Improvement in the performance and inflammatory reaction of Ross 708 broilers in response to the *in ovo* injection of 25-hydroxyvitamin D_3[1,2,3]

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ABSTRACT The effects of the *in ovo* administration of vitamin D_3 (**D_3**) and its metabolite, 25-hydroxyvitamin D_3 (**25OHD_3**), on the performance, breast meat yield, and inflammatory responses of broilers fed commercial diets were investigated. Live embryonated Ross 708 broiler hatching eggs were randomly assigned to one of the following 5 *in ovo* injection treatments at 18 d of incubation: 1) noninjected; 2) diluent; diluent containing 3) 2.4-μg D_3, 4) 2.4-μg 25OHD_3, or 5) 2.4-μg D_3 + 2.4-μg 25OHD_3. A 50-μL solution volume of each prespecified treatment was injected into each egg using an Inovoject multiegg injector. At hatch, 18 male chicks were randomly assigned to each of 30 floor pens. The BW, BW gain, feed intake, and feed conversion ratio of the birds were determined in each dietary phase. At 14, 28, and 39 d of posthatch age (**doa**), plasma α-1-acid glycoprotein (**AGP**) levels in 1 bird in each of 6 replicate pens per treatment were determined at 14 and 39 doa. The pectoralis major and minor weights of those same birds were also determined. The remaining birds were processed at 43 doa, and the weights of their processing parts were determined. At 39 doa, the *in ovo* injection of 25OHD_3 alone decreased plasma AGP concentrations in comparison with the noninjected, diluent, and D_3-alone treatment groups. In addition, birds that received 25OHD_3 alone had a greater BW at 42 doa than birds in the noninjected, diluent, and D_3-alone treatment groups. At 39 and 43 doa, breast meat yield was increased in response to the *in ovo* injection of 25OHD_3 alone in comparison to all other treatments. These results indicate that the *in ovo* injection of 2.4 μg of 25OHD_3 resulted in an improvement in the performance and inflammatory responses of broilers. A reduction in the inflammatory response subsequent to the *in ovo* injection of 2.4 μg of 25OHD_3 may have led to an increase in broiler performance.

Key words: 25-Hydroxyvitamin D_3, Breast meat yield, Broiler performance, Inflammatory response, *In ovo* injection

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INTRODUCTION

In ovo administration is an alternative approach to the posthatch vaccination of chickens, particularly in broilers. *In ovo* injection is used to deliver a particular solution between 17.5 and 19.25 d of injection (**doi**) into the amniotic sac surrounding the broiler embryo (Williams, 2007 and 2011). *In ovo* injection is widely used in the U.S. commercial broiler industry and has allowed for the direct administration of particular nutrients or vaccines to embryos. It is less labor intensive and is relatively less stressful for the embryo. *In ovo* injection also uniformly delivers injected material with limited contamination for the initiation of an early immune response in broilers. The poultry industry commercially uses *in ovo* injection against Marek's disease (Williams, 2007). In addition, *in ovo* injection has been used to successfully deliver various nutrients such as vitamins, minerals, carbohydrates, and amino acids into broiler embryos (Peebles, 2018).

Vitamin D is a secosteroid that has a similar structure to steroid hormones such as estradiol and cortisol (Norman, 2008). Vitamin D is largely involved in bone

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development and preservation through its maintenance of plasma calcium (**Ca**) and phosphorus homeostasis (Soares et al., 1995; Khan et al., 2010). In addition, vitamin D plays vital roles in broiler performance (Fritts and Waldroup, 2003), reproduction (Saunders-Blades and Korver, 2015), muscle development (Vignale et al., 2015), and immunity (Chou et al., 2009). Thus, vitamin D is not only a fat-soluble vitamin (Abawi and Sullivan, 1989) but also considered as a prohormone (Norman, 2008) and an immunomodulatory nutrient (Correale et al., 2009). In addition, the presence of vitamin D_3 (**D_3**) in eggs is very important to support embryo Ca metabolism during incubation (Landauer, 1967). Therefore, a deficiency of this vitamin can lead to reduced hatchability, which can be specifically related to late embryo mortality (Stevens et al., 1984).

