Effects of the in ovo injection of vitamin D₃ and 25-hydroxyvitamin D₃ in Ross 708 broilers subsequently fed commercial or calcium and phosphorus-restricted diets. I. Performance, carcass characteristics, and incidence of woody breast myopathy¹,²,³

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ABSTRACT Effects of the in ovo-injection of vitamin D₃ (D₃) and 25-hydroxyvitamin D₃ (25OHD₃) 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 D₃; (3) 2.4 μg 25OHD₃; or (4) 2.4 μg D₃ + 2.4 μg 25OHD₃. 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 25OHD₃ alone was higher compared to birds that received diluent alone or a combination of D₃ and 25OHD₃. Lower WBM incidence in ReCaP-fed birds was associated with a lower breast weight. An increase in breast meat yield in response to 25OHD₃ 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

INTRODUCTION

The absorption of vitamin D₃ (D₃), a fat-soluble vitamin, is facilitated by bile salts in the upper small intestine of chickens (Bar et al., 1980). Vitamin D₃ is a multifunctional prehormone which requires 2 hydroxylation steps in order to become the active hormone, 1, 25-dihydroxyvitamin D₃ [1, 25-(OH)₂ D₃]. After intestinal absorption, D₃ is delivered to the liver for the first hydroxylation which converts it to 25-hydroxyvitamin D₃ (25OHD₃) by 25-hydroxylase. The second hydroxylation takes place in renal epithelial cells which converts 25OHD₃ to 1, 25-(OH)₂ D₃ via 1 α-hydroxylase activity (Booth et al., 1985). Inclusion of dietary D₃ and its metabolites are essential for proper growth in commercial broilers. Vitamin D₃ 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, D₃ has strong immunomodulatory activity that promotes broiler immunity during pathogenic infections (Morris et al., 2014; Chou et al., 2009). Dietary
25OHD₃ in combination with D₃ has also been shown to enhance broiler performance and bone quality in comparison to D₃ alone at the same level of inclusion (Papesova et al., 2008). Furthermore, 25OHD₃ is more effective than D₃, particularly in birds fed Ca and P-restricted diets (Bar et al., 2003). However, the first metabolite of D₃, 25OHD₃, has been shown to increase the rate of Ca and P intestinal absorption more than D₃ (Bar et al., 1980), and the first hydroxylation step of D₃ in the liver can be bypassed. In young embryos and hatchlings, the conversion of D₃ to 25OHD₃ is low due to the immaturity of their livers. This has been found to restrict an increase in serum 25OHD₃ when D₃ 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; Silvo et al., 2014). Dietary 25OHD₃ 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 D₃ 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 D₃ (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 25OHD₃ 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 25OHD₃ has been observed to result in an improvement in rooster 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 D₃, an improvement in the inflammatory response of 39 doa broilers was observed when they received 2.4 μg of 25OHD₃ by in ovo administration (Fatemi et al., 2021). However, there is limited information concerning the effects of the in-ovo injection of D₃ alone or in combination with 25OHD₃ on broiler posthatch performance and meat yield. Therefore, the objective of this study was to investigate the effects of the in ovo injection of D₃ and 25OHD₃ 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-old commercial Ross 708 broiler breeder hens and stored under commercial conditions as 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 Inovject 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) D₃ (50 μL of commercial diluent containing 2.4 μg D₃), (3) 25OHD₃ (50 μL of commercial diluent containing 2.4 μg 25OHD₃), and D₃+25OHD₃ (50 μL of commercial diluent containing 2.4 μg of D₃ and 2.4 μg of 25OHD₃). 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 m²/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
| Item | Ingredient (%) | Commercial diet Starter (0-14 doa) | Calcium and available phosphorus restricted (ReCap) diet |
|------|---------------|----------------------------------|------------------------------------------------------|
|      |               | Pct                             | Pct                                                 |
|      |               | Yellow corn                     | 53.23                                               |
|      |               | Soybean meal                    | 38.23                                               |
|      |               | Animal fat                      | 2.60                                                |
|      |               | Dicalcium phosphate             | 2.23                                                |
|      |               | Limestone                       | 1.27                                                |
|      |               | Salt                            | 0.34                                                |
|      |               | Choline chloride 60%            | 1.00                                                |
|      |               | Lysine                          | 0.28                                                |
|      |               | DL-Methionine                   | 0.37                                                |
|      |               | L-Threonine                     | 0.15                                                |
|      |               | Premix                          | 0.25                                                |
|      |               | Cellulose                       | 0.78                                                |
|      |               | Total                           | 100                                                 |
|      |               | Calculated nutrients            |                                                     |
|      |               | Crude protein                   | 23                                                  |
|      |               | Calcium                         | 0.96                                                |
|      |               | Available phosphorus            | 0.48                                                |
|      |               | Apparent metabolizable energy (AME, Kcal/kg) | 3,000                                                |
|      |               | Digestible Methionine           | 0.51                                                |
|      |               | Digestible Lysine               | 1.28                                                |
|      |               | Digestible Threonine            | 0.86                                                |
|      |               | Digestible total sulfur amino acids (TSAA) | 0.95                                                |
|      |               | Sodium                          | 0.16                                                |
|      |               | Choline                         | 0.16                                                |
|      |               | Grower (15-28 doa)              |                                                     |
|      |               | Pct                             |                                                     |
|      |               | Yellow corn                     | 57.13                                               |
|      |               | Soybean meal                    | 34.80                                               |
|      |               | Animal fat                      | 3.50                                                |
|      |               | Dicalcium phosphate             | 2.00                                                |
|      |               | 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                                                |
|      |               | Cellulose                       | 0.71                                                |
|      |               | Total                           | 100                                                 |
|      |               | Calculated nutrients            |                                                     |
|      |               | Crude protein                   | 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)            |                                                     |
|      |               | Pct                             |                                                     |
|      |               | Yellow corn                     | 54.23                                               |
|      |               | Soybean meal                    | 38.23                                               |
|      |               | Animal fat                      | 2.50                                                |
|      |               | 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                          | 0.25                                                |
|      |               | Coccidiostat                    | 0.05                                                |
|      |               | Cellulose                       | 0.78                                                |
|      |               | Total                           | 100                                                 |
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 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:

