietary 25-OH-D$_3$ was able to maintain tibia health and to mitigate the negative impact of high stocking density on productive performance.

Key words: 25-hydroxycholecalciferol, stocking density, production performance, egg quality, tibia quality

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INTRODUCTION

The welfare of laying hens is closely related to their stocking density. Under Council Directive 1999/74/EC, the minimum space allowances per laying hen ranges from 550 cm^2 in unfurnished cages to 1,111 cm^2 in alternative housing (Savory et al., 2006). The American Egg Producers Association recommends that the bottom area of layers' cages to be 432 to 555 cm^2 per cage (UEP, 2016). After the 2012 EU ban on conventional cages was implemented, the standard for laying hens reared in large cages has been changed to 750 cm^2 per hen. Several studies have shown that high stocking density reduces layers' production performances (Leeson and Summers, 1984; Hester and Wilson, 1986; Carey, 1987; Kang et al., 2016). It has also been reported that in caged laying hens skeletal health

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might be compromised as reflected in the higher incidence of pathologies such as weak bones, bone deformities, fractures, infections, and osteoporosis (Rath et al., 2000). It has been shown that high stocking density can negatively affect the development of the tibia and reduce the quality of the tibia (Sanotra et al., 2001; Buijs et al., 2009; Ventura et al., 2010; Zhang et al., 2018). In addition, the quality and color of the eggshell can also be affected by stocking density (Saki et al., 2012; Samiullah et al., 2015; Campbell et al., 2017). Moreover, as the layer become older, the egg production rate was decreased, the egg weight was increased, and the eggshell quality was also reduced. This may increase the sensitivity of layers to environmental stress (such as high density).

The 25-hydroxycholecalciferol (**25-OH-D_3**) is an active metabolite of vitamin D_3 (Holick and Chen, 2008) and has a wide range of applications in the poultry industry. Dietary supplementation of 25-OH-D_3 can increase the body weight gain of broilers (Yarger et al., 1995; Bello et al., 2014) and improve the hatching rate and immunity of hens (Saunders-Blades and Korver, 2015). In addition, dietary 25-OH-D_3 supplementation can effectively improve meat quality in broilers and reduce tibia dysplasia (Rennie and Whitehead, 1996; Ledwaba and Roberson, 2003; Swiatkiewicz et al., 2006) and improve eggshell quality (McLoughlin and Soares, 1976; Keshavarz, 2003). However, the results are not always consistent in laying hens (Keshavarz, 2003; Peebles et al., 2008). In our previous study, we found that dietary administration of 69 μg/kg 25-OH-D_3 improved the egg quality of layers under high stocking density (data not published); however, whether 25-OH-D_3 can alleviate the adverse effects of high stocking density on tibia quality is not clear.

Therefore, the objective of this study was to investigate the effect of 25-OH-D_3 on performance, egg quality, and tibia quality in laying hens (late laying stage) reared under high stocking density.

MATERIALS AND METHODS

Birds, Diets, and Management

The experimental protocol used in the study was approved by the Animal Care and Use Committee of the Sichuan Agricultural University, China. At 45 wk of age, 800 Lohman pink-shell laying hens were randomly assigned to 4 treatments with 10 replicates per treatment (20 layers/replicate). A 2 × 2 factorial design with 2 levels of 25-OH-D_3 (0 and 69 μg/kg) and 2 stocking densities (506 [low density] and 338 [high density] cm^2/hen) was used, resulting in 4 experimental groups: (1) L + 0 (low density + 0 μg/kg 25-OH-D_3), (2) L + 25-OH-D_3 (low density + 69 μg/kg 25-OH-D_3), (3) H + 0 (high density + 0 μg/kg 25-OH-D_3), and (4) H + 25-OH-D_3 high density + 69 μg/kg 25-OH-D_3). Laying hens were fed a complete feeding mixture in a mash form. Basal diet is shown in Table 1. The total experimental period was 16 wk. Room environment was controlled at 22°C by a daily lighting schedule of 16 h light and 8 h dark. Birds were allowed *ad libitum* access to water and feed.

Table 1. Composition and nutrient level of basal diet (as-fed basis).

Item, %	Amount
Corn	59.06
Wheat bran	3.87
Soybean oil	1.50
Soybean meal (CP, 43%)	15.24
Corn gluten (CP, 60%)	5.00
Corn DDGS	5.00
Calcium carbonate (granular)	6.10
Calcium carbonate (powder)	2.50
Calcium hydrophosphate (powder)	0.94
NaCl	0.25
$NaHCO_3$	0.10
L-Lysine HCl	0.16
DL-Methionine	0.01
Choline chloride, 60%	0.10
Vitamin premix[1]	0.02
Mineral premix[2]	0.15
Total	100.00
Calculated nutrient content, %	
ME[3], kcal/kg	2,690
Analyzed nutrient levels, %	
CP	15.82
Calcium	3.65
Total phosphorus	0.64
Lysine	0.65
Methionine	0.33

Abbreviation: DDGS, distillers dried grains with solubles.

[1]Provided per kilogram of diets: VA 9950 IU, VB_1 37.7 mg, VB_2 12 mg, D-pantothenate 18.2 mg, VB_6 7.55 mg, VB_{12} 0.5 mg, VD_3 5000 IU, VE 70 IU, VK_3 4.47 mg, Biotin 4 mg, VC 195 mg, niacin acid 70.35 mg.

