INRODUCTION

In ovo injection of broiler hatching eggs is widely accepted in the poultry industry. In comparison with the traditional method of broiler vaccination, in ovo injection offers a less stressful, faster, and more uniform delivery of vaccines and nutrient mixtures to developing embryos (Williams, 2007). Included among the various embryological and physiological studies that are made possible by in ovo injection technology are investigations concerning the effects of substances such as amino acids (Ohta et al., 1999; Kadam et al., 2008), carbohydrates (Tako et al., 2004; Uni et al., 2005; Zhai et al., 2011c), electrolyte solutions (McGruder et al., 2011), hormones (Henry and Burke, 1999; Kocamis et al., 1999, 2000), nucleotides (Dalloul et al., 2005), stimulants (Zhai et al., 2008; Keralapurath et al., 2010), and vitamins (Gore and Qureshi, 1997; Bhanja et al., 2007).

Because toxic levels of vitamin D₃, especially in the forms of its more bio-potent metabolites, are transferable from hens to embryos, its excessive supplementation in diets fed to hens may result in the possible calcification of soft bone, a compromised hatchability, and an increase in embryonic mortality (Johnson and Ivey, 2002; de Matos, 2008). However, 25-hydroxycholecalciferol \([25(OH)D_3]\) is more biologically active in comparison with vitamin D₃ and is less toxic and more stable compared with 1,25-dihydroxycholecalciferol \([1,25(OH)_2D_3]\) (Soares et al., 1995). Furthermore, the use of 25(OH)D₃ has been generally recognized as safe for inclusion in commercial broiler diets (Ward, 1995). Approximately 18.3 IU of vitamin D naturally occur in eggs, particularly in the yolk. However, Yarger et al. (1995) recommended that a minimum of 69 µg of 25(OH)D₃ should be supplemented in each kilogram of diet fed to broilers beginning on d 1 posthatch for maximum performance. Coto et al. (2010) reported that the hatchability of fertilized eggs was improved when the

Key words: 25-hydroxycholecalciferol, chick quality, embryonic, hatchability, in ovo injection

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ABSTRACT The effects of the in ovo injection of commercial diluent containing various levels of 25-hydroxycholecalciferol \([25(OH)D_3]\) on hatchability and hatching chick quality variables in Ross × Ross 708 broilers were examined in 2 trials. All treatment groups, each containing 21 and 40 eggs in trials 1 and 2, respectively, were randomly represented on each of 10 replicate tray levels of a single-stage incubator. On 18 d of incubation (doi), eggs were subjected to 1 of 6 treatments using a commercial multi-egg injector. Treatments included noninjected and diluent-injected controls, along with those that received diluent containing 0.15, 0.30, 0.60, or 1.20 µg of 25(OH)D₃ in trial 1 and 0.20, 0.60, 1.80, or 5.4 µg of 25(OH)D₂ in trial 2. Hatchability of injected eggs (HI) was recorded on 20.5, 21.0, and 21.5 doi, and embryonic mortalities through 21.5 doi were determined. On 21.0 doi in each trial, the BW, body length, and weights and moisture concentrations of the livers and yolk sacs of male and female chicks in each replicate group were determined. In a preliminary trial, the in ovo injection of 0.60 µg of 25(OH)D₃ on 18 doi significantly elevated its serum level concentrations in embryos on 19.25 doi. In both trials, the HI of noninjected controls through 21.0 doi was higher than that of diluent-injected controls. In trial 1, the HI of eggs on 21.0 doi after being injected with 0.30, 0.60, or 1.20 µg of 25(OH)D₃ was higher compared with that of diluent-injected controls, and in trial 2, the HI of eggs on 21.0 and 21.5 doi after being injected with 0.60 µg of 25(OH)D₃ was higher compared with that of diluent-injected controls. In conclusion, the in ovo injection of 0.60 µg of 25(OH)D₃ may be used to alleviate depressions in HI in Ross × Ross 708 broiler hatching eggs that can occur in response to the in ovo injection of commercial diluent.

