Effects of the in ovo injection of vitamin D_3 and 25-hydroxyvitamin D_3 in Ross 708 broilers subsequently fed commercial or calcium and phosphorus-restricted diets. I. Performance, carcass characteristics, and incidence of woody breast myopathy[1,2,3]

S. A. Fatemi,[*,4] A. Alqhtani,[*] K. E. C. Elliott,[*] A. Bello,[†] H. Zhang,[‡] and E. D. Peebles[*]

[*]*Department of Poultry Science, Mississippi State University, Mississippi State 39762, USA;* [†]*Department of Agricultural, Food, and Nutritional Science, University of Alberta, Edmonton T6G 2P5, Canada; and* [‡]*Feed Research Institute of Chinese Academy of Agricultural Sciences, Beijing, China*

ABSTRACT Effects of the in ovo-injection of vitamin D_3 (**D_3**) and 25-hydroxyvitamin D_3 (**25OHD_3**) on broiler performance, carcass characteristics, and woody breast myopathy (**WBM**) incidence were investigated. Live embryonated Ross 708 broiler hatching eggs (2,880) were randomly assigned to one of the following in ovo injection treatments: (1) diluent (50 μL); diluent (50 μL) containing either (2) 2.4 μg D_3; (3) 2.4 μg 25OHD_3; or (4) 2.4 μg D_3 + 2.4 μg 25OHD_3. Eggs were injected at 18 d of incubation (doi) using an Inovoject multiegg injector. At hatch, 18 male chicks were randomly placed in each of 6 replicate pens belonging to each in ovo injection and, dietary treatment combination. Birds were fed either a commercial diet or a diet restricted in calcium and phosphorous (**ReCaP**) content by 20% for the starter, grower and finisher dietary phases. Broiler performance was determined in each dietary phase and breast muscle yield was also determined at 14 and 40 d of age (**doa**). At 41 and 46 doa, birds were processed for determination of WBM, carcass weight, and the absolute and relative (% of carcass weight) weights of various carcass parts. Compared to birds fed the commercial diet, birds fed ReCaP diets experienced a reduction in performance from 14 to 40 doa, in breast meat yield at 41 and 46 doa, and in WBM at 41 and 46 doa. At 14 and 40 doa, breast meat yield in birds that received an in ovo injection of 25OHD_3 alone was higher compared to birds that received diluent alone or a combination of D_3 and 25OHD_3. Lower WBM incidence in ReCaP-fed birds was associated with a lower breast weight. An increase in breast meat yield in response to 25OHD_3 alone may be due to improved immunity and small intestine morphology. However, further study is needed to determine the aforementioned effects.

Key words: vitamin D source, in ovo injection, woody breast, broiler

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INTRODUCTION

The absorption of vitamin D_3 (**D_3**), a fat-soluble vitamin, is facilitated by bile salts in the upper small intestine of chickens (Bar et al., 1980). Vitamin D_3 is a multifunctional prehormone which requires 2 hydroxylation steps in order to become the active hormone, 1, 25-dihydroxyvitamin D_3 [**1, 25-$(OH)_2$ D_3**]. After intestinal absorption, D_3 is delivered to the liver for the first hydroxylation which converts it to 25-hydroxyvitamin D_3 (**25OHD_3**) by 25-hydroxylase. The second hydroxylation takes place in renal epithelial cells which converts 25OHD_3 to 1, 25-$(OH)_2$ D_3 via 1 α-hydroxylase activity (Booth et al., 1985). Inclusion of dietary D_3 and its metabolites are essential for proper growth in commercial broilers. Vitamin D_3 is well-known for its functions in intestinal calcium (**Ca**) and phosphorous (**P**) absorption (Bar et al., 1980), which is essential for bone (Fritts and Waldroup, 2003) and muscle (Vignale et al., 2015) formation and their development in broilers. In addition, D_3 has strong immunomodulatory activity that promotes broiler immunity during pathogenic infections (Morris et al., 2014; Chou et al., 2009). Dietary

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[4]Corresponding author: sf1006@msstate.edu

$25OHD_3$ in combination with D_3 has also been shown to enhance broiler performance and bone quality in comparison to D_3 alone at the same level of inclusion (Papešova et al., 2008). Furthermore, $25OHD_3$ is more effective than D_3, particularly in birds fed Ca and P-restricted diets (Bar et al., 2003). However, the first metabolite of D_3, $25OHD_3$, has been shown to increase the rate of Ca and P intestinal absorption more than D_3 (Bar et al., 1980), and the first hydroxylation step of D_3 in the liver can be bypassed. In young embryos and hatchlings, the conversion of D_3 to $25OHD_3$ is low due to the immaturity of their livers. This has been found to restrict an increase in serum $25OHD_3$ when D_3 alone is supplemented in the diets of broilers during early post-hatch life (Saunders-Blades and Korver, 2014).

