Efficacy of 25-OH Vitamin D_3 prophylactic administration for reducing lameness in broilers grown on wire flooring

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ABSTRACT Bacterial chondronecrosis with osteomyelitis (BCO) is the most common cause of lameness in commercial broilers. Growing broilers on wire flooring provides an excellent experimental model for reproducibly triggering significant levels of lameness attributable to BCO. In the present study we evaluated the efficacy of adding HyD (25-OH vitamin D_3) to the drinking water as a preventative/prophylactic treatment for lameness. Broiler chicks were reared on 5 x 10 ft flat wire floor panels within 6 environmental chambers. Three chambers were supplied with tap water (Control group) and the remaining chambers were supplied with HyD (HyD group: 0.06 mL HyD solution/L water; dosing based on the HyD Solution label to provide 33.9 μg 25-OHD_3/L) from d 1 through 56. Feed was provided ad libitum and was formulated to meet or exceed minimum standards for all ingredients, including 5,500 IU vitamin D_3/kg. Lameness initially was detected on d 28, and the cumulative incidence of lameness on d 56 was higher in the Control group than in the HyD group (34.7 vs. 22.7%, respectively; $P = 0.03$; Z-test of proportions; chambers pooled). The most prevalent diagnoses for lame birds were osteochondrosis and osteomyelitis (BCO) of the proximal femora (52%) and tibiae (79%), accompanied by minor incidences of tibial dyschondroplasia (0.33%), spondylolisthesis, or kinky back (0.67%), and twisted legs (1%). Broilers that survived to d 56 without developing lameness did not differ in BW when compared by group within a gender. The wire flooring model imposes a rigorous, sustained challenge that undoubtedly is much more severe than typically would be experienced by broilers under normal commercial conditions. Therefore the encouraging response to HyD supplementation in the present study supports the potential for 25-OH vitamin D_3 to attenuate outbreaks of lameness caused by BCO in commercial broiler flocks.

Key words: lameness, vitamin D, osteomyelitis

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INTRODUCTION

Bacteria entering the blood via translocation across the intestinal or respiratory epithelial barriers can trigger bacterial chondronecrosis with osteomyelitis (**BCO**) by infecting osteochondrotic microfractures in the epiphyseal-physeal growth plates in the femora, tibiae, and flexible thoracic vertebrae of rapidly growing broilers. BCO is an important cause of lameness that has been estimated to affect approximately 1.5% all broilers grown past 42 d age in the United States (Carnaghan, 1966; Wise, 1971; McCaskey et al., 1982; Emslie and Nade, 1983; Emslie et al., 1983; Riddell et al., 1983; Duff, 1989b,a; Alderson et al., 1986; Alderson and Nade, 1987; Duff and Randall, 1987; Thorp et al., 1993; McNamee et al., 1998, 1999; McNamee and Smyth, 2000; Bradshaw et al., 2002; Dinev, 2009; Wideman and Prisby, 2013). Rearing broilers on wire flooring reliably triggers high incidences of BCO in research flocks without purposefully exposing the birds to known pathogens. The footing instability created by wire flooring appears to amplify the mechanical stresses exerted on the growth plates and accelerates the formation of osteochondrotic microfractures and clefts in the proximal epiphyseal–physeal cartilage (Wideman et al., 2012, 2013, 2014, 2015; Gilley et al., 2014). Wire flooring per se, or the lack of access to litter, also triggers physiological stress leading to elevated blood corticosterone levels and immunosuppression (El-Lethey et al., 2003; Nagarajan et al., 2011; Wideman and Pevzner, 2012; Wideman and Prisby, 2013). Stress and immunosuppression have been implicated in the etiology of spontaneous BCO outbreaks (Mutalib et al., 1983; Andreasen et al., 1993; Butterworth, 1999; McNamee et al., 1998, 1999; McNamee and Smyth, 2000; Wideman and Prisby, 2013), perhaps by increasing the permeability of the intestinal epithelial barrier and facilitating bacterial translocation and bacteraemia (Quinteiro-Filho et al., 2010, 2012b,a; Sohail et al., 2010, 2012; Murugesan et al., 2014; Song et al., 2014).

