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Effect of Different Vitamin D Sources and Calcium Levels in the Diet of Layers in the Second Laying Cycle

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ABSTRACT

This study evaluated the effects of different sources of vitamin D and calcium levels on performance, egg quality and bone strength of hens in the second production cycle. A total of 384 Hy-Line W36 birds with 80 weeks of age were used, alloted into 3 x 4 factorial design (sources of vitamin D: cholecalciferol; 25(OH)D_3 and 1.25$(OH)_2D_3$ x calcium levels: 2.85, 3.65; 4.45 and 5.25%) with four replicates and eight birds each. The performance was evaluated for three cycles of 28 days each, egg quality was evaluated in the last four days of each cycle and bone strength on the last day of the experimental period. There was no interaction ($p> 0.05$) between the different sources of vitamin D and calcium levels in all parameters evaluated. There was a quadratic effect ($p<0.05$) of calcium levels in egg production and feed conversion (kg/kg and kg/dz), with better results at levels of 4.12%, 4.09% and 4.14%, respectively. Calcium levels had no effect ($p> 0.05$) in the egg weight and Haugh unit, but there was a linear increase ($p <0.05$) in the percentage and eggshell thickness, in specific gravity and bone strength. The different sources of vitamin D influenced ($p<0.05$) the egg production rate, feed conversion, egg weight and Haugh unit. Thus, the results of this study suggest that the recommended calcium level for laying on second cycle is between 4.09% and 4.14% and that the metabolites cholecalciferol and 25(OH)D_3 improved the performance and egg quality. Regarding bone strength was improved as the calcium levels were increased in diets.

INTRODUCTION

During the second laying cycle, after forced molting, layers restore their calcium absorption levels, with resulting eggshell quality improvement. However, this effect is independent of egg size, and therefore, eggshell quality worsens as birds age (Albano Jr *et al*., 2000).

During the second laying cycle, commercial layers present high egg loss due to poor eggshell quality and weak bones due to calcium mobilization. Therefore, their calcium and vitamin D requirements need to be updated to improve egg quality and the productivity of the egg industry.

Calcium is required for several metabolic functions in poultry (Nunes *et al*., 2006) and to ensure good eggshell quality. Its functions are also associated with phosphorus and vitamin D_3. During eggshell synthesis, blood calcium is rapidly mobilized, thereby reducing its levels. This stimulates the secretion of the parathyroid hormone (PTH), which promotes bone resorption to reestablish calcium homeostasis (Pelícia *et al*. 2009). The high rates of bone resorption during lay result in bone weakness in layers by the end of their production cycle (Whitehead, 2004).

Vitamin D_3 can be synthesized in the skin, catalyzed by ultraviolet radiation, from 7-dihydrocholesterol present in the dermis and epidermis (Pedrosa & Castro, 2005) or can be supplied in the feed. Commercial layers are usually maintained indoors, and do not receive enough solar radiation to convert 7-dihydrocholesterol in sufficient levels to supply their vitamin D_3 requirements. This is why vitamin D_3 is routinely added to layer feeds, which is essential for the maintenance of egg production, eggshell formation, and calcium homeostasis.

Cholecalciferol (D_3) is the most common form of vitamin D added to feeds. After absorption by the intestinal mucosa, it is transported to the liver, where it is hydroxylated in the position 25, resulting in 25-hydroxycholecalciferol ($25(OH)D_3$).This metabolite is directed to the kidneys and hydroxylated at carbon 1, originating the active metabolite of vitamin D, 1,25-dihydroxycholecalciferol ($1,25(OH)_2D_3$) (Leeson & Summers, 2001).

The inclusion of different vitamin D metabolites in the diet may enhance the effect of vitamin D due to their ready availability, sparing the chain of reactions required for the synthesis of the active metabolite.

Therefore, this study aimed at the evaluating the effects of different dietary vitamin D sources and calcium levels on the production performance, egg quality, and bone strength of layers in their second laying cycle.

MATERIALS AND METHODS

The experiment was carried out at the poultry sector of the experimental farm of Iguatemi, State University of Maringá, Brazil. The experiment was approved by the Committee of Ethics and Animal Welfare of that university, under protocol n. 023/2013.

In the trial, 384 80-wk-old Hy-line® W36 layers were housed in battery cages (100 x 40 x 45 cm^3), at a density of 500 cm^2/bird, an open-sided layer house covered with clay tiles.