Vitamin D_3 must undergo 2 sequential hydroxylation steps to become active. The first hydroxylation occurs through 25-hydroxylase activity in liver microsomes and mitochondria (Henry, 1980). This first hydroxylation produces $25OHD_3$. In addition, conversion of D_3 to $25OHD_3$ takes place in the intestine or kidney, but at a lower rate (Norman, 1987). Then, $25OHD_3$ is transported via vitamin D–binding protein to the kidneys, where $25OHD_3$ is hydroxylated to 1,25-dihydroxyvitamin D_3 (**$1,25(OH)_2 D_3$**) by 1α-hydroxylase (Henry, 1980). As compared with D_3 at the same level of inclusion, $25OHD_3$ is more potent in its ability to improve performance (Yarger et al., 1995) and breast meat yield (Vignale et al., 2015) and to reduce an inflammatory response (Morris et al., 2014; Fatemi, 2016). Its effects may be due to its longer half-life (Smith and Goodman, 1971; Hollis and Wagner, 2013), its enhancement of Ca intestinal absorption (Bar et al., 1980), or its greater storage in muscle tissue (Burild et al., 2016).

The *in ovo* injection of D_3 has been shown to increase the concentrations of Ca and phosphorous in the yolk and of embryos at 17 doi. In addition, *in ovo* injection of 2.4 μg of $25OHD_3$ has been shown to increase embryonic serum $25OHD_3$ concentrations (Fatemi et al., 2020b), decrease embryonic mortality, and improve early posthatch broiler performance when compared with the *in ovo* injection of diluent with or without 2.4 μg of D_3 (Fatemi et al., 2020a). However, the long-term effects of the *in ovo* injection of individual or combined sources of D_3 on the breast meat yield and immunity of broilers have not been previously reported. Therefore, the objective of this study was to determine the effects of the *in ovo* administration of D_3 and its metabolite, $25OHD_3$, alone or in combination, on the performance, breast meat yield, and inflammatory responses of broilers fed commercial diets.

MATERIAL AND METHODS

Experiment Design and Egg Incubation

This study was conducted according to a protocol approved by the Institutional Animal Care and Use Committee of Mississippi State University. Twelve Ross 708 broiler hatching eggs were set in each of 5 treatment groups that were randomly arranged on each of 20 replicate tray levels (1,200 total eggs) in a single-stage incubator (Chick Master Incubator Company, Medina, OH). Positional effects were removed by rerandomizing all treatments between each incubator level. The same incubator served as both a setter and hatcher unit. The setter was set at 37.5°C dry bulb and 29.0°C wet bulb temperatures and the hatcher at 36.9°C dry bulb and 29.9°C wet bulb temperatures. All eggs were candled at 12 and 18 doi in accordance with the procedures described by Ernst et al. (2004). At 18 doi, 50-μL solution volumes of each prespecified treatment were injected into eggs using a Zoetis Inovoject M (Zoetis Animal Health, Research Triangle Park, NC) multiegg injection machine. Prespecified treatments were 1) **non-injected** (control); or a 50-μL solution volume of the 2) **diluent** (commercial diluent; control); 3) **D_3** (commercial diluent containing 2.4-μg D_3); 4) **$25OHD_3$** (commercial diluent containing 2.4-μg $25OHD_3$); and 5) **D_3 + $25OHD_3$** (commercial diluent containing 2.4 μg of D_3 and $25OHD_3$). The form and source of D_3 and $25OHD_3$ used in this study were the same as those used by Fatemi et al. (2020a,b). All *in ovo* injection solutions were also prepared and injected according to the procedures of Fatemi et al. (2020a,b).

After injection, eggs were transferred to hatching baskets that were arranged in the hatcher unit that coincided with the arrangement of the trays for each respective treatment replicate in the setter unit. At hatch, all chicks belonging to a replicate basket in each treatment group were weighed and counted to determine hatchling BW and the hatchability of injected live embryonated eggs. In addition, hatch residue was analyzed as described by Ernst et al. (2004). All chicks were feather-sexed to select for male broilers in their prespecified treatment, and then male chicks from each replicate basket were pooled within their respective treatment group. Eighteen male broilers were randomly selected from each pooled treatment group and were placed in each of 6 replicate floor pens in each of the 5 treatment groups (540 total birds). All birds received a Mississippi State University basal corn-soybean diet (Table 1) formulated to meet Ross 708 commercial guidelines (Aviagen, 2015). Feed and water were provided for ad libitum consumption throughout the 42 d of posthatch age (**doa**) period.

Growth Performance

All birds were fed a starter diet from 0 to 14 doa, a grower diet from 15 to 28 doa, and a finisher diet from 29 to 41 doa. The BW, BW gain, ADG, feed intake, and ADFI of the birds were determined in each dietary phase. The percentage mortality and feed conversion ratio (g feed/g gain) adjusted for bird mortality were calculated for the same time periods.