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

Where \(\mu\) was the population mean; \(B_i\) was the block factor \((i = 1–2)\); \(D_j\) was the effect of each dietary treatments \((j = 1–2)\); \(I_k\) was the effect of in ovo injection treatment \((k = 1–4)\); \((DI)_{ijk}\) 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\(^1\)    | 0.696         | 0.689       |    | 0.348         | 0.343       |
| **Finisher**   |               |             |    |               |             |
| Control        | 0.780         | 0.775       |    | 0.390         | 0.403       |
| ReCaP\(^1\)    | 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 NPAR1WAY) 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. 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 (%) | Late embryo mortality (%) | Dead piping embryos (%) | Dead post-pipped embryos (%) | Dead hatchlings (%) |
|----------------------------|-----|--------|---------------------------|------------------------|----------------------------|--------------------|
| In ovo injection           |     |        |                           |                        |                            |                    |
| Diluent                    | 12  | 92.9   | 4.85                      | 1.04                   | 1.19                       | 0.74               |
| D$_3$                      | 12  | 92.4   | 3.55                      | 0.78                   | 2.13                       | 1.11               |
| D$_3$ + 25OHD$_3$           | 12  | 95.4   | 2.45                      | 0.45                   | 1.36                       | 0.30               |
| D$_3$ + 25OHD$_3$           | 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                      |                    |

*1Hatchability of live embryonated eggs.  
*2Mortality between 18 and 21 doi, prior to pip.  
*3Mortality during the pipping process.  
*4Mortality after the pipping process.  
*5Mortality immediately after complete emergence of hatchlings from the shell.  
*6Eggs injected at 18 doi with 50 μl of commercial diluent.  
*7Eggs injected at 18 doi with 50 μl of commercial diluent containing 2.4 μg of vitamin D$_3$.  
*8Eggs injected at 18 doi with 50 μl of commercial diluent containing 2.4 μg of 25OHD$_3$.  
*9Eggs injected at 18 doi with 50 μl of commercial diluent containing 2.4 μg of D$_3$ and 2.4 μg of 25OHD$_3$.  

See page 5 for more details.
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 D3 sources on the performance of pigs fed diets lacking supplemental phytase (O'Doherty et al., 2010). However, the effects of supplemental vitamin D3 sources in broiler diets deficient in Ca and P and without supplemental phytase have not been previously reported.