[2]Provided per kilogram of diets: Cu (as copper sulfate) 9.6 mg, Fe (as ferrous sulfate) 64 mg, Mn (as manganese sulfate) 121.5 mg, Zn (as zinc sulfate) 57 mg, I (as potassium iodide) 0.60 mg, Se (as sodium selenite) 0.36 mg.

[3]Calculated according to NRC (1994).

Sample Collection and Measurements

Egg number, total egg weight, broken eggs, and unqualified eggs (egg weight <50 g or >75 g, misshaped egg, dirty egg, and sand-shelled egg) were recorded daily for each replicate. Feed conversion ratio was calculated as the ratio of grams of total feed intake to grams of total egg weight. Egg production was expressed as an average day production. At 16 wk, a total of 30 eggs with the exception of unqualified eggs and broken eggs were randomly collected from each treatment and assessed for egg quality traits. At the end of 8 and 16 wk, 40 hens (10 replicates for each treatment) were individually weighted, and blood samples were collected from the wing vein into a sterile syringe. Samples were then centrifuged at 3,000 × *g* for 15 min, and then, serum was stored at −20°C pending analysis. The same hens were then sacrificed with an overdose intravenous injection of sodium pentobarbital, and the left tibia was taken and stored at 4°C for tibia strength analysis, and the right tibia was taken and stored in 4% polyformaldehyde for histopathology.

Table 2. Effect of dietary 25-OH-D_3 on performance of laying hens under high or low stocking density (1–8 wk)[1].

Item	Laying rate, %	Egg weight, g	FCR	Feed intake, g	Broken egg, %	Unqualified egg,%
Density 25-OH-D_3						
Low−	95.73 ± 0.47[a]	64.55 ± 0.24[a]	1.91 ± 0.01	117.87 ± 1.04[a]	1.14 ± 0.11	0.98 ± 0.17
Low+	95.75 ± 0.59[a]	63.93 ± 0.31[a,b]	1.94 ± 0.03	118.85 ± 1.53[a]	0.98 ± 0.17	0.97 ± 0.09
High−	93.76 ± 0.64[b]	63.21 ± 0.37[b]	1.94 ± 0.02	114.83 ± 0.97[b]	1.55 ± 0.21	1.64 ± 0.21
High+	93.74 ± 0.61[b]	63.25 ± 0.41[b]	1.91 ± 0.01	112.78 ± 0.88[b]	1.44 ± 0.25	1.31 ± 0.22
P-value	<0.01	<0.01	0.26	<0.01	0.54	0.31
Main effect *P*-value						
Density	<0.01	<0.01	0.96	<0.01	0.21	0.44
25-OH-D_3	0.87	0.24	0.77	0.66	0.74	0.33
Density*25-OH-D_3	0.88	0.32	0.13	0.11	0.66	0.57

[a-b]Means with different superscripts within a column differ significantly ($P < 0.05$).
Abbreviations: 25-OH-D_3, 25-hydroxycholecalciferol; FCR, feed conversion ratio.
[1]Each mean represents 10 replicates per treatment, with 20 layer per replicate.

Egg Quality

Egg yolk color, albumen height, and Haugh unit were evaluated using an egg multitester (EMT-7,300, Robotmation Co., Ltd., Tokyo, Japan). Eggshell strength was evaluated using an eggshell force gauge model II (Robotmation Co., Ltd.). Eggshell thickness was measured on the large end, equatorial region, and small end, using an eggshell thickness gauge (Robotmation Co., Ltd.). Eggshell lightness (L*), redness (a*), and yellowness (b*) were measured as reported by Odabasi et al. (2007).

Tibia Quality

Tibia (8 replicate per treatment group) were fixed with 4% paraformaldehyde, decalcified with 10% ethylenediamine tetraacetic acid (**EDTA**), dehydrated, and embedded in paraffin wax. The paraffin blocks were cut into 5-μm-thick sections, stained with toluidine blue, mounted on slides, and observed for histological observation.

The tibias were cleaned of surrounding muscles and soft tissues and measured the strength by the texture analyzer (TAXTPlus, Stable MicroSystems corp. , England). Then tibias were cut into pieces to fit into a soxhlet for ether extraction. Ether extracted bone pieces were dried and weighed and placed in crucibles and further dried at 105°C for 24 h in a DN-81 constant-temperature oven (American Scientific, Portland, OR). Finally, bones were placed in an ash oven (Thermolyne 30,400, Barnstead International, Dubuque, IA) at 600°C for 6 h, and the ash percentage was determined. The content of phosphorus and calcium were determined by ammonium metavanadate colorimetric and ethylenediamine tetraacetic acid titration method respectively (Manobhavan et al., 2016).

Serum Hormones Analysis

Serum concentration of 25-OH-D_3, corticosterone (**CORT**), lipopolysaccharide (**LPS**), carbonic anhydrase (**CA**), osteocalcin (**OC**), parathyroid hormone (**PTH**), and calcitonin (**CT**) were assessed by enzyme-linked immunosorbent assay kits obtained from LifeSpan BioSciences Inc.

