Effect of hen age and maternal vitamin D source on performance, hatchability, bone mineral density, and progeny in vitro early innate immune function

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ABSTRACT The metabolite 25-hydroxy vitamin D_3 (25-OHD) can complement or replace vitamin D_3 in poultry rations, and may influence broiler production and immune function traits. The effect of broiler breeder dietary 25-OHD on egg production, hatchability, and chick early innate immune function was studied. We hypothesized that maternal dietary 25-OHD would support normal broiler breeder production and a more mature innate immune system of young chicks. Twenty-three-week-old Ross 308 hens (n = 98) were placed in 4 floor pens and fed either 2,760 IU vitamin D_3 (D) or 69 μg 25-OHD/kg feed. Hen weights were managed according to the primary breeder management guide. At 29 to 31 wk (Early), 46 to 48 wk (Mid), and 61 to 63 wk (Late), hens were artificially inseminated and fertile eggs incubated and hatched. Chicks were placed in cages based on maternal treatment and grown to 7 d age. Innate immune function and plasma 25-OHD were assessed at 1 and 4 d post-hatch on 15 chicks/treatment. Egg production, hen BW, and chick hatch weight were not affected by diet ($P > 0.05$). Total in vitro *Escherichia coli* (*E. coli*) killing by 25-OHD chicks was greater than the D chicks at 4 d for the Early and Mid hatches, and 1 and 4 d for the Late hatch. This can be partly explained by the 25-OHD chicks from the Late hatch also having a greater *E. coli* phagocytic capability. No consistent pattern of oxidative burst response was observed. Chicks from the Mid hatch had greater percent phagocytosis, phagocytic capability, and *E. coli* killing than chicks from Early and Late hatches. Overall, maternal 25-OHD increased hatchability and in vitro chick innate immunity towards *E. coli*. Regardless of treatment, chicks from Late and Early hens had weaker early innate immune responses than chicks from Mid hens. The hen age effect tended to be the greatest factor influencing early chick innate immunity, but maternal 25-OHD also increased several measures relative to D.

Key words: broiler, broiler breeder, 25-hydroxy vitamin D_3, innate immune function, bone mineral density

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INTRODUCTION

Vitamin D is required for normal embryonic development of chickens. The vitamin D_3 level in the maternal diet is positively correlated with the vitamin D_3 and 25-hydroxy vitamin D_3 (**25-OH D_3**) contents within the egg yolk (Mattila et al., 1999). The enzyme 25-hydroxyvitamin D-1α-hydroxylase, which is responsible for the hydroxylation of 25-OH D_3 to 1,25$(OH)_2D_3$, is present as early as 12 d incubation and increases in specific activity during further embryonic development (Turner et al., 1987). The early development of vitamin D_3 metabolism within the chick signifies the importance of this nutrient to the developing embryo. However, even with diets sufficient in vitamin D (2,200 IU/kg), the addition of 1,100 IU/kg 25-OH D_3 to the diet of turkey breeders increased egg hatchability as compared to dietary vitamin D_3 alone (Manley et al., 1978). This may be related to the fact that 25-OH D_3 is more efficiently utilized by the bird than vitamin D_3 (Bar et al., 1980) which may also be the case for the chick embryo.

The innate immune system is the first line of defense of the bird. The cells of the innate immune system work to recognize, phagocytose, and (using nonspecific properties) kill the invading pathogen as well as working to signal the acquired immune response (Zekarias et al., 2002). The young, newly hatched chick has an immature immune system that could leave them more susceptible to infection and disease than more mature birds (Lowenthal et al., 1994; Wells et al., 1998).

The maternal diet could have significant effects on the immunocompetence of the chick at hatch, as it is the nutrients from the egg that will enable the chick to develop. The abundant number of studies indicating a regulatory role for vitamin D_3 and its metabolites within the immune system of various other species (Manolagas et al., 1985; Provvedini et al., 1986; Rockett et al., 1998; Binder et al., 1999; Waters et al., 2001) suggests the possibility of similar roles in chickens, although

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limited research has been done to support this. Vitamin D deficient chicks have decreased cellular immune responses, with decreased cutaneous basophil response, as well as decreased macrophage phagocytic capability (Aslam et al., 1998). In addition, maternal supplementation of 25-OH D_3 increases deposition of 25-OH D_3 into the egg (Mattila et al., 2011), and therefore may result in increased egg hatchability and improved chick quality, which could in turn result in better growth and feed efficiency of the broiler chick. Therefore the objectives of this study were to investigate the effects of maternal dietary 25-OH D_3 on broiler breeder production traits and bone mineral density (**BMD**) as well as in vitro innate immune function of the progeny. We hypothesized that maternal dietary 25-OH D_3 would support normal broiler breeder production, increase broiler breeder BMD, as well as lead to a more mature innate immune system of their progeny at hatch.

MATERIALS AND METHODS

Experimental Diets

Broiler breeder hens were fed nutritionally complete broiler breeder rations (16.2% CP, 3.1% Ca, 0.43% available phosphorus, and 2,870 kcal/kg ME) from 23 to 44 wk age. From 45 to 65 wk age hens were fed a ration containing 15.8% CP, 3.3% Ca, 0.37% available phosphorus, and 2,870 kcal/kg ME. Wheat-based basal maternal diets devoid of supplemental vitamin D were formulated for each breeder phase to meet or exceed primary broiler breeder recommendations (Aviagen, Huntsville, AL) and National Research Council (**NRC**) recommendations (National Research Council, 1994). Each basal diet was subdivided and supplemented with either 2,760 IU dietary vitamin D_3 for the D treatment (Rovimix D_3 500, DSM Nutritional Products, Parsippany, NJ) per kg feed or 69 μg dietary 25-OH D_3 per kg feed for the 25-OHD treatment (Rovimix HyD, DSM Nutritional Products, Parsippany, NJ, based on the manufacturer's recommended level of supplementation) per kg feed, which according to the manufacturer is the equivalent of 2,760 IU vitamin D_3 activity, as the sole source of vitamin D activity. Each batch of diet mixed in the study was analyzed for vitamin D and 25-OH D_3 by a commercial lab. The vitamin D_3 and 25-OH D_3 levels for the D treatment were (mean $\pm$ SEM) 4,097 $\pm$ 894 IU and 0.0 $\pm$ 0 μg/kg, and 3,030 $\pm$ 220 IU and 0.0 $\pm$ 0 μg/kg for the Phase 1 and 2 diets, respectively. The analyzed vitamin D_3 and 25-OH D_3 levels for the 25-OHD treatment were 988 $\pm$ 674 IU and 70.9 $\pm$ 8.2 μg/kg, and 40 $\pm$ 45 IU and 74.3 $\pm$ 7.4 μg/kg, for the Phase 1 and 2 diets, respectively. The relatively high levels of vitamin D_3 in the 25-OHD diets were in each case due to a single feed sample having higher than formulated values of vitamin D_3. However, the differences in the actual levels of the two forms of vitamin D between treatments were appropriate for the purposes of the study.

Experimental Design and Procedures

The experimental protocols were conducted according to the Canadian Council on Animal Care guidelines (Canadian Council on Animal Care, 2009). At 23 wk age, 98 Ross 308 broiler breeder hens were randomly allocated to 4 floor pens (24 to 25 birds/pen; 2 pens/treatment; average BW 2,273 $\pm$ 26.6 g). Birds were weighed and feed allocation adjusted on a weekly basis for the average BW of the 4 pens to maintain the breeder-recommended BW curve (Aviagen, 2002). Egg production was recorded on a daily basis for each pen. Eggs with a normal shell were deemed as settable eggs as compared to soft shell, shell-less, and double-yolk eggs. Fresh egg quality traits (egg weight, specific gravity, shell weight and thickness, yolk weight, and albumen height and weight) were assessed every 6 wk starting at 29 wk age on all eggs from 2 consecutive days of egg production per period. Egg specific gravity was measured by the floatation method (Hamilton, 1982). Individual eggs were weighed, and albumen height was measured with an albumen height gauge. The individual weights of yolk, albumen, and shell were recorded. Eggshell weight (with membranes attached) was measured after the eggshells were washed and air-dried overnight. Eggshell thickness was determined from the middle of the egg using a micrometer. Eggshell conductance was determined using the method described by O'Dea et al. (2004). Briefly, the rate of egg weight loss (presumed to be moisture loss) was determined daily on eggs (N = 15 per treatment) that were placed in desiccators and covered in desiccant for a 9 d period. Room temperature was recorded daily for the determination of the saturation vapor pressure. At 64 wk age, blood from 10 birds per pen was obtained by brachial venipuncture, plasma was separated by centrifugation at 894 $\times$ g for 15 min, and frozen at -20°C for subsequent analysis of 25-OH D_3 by HPLC as described by Aksnes (1992). A standard curve was obtained using dilutions of a 25-OH D_3 standard (Fluka Biochemika, Buchs, Switzerland).

