

# Effect of high concentrations of dietary vitamin D<sub>3</sub> on pullet and laying hen performance, skeleton health, eggshell quality, and yolk vitamin D<sub>3</sub> content when fed to W36 laying hens from day of hatch until 68 wk of age

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**ABSTRACT** The objective of this experiment was to investigate the effects of various dietary concentrations of vitamin D<sub>3</sub> (D<sub>3</sub>) on pullet and laying hen performance, eggshell quality, bone health, and yolk D<sub>3</sub> content from day of hatch until 68 wk of age. Initially, 440 Hy-line W36-day-old chicks were randomly assigned to 5 dietary treatments: 1,681 (control); 8,348; 18,348; 35,014; 68,348 IU D<sub>3</sub>/kg. At 17 wk of age, pullets were assigned to experimental diets with 12 replicate groups of 6 birds. At 17 wk of age, pullets fed diets containing 8,348 and 35,014 IU D<sub>3</sub>/kg had an increased bone mineral density in comparison to the control fed birds ( $P \leq 0.01$ ). Body weights of pullets fed the diet with 68,348 IU D<sub>3</sub>/kg were lower than other treatments ( $P \leq 0.01$ ). Hen-housed egg production (HHEP) of hens fed the 35,014 IU D<sub>3</sub>/kg diet was increased in comparison to control-fed hens ( $P \leq 0.01$ ), whereas HHEP of those fed 68,348 IU D<sub>3</sub>/kg diet was reduced in compar-

ison to all other treatments ( $P \leq 0.01$ ). Shell breaking strength of eggs from hens fed 8,348, 35,014 and 68,348 IU D<sub>3</sub>/kg was increased in comparison to eggs from control-fed birds ( $P \leq 0.01$ ). Fat-free tibia ash content of hens fed any of the diets supplemented with D<sub>3</sub> (8,348 to 68,348 IU D<sub>3</sub>/kg) was increased in comparison to control-fed hens ( $P \leq 0.05$ ). Yolk D<sub>3</sub> content increased linearly with dietary D<sub>3</sub> and the D<sub>3</sub> transfer efficiency for the control, 8,348 IU, 18,348 IU, 35,014 IU, and 68,348 IU D<sub>3</sub> treatments were 8.24, 10.29, 11.27, 12.42, and 12.06%, respectively. These data suggest that supplementation of dietary D<sub>3</sub> up to 35,014 IU D<sub>3</sub>/kg feed maintained if not increased laying hen performance and enhanced pullet and laying hen skeletal quality as well as yolk D<sub>3</sub> content and eggshell quality. Feeding pullets at a higher level 68,348 IU of D<sub>3</sub> resulted in reduced growth and ultimately decreased performance of laying hens.

**Key words:** laying hen, vitamin D<sub>3</sub>, skeleton health, eggshell quality, yolk vitamin D<sub>3</sub> concentration

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## INTRODUCTION

Vitamin D is a group of closely related compounds that have antirachitic activity. It has been estimated that nearly 50% of the United States population is at risk for vitamin D deficiency or insufficiency (Holick et al., 2011). Vitamin D<sub>3</sub> (D<sub>3</sub>) deficiency can cause rickets in young children or increase the risk of osteoporosis and osteomalacia in adults (Holick, 2005). One approach to increase D<sub>3</sub> intake in a population without changing eating habits is to produce D<sub>3</sub>-fortified eggs (Mattila et al., 2004). As a fat-soluble vitamin, D<sub>3</sub> from the hen diet is absorbed into the blood stream and transferred to egg yolk via the ovary. Previous reports have demonstrated a linear increase on egg D<sub>3</sub> concentration with increasing dietary D<sub>3</sub>, with no changes in

physical, functional and sensory quality properties in the high D<sub>3</sub> eggs (Yao et al., 2013).

In addition to fortifying eggs, high dietary D<sub>3</sub> may potentially affect calcium and phosphorus metabolism and related biological characteristics in laying hens. Tibia breaking strength increased when hens were fed diets with 6,000 or 15,000 IU D<sub>3</sub>/kg in comparison to control-fed birds (2,500 IU D<sub>3</sub>/kg), in laying hens from 20 to 67 wk of age (Mattila et al., 2004). In contrast, neither egg specific gravity nor eggshell strength was affected by diet supplementation with D<sub>3</sub> from 2,500 to 15,000 IU/kg. Egg specific gravity and eggshell breaking strength increased in hens fed diets containing 2,400 IU D<sub>3</sub>/kg in comparison to those fed a diet containing 300 IU D<sub>3</sub>/kg (Zang et al., 2011). However, another experiment using higher supplementation with D<sub>3</sub> (2,200 to 102,200 IU/kg of diet) found no effect on either eggshell or bone quality in Hy-line W36 laying hens from 19 to 58 wk of age (Persia et al., 2013). Whereas the focus of previous research has been improving bone health of laying hens or egg shell quality by increasing D<sub>3</sub> during the egg laying phase, limited research

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has been conducted on the effect of increased dietary D<sub>3</sub> of pullets as well as laying hens on pullet bone development and the subsequent impact on laying hen skeletal health and eggshell quality. The objective of the current experiment was to determine the effects of feeding various concentrations of dietary D<sub>3</sub> to birds (day of hatch to 68 wk) on pullet and laying hen bone health, hen performance, and eggshell quality. The hypothesis was that supplementation of laying hens with high concentration of vitamin D from day of hatch to the end of the experimental feeding period would not only increase the amount of vitamin D in the egg, it would also increase skeletal mineral status and egg shell quality.