Woody breast myopathy (**WBM**) is an abnormality in breast fillets that results in hard and thick breast meat. The occurrence of WBM is due to lymphocyte and macrophage infiltration, fibrosis (inflammation or necrosis in connective tissue), and lipidosis in muscle fibers (Kuttappan et al., 2013; Sihvo et al., 2014). Dietary $25OHD_3$ has been shown to increase the rate of protein synthesis (Hutton et al., 2014) and reduce inflammation (Fatemi, 2016) in the breast fillets of broilers. These effects may contribute to a reduction in WBM incidence in breast fillets. In ovo injection technology has emerged as a means to accelerate embryonic development (Bello et al., 2013) as well as a means to confer early immunity in broiler embryos against pathogenic viral infections such as Marek's disease (Williams, 2007). The in ovo injection of various vitamin D_3 sources in broilers has largely focused on their effects on hatchability and embryonic development (Gonzales et al., 2013; Bello et al 2013, 2015; Mansour et al., 2017). In ovo injection of vitamin D_3 (0.2 μg) into the amnion surrounding the embryo at 12 d of incubation (**doi)** has been reported to increase yolk and embryonic tissue concentrations of Ca and P at 17 doi (Mansour et al., 2017). In comparison to uninjected controls, the in ovo injection of 0.6 μg of $25OHD_3$ increased hatchability and bone quality in broilers (Bello et al., 2013; Bello et al., 2014). More recently, the in ovo injection of 2.4 μg of $25OHD_3$ has been observed to result in an improvement in broiler hatch quality (Fatemi et al., 2020b) as well as a decrease in feed conversion ratio (**FCR**) in broilers from 0 to 14 d of age (**doa**; Fatemi et al., 2020a). Additionally, in comparison to the in ovo injection of diluent containing or not containing D_3, an improvement in the inflammatory response of 39 doa broilers was observed when they received 2.4 μg of $25OHD_3$ by in ovo administration (Fatemi et al., 2021). However, there is limited information concerning the effects of the in-ovo injection of D_3 alone or in combination with $25OHD_3$ on broiler posthatch performance and meat yield. Therefore, the objective of this study was to investigate the effects of the in ovo injection of D_3 and $25OHD_3$ alone or in combination on performance, breast meat yield, and incidence of WBM in broilers fed commercial or Ca and P-restricted diets.

MATERIAL AND METHODS

Experiment design and broiler performance

All experimental procedures were approved by the Institutional Animal Care and Use Committee of Mississippi State University. Fertile eggs were collected from 35-wk-oldcommercial Ross 708 broiler breeder hens and stored under commercial conditions as is described by Fatemi et al (2020a). Thirty eggs were assigned to each of 4 treatment groups on each of 12 incubator tray levels (blocks) in a Jamesway model PS 500 setter unit (Jamesway Incubator Company Inc., Cambridge, Ontario, Canada) set at 37.5°C dry bulb and 29°C wet bulb temperatures. Positional effects were prevented by re-randomizing all treatments between each incubator level. Eggs were stored and incubated under standard conditions as described by Zhang et al. (2018). At 18 doi, 50 μL solution volumes of pre-specified treatments were injected into eggs using a Zoetis Inovoject m (Zoetis Animal Health, Research Triangle Park, NC) in ovo injection machine. The pre-specified 4 treatments solutions were: (1) **diluent** (control; 50 μL of commercial diluent (commercial Marek's Disease vaccine diluent; Merial Co., Duluth, GA)); (2) **D_3** (50 μL of commercial diluent containing 2.4 μg D_3), (3) **$25OHD_3$** (50 μL of commercial diluent containing 2.4 μg $25OHD_3$), and **D_3+25OHD3** (50 μL of commercial diluent containing 2.4 μg of D_3 and 2.4 μg of $25OHD_3$). All in ovo injection solutions were prepared and injected according to the procedure described by Fatemi et al. (2020a,b). At hatch (21 doi), all chicks were feather-sexed to select for male broilers in their pre-specified treatment, and then 18 male broilers were placed at a 0.062 m^2/bird stocking density in each of 48 floor pens (12 replicates per in ovo treatment) containing used litter top dressed with fresh wood shavings. All birds received either a Mississippi State University basal corn-soybean diet formulated to meet Ross 708 commercial guidelines (Aviagen, 2015), or the same diet with a 20% reduction in Ca and available P content (**ReCaP**; Table 1). Diets were analyzed for Ca and available P content in each dietary phase and all were close to calculated values (Table 2). A three phase feeding program with starter (0−14 d), grower (15−28 d) and finisher (29−40 d) phases was used. For each pen at 14, 28, and 40 doa, mean bird BW, feed intake (**FI**; g/bird), and BW gain (**BWG**) were determined for the starter, grower and finisher phases, respectively. Average daily gain (**ADG**), and average daily FI (**ADFI**) were further calculated. Feed conversion ratio (g feed intake/g BW gain) for the same time periods was calculated and adjusted for bird mortality.

Meat yield and processing

Six birds per treatment (1 bird per treatment replicate pen) were randomly selected for determination of the weights of their pectoralis major (**P. major**) and pectoralis minor (**P. minor**) muscles at 14 and 40 doa. The

Table 1. Feed composition of the experimental diets from 0 to 45 d of age (doa).

	Commercial diet Starter (0-14 doa)	Calcium and available phosphorus restricted (ReCap) diet
Item		
Ingredient (%)	Pct	Pct
Yellow corn	53.23	53.23
Soybean meal	38.23	38.23
Animal fat	2.60	2.60
Dicalcium phosphate	2.23	1.71
Limestone	1.27	1.01
Salt	0.34	0.34
Choline chloride 60%	1.00	1.00
Lysine	0.28	0.28
DL-Methionine	0.37	0.37
L-threonine	0.15	0.15
Premix[1]	0.25	0.25
Coccidiostat[2]	0.05	0.05
Cellulose	0	0.78
Total	100	100
Calculated nutrients		
Crude protein	23	23
Calcium	0.96	0.768
Available phosphorus	0.48	0.384
Apparent metabolizable energy (**AME**; Kcal/kg)	3,000	3,000
Digestible Methionine	0.51	0.51
Digestible Lysine	1.28	1.28
Digestible Threonine	0.86	0.86
Digestible total sulfur amino acids (**TSAA**)	0.95	0.95
Sodium	0.16	0.16
Choline	0.16	0.16
	Grower (15-28 doa)	
Item		
Ingredient (%)	Pct	Pct
Yellow corn	57.13	57.13
Soybean meal	34.80	34.80
Animal fat	3.50	3.50
Dicalcium phosphate	2.00	1.52
Limestone	1.17	0.94
Salt	0.34	0.34
Choline chloride 60%	0.10	0.10
Lysine	0.21	0.21
DL-Methionine	0.32	0.32
L-threonine	0.16	0.16
Premix	0.25	0.25
Coccidiostat	0.05	0.05
Cellulose	0	0.71
Total	100	100
Calculated nutrients		
Crude protein	21.5	21.5
Calcium	0.87	0.696
Available phosphorus	0.435	0.348
AME (Kcal/kg)	3,100	3,100
Digestible Methionine	0.47	0.47
Digestible Lysine	1.15	1.15
Digestible Threonine	0.77	0.77
Digestible TSAA	0.87	0.87
Sodium	0.16	0.16
Choline	0.16	0.16
	Finisher (29−45 doa)	
Item		
Ingredient (%)	Pct	Pct
Yellow corn	54.23	54.23
Soybean meal	38.23	38.23
Animal fat	2.50	2.50
Dicalcium phosphate	2.23	1.71
Limestone	1.27	1.01
Salt	0.34	0.34
Choline chloride 60%	0.10	0.10
Lysine	0.28	0.28
DL-Methionine	0.37	0.37
L-threonine	0.15	0.15
Premix	0.25	0.25
Coccidiostat	0.05	0.05
Cellulose	0	0.78
Total	100	100