A completely randomized experimental design in a 3 x 4 factorial arrangement, consisting of three vitamin D sources (cholecalciferol;$25(OH)D_3$ and $1,25(OH)_2D_3$) and four dietary calcium levels (2.85, 3.65, 4.45, 5.25), with four replicates of eight birds per experimental unit, was applied.

Average maximum and minimum environmental temperatures recorded during the experimental period were 29.2 °C and 16.6 °C, respectively.

Feeds were based on corn and soybean meal, supplemented with vitamins and trace minerals, and formulated according to the recommendations of Rostagno *et al*. (2011), except for calcium levels. The different vitamin D sources were included in the diet to supply 2000 IU of vitamin D_3. The vitamin supplement did not contain vitamin D. The experimental diets are shown in Table 1.

Table 1 – Ingredients and nutritional composition of the experimental diets.

Ingredients	Calcium levels			
	2.85	3.65	4.45	5.25
Corn	70	70	68.74	64.47
Soybean meal 45%	15.98	15.98	16.27	16.99
Soybean oil	0.605	0.605	1.021	2.48
Dicalcium phosphate	2.082	2.082	2.080	2.099
Limestone	5.94	8.02	10.1	12.18
Salt	0.420	0.420	0.420	0.420
Vitamin and mineral suppl.[1]	0.250	0.250	0.250	0.250
Inert material[2]	3.846	1.762	0.250	0.250
Sodium bicarbonate	0.420	0.420	0.420	0.420
L-Lysine HCL 78.5%	0.123	0.123	0.117	0.105
DL-Methionine 99%	0.185	0.185	0.186	0.192
L-threonine 98%	0.051	0.051	0.05	0.05
L-valine 98%	0.068	0.068	0.067	0.068
L-tryptophan 98%	0.02	0.02	0.019	0.017
BHT[3]	0.01	0.01	0.01	0.01
Calculated nutritional composition				
Metabolizable energy (kcal/kg)	2800	2800	2800	2800
Crude protein (%)	13.35	13.35	13.37	13.34
Digestible lysine (%)	0.650	0.650	0.650	0.650
Digestible Met + Cys (%)	0.590	0.590	0.590	0.590
Digestible valine (%)	0.620	0.620	0.620	0.620
Dig. tryptophan (%)	0.150	0.150	0.150	0.150
Calcium (%)	2.850	3.650	4.450	5.250
Available phosphorus (%)	0.470	0.470	0.470	0.470
Chlorine (%)	0.293	0.2933	0.2929	0.291
Sodium (%)	0.297	0.2973	0.297	0.2964
Potassium (%)	0.489	0.4885	0.49	0.4915
Electrolyte balance (mEq/kg)	172	172	172	173

[1] Vitamin and mineral supplement (content/kg product): vit. A – 3600 IU; vit. E – 3200 IU; vit. K – 800 mg; vit. B1 – 500 mg; vit. B2 – 1600 mg; Vit. B6 – 500 mg; Vit. B12 - 4000 mcg; niacin – 8000 mg; calcium pantothenate – 3200 mg; Se – 100 mg; Mn – 24 g; Zn – 20 g; Cu – 4.800 mg; I – 400 mg; Fe – 20 g; Co – 80 mg.

[2]Vitamins D_3, $25(OH)D_3$ and $1.25(OH)2D_3$ were included at the expense of inert material at 2000 IU at the following levels: 400mg, 5mg, and 200,000mg, respectively.

[3]BHT= antioxidant butyl-hidroxytoluene at 4000 mg.

A lighting program of 17 h of light (natural + artificial) per day was adopted. Feed and water were supplied *ad libitum.* Birds were submitted to a 14-day adaptation period and evaluated for three periods of 28 days each. Feed intake (g/bird/day) and feed conversion ratio (kg/kg and kg/dz) were evaluated in each period by weighing feed offer in the beginning and at the end of each period. Eggs were collected daily to determine egg production.

Eggs were collected during the last four days of each period to determine average egg weight and egg specific gravity. A sample of three eggs per replicate out of the total eggs produced per cage as collected to determine albumen height, and eggshell percentage and thickness.