## MATERIALS AND METHODS

### Diets

In total, 7 phases of diet were formulated for each treatment including starter 1 (week 0–4), starter 2 (week 5–8), grower (week 9–12), developer (week 13–15), pre-laying (week 16–17), peak (week 18–24) and post-peak diets (week 25–68), respectively. The vitamin and mineral premix utilized produced basal diets containing 1,681 IU D<sub>3</sub>/kg (Table 1). Experimental diets were prepared by adding D<sub>3</sub> (DSM Nutritional Products Inc, Parsippany, NJ) at a concentration of 6,667, 16,667, 33,333, 66,667 IU/kg resulting in diets containing 1,681, 8,348, 18,348, 35,014, 68,348 IU D<sub>3</sub>/kg, respectively. These concentrations of supplemental Vitamin D were selection in an attempt to generate a range of egg vitamin D concentrations that can be used in conjunction with the previous literature and current commercial production to better understand the both the relationship between supplement and egg vitamin D concentrations and the toxicity of Vitamin D. Vitamin D<sub>3</sub> premix was stored at 4°C throughout the experimental period. Starter 1, starter 2, grower, developer, and pre-laying diets were manufactured as single batch diets whereas peak and post peak diets were manufactured every 2 wk due to longer feeding periods. Diet samples were collected over each mixing period and pooled into one sample per treatment for D<sub>3</sub> analysis. With the exception of Ca and P, nutrient concentrations of the diets generally followed Hy-Line W-36 Commercial Layer Management Guidelines (2016). Calcium and P concentrations for starter 1, starter 2, grower, developer, pre-laying diets were set at 95% of NRC (1994) recommendation to simulate a potential commercial pullet flock that might be exposed to limited Ca and P availability. For peak and post peak diets, Ca and P concentrations were set at 100% of NRC (1994) recommendations.

### Animals and Housing

All animal work was approved by the Institutional Animal Care and Use Committee at Virginia Tech (Blacksburg, VA). Initially, 440-day-old chicks (Hy-line

W36) were randomly assigned to one of 5 treatments with 2 replicates per treatment (84.5 cm<sup>2</sup>/bird) housed across the top tier of a three-tiered housing system. Initial chick body weight ranged between 39.1 and 39.6 g/bird on an average cage basis ( $P > 0.05$ ). Birds in each treatment were subsequently split into 4 replicates (169.0 cm<sup>2</sup>/bird) at 2 wk of age and into 8 replicates (338.0 cm<sup>2</sup>/bird) at 6 wk of age, decreasing stocking density over time to maintain body mass per cage early in life. Increasing the replication was done by tier as well with the top 2 tiers used at 2 wk of age and all 3 tiers at 6 wk of age. In the pullet house, the environment was controlled according to Hy-Line W-36 Commercial Layer Management Guide (2016). The temperature was 32 to 33°C at day of hatch and gradually decreased to 21°C when the chicks reached 36 D of age. Light exposure was 20 h at day of hatch and decreased over time to 12 h at 11 wk of age. Pullets were allowed ad libitum access to feed and water. At 14 wk of age, pullets received a killed *Salmonella enteritidis* vaccination (subcutaneous injection, 0.25 mL/bird). At 17 wk of age, 72 pullets in each treatment (those closest to the average body weight within each treatment) were moved into a multi-tiered, A-frame cage system resulting in 12 replicates of 6 birds per experimental unit (154.8 cm<sup>2</sup>/bird) for each of the 5 dietary treatments. Each experimental unit consisted of two consecutive cages with 3 birds per cage. The remaining 16 pullets from each treatment were euthanized, defeathered and the intact carcasses were stored at –20°C until bone mineral analysis. During the egg laying phase, 95 to 97 g of feed was provided per hen per day and feed was provided at approximately 10 AM. Hens were housed in dark-out house conditions and light exposure increased from 12 to 16 h over 17 to 32 wk of age. House temperature was controlled via a fan and air inlet ventilation system and ranged from 21 to 27°C over the duration of the experiment. Birds were monitored at least twice daily.

### Data Collection

Pullets were weighed at 2, 4, 6, 8, 12, 15, and 17 wk of age. Feed offered and refused was recorded weekly in both the pullet and egg production phases. Egg numbers and gross egg weight per pen were recorded daily. After the onset of lay, hens were weighed every 4 wk and at the end of the experiment. Feed intake, HHEP, egg weight, egg mass, and FCR were determined to correspond with the bi-weekly feed manufacture periods. Starting at 22 wk of age, internal and external egg quality were determined every 4 wk. Two eggs per experimental unit were collected and stored at 4°C for 7 D until egg quality determination. Egg quality measurements included Haugh unit, egg composition, eggshell thickness, egg specific gravity, and eggshell breaking strength. During week 38, one egg from each pen was collected over 2 consecutive days and pooled by treatment (6 eggs total) for yolk vitamin D<sub>3</sub> analysis. At

**Table 1.** Pullet and laying hen basal diets<sup>1</sup> for week 0–4, week 5–8, week 9–12, week 13–15, week 16–17, week 18–24, and week 25–68.