Statistical Analysis

Data were analyzed as a 2 × 2 factorial design by two-way ANOVA with a model that included the main effects of stocking density and 25-OH-D_3, as well as their interaction. When the interaction was significant ($P \leq 0.05$), means were compared by using Tukey's range test to determine significant differences among means.

RESULTS

Production Performance

Overall, high stocking density resulted in lower laying rate and feed intake, whereas it decreased egg weight during 1 to 8 and 1 to 16 wk compared with low stocking density (Table 2, Table 3 and Table 4; $P < 0.01$). The broken egg rate was also higher in high stocking density during 9 to 16 and 1 to 16 wk compared with low stocking density ($P < 0.01$). Dietary 25-OH-D_3 did not affect production performance (egg laying rate, feed intake, egg weight, and feed efficiency) during the experimental period ($P > 0.05$), whereas it decreased the broken egg rate under high density (interaction, $P < 0.05$). Feed efficiency and unqualified egg rate was not affected by either dietary 25-OH-D_3 or stocking density ($P > 0.05$).

Egg Quality

As shown in Table 5, high stocking density resulted in an increased eggshell lightness (L*) and a reduction in shell redness (a*), yellowness (b*) value, shell strength, thickness, and relative weigh compared with low stocking density ($P < 0.05$) at 16 wk. Dietary supplementation with 25-OH-D_3 reduced the L* of the eggshell and enhanced the b* value and the weight of eggshells compared with no addition ($P \leq 0.05$). The increase in eggshell thickness was more pronounced when layers received a 25-OH-D_3 supplemented diet under high stocking density ($P < 0.05$).

Tibia Quality

Hens under high stocking density had lower tibia ash and calcium content than hens under low stocking

Table 3. Effect of dietary 25-OH-D_3 on performance of laying hens under high or low stocking density (9–16 wk)[1].

Item	Laying rate, %	Egg weight, g	FCR	Feed intake, g	Broken egg, %	Unqualified egg,%
Density 25-OH-D_3						
Low−	94.74 ± 0.88[a]	65.47 ± 0.55	1.95 ± 0.03	120.68 ± 1.43[a]	1.54 ± 0.17[b]	1.20 ± 0.07
Low+	95.42 ± 0.77[a]	64.58 ± 0.67	1.96 ± 0.06	120.67 ± 1.23[a]	1.33 ± 0.20[b]	1.14 ± 0.17
High−	92.99 ± 0.43[b]	64.52 ± 0.32	1.98 ± 0.05	118.68 ± 1.45[a,b]	2.44 ± 0.11[a]	1.65 ± 0.11
High+	91.66 ± 0.66[b]	64.32 ± 0.25	1.99 ± 0.04	116.44 ± 1.91[b]	1.41 ± 0.14[b]	1.77 ± 0.23
P-value	<0.01	0.09	0.67	0.04	<0.01	0.41
Main effect *P*-value						
Density	<0.01	0.10	0.40	<0.01	<0.01	0.54
25-OH-D_3	0.53	0.24	0.94	0.23	0.02	0.33
Density*25-OH-D_3	0.08	0.40	0.90	0.51	0.04	0.21

[a-b]Means with different superscripts within a column differ significantly ($P < 0.05$).
Abbreviations: 25-OH-D_3, 25-hydroxycholecalciferol; FCR, feed conversion ratio.
[1]Each mean represents 10 replicates per treatment, with 20 layer per replicate.

density (Table 6; $P < 0.05$), whereas 25-OH-D_3 dietary supplementation increased tibial strength compared with no addition ($P = 0.05$).

Histopathology evaluation of the tibia (Figure 1) revealed the presence of a dense cortical bone structure and good presence of bone cells in the L + 0 group and L + 25-OH-D_3 group, whereas the medullary cavity was filled with a large number of medullary trabeculae, which were well connected to each other, resulting in a good tibia structure. In the H + 0 group, several fractures were shown, and the trabecular area was also lower than the control treatment (L + 0), whereas dietary 25-OH-D3 increased the trabecular area in layers under high stocking density ($P < 0.05$), resulting in a comparative good tibia structure as the control treatment.

Serum Hormones Analysis

Overall, compared with the layers kept under low stocking density, layers kept under high stocking density had a higher serum concentration of 25-OH-D_3, CORT, LPS, and OC (Table 7; $P < 0.05$) and lower serum PTH concentration ($P < 0.01$). As expected, dietary 25-OH-D_3 increased serum concentration of 25-OH-D_3, CA, and CT ($P < 0.01$) and reduced the concentration of CORT ($P < 0.01$), LPS ($P < 0.05$), and OC ($P < 0.01$). There was a tendency for the stocking density and 25-OH-D_3 interaction on 25-OH-D_3 ($P = 0.09$), CORT ($P = 0.06$), and OC ($P = 0.07$) concentration, whereas a significant effect was noted on PTH concentration ($P < 0.05$), in that the observed increase in serum 25-OH-D_3 and decrease in COR and PTH by dietary 25-OH-D_3 was higher when layers were under high stocking density and that the decrease in serum OC concentration was higher when the layers were under low stocking density.