At 65 wk age, all broiler breeders were euthanized by cervical dislocation and body composition (weights of whole breast, Pectoralis major, Pectoralis minor, fat pad, liver, and spleen) and ovarian morphology (weight of the ovary, oviduct, stroma, and number of large, small, and atretic yellow follicles) were assessed as described by Joseph et al. (2002).

Initial (23 wk) right tarsometatarsus BMD was assessed on 12 live birds (6 per treatment group) prior to receiving any dietary treatment. Quantitative computed tomography (**QCT**) was conducted using a Stratec Norland XCT (XCT Reseach SA, Norland, Fort Atkinson, WI) scanner having a 50 kV X-ray tube (Saunders-Blades et al., 2009). Bone mineral density was again assessed on 8 live birds per pen (including the 6 birds previously scanned at 23 wk) at 30 and 49 wk age and on excised bones of those same birds at 65 wk age.

From 31 to 33 wk age (Early production), 46 to 48 wk age (Mid production), and 61 to 63 wk age (Late production) hens were artificially inseminated with 50 μL pooled semen from approximately 15 Ross 344 roosters. For each age, hens were inseminated 3 times over a 2 wk period (2 consecutive days at the beginning and once 7 d later). Eggs from each of these time periods were collected 2 days after hens were first inseminated, incubated, and hatched. At each breeder age, eggs were collected for 7 d and incubated as a single group; eggs were then collected for 7 d (additional) before being incubated, thus resulting in 2 complement hatches for each broiler breeder age. Eggs were incubated (Jamesway single-stage incubator, Jamesway Incubator, Cambridge, ON, Canada) for 21.5 d (temperature = 37.5°C and relative humidity = 85%). Eggs were weighed and transferred to a hatcher (Jamesway single-stage hatcher, Jamesway Incubator, Cambridge, ON, Canada) at 18 d incubation, and placed in hatch trays divided into 8 sections, which held 18 eggs per section. At hatch, the phase of embryonic mortality (Hamburger and Hamilton, 1951), fertility, hatchability, and chick BW were assessed for each maternal dietary treatment group. Early (0 to 7 d) and late (8 to 18 d) embryonic mortality (Mid embryonic mortality was combined with late embryonic mortality due to low mortality in both phases), late hatch (chicks requiring longer than 21.5 d to hatch), percent internal pip live and dead (**IPL**, and **IPD**, respectively; those chicks that pipped through the shell membrane), and percent external pip live and dead (**EPL** and **EPD**, respectively; those chicks that pipped through the shell) were determined as a percentage of fertile eggs. Eggshell conductance was determined as previously described on a subset of eggs (15 per maternal treatment) collected the day after the final eggs were collected for hatching at each broiler breeder age. After hatch, chicks were separated based on maternal vitamin D treatment and housed in Petersime battery brooders (Petersime Incubator, Gettysburg, OH) for 1 wk posthatch and fed a standard broiler mash ration (23.25% CP, 1.1% Ca, 0.55% aP, 3,134 kcal/kg ME, and 2,500 IU/kg vitamin D_3) devoid of any antibiotic supplementation. At 1 and 4 d posthatch, blood was collected from 15 female chicks per maternal treatment per day and plasma 25-OHD was determined by HPLC as described previously.

At 1 and 4 d posthatch, male chicks (n = 15 chicks per maternal treatment) were assessed for in vitro innate immune function. Approximately 1 mL whole blood was collected into heparinized 5 mL vacutainer tubes through decapitation. Forty microliters of whole blood was removed for each of the phagocytosis and bactericidal assays. The remainder of the whole blood was used to assess oxidative burst (**OB**) response.

Phagocytosis Assay

White blood cells were assessed for capability to phagocytose fluorescently-labeled *Escherichia coli* (***E. coli***) bioparticles as outlined in Millet et al. (2007) with some modification for analysis by flow cytometry. Briefly, 40 μL whole blood was diluted 1:20 with CO_2-independent media (Gibco, Invitrogen, Burlington, ON, Canada) supplemented with 100 μg/mL penicillin, 100 μg/mL streptomycin, and 4 mM L-glutamine. Diluted blood and *E. coli* bioparticles [K-12 strain; Molecular Probes (E-2864), Invitrogen, Burlington, ON, Canada] were mixed together at a white blood cell to *E. coli* particle ratio of 1:100 and allowed to incubate for 15 min at 41°C. After submerging in an ice bath for 5 min to stop the phagocytosis reaction, samples were centrifuged (10,600 × g for 10 min), each sample was then washed twice with 300 μL CO_2-independent media supplemented with 100 μg/mL penicillin and 100 μg/mL streptomycin, and centrifuged (3,800 × g for 8 min) to remove all nonphagocytosed bacteria. Cells were lysed with 300 μL lysing buffer [0.07-M ammonium chloride, 0.01-M sodium bicarbonate, and 1 mL ethylenediaminetetraacetic acid (**EDTA**), in 500 mL ultrapure water], and centrifuged (110 × g for 5 min) to remove red blood cells from the mixture. Cells were then fixed with methanol for 5 min on ice. After centrifuging (6,800 × g for 5 min) to remove methanol, samples were then reconstituted in clear wash buffer (0.5-g bovine serum albumin, 1 mL EDTA, and 500 mL Hanks balanced salt solution), and transferred to a sterile tube and stored on ice for subsequent measurement of fluorescence by flow cytometry (Becton-Dickinson FacScan Flowcytometer, Sunnyvale, CA). Cells were separated using forward (cell size) and side (granularity) scatter characteristics. As it was difficult to distinguish between leukocyte subpopulations for this assay, the analysis included the entire white blood cell population after 10,000 cells had been acquired. The percent of cells phagocytosing was assessed as the number of cells that had taken up (or bound) at least one *E. coli* particle (i.e., exhibiting increased fluorescence). Fluorescence of nonphagocytosing cells was determined from a control sample to which no fluorescent bacteria was added. The amount of bacteria taken up by each white blood cell (phagocytic capability) was assessed using the mean fluorescence which was indicative of the amount of *E. coli* particles that were engulfed by an individual cell. An increased fluorescence indicated a greater number of bacteria being engulfed per cell.

Oxidative Burst Assay

Whole blood samples were diluted in 1% bovine serum albumin (**BSA**) (1:2 v/v) and separated over a 1.119 density gradient (Sigma-Aldrich, Oakville, ON, Canada) by centrifugation (674 × g) for 30 min. After centrifugation, the 1.119 band was collected and cells washed with 1% BSA in phosphate-buffered saline (**PBS**) solution and centrifuged again (453 × g). Cell pellets were then resuspended to 1 mL Hanks balanced salt solution supplemented with BSA and EDTA. Cell

viability was determined by trypan blue exclusion assay as described by Holloway et al. (2003). Heterophil oxidative burst was measured using a modified version of that given by He et al. (2003). Briefly, 10 μL 2′,7′ dichlorodihydrofluorescein diacetate (Sigma-Aldridch, Oakville, ON, Canada) at a concentration of 10 μL/mL was added to resuspended white blood cells and incubated at 37°C for 5 min. After 5 min, 200 μL were removed and placed in a FACScan tube, and stored on ice in the dark for subsequent analysis of cell background fluorescence by flow cytometry. Ten microliters phorbol 12-myristate-13-acetate (**PMA**; Biomol, Plymouth Meeting, PA) at a concentration 10 μg/mL were then added to each sample and 200 μL were removed at 5 min intervals for 20 min, and placed on ice for subsequent analysis of fluorescence by flow cytometry. Heterophils were determined by the separation of white blood cells based on forward (size) and side (granularity) scatter characteristics which allowed for the separation of the leukocyte subpopulations (Holloway et al., 2003). As heterophils are one of the major circulating innate immune cells in the bird (Maxwell and Robertson, 1998), these cells were gated and used for the measurement of the oxidative burst response, after 20,000 cells had been acquired. The extent of the oxidative burst response was measured as a ratio of increase in fluorescence from the nonstimulated background fluorescence of each sample.