Ingredient (%)	week 0–4	week 5–8	week 9–12	week 13–15	week 16–17	week 18–24	week 25–68
Corn	65.65	67.14	67.99	69.35	64.65	48.47	59.49
Soybean meal	28.73	26.27	19.84	16.83	18.51	27.55	22.79
Dried distillers grains	–	1.00	6.00	8.00	8.00	8.00	5.00
Poultry byproduct meal	2.00	1.98	3.00	2.78	2.50	2.00	2.00
Soy oil	0.20	0.20	0.30	0.30	0.30	2.92	0.94
Salt	0.40	0.40	0.40	0.40	0.40	0.43	0.38
DL-methionine	0.18	0.20	0.14	0.08	0.14	0.25	0.20
L-lysine HCL	0.07	0.05	0.06	–	–	–	–
L-threonine	0.06	0.06	0.03	–	0.01	0.03	0.03
Limestone	1.03	1.03	1.00	1.30	2.19	4.68	4.14
Oyster shell	–	–	–	–	2.19	4.68	4.14
Dicalcium phosphate	1.02	1.02	0.59	0.31	0.47	0.34	0.23
Choline chloride	0.10	0.10	0.10	0.10	0.10	0.10	0.10
Vitamin & mineral premix <sup>2</sup>	0.50	0.50	0.50	0.50	0.50	0.50	0.50
Phytase <sup>3</sup>	0.06	0.06	0.06	0.06	0.06	0.06	0.06
Calculated content (%)							
Crude protein (%)	20.00	19.24	18.26	17.29	17.55	20.18	18.04
ME (kcal/kg)	3019	3029	3050	3055	2935	2844	2844
Calcium (%)	0.78	0.77	0.68	0.72	1.92	3.79	3.34
Non-phytate P (%)	0.32	0.32	0.27	0.22	0.24	0.22	0.18
Fat (%)	3.42	3.54	4.17	4.34	4.14	6.03	4.26
Digestible Lys (%)	1.05	0.98	0.88	0.76	0.79	0.97	0.85
Digestible Met+Cys (%)	0.74	0.74	0.67	0.60	0.66	0.80	0.71
Digestible Thr (%)	0.69	0.66	0.60	0.54	0.55	0.67	0.59

<sup>1</sup>Cholecalciferol was added to basal diets to obtain dietary cholecalciferol concentrations of 8,348, 18,348, 35,014, and 68,348 IU of D<sub>3</sub>/kg of diet. Measured cholecalciferol concentrations across all diet phases were 1,681, 7,987, 16,369, 39,802, and 72,529 IU of D<sub>3</sub>/kg, respectively.

<sup>2</sup>Provided per kg of diet: vitamin A, 4,403 IU; vitamin D<sub>3</sub>, 1,457 IU; vitamin E, 1.10 IU; menadione, 0.77 mg; vitamin B<sub>12</sub>, 4.40 µg; choline, 254.79 mg; niacin, 13.21 mg; pantothenic acid, 4.05 mg; riboflavin, 2.75 mg; Cu, 2.70 mg; Fe, 33.75 mg; I, 0.67 mg; Mn, 42.90 mg; Zn, 32.50 mg; Co, 0.17 mg.

<sup>3</sup>Supplementation of 0.06% phytase (300 FTU) to release 0.08% calcium and 0.08% non-phytate P in the complete diet.

68 wk of age, 5 hens from each experimental unit were euthanized to collect keel bone and right and left tibia. Once collected, keel bones were stored in plastic bags and held at 4°C until scored the next day. Both tibia were frozen at –20°C until analysis.

### Bone Mineral Density and Content

Dual X-ray absorptiometry (DXA) with a Lunar Prodigy machine (GE Lunar, GE Healthcare, Wauke-sha, WI) was utilized to measure bone mineral density and mineral content of carcasses and tibia. The pullets euthanized at 17 wk of age were thawed and arranged on the target pad of the DXA analysis platform. Ten carcasses were scanned using a single pass of the machine using the small animal mode. The tibia collected from the hens at 68 wk of age were thawed, defleshed of adhering tissue, and placed on the same analysis platform to measure bone mineral density and bone mineral content using the small animal mode. For scanning, 5 tibias from each experimental unit were pooled to generate one replicate sample.