Egg specific gravity was determined by immersing eggs in saline solutions at different concentrations (1.070, 1.074, 1.078, 1.082, and 1.086 g/mL). Saline solutions were adjusted using an oil densitometer and regularly calibrated.

Egg internal quality was evaluate relative to Haugh units, as described by Brant & Shrader (1958), where $HU = 100 \log (H + 7.57 - 1.7 W^{0.37})$, where H = albumen height (mm) and W = egg weight (g).

Eggshells with the internal membrane were washed under running water and then dried at environmental temperature for 72 hours (Maia *et al*., 2002; Costa *et al*., 2011), and dried in a digital scale to determine its weight relative to egg weight.

Eggshell thickness was determined using a digital micrometer (Mitutoyo®) with 0.001-mm precision. Measures were taken at the egg equator, where the distribution of calcium carbonate crystals is homogenous (Murakami *et al*., 2006).

Bone strength was evaluated in the left tibia of two hens per experimental unit, which was collected and frozen (-18° C) until analyses. Legs were thawed, the muscle tissue adhered to the bone was removed with the aid of scissors and forceps, and the tibiae were separated. Bone strength was analyzed in thawed bones at the Laboratory of Construction Materials and Soil Mechanics of the Technology Center of UEM. The mechanism consisted of a base that supported bone epiphyses and the force was applied in the central region of the bone.Values are expressed in kilograms-force (kgf).

In order to evaluate the influence of vitamin D sources on calcium metabolism, blood samples were collected every six hours for 24 hours, starting one hour after egg lay. Blood was collected by wing-vein puncture from one hen per experimental unit in each of the five blood collections. Serum calcium level was determined using a commercial kit.

Orthogonal polynomials were calculated for the obtained data for analysis of variance and analysis of regression. Means were compared by the test of Tukey at 5% probability level using the System of Statistical and Genetic Analyses (SAEG-8.0), developed by the Federal University of Viçosa (2005).

RESULTS AND DISCUSSION

There was no interaction ($p>0.05$) between vitamin D sources and calcium levels for none of the evaluated performance and egg quality parameters, or bone strength (Tables 2 and 3).

Dietary calcium levels did not influence ($p> 0.05$) feed intake. Costa *et al*. (2008) tested 3.0-5.0% dietary calcium levels and did not find any effects on feed intake. On the other hand, Araújo *et al*. (2011) verified lower feed intake when calcium level was increased from 3.5 to 4.2% in the diet of commercial layers in the post-molting period.

Egg production and feed conversion ratio (kg/kg and kg/dz) presented quadratic responses ($p < 0.05$). The resulting calcium requirement estimates of 4.12, 4.09, and 4.14% for second-cycle layers are higher than that reported by Rodrigues *et al*. (2005), who recommended a level of 3.5% calcium in the diet of these layers.

The obtained calcium values are close to that recommended by Rostagno et al. (2011), of 3.9% calcium for commercial layers and below the recommendations of the Hy-Line W36 manual (2011), of 4.67% calcium in the diet of layers producing less than 75% eggs.

Among the tested vitamin D sources, the hens fed D_3 presented higher egg production ($p<0.05$) compared with those fed $1,25(OH)_2D_3$, which, however, was not different from those fed $25(OH)D_3$. The hens fed $25(OH)D_3$ presented better feed conversion ratio (kg/kg and kg/dz; $p<0.05$) relative to $1,25(OH)_2D_3$, but similar results ($p>0.05$) as the group fed D_3.

Evaluating the effects of the inclusion of vitamin D_3 or metabolite $25(OH)D_3$ in layer diets, Salvador *et al*. (2009) obtained better feed conversion ratio with $25(OH)D_3$. On the other hand, Hamilton (1980) observed that D_3 promoted better egg production and feed conversion ratio (kg/kg) results relative to $25(OH)D_3$.

Egg weight (g) was not influenced by dietary calcium levels. However, the hens fed the metabolite $25(OH)D_3$

Table 2 – Performance of commercial layers in their second laying cycle fed diets with different vitamin D sources and calcium levels.