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Turkey osteomyelitis complex (**TOC**) shares many pathogenic similarities with BCO. Turkeys with TOC succumb to lameness attributable to bacterial arthritis and osteomyelitis of the proximal tibiae caused by opportunistic microorganisms such as *Staphylococcus aureus* and *Escherichia coli*. Stress and immunosuppression clearly contribute to the pathogenesis of TOC. In an experimental setting, all TOC lesions are readily triggered by injecting young turkey poults with repeated immunosuppressive doses of the synthetic glucocorticoid dexamethasone, and by inoculating *E. coli* into their air sacs (Wyers et al., 1991; Huff et al., 1998, 1999, 2000, 2006). Dexamethasone injections also cause lameness and pathognomonic BCO lesions of the proximal tibiae in broilers (Wideman and Pevzner, 2012). Huff et al. (2000) used the dexamethasone injection TOC model to evaluate the potential protective efficacy of vitamin D_3, based on evidence in mammals that supplemental vitamin D_3 can ameliorate the immunosuppression and heightened disease susceptibility caused by stress. Supplementing the poults' drinking water with vitamin D_3, in addition to providing typical levels of vitamin D_3 in the feed, reduced the incidence of TOC, minimized immunosuppression, and diminished bacterial colonization of air sacs caused by repeated injections of dexamethasone (Huff et al., 2000). Accordingly, because TOC and BCO share etiological similarities, the present study was conducted to determine if prophylactic administration of HyD via the drinking water might reduce the incidence of BCO in broilers reared on wire flooring. The commercially available product HyD (DSM Nutritional Products LLC, Parsippany NJ) is an additive that contains 25-hydroxy vitamin D_3 (**25-OHD$_3$**) which is an intermediate metabolite of vitamin D_3 and is considered to be the body's storage form for the vitamin. In poultry 25-OH D_3 is better absorbed than vitamin D_3, especially during periods of malabsorption, and providing vitamin D in this form eliminates the need for enzymatic conversion of vitamin D_3 to 25-OHD$_3$ by the liver (Bar et al., 1980; Soares et al., 1995; Yarger et al., 1995; Rebel and Weber, 2009).

MATERIALS AND METHODS

Animal procedures were approved by the University of Arkansas Institutional Animal Care and Use Committee (Protocol #11002). Six environmental chambers (dimensions: 3.7-m long × 2.5-m wide × 2.5-m high) within the Poultry Environmental Research Lab at the University of Arkansas Poultry Research Farm were used to conduct the study. Each chamber contained a rectangular pen with dimensions of 3 × 1.5 m with flooring consisting of flat wire panels. The wire panels were constructed from 5 × 5 cm lumber and were 3-m long and 1.5-m wide, with 5 × 5 cm cross members added for support. Hardware cloth (1.3 × 2.54 cm mesh = 0.5 × 1 in., 0.063 gauge, galvanized welded wire cloth; Direct Metals, Kennesaw, GA) was fastened to the top of the frame and cross-members. The panels were elevated on 30-cm high masonry blocks to permit manure to pass through and accumulate underneath the wire surface. Tube feeders were positioned at the front and nipple waterers were positioned at the rear of the pen, thereby forcing birds to traverse the length of the floor to eat and drink. Straight run chicks from a commercial broiler line were wing banded and placed initially at 60/chamber at 1 d age. All chicks received standard hatchery vaccinations for Marek's and Newcastle disease. At 14 d age the population in all pens was reduced to 50 of the largest clinically healthy chicks per pen, yielding a density of 1 ft^2/chick. The early culling protocol was instituted because necropsies of runts and culls during the first 2 wk often reveal macroscopic evidence of systemic bacterial infection including osteomyelitis and BCO lesions (Wideman et al., 2012). The chambers utilize single-pass ventilation at a constant rate of 6 m^3/min. The photoperiod was set for 23L:1D throughout the study. Thermoneutral temperatures were maintained throughout: 32°C for d 1 to 3, 31°C for d 4 to 6, 29°C for d 7 to 10, 26°C for d 11 to 14, and 24°C thereafter. Chicks reared on wire flooring are exposed to circulating air on their ventral surfaces and therefore require somewhat elevated brooding temperatures. The nipple waterers in Chambers 1, 3, and 5 were supplied with Fayetteville, AR municipal water [tap water (**TW**) chambers] throughout the study. The nipple waterers in Chambers 2, 4, and 6 (HyD; termed HD chambers) were supplied from 20-L water carboys elevated to a height of 2 m to create a hydrostatic pressure head sufficient to operate the pressure regulators for the nipple water lines. The carboys contained HyD mixed at 0.6 mL concentrate per 10 L TW beginning on d 1 and continuing through the end of the trial. This dosing is based on the HyD solution label to provide 33.9 μg 25-OHD$_3$/L. The HyD and Fayetteville municipal water were mixed in the carboys and no more than a 2-d supply was in a carboy at any one time. Commercial broiler feed formulated to meet or exceed minimum NRC (1994) standards for all ingredients including 5,500 IU vitamin D_3/kg was provided as starter crumbles on d 1 through 35, and as pelleted finisher thereafter. Feed and water were provided ad libitum.