### Yolk Vitamin D<sub>3</sub> Concentration

Eggs collected at 38 wk of age were stored at 4°C for no more than 3 D before they were analyzed. Yolks were separated from albumen by a yolk separator. All yolks from the same treatment were pooled and then homogenized using a spatula. The homogenized yolk sample for each of the 5 treatment was

lyophilized and vacuum packed. Egg yolk subsample (100 g) from each treatment was sent for D<sub>3</sub> analysis by liquid chromatography–mass spectrometry (LC-MS) method (Heartland Assay LLC, Ames, Iowa). Vitamin D<sub>3</sub> transfer efficiency was calculated (Yao et al., 2013) according to the following equation:

$$\begin{aligned} \text{Transfer efficiency (\%)} &= 100 \\ &\times [\text{yolk cholecalciferol concentration (IU/g)} \\ &\times \text{yolk mass (g)} \times \text{egg production (\% per hen per day)}] \\ &/ [\text{feed cholecalciferol concentration (IU/g)} \\ &\times \text{feed intake (g/D per hen)}]. \end{aligned}$$

### Eggshell and Tibia Breaking Strength

Eggshell and tibia breaking strength were measured with an Instron Universal Testing Machine Model 1011 (Instron Corp, Canton, MA). Each egg was placed on a base with the air cell or blunt end of the egg located to the bottom of the machine. A round steel head was used and lowered to the top of the egg (<1 mm) before compressing. During testing, the steel head was advanced towards the egg at 10 mm/min to begin with, and after the compression load reached 0.3 kg, the speed was reduced to 2 mm/min until the extension of the steel was 1.5 mm. Maximum compression load during this process was recorded.

Bone breaking strength was measured using a 3 point bending method. Tibia length was measured and middle point was marked. The tibia was placed on two parallel vertical holders with a 42 mm span positioned so the middle point of the tibia was underneath the bottom of the loading head. Before testing, the loading head was lowered to barely touch the middle point of tibia with a preload compression level of around 0.05 kg. The loading head descended at rate of 5 mm/min until bone fracture. Maximum compression load before fracture was recorded.

### **Fat-Free Tibia Ash Content**

Tibias were thawed, wrapped with aluminum foil, and autoclaved for 20 min. Tibias were then de-fleshed, had all cartilage removed, were placed into a glass beaker and then dried at 100°C for 24 h. Dried tibias were wrapped with cheese cloth and placed in a soxhlet apparatus. Hexane was used to extract fat from the tibia bones for 48 h. Tibias were then taken out of the soxhlet apparatus and allowed to dry in a hood overnight to allow residual hexane to evaporate before being dried at 100°C for 24 h. Tibias were weighed to determine dry fat-free weight, placed into a muffle furnace at 600°C for 24 h and then reweighed to determine ash weight. Fat-free tibia ash content was calculated as below.

$$\begin{aligned} &\text{Fat - free tibia ash content(\%)} \\ &= (\text{tibia ash weight / fat - free tibia dry weight})^* \\ &\quad \times 100\% \end{aligned}$$

### **Keel Bone Score**

Keel bones collected from 5 hens per experimental unit were scored for damage using the method described by Donaldson et al. (2012). Bones were scored from 0 (no damage) to 3 (severe damage and multiple fractures):

0 = one fracture, usually toward the tip of the keel;  
1 = 2–3 fractures or callus formation; 2 = multiple fractures or callus formation plus slight deformation at the fracture sites; 3 = multiple fractures or callus formation and severe deformations of the ventral edge

### **Statistical Analysis**

SAS 9.4 (SAS Institute Inc, Cary, NC) was used to analyze HHEP, feed intake, egg weight, egg mass, feed conversion ratio (FCR), Haugh unit, relative albumen weight, relative yolk weight, relative shell weight, shell thickness, egg specific gravity, eggshell breaking strength using a one-way ANOVA by repeated measures, with means separated using a Tukey's adjustment. Single measurement response variables, including body weight, bone mineral density, and mineral content

of pullets, tibia mineral density and mineral content of laying hens, bone breaking strength of laying hens, and fat-free tibia ash content of laying hens were analyzed using a one-way ANOVA with means separated using a Fisher's LSD test. If significant differences were detected in the bone quality parameters (bone mineral density, bone mineral content, tibia breaking strength, and fat-free tibia ash), linear and quadratic responses were fitted on parameters. Keel bone scoring was analyzed using a non-parametric test (Wilcoxon/Kruskal-Wallis). Significance was accepted at  $P \leq 0.05$ .

## **RESULTS AND DISCUSSION**

### **Pullet Growth Performance**

Generally, dietary D<sub>3</sub> concentrations did not affect pullet growth performance or feed intake except for pullets fed 68,348 IU D<sub>3</sub>/kg. Feed intake of pullets from the current experiment was generally in the recommended range in the Hy-Line W-36 Commercial Layer Management Guide (in the Hyline W-36 Commercial Layers Management Guide, 2016). However, at 17 wk of age, body weight of pullets fed 68,348 IU D<sub>3</sub>/kg was reduced in comparison to body weights of pullets fed the other treatments ( $P \leq 0.05$ ), 1164 g/bird versus 1,225 to 1,241g/bird. At 15 wk of age, feed intake of all treatments dropped below the recommended feed intake in the Hy-Line Guide (Hy-Line, 2016), but recovered at 16 wk of age. This reduction in feed intake was most likely associated with the SE vaccination at 14 wk. Morrissey et al. (1977) studied the toxicity of high-D<sub>3</sub> diets (400, 4,000, 40,000, 400,000, 4,000,000 IU D<sub>3</sub>/kg) in pullets 14 to 28 D of age. These authors found reduced growth rate and feed intake in chicks fed 400,000 and 4,000,000 IU D<sub>3</sub>/kg at 6 and 14 D after the initiation of experiment, but no inhibition of growth in chicks fed 40,000 IU D<sub>3</sub>/kg or less. The results of the current experiment are in line with and further refine those of Morrissey et al. (1977), suggesting that reduced growth may occur at an even lower level of 68,348 IU D<sub>3</sub>/kg. Increased serum calcium, defined as a sign of toxicity, was observed by Morrissey *et al.* (1977) in the 400,000 and 4,000,000 IU treatments. Serum calcium was not measured in the current experiment, but the reduced body weight of pullets fed 68,348 IU D<sub>3</sub> diet suggest it had a toxic effect on pullet growth.