Table 1. Feed composition of the experimental diets from 0 to 42 d of age.

Starter (0-14 doa)	
Item	Commercial diet
Ingredient (%)	
Yellow corn	53.23
Soybean meal	38.23
Animal fat	2.6
Dicalcium phosphate	2.23
Limestone	1.27
Salt	0.34
Choline chloride 60%	0.10
Lysine	0.28
DL-Methionine	0.37
L-Threonine	0.15
Premix[1]	0.25
Coccidiostat[2]	0.05
BMD[3]	0.05
Total	100
Calculated nutrients	
CP	23
Calcium	0.96
Available phosphorus	0.48
AME; Kcal/kg	3,000
Digestible methionine	0.51
Digestible lysine	1.28
Digestible threonine	0.86
Digestible TSAA	0.95
Sodium	0.16
Choline	0.16

Grower (15-28 doa)	
Item	Commercial diet
Ingredient (%)	
Yellow corn	57.13
Soybean meal	34.8
Animal fat	3.5
Dicalcium phosphate	2
Limestone	1.17
Salt	0.34
Choline chloride 60%	0.10
Lysine	0.21
DL-Methionine	0.32
L-Threonine	0.16
Premix	0.25
Coccidiostat	0.05
BMD	0.05
Total	100
Calculated nutrients	
CP	21.5
Calcium	0.87
Available phosphorus	0.435
AME (Kcal/kg)	3,100
Digestible methionine	0.47
Digestible lysine	1.15
Digestible threonine	0.77
Digestible TSAA	0.87
Sodium	0.16
Choline	0.16

Finisher (29-45 doa)	
Item	Commercial diet
Ingredient (%)	
Yellow corn	54.23
Soybean meal	38.23
Animal fat	2.5
Dicalcium phosphate	2.23
Limestone	1.27
Salt	0.34
Choline chloride 60%	0.10
Lysine	0.28
DL-Methionine	0.37
L-Threonine	0.15

(continued on next column)

Table 1. (*continued*)

Finisher (29-45 doa)	
Item	Commercial diet
Premix	0.25
Coccidiostat	0.05
BMD[3]	0.05
Total	100
Calculated nutrients	
CP	19.5
Calcium	0.78
Available phosphorus	0.39
AME (Kcal/kg)	3,200
Digestible methionine	0.43
Digestible lysine	1.02
Digestible threonine	0.68
Digestible TSAA	0.8
Sodium	0.16
Choline	0.16

Abbreviation: doa, days of age.

[1]The broiler premix provided per kilogram of diet: vitamin A (retinyl acetate), 10,000 IU; cholecalciferol, 4,000 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]Deccox (Zoetis, Parsippany, NJ).

[3]Bacitracin methylene disalicylate (BMD 110; Zoetis, Parsippany, NJ): containing 55 mg of BMD per kg.

Meat Yield and Processing

One bird from each of the 6 replicate pens per treatment was randomly selected for determination of pectoralis major (***P. major***) and pectoralis minor (***P. minor***) muscle weight at 14 and 39 doa. The birds that remained in each pen (approximately 11 birds/treatment replicate pen) were processed at 43 doa according to the method described by Wang et al. (2018). Weights of the whole carcass, *P. major* and *P. minor*, and leg, thigh, and wing parts were determined. Part yields were calculated as percentages of the carcass weight. At processing, the *P. major* samples were scored for incidence of woody breast myopathy (**WBM**) according to the procedures of Tijare et al. (2016).

Inflammatory Response

At 14, 28, and 39 doa, one bird from each of the 6 replicate pens per treatment was randomly selected and individually weighed and bled by venipuncture of the wing brachial artery. Plasma (approximately 1 mL) was subsequently extracted for immunological assay. Plasma α-1-acid glycoprotein (**AGP**) concentration was determined according to the procedure of Kaab et al. (2018). The optical density (450 nm) for each AGP sample was measured with a SpectraMax M5 Microplate Reader (Molecular Devices, San Jose, CA).