(*continued*)

Table 1 (*Continued*)

	Commercial diet Starter (0-14 doa)	Calcium and available phosphorus restricted (ReCap) diet
Calculated nutrients		
Crude protein	19.5	19.5
Calcium	0.78	0.624
Available phosphorus	0.39	0.312
AME (Kcal/kg)	3,200	3,200
Digestible Methionine	0.43	0.43
Digestible Lysine	1.02	1.02
Digestible Threonine	0.68	0.68
Digestible TSAA	0.80	0.80
Sodium	0.16	0.16
Choline	0.16	0.16

[1]The broiler premix provided per kilogram of diet: vitamin A (retinyl acetate), 10,000 IU; Cholecalciferol, 250 IU; vitamin E (DL-α-tocopheryl acetate), 50 IU; vitamin K, 4.0 mg; thiamine mononitrate (B_1), 4.0 mg; riboflavin (B_2), 10 mg; pyridoxine HCL (B_6), 5.0 mg; vitamin B_{12} (cobalamin), 0.02 mg; D-pantothenic acid, 15 mg; folic acid, 0.2 mg; niacin, 65 mg;biotin, 1.65 mg; iodine (ethylene diamine dihydroiodide), 1.65 mg; Mn ($MnSO_4H_2O$), 120 mg; Cu, 20 mg; Zn, 100 mg, Se, 0.3 mg; Fe ($FeSO_4.7H_2O$), 800 mg.
[2]Decocx (Zoetis, Parsippany, NJ).

remaining birds (approximately 5) in each pen were processed at 41 and 46 doa due to limitations in processing all the remaining birds at one time. Prior to slaughter, birds did not have access to feed or water for at least 12 h. The birds were processed according to the method described by Wang et al. (2018). Carcasses were mechanically defeathered, manually eviscerated, and carcass traits assessed. Whole carcass, and P. major, P. minor, drumstick, thigh, and wing weights and yields (percentage of carcass weight) were determined.

Woody breast score

At 41 and 46 doa, the P. major were scored for incidence of WBM according to the procedures of Tijare et al. (2016). Briefly, breasts with a score of 0 were considered normal, a score of 1 was considered mild, a score of 2 was considered moderate, and a score of 3 was considered as severe. All normal breasts exhibited some degree of flexibility throughout (from the cranial to caudal tip region). However, those having a mild score exhibited hardness that was restricted to the cranial region, whereas those fillets with moderate scores possessed some hardness throughout, with flexibility restricted to the mid to caudal region. Finally, those fillets with a severe score were extremely hard and rigid throughout (from the cranial to caudal tip region).

Statistical analysis

The experimental unit was incubator tray for the hatch data and was floor pen for the performance, meat yield, and woody breast data. The experimental design was a randomized complete block for both the incubational and rearing periods. Incubator tray level was the blocking factor, with all in ovo injection treatments randomly represented on each of 12 levels (blocks). A group of pens was the blocking factor, with both the dietary and in ovo injection treatments (2 × 4) being randomly represented in each of 6 pens (blocks). The hatch data were analyzed using a one-way ANOVA to test for the effects of the 4 in ovo injection treatments. Performance, meat yield, and WBM data were analyzed using two-way ANOVA in a 2 × 4 factorial arrangement of treatments to test for the main and interactive effects of the 2 dietary treatments, and the 4 in ovo injection treatments. The following model was used for analysis of the posthatch data:

$$Y_{ijk} = \mu + B_i + D_j + I_k + (DI)_{jk} + E_{ijk},$$

Where μ was the population mean; B_i was the block factor (i = 1−2); D_i was the effect of each dietary treatments (j = 1−2); Ik was the effect of in ovo injection treatment (k = 1−4); (DI)ij was the interaction of each

Table 2. The analyzed values of percentage calcium (Ca) and available phosphorus (aP) of 2 dietary treatments in starter, grower, and finisher dietary phases.

		Ca calculated	Ca observed	aP calculated	aP observed
		——————————————————%——————————————————			
Starter					
	Control	0.960	1.010	0.480	0.502
	ReCaP[1]	0.768	0.775	0.384	0.377
Grower					
	Control	0.870	0.882	0.435	0.432
	ReCaP	0.696	0.689	0.348	0.343
Finisher					
	Control	0.780	0.775	0.390	0.403
	ReCaP	0.624	0.618	0.312	0.306

[1]A diet restricted in Ca and available P by 20% throughout the rearing period.

dietary treatment with in ovo injection treatment; and E_{ij} was the residual error.

The procedure for general linear mixed models (PROC GLIMMIX) of SAS 9.4© (SAS Institute, 2013) was used for all the above data analysis. Differences were considered significant at $P \leq 0.05$. Differences among mean WBM scores were also analyzed using the procedure for nonparametric models (PROC NPAR1-WAY) and general linear mixed models (PROC GLIMMIX) of SAS 9.4© (SAS Institute, 2013). Means separations were performed by Fisher's protected least significant difference (Steel and Torrie, 1980). Differences among means were deemed significant at $P \leq 0.05$.