The in ovo injection of vitamin D3 at 12 doi has been reported to increase Ca and P serum levels in broiler embryos (Mansour et al., 2017). The influence of vitamin D3 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 D3 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 25OHD3 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 D3 sources may have the potential to increase

### Table 4. Live performance variables within in ovo treatment: diluent-injected control, and diluent containing 2.4 μg of vitamin D3 (D3) or 25-hydroxycholecalciferol (25OHD3) alone or in combination (D3 + 25OHD3) 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 (g) | ADG (g) | FI (g) | ADFI (g) | FCR (g/g) |
|----------------------------|------|--------|---------|---------|--------|----------|-----------|
| **Starter (0 to 14 doa)**  |      |        |         |         |        |          |           |
| In ovo injection           |      |        |         |         |        |          |           |
| Diluent                   | 12   | 427.6  | 386.8   | 27.63   | 445.8  | 31.81    | 1.151     |
| D3                        | 12   | 440.7  | 400.3   | 28.59   | 443.6  | 31.68    | 1.142     |
| 25OHD3                    | 12   | 434.8  | 393.7   | 28.12   | 449.6  | 32.12    | 1.142     |
| D3 + 25OHD3               | 12   | 429.6  | 389.3   | 27.81   | 441.6  | 31.57    | 1.138     |
| Diet                      |      |        |         |         |        |          |           |
| Commercial                | 24   | 431    | 395.2   | 28.23   | 448    | 31.99    | 1.134     |
| ReCaP                     | 24   | 436    | 390.2   | 28.23   | 448    | 31.99    | 1.134     |
| Pooled SEM                | 6.17 | 8.16   | 0.583   | 7.54    | 0.539  | 0.0186   |            |
| **Grower (15−28 doa)**    |      |        |         |         |        |          |           |
| In ovo injection           |      |        |         |         |        |          |           |
| Diluent                   | 12   | 1,387  | 959     | 68.51   | 1,404  | 100.3    | 1.481     |
| D3                        | 12   | 1,364  | 922     | 65.88   | 1,359  | 97.1     | 1.489     |
| 25OHD3                    | 12   | 1,393  | 959     | 68.49   | 1,395  | 99.6     | 1.475     |
| D3 + 25OHD3               | 12   | 1,384  | 954     | 68.11   | 1,387  | 99.1     | 1.470     |
| Diet                      |      |        |         |         |        |          |           |
| Commercial                | 24   | 1,502a | 1,071a  | 76.53a  | 1,466a | 104.7a   | 1.370a    |
| ReCaP                     | 24   | 1,262b | 826b    | 58.97b  | 1,307b | 93.4b    | 1.588b    |
| Pooled SEM                | 24.6 | 21.1   | 1.508   | 22.8    | 1.63   | 0.0291   |            |
| **Finisher (29−40 doa)**  |      |        |         |         |        |          |           |
| In ovo injection           |      |        |         |         |        |          |           |
| Diluent                   | 12   | 2,348  | 961     | 80.10   | 1,792  | 149.3    | 1.888     |
| D3                        | 12   | 2,337  | 974     | 81.15   | 1,762  | 155.2    | 1.915     |
| 25OHD3                    | 12   | 2,370  | 978     | 81.47   | 1,768  | 147.4    | 1.827     |
| D3 + 25OHD3               | 12   | 2,334  | 951     | 79.26   | 1,790  | 149.1    | 1.889     |
| Diet                      |      |        |         |         |        |          |           |
| Commercial                | 24   | 2,558a | 1,056a  | 87.98a  | 1,950a | 162.5a   | 1.867     |
| ReCaP                     | 24   | 2,138b | 876b    | 73.01b  | 1,657b | 138.0b   | 1.893     |
| Pooled SEM                | 35.0 | 34.9   | 36.4    | 4.12    | 0.0816 | 0.0457   | 2.02      |
| **Total**                 |      |        |         |         |        |          |           |
| 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   | 0.741   | 0.157  | 0.157    | 0.741     |
| **Mortality**             |      | 0.771  | 0.888   | 0.888   | 0.296  | 0.296    | 0.773     |
| In ovo                     |      | 0.001  | 0.001   | 0.001   | 0.001  | 0.001    | 0.003     |
| In ovo x Diet              |      | 0.914  | 0.914   | 0.914   | 0.446  | 0.446    | 0.361     |

a,bTreatment means within the same variable column within type of treatment with no common superscript differ significantly (P < 0.05).