### **Pullet Bone Quality**

Bone mineral density of 17-wk old pullets fed high D<sub>3</sub>-diets was increased in comparison to the control-fed pullets ( $P \leq 0.01$ ; Table 2). Bone mineral density was increased in pullets fed 35,014 IU D<sub>3</sub>/kg in comparison to those fed control, 8,348, 18,348, and 68,348 IU D<sub>3</sub>/kg diets. In terms of mineral content, pullets fed diets with intermediate D<sub>3</sub> (18,348 or 35,014 IU D<sub>3</sub>/kg) showed an increased bone mineral content in comparison to those fed control or 68,348 IU D<sub>3</sub>/kg diets ( $P \leq 0.01$ ,



**Table 2.** Body weight, bone mineral density, and bone mineral content of 17 wk old pullets fed diets containing 1,681, 8,348, 18,348, 35,014, or 68,348 IU of D<sub>3</sub>/kg of diet.

Dietary cholecalciferol	Body weight	Pullet bone mineral density	Pullet bone mineral content
(IU of D <sub>3</sub> /kg of diet)	(g)	(g/cm <sup>2</sup> )	(g)
1,681 (Control)	1241.4 <sup>a</sup>	0.195 <sup>c</sup>	15.08 <sup>c</sup>
8,348	1225.3 <sup>a</sup>	0.205 <sup>b</sup>	16.35 <sup>a,b</sup>
18,348	1241.3 <sup>a</sup>	0.200 <sup>b,c</sup>	17.24 <sup>a</sup>
35,014	1236.1 <sup>a</sup>	0.213 <sup>a</sup>	17.87 <sup>a</sup>
68,348	1163.6 <sup>b</sup>	0.199 <sup>b,c</sup>	15.96 <sup>b</sup>
Pooled SEM	13.19	0.0028	0.553
P-value (ANOVA)	≤ 0.01	≤ 0.01	≤ 0.01

<sup>a-c</sup>Least square means without a common superscript differ ( $P \leq 0.05$ ).

**Table 3.** Hen-housed egg production (HHEP), feed intake, egg weight, egg mass, feed conversion ratio<sup>1</sup> (FCR) from hens fed diets containing 1,681, 8,348, 18,348, 35,014, and 68,348 IU of D<sub>3</sub>/kg of diet during first cycle laying phase.

Dietary cholecalciferol	HHEP	Feed Intake	Egg mass	FCR <sup>1</sup>
IU of D <sub>3</sub> /kg	(%)	(g/h/d)	(g/h/d)	(g/g)
1,681 (control)	83.5 <sup>b</sup>	93.1 <sup>b</sup>	51.2 <sup>a,b</sup>	1.865 <sup>b</sup>
8,348	83.8 <sup>a,b</sup>	93.5 <sup>a,b</sup>	51.0 <sup>a,b</sup>	1.878 <sup>a,b</sup>
18,348	83.8 <sup>a,b</sup>	93.8 <sup>a</sup>	51.0 <sup>a,b</sup>	1.888 <sup>a,b</sup>
35,014	85.2 <sup>a</sup>	93.6 <sup>a,b</sup>	51.8 <sup>a</sup>	1.850 <sup>b</sup>
68,348	81.9 <sup>c</sup>	93.7 <sup>a,b</sup>	50.4 <sup>b</sup>	1.918 <sup>a</sup>
Pooled SEM	0.37	0.19	0.24	0.010
		<i>P</i> -Value		
Treatment (ANOVA)	≤0.01	≤0.05	≤0.01	≤0.01
Time	≤0.01	≤0.01	≤0.01	≤0.01
Treatment ×Time	0.14	≤0.05	0.18	≤0.01

<sup>1</sup>Gram of feed / gram of egg.

<sup>a-c</sup>Least square means without a common superscript differ in post hoc analysis ( $P \leq 0.05$ ).

Table 2). The previous literature is sparse concerning the effects of high dietary D<sub>3</sub> on pullet skeletal health, but results from broiler experiments may provide valid comparisons, although differences in growth rate need to be considered between the fast growing broiler and slower growing laying hen pullet, these differences are somewhat mitigated by reduced feed intake in the absence of laying hen pullet data are acceptable. Feeding broilers increased D<sub>3</sub>-diets (200, 800, 5,000, and 10,000 IU D<sub>3</sub>/kg) from day of hatch to 14 D of age, tibia breaking strength was maximized with 10,000 IU D<sub>3</sub>, and tibia ash was maximized in the 5,000 IU D<sub>3</sub> treatment (Whitehead et al., 2004). In another experiment, tibia ash and toe ash increased as broilers were fed D<sub>3</sub>-diets increasing from 200 to 3,000 IU D<sub>3</sub>/kg diet from day of hatch to 42 D of age (Khan et al., 2010). The data in these two reports with broiler chicks are consistent with the results in this study which suggests that increased dietary D<sub>3</sub> concentration can enhance bone mineral deposition during skeletal development in laying hen pullets.