Beginning on d 15 all birds were observed daily to detect the onset of lameness. Lameness began on d 28 and progressed rapidly in birds that previously appeared to be healthy. Affected broilers had difficulty standing, exhibited an obvious limping gait while dipping one or both wing tips and, if not removed, became completely immobilized within 48 h. Birds were humanely euthanized as soon as the onset of lameness was noticed, and were necropsied within 20 min postmortem. Lame birds with BCO can die quickly because they have difficulty accessing food and water, and they can be trampled by their flock mates. Therefore birds found dead also were necropsied to ascertain the cause of death and assess leg lesions. The survivors on d 56 were

considered to be clinically healthy. Ten male and 10 female survivors from each chamber were weighed, euthanized via CO_2 gas inhalation, and necropsied to assess subclinical lesion incidences in the proximal heads of the femora and tibiae. All birds that died or developed clinical lameness were recorded by date and pen number, and then they were necropsied and assigned to one of the following categories: Cull (runts and moribund individuals that failed to thrive); unknown (**UNK**) cause of death; sudden death syndrome (flipover, heart attacks); pulmonary hypertension syndrome (ascites); kinky back (**KB**), spondylolisthesis, or vertebral BCO: diagnosed based on the characteristic posterior paraparesis and hock-resting posture, and the absence of macroscopic severe BCO lesions of the femora and tibiae; Martin et al., 2011); twisted leg or slipped tendon (**TST**), perosis, chondrodystrophy); normal femur (no macroscopic abnormalities of the proximal femur); proximal femoral head separation (**FHS**) or epiphyseolysis; proximal femoral head transitional (**FHT**) degeneration; proximal femoral head necrosis (**FHN**); normal tibia (no macroscopic abnormalities of the proximal tibia); tibial head necrosis (**THN**), a subcategory of BCO in the tibiotarsus); tibial dyschondroplasia (**TD**); and lame–UNK (lameness for unknown/undetermined reasons). Previously published photographs illustrate typical BCO lesions of the proximal femora and tibiae (Wideman et al., 2012, 2014; Wideman and Prisby, 2013). Proximal femora and tibiae that appeared to be normal macroscopically were not routinely evaluated microscopically.

Statistical Analysis

The total incidence of femoral BCO lesions was calculated as: all femur = FHS + FHT + FHN. The total incidence of lameness was calculated as: total lame = KB + TST + TD + lame UNK + all femur + THN. For comparisons of lameness incidences the individual bird was used as the study unit, and the SigmaStat Z-test procedure was used to compare proportions (Jandel Scientific, 1994). For comparisons of lesion incidences the number of legs evaluated was used as the sample size, and the percentage of affected legs was used as the proportion for all Z-tests. The SigmaStat ANOVA package was used to compare BW among treatments.