1ADG, average daily gain; ADFI, average daily feed intake; BWG, BW gain; FCR, feed conversion ratio; FI, feed intake.

2Eggs injected at 18 d of incubation (doi) with 50 μl of commercial diluent.

3Eggs injected at 18 doi with 50 μl of commercial diluent containing 2.4 μg of vitamin D3.

4Eggs injected at 18 doi with 50 μl of commercial diluent containing 2.4 μg of 25OHD3.

5Eggs injected at 18 doi with 50 μl of commercial diluent containing 2.4 μg of 25OHD3 and 2.4 μg of vitamin D3.

6A diet restricted in Ca and available P by 20% throughout the rearing period.

7Cumulative FCR from 0 to 40 d of age.

8Cumulative mortality from 0 to 40 d of age.
Table 6. Carcass weight and weights of pectoralis major (P. major) and minor (P. minor), and breast meat yield (Breast) relative to carcass weight within in ovo treatment: diluent-injected control, and diluent containing 2.4 μg of vitamin D₃ (D₃) or 25-hydroxycholecalciferol (25OHD₃) alone or in combination (D₃ + 25OHD₃) 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 (%) | P. major (%) | P. minor (%) | Breast (%) |
|-----------|-----|-------------|--------------|--------------|------------|--------------|--------------|------------|
| In ovo injection |     |                          |              |              |            |              |              |            |
| Diluent¹ | 60  | 1,758       | 29.2         | 6.0          | 35.2       | 10.9         | 12.6         | 15.7       |
| D₃²      | 60  | 1,749       | 29.5         | 6.1          | 35.6       | 10.9         | 12.5         | 15.7       |
| 25OHD₃³  | 60  | 1,725       | 30.1         | 6.0          | 36.1       | 11.0         | 12.5         | 15.9       |
| D₃+25OHD₃⁴ | 60  | 1,749       | 30.0         | 6.0          | 36.0       | 11.0         | 12.4         | 15.5       |
| Diet     |     |                          |              |              |            |              |              |            |
| Commercial | 24  | 12.4         | 2.6          | 15.0         | 11.0       | 12.5         | 15.9         | 15.5       |
| ReCaP⁵   | 24  | 12.8         | 2.6          | 15.4         | 10.4       | 12.1         | 15.4         | 15.8       |
| Pooled SEM | 0.22 | 0.13       | 0.31          | 0.96         | 0.18       | 1.03        |             |            |
| In ovo   |     |                          |              |              |            |              |              |            |
| Diet     |     |                          |              |              |            |              |              |            |
| Commercial | 120 | 1,894       | 30.4         | 6.2          | 36.2       | 11.2         | 12.3         | 15.6       |
| ReCaP⁵   | 120 | 1,597       | 29.0          | 5.9          | 34.9       | 10.7         | 12.6         | 15.8       |
| Pooled SEM | 19.0 | 0.31     | 0.07          | 0.08         | 0.16       | 0.21        | 0.019       |            |

**Note:** Treatment means within the same variable column within type of treatment with no common superscript differ significantly (P < 0.05).

¹Eggs injected at 18 d of incubation (dOi) with 50 μl of commercial diluent.
²Eggs injected at 18 dOi with 50 μl of commercial diluent containing 2.4 μg of vitamin D₃.
³Eggs injected at 18 dOi with 50 μl of commercial diluent containing 2.4 μg of 25OHD₃.
⁴Eggs injected at 18 dOi with 50 μl of commercial diluent containing 2.4 μg of D₃ and 2.4 μg of 25OHD₃.
⁵A 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 D₃ sources in broiler embryos was not sufficient to overcome the subsequent negative effects of a 20% dietary reduction in Ca and available P by 20% throughout the rearing period. In the current study, broiler performance did not significantly differ among in ovo injection treatments with no common superscripts are significantly different (P < 0.05).