INTRODUCTION

In ovo injection of broiler hatching eggs is widely accepted in the poultry industry. In comparison with the traditional method of broiler vaccination, in ovo injection offers a less stressful, faster, and more uniform delivery of vaccines and nutrient mixtures to developing embryos (Williams, 2007). Included among the various embryological and physiological studies that are made possible by in ovo injection technology are investigations concerning the effects of substances such as amino acids (Ohta et al., 1999; Kadam et al., 2008), carbohydrates (Tako et al., 2004; Uni et al., 2005; Zhai et al., 2011c), electrolyte solutions (McGruder et al., 2011), hormones (Henry and Burke, 1999; Kocamis et al., 1999, 2000), nucleotides (Dalloul et al., 2005), stimulants (Zhai et al., 2008; Keralapurath et al., 2010), and vitamins (Gore and Qureshi, 1997; Bhanja et al., 2007).

Because toxic levels of vitamin D₃, especially in the forms of its more bio-potent metabolites, are transferable from hens to embryos, its excessive supplementation in diets fed to hens may result in the possible calcification of soft bone, a compromised hatchability, and an increase in embryonic mortality (Johnson and Ivey, 2002; de Matos, 2008). However, 25-hydroxycholecalciferol \([25(OH)D_3]\) is more biologically active in comparison with vitamin D₃ and is less toxic and more stable compared with 1,25-dihydroxycholecalciferol \([1,25(OH)_2D_3]\) (Soares et al., 1995). Furthermore, the use of 25(OH)D₃ has been generally recognized as safe for inclusion in commercial broiler diets (Ward, 1995). Approximately 18.3 IU of vitamin D naturally occur in eggs, particularly in the yolk. However, Yarger et al. (1995) recommended that a minimum of 69 µg of 25(OH)D₃ should be supplemented in each kilogram of diet fed to broilers beginning on d 1 posthatch for maximum performance. Coto et al. (2010) reported that the hatchability of fertilized eggs was improved when the
broiler breeder hens from which the eggs were collected were fed diets supplemented with 25(OH)D₃ as their main source of vitamin D₃. Vitamin D₃ binding protein is required to facilitate adequate mineral transport from the eggshell to the developing embryo. On a more specific note, it has been affirmed that 25(OH)D₃ possesses a greater affinity for the vitamin D₃ binding protein, therefore fortifying its ability to improve embryonic development and hatchability (Soares et al., 1995). Sindel et al. (1978) improved the hatchability of fertilized eggs of vitamin D-deficient layer embryos by the in ovo injection of 25(OH)D₃ before incubation. Similarly, the in ovo injection of various forms of vitamin D₃ has been used as a means to support the skeletal development, mobilization of shell calcium, and prevention of hypocalcaemia in vitamin D-deficient quail embryos (Elaroussi et al., 1993).

However, to achieve an improvement in hatchability of fertilized eggs, Coto et al. (2010) supplied 68.0 µg of 25(OH)D₃ per kg to the diets of broiler breeder hens. Likewise, for the benefits to be realized, which included an increase in hatchability of fertilized eggs, hens were continuously fed the diets supplemented with 25(OH)D₃. An additional limitation to the commercial use of 25(OH)D₃ in poultry diets is that its use as a supplemental macro-ingredient is not cost effective (Soares et al., 1995). Also, it is important to point out that this Marek’s disease vaccine is delivered to the embryos in the commercial diluent that was used in this study. We hypothesized that a one-time in ovo injection of a much lower amount of 25(OH)D₃ may be more cost effective in achieving comparable embryonic development and hatchability of fertilized egg results in comparison with its use in diets. Furthermore, because the eggs in this current study were injected late in incubation (d 18), hatchability of live embryonated injected eggs (HI) was assessed. Therefore, in 2 main trials, the objectives of this current study were to investigate effects of various concentrations of supplementary 25(OH)D₃ provided by in ovo injection on 18 d of incubation (doy) on HI and on various hatching chick quality variables.