Statistical Analysis

There were 5 *in ovo* injection treatments for the incubation and grow-out periods. The experimental design

was a randomized complete block for both the hatch and rearing periods. The incubator level in the setter and hatcher served as the unit of treatment replication for the hatch data, and the floor pen served as the unit of treatment replication for the performance, meat yield, and immunity data. For the incubation and grow-out phases, the blocking factor was a group of the 5 treatments. The treatments were rerandomized between blocks. All data were analyzed separately within each time period by one-way ANOVA using the procedure for linear mixed models (PROC GLIMMIX) of SAS©, version 9.4 (SAS Institute Inc., Cary, NC). Differences were considered significant at $P < 0.05$. The following model was used for analysis of the incubation and posthatch data:

$$Yij = \mu + B_i + T_j + E_{ij}$$

where μ was the population mean; B_i was the block factor (i = 1 or 2); T_i was the effect of each *in ovo* injection treatments (j = 1 to 5); and E_{ij} was the residual error.

RESULTS AND DISCUSSION

Hatch

A number of studies have clearly demonstrated the importance and requirement of D_3 for chicken embryonic development. The presence of D_3 in eggs is very important to support embryo Ca metabolism during incubation (Landauer, 1967). Therefore, a deficiency of this vitamin can lead to reduced hatchability, which can be specifically related to late embryo mortality (Stevens et al., 1984). The *in ovo* injection of D_3 (0.2 μg) in the amniotic fluid at 12 doi has been shown to increase the concentrations of Ca and P in the yolk and the bodily tissues of embryos at 17 doi (Mansour et al., 2017). More recently, the *in ovo* injection of $25OHD_3$ at 18 doi has been shown to increase broiler hatchability (Bello et al., 2013) and to reduce the mortality of broilers embryos (Fatemi et al., 2020a).

A possible mechanism by which the *in ovo* injection of D_3 sources improve broiler performance may be linked to an increase in the demand of Ca during the last phase of incubation. Calcium absorption through the vitelline membrane or gut cells is facilitated by Ca-binding protein expression that is stimulated by the renal metabolite, $1,25(OH)_2$ D_3 (Hurwitz, 1992). In the renal cells, $1,25(OH)_2$ D_3 is produced by the hydrolysis of $25OHD_3$, which is available in the yolk. It can be also obtained by D_3 metabolism in hepatic cells (Hurwitz, 1992). The abdominal internalization of the yolk sac in embryos occurs at 20 doi, with the only source of nutrients, including Ca, being that which is derived from the yolk remaining in the gut of the embryo (Noy and Sklan, 2001). Therefore, a greater demand for D_3 may occur during the last stage of incubation, particularly if the level of $25OHD_3$ is insufficient or there is limited mobilization of D_3 and $25OHD_3$ by immature embryonic liver and kidney cells. Nevertheless, all the hatch variables examined in the present study were not significantly affected by treatment (Table 2). The limited amount of time (approximately 3 d) between the doi and time of hatch is the probable basis for the lack of effects on the hatch results. There are also several possible reasons for the contrast in the hatch results between this study and that of Bello et al. (2013) and Fatemi et al. (2020a). The facilities and the sources of D_3 used were different among the 3 studies, and eggs in the 3 studies were obtained from different broiler breeder flocks. An increase in hatchability was only observed when the crystalline form of $25OHD_3$ was used in the study by Bello et al. (2013). In addition, the incubation system used in this study was different from that used in the study conducted by Fatemi et al. (2020a). Fatemi et al. (2020a) used Jamesway setter and hatcher units (Jamesway Incubator Company Inc., Cambridge, Ontario, Canada), whereas the same Chick Master incubator (Chick Master Incubator Company, Medina, OH) was used as both a setter and hatcher unit in this study.

Performance and Meat Yield

Although the *in ovo* applications used at 18 doi did not have an immediate impact on hatch performance, subsequent effects in the posthatch phase were observed. The broiler performance data within each time period through 28 doa was not significantly affected by treatment, but the positive impact of $25OHD_3$ was observed after 28 doa. Birds in the $25OHD_3$ treatment had a higher BW and BWG between 29 and 41 doa than those in the noninjected, diluent, and D_3 treatments (Table 3). At 39 doa, birds in the $25OHD_3$ treatment had a higher *P. major* and breast meat yield than birds belonging to all the other treatments (Table 4), and at processing, $25OHD_3$ treatment resulted in an increase in *P. major* and breast meat yield in comparison with all other treatment groups (Table 5). Conversely, no significant differences were observed between the treatments for WBM at 43 doa (Table 6). In previous studies, the *in ovo* injection of $25OHD_3$ has likewise been shown to improve the early posthatch performance (Fatemi et al., 2020a) and immunity (Abbasi et al., 2017) of broilers. Vitamin D_3 sources can be stored in several organs, including white and red muscles, the liver, and adipose tissue (Burild et al., 2016). The storage of D_3 sources in the aforementioned tissues can assist in improving the performance of broiler chicks selected for high growth rates during grow out. A 24-hour delay in posthatch access to feed can result in decreased broiler finisher performance at 42 doa (Gonzales et al., 2008a,b). Furthermore, the *in ovo* injection of 36 mg/mL of L-ascorbic acid has been shown to have no positive effect on hatch (Zhang et al., 2018), but increased the BW and antioxidant activity of broilers in comparison with control groups at 40 doa (Zhang et al., 2019). These earlier observations along with the current one would confirm that delayed effects that are noted in the later posthatch phase may occur in response to events in the incubational and early posthatch phases.