RESULTS

Hatch and posthatch performance

No significant treatment differences were observed for the hatchability and hatch residue data, but there was a notable trend that approached significance ($P = 0.077$) concerning the effects of treatment on the hatchability of fertile eggs (Table 3). The in ovo injection of $25OHD_3$ alone tended to increase the hatchability of fertile eggs in comparison to the D_3 and diluent-injected treatments. There were no significant main effects due to in ovo injection treatment and no diet x *in* ovo injection treatment interactions for any of the observed performance variables throughout the rearing period (Table 4). Furthermore, broiler performance did not differ between commercial and ReCap treatments from 0 to 14 doa. However, in comparison to birds in the ReCaP treatment, those fed commercial diets had a higher BW, BWG, ADG, FI, and ADFI, and a lower FCR from 15 to 28 doa. Also, a similar pattern among the performance measurements was observed from 29 to 40 doa. The exception to this was FCR for the ReCap and commercial fed birds from 29 to 40 doa (Table 4). However, total FCR and total mortality were lower between 0 and 40 doa for birds fed commercial diets as compared to those fed ReCaP diets (Table 4).

Meat yield and processing

No significant interaction was observed between diet and in ovo injection treatment for the breast meat yield and processing measurements (Tables 5 and 6). At 14 doa, in ovo injection of $25OHD_3$ alone resulted in higher P. major weights in comparison to all other treatments, and the diluent-injected treatment resulted in lower P. major weights compared to the D_3 and D_3 + $25OHD_3$ treatments. Also, total breast meat yield was greater for birds that received $25OHD_3$ alone in comparison to those that were injected with diluent or D_3 + $25OHD_3$ (Table 5). At 40 doa, P. major and total breast meat yield was greater for birds that received $25OHD_3$ alone in comparison to those that were injected with diluent or D_3 + $25OHD_3$ (Table 5). At 41 doa, birds fed commercial diets had a higher carcass weight, and higher P. major, P. minor, and wing weights relative to carcass weight in comparison to those birds fed ReCap diets (Table 6). In comparison to the commercial diet, the ReCap diet resulted in lower carcass and wing weights relative to carcass weight at 46 doa (Table 6).

Woody breast myopathy score

No significant interaction was observed between diet and in ovo injection treatment for WBM at both 41 and 46 doa (Table 7). At 41 doa, birds fed a commercial diet had higher percentages of mid and moderate WBM scores in comparison to those fed ReCap diets (Table 6). The feeding of commercial diets resulted in birds with more 1 and 2 scores for WBM, and lower numbers of 0 scores for WBM than in the birds fed ReCap diets. Additionally, birds that received $25OHD_3$ alone had lower WBM scores of 3 in comparison to birds in the D_3 and the D_3 + $25OHD_3$ treatments. At 46 doa, overall WBM scores were greater for birds fed commercial diets in comparison to those fed ReCap diets. Furthermore, the commercial diet resulted in birds with lower scores of 0 and higher scores of 2 than did the ReCap diet.

Table 3. Hatchability and hatch residue variables at 21 d of incubation (doi) within in ovo treatment: diluent-injected control, and diluent containing 2.4 μg of vitamin D_3 (D_3) or 25-hydroxycholecalciferol ($25OHD_3$) alone or in combination (D_3 + $25OHD_3$).

Treatment	N	HF[1] (%)	Late embryo mortality[2] (%)	Dead piping embryos[3] (%)	Dead post-pipped embryos[4] (%)	Dead hatchlings[5] (%)
In ovo injection						
Diluent[6]	12	92.0	4.85	1.04	1.19	0.74
D_3[7]	12	92.4	3.55	0.78	2.13	1.11
$25OHD_3$[8]	12	95.4	2.45	0.45	1.36	0.30
D_3+$25OHD_3$[9]	12	94.0	3.84	0.88	0.92	0.17
P-value		0.077	0.140	0.729	0.527	0.119
Pooled SEM		1.48	0.960	0.408	0.610	0.960

[1]Hatchability of live embryonated eggs.
[2]Mortality between 18 and 21 doi, prior to pip.
[3]Mortality during the pipping process.
[4]Mortality after the pipping process.
[5]Mortality immediately after complete emergence of hatchlings from the shell.
[6]Eggs injected at 18 doi with 50 μl of commercial diluent.
[7]Eggs injected at 18 doi with 50 μl of commercial diluent containing 2.4 μg of vitamin D_3.
[8]Eggs injected at 18 doi with 50 μl of commercial diluent containing 2.4 μg of $25OHD_3$.
[9]Eggs injected at 18 doi with 50 μl of commercial diluent containing 2.4 μg of D_3 and 2.4 μg of $25OHD_3$.

Table 4. Live performance variables within in ovo treatment: diluent-injected control, and diluent containing 2.4 μg of vitamin D_3 (D_3) or 25-hydroxycholecalciferol ($25OHD_3$) alone or in combination (D_3 + $25OHD_3$) and dietary treatment: commercial diet or calcium and available phosphorus restricted (ReCaP) diets throughout the 40 d of age (doa) rearing period.