**MATERIALS AND METHODS**

**General**

Protocols for a preliminary trial and the 2 consecutive main trials conducted in this current study were approved by the Institutional Animal Care and Use Committee of Mississippi State University. In the preliminary and the 2 main trials, Ross × Ross 708 hatching eggs were collected and then stored under commercial conditions for a maximum of 3 d before weighing (for selection of eggs within 10% of set egg weight) and setting for incubation (Zhai et al., 2011a,b,c). In all trials, pure crystalline 25(OH)D₃ (DSM Nutritional Products Inc., Parsippany, NJ) was dissolved in 95% ethanol. To accomplish the delivery of presupervised concentrations of 25(OH)D₃ to individual eggs in each trial, serial dilutions using ethanol as the solvent were performed to *quantum satis* the 25(OH)D₃-ethanol mixture to an 8-mL volume. An equal volume of diluent (commercial MD vaccine diluent, Merial Co., Duluth, GA) in each infusion bag (1,200 mL of total volume) used for injection was removed to accommodate the added 8 mL of 25(OH)D₃-ethanol mixture. On 18 doy, all eggs that were designated for injection received their presupervised treatments using an Intelliject multi-egg injector (Avitech LLC, Salisbury, MD). Details of the automated in ovo injection procedures, needle dimensions and injection depth, egg handling, and verification of the amnion as the site of injection were as described by Zhai et al. (2011c). All eggs including those that belonged to the noninjected control group remained outside the setter for a maximum of 2 min during the injection procedure.

**Preliminary Trial**

Eggs used were obtained from a commercial broiler breeder flock at 30 wk of age. A total of 300 eggs were randomly distributed into 2 treatments that were equally and randomly represented on each of 3 tray levels (blocks). The eggs were incubated under standard incubation conditions in a single stage incubator (model NMC-1000, Natureform, Jacksonville, FL). The 2 treatments included a diluent-injected control (100 µL of commercial diluent) and an experimental treatment in which 100 µL of commercial diluent containing 0.60 µg of 25(OH)D₃ was injected. On 19.25 doy, blood was collected from the chorio-allantoic vasculature and serum was extracted as specified by Peebles et al. (1996). The embryos were subsequently euthanized to determine their sex. Equal volumes of serum samples from 2 to 3 females were pooled within each treatment group on each tray level (blocks). The 25(OH)D₃ concentrations in the 3 resulting serum samples within each treatment group were subsequently assayed by RIA using the method described by Hollis et al. (1993).

**Main Trials**

Eggs were collected from 30-wk-old (trial 1) and 34-wk-old (trial 2) broiler breeder flocks housed in a commercial facility. In trials 1 and 2, 1,260 and 2,400 eggs, respectively, were selected according to weight and quality as specified by Zhai et al. (2011c). The eggs were set and then incubated under standard conditions (Peebles and Brake, 1987) in a Jamesway model PS 500 setter unit (Jamesway Incubator Company Inc., Cambridge, Ontario, Canada). In each of the main trials, 6 treatment groups, each containing 21 eggs (trial 1) and 40 eggs (trial 2), were represented on each of 10 tray levels (experimental blocks) in the setter. On 10 doy, eggs with shells that were cracked were discarded and all others were candled to remove those that were unfertilized or contained dead embryos as described by Ernst et al. (2004).
**Experimental Layout**

In trials 1 and 2, the treatments included noninjected (treatment 1) and diluent-injected (sham-injected with 100 µL of commercial diluent; treatment 2) controls. Experimental treatments with 100 µL of diluent contained 0.15, 0.30, 0.60, or 1.20 µg of 25(OH)D3 in trial 1 and 0.20, 0.60, 1.80, or 5.40 µg of 25(OH)D3 in trial 2. The experimental treatments corresponded to vitamin D3 activity levels of 6, 12, 24, and 48 IU, respectively, in their respective treatment replicate groups were assigned the hatcher basket that corresponded to their positions in the setter.

**Data Collection**

Every 12 h between 20.5 and 21.5 doi in each trial, HI was monitored. Hatch residues were analyzed according to the procedures of Ernst et al. (2004) in both trials on d 21.5 for determination of postinjection embryonic mortality (PIM) at 21.5 doi. Only embryos that were observed to have died on or after 18 doi were included along with those that hatched in the determination of PIM and HI, respectively. On day of hatch, 3 chicks in trial 1 (180 total) and 4 chicks in trial 2 (240 total) were randomly selected from each treatment replicate group. In trial 1, at least 1 chick from each sex, and in trial 2, 2 chicks from each sex as determined by feather sexing were sampled from each treatment replicate group. After obtaining BW and body length (BL) measurements (Molenaar et al., 2008), birds were necropsied to confirm sex and for yolk sac and liver extraction. The BW of birds relative to the square of their BL was further calculated as described by Willemsen et al. (2008).

Absolute yolk sac and liver weights and yolk-free BW were determined. Subsequently, yolk sac and liver weights relative to BW were calculated. Furthermore, liver weight was calculated relative to yolk-free BW, whereas BW and yolk-free BW were calculated relative to set egg weight. Extracted yolk and liver samples were oven-dried for determination of percentage yolk moisture and percentage liver moisture according to the procedure of Zhai et al. (2011c).