DISCUSSION

Increasing stocking density reduced the space allowances which may negatively affect the production performance (body weight gain, feed efficiency, egg laying rate, and egg weight) by decreasing feed assumption in broilers and layers (Leeson and Summers, 1984; Hester and Wilson, 1986; Carey, 1987; Kang et al., 2016). In this study, we did find that high stocking density significantly reduced egg production rate, egg weight, and feed intake of laying hens. On the same time, the administration of 25-OH-D3 diet had no significant effect on production, which is also consistent with previous observations (Keshavarz, 2003; Peeble et al., 2008). The reason may contribute to that the content of vitamin D_3 (5000 IU) in diet had already met the layers' requirements in current study. It has been indicated that only when vitamin D_3 was deficient in the diet, the exogenous addition of vitamin D_3 or its analog can exert its improving effect on performance of laying hens (Shen et al., 1981; Goodson-Williams et al., 1986; Geng et al., 2018).

Table 4. Effect of dietary 25-OH-D_3 on performance of laying hens under high or low stocking density (1–16 wk)[1].

Item	Laying rate, %	Egg weight, g	FCR	Feed intake, g	Broken egg, %	Unqualified egg,%
Density 25-OH-D_3						
Low−	94.92 ± 0.37[a]	65.01 ± 0.27[a]	1.93 ± 0.04	119.27 ± 0.97[a]	1.34 ± 0.11[b]	1.09 ± 0.11
Low+	95.58 ± 0.45[a]	64.26 ± 0.31[a,b]	1.95 ± 0.01	119.77 ± 1.10[a]	1.16 ± 0.21[b]	1.06 ± 0.14
High−	93.37 ± 0.68[b]	63.86 ± 0.33[b]	1.95 ± 0.04	116.75 ± 0.88[b]	2.00 ± 0.17[s]	1.65 ± 0.21
High+	92.69 ± 0.73[b]	63.60 ± 0.21[b]	1.94 ± 0.03	114.59 ± 0.95[b]	1.43 ± 0.14[b]	1.54 ± 0.09
P-value	<0.01	<0.01	0.82	<0.01	0.02	0.44
Main effect *P*-value						
Density	<0.01	<0.01	0.67	<0.01	<0.01	0.54
25-OH-D_3	0.64	0.11	0.78	0.37	0.04	0.37
Density*25-OH-D_3	0.09	0.57	0.44	0.16	0.01	0.81

[a-b]Means with different superscripts within a column differ significantly ($P < 0.05$).
Abbreviations: 25-OH-D_3, 25-hydroxycholecalciferol; FCR, feed conversion ratio.
[1]Each mean represents 10 replicates per treatment, with 20 layer per replicate.

Table 5. Effect of dietary 25-OH-D_3 on egg quality of laying hens under high or low stocking density[1].

Item	Eggshell color			Eggshell strength, kg/cm^3	Eggshell thickness, mm^{-2}	Eggshell relative weight, %	Yolk relative weight, %	Yolk color	Albumen height, mm	Haugh unit
	L*	a*	b*							
Density25-OH-D_3										
Low−	82.35 ± 0.33[b]	6.51 ± 0.19[a]	17.21 ± 0.31[a]	4.37 ± 0.11	40.88 ± 0.07[a]	11.25 ± 0.61	28.46 ± 0.76	13.33 ± 0.76	7.14 ± 0.09	82.94 ± 1.98
Low+	82.07 ± 0.29[b]	6.47 ± 0.21[a,b]	17.93 ± 0.29[a]	4.27 ± 0.21	39.63 ± 0.04[a]	11.24 ± 0.48	28.38 ± 0.54	13.41 ± 0.11	7.07 ± 0.11	82.55 ± 0.97
High−	83.23 ± 0.22[a]	5.56 ± 0.27[c]	16.30 ± 0.37[b]	4.05 ± 0.28	36.73 ± 0.08[b]	10.85 ± 0.55	28.07 ± 0.66	13.30 ± 0.27	7.13 ± 0.27	82.66 ± 0.87
High+	82.25 ± 0.43[b]	6.02 ± 0.18[b,c]	17.46 ± 0.19[a]	4.11 ± 0.17	37.63 ± 0.05[b]	11.07 ± 0.87	27.47 ± 0.57	13.27 ± 0.55	7.08 ± 0.32	82.37 ± 1.49
SEM	0.35	0.24	0.38	0.15	0.06	0.63	0.50	0.15	0.21	1.38
P-value	<0.01	<0.01	<0.01	0.12	<0.01	<0.01	0.18	0.82	0.98	0.98
Main effect *P*-value										
Density	0.04	<0.01	0.04	0.04	<0.01	0.01	0.08	0.46	0.86	0.92
25-OH-D_3	0.04	0.22	<0.01	0.92	0.79	0.25	0.37	0.84	0.57	0.63
Density*25-OH-D_3	0.16	0.13	0.42	0.49	0.01	0.25	0.50	0.62	0.84	0.86

[a-b]Means with different superscripts within a column differ significantly ($P < 0.05$).
Abbreviation: 25-OH-D_3, 25-hydroxycholecalciferol.
[1]Each mean represents 10 replicates per treatment, with 30 eggs per replicate.