Bactericidal Assay

Forty microliters of heparinized blood was assessed for bactericidal capability to *E. coli* as described by Millet et al. (2007). It was assumed that the reduction in live *E. coli* was due to killing by the white blood cells.

Statistical Analysis

The experimental unit for the broiler breeder data was the pen, for the hatch data was the egg, for the chick production data was the pen and for the chick immune function data was the individual chick. Breeder BW, egg production and quality, BMD and cross-sectional area, carcass characteristics and ovarian morphology, and progeny BW, growth and production efficiency, plasma 25-OH D_3, ex-vivo phagocytosis, and oxidative burst were analyzed as a 1-way analysis of variance (**ANOVA**) with maternal dietary treatment as the main factor using the Mixed Model analysis in SAS (SAS Institute, 1999). The hatch data (percent fertility, percent hatch, percent hatch of fertile eggs, percent early and late mortality, percent late hatch, percent internal and external pips (live and dead), percent dead, and percent culls) and percent *E. coli* killed data was analyzed as a chi-square analysis using SAS (SAS Institute, 1999). Repeated measures analysis of SAS (SAS Institute, 1999) was performed on the plasma 25-OH D_3 and immune assay results to determine effects of broiler breeder age. Probability of differences was considered significant at $P < 0.05$. Means were separated using LSMeans comparisons (SAS Institute, 1999).

RESULTS AND DISCUSSION

Broiler Breeder BW, Egg Production, and Quality

Dietary 25-OHD did not significantly affect breeder hen BW (data not shown). This was expected as broiler breeders were feed-restricted to a specific target BW curve recommended by the Ross 308 breeder management guide to maximize egg production (Aviagen, 2002). Throughout the entire study, the broiler breeders were 7 to 10% below the specific target BW but were fed to maintain the target BW curve and egg production.

Dietary 25-OHD did not affect total settable egg production (185 vs 186 ± 3.5 total eggs per bird from 25 to 64 wk for the D-fed and 25-OHD-fed breeders, respectively). The breeder hens that received dietary 25-OHD reached peak production faster than D hens, with a significantly greater egg production at 28 wk whereas the D hens peaked at approximately 30 wk (data not shown). Although peaking earlier, the 25-OHD birds maintained the same weekly egg production as the D birds throughout the rest of the trial (data not shown). Egg weight, egg specific gravity, percent yolk, and percent egg shell were not different between the 2 treatments at any age (Table 1), possibly due to the small replication in number of pens (n = 2 per treatment). Eggshell thickness at 29 wk age was greater for eggs from the hens on the 25 OHD treatment, but not at any other time. As this effect was not carried forward for the rest of the laying cycle, it therefore would not be expected to impact broiler breeder production traits. Previous research has also shown that at similar levels of supplementation in excess of the requirement for vitamin D, supplementation of either vitamin D_3 or 25-OH D_3 results in similar levels of egg production and shell quality traits, in broiler breeders (Atencio et al., 2005) and laying hens (Keshavarz, 2003).

One of the difficulties in interpreting research results in this field regards assumptions made about the relative biopotency of vitamin D_3 and 25-OH D_3. The manufacturer of the commercially available 25-OH D_3 indicates that 1 μg 25-OH D_3 has a potency of 40 IU (DSM Nutritional Products, Parsippany, NJ), which is consistent with the reported potency of D_3. The potency per unit weight of 25-OH D_3 is greater than D_3, particularly at lower levels of supplementation (Atencio et al., 2005). Vitamin D_3 is involved in Ca metabolism and therefore eggshell formation, but the age-related response to vitamin D source has not been previously reported. McLoughlin and Soares (1976) reported no benefit of dietary 25-OH D_3 (600 IU/kg) on eggshell

Table 1. Effect of dietary vitamin D source on broiler breeder egg quality from 29 to 64 wk age.

Treatment	N	Egg wt (g)	SG[1]	Yolk (%)[1]	Albumen (%)[1]	Albumen height[1] (mm)	Shell (%)[1]	Shell thickness[1] (mm)
					29 wk[2]			
D[3]	2	53.93	1.086	27.43	60.67	8.3	9.57	0.355[b]
25-OHD[4]	2	54.07	1.087	27.55	59.55	8.5	9.76	0.384[a]
SEM		0.26	0.001	0.29	0.47	0.07	0.16	0.001
ANOVA	DF					Probabilities		
Treatment	1	0.7315	0.4192	0.7970	0.0962	0.2083	0.5166	0.0011
					36 wk[2]			
D	2	59.87	1.081	29.58	57.17	6.67	8.74	0.339
25-OHD	2	60.70	1.083	29.76	56.81	7.29	9.01	0.388
SEM		0.69	0.001	0.27	0.36	0.16	0.09	0.028
ANOVA	DF					Probabilities		
Treatment	1	0.4854	0.2796	0.6844	0.5567	0.1082	0.1921	0.3424
					42 wk[2]			
D	2	63.03	1.080	31.00	56.17	6.67	8.61	0.311
25-OHD	2	63.07	1.081	31.14	55.33	7.11	8.79	0.326
SEM		0.70	0.001	0.21	0.31	0.12	0.10	0.004
ANOVA	DF					Probabilities		
Treatment	1	0.9625	0.3348	0.6855	0.1914	0.1283	0.3473	0.1367
					46 wk[2]			
D	2	62.96	1.080	31.67	55.56	6.37	8.66	0.332
25-OHD	2	63.53	1.080	31.51	55.13	6.88	8.76	0.336
SEM		1.05	0.001	0.07	0.48	0.15	0.11	0.004
ANOVA	DF					Probabilities		
Treatment	1	0.7372	0.7274	0.2690	0.5923	0.1396	0.5798	0.4798
					52 wk[2]			
D	2	65.85	1.08	32.22	59.13	6.01	8.84	0.331
25-OHD	2	66.46	1.08	31.92	59.23	6.65	8.70	0.319
SEM		1.10	0.001	0.26	0.51	0.13	0.21	0.005
ANOVA	DF					Probabilities		
Treatment	1	0.7322	0.7542	0.4927	0.8946	0.0696	0.6918	0.2765
					58 wk[2]			
D	2	67.42	1.079	31.99	59.58[a]	5.33[b]	8.51	0.347
25-OHD	2	69.74	1.080	32.43	58.64[b]	6.11[a]	8.65	0.362
SEM		1.08	0.0004	0.30	0.15	0.11	0.12	0.007
ANOVA	DF					Probabilities		
Treatment	1	0.2697	0.2727	0.0915	0.0494	0.0398	0.4996	0.2923
					64 wk[2]			
D	2	68.97	1.077	31.96	59.54	5.29	8.43	0.316
25-OHD	2	69.14	1.078	32.86	58.63	6.84	8.54	0.319
SEM		2.00	0.001	0.40	0.41	0.63	0.17	0.004
ANOVA	DF					Probabilities		
Treatment	1	0.9576	0.7319	0.2542	0.2595	0.2241	0.6981	0.5441

[a–b]Treatment means within a column and breeder age with different superscripts are significantly different.