**Statistical Description**

A randomized complete block design was employed with each of the 3 tray levels in the incubator of the preliminary trial and each of the 10 tray levels of the setter and each of the 10 hatching basket levels in the hatcher of the 2 main trials representing a block. The 2 treatment groups in the preliminary trial and all of the 6 treatment groups in each of the 2 main trials were randomly arranged and represented within each level (block). All variables in each trial were analyzed using the MIXED procedure of SAS software 9.2 (SAS Institute, 2010). Treatments were viewed as fixed effects and blocks as random effects to analyze for the effects of treatment on serum 25(OH)D3 in the preliminary, and HI and PIM in the 2 main trials using ANOVA. A split-plot analysis of the d 0 chick quality variables was performed with treatment, sex, and their interaction designated as a fixed effect and block as a random effect in the 2 main trials. Least squares means were compared in the event of significant global effects. Global and least squares means differences were considered significant at \( P \leq 0.05 \).

**RESULTS**

**Preliminary Trial**

There was a significant treatment effect on serum 25(OH)D3 concentration \( (P \leq 0.02) \). The concentration of 25(OH)D3 in the serum of embryos injected with 0.60 µg of 25(OH)D3 \((33.8 \pm 3.59 \text{ ng/mL})\) was significantly higher in comparison with that of embryos in the diluent-injected control group \((13.7 \pm 3.59 \text{ ng/mL})\).

**Main Trials**

**Trial 1.** In trial 1, mean set egg weight ± SEM was 57.3 ± 0.52 g. Injection treatment had no significant effect on PIM at 21.5 doi or on HI at 20.5 and 21.5 doi. However, there was a significant \( (P \leq 0.01) \) injection treatment effect on HI on 21.0 doi (Figure 1). The HI on 21.0 doi in the noninjected controls was higher compared with that of the diluent-injected controls and the 0.15 µg of 25(OH)D3 treatment. Furthermore, the HI on 21.0 doi was higher in eggs injected with 0.30, 0.60, or 1.20 µg of 25(OH)D3 in comparison with that of diluent-injected controls (Figure 1). For further informational reference, the 21.5 HI means in noninjected, diluent-injected, and 0.15, 0.30, 0.60, and 1.20 µg of 25(OH)D3 treatment groups was 98.0, 92.3, 93.4, 94.4, 93.3, and 92.7%, respectively (pooled SEM = 1.75%). There were no main or interactive effects involving injection treatment on any of the hatching chick quality variables except for yolk-free BW relative to set egg weight (RYFWSW; Figure 2). There was a significant \( (P \leq 0.03) \) treatment \( \times \) sex interaction for RYFWSW (Figure 2). In female chicks, RYFWSW was increased by the injection of 0.60 µg of 25(OH)D3 compared with that of eggs injected with the 1.20-µg dose. Whereas in male chicks, RYFWSW in the diluent-injected controls was higher compared with that in the 0.30 and 0.60 µg of 25(OH)D3-injected groups. Furthermore, male chicks in the 1.50 µg of 25(OH)D3-injected group had a greater RYFWSW in comparison with that in the males belonging to the 0.60-µg injected group (Figure 2).

**Trial 2.** In trial 2, mean set egg weight ± SEM was 60.5 ± 0.44 g. Injection treatment had no significant effect on HI at 20.5 doi. On the other hand, there were significant injection treatment effects for HI on 21.0 \( (P \leq 0.01) \).
Furthermore, there was a significant treatment effect on PIM at 21.5 doi ($P \leq 0.03$). The HI on 21.0 doi in noninjected controls was higher compared with that of diluent-injected controls and compared with the 1.80 and 5.40 µg of 25(OH)D$_3$-injected groups. Also, the d 21.0 HI of eggs injected with 0.60 µg of 25(OH)D$_3$ was higher in comparison with that of diluent-injected controls and eggs given the 5.40-µg dose (Figure 3a). Furthermore, HI on 21.5 doi in the noninjected controls was higher in comparison with those in the 0.20, 1.80, and 5.40 µg of 25(OH)D$_3$-injected groups. The HI on 21.5 doi in the group injected with 0.60 µg of 25(OH)D$_3$ was also higher compared with that of the group injected with the 1.80-µg dose of 25(OH)D$_3$. The PIM at 21.5 doi in the group injected with 0.20 or 5.40 µg of 25(OH)D$_3$ was also greater compared with that of noninjected controls. Mean PIM in the noninjected control, diluent-injected, and 0.20, 0.60, 1.80, and 5.40 µg 25(OH)D$_3$ treatment groups was 3.08, 5.76, 7.45, 4.91, 9.38, and 8.24%, respectively (pooled SEM = 1.431%).