Eggshell pigmentation has been widely used as a potential indicator for stress and disease conditions in commercial laying hens (Samiullah et al., 2015). Stress factors, such as high density, fear, and illness can cause laying hens to lay pale coloration or extraneous calcium eggshells eggs (Hughes et al., 1986; Walker, 1998; Samiullah et al., 2015). We found that high stocking density increased the lightness (higher L* value) of the eggshell and reduced the redness and yellowness (lower b* values), indicating that the high stocking density reduces the eggshell color (become lighter). Dietary 25-OH-D_3 significantly reduced the L* of the eggshell and increased the yellowness (b*), suggesting that dietary 25-OH-D_3 recovered the eggshell color. In addition, we observed that high stocking density reduced the strength, thickness, and relative weight of the eggshell, which is consistent with previous reports (Kang et al., 2016). On the same time, we also found that 25-OH-D_3 increased the relative weight of the eggshell and increased the thickness of the eggshell under high stocking density, which is inconsistent with the previous studies (McLoughlin and Soares, 1976; Keshavarz, 2003). This inconsistency could be attributed to the diet composition (the dietary calcium or the VD_3 levels) in these previous researches.

Serum 25-OH-D_3 concentration is considered as the important index for vitamin D status in the body. We observed that the addition of 25-OH-D_3 in the diet significantly increased the serum 25-OH-D_3 level, indicating higher serum 25-OH-D_3 is advantageous for optimal calcium and skeletal homeostasis in layers under high stocking density. Corticosterone is one of the glucocorticoids isolated from the adrenal gland, and it can be used as a stress marker to assess whether birds' welfare has been impaired. Similarly, Siegel (1995) also reported the increase of serum CORT in layers under high stocking density. Lipopolysaccharide, a molecule present on the outer surface of gram-negative bacteria, is a powerful inflammatory mediator. Lipopolysaccharide concentration can be indicative of excessive immune activation, compromised gastrointestinal functionality (Celi et al., 2019), and overall health status of the animal (Ahola et al., 2017). In our study, we observed that high stocking density increased both CORT and LPS concentration in serum, indicating that high stocking density can cause stress and induce inflammation in laying hens. The reduced CORT and LPS levels by dietary 25-OH-D_3 support the role of 25-hydroxycholecalciferol in decreasing inflammation in poultry (Morris et al., 2014, 2015).

Tibia is an important organ for both growth and production of laying hens, because 35 to 40% of the calcium comes from bone deposition and rest of calcium comes from the diet during eggshell development. Selection for early sexual maturity and high egg production in commercial layer lines required high calcium deposition because of the formation of eggshells (Sandilands et al., 2009). High stocking density can affect the development of the tibia and reduce its quality (Sanotra et al., 2001; Buijs et al., 2009; Ventura et al., 2010;

Table 6. Effect of dietary 25-OH-D_3 on tibia quality of laying hens under high or low stocking density[1].

Item	Strength, kgf	Ash, %	Ca, %	P, %
Density 25-OH-D_3				
Low−	16.88 ± 1.40	52.96 ± 1.35	19.50 ± 0.42	8.10 ± 0.21
Low+	18.95 ± 1.65	53.49 ± 2.09	19.68 ± 0.55	7.90 ± 0.11
High−	15.80 ± 0.97	51.76 ± 1.01	18.87 ± 0.31	7.90 ± 0.22
High+	17.23 ± 1.19	51.37 ± 0.95	18.92 ± 0.42	7.80 ± 0.13
P-value	0.15	0.16	0.30	0.40
Main effect *P*-value				
Density	0.16	0.04	0.04	0.07
25-OH-D_3	0.05	0.79	0.96	0.07
Density*25-OH-D_3	0.74	0.77	0.91	0.30

[a-b]Means with different superscripts within a column differ significantly ($P < 0.05$).
Abbreviation: 25-OH-D_3, 25-hydroxycholecalciferol.
[1]Each mean represents 10 replicates per treatment, with 10 layer per replicate.

Zhang et al., 2018). Studies have shown that 25-OH-D_3 can reduce broiler tibia dyschondroplasia (Rennie and Whitehead, 1996; Ledwaba and Roberson, 2003). Swiatkiewicz et al., (2006) also showed that tibia strength of broilers were significantly increased when Vitamin D3 were replaced 80 and 100% with 25-OH-D_3. In our study, the histological valuation of the tibia suggests that 25-OH-D_3 in the diet resulted in better connectivity of the medullary trabecular bone of the tibia and a more complete tibia structure. We also found that high stocking density reduced ash and calcium content of the tibia, whereas 25-OH-D_3 supplementation increased the strength of the tibia. These results proved that 25-OH-D_3 can improve the adverse effects of high stocking density on tibia quality. Moreover, lower serum PTH concentration was observed in layers under high stocking density, indicating absorption of calcium in the body is impaired by the higher stocking density in our study, which is in agreement with (MacDonald et al., 1986), who suggested that PTH is able to absorb bone and increase osteoclasts activity, allowing the laying hen to complete the process of bone calcium activation. Studies also suggested that serum OC is indicator for bone turnover rate (Pocock et al., 1987; Kelly et al., 1993). Genetic polymorphisms located in the OC gene have reported to be linked with serum OC levels and bone fractures (McGuigan et al., 2010). Our studies suggested dietary 25-OH-D_3 increased serum CT and PTH concentration, indicating that 25-OH-D_3 can promote calcium absorption and maintain skeletal health during these conditions. In addition, we found that 25-OH-D_3 increased the content of CA in serum. Carbonic anhydrase is considered a pivotal enzyme involved in the deposition of calcium carbonate during shell formation (Zhang et al., 2017). Carbonic anhydrase generates protons and $CO3^{2-}$ from H_2O_2 and CO2 and is involved in skeletal health and acid-base regulation (Billecocq et al., 1990). Therefore, it is plausible that the beneficial effects of dietary 25-OH-D3 on eggshell quality and skeletal health could be partially ascribed to the observed higher CA concentration in our study.