### Laying Hen Performance

Hens started to produce eggs at 19 wk of age. From 19 to 24 wk of age, HHEP dramatically increased from 0 to 95% at peak production. During this period, the

increase of HHEP of hens fed 68,348 IU D<sub>3</sub>/kg was reduced in comparison to the other treatments, but reached the peak of egg production of the other treatments at 24 wk of age (Table 3). This response is likely explained by the reduced body weight of those hens as light hens produce fewer eggs at the initiation of the laying period (Leeson et al., 1997; Perez-Bonilla et al., 2012). Hen-housed egg production was similar across treatments during the early to peak production; however, HHEP among treatments started to diverge after 54 wk of age, resulting in differences across treatments. Hen-housed egg production of hens fed 35,014 IU D<sub>3</sub>/kg was significantly increased in comparison to the control fed birds with 8,348 and 18,348 IU D<sub>3</sub>/kg fed birds being intermediate. Hen-housed egg production of hens fed 68,348 IU D<sub>3</sub>/kg diet was significantly reduced in comparison to all other treatments. Egg mass of hens fed 35,014 IU D<sub>3</sub> diet was significantly increased in comparison to hens fed 68,348 IU D<sub>3</sub> with the other treatments being intermediate. Feed conversion ratio of control and 35,014 IU D<sub>3</sub> fed birds was reduced when compared to that of 68,348 IU/kg-fed birds ( $P \leq 0.01$ ). The decrease in laying hen performance between 35,014 IU and 68,348 IU/kg treatments is inconsistent with previous reports (Persia et al., 2013) in which laying hen performance from 19 to 58 wk of age was not affected by dietary D<sub>3</sub> up to 102,200

**Table 4.** Egg characteristics from hens fed diets containing 1,681, 8,348, 18,348, 35,014, or 68,348 IU of D<sub>3</sub>/kg of diet during first cycle laying phase.

Dietary cholecalciferol	Egg weight	Haugh unit <sup>1</sup>	Relative yolk weight	Relative albumen weight	Relative shell weight	Eggshell thickness	Egg specific gravity	Eggshell breaking strength
(IU of D <sub>3</sub> /kg of diet)	(g/h)		(%)	(%)	(%)	(mm)		(kg)
1,681 (control)	59.0	85.8 <sup>a</sup>	27.2	57.7	9.5	0.399	1.072 <sup>b</sup>	4.32 <sup>b</sup>
8,348	58.5	84.5 <sup>b</sup>	27.3	57.7	9.7	0.415	1.073 <sup>a,b</sup>	4.56 <sup>a</sup>
18,348	58.5	86.5 <sup>a</sup>	27.5	57.6	9.6	0.402	1.073 <sup>b</sup>	4.38 <sup>a,b</sup>
35,014	58.5	85.8 <sup>a</sup>	27.4	57.7	9.6	0.397	1.074 <sup>a,b</sup>	4.51 <sup>a</sup>
68,348	59.1	84.5 <sup>b</sup>	27.3	57.9	9.7	0.405	1.075 <sup>a</sup>	4.56 <sup>a</sup>
Pooled SEM	0.19	0.31	0.10	0.15	0.04	0.0057	0.0004	0.045
					<i>P</i> Value			
Treatment (ANOVA)	≤ 0.05	≤ 0.01	0.22	0.69	≤ 0.05	0.28	≤ 0.01	≤ 0.01
Time	≤ 0.01	≤ 0.01	≤ 0.01	≤ 0.01	≤ 0.01	≤ 0.01	≤ 0.01	≤ 0.01
Treatment × time	≤ 0.01	0.76	0.89	0.65	0.32	0.55	≤ 0.05	0.45

<sup>1</sup>Haugh unit = 100\* log (h-1.7\*w<sup>3.7</sup>+ 7.6); h = observed height of the albumen in millimeters; w = egg weight in grams

<sup>a-b</sup>Least square means without a common superscript differ in post hoc analysis (*P* ≤ 0.05).

IU/kg. The major difference between the current experiment and previous reports is the timing of D<sub>3</sub> supplementation; in the previous experiment D<sub>3</sub> supplementation started at point of lay whereas Vitamin D<sub>3</sub> treatment started at day of hatch in the current experiment. In the current experiment, reduced HHEP and egg mass in the 68,348 IU D<sub>3</sub>-fed hens may be explained by the reduced body weight of the pullets as they entered egg production; pullets with reduced body weight produce less total egg mass to 70 wk of age (Leeson et al., 1997) and produce fewer eggs at the start of the laying period (Perez-Bonilla et al., 2012).