Among the hatching chick quality variables, there were no main or interactive effects involving injection treatment except for treatment main effects on percent yolk moisture (PYM; $P \leq 0.01$), yolk sac weight relative to BW (RYBW; $P \leq 0.02$), RYFWSW ($P \leq 0.05$), and BL ($P \leq 0.05$; Table 1). The PYM in the 5.40 µg of 25(OH)D$_3$-injected group was higher compared with that in noninjected controls and compared with the groups injected with 0.20, 0.60, or 1.80 µg of 25(OH)D$_3$. Furthermore, the PYM in the diluent-injected controls was increased in comparison with that of the 0.20 and 0.60 µg of 25(OH)D$_3$-injected groups. Similarly, RYBW in the group injected with a 5.40 µg dose of 25(OH)D$_3$ was higher compared with that of the noninjected and diluent-injected control groups as well as the group that received a 1.80-µg dose of 25(OH)D$_3$. The RYBW in eggs injected with 0.20 µg of 25(OH)D$_3$ was also higher in comparison with that in the 2 control groups. The RYFWSW in the diluent-injected controls was greater compared with that in all the 25(OH)D$_3$-injected groups, whereas RYFWSW in the noninjected controls was higher only in comparison with that of the 5.40 µg of 25(OH)D$_3$-injected group. Finally, BL in the diluent-injected control and 1.80 µg of 25(OH)D$_3$-injected groups was greater in comparison with that of the 5.40-µg treatment group (Table 1).

**DISCUSSION**

The results of the preliminary trial in this study showed that the in ovo injection of 0.60 µg of 25(OH)D$_3$ into the amnion of d 18 embryos increased their subsequent circulating concentrations of 25(OH)D$_3$ by approximately 2.5-fold on 19.25 doi. This result suggests that the in ovo injection of 25(OH)D$_3$ is capable of increasing its blood concentrations in broiler embryos. Of the various forms of vitamin D$_3$, 25(OH)D$_3$ is the most stable, and the hydroxylation from 25(OH)D$_3$ to 1,25(OH)$_2$D$_3$ is highly regulated (de Matos, 2008). Therefore, the elevated concentrations of 25(OH)D$_3$ may be more readily available to the bird’s tissues during the late embryonic and posthatch developmental periods.

It is important to point out that because the in ovo injection of Marek’s disease vaccine is also commercially delivered in the same diluent used in this study, the diluent-injected control group was considered as the primary control in this study. Upon evaluation of the results of HI on 21.0 doi across trials, it is apparent that the in ovo injection of Ross × Ross 708 broiler...
Hatching eggs with 25(OH)D3 between 0.30 to 1.20 µg in 100 µL of commercial diluent may positively influence late embryogenesis. This is based on the observation that this dosage range was able to alleviate depressions or delays in HI on 21.0 doi that were caused by the in ovo injection of commercial diluent. On further evaluation of the results of HI, including that on 21.5 doi, it is further suggested that the in ovo injection of 25(OH)D3 at 0.60 µg is sufficient to improve the hatchability of broiler embryos. In support of these findings, it was reported in a previous study in which the in ovo injection of 0.50 µg of 25-hydroxycholecalciferol [25(OH)D3] was given before 100 µL of injected diluent (trial 1). Means among treatment groups within each sex with no common letter (a-c) differ \((P \leq 0.05)\). At least 1 chick of each sex in each of 10 replicate units was used to calculate each treatment mean.

Figure 2. Yolk-free BW as a percentage of set egg weight (RYFWSW) in (A) female and (B) male hatchlings in noninjected and diluent-injected (100 µL) controls and in treatments in which 0.15, 0.30, 0.60, or 1.20 µg of 25-hydroxycholecalciferol [25(OH)D3] was added to 100 µL of injected diluent (trial 1). Means among treatment groups within each sex with no common letter (a-c) differ \((P \leq 0.05)\). At least 1 chick of each sex in each of 10 replicate units was used to calculate each treatment mean.