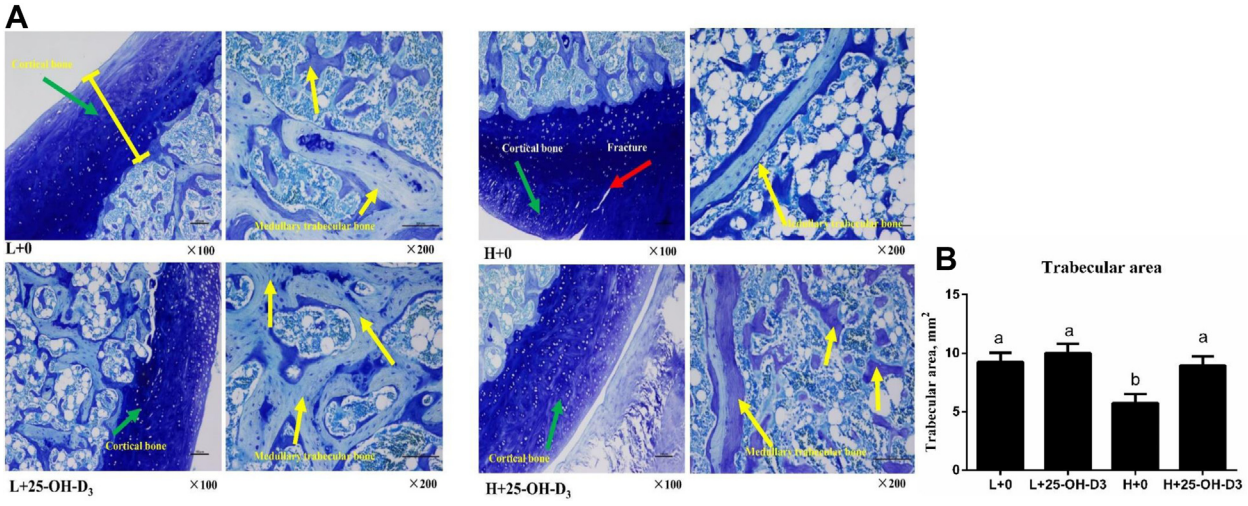

Figure 1. Effect of dietary 25-OH-D_3 on tibia histopathology of laying hens under high or low stocking density (×100 and 200). (A) The tibia histopathology. The cortical bone and medullary trabecular bone were indicated by green arrow and yellow arrow respectively, with the red arrow presented the fracture of cortical bone. A dense cortical bone structure and good presence of bone cells were shown in the L + 0 group and L + 25-OH-D_3 group, whereas several fracture was shown in H + 0 group layers (red arrow). (B) The trabecular area. The layers from high stocking density had lower trabecular area than the control treatment (L + 0), whereas dietary 25-OH-D3 increased the trabecular area in layers under high stocking density ($P < 0.05$). Abbreviation: 25-OH-D_3, 25-hydroxycholecalciferol.

Table 7. Effect of dietary 25-OH-D_3 on serum hormone concentrations of laying hens under high or low stocking density[1].

	25-OH-D_3	CORT	LPS	CA	OC	PTH	CT
Item	ng/mL	ng/L	ng/L	ng/L	ng/L	ng/L	ng/L
Density 25-OH-D_3							
Low−	7.65 ± 0.34^{b}	455.13 ± 12.57^{a}	195.09 ± 0.47^{a,b}	311.91 ± 16.54^{c}	498.94 ± 20.56^{b}	217.23 ± 10.56^{a}	69.87 ± 3.21^{b}
Low+	8.15 ± 0.51^{b}	367.00 ± 17.86^{b}	181.71 ± 0.47^{b}	354.80 ± 19.65^{a,b}	469.52 ± 18.65^{b}	200.66 ± 12.89^{a,b}	77.93 ± 2.99^{a,b}
High−	7.86 ± 0.61^{b}	459.78 ± 9.67^{a}	212.10 ± 0.47^{a}	328.87 ± 12.56^{b,c}	579.30 ± 26.89^{a}	160.36 ± 7.48^{c}	68.78 ± 4.98^{b}
High+	9.20 ± 0.47^{a}	427.93 ± 19.21^{a}	195.19 ± 0.47^{a,b}	373.86 ± 21.44^{a}	481.80 ± 16.98^{b}	182.26 ± 10.55^{b,c}	83.21 ± 5.07^{a}
P-value	<0.01	<0.01	0.05	0.05	<0.01	<0.01	0.05
Main effect *P*-value							
Density	0.05	0.05	0.05	0.19	0.05	<0.01	0.53
25-OH-D_3	<0.01	<0.01	0.05	<0.01	<0.01	0.74	<0.01
Density*25-OH-D_3	0.09	0.06	0.81	0.94	0.07	0.05	0.34

[a-b]Means with different superscripts within a column differ significantly ($P < 0.05$).