[1]Specific gravity was measured by the floatation method with a series of saline solutions of increasing specific gravity ranging from 1.060 to 1.010 in increments of 0.002. Shell wt = weight of washed and air-dried egg shell (with membrane). Shell thickness was determined on the eggshell from the middle of the egg using a micrometer. Percent shell, yolk, and albumen were determined as a percentage of the total egg weight. Albumen height was measured using an albumen height gauge.

[2]Broiler breeder age (wk).

[3]Broiler breeders fed a diet containing 2,760 IU/kg vitamin D_3 as the sole supplemental source of vitamin D activity from 23 to 64 wk age.

[4]Broiler breeders fed a diet containing 69 μg/kg 25-OH vitamin D_3 as the sole supplemental source of vitamin D activity from 23 to 64 wk age.

quality of young laying hens; however, in older hens the addition of dietary 25-OH D_3 increased eggshell quality over those eggs from hens fed 600 IU/kg dietary vitamin D_3. In addition, at a broiler breeder age of 58 wk the percent albumen was greatest for the eggs from birds on the D diet. However, the albumen height was greatest for the eggs from hens on the 25-OHD treatment at 52 wk ($P = 0.07$) and 58 wk ($P = 0.04$; Table 1). A greater albumen height decreases embryo weight but increases hatchability of the developing embryo in hatching eggs (Hurnik et al., 1978; Deeming, 1989; Lapao et al., 1999), which could explain the greater percent hatch of fertile eggs for the eggs from breeders fed the 25-OHD in the current study (Table 3). As albumen height decreases with egg storage time (Lapao et al., 1999), the eggs from the hens fed the 25-OHD may therefore be able to withstand longer egg storage time with less effect on embryo viability than eggs from the hens fed D_3. In a commercial broiler breeder study, our lab found significantly lower embryonic mortality in eggs from breeder hens supplemented with liquid 25-OHD plus dietary vitamin D_3 than in hens fed solely vitamin D_3 (Saunders-Blades and Korver, 2014).

Laying hens have increased levels of plasma $1,25(OH)_2$ D_3 during shell calcification (Abe et al.,

Table 2. Effect of dietary vitamin D source on bone mineralization of broiler breeders from 21 to 65 wk age.

		Femur density (mg/cm^3)			Femur cross-sectional area (mm^2)		
	N	Total[1]	Cortical[2]	Trabecular[3]	Total	Cortical	Trabecular
				21 wk[4]			
Initial Scan[5]	12	487.68	984.36	52.13	39.82	17.84	20.28
SEM		12.37	8.52	3.69	1.64	0.58	1.29
				32 wk[4]			
D[6]	8	527.68	1,016.82	48.27	43.88	20.93	21.08
25-OHD[7]	8	527.46	1,008.94	47.16	43.63	20.89	20.95
SEM		10.50	4.21	2.08	1.08	0.31	0.93
ANOVA				Probabilities			
Treatment		0.8301	0.1956	0.7097	0.8666	0.9289	0.9215
				52 wk[4]			
D	8	515.11	984.63	86.18	44.95	20.85	22.07
25-OHD	8	514.26	982.29	75.16	43.51	20.51	21.41
SEM		11.30	7.59	4.19	1.21	0.31	1.02
ANOVA				Probabilities			
Treatment		0.9676	0.8287	0.0731	0.4045	0.4391	0.6490
				65 wk[4]			
D	8	555.45	1044.42	87.57	44.60	21.04	21.44
25-OHD	8	550.36	1035.07	88.68	44.07	20.74	20.99
SEM		13.11	11.49	6.69	1.20	0.28	1.02
ANOVA				Probabilities			
Treatment		0.7819	0.5635	0.9066	0.7519	0.4542	0.7525

[1]Total =For the entire bone.

[2]Cortical = Outer shell of the femur with a density >500 mg/cm^3.

[3]Trabecular = Measurements taken in the inner part of the bone in the trabecular space.

[4]Broiler breeder age (wk).

[5]Bone scans were conducted prior to start of experimental treatments, therefore no treatment effect.

[6]Broiler breeders fed a diet containing 2,760 IU/kg vitamin D_3 as the sole supplemental source of vitamin D activity from 23 to 64 wk age.

[7]Broiler breeders fed a diet containing 69 μg/kg 25-OH vitamin D_3 as the sole supplemental source of vitamin D activity from 23 to 64 wk age.

1979; Castillo et al., 1979). In addition, it has been speculated that 1,25$(OH)_2D_3$ is involved in the transport of Ca across the uterine membrane (Bar and Hurwitz, 1973), thereby potentially increasing the amount of Ca available for eggshell deposition. It was hypothesized that dietary 25-OH D_3 would increase eggshell quality as it would reduce the reliance on the liver hydroxylation of vitamin D_3 to 25-OH D_3 and increase the precursor pool of the active hormone. The results of the current study show that feeding 69 μg/kg 25-OH D_3 in the feed in place of the equivalent amount of vitamin D_3 supported production of eggs of the same quality.

Broiler Breeder Bone Mineral Density, Carcass Characteristics, and Ovarian Morphology

Live and excised tarsometatarsus BMD were not different between the treatment groups from 32 to 65 wk (Table 2). In the current study, it was hypothesized that the effects of 25-OHD on broiler breeder bone quality would be especially important because they are feed-restricted and do not have the option to consume additional Ca in response to anticipated need for eggshell production as has been observed in the laying hen (Roland et al., 1973). As approximately 2 g Ca goes into an egg (Roland, 1986), breeder hens would have a greater Ca reserve, due to the greater body size and lower egg production of the breeder hen in comparison with the laying hen. However, Ca demand on the broiler breeder would be much less than that of the laying hen as the egg production of the broiler breeder is only slightly more than half that of a laying hen, in addition to also having a greater Ca reserve with the greater body size.

Dietary vitamin D source did not affect broiler breeder body composition (total percent breast yield, percent pectoralis major and minor, percent fat pad, and liver and spleen weight, with averages between the treatment groups of 16.8%, 13.1%, 3.8%, 2.7%, 1.7 g, and 3.2 g, respectively; $P > 0.05$) or ovarian morphology (ovary, oviduct, and stroma weights (57.1, 63.1, and 9.9 g, respectively; $P > 0.05$), and numbers of large, small and atretic yellow follicles (4.4, 13.0, and 15.8, respectively; $P > 0.05$). As both dietary treatments provided adequate levels of vitamin D (Aviagen, 2002), no deficiency signs were expected. Providing dietary 25-OH D_3 to broilers has resulted in increased growth and breast muscle yield as compared to broilers fed similar levels of vitamin D_3 (Yarger et al., 1995). The reasons for this increased body weight and breast yield in broilers are still unknown but could be linked to the role vitamin D plays in the immune response. Less immunological stress means the bird can divert more energy and resources to growth. However, this was not the case with these broiler breeders which is likely due to

the fact that they are feed-restricted whereas broilers are full-fed.

Fertility, Hatchability, Chick BW, and Early Chick Performance

At the Early breeder age, hens that received the D diet had greater percent fertility (91.88 vs 85.62%) than the 25-OHD treatment (Table 3). However, there was no difference between the two maternal dietary treatments in percent hatch of fertile eggs (76.28 vs 76.97; Table 3) for the D and 25-OHD treatments, respectively. This was due to the reduced percent total embryonic mortality of the 25-OHD treatment among the Early hens (27.41 vs 18.43; Table 3), for the D and 25-OHD treatments, respectively. There were no treatment effect on IPL, IPD, EPL, EPD, culls, and dead chicks at the Early hatch (Table 3). Although there were no differences in egg set or transfer weights or eggshell conductance, chick BW at hatch was significantly greater for the chicks from broiler breeders fed 25-OHD at the Early age (37.54 vs 38.17; Table 4), for the D and 25-OHD treatments, respectively. At the Mid breeder age, there were no treatment effects on any of the hatch variables (fertility, hatchability, embryonic mortality, and late hatch; Table 3) or chick BW (Table 4). At the Late breeder age, there was a difference in percent hatch of total eggs and percent hatch of fertile eggs (78.38 vs 84.62 and 86.23 vs 91.07; Table 3) for the D and 25-OHD treatments, respectively. However, there were no significant differences between the treatments in embryo mortality, late hatches, internal and external pips, as well as culls and dead chicks at the Late hatch (Table 3). In addition, there were no treatment effects on set and transfer egg weight, percent egg weight loss from set to transfer, eggshell conductance, as well as chick BW at the Late hatch (Table 4).