The reason why the in ovo injection of 25(OH)D3 at dosages of 1.80 µg or higher failed to influence HI is not completely clear. Nevertheless, as 25(OH)D3 is converted to 1,25(OH)2D3 by renal cells, these metabolites, particularly the 1,25(OH)2D3, are actively involved with calcium-phosphorus balance and calcium mobilization. In light of this information, it was reported in a previous study that the in ovo injection of 1,25(OH)2D3 in the yolk sacs of 9, 12, and 15 doi embryos increased yolk sac calcium concentration (Lee et al., 1990). Klasing (1998) affirmed that when excessive circulating calcium concentrations occur in developing...
embryos toward the end of incubation, the excess calcium is transferred to the yolk sac. Hence, the in ovo injection of 25(OH)D₃ at a dosage of 1.80 µg or higher may have caused a hypercalcemic condition due to an excessive influx of calcium into the circulation and tissues of the embryos. It is worth mentioning that such an imbalance may have interfered in some way with normal embryogenesis.

The observed higher PYM in the 5.40-µg dosage group in comparison with all the other 25(OH)D₃ dosages as well as the noninjected control treatment may have also been a result of a hypercalcemic property of the yolk sac. This may have likewise led to an increased osmotic pressure in the yolk sac so as to increase the diffusion of water back into the yolk sac, causing the relative proportion of water to total lipid in its contents to increase. Conversely, the in ovo injection of 0.20 and 0.60 µg of 25(OH)D₃ stimulated an increase in the total lipid and DM contents of the yolk sac. This was apparent by the fact that the 0.20 and 0.60 µg dosages of 25(OH)D₃ decreased PYM in comparison with the 5.40 µg of 25(OH)D₃-injected group. Nevertheless, RYBW was not different in the 0.20- or 0.60-µg treatment groups compared with diluent-injected controls and the group injected with 5.40 µg of 25(OH)D₃. Because PYM was affected differently by the 0.20- and 0.60-µg dosages in comparison with the 5.40-µg dosage, whereas RYBW was similar for those dosage levels, it is

Figure 3. Hatchability as a percentage of fertilized injected eggs (HI) on (A) d 21.0 and (B) d 21.5 of incubation in noninjected and diluent-injected (100 µL) controls and in eggs injected with 0.20, 0.60, 1.80, or 5.40 µg of 25-hydroxycholecalciferol [25(OH)D₃] in 100 µL of diluent in trial 2. Means within a variable with no common letter (a–c) differ (P ≤ 0.05). Data from 10 replicate units were used for calculation of means for each variable.
suggested that the differential effects of the lower and higher levels of 25(OH)D₃ may be due to an opposing shift in the relative concentrations of the various yolk constituents, including water.

The in ovo injection of 0.15 to 5.40 µg of 25(OH)D₃ did not adversely affect embryonic survivability observed on 21.5 doi. This was evidenced by the fact that average HI on d 21.5 across all treatments was 94.0% and that there was no significant treatment effect on HI or PIM on 21.5 doi in trial 1. Also, there were no differences among the injection treatment means for HI and PIM on 21.5 doi in trial 2. Chickens have the capability of eventually compensating for excessive circulating calcium concentrations, and circulating concentrations of 1,25(OH)₂D₃ are regulated by the conversion of 25(OH)D₃ to its excretory form (24,25-dihydrocholecalciferol) in renal cells (de Matos, 2008). Within the 12 h between 21.0 and 21.5 doi, embryos apparently used these regulatory mechanism so that HI and PIM on 21.5 doi were unaffected by the 25(OH)D₃, even at dosages of 1.80 µg or higher.