Treatment	N	BW (g)	BWG[1] (g)	ADG[1] (g)	FI[1] (g)	ADFI[1] (g)	FCR[1] (g/g)	Total[7] FCR (g/g) (0 to 40 doa)	Mortality[8] (%)
				Starter (0 to 14 doa)					
In ovo injection									
Diluent[2]	12	427.6	386.8	27.63	445.8	31.81	1.151		
D_3[3]	12	440.7	400.3	28.59	443.6	31.68	1.109		
$25OHD_3$[4]	12	434.8	393.7	28.12	449.6	32.12	1.142		
D_3+$25OHD_3$[5]	12	429.6	389.3	27.81	441.6	31.57	1.138		
Diet									
Commercial	24	431	389.9	27.85	442	31.59	1.136		
ReCaP[6]	24	436	395.2	28.23	448	31.99	1.134		
Pooled SEM		6.17	8.16	0.583	7.54	0.539	0.0186		
				P value					
In ovo		0.444	0.445	0.446	0.784	0.783	0.195		
Diet		0.447	0.400	0.401	0.340	0.338	0.881		
In ovo x Diet		0.631	0.738	0.737	0.066	0.066	0.196		
		BW (g)	BWG (g)	ADG (g)	FI (g)	ADFI (g)	FCR (g/g)		
				Grower (15–28 doa)					
In ovo injection									
Diluent	12	1,387	959	68.51	1,404	100.3	1.481		
D_3	12	1,364	922	65.88	1,359	97.1	1.489		
$25OHD_3$	12	1,393	959	68.49	1,395	99.6	1.475		
D_3+$25OHD_3$	12	1,384	954	68.11	1,387	99.1	1.470		
Diet									
Commercial	24	1,502[a]	1,071[a]	76.53[a]	1,466[a]	104.7[a]	1.370[a]		
ReCaP	24	1,262[b]	826[b]	58.97[b]	1,307[b]	93.4[b]	1.588[b]		
Pooled SEM		24.6	21.1	1.508	22.8	1.63	0.0291		
				P value					
In ovo		0.709	0.323	0.319	0.304	0.304	0.937		
Diet		0.001	0.001	0.001	0.001	0.001	0.001		
In ovo x Diet		0.738	0.736	0.731	0.157	0.157	0.741		
		BW (g)	BWG (g)	ADG (g)	FI (g)	ADFI (g)	FCR (g/g)		
				Finisher (29–40 doa)					
In ovo injection									
Diluent	12	2,348	961	80.10	1,792	149.3	1.888	1.648	3.2
D_3	12	2,337	974	81.15	1,862	155.2	1.915	1.667	5.1
$25OHD_3$	12	2,370	978	81.47	1,768	147.4	1.827	1.495	4.2
D_3+$25OHD_3$	12	2,334	951	79.26	1,790	149.1	1.889	1.585	6.0
Diet									
Commercial	24	2,558[a]	1056[a]	87.98[a]	1,950[a]	162.5[a]	1.867	1.554[b]	2.8[b]
ReCaP	24	2,138[b]	876[b]	73.01[b]	1,657[b]	138.0[b]	1.893	1.633[a]	6.5[a]
Pooled SEM		35.0	34.9	2.910	36.4	4.12	0.0816	0.0457	2.02
				P value					
In ovo		0.771	0.888	0.888	0.296	0.296	0.773	0.491	0.612
Diet		0.001	0.001	0.001	0.001	0.001	0.675	0.003	0.021
In ovo x Diet		0.914	0.982	0.982	0.150	0.150	0.446	0.094	0.361

[a,b]Treatment means within the same variable column within type of treatment with no common superscript differ significantly ($P < 0.05$).
[1]ADG, average daily gain; ADFI, average daily feed intake; BWG, BW gain; FCR, feed conversion ratio; FI, feed intake.
[2]Eggs injected at 18 d of incubation (**doi**) with 50 μl of commercial diluent.
[3]Eggs injected at 18 doi with 50 μl of commercial diluent containing 2.4 μg of vitamin D_3.
[4]Eggs injected at 18 doi with 50 μl of commercial diluent containing 2.4 μg of $25OHD_3$.
[5]Eggs injected at 18 doi with 50 μl of commercial diluent containing 2.4 μg of D_3 and 2.4 μg of $25OHD_3$.
[6]A diet restricted in Ca and available P by 20% throughout the rearing period.
[7]Cumulative FCR from 0 to 40 d of age.
[8]Cumulative mortality from 0 to 40 d of age.

DISCUSSION

An antibiotic growth promoter was not used in the diets, but a coccidiostat (Decocx, Zoetis, Parsippany, NJ) was included in both commercial and ReCaP diets in order to reduce the risk of a coccidiosis infection. Also, phytase was not included in the broiler diets. This was due to earlier observations showing greater effects of various vitamin D_3 sources on the performance of pigs fed diets lacking supplemental phytase (O'Doherty et al., 2010). However, the effects of supplemental vitamin D_3 sources in broiler diets deficient in Ca and P and without supplemental phytase have not been previously reported.

The in ovo injection of vitamin D_3 at 12 doi has been reported to increase Ca and P serum levels in broiler embryos (Mansour et al., 2017). The influence of vitamin D_3 on embryonic development is well understood (Narbaitz et al., 1987; Stevens et al., 1984; Tuan and Suyama, 1996). Furthermore, it is also well documented that vitamin D_3 sources have a greater effect on broiler performance when Ca and P are restricted in commercial diets (Bar et al., 2003). Additionally, the amniotic in ovo injection of $25OHD_3$ at 18 doi has been observed to decrease Ca content in the yolk sac (Bello et al., 2015). These results indicate that the in ovo injection of the 2 vitamin D_3 sources may have the potential to increase

Table 5. Pectoralis major (P. major) and minor (P. minor), and breast meat (Breast) relative to BW within in ovo treatment: diluent-injected control, and diluent containing 2.4 μg of vitamin D_3 (D_3) or 25-hydroxycholecalciferol ($25OHD_3$) alone or in combination (D_3 + $25OHD_3$) and dietary treatment: commercial diet or calcium and available phosphorus restricted (ReCaP) diets at 14 and 40 d of age (doa).