RESULTS

Over the course of the study 2 birds died of sudden death syndrome. No mortality was attributed to pulmonary hypertension syndrome or unknown causes. Two lame birds were diagnosed with KB (0.67%), 3 with TST (1.0%), and one with TD (0.33%). Accordingly, lameness overwhelmingly was attributable to BCO lesions of the proximal femora and tibiae. Lameness incidences for each environmental chamber (TW1, TW3, TW5, HD2, HD4, and HD6) and for

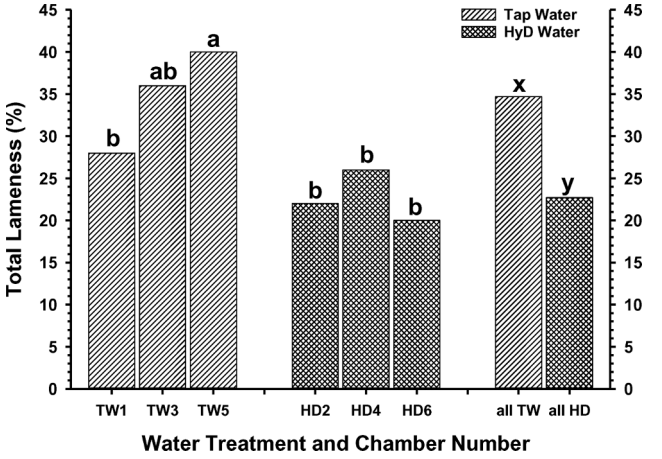

Figure 1. Cumulative bacterial chondronecrosis with osteomyelitis (BCO) lameness for broilers that were provided on d 1 through 56 with tap water (TW) in odd-numbered environmental chambers (1,3, and 5), or tap water containing HyD (HD) in even-numbered chambers (2,4, and 6), or for the entire study combined (all TW, all HD). [a,b]Lameness incidences with different superscripts differed significantly ($P \leq 0.05$) among individual chambers based on repeated SigmaStat Z-tests. [x,y]Values for all pooled by water treatment with different superscripts differed significantly ($P = 0.03$; SigmaStat Z-test).

chambers pooled by water treatment (all TW vs. all HD) are shown in Figure 1. The incidences were calculated as percentages of 50 birds/chamber, or 150/water treatment, after culling on d 14. Lameness incidences in the TW chambers ranged between 28% (TW1) and 40% (TW5). Lameness in the HD chambers ranged between 20% (HD6) and 26% (HD4). The lowest incidence among the chambers assigned to the TW treatment (28%, TW1) numerically exceeded the highest incidence among the chambers assigned to the HD treatment (26%, HD4). This range of pen-to-pen variability is typical based on prior experience with the wire flooring model. The difference in lameness between the water treatments (chambers pooled) was significant ($P = 0.03$), with 22.7% of the broilers consuming HyD becoming lame as compared with 34.7% of the broilers consuming tap water. The time course for cumulative lameness is shown in Figure 2. Lameness initially was detected on d 28 in both water treatment groups. Thereafter the cumulative incidence consistently was numerically higher for broilers in the TW treatment than in the HD treatment, with the difference attaining significance at $P \leq 0.05$ after d 50.

Figure 3 illustrates the lesion incidences within each diagnostic category for the proximal femora and tibiae of lame birds from the TW and HD groups. When pooled by diagnostic category independent of water treatment, no tendency was revealed for lesions to form preferentially in either leg (left vs. right leg, genders pooled; data not shown) or sex (males vs. females, left and right legs pooled; data not shown). Accordingly, the incidences shown in Figure 3 reflect the percentages of all legs evaluated within each treatment group (genders pooled, left and right legs pooled). Lame broilers in the HD group exhibited a lower incidence of normal tibiae