Table 2. Effects of treatment [noninjected; diluent-injected; diluent containing 2.4 μg of vitamin D_3 alone, 25-hydroxyvitamin D_3 alone, or 2.4 μL of D_3 and $25OHD_3$ together] on the percentage egg weight loss between 0 and 12, and 0 and 18 d of incubation, hatchability of injected live embryonated eggs, and hatchling BW at 21 doi.

Treatment	0-12 PEWL (%)	0-18 PEWL (%)	HI (%)	Hatchling BW (g)
Noninjected[1]	5.01	12.9	96.3	42.5
Diluent[2,3]	5.01	12.1	92.2	42.9
D_3[2,4]	6.03	12.9	97.2	43.2
$25OHD_3$[2,5]	5.03	12.8	94.5	43.6
$25OHD_3$ + D_3[2,6]	5.04	12.5	95.5	43.5
Pooled SEM	0.695	0.70	2.33	0.40
P-value	0.522	0.758	0.309	0.120

No treatment means were significantly different at $P \leq 0.05$.

Abbreviations: $25OHD_3$, 25-hydroxycholecalciferol; D_3, vitamin D_3; doi, days of incubation; HI, hatchability of injected live embryonated eggs; PEWL, percentage egg weight loss.

[1]Embryos that were not injected with a solution.

[2]Received a 50-μL solution volume injected at 18 doi.

[3]Embryos injected with the commercial diluent.

[4]Embryos injected with the commercial diluent containing vitamin D_3 at 2.4 μg.

[5]Embryos injected with the commercial diluent containing $25OHD_3$ at 2.4 μg.

[6]Embryos injected with the commercial diluent containing D_3 at 2.4 μg and $25OHD_3$ at 2.4 μg.

Vitamin D_3 levels in birds are best assessed by an evaluation of the concentration of $25OHD_3$ in the blood because $25OHD_3$ has a longer half-life (approximately 2–3 wk) (Smith and Goodman, 1971; Hollis and Wagner, 2013) than D_3 (approximately 12–24 h) (Smith and Goodman, 1971; Haddad et al., 1993). In addition, dietary $25OHD_3$ increases circulating $25OHD_3$ concentrations to a greater extent than D_3 at the same level of inclusion in both broilers (Vignale et al., 2015) and laying hens (Käppeli et al., 2011). The longer half-life of $25OHD_3$ allows this metabolite to reside longer in the blood, thereby allowing more time for its conversion to the active form of vitamin D in response to depressed serum Ca or $25OHD_3$ levels (Taylor and Dacke, 1984; Rosol et al., 2000). A longer half-life of $25OHD_3$ can be beneficial in the newly

Table 3. Effects of treatment [noninjected; diluent-injected; diluent containing 2.4 μg of vitamin D_3 alone, 25-hydroxyvitamin D_3 alone, or 2.4 μL of D_3 and $25OHD_3$ together] on Ross 708 broiler BW, BW gain, average daily gain, feed intake, average daily feed intake, and percentage of mortality through 42 d of posthatch age.