Embryonic mortality and chick deformation increase when hatching eggs are deficient in vitamin D (Sunde et al., 1978; Stevens et al., 1984a; Elaroussi et al., 1993). In the current study, both treatments provided well above the NRC-recommended level of vitamin D activity of 300 IU/kg (National Research Council, 1994) for egg-laying hens. In contrast to the current study, maternal supplementation of 3.125 μg/kg (125 IU) 25-OH D_3 reduced late embryonic mortality as compared to 3.125 μg/kg (125 IU) vitamin D_3 (Atencio et al., 2005). However, the greater embryonic mortality can be explained by the deficient vitamin D activity (125 IU/kg) provided to the vitamin D_3-fed hens (Atencio et al., 2005). The molecule 25-OH D_3 has a greater biopotency than vitamin D_3 (Fritts and Waldroup, 2003; Atencio et al., 2005), so although providing the same amount in terms of micrograms, 25-OH D_3 would provide a greater amount of vitamin D activity, which may have been sufficient to support normal embryonic development. This is further supported by the fact that when the dose of 25-OH D_3 and vitamin D was increased to

Table 3. Effect of maternal vitamin D source on hatchability and fertility from 30 to 32, 47 to 49, and 61 to 63-week-old broiler breeders.

Treatment		Fertility (%)[1]	Hatch (% of total eggs)[1]	Hatch (% of fert. eggs)[1]	Early mortality (%)[1]	Late mortality (%)[1]	Total mortality (%)	IPL (%)[1]	IPD (%)[1]	EPL (%)[1]	EPD (%)[1]	Dead (%)[1]	Cull (%)[1]
	N					Early (31 to 33 wk)[2]							
D[3]	49	91.88[a]	70.64	76.28	4.49	22.91	27.41[a]	1.18	0.45	0.99	0	0	0.01
25-OHD[4]	47	85.62[b]	65.90	76.97	6.61	11.82	18.43[b]	0.79	0.79	0.41	0	0	0.21
Chi square	DF					Probabilities							
Treatment	1	0.0110	0.1635	0.9874	0.2698	0.1545	0.0024	0.8634	0.2188	0.4981	–	–	0.9795
						Mid (46 to 48 wk)[2]							
D	34	92.04	85.60	92.84	3.33	1.64	4.97	0	0.49	0.01	0.01	0.21	0.01
25-OHD	35	94.24	82.02	87.18	4.31	2.74	7.04	0	0.01	0.88	0.20	0.01	0.76
Chi square	DF					Probabilities							
Treatment	1	0.3020	0.3814	0.0649	0.3248	0.2275	0.1200	–	0.9803	0.9806	0.9680	0.9803	0.9806
						Late (61 to 63 wk)[2]							
D	26	91.09	78.38[b]	86.23[b]	5.05	4.31	9.35	0	0.27	0.87	0.88	0.56	0.25
25-OHD	25	92.56	84.62[a]	91.07[a]	3.47	3.24	6.72	0	0.01	1.37	0.01	0.01	0.55
Chi square	DF					Probabilities							
Treatment	1	0.3503	0.0217	0.0291	0.4200	0.2430	0.2226	–	0.9724	0.9500	0.9832	0.9724	0.5425

[a-b]Treatment means within same column with different superscripts are significantly different ($P < 0.05$).

[1]Percent fertility = percent of set eggs that were fertile; percent hatch = percent of chicks that hatched from all set eggs; percent hatch (fert. eggs) = percent of chicks that hatched from all fertile eggs; percent Early mortality = percent of embryonic mortality fertile eggs from 0 to 7 d; percent Late mortality = percent of embryonic mortality fertile eggs from 8 to 18 d; percent Late hatch = percent of chicks from fertile eggs taking longer than 21.5 days to hatch; percent IPL = percent internal pip of live fertile eggs (chick pips through only membrane and is alive); percent IPD = percent internal pip dead of fertile eggs (chick pips through only membrane and is dead); percent EPL = percent external pip of live fertile eggs (chick pips through shell and is alive); percent EPD = percent external pip dead of fertile eggs (chick pips through shell and is dead); percent dead = percent dead chicks at hatch of fertile eggs; and percent cull = percent chicks culled at hatch of fertile eggs.

[2]Broiler breeder age (wk).

[3]Broiler breeders fed a diet containing 2,760 IU/kg vitamin D_3 as the sole supplemental source of vitamin D activity from 23 to 64 wk age.

[4]Broiler breeders fed a diet containing 69 μg/kg 25-OH vitamin D_3 as the sole supplemental source of vitamin D activity from 23 to 64 wk age.

Table 4. Effect of maternal vitamin D source on set, transfer, and weight loss of hatching eggs during incubation and chick hatch BW.

Treatment	Set egg weight[1] (g)	Transfer egg weight[1] (g)	Weight loss (%)[1]	Eggshell conductance[1] (mg H_2O/d/mm Hg)	Chick BW (g)
			Early (31 to 33 wk)[2]		
D[3]	54.41	47.87	12.25	10.64	37.54[b]
25-OHD[4]	54.53	48.24	12.94	10.34	38.17[a]
SEM	0.186	0.183	0.406	0.201	0.198
ANOVA			Probabilities		
Treatment	0.6544	0.1575	0.2360	0.2873	0.0293
			Mid (46 to 48 wk)[2]		
D	63.69	56.39	11.74	14.64	44.10
25-OHD	64.39	57.00	11.61	14.63	44.21
SEM	0.283	0.296	0.184	0.449	0.266
ANOVA			Probabilities		
Treatment	0.0863	0.1520	0.6177	0.9863	0.7416
			Late (61 to 63 wk)[2]		
D	67.66	58.90	13.14	13.81	46.17
25-OHD	68.26	59.41	13.13	13.87	46.05
SEM	0.348	0.328	0.19	0.717	0.335
ANOVA			Probabilities		
Treatment	0.2385	0.2796	0.9598	0.8896	0.7955

[a–b]Treatment means within same column with different superscripts are significantly different ($P < 0.05$).

Set egg weight = weight of egg when first put in incubator; transfer egg weight = weight of egg after 18 d incubation; percent weight loss = percent of weight loss of the egg from 0 to 18 d incubation; and eggshell conductance = rate of water loss from egg when stored for 7 d covered with desiccant.

[2]Broiler breeder age (wk).

[3]Broiler breeders fed a diet containing 2,760 IU/kg vitamin D_3 as the sole supplemental source of vitamin D activity from 23 to 64 wk age.

[4]Broiler breeders fed a diet containing 69 μg/kg 25-OH vitamin D_3 as the sole supplemental source of vitamin D activity from 23 to 64 wk age.

12.5 μg/kg (500 IU/kg) there was no difference in embryonic mortality between the forms of dietary vitamin D supplementation (Atencio et al., 2005). The addition of 1,100 IU/kg 25-OH D_3 to a diet already containing 2,200 IU/kg vitamin D_3 increased hatchability from 19.7 to 37.5% relative to turkey hens not supplemented with additional 25-OH D_3 (Manley et al., 1978). That study did not report at what stage of embryo development the losses occurred or a reason for the already low hatchability. In a previous study in our lab, water supplementation with 34.5 μg 25-OH D_3 per liter of broiler breeders fed a diet containing 3,000 IU/kg D_3 reduced early embryonic mortality to 4.37 from 6.22% compared to hens not provided with supplemental 25-OH D_3 (Saunders-Blades and Korver, 2014). Thus, there appears to be some protective effect of 25-OH D_3 during early embryonic development.