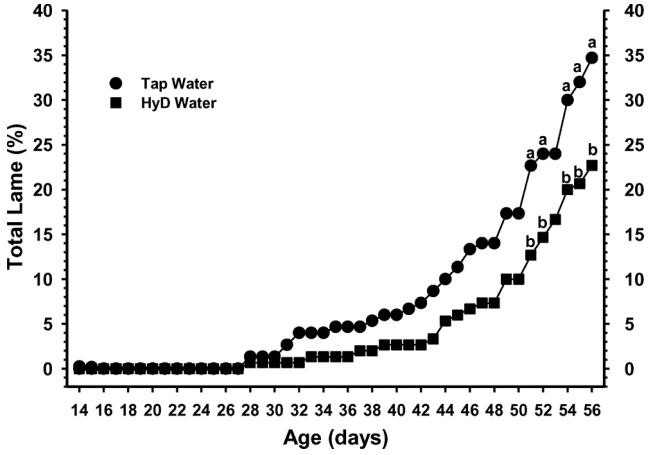

Figure 2. Time course of cumulative lameness from d 14 through 56 for broilers that were supplied with either tap water (TW) alone (circles) or TW containing HyD (squares). [a,b]Lameness incidences with different superscripts differed significantly between water treatments on d 51 through 56 (SigmaStat Z-test used to compare proportions).

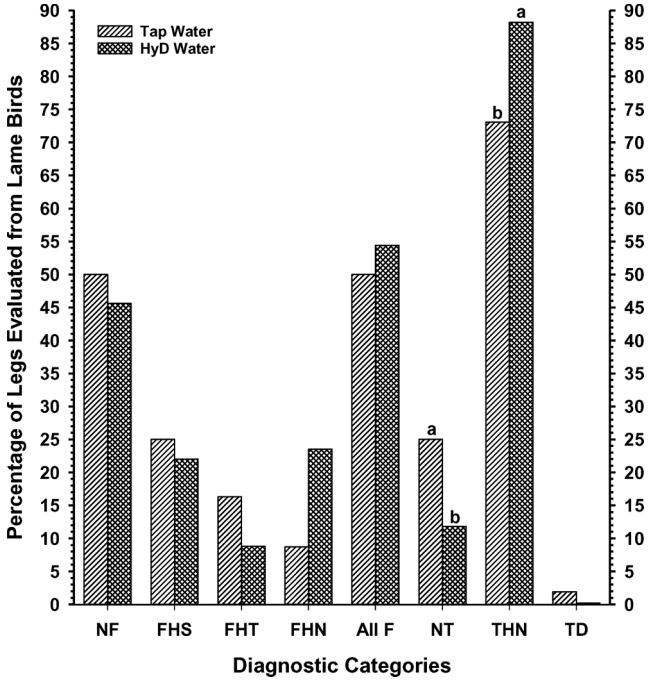

Figure 3. Incidences within proximal femoral or tibial diagnostic categories for broilers that became lame on d 14 through 56 while being provided with either tap water (TW) alone or tap water containing HyD. The proximal femora and tibiae of both legs were evaluated and were diagnosed as being macroscopically normal (NF, NT; no apparent abnormalities) or they exhibited femoral head separation (FHS), femoral head transitional (FHT) degeneration, femoral head necrosis (FHN), tibial head necrosis (THN), or tibial dyschondroplasia (TD). All F = FHS+FHT+FHN (total femoral head lesions). Values reflect the percentages of all legs evaluated within each treatment group (genders pooled, left and right legs pooled). [a,b]Within a diagnostic category incidences with different superscripts differed significantly ($P \leq 0.05$; SigmaStat Z-test).

and a higher incidence of THN when compared with lame broilers from the TW group.

Broilers that survived and appeared to be clinically healthy on d 56 were weighed and necropsied. BW (mean ± SEM) for males in the TW and HD groups