The lack of interactive effects involving treatment and sex on most of the hatching chick quality variables indicate that possible differences in sex hormone levels in male and female Ross × Ross 708 broiler embryos have a limited influence on the effects of the in ovo injection of 25(OH)D₃ on the hatching chick quality variables examined. However, the contrasting effects of the 0.60-µg dosage of 25(OH)D₃ on RYFWSW in male and female hatchlings suggests that sex may influence this particular posthatch variable. Nevertheless, the absence of significant effects by the 0.30- and 1.20-µg dosages make the observed specific effect by the 0.60-µg dosage difficult to explain. Progressive increases in 25(OH)D₃ dosages up to 1.88 µg that were injected into the allantois of d 17 embryos were shown by Gonzales et al. (2003) to exhibit a negative linear relationship to hatchling BW. This result is consistent with the current finding of numerical consecutive decreases in RYFWSW in male chicks that were injected with dosages of 25(OH)D₃ between 0.15 and 0.60 µg in trial 1. However, a subsequent numerical decrease was not shown for RYFWSW in response to the 1.20-µg dosage. It was, nevertheless, observed that RYFWSW in all 25(OH)D₃-injected treatment groups were lower across sex in comparison with diluent-injected controls in trial 2, which further supports the contention that the addition of 0.20 to 5.40 µg of 25(OH)D₃ to commercial diluents may exert a negative effect on hatching BW. However, although broiler hatching BW may be compromised by various factors during incubation, optimal conditions during brooding may enable birds to compensate in growth during the posthatch period. This was demonstrated in a report by Bruzual et al. (2000), in which optimal brooding conditions ameliorated the negative influences of a suboptimal RH during incubation on posthatch performance. Furthermore, it may be suggested that the in ovo injection of 25(OH)D₃ at 0.20- to 1.80-µg dosages had no adverse effect on skeletal

### Table 1

<table>
<thead>
<tr>
<th>Treatment</th>
<th>BW (g)</th>
<th>YFBW (g)</th>
<th>RBSW (%)</th>
<th>BL (cm)</th>
<th>RBWBL (g/cm²)</th>
<th>LW (g)</th>
<th>RLBW (%)</th>
<th>RLYFW (%)</th>
<th>PYM (%)</th>
<th>YSW (g)</th>
<th>RYW (%)</th>
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<tr>
<td>Noninjected control</td>
<td>43.44</td>
<td>38.29</td>
<td>71.93</td>
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<td>1.03</td>
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<td>5.15</td>
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<td>38.56</td>
<td>71.91</td>
<td>17.84</td>
<td>2.44</td>
<td>1.01</td>
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<td>37.51</td>
<td>71.52</td>
<td>17.62</td>
<td>2.45</td>
<td>1.04</td>
<td>1.02</td>
<td>2.49</td>
<td>1.01</td>
<td>50.02</td>
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<td>71.34</td>
<td>17.62</td>
<td>2.45</td>
<td>1.01</td>
<td>1.03</td>
<td>2.43</td>
<td>1.01</td>
<td>50.75</td>
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<td>1.80 µg of 25(OH)D₃</td>
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<tr>
<td>SEM</td>
<td>0.45</td>
<td>0.39</td>
<td>0.48</td>
<td>0.51</td>
<td>0.09</td>
<td>0.03</td>
<td>0.02</td>
<td>0.04</td>
<td>0.04</td>
<td>0.84</td>
<td>0.07</td>
</tr>
<tr>
<td>P-value</td>
<td>0.94</td>
<td>0.1</td>
<td>0.82</td>
<td>0.3</td>
<td>0.04</td>
<td>0.61</td>
<td>0.82</td>
<td>0.82</td>
<td>0.82</td>
<td>0.01</td>
<td>0.01</td>
</tr>
</tbody>
</table>

*Means within a variable with no common superscript differ (P ≤ 0.05).*

**Note:** For two male and two female birds in each of 10 replicate units (240 total) were used to calculate each treatment mean.
tional development, as evidenced by the lack of differences in the BL of hatchlings from eggs injected with diluent with or without the various dosages of 25(OH)D3.

In conclusion, the in ovo injection of 0.60 µg of 25(OH)D3 in 100 µL of commercial diluent on 18 doi was capable of significantly elevating its circulating concentration in Ross × Ross 708 broilers. Furthermore, it is suggested that a 0.60-µg dosage of 25(OH)D3 may subsequently alleviate a depression or delay in HI through 21.0 doi, which may have resulted in the reduction of a stress response to the invasive process of injecting commercial diluent into broiler hatching eggs. The use of 0.60 µg of 25(OH)D3, likewise, had no detrimental effect on embryogenesis, skeletal development, embryonic survivability, or HI by 21.5 doi. Further research concerning effects of the in ovo injection of 25(OH)D3 in conjunction with Marek’s disease vaccine in 100 µL of commercial diluent on embryogenesis and hatchability in broiler hatching eggs should be considered.

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REFERENCES