Breeder and Chick Plasma 25-OH D_3 and Early Chick Growth

Breeder plasma 25-OH D_3 levels at 64 wk age was significantly greater for the 25-OHD hens (60.54 ± 5.72 ng/mL) than the D hens (38.44 ± 3.60 ng/mL; $P = 0.0030$; SE = 5.03). Previous research has shown adding dietary 25-OH D_3 on top of 1,100 IU/kg D_3 was also effective at increasing plasma 25-OH D_3 in broilers (Mitchell et al., 1997). Vitamin D_3 level in the maternal diet is positively correlated with the vitamin D_3 and 25-OH D_3 content within the egg yolk (Mattila et al., 1999). Therefore, with the increased amount of plasma 25-OH D_3 of the broiler breeders on the 25-OHD treatment, it is probable that more 25-OH D_3 would get passed into the egg yolk.

There was a significant interaction effect of breeder hen age and dietary treatment on progeny plasma 25-OH D_3 levels at 1 d posthatch ($P = 0.0018$; Table 5). Chicks from Mid hens had a significantly greater plasma 25-OH D_3 than those from the Early hatch for both maternal treatments. For the D treatment the chicks from the Mid hens also had greater plasma 25-OH D_3 than chicks from the Late hens, while there was no difference for the 25-OHD treatment. At 4 d posthatch, plasma 25-OH D_3 levels were greater in chicks from the D maternal treatment for the Early and Mid hens ($P = 0.01$ and 0.03, respectively; Table 5). Chicks from the Late hatch had the greatest plasma 25-OH D_3 level at 4 d posthatch compared to the other breeder ages ($P < 0.0001$).

These results indicate that the maternal effect on broiler plasma 25-OH D_3 is transient. The reason that plasma 25-OH D_3 would be greater for the D than the 25-OHD maternal treatment, in 4 day-old chicks from Early and Mid hens, is not known. Chick plasma 25-OH D_3 levels decreased from 1 to 4 d age for the broilers from Early and Mid, but not Late hens. Decreases in plasma 25-OH D_3 levels have previously been observed to occur in the chick after 1 d age when chicks received only dietary vitamin D_3 for the first 10 d posthatch (Saunders-Blades and Korver, 2014). The yolk sac is intensively absorbed during the first 5 d posthatch

Table 5. Effect of maternal vitamin D source on broiler plasma 25-OH-D_3 at 1 and 4 d posthatch from broiler breeders at 31 to 33, 46 to 48, and 61 to 63 wk of age.

Maternal treatment		Plasma 25-OH-D_3 (ng/mL) 1 d	4 d
		Early (31 to 33 wk)[1]	
	D[2]	15.95 (21)[4]	12.91[a] (27)
	25-OHD[3]	16.10 (18)	10.38[b] (28)
SEM		0.86	0.67
ANOVA[5]		Probabilities	
Treatment		0.9011	0.0100
		Mid (46 to 48 wk)[1]	
	D	38.74 (20)	12.36[a] (25)
	25-OHD	27.33 (13)	7.98[b] (27)
SEM		4.31	1.41
ANOVA		Probabilities	
Treatment		0.0564	0.0272
		Late (61 to 63 wk)[1]	
	D	25.94[b] (26)	55.38 (28)
	25-OHD	36.40[a] (27)	50.72 (29)
SEM		3.40	4.00
ANOVA		Probabilities	
Treatment		0.0345	0.7129

[a–b]Means within the same column and maternal age with different superscripts are significantly different ($P < 0.05$).

[1]Broiler breeder age (wk).

[2]Chicks from broiler breeders fed a diet containing 2,760 IU/kg vitamin D_3 as the sole supplemental source of vitamin D activity from 23 to 64 wk age.

[3]Chicks from broiler breeders fed a diet containing 69 μg/kg 25-OH vitamin D_3 as the sole supplemental source of vitamin D activity from 23 to 64 wk age.

[4]Treatment mean followed by N in parentheses.

[5]ANOVA for repeated measures with breeder hen age; for 1 d: age P-value (SE) = 0.0018(2.59); age×treatment $P = 0.0018$ (4.11). For 4 d: age $P = <0.0001$(3.99); age×treatment $P = 0.9322$ (5.73).

(Jamroz et al., 2004) suggesting that stored 25-OH D_3 (either tissue or yolk sac) may be readily available to the fast-growing chick and may indicate a delay in the maturation of the mechanisms for liver 25-OH D_3 production from dietary vitamin D_3. Chicks from older hens have larger yolk sacs and more yolk fat (where the vitamin D and metabolites would be found) at hatch (Yadgary et al., 2010), so this may represent a greater reserve of 25-OH D_3 at hatch. Kidney 1α-hydroxylase activity peaked at 8 to 12 d posthatch in the progeny of poults from turkey hens consuming 2,700 IU/kg vitamin D_3 (Stevens et al., 1984b). This shows that chicks from hens consuming adequate vitamin D_3 may become deficient in vitamin D_3 8 d after hatch, perhaps after yolk stores of 25-OHD have been used up. The reason the opposite effect was found in the Late chicks is unknown. The yolk sac storage of 25-OH D_3 may be greater in these chicks as they are larger chicks (Table 4) and therefore the decrease in plasma 25-OH D_3 is delayed until after the yolk sac is absorbed.

There were no significant effects of maternal dietary vitamin D_3 source on chick growth (7 d average BW of 115.4 ± 2.77, 142.5 ± 4.63, and 184.3 ± 3.33 g for the Early, Mid, and Late hatches, respectively; $P > 0.05$) and feed efficiency to 7 d (0 to 7 d average feed efficiency of 1.17 ± 0.05, 1.18 ± 0.02, and 0.85 ± 0.05 for the Early, Mid and Late hatches, respectively; $P > 0.05$) for any of the maternal broiler breeder ages. This is in agreement with other work in our lab (Saunders-Blades and Korver, 2014) that found no difference in 7 d BW, gain, and feed efficiency of chicks from broiler breeders fed 34.5 μg 25-OH D_3 per liter water given in addition to 3,000 IU/kg dietary vitamin D_3. Previous studies have shown direct feeding of 25-OH D_3 to increase BW (Yarger et al., 1995; Mitchell et al., 1997; Aburto et al., 1998), improve feed efficiency, and to increase breast muscle yield in broilers (Yarger et al., 1995) in comparison with vitamin D_3. Although dietary 25-OH D_3 has a positive effect on plasma 25-OH D_3 of the bird to which it is being fed (Yarger et al., 1995), the effect of maternal 25-OH D_3 supplementation on subsequent broiler performance has not been reported. It appears that 25-OH D_3 is most beneficial to performance of the broiler chick when fed directly to them.

Early Chick Innate Immune Function

There was no effect of maternal vitamin D source on the percentage of phagocytosing leukocytes (percent phagocytosis) as well as no difference in the number of *E. coli* engulfed per cell (phagocytic capability) at either 1 or 4 d posthatch, for the Early or Mid breeder ages (Table 6). However, the maternal breeder age had a significant effect on phagocytic function of the progeny. The percent phagocytosing cells was greatest for chicks from the Mid hatch at both 1 d ($P < 0.0001$) and 4 d ($P = 0.0003$) posthatch as compared the Early and Late hatches (Table 6). In addition, at 1 d posthatch, percent phagocytosis was lowest in chicks from the Early hens, while at 4 d posthatch there was no difference between the Early and Late hatches.

At 1 d posthatch, there was an interaction effect of hen age and dietary treatment on phagocytic capacity as cells from chicks hatched from the Late age had a lower phagocytic capability than those from the Mid hatch; however, phagocytic capability from chicks on the D treatment was lower than the 25-OHD chicks at this age ($P = 0.0152$; Table 6). At 4 d posthatch phagocytic capability was greatest for the chicks from the Mid hatch and lowest for the chicks from the Early hatch ($P = <0.0001$; Table 6).