Treatment	BW (g)	FI (g)	ADFI (g)	BWG (g)	ADG (g)	FCR (g/g)	Mortality (%)
Day 1–14							
Noninjected	441	501	35.8	398	28.5	1.26	3.13
Diluent[1,2]	441	501	35.0	398	28.4	1.23	2.08
D_3[1,3]	445	497	35.5	402	28.7	1.24	1.04
$25OHD_3$[1,4]	456	509	36.3	414	29.6	1.23	1.04
$25OHD_3$ + D_3[1,5]	453	500	35.7	411	29.3	1.22	1.04
Pooled SEM	5.5	8.1	0.54	5.5	0.39	0.016	1.179
P-value	0.201	0.872	0.536	0.173	0.172	0.544	0.650
Day 15–28							
Noninjected	1,490	1,516	108	1,091	77.9	1.38	0.00
Diluent	1,552	1,586	112	1,155	82.5	1.35	3.26
D_3	1,534	1,554	110	1,132	80.9	1.35	0.00
$25OHD_3$	1,579	1,590	113	1,165	83.2	1.35	0.00
D_3 + $25OHD_3$	1,531	1,563	109	1,120	80.0	1.39	2.08
Pooled SEM	26.4	28.7	1.7	23.7	1.70	0.015	1.226
P-value	0.228	0.401	0.294	0.236	0.237	0.327	0.228
Day 29–42							
Noninjected	2,968[b]	2,520	210	1487[b]	106	1.99	1.11
Diluent	3,011[b]	2,471	206	1481[b]	103	2.01	0.00
D_3	3,015[b]	2,541	212	1440[b]	106	2.01	0.00
$25OHD_3$	3,210[a]	2,563	214	1630[a]	116	1.84	2.22
D_3 + $25OHD_3$	3,090[a,b]	2,532	211	1559[a,b]	111	1.91	1.11
Pooled SEM	45.7	73.5	6.1	45.2	3.2	0.081	1.007
P-value	0.011	0.923	0.923	0.050	0.056	0.512	0.508

[a-b]Treatment means within the same column within the effect with no common superscripts are significantly different ($P < 0.05$).

Abbreviations: $25OHD_3$, 25-hydroxyvitamin D_3; BWG, BW gain; D_3, vitamin D_3; FCR, feed conversion ratio; FI, feed intake.

[1]Received a 50-μL solution volume injected at 18 d of incubation (doi).

[2]Embryos injected with the commercial diluent.

[3]Embryos injected with the commercial diluent containing vitamin D_3 at 2.4 μg.

[4]Embryos injected with the commercial diluent containing $25OHD_3$ at 2.4 μg.

[5]Embryos injected with the commercial diluent containing D_3 at 2.4 μg and $25OHD_3$ at 2.4 μg.

Table 4. Effects of treatment [noninjected; diluent-injected; diluent containing 2.4 µg of vitamin D_3 alone, 25-hydroxyvitamin D_3 alone, or 2.4 µL of D_3 and $25OHD_3$ together] on Ross 708 broiler BW and weights of pectoralis major, pectoralis minor, and breast muscle as a percentage of BW at 14 and 39 d of posthatch age.

	BW	*P. major*	*P. minor*	Breast
	14 doa			
Treatment	G	%		
Noninjected[1]	466	13.51	2.81	16.33
Diluent[2,3]	451	13.57	2.90	16.48
D_3[2,4]	460	12.82	2.85	15.68
$25OHD_3$[2,5]	448	13.56	2.80	16.36
$25OHD_3$ + D_3[2,6]	450	12.95	2.87	15.82
Pooled SEM	13.73	0.476	0.205	0.602
P-value	0.868	0.677	0.996	0.838
	39 doa			
Noninjected	2,807	20.16[b]	4.09	24.24[b]
Diluent	3,002	20.65[b]	3.79	24.45[b]
D_3	2,842	20.89[b]	3.85	24.74[b]
$25OHD_3$	2,892	22.74[a]	4.12	26.86[a]
$25OHD_3$ + D_3	2,869	20.99[b]	3.90	24.89[b]
Pooled SEM	119.6	0.578	0.15	0.603
P-value	0.818	0.050	0.454	0.041

[a-b]Treatment means within the same column within the effect with no common superscripts are significantly different ($P < 0.05$).

Abbreviations: $25OHD_3$, 25-hydroxyvitamin D_3; BWG, BW gain; D_3, vitamin D_3; doa, days of age; *P. major*, pectoralis major; *P. minor*, pectoralis minor.

[1]Embryos that were not injected with a solution.

[2]Received a 50-µL solution volume injected at 18 d of incubation (doi).

[3]Embryos injected with the commercial diluent.

[4]Embryos injected with the commercial diluent containing vitamin D_3 at 2.4 µg.

[5]Embryos injected with the commercial diluent containing $25OHD_3$ at 2.4 µg.