### Egg Characteristics

Haugh unit measurement of eggs from the 8,348 IU and 68,348 IU D<sub>3</sub> treatments were lower than those from control, 18,348 and 35,014 IU D<sub>3</sub>-fed hens (*P* ≤ 0.01) (Table 4). These results are contrary to previous reports. Persia *et al.* (2013) indicated no difference in Haugh unit of eggs of hens fed various dietary D<sub>3</sub> concentrations from 2,200 IU to 102,200 IU/kg feed whereas Park et al. (2005) demonstrated decreased Haugh unit with increased dietary D<sub>3</sub> up to 20,000 IU/kg. The contradictory responses among experiments of egg Haugh unit to variation in concentrations of dietary D<sub>3</sub> may indicate that Haugh units are not directly associated with or are only slightly related to dietary D<sub>3</sub> and that the differences noted may not be biologically relevant.

In the current experiment, no differences were observed in egg weight, relative shell weight, relative yolk weight, relative albumen weight, or eggshell thickness across treatments. In contrast, Ameenuddin et al. (1986) found that egg weight decreased with elevated D<sub>3</sub>-diets relative to the those of control-fed (960 IU D<sub>3</sub>) hens, but not until hens were fed 100,000 or 200,000 IU D<sub>3</sub>/kg, concentrations exceeding the maximum used in the current study. The previously reported results suggest that higher concentrations of D<sub>3</sub> may be needed

to alter egg weights. Specific gravity of eggs from the 68,348 IU D<sub>3</sub>-fed hens was increased in comparison to the control and 18,348 IU D<sub>3</sub>-treatments with the other treatments being intermediate (*P* ≤ 0.01). Eggshell breaking strength in the 8,348, 35,014, or 68,348 IU D<sub>3</sub> treatments was significantly increased relative to control-fed birds. Both indicators of eggshell quality, egg specific gravity and eggshell breaking strength, increased in high dietary D<sub>3</sub> treatments relative to the control indicating that increased dietary D<sub>3</sub> improved eggshell quality between 1,681 and 68,348 IU D<sub>3</sub>/kg. In contrast, previous studies found that feeding enriched-D<sub>3</sub> diets to laying hens didn't affect either egg specific gravity or eggshell breaking strength (Mattila et al., 2003; Park et al., 2005). The difference in responses of eggshell quality to elevated D<sub>3</sub>-diets in this versus the other studies probably stem from variation in the age when the experimental diets were started. The previously reported experiments did not start to feed high D<sub>3</sub> diets until after the onset of egg production; until then, pullets received diets with lower D<sub>3</sub> concentrations. In contrast, hens in the current experiment received high D<sub>3</sub> diets throughout pullet and egg laying phases.

### Laying Hen Bone Quality

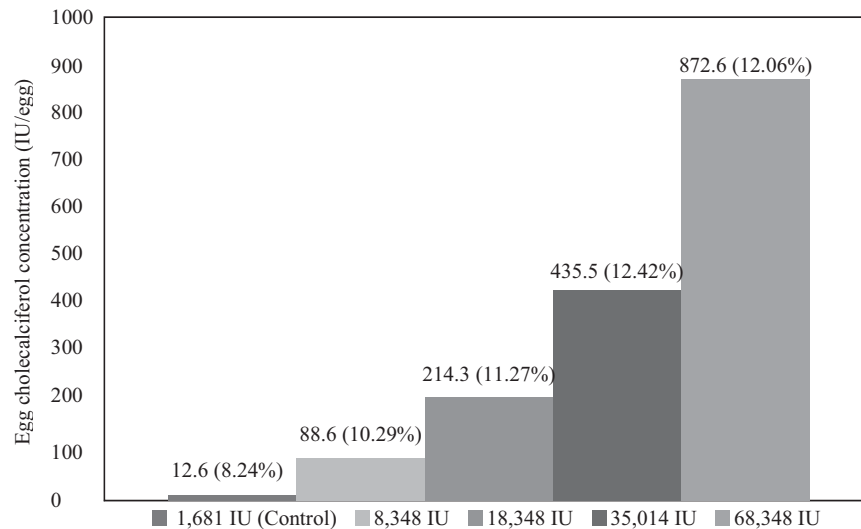
In terms of keel bone damage, no differences were observed across treatments. At the conclusion of the egg laying phase of the experiment (68 wk of feeding), tibia bone mineral content of hens in the 68,348 IU D<sub>3</sub> treatment was increased in comparison to the control, 8,348 and 18,348 IU D<sub>3</sub> fed hens (Table 5). Generally, the increased bone mineral content would be viewed as a positive outcome, but it is probably the result of the negative effect of the 68,348 IU D<sub>3</sub> treatment on egg production; the increased tibia mineral content is expected due to reduced Ca and P utilized for egg shell production given the overall reduction in egg production. Fat-free tibia ash content of hens in the 8,348, 18,348, 35,014, and 68,348 IU D<sub>3</sub> treatments were all

**Table 5.** Bone quality of hens fed diets containing 1,681, 8,348, 18,348, 35,014 or 68,348 IU of D<sub>3</sub>/kg of diet.