		P. major (%)	P. minor (%)	Breast (%)	P. major (%)	P. minor (%)	Breast (%)
Treatment	N	14 doa			40 doa		
In ovo injection							
Diluent[1]	12	11.7[c]	2.5	14.3[c]	20.1[b]	3.7	23.8[b]
D_3[2]	12	12.7[b]	2.6	15.3[ab]	21.3[ab]	3.9	25.2[ab]
$25OHD_3$[3]	12	13.6[a]	2.8	16.3[a]	23.5[a]	4.4	27.9[a]
D_3+$25OHD_3$[4]	12	12.6[b]	2.4	15.0[bc]	19.6[b]	3.8	23.5[b]
Diet							
Commercial	24	12.4	2.6	15.03	21.9	3.9	25.8
ReCaP[5]	24	12.8	2.6	15.40	20.4	4.0	24.4
Pooled SEM	0.22	0.13	0.31		0.96	0.18	1.03
		——————————————————*P* value——————————————————					
In ovo		0.001	0.614	0.003	0.047	0.107	0.028
Diet		0.150	0.773	0.318	0.151	0.396	0.233
In ovo x Diet	0.947	0.430	0.865		0.565	0.926	0.573

[a-c]Treatment means within the same variable column within type of treatment with no common superscript differ significantly ($P < 0.05$).
[1]Eggs injected at 18 d of incubation (**doi**) with 50 μl of commercial diluent.
[2]Eggs injected at 18 doi with 50 μl of commercial diluent containing 2.4 μg of vitamin D_3.
[3]Eggs injected at 18 doi with 50 μl of commercial diluent containing 2.4 μg of $25OHD_3$.
[4]Eggs injected at 18 doi with 50 μl of commercial diluent containing 2.4 μg of D_3 and 2.4 μg of $25OHD_3$.
[5]A diet restricted in Ca and available P by 20% throughout the rearing period.

Table 6. Carcass weight and weights of pectoralis major (P. major) and minor (P. minor), breast meat yield (Breast), and wing, drumsticks, thighs, and fat pad weights relative to carcass weight within in ovo treatment: diluent-injected control, and diluent containing 2.4 μg of vitamin D_3 (D_3) or 25-hydroxycholecalciferol ($25OHD_3$) alone or in combination (D_3 + $25OHD_3$) and dietary treatment: commercial diet or calcium and available phosphorus restricted (ReCaP) diets at 41 and 46 d of age (doa).

Treatment	N	Carcass (kg)	P.major (%)	P.minor (%)	Breast (%)	Wing (%)	Drumstick (%)	Thigh (%)	Fat pad (%)
					41 doa				
In ovo injection									
Diluent[1]	60	1,758	29.2	6.0	35.2	10.9	12.6	15.7	0.68
D_3[1]	60	1,749	29.5	6.1	35.6	10.9	12.5	15.7	0.64
$25OHD_3$[3]	60	1,725	30.1	6.0	36.1	11.0	12.5	15.9	0.66
D_3+$25OHD_3$[4]	60	1,749	30.0	6.0	36.0	11.0	12.4	15.5	0.68
Diet									
Commercial	120	1,894[a]	30.4[a]	6.2[a]	36.2	11.2[a]	12.3	15.6	0.67
ReCaP[5]	120	1,597[b]	29.0[b]	5.9[b]	34.9	10.7[b]	12.6	15.8	0.65
Pooled SEM		19.0	0.31	0.07	0.53	0.08	0.16	0.21	0.019
		P value							
In ovo		0.647	0.511	0.979	0.620	0.887	0.879	0.603	0.629
Diet		0.001	0.006	0.004	0.070	0.001	0.074	0.204	0.389
In ovo x Diet		0.268	0.688	0.872	0.803	0.323	0.952	0.800	0.368
					46 doa				
In ovo injection									
Diluent	60	2,055	32.2	6.0	38.2	10.8	12.0	15.5	1.27
D_3	60	2,048	29.2	6.1	35.2	10.5	11.7	15.5	1.31
$25OHD_3$	60	2,073	30.9	6.1	36.9	10.9	12.1	15.6	1.34
D_3+$25OHD_3$	60	2,073	28.5	5.9	34.4	10.6	11.7	15.3	1.23
Diet									
Commercial	120	2,223[a]	29.5	6.1	35.5	10.9[a]	12.0	15.5	1.29
ReCaP	120	1,902[b]	30.9	6.0	36.9	10.4[b]	11.8	15.5	1.28
Pooled SEM		27.2	1.94	0.11	1.95	0.13	0.14	0.18	0.090
In ovo		0.946	0.508	0.815	0.505	0.420	0.325	0.787	0.828
Diet		0.001	0.509	0.603	0.531	0.020	0.307	0.946	0.891
In ovo x Diet		0.327	0.559	0.832	0.570	0.775	0.541	0.271	0.756

[a-b]Treatment means within the same variable column within type of treatment with no common superscript differ significantly ($P < 0.05$).
[1]Eggs injected at 18 d of incubation (**doi**) with 50 μl of commercial diluent.
[2]Eggs injected at 18 doi with 50 μl of commercial diluent containing 2.4 μg of vitamin D_3.
[3]Eggs injected at 18 doi with 50 μl of commercial diluent containing 2.4 μg of $25OHD_3$.
[4]Eggs injected at 18 doi with 50 μl of commercial diluent containing 2.4 μg of D_3 and 2.4 μg of $25OHD_3$.
[5]A diet restricted in Ca and available P by 20% throughout the rearing period.

Table 7. Incidence of woody breast within in ovo treatment: diluent-injected control, and diluent containing 2.4 μg of vitamin D_3 (D_3) or 25-hydroxycholecalciferol ($25OHD_3$) alone or in combination (D_3 + $25OHD_3$) and dietary treatment: commercial diet or calcium and available phosphorus restricted (ReCaP) diets at 41 and 46 d of age (doa).