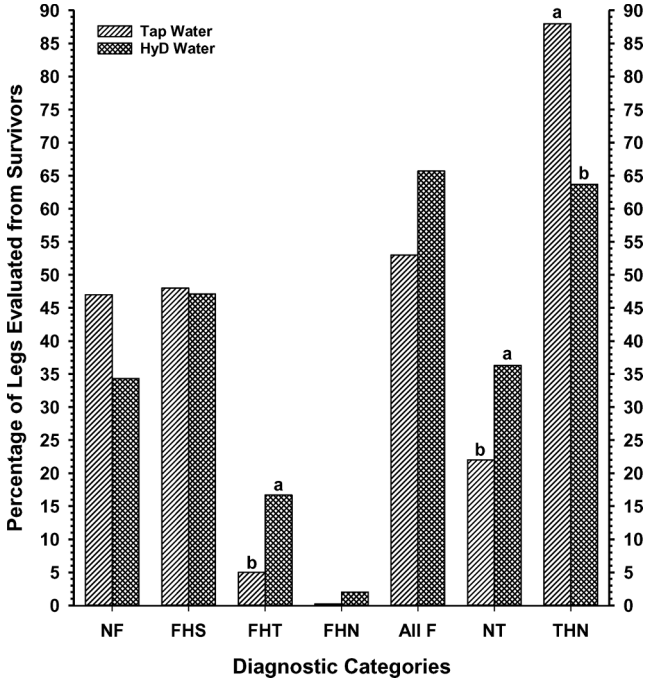

Figure 4. Incidences within proximal femoral or tibial diagnostic categories for broilers that survived and appeared to be clinically healthy on d 56 after consuming tap water (TW) alone or tap water containing HyD. The proximal femora and tibiae of both legs were evaluated and were diagnosed as being macroscopically normal (NF, NT; no apparent abnormalities) or they exhibited femoral head separation (FHS), femoral head transitional (FHT) degeneration, femoral head necrosis (FHN), or tibial head necrosis (THN). All F = FHS+FHT+FHN (total femoral head lesions). Values reflect the percentages of all legs evaluated within each treatment group (genders pooled, left and right legs pooled). [a,b]Within a diagnostic category incidences with different superscripts differed significantly ($P \leq 0.05$; SigmaStat Z-test).

averaged 3,711 ± 42 and 3,752 ± 57 g, respectively (not significant), and BW for females in the TW and HD groups averaged 3,213 ± 54 and 3,243 ± 56 g, respectively (not significant). Males were heavier than females ($P < 0.001$), but within each gender the d 56 BW were not affected by the water treatments ($P < 0.10$). Lesion incidences for the proximal femora and proximal tibiae from survivors on d 56 are summarized in Figure 4 (genders pooled, left and right legs pooled). The HD group had higher incidences of FHT and normal tibiae, and lower incidences of THN when compared with survivors in the TW group.

DISCUSSION

BCO and TOC share a similar pathogenesis, including the translocation of opportunistic bacteria across the intestinal and respiratory epithelial barriers, and the onset of lameness attributable to osteomyelitis within the epiphysis and metaphysis of rapidly growing proximal tibiae. Stress and immunosuppression also contribute to the pathogenesis of both BCO and TOC. Indeed, BCO and TOC are readily induced by injecting broilers or poults with repeated immunosuppressive doses of the synthetic glucocorticoid dexamethasone. A

previous study demonstrated that the incidence of TOC induced by dexamethasone injections could be reduced by prophylactically supplementing the drinking water with vitamin D_3, in spite of the presence of vitamin D_3 levels in the poult starter feed that met NRC (1994) requirements (Huff et al., 2000). It is within this context that the present study was conducted to evaluate the potential for HyD supplementation to reduce the incidence of lameness attributable to BCO in broilers. The lesions associated with clinical lameness in the present study overwhelmingly were associated with BCO of the proximal tibiae and femora, with negligible contributions attributable to KB, TST, or TD. Prophylactic supplementation of HyD in the drinking water clearly reduced the incidence of clinical lameness and thus BCO. Broilers in the chambers in which supplemental HyD was supplied consistently developed the lowest incidences of lameness when compared with broilers in the chambers supplied with unsupplemented tap water. The time course for cumulative lameness demonstrated that HyD elicited a protective response from d 28 through 56, and this response did not depend on reduced body weights for survivors on d 56. The wire flooring model imposes a rigorous, sustained challenge that undoubtedly is much more severe than typically would be experienced by broilers under normal commercial conditions. Indeed, the high incidences of BCO induced by rearing broilers on wire flooring are only partly suppressed by administering therapeutic doses of the potent fluoroquinolone antimicrobial enrofloxacin (Wideman et al., 2015). Therefore the positive response to HyD supplementation in the present study strongly supports the potential for HyD supplementation to attenuate outbreaks of lameness caused by BCO in commercial broiler flocks.