Table 7. Incidence of woody breast within in ovo treatment: diluent-injected control, and diluent containing 2.4 μg of vitamin D₃ (D₃) or 25-hydroxycholecalciferol (25OHD₃) alone or in combination (D₃ + 25OHD₃) 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 | P-value |
|-----------|----|-------------|-------------|-------------|-------------|---------------|---------|
| In ovo injection | | | | | | | |
| Diluent¹ | 60 | 41.8 | 37.6 | 19.7 | 0.9ᵃᵇ | 0.65 | 0.042 |
| D₃² | 60 | 41.0 | 42.3 | 10.4 | 6.3ᵃ | 0.75 | 0.001 |
| 25OHD₃³ | 60 | 41.8 | 48.5 | 9.6 | 0.1ᵇ | 0.55 | 0.024 |
| D₃ + 25OHD₃⁴ | 60 | 36.7 | 41.0 | 15.8 | 6.4ᵇ | 0.79 | 0.013 |
| Diet | | | | | | | |
| Commercial | 120 | 18.6ᵇ | 57.5ᵇ | 19.0ᵇ | 4.9 | 1.0³ᵇ | 0.099 |
| ReCaP⁵ | 120 | 62.0ᵃ | 27.3ᵃ | 8.8ᵇ | 1.9 | 0.34ᵇ | 0.078 |
| Pooled SEM | 4.47 | 3.08 | 2.27 | 1.98 | 0.078 | | |
| In ovo | | | | | | | |
| Health | 0.870 | 0.495 | 0.197 | 0.042 | 0.117 | | |
| Diet | 0.001 | 0.001 | 0.024 | 0.001 | 0.001 | | |
| In ovo x Diet | 0.903 | 0.509 | 0.153 | 0.079 | 0.800 | | |
| In ovo injection | | | | | | | 46 doa |
| Diluent | 60 | 41.8 | 25.6 | 18.3 | 14.3 | 0.86 | |
| D₃ | 60 | 48.8 | 18.6 | 14.6 | 18.0 | 0.90 | |
| 25OHD₃ | 60 | 49.0 | 19.1 | 21.6 | 10.3 | 0.76 | |
| D₃ + 25OHD₃ | 60 | 36.8 | 30.5 | 24.2 | 8.4 | 0.84 | |
| Diet | | | | | | | |
| Commercial | 120 | 31.6ᵇ | 24.5 | 27.5ᵇ | 16.4 | 1.10ᵇ | 0.099 |
| ReCaP | 120 | 56.6ᵃ | 22.5 | 11.9ᵇ | 9.0 | 0.59ᵇ | | |
| Pooled SEM | 3.50 | 3.38 | 2.64 | 3.61 | | | |
| In ovo | | | | | | | |
| Health | 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 | | |

¹ᵇTreatment means within the same variable column within type of treatment with no common superscripts are significantly different (P < 0.05).
²Eggs injected at 18 do of incubation (doi) with 50 μl of commercial diluent.
³Eggs injected at 18 doi with 50 μl of commercial diluent containing 2.4 μg of vitamin D₃.
⁴Eggs injected at 18 doi with 50 μl of commercial diluent containing 2.4 μg of 25OHD₃.
⁵Eggs injected at 18 doi with 50 μl of commercial diluent containing 2.4 μg of D₃ and 2.4 μg of 25OHD₃.
⁶A diet restricted in Ca and available P by 20% throughout the rearing period.
small intestine morphology, and longer half-life and a higher rate of 25OHD₃ absorption relative to D₃. Furthermore, it may be linked to differences in the duration and levels of storage of the 2 vitamin D₃ sources in the tissues of the birds. In pigs, dietary 25OHD₃ 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 D₃ 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 25OHD₃ stored in muscle tissue may be another reason for the increased P₃ major yield in birds belonging to the 25OHD₃-injected treatments in comparison to those in the D₃-injected treatments. The expression of 1α-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 1α-hydroxylase in muscle tissue, which can only convert 25OHD₃ to the, 1, 25-(OH)₂ D₃ (the active form of D₃), results in an increase in protein synthesis and muscle hypertrophy of muscle tissue (Hutton et al., 2014). Conversely, 1α-hydroxylase cannot convert D₃ to 1, 25-(OH)₂ D₃. Therefore, at the level of activity equal to 25OHD₃, D₃ 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 D₃-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 D₃ and 25OHD₃ 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 D₃, 25OHD₃, 1α-hydroxy vitamin D₃, and 1, 25-(OH)₂ D₃ 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 D₃, the in ovo injection of 25OHD₃ alone tended to decrease the inflammatory response in broilers. Thus, lower proportions of severe WBM scores in response to the in ovo injection of 25OHD₃ 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 D₃ 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 D₃ sources did not affect broiler performance, meat yield, or quality when Ca and P were restricted in the diet. Furthermore, in comparison to D₃, the in ovo injection of 25OHD₃ 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 25OHD₃ 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 D₃ 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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