[6]Embryos injected with the commercial diluent containing D_3 at 2.4 µg and $25OHD_3$ at 2.4 µg.

hatched chick because of its impaired absorption of D_3. During the first 2 wk of life, the absorption of D_3 by the chick is low because of the immaturity of its digestive tract and the low activity of enzymes involved in lipid absorption (Noy and Sklan, 1995). In a more recent study in broiler chickens, plasma $25OHD_3$ levels decreased from 0 to 6 doa and remained low until 10 doa when D_3 was supplemented at the recommended level in the diet (Saunders-Blades and Korver, 2014). This indicates that birds are not able to properly absorb D_3 from the diet during the first 10 d of posthatch life. In the present study, the *in ovo* injection of $25OHD_3$ resulted in an increase in the breast meat yield and BW of mature broilers in comparison with the control and D_3 treatment groups. Therefore, the improvement in broiler performance and breast meat yield in response to the *in ovo* injection of $25OHD_3$ could be due to its greater half-life and greater storage efficiency in muscle tissue.

Woody breast myopathy 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). An increase in severity of WBM is associated with an increase in broiler age (Bodle et al., 2018), in broiler sex (Kuttappan et al., 2013), and with broiler diets containing high protein and energy levels (Kuttappan et al., 2012; Bodle et al., 2018). In addition, it is reported that WBM is mainly correlated to environmental and management factors that contribute 90% of the variance of the incidence of WBM in broiler chickens (Bailey et al., 2015). In the present study, we did not observe a significant difference among treatments for WBM scores. However, a greater breast meat yield was observed in birds belonging to the $25OHD_3$ treatment. This result may indicate that the *in ovo* injection of $25OHD_3$ improved the quality of the broiler meat. Nevertheless, further research is required to determine the specific effects of the *in ovo* injection of various D_3 sources on muscle developmental characteristics.

Table 5. Effects of treatment [noninjected; diluent-injected; diluent containing 2.4 µg of vitamin D_3 alone, 25-hydroxyvitamin D_3 alone, or 2.4 µL of D_3 and $25OHD_3$ together] on Ross 708 broiler carcass weight and weights of pectoralis major and minor muscle, breast, wing, leg, thighs, and fat pad parts relative to the carcass weight at 43 d of posthatch age.

Treatment	Carcass	*P. major*	*P. minor*	Breast	Wings	Drumstick	Thigh	Fat
	G	%						
Noninjected[1]	2,173	29.5[b]	5.77	35.2[b]	11.5	13.0	16.3	1.52
Diluent[2,3]	2,193	29.7[b]	5.85	35.6[b]	10.9	13.0	16.3	1.71
D_3[2,4]	2,140	29.2[b]	5.75	35.0[b]	11.2	13.2	16.3	1.64
$25OHD_3$[2,5]	2,204	31.3[a]	5.90	37.2[a]	12.1	13.5	16.8	1.51
$25OHD_3$ + D_3[2,6]	2,341	29.6[b]	5.70	35.3[b]	11.3	13.1	16.1	1.47
Pooled SEM	59.4	0.562	0.136	0.64	0.48	0.22	0.24	0.141
P-value	0.073	0.029	0.759	0.044	0.388	0.311	0.271	0.610

[a-b]Treatment means within the same column within the effect with no common superscripts are significantly different ($P < 0.05$).

Abbreviations: $25OHD_3$, 25-hydroxyvitamin D_3; BWG, BW gain; D_3, vitamin D_3; doa, days of age; *P. major*, pectoralis major; *P. minor*, pectoralis minor.

[1]Embryos that were not injected with a solution.

[2]Received a 50-µL solution volume injected at 18 d of incubation (doi).

[3]Embryos injected with the commercial diluent.

[4]Embryos injected with the commercial diluent containing vitamin D_3 at 2.4 µg.

[5]Embryos injected with the commercial diluent containing $25OHD_3$ at 2.4 µg.

[6]Embryos injected with the commercial diluent containing D_3 at 2.4 µg and $25OHD_3$ at 2.4 µg.

Table 6. Effects of treatment [noninjected; diluent-injected; diluent containing 2.4 μg of vitamin D_3 alone, 25-hydroxyvitamin D_3 alone, or 2.4 μL of D_3 and $25OHD_3$ together] on Ross 708 broiler woody breast incidence percentage in normal (score 0), low (score 1), mild (score 2), and severe (score 3) conditions at 43 d of posthatch age.