Treatment	N	Score 0 (%)	Score 1 (%)	Score 2 (%)	Score 3 (%)	Overall score
			41 doa			
In ovo injection						
Diluent[1]	60	41.8	37.6	19.7	0.9[ab]	0.65
D_3[2]	60	41.0	42.3	10.4	6.3[a]	0.75
$25OHD_3$[3]	60	41.8	48.5	9.6	0.1[b]	0.55
D_3+$25OHD_3$[4]	60	36.7	41.0	15.8	6.4[a]	0.79
Diet						
Commercial	120	18.6[b]	57.5[a]	19.0[a]	4.9	1.03[a]
ReCaP[5]	120	62.0[a]	27.3[b]	8.8[b]	1.9	0.34[b]
Pooled SEM		4.47	3.08	2.27	1.98	0.078
				P value		
In ovo		0.870	0.495	0.197	0.042	0.117
Diet		0.001	0.001	0.024	0.193	0.001
In ovo x Diet		0.903	0.509	0.153	0.379	0.800
			46 doa			
In ovo injection						
Diluent	60	41.8	25.6	18.3	14.3	0.86
D_3	60	48.8	18.6	14.6	18.0	0.90
$25OHD_3$	60	49.0	19.1	21.6	10.3	0.76
D_3+$25OHD_3$	60	36.8	30.5	24.2	8.4	0.84
Diet						
Commercial	120	31.6[b]	24.5	27.5[a]	16.4	1.10[a]
ReCaP	120	56.6[a]	22.5	11.9[b]	9.0	0.59[b]
Pooled SEM		3.50	3.38	2.64	3.61	0.099
				P value		
In ovo		0.380	0.383	0.439	0.352	0.865
Diet		0.001	0.756	0.004	0.120	0.001
In ovo x Diet		0.440	0.629	0.151	0.579	0.758

[a,b]Treatment means within the same variable column within type of treatment with no common superscripts are significantly different ($P < 0.05$).
[1]Eggs injected at 18 d of incubation (**doi**) with 50 μl of commercial diluent.
[2]Eggs injected at 18 doi with 50 μl of commercial diluent containing 2.4 μg of vitamin D_3.
[3]Eggs injected at 18 doi with 50 μl of commercial diluent containing 2.4 μg of $25OHD_3$.
[4]Eggs injected at 18 doi with 50 μl of commercial diluent containing 2.4 μg of D_3 and 2.4 μg of $25OHD_3$.
[5]A diet restricted in Ca and available P by 20% throughout the rearing period.

the rate of absorption of Ca and P in broiler embryos, which could be beneficial for birds fed diets with reduced levels of Ca and P. However, the results of the current study indicate that the carry over effect of vitamin D_3 sources in broiler embryos was not sufficient to overcome the subsequent negative effects of a 20% dietary reduction in Ca and P on broiler performance and breast meat yield. In comparison to D_3, $25OHD_3$ is more efficient in increasing Ca and P absorption (Bar et al., 1980). Also, $25OHD_3$ has a longer half-life, which is approximately 2 to 3 wk (Smith and Goodman, 1971; Hollis and Wagner, 2013). This is in contrast to D_3, which has a half-life approximately 12 to 24 h (Smith and Goodman, 1971; Haddad et al., 1993). Additionally, at the same level of inclusion in both broilers (Vignale et al., 2015; Yarger et al., 1995) and laying hens (Käppeli et al., 2011), dietary $25OHD_3$ significantly increases circulating $25OHD_3$ concentrations in comparison to D_3. These data indicate that $25OHD_3$ stays in the blood for a longer period of time. A longer half-life of $25OHD_3$ can be beneficial in the newly hatched chick due to their impaired absorption of D_3 (Saunders-Blades and Korver, 2014). During the first 2 wk of life, the absorption of D_3 by the chick is low due to the immaturity of the digestive tract and low activity of enzymes involved in lipid absorption (Noy and Sklan, 1995). However, the longer half-life of $25OHD_3$ may be used to promote the performance of broilers selected for a high growth rate in the very early phase of life. Furthermore, an improvement in small intestine morphology has been shown is associated with increased breast meat yield in Ross 708 broilers (Wang et al., 2019). Dietary supplementation of $25OHD_3$ has been shown to increase villus length (**VL**) and decrease crypt depth (**CD**) in broilers at 28 and 35 doa (Chou et al., 2009). An increase in VL is associated with an increase in nutrient absorption (Onderci et al., 2006) and a decreased CD is linked to a lower energy requirement in the gut (Yang et al., 2008). In addition to small intestine morphology, the inflammatory response of broilers has been shown to decease in response to dietary (Fatemi, 2016) and in ovo supplementation (Fatemi et al., 2021) of $25OHD_3$. Additionally, the increase in breast meat yield in response to in ovo supplementation of $25OHD_3$ has been shown to be associated with a reduced inflammatory response (Fatemi et al., 2021).

In the current study, broiler performance did not significantly differ among in ovo injection treatments throughout the rearing period. However, the in ovo injection of $25OHD_3$ at a 2.4 μg dose increased P. major weight over that in birds injected with either D_3 or diluent alone. This improvement in meat yield in the response to the in ovo injection of $25OHD_3$ alone is likely due to an improvement in the inflammatory response,

small intestine morphology, and longer half-life and a higher rate of $25OHD_3$ absorption relative to D_3. Furthermore, it may be linked to differences in the duration and levels of storage of the 2 vitamin D_3 sources in the tissues of the birds. In pigs, dietary $25OHD_3$ at low levels of inclusion (5 μg) is mainly stored in white and red muscle more than in adipose tissue (Burild et al., 2016). However, although D_3 at the same level of inclusion is mostly stored in adipose tissue with only small amounts stored in the liver or muscle tissues (Burild et al., 2016), the greater amount of $25OHD_3$ stored in muscle tissue may be another reason for the increased P. major yield in birds belonging to the $25OHD_3$-injected treatments in comparison to those in the D_3-injected treatments. The expression of 1 α-hydroxylase occurs in high amounts in the kidney as well as the thigh and breast muscles in chickens (Shanmugasundaram and Selvaraj, 2012). Moreover, considerable level of 1 α-hydroxylase in muscle tissue, which can only convert $25OHD_3$ to the, 1, 25-$(OH)_2$ D_3 (the active form of D_3), results in an increase in protein synthesis and muscle hypertrophy of muscle tissue (Hutton et al., 2014). Conversely, 1 α-hydroxylase cannot convert D_3 to 1, 25-$(OH)_2$ D_3. Therefore, at the level of activity equal to $25OHD_3$, D_3 cannot able to cause muscle hypertrophy or protein synthesis (Hutton et al., 2014). Additionally, the in ovo injection of both vitamin D3 sources proved to be more effective in terms of increased breast meat yield during the first 2 wk of posthatch life, but after 2 wk, this effectiveness was more quickly ameliorated in the D_3-injected broilers in comparison to the diluent-injected broilers.