Previous studies have shown that when chicks were deficient in vitamin D, in vitro phagocytic potential of macrophages to sheep red blood cells was reduced (Aslam et al., 1998). Vitamin D-deficient mice have reduced macrophage phagocytic capability to yeast (Bar-Shavit et al., 1981). Although reporting a 10% difference between vitamin D and 25-OH D_3 treatments, Chou et al. (2009) found no significant difference in percent phagocytosis when feeding vitamin D and 25-OH D_3 levels similar to those used in the current study. The results of the current study indicate that maternal dietary 25-OH D_3 supports chick phagocytic immune function at least to the same extent, and in some cases to a greater extent than vitamin D when fed at levels

Table 6. Effect of maternal dietary vitamin D source on in vitro innate immune cell phagocytosis of *E. coli* at 1 and 4 d posthatch from broiler breeders at 31 to 33, 46 to 48, and 61 to 63 wk age.

Maternal treatment		Cells phagocytosing (%)[1]		Phagocytic capability (mean fluorescence)[2]	
		1 d	4 d	1 d	4 d
			Early (31 to 33 wk)[3]		
	D[4]	35.89 (19)[6]	49.92 (22)	1,380.65 (19)	1,365.15 (22)
	25-OHD[5]	32.59 (19)	44.43 (21)	1,441.38 (19)	1,302.48 (21)
SEM		1.89	2.25	40.95	28.08
ANOVA[7]			Probabilities		
Maternal treatment		0.0839	0.0898	0.2952	0.1181
			Mid (46 to 48 wk)[3]		
	D	49.53 (25)	53.50 (24)	2,837.36 (25)	2,617.56 (24)
	25-OHD	49.46 (25)	51.05 (27)	2,717.59 (25)	2,711.23 (27)
SEM		1.37	1.53	76.13	80.64
ANOVA			Probabilities		
Maternal treatment		0.9687	0.2484	0.2765	0.4021
			Late (61 to 63 wk)[3]		
	D	43.87 (26)	47.11 (27)	2,011.34[b] (26)	1,995.77 (27)
	25-OHD	47.42 (26)	46.23 (28)	2,223.38[a] (26)	1,964.29 (28)
SEM		1.53	1.44	60.76	56.32
ANOVA			Probabilities		
Maternal treatment		0.1057	0.6625	0.0171	0.6916

[a–b]Means within the same column and maternal age with different superscripts are significantly different ($P < 0.05$).

[1]Percent of cells phagocytosing = Number of cells that had taken up (or bound) at least one *E. coli* particle (i.e., exhibiting increased fluorescence).

[2]Phagocytic capability = Mean fluorescence which was indicative of the amount of *E. coli* particles that were engulfed by an individual cell.

[3]Broiler breeder age (wk).

[4]Chicks from broiler breeders fed a diet containing 2,760 IU/kg vitamin D_3 as the sole supplemental source of vitamin D activity from 23 to 64 wk age.

[5]Chicks from broiler breeders fed a diet containing 69 μg/kg 25-OH vitamin D_3 as the sole supplemental source of vitamin D activity from 23 to 64 wk age.

[6]Treatment mean followed n in parentheses.

[7]Percent phagocytosis ANOVA with repeated measures for breeder hen age; for 1 d: age *P*-value (SEM) = <0.0001(1.12); age×treatment P = 0.0705(1.59). For 4 d: age P = 0.0003(1.30); age×treatment P = 0.3558 (1.84). Phagocytic capability ANOVA with repeated measures for breeder hen age; for 1 d: age *P*-value (SEM) = <0.0001(52.90); age×treatment P = 0.0152(75.96). For 4 d: age P = <0.0001(40.97); age×treatment P = 0.1269(59.66).

well above NRC (1994) recommendations. Vitamin D and its metabolites play a role in phagocytosis in the chick, but the mechanisms of the effect are still not clear. In addition, maternal age seems to play a role in the effect of vitamin D on early phagocytic capability of chicks.

At the Early hatch, heterophil OB response at 1 d posthatch was similar between treatments from 5 to 15 min after stimulation with PMA (Figure 1A); however, at 20 min poststimulation, cells from the D chicks still exhibited an increased OB response while there was no significant increase for the 25-OHD chicks (Figure 1A). At 4 d posthatch there were no treatment effects on the heterophil OB response (Figure 1A). For the Mid hatch chicks, the OB of the maternal 25-OHD chicks at 1 d posthatch was significantly greater from 10 to 20 min poststimulation (Figure 1B). At this same breeder age, the chicks at 4 d posthatch exhibited no treatment effect on OB response until 20 min poststimulation at which time the D chicks had a significantly greater OB (Figure 1B). For the Late hatch there were no significant differences between treatments in the OB response at either 1 or 4 d posthatch (Figure 1C).

The most significant effect of maternal 25-OH D_3 on OB response of cells was observed in cells from 1-day-old chicks from the Mid breeder age, in which the cells of the 25-OH D_3 chicks had a greater response just 10 min after stimulation (Figure 1B). The extent to which this difference would be to the bird's ability to combat the invading pathogen is unknown. However, the phagocytosis and *E. coli* killing results indicate no effect of maternal treatment at 1 d posthatch for chicks from the Mid breeder age. To the authors' knowledge, no previous studies have investigated the effect of vitamin D source on heterophil OB response. Oxidative burst is one method chicken heterophils use to kill pathogens (Kogut et al., 2001; Swaggerty et al., 2003). Genetic strain has been shown to affect strength of the OB response (Swaggerty et al., 2003); however, there are currently no reports on the effects of vitamin D on this response. However, Fritts et al., (2004) reported that nitric oxide production of macrophages from 3 wk broilers was affected by neither level of dietary vitamin D_3 nor 25-OH D_3 when fed at levels ranging from 125 IU to 4,000 IU/kg (3.125 to 100 μg/kg feed for 25-OH D_3).

Leukocytes from chicks in the maternal 25-OHD treatment consistently killed more *E. coli* than those from the D group at all broiler breeder and chick ages, except at 1 d posthatch of the Mid broiler breeder age (Table 7). There is no killing data presented for 1 d posthatch at the Early broiler breeder age as the bacteria was not available. Broiler breeder age did not