Abbreviations: 25-OH-D3, 25-hydroxycholecalciferol; CA, carbonic anhydrase; CORT, corticosteron; CT, calcitonine; LPS, lipopolysaccharide; OC, osteocalcin; PTH, parathyroid hormone.

[1]Each mean represents 10 replicates per treatment, with 20 layer per replicate.

CONCLUSION

It could be concluded that high stocking density reduce production performances (egg laying rate, egg weight, and feed intake), shell quality, and tibia quality. Dietary 25-OH-D_3 supplementation is good for the skeletal health and can mitigate the adverse effects of high stocking density on production performance.

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REFERENCES

Ahola, A. J., M. I. Lassenius, C. Forsblom, V. Harjutsalo, M. Lehto, and P. H. Groop. 2017. Dietary patterns reflecting healthy food choices are associated with lower serum LPS activity. Sci. Rep. 7:6511.

Bello, A., W. Zhai, P. D. Gerard, and E. D. Peebles. 2014. Effects of the commercial in ovo injection of 25-hydroxycholecalciferol on broiler posthatch performance and carcass characteristics. Poult. Sci. 93:155–162.

Billecocq, A., J. R. Emanuel, R. Levenson, and R. Baron. 1990. 1 alpha,25-dihydroxyvitamin D3 regulates the expression of carbonic anhydrase II in nonerythroid avian bone marrow cells. Proc. Nat. Aca. Sci. 87:6470.

Buijs, S., L. Keeling, S. Rettenbacher, E. Van Poucke, and F. A. Tuyttens. 2009. Stocking density effects on broiler welfare: identifying sensitive ranges for different indicators. Poult. Sci. 88:1536–1543.

Carey, J. B. 1987. Effects of pullet-stocking density on performance of laying hens. Poult. Sci. 66:1283–1287.

Campbell, D. L. M., C. Lee, G. N. Hinch, and J. R. Roberts. 2017. Egg production and egg quality in free-range laying hens housed at different outdoor stocking densities. Poult. Sci. 96:3128.

Celi, P., V. Verlhac, E. Pérez Calvo, J. Schmeisser, and A.-M. Kluenter. 2019. Biomarkers of gastrointestinal functionality in animal nutrition and health. Anim. Feed Sci. Technol. 250:9–31.

Geng, Y., Q. Ma, Z. Wang, and Y. Guo. 2018. Dietary vitamin D(3) supplementation protects laying hens against lipopolysaccharide-induced immunological stress. Nutr. Metab. (Lond). 15:58.

Goodson-Williams, R., D. A. Roland, and J. A. McGuire. 1986. Effects of feeding Graded levels of vitamin D3 on egg shell Pimpling in aged hens. Poult. Sci. 65(8):1556–1560.

Hester, P. Y., and E. K. Wilson. 1986. Performance of White Leghorn hens in response to cage density and the introduction of cage mates. Poult. Sci. 65:2029–2033.

Holick, M. F., and T. C. Chen. 2008. Vitamin D deficiency: a worldwide problem with health consequences. Am. J. Clin. Nutr. 87:1080S–1086S.

Hughes, B. O., A. B. Gilbert, and M. F. Brown. 1986. Categorisation and causes of abnormal egg shells: Relationship with stress. Br. Poult. Sci. 27(2):325–337.

Kang, H. K., S. B. Park, S. H. Kim, and C. H. Kim. 2016. Effects of stock density on the laying performance, blood parameter, corticosterone, litter quality, gas emission and bone mineral density of laying hens in floor pens. Poult. Sci. 95:2764–2770.

Kelly, P. J., T. Nguyen, J. Hopper, N. Pocock, P. Sambrook, and J. Eisman. 1993. Changes in axial bone density with age: a twin study. J. Bone Miner. Res. 8:11–17.

Keshavarz, K. 2003. A comparison between cholecalciferol and 25-OH-cholecalciferol on performance and eggshell quality of hens fed different levels of calcium and phosphorus. Poult. Sci. 82:1415–1422.

Ledwaba, M. F., and K. D. Roberson. 2003. Effectiveness of twenty-five-hydroxycholecalciferol in the prevention of tibial dyschondroplasia in Ross cockerels depends on dietary calcium level. Poult. Sci. 82:1769–1777.

Leeson, S., and J. D. Summers. 1984. Effects of cage density and diet energy concentration on the performance of growing Leghorn pullets subjected to early induced maturity. Poult. Sci. 63:875–882.

MacDonald, B. R., J. Gallagher, and R. Russell. 1986. Parathyroid hormone stimulates the proliferation of cells derived from human bone. Endocrinology 118(6):2445–2449.

Manobhavan, M., A. V. Elangovan, M. Sridhar, D. Shet, S. Ajith, D. T. Pal, and N. K. Gowda. 2016. Effect of super dosing of phytase on growth performance, ileal digestibility and bone characteristics in broilers fed corn-soya-based diets. J. Anim. Physiol. Anim. Nutr. (Berl.). 100:93–100.

McGuigan, F., J. Kumar, K. K. Ivaska, K. J. Obrant, P. Gerdhem, and K. Åkesson. 2010. Osteocalcin gene polymorphisms influence concentration of serum osteocalcin and enhance fracture identification. J. Bone Miner. Res. 25:1392–1399.