Parameters	Egg production (%)	Egg weight (g)	Feed intake (g/bird/day)	Feed conversion ratio (kg/kg)	Feed conversion ratio (kg/dz)
Vitamin D					
Cholecalciferol	73.27 ± 0.58a	69.77 ± 0.21ab	107.13 ± 0.34	2.135 ± 0.01ab	1.858 ± 0.03ab
25-hydroxycholecalciferol	72.08 ± 0.75ab	69.97 ± 0.33a	106.44 ± 0.28	2.092 ± 0.02a	1.781 ± 0.03a
1,25-dihydroxycholecalciferol	70.17 ± 1.04b	68.95 ± 0.26b	107.35 ± 0.53	2.215 ± 0.04b	1.894 ± 0.04b
Calcium levels (%)					
2.85	70.46 ± 1.10	69.07 ± 0.32	107.78 ± 0.46	2.208 ± 0.04	1.935 ± 0.05
3.65	71.93 ± 0.94	69.66 ± 0.30	106.51 ± 0.57	2.095 ± 0.02	1.780 ± 0.02
4.45	74.59 ± 0.63	69.37 ± 0.37	106.51 ± 0.49	2.093 ± 0.01	1.776 ± 0.03
5.25	70.38 ± 0.80	70.15 ± 0.30	107.10 ± 0.29	2.192 ± 0.03	1.886 ± 0.03
Vitamin D	*	*	NS	*	*
Calcium levels	*	NS	NS	*	*
Interaction	NS	NS	NS	NS	NS
Regression	$Q^1 = 4.12\%$	NS	NS	$Q^2 = 4.09\%$	$Q^3 = 4.14\%$
CV (%)	3.74	1.58	1.53	4.44	3.74

[a,b] Means followed by different letters in the same column are different by the test of Tukey at 5% probability level. *=Significant (P<0.05). NS= not significant.

Q^1 - $\hat{Y} = 36.06 + 18.241x - 22148x^2$ ($R^2 = 0.72$); Q^2 - $\hat{Y} = 3.464 - 0.677x + 0.0828x^2$ ($R^2 = 0.99$); Q^3 - $\hat{Y} = 3.5358 - 0.8574x + 0.1035x^2$ ($R^2 = 0.99$).

produced heavier eggs (p<0.05) compared with those fed $1,25(OH)_2D_3$, but not with those fed D_3 (p>0.05).

Eggshell percentage and thickness, and egg specific gravity linearly increased (p<0.05) with increasing dietary calcium levels. Calcium plays an essential role in eggshell formation, and therefore, the increase in its dietary level contributed to better eggshell synthesis (Costa *et al*., 2008).

Albano Jr. *et al.* (2000) verified higher eggshell percentage and egg specific gravity as dietary calcium levels increased from 2 to 6%. On the other hand, the tested vitamin D sources did not affect (p> 0.05) eggshell percentage and thickness or egg specific gravity.

Haugh units were not influenced (p>0.05) by dietary calcium levels. However, the hens fed vitamin

Table 3 – Egg quality and bone strength of commercial layers in their second laying cycle fed diets with different vitamin D sources and calcium levels.

Parameters	Eggshell %	Eggshell thickness (mm)	Egg specific gravity (g/mL)	Haugh units	Bone strength (kgf/cm²)
Vitamin D					
Cholecalciferol	8.16±0.07	0.35±0.004	1.075 ± 0.0005	97.64 ± 0.22a	16.20 ± 0.48
25-hydroxycholecalciferol	8.08±0.07	0.36±0.003	1.075 ± 0.0005	97.27 ± 0.16ab	15.53 ± 0.41
1,25-dihydroxycholecalciferol	8.16±0.09	0.35 ± 0.004	1.076 ± 0.0006	96.96 ± 0.20b	15.65 ± 0.41
Calcium levels (%)					
2.85	7.81 ± 0.07	0.34 ± 0.003	1.073 ± 0.001	97.63 ± 0.22	14.96 ± 0.41
3.65	8.08 ± 0.05	0.35 ± 0.003	1.075 ± 0.004	97.43 ± 0.22	15.55 ± 0.29
4.45	8.36 ± 0.05	0.36 ± 0.002	1.077 ± 0.003	97.20 ± 0.25	15.75 ± 0.57
5.25	8.28 ± 0.06	0.37 ± 0.004	1.078 ± 0.005	96.86 ± 0.22	16.91 ± 0.47
Vitamin D	NS	NS	NS	*	NS
Calcium levels	*	*	*	NS	*
Interaction	NS	NS	NS	NS	NS
Regression	L^1	L^2	L^3	NS	L^4
CV (%)	2.64	3.16	0.12	0.76	3.74

[a,b] Means followed by different letters in the same column are different by the test of Tukey at 5% probability level.