Dietary cholecalciferol	Keel score <sup>1</sup>	Tibia mineral density	Tibia mineral content	Tibia breaking strength	Fat-free tibia ash
(IU of D <sub>3</sub> /kg of diet)		(g/cm <sup>2</sup> )	(g)	(kg)	(%)
1,681 (control)	0.43	0.226	1.41 <sup>b</sup>	17.99	60.97 <sup>b</sup>
8,348	0.32	0.236	1.46 <sup>b</sup>	18.55	61.57 <sup>a</sup>
18,348	0.40	0.233	1.46 <sup>b</sup>	19.21	61.49 <sup>a</sup>
35,014	0.27	0.235	1.46 <sup>a,b</sup>	18.49	61.74 <sup>a</sup>
68,348	0.37	0.241	1.55 <sup>a</sup>	19.22	61.58 <sup>a</sup>
Pooled SEM	0.093	0.0042	0.030	0.361	0.161
<i>P</i> -value (ANOVA)	0.74	0.13	≤ 0.05	0.09	≤ 0.05
Quadratic	–	–	≤ 0.05	–	0.20
Linear	–	–	≤ 0.01	–	0.17

<sup>1</sup>Score 0: a bone with one fracture, usually toward the tip of keel; Score 1: a bone with between 2 and 3 fractures or callus formation. Score 2: a bone with multiple fractures or callus formation plus slight deformation at the fracture sites. Score 3: a bone with multiple fractures or callus formation and severe deformations of the ventral edge.

<sup>a-b</sup>Least square means without a common superscript differ ( $P \leq 0.05$ ).



**Figure 1.** Cholecalciferol concentrations of and vitamin D<sub>3</sub> transfer efficiency to eggs from hens fed diets containing 1,681; 8,348; 18,348; 35,014; and 68,348 IU of D<sub>3</sub>/kg of diet, respectively. Vitamin D<sub>3</sub> transfer efficiency presented in parenthesis.

increased in comparison to the control-fed hens ( $P \leq 0.05$ ). Unlike the response in this experiment, there was no difference in tibia ash content among laying hens fed D<sub>3</sub> up to 102,200 IU/kg from 19 to 58 wk of age (Persia et al., 2013). Similarly, 34-wk-old white leghorns fed experimental diets with D<sub>3</sub> up to 200,000 IU/kg of diet for 16 wk showed no difference in bone ash among treatments (Ameenuddin et al., 1986). The difference in results between the current and previous experiments suggest that bone development and skeletal status of pullets may play a more important role in maintaining skeleton bone health of layers than supplying higher dietary D<sub>3</sub> during the egg production phase.

Despite the effect seen in this experiment of increasing D<sub>3</sub>-diets on the bone mineral content of layers, there was no difference in tibia breaking strength across treatments; however, there was a trend suggesting that high dietary D<sub>3</sub> might improve tibia breaking strength ( $P = 0.09$ ). Previous experiments have reported that tibia breaking strength of layers was significantly increased in hens fed 6,000 IU or 15,000 IU D<sub>3</sub> diets relative to those fed 2,500 IU D<sub>3</sub> from 20 to 65 wk of age

(Mattila et al., 2004). Yet, in the current experiment no differences were observed in tibia bone mineral density among hens fed the different D<sub>3</sub> diets ( $P = 0.13$ ). Nonetheless, there was a linear increase in tibia bone mineral density and tibia bone mineral content as dietary D<sub>3</sub> concentration increased ( $P \leq 0.05$ ). There was also a quadratic relationship between tibia bone breaking strength and D<sub>3</sub> concentration. Therefore, fat-free tibia ash could be a better candidate than other bone quality indices to distinguish the difference between low and high dietary D<sub>3</sub> in laying hen diets.

### Egg Vitamin D<sub>3</sub> Concentration

The concentration of D<sub>3</sub> in egg yolks from the control, 8,348, 18,348, 35,014, and 68,348 IU D<sub>3</sub> treatments was 12.6, 88.6, 214.3, 435.5, and 872.6 IU D<sub>3</sub>/egg, respectively (Figure 1). The increase of yolk D<sub>3</sub> concentration with increasing dietary D<sub>3</sub> in the current experiment was consistent with previous scientific reports (Yao et al., 2013). The transfer efficiency of vitamin D<sub>3</sub> for

the control, 8,348 IU, 18,348 IU, 35,014 IU, 68,348 IU D<sub>3</sub> treatments was 8.24, 10.29, 11.27, 12.42, 12.06%, respectively. Current results are consistent with those of Yao et al. (2013) who reported that; the transfer efficiency of hens fed 9,700 to 24,700 IU D<sub>3</sub> diets was between 11% to 14% which was 4 to 6 percent higher than control (2,200 IU D<sub>3</sub>). In the current experiment, the D<sub>3</sub> transfer efficiency did not increase between the 35,014 IU, 68,348 IU D<sub>3</sub> treatments (Figure 1). The results of Yao et al. (2013) suggest that 102,000 IU might be toxic to hens and caused mistmetabolism of the vitamin D as the hens tried to reduce vitamin D exposure, but this phenomenon was not observed in the current experiment with 68,348 IU D<sub>3</sub>.

Data from current experiment indicate that feeding pullets and hens (day old to 68 wk of age) increased dietary D<sub>3</sub>, up to 35,014 IU /kg, would improve pullet skeletal quality and further enhance skeletal health and eggshell quality of the laying hens during first cycle production without decreasing performance of laying hens. Feeding pullet diets containing 68,348 IU D<sub>3</sub>/kg decreased body weight of pullets and negatively affected laying hen performance from 19 to 68 wk of age.

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