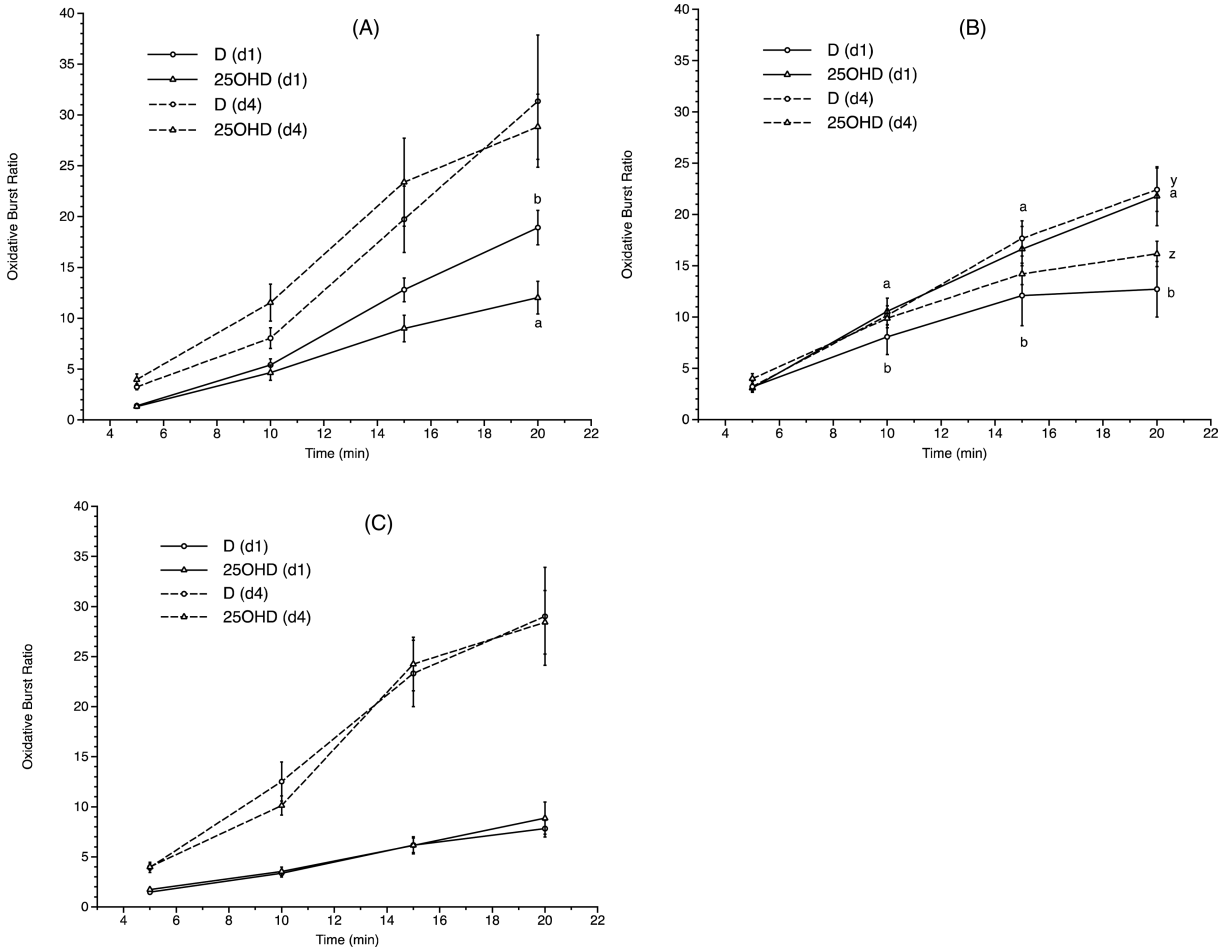

Figure 1. Effect of maternal vitamin D source on oxidative burst response of broiler chicks at 1 and 4 d posthatch. A) Chicks hatched from broiler breeders at 31 to 33 wk. B) Chicks hatched from broiler breeders at 45 to 47 wk. C) Chicks hatched from broiler breeders at 61 to 63 wk. The D treatment was the chicks from broiler breeders fed a diet containing 2,760 IU/kg vitamin D_3 as the sole supplemental source of vitamin D activity from 23 to 64 wk age. The 25-OHD treatment was the chicks from broiler breeders fed a diet containing 69 μg/kg 25-OH vitamin D_3 as the sole supplemental source of vitamin D activity from 23 to 64 wk age. Peripheral blood from males chicks were assessed for the strength of heterophil oxidative burst response over 20 min, measured as the ratio of increase in fluorescence from nonstimulated heterophils from the same chick. Small letters (a, b) indicate a significant treatment effect ($P < 0.05$) at 1 d posthatch. Small letters (y, z) indicate a significant treatment effect ($P < 0.05$) at 4 d posthatch.

have an effect on *E. coli* killing at 1 d posthatch ($P = 0.5965$; Table 7). At 4 d posthatch, chicks from the Early breeder age had the greatest percentage of killed bacteria while the chicks from the Late hatch had the lowest ($P < 0.0001$; Table 7).

Dietary 25-OH D_3 is transported into the fertile egg (Sunde et al., 1978), potentially increasing embryo 25-OH D_3 status and in turn influencing vitamin D-dependent functions. In the current study, maternal dietary 25-OH D_3 increased broiler chick bactericidal capability (Table 7). In the chicken, increasing dietary vitamin D level (from 200 to 3,500 IU/kg) increased weights of the bursa of Fabricius, thymus, and spleen (Khan et al., 2010), but had no effect on antibody titres to Newcastle disease virus. Supplementing a vitamin D-adequate turkey diet with additional vitamin D_3 in the drinking water increased resistance to repeated bacterial infections (Huff et al., 2000). Together, these studies suggest vitamin D_3 levels and metabolites play a role in chicken immune function.

Although limited research has been done in poultry, human research has shown that $1,25(OH)_2D_3$ stimulates antimicrobial activity in neutrophils and macropahges (Wang et al., 2004; Gombart et al., 2005). A vitamin D deficiency in the chicken reduces both cellular and innate immune responses (Aslam et al., 1998). However, when fed adequate levels of the vitamin, no difference between vitamin D or 25-OHD fed chicks on macrophage nitric oxide production and cytotoxicity was found (Fritts et al., 2004). Although the current study did not investigate molecular mechanisms of the enhanced bactericidal capability observed in the 25-OH D_3 chicks, this could explain the difference in bacteria killing capability while few differences in phagocytosis were observed between the maternal treatments. If the ex vivo results hold true in vivo, than this may result in less resources used by the bird to fight an infection and therefore a reduction in the metabolic consequences of the inflammatory response.

Table 7. Effect of maternal dietary vitamin D source on innate immune cell killing of *E. coli* at 1 and 4 d posthatch from broiler breeders at 31 to 33, 46 to 48, and 61 to 63 wk age.

Maternal treatment			*E. coli* killed (%) 1 d	4 d
			Early (31 to 33 wk)[1]	
		N		
	D[2]	15	–[4]	80.76[b]
	25-OHD[3]	15	–	88.28[a]
Chi square			Probabilities	
Treatment			–	<0.0001
			Mid (46 to 48 wk)[1]	
		N		
	D	30	54.68	72.12[b]
	25-OHD	30	52.12	74.34[a]
Chi square			Probabilities	
Treatment			0.6679	0.0352
			Late (61 to 63 wk)[1]	
		N		
	D	30	51.04[b]	51.34[b]
	25-OHD	30	54.40[a]	57.30[a]
Chi square			Probabilities	
Treatment			<0.0001	<0.0001

[a–b]Means within the same column and maternal age with different superscripts are significantly different ($P < 0.05$).

[1]Broiler breeder age (wk); ANOVA with repeated measures for breeder hen age; for 1 d: age *P*-value (SE) = 0.5965(2.40); age×treatment P = 0.3714 (3.58). For 4 d: age P = <0.0001 (2.71); age×treatment P = 0.7111 (3.95).

[2]Chicks from broiler breeders fed a diet containing 2,760 IU/kg vitamin D_3 as the sole supplemental source of vitamin D activity from 23 to 64 wk age.

[3]Chicks from broiler breeders fed a diet containing 69 μg/kg 25-OH vitamin D_3 as the sole supplemental source of vitamin D activity from 23 to 64 wk age.

[4]Data not available for 1 d posthatch at broiler breeder age of 31 to 33 wk as bacteria was not available at the time of sample collection.

Supplementation of breeder hens with 25-OH D_3 did not affect egg production, which may not be surprising given that the differences in potency between D_3 and 25-OH D_3 are most apparent at low levels of supplementation. Maternal dietary 25-OH D_3 increased bactericidal activity of leukocytes from the 25-OHD chicks at all broiler breeder and chick ages studied, as well as increased phagocytic and oxidative burst response at some ages measured. Therefore, the immune system in these young chicks is potentially more mature and better equipped to handle an infectious challenge when placed in the broiler barn. In addition, 25-OH D_3 appears to have some protective effect on early embryonic survival, resulting in a greater percent hatch, which has not been previously reported. The lack of effect on early broiler growth after hatch indicates that supplementation of the broiler diet may be necessary to see the effects of 25-OH D_3 on broiler production traits.

Broiler breeder age was also found to have a significant effect on how vitamin D metabolites affect circulating levels of 25-OH D_3 and innate immune function of the chick. While 25-OH D_3 was greatest in chicks from the Late age hens, it was the chicks from the Mid age hens that had the greater innate immune responses with the greatest percent phagocytosis, phagocytic capability, and greatest bactericidal responses. To our knowledge this is the first time this is being reported but seems to be consistent with what is seen in the field, where it is the chicks from the Early and Late age breeders that tend to not be as robust and may be more susceptible to infection and disease, which in turn could affect overall flock performance. It has been reported that first-week mortality is lowest in flocks from broiler breeders of 38 to 44 wk age, where mortality is greatest in the chicks from young hens (25 wk) and also greater in chicks from old breeder hens (60 wk; Yassin et al., 2009). This opens up an area of opportunity to look further into the differences in chicks from broiler breeders of differing ages in order to improve the ability of chicks from the Early and Late broiler breeders, and be able to grow to their potential.

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