McLoughlin, C. P., and J.H. Soares, Jr. 1976. A study of the effects of 25 hydroxycholecalciferol and calcium source on egg shell Quality1. Poult. Sci. 55:1400–1410.

Morris, A., R. Shanmugasundaram, M. S. Lilburn, and R. K. Selvaraj. 2014. 25-Hydroxycholecalciferol supplementation

improves growth performance and decreases inflammation during an experimental lipopolysaccharide injection. Poult. Sci. 93:1951–1956.
Morris, A., R. Shanmugasundaram, J. McDonald, and R. K. Selvaraj. 2015. Effect of in vitro and in vivo 25-hydroxyvitamin D treatment on macrophages, T cells, and layer chickens during a coccidia challenge. J. Anim. Sci. 93:2894–2903.
Odabasi, A. Z., R. D. Miles, M. O. Balaban, and K. M. Portier. 2007. Changes in brown eggshell color as the hen ages. Poult. Sci. 86:356–363.
Peebles, E. D., S. L. Branton, M. R. Burnham, S. K. Whitmarsh, and P. D. Gerard. 2008. Effects of supplemental dietary phytase and 25-hydroxycholecalciferol on the performance characteristics of commercial layers inoculated before or at the onset of lay with the F-strain of Mycoplasma gallisepticum. Poult. Sci. 87:598–601.
Pocock, N. A., J. A. Eisman, J. L. Hopper, M. G. Yeates, P. N. Sambrook, and S. Eberl. 1987. Genetic determinants of bone mass in adults. A twin study. J. Clin. Invest. 80:706–710.
Rath, N. C., G. R. Huff, W. E. Huff, and J. M. Balog. 2000. Factors regulating bone maturity and strength in poultry. Poult. Sci. 79:1024–1032.
Rennie, J. S., and C. C. Whitehead. 1996. Effectiveness of dietary 25- and 1-hydroxycholecalciferol in combating tibial dyschondroplasia in broiler chickens. Br. Poult. Sci. 37:413–421.
Saki, A. A., P. Zamani, M. Rahmati, and H. Mahmoudi. 2012. The effect of cage density on laying hen performance, egg quality, and excreta minerals. J. Appl. Poult. Res. 21:467–475.
Samiullah, S., J. R. Roberts, and K. Chousalkar. 2015. Eggshell color in brown-egg laying hens - a review. Poult. Sci. 94:2566–2575.
Sandilands, V., C. Moinard, and N. H. C. Sparks. 2009. Providing laying hens with perches: fulfilling behavioural needs but causing injury? Br. Poult. Sci. 50:395–406.
Sanotra, G. S., L. G. Lawson, K. S. Vestergaard, and M. G. Thomsen. 2001. Influence of stocking density on tonic immobility, lameness, and tibial dyschondroplasia in broilers. J. Appl. Anim. Welf. Sci. 4:71–87.
Saunders-Blades, J. L., and D. R. Korver. 2015. Effect of hen age and maternal vitamin D source on performance, hatchability, bone mineral density, and progeny in vitro early innate immune function. Poult. Sci. 94:1233–1246.
Savory, C. J., M. C. Jack, and V. Sandilands. 2006. Behavioural responses to different floor space allowances in small groups of laying hens. Br. Poult. Sci. 47:120–124.
Shen, H., J. D. Summers, and S. Leeson. 1981. Egg production and shell quality of layers fed Various levels of vitamin D3. Poult. Sci. 60(7):1485–1490.
Siegel, H. S. 1995. Gordon Memorial Lecture. Stress, strains and resistance. Br. Poult. Sci. 36:3–22.
Swiatkiewicz, S., J. Koreleski, and J. Kopowski. 2006. Effect of phytase and 25-hydroxycholecalciferol on performance and bone quality in broiler chickens. Med. Weter. 62(1):81–84.
United Egg Producers. 2016. Animal Husbandry Guidelines for U.S. Egg Laying Flocks. Accessed Nov. 2019. http://www.unitedegg.com/information/pdf/UEP-Animal-Welfare-Guidelines2016.pdf.
Ventura, B. A., F. Siewerdt, and I. Estevez. 2010. Effects of barrier perches and density on broiler leg health, fear, and performance. Poult. Sci. 89:1574–1583.
Walker, A. W. 1998. Egg shell colour is affected by laying cage design. Br. Poult. Sci. 39:696–699.
Yarger, J. G., C. A. Saunders, J. L. McNaughton, C. L. Quarles, B. W. Hollis, and R. W. Gray. 1995. Comparison of dietary 25-hydroxycholecalciferol and cholecalciferol in broiler chickens. Poult. Sci. 74:1159–1167.
Zhang, Y. N., H. J. Zhang, J. Wang, H. Y. Yue, X. L. Qi, S. G. Wu, and G. H. Qi. 2017. Effect of dietary supplementation of organic or inorganic zinc on carbonic anhydrase activity in eggshell formation and quality of aged laying hens. Poult. Sci. 96:2176–2183.
Zhang, Y. R., L. S. Zhang, Z. Wang, Y. Liu, F. H. Li, J. M. Yuan, and Z. F. Xia. 2018. Effects of stocking density on growth performance, meat quality and tibia development of Pekin ducks. Anim. Sci. J. 89(6):925–930.