Treatment	Score 0	Score 1	Score 2	Score 3
	(%)			
Noninjected[1]	42.5	40.37	11.9	5.18
Diluent[2,3]	43.1	31.95	20.8	4.15
D_3[2,4]	41.7	33.42	22.6	2.36
$25OHD_3$[2,5]	54.2	25.0	18.0	2.77
$25OHD_3$ + D_3[2,6]	43.1	44.45	11.1	1.38
Pooled SEM	6.06	5.819	5.81	2.242
P-Value	0.509	0.125	0.448	0.733

No treatment means were significantly different at $P \leq 0.05$.
Abbreviations: $25OHD_3$, 25-hydroxyvitamin D_3; D_3, vitamin D_3.
[1]Embryos that were not injected with a solution.
[2]Received a 50-μL solution volume injected at 18 d of incubation (doi).
[3]Embryos injected with the commercial diluent.
[4]Embryos injected with the commercial diluent containing vitamin D_3 at 2.4 μg.
[5]Embryos injected with the commercial diluent containing $25OHD_3$ at 2.4 μg.
[6]Embryos injected with the commercial diluent containing D_3 at 2.4 μg and $25OHD_3$ at 2.4 μg.

Inflammatory Response

In the chicken, it has been proposed in several reports that AGP can be used to monitor inflammation (Lee et al., 2010; Asasi et al., 2013). Plasma AGP level is an inflammatory response indicator, and its concentrations have been observed to be higher in modern broiler lines than broiler lines used in the 1990s (O'Reilly et al., 2018). Therefore, AGP was investigated to establish the relative degree of inflammation experienced by the chickens in this experiment. Plasma AGP did not differ among the treatments at 14 and 28 doa. However, birds in the $25OHD_3$ treatment had lower plasma AGP levels than those in the noninjected, diluent, and D_3 treatment groups at 39 doa (Table 7). Therefore, it can be extrapolated that the *in ovo* injection of $25OHD_3$ may result in a lower inflammatory response in grown broilers.

Table 7. Effects of treatment [noninjected; diluent-injected; diluent containing 2.4 μg of vitamin D_3 alone, 25-hydroxyvitamin D_3 alone, or 2.4 μL of D_3 and $25OHD_3$ together] on Ross 708 broiler plasma α-1-acid glycoprotein levels at 14, 28, and 39 d of age.

Treatment	Day 14	Day 28	Day 39
	(μmol)		
Noninjection[1]	9.76	10.12	13.50^{a}
Diluent[2,3]	8.27	10.18	212.45^{a}
D_3[2,4]	8.57	10.20	12.90^{a}
$25OHD_3$[2,5]	8.84	9.86	9.63^{b}
$25OHD_3$ + D_3[2,6]	8.25	9.76	$12.15^{a,b}$
Pooled SEM	0.734	0.301	0.873
P-value	0.589	0.788	0.049

[a-b]Treatment means within the same column within the effect with no common superscripts are significantly different ($P < 0.05$).
Abbreviations: $25OHD_3$, 25-hydroxyvitamin D_3; D_3, vitamin D_3; doa, days of age.
[1]Embryos that were not injected with a solution.
[2]Received a 50-μL solution volume injected at 18 d of incubation (doi).
[3]Embryos injected with the commercial diluent.
[4]Embryos injected with the commercial diluent containing vitamin D_3 at 2.4 μg.
[5]Embryos injected with the commercial diluent containing $25OHD_3$ at 2.4 μg.
[6]Embryos injected with the commercial diluent containing D_3 at 2.4 μg and $25OHD_3$ at 2.4 μg.

In conclusion, the impact of the *in ovo* injection of 2.4 μg of D_3 and $25OHD_3$ alone or in combination on the performance, meat yield, and inflammatory response of Ross 708 broilers reared under commercial conditions was investigated. Our findings revealed that 2.4 μg of $25OHD_3$ exhibited a potential to increase the BW and breast meat yield and decrease the circulating AGP concentrations of broilers. The improvement in breast meat yield and performance of Ross 708 broilers in response to the *in ovo* injection of 2.4 μg of $25OHD_3$ may be due to its longer half-life, the greater storage of $25OHD_3$ in breast meat tissue, and a subsequent reduction in inflammation. Further research is required to determine the morphological and molecular mechanisms that might have been involved with the effects of the *in ovo* injection of the 2 D_3 sources used in this study on the meat yield and immunity of the broilers. Furthermore, more research is needed to determine the safety and efficacy of *in ovo* administration of $25OHD_3$ for this intended use.

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DISCLOSURES

The authors declare no conflicts of interest.

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