These current results show that a 20% reduction of dietary Ca and available P resulted in a decrease in breast meat yield in 2 and 6-wk-old broilers. Effects of different levels of dietary Ca or P on the breast meat yield of broilers have not been reported to-date. However, increased leg meat yield has been previously observed in broilers at 41 doa when the percentage of Ca in the diet increased from 0.95 to 1.05 % (Xing et al., 2020). Effects of a severe reduction in dietary Ca and P content on broiler performance and bone quality have likewise been previously reported, but there is limited information about this restriction on meat yield (Delezie et al., 2015; Ribeiro et al., 2018). Delezie et al. (2015) reported that a 20% reduction in the Ca and available P content of corn-soybean meal diets in the absence of phytase reduced BW and ADFI from 13 to 39 doa, with no effect on FCR. Additionally, similar results were reported for BWG and FCR from 1 to 41 doa in broilers fed Ca and P- restricted diets (Ribeiro et al., 2018). In previous studies, the inclusion of dietary phytase allowed the negative effects of lower dietary levels of Ca and P on broiler performance to be overcome. It is well documented that supplemental dietary phytase can improve the performance (Delezie et al 2015; Ribeiro et al., 2018) of broilers fed Ca and P-restricted diets. This improvement in response to dietary phytase could be due to a higher availability of P and Ca leading to a reduction of phytates and anti-nutritional factors, and an increased digestibility of amino acids (Manobhavan et al., 2016). It is because of these documented effects in response to phytase, that phytase was not included in the diets of the current study. Nevertheless, a decline in broiler performance and meat yield is not only linked to other components of the diet such as phytase, but may also be due to the important functions of Ca, including its role in muscle synthesis and nutrient absorption.

Both dietary D_3 and $25OHD_3$ have been shown to increase the rate of absorption of Ca and P in the jejunum (Bar et al., 1980). However, posthatch increases in serum Ca and P levels in response to various vitamin D sources administrated by in ovo injection have not been previously investigated. Nevertheless, previous studies have reported the effects of the in ovo injection of D_3, $25OHD_3$, 1α-hydroxy vitamin D_3, and 1, 25-$(OH)_2$ D_3 in broiler embryos during the incubation period (Bello et al., 2013; Mansour et al., 2017). Intracellular Ca promotes the release of hepatocyte growth factor from the extra cellular matrix, leading to an increase in the number of satellite cells (Allen et al., 1995). Muscle fiber formation is completed at hatch (Smith, 1963) and subsequent muscle growth is facilitated by myoblast or satellite cell activity (Mauro, 1961). An increase in the number of satellite cells is associated with an increase in protein synthesis and muscle fiber growth through hypertrophy (Moss and LeBlond, 1971). In addition to muscle formation, dietary Ca can also improve the small intestine morphology of broilers (Xing et al., 2020). In unpublished data in our laboratory, an increase in VL and a decrease in CD were observed in response to increased dietary Ca levels, which subsequently led to decreased FCR, and increased BWG and leg meat yield. A 20% reduction in the Ca and available P levels in broiler diets resulted in a decline in small intestine morphology of broilers at 14 and 40 doa. These data indicate that a decline in small intestine morphology and satellite cell numbers could be the reasons for the lower meat yield in ReCaP-fed birds when compared to those fed commercial diets.

The increased incidence of WBM is a recent major concern in the poultry industry. It is well documented that the rate of protein synthesis is reduced and that the fat content is increased in the breast fillets of broilers exhibiting WBM (Kuttappan et al., 2013; Trocino et al., 2015). Furthermore, RNA sequencing results in WBM breast fillets has revealed that there is a greater expression of genes involved in oxidative stress, and that there are higher levels of intracellular Ca as well as an increase in the inflammatory response in fast-growing broilers (Mutryn et al., 2015). In other unpublished data from our laboratory, it was observed that in comparison to D_3, the in ovo injection of $25OHD_3$ alone tended to decrease the inflammatory response in broilers. Thus, lower proportions of severe WBM scores in response to the in ovo injection of $25OHD_3$ could be also due to a reduced inflammatory response. An increase in the amount of Ca in the sarcoplasmic reticulum in skeletal muscle can stimulate enzymatic activity in association with protein denaturation (Sandercock and

Mitchell, 2003; Whitehead et al., 2006). Thus, one of the possible reasons for the increased incidence of WBM in the broilers fed the commercial diets may be due to increased intracellular Ca levels, thereby causing the occurrence of WBM to be higher than that of broilers fed ReCaP diets.

In conclusion, effects of the in ovo injection of 2 vitamin D_3 sources on breast meat yield, incidence of WBM, and the overall performance of broilers fed diets restricted in Ca and available P were investigated. Our findings revealed that the in ovo injection of those vitamin D_3 sources did not affect broiler performance, meat yield, or quality when Ca and P were restricted in the diet. Furthermore, in comparison to D_3, the in ovo injection of $25OHD_3$ increased the breast meat yield of early posthatch broilers and decreased the severity of WBM of the broilers at 41 doa in this study. The changes in these observed factors may be due to a greater storage efficiency of $25OHD_3$ in muscle tissue, and an improvement in small intestine morphology. Severe reductions in dietary Ca and available P resulted in a decline in overall performance and breast meat yield, and reduced incidence of WBM. The disadvantages caused by the ReCaP diet could be due to a reduction in Ca and P uptake, which is essential for growth and muscle development. This could also be the reason for a decrease in intracellular Ca in association with lower WBM scores. Further study is required to determine the effects of the in ovo injection of various vitamin D_3 sources on the small intestine morphology and inflammatory response of broiler chickens.

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DISCLOSURES

There is no conflict of interest.

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