*=Significant (p<0.05). NS= not significant. L^1 - $\hat{Y} = 7.2769 + 0.2113x$ ($R^2 = 0.79$); L^2 - $\hat{Y} = 0.3044 + 0.0125x$ ($R^2 = 1$); L^3 - $\hat{Y} = 1.0671 + 0.0021x$ ($R^2 = 0.97$); L^4 - $\hat{Y} = 12.73 + 0.7563x$ ($R^2 = 0.91$).

D_3 presented better HU results ($p<0.05$) compared with those fed $1,25(OH)_2D_3$, but similar HU as those fed $25(OH)D_3$. The results obtained with $1,25(OH)_2D_3$ may be attributed to the shorter half-life of this metabolite (4-6 hours), compared with $25(OH)D_3$ (2-3 weeks) (Castro, 2011), and therefore its utilization may have been impaired due to the lack of body reserves.

Bone strength linearly increased ($p<0.05$) with calcium levels. When calcium blood levels are high, the C cells of the ultimobranchial glands are stimulated to secrete calcitonin, reducing bone resorption and consequently, increasing bone strength (Whitehead, 2004; Nunes *et al*., 2006).

Bone strength was not affected ($p>0.05$) by the tested vitamin D sources, despite the absorption rate of $25(OH)D_3$ is higher than that of vitamin D_3 (Applegate & Angel, 2005) and $1,25(OH)_2D_3$ increased calcium absorption by the intestine (Grüdtner *et al*., 1997). This result may be attributed to the fact that the activity of cholecalciferol, $25(OH)D_3$, and $1,25(OH)_2D_3$ depend on the biological response of birds (Aburto *et al*., 1998). Sahin *et al.* (2009) obtained better bone mineralization in laying quails when $25(OH)D_3$ was added to the diet.

Vitamin D sources did not influence ($p>0.05$) blood calcium levels (mg/dL) in none of the blood collections performed at different times after lay, as shown in Table 4.

Layers maintain balanced calcium and phosphorus blood levels for eggshell formation (Junqueira *et al*., 2002), independently of vitamin D source. Considering the measurement of vitamin D sources, the level of the metabolite $25(OH)D_3$ provides the best indication of vitamin D blood levels (Barral *et al*., 2007), as the $1,25(OH)_2D_3$ level reflects calcium homeostasis.

The different metabolite types did not influence the concentration of $1,25(OH)_2D_3$, which induces the release of the Ca^{2+} ion for calcium deposition in the eggshell, thereby affecting eggshell quality (Bar *et al*., 1999). This effect on calcium deposition characteristics was observed in the present study, as shown by the lack of effect of the tested vitamin D metabolites on the eggshell quality of layers in their second laying cycle.

CONCLUSIONS

Considering the egg production and feed conversion ratio (kg/kg and kg/dz) results obtained in the present study, 4.09 and 4.14% dietary calcium levels are recommended for layers in their second laying cycle, respectively. Cholecalciferol and 25-hydroxycholecalciferol improved production performance and egg quality. Bone strength and serum calcium levels were not influenced by the tested vitamin D metabolites. Bone strength increased with increasing dietary calcium levels.

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Table 4 – Blood calcium levels (mg/dL) of layers fed different vitamin D sources as evaluated at different times after lay.

Hours after lay	Calcium (mg/dL)			P	CV (%)
	Cholecalciferol	25-hydroxycholecalciferol	1,25-dihydroxycholecalciferol		
1	12.32 ± 0.97	13.48 ± 0.74	13.44 ± 1.11	NS	8.88
6	14.20 ± 0.23	14.60 ± 0.23	14.41 ± 0.26	NS	6.64
12	19.68 ± 0.22	20.08 ± 0.28	19.58 ± 0.22	NS	4.84
18	25.33 ± 0.66	25.52 ± 1.39	25.51 ± 0.72	NS	15.14
24	13.89 ± 0.43	13.35 ± 0.42	14.16 ± 0.34	NS	11.52
Mean	17.09 ± 0.60	17.40 ± 0.63	17.42 ± 0.59	NS	21.06

*Test of Tukey ($p > 0.05$). NS = not significant.

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