Lame birds in the TW and HyD treatment groups tended to have a higher prevalence of proximal tibial head pathology (approximately 75 to 80% incidences) and a somewhat lower prevalence of proximal femoral head pathology (approximately 50 to 55% incidences; Figure 3). Lame birds from the HyD treatment group had higher incidences of THN when compared with lame birds from the TW treatment group (Figure 3), whereas just the opposite was true for clinically healthy survivors on d 56 (Figure 4). These opposing patterns of tibial lesion incidences in lame birds vs. survivors are difficult to reconcile mechanistically; however, they do suggest that HyD supplementation preferentially influenced the development of proximal tibial rather than proximal femoral BCO lesions. In this context it may be relevant to note that dexamethasone injections previously were demonstrated to preferentially increase the incidence of severe necrosis in the proximal tibiae rather than in the proximal femora of broilers, perhaps indicating a differential responsiveness to stress by the tibial and femoral growth plates (Wideman and Pevzner, 2012).

The mechanisms by which HyD supplementation reduced lameness remain to be identified. Vitamin D_3 plays a key role in regulating calcium and phosphorus metabolism and thus bone mineralization, however no overt symptoms of a primary vitamin D_3 deficiency (e.g., growth depression, skeletal deformity, macroscopic bone fractures, and rickets) were detected in the present or previous studies in which high incidences of BCO were induced on wire flooring (Wideman, personal observations). The starter and finisher feeds in the present study were formulated to contain levels of vitamin D_3 far in excess of the NRC (1994) recommendations. Accordingly, the results of the present study cannot be attributed to an overt deficiency of vitamin D_3 per se, but instead must be attributed to the specific characteristics of 25-OH vitamin D_3. The 25-OH D_3 metabolite is better absorbed than vitamin D_3, and providing vitamin D in this form eliminates the need for hepatic conversion of vitamin D_3 to 25-OHD_3 (see the Introduction). High circulating levels of vitamin D_3 can inhibit the activity of hepatic vitamin D_3-25-hydroxylase in chickens, and inhibition of 25-hydroxylase may prevent the hepatic synthesis of 25-OH vitamin D_3 in quantities sufficient to support the production of the active metabolite 1,25-$(OH)_2$ vitamin D_3 by the enzyme 25-hydroxyvitamin D-1α-hydroxylase (Bhattacharyya and DeLuca, 1974). Administering 25-OH vitamin D_3 (e.g., HyD) as a supplement in the drinking water would serve to bypass the 25-hydroxylation step in the liver, thereby directly providing additional substrate for the synthesis of biologically active 1,25-$(OH)_2$ vitamin D_3. Higher circulating levels of 1,25-$(OH)_2$ vitamin D_3 may in turn modulate the response of the immune system to counteract stress-mediated immunosuppression, inhibit bacterial translocation across the gastrointestinal tract, and attenuate the microbial infection and pathological deterioration of proximal growth plates associated with BCO in broilers and TOC in turkeys (Huff et al., 2000; Baeke et al., 2010; Hewison, 2010; Lagishetty et al., 2010; Zhang et al., 2011; Pastorelli et al., 2013; Morris et al., 2014; Mouli and Ananthakrishnan, 2014). Another potential mechanism is a localized effect of 25-OHD_3 in specific tissues. The kidneys are the main source for 1α-hydroxylase production, but many tissues, including the immune system, have this enzyme indicating the potential for producing 1,25-$(OH)_2$ vitamin D_3 locally from circulating 25-OHD_3 (Shanmugasundaram and Selvaraj, 2012). Therefore, 25-OHD_3 may be playing a role not only in the systemic immune response as noted previously, but also at the level of the intestine and growth plates, which are the source of bacterial translocation and colonization respectively. As systemic 1,25-$(OH)_2$ vitamin D_3 production is tightly regulated due its impact on calcium and phosphorus levels, this paracrine and autocrine activity of vitamin D may be critical as it is independent of calcium status and is solely dependent on adequate circulating 25-OHD_3. Regardless of the possible mechanism, the present study demonstrates that prophylactic administration of HyD via the drinking water can reduce the

incidence of BCO and clinical lameness in broilers reared on wire flooring.

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