Effects of in ovo injection of organic trace minerals and post-hatch holding time on broiler performance and bone characteristics[1]

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ABSTRACT Effects of the in ovo injection of organic Mn, Zn, and Cu in association with post-hatch (POH) feed and water restriction on the performance and physical-chemical bone parameters of male Ross × Ross 708 broilers were examined. On 17 d of incubation, a total of 1,872 eggs were subjected to in ovo injection using a commercial multi-egg injector. Treatments (TRT) includingd non-injected and diluent-injected controls. The respective Zn, Mn, and Cu levels (mg/mL) added to the diluent of the low (LMD) and high mineral (HMD) TRT groups were 0.181, 0.087, and 0.010, and 0.544, 0.260, and 0.030, respectively. The 4 TRT groups were then sub-divided into 2 POH holding time (HT) groups, with 15 birds randomly allocated to each of 6 replicate pens in each of the 8 groups. The first HT group (0HT) had immediate access to water and feed, and the second HT group (24HT) contained birds that were kept in transport baskets for 24 h before being released. Performance was determined and selected birds were subsequently necropsied and their tibiae extracted for analysis. In comparison to birds from 24HT group, those in the 0HT group had a higher BW gain and feed intake, and a lower FCR through 21 d POH. The percentage of bone ash of the birds belonging to the HMD group was higher than all other TRT on d 1 POH and was higher than the non-injection control group on d 21 POH. On d 1, the LMD and HMD groups had higher tibial Mn concentrations than those of the control groups. On d 7, bones from the HMD group had a higher concentration of Mn than did the non-injected control group, and likewise, on d 21 POH, had a higher concentration of Zn than did the control groups. In conclusion, a 24HT negatively affected the performance of the birds during the first 2 wk POH; however, the LMD and HMD TRT had a positive influence on bone mineralization.

Key words: bone quality, in ovo supplementation, mineralization, posthatch

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INTRODUCTION

In the last decade, genetic selection for fast growth rate in broilers has led to numerable problems including skeletal disorders. At hatch, the bones of chicks are not completely formed, which means that there is a high demand for minerals during the initial stages of posthatch (**POH**) growth. Poor mineralization during bone ossification can lead to compromised leg development that can culminate in immobility or condemnation. These factors contribute to major economic losses in the poultry industry (Dibner et al., 2007). Furthermore, other factors such as growth rate and nutrient availability are associated with leg problems.

The yolk along with the eggshell constitute the extraembryonic sources of calcium (Simkiss, 1961), and Tuan and Ono (1986) noted that early calcium tracer studies conducted by Johnston and Comar (1955) confirmed that calcium is sequentially mobilized from the yolk first and then later from the eggshell. Towards the end of the incubation period, yolk is internalized into the abdominal cavity and continues to be the main source of nutrients. The yolk comprises approximately 20 to 25% of the BW of posthatch chicks and provides immediate nutrition for maintenance and growth (Romanoff, 1960; Sklan and Noy, 2000; Khan et al., 2004). During this period, chicks make a nutrient transition from a yolk-based to an exogenous feed-based diet. Yair and Uni (2011) reported that the concentration of

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microminerals (Zn, Cu, and Mn) in the yolk at hatch is very low.

Different strategies have been tested experimentally in an effort to prevent leg problems. Changing the source of minerals used in the feed of breeders is one attempt to improve the bone parameters of broilers. Favero et al. (2013) substituted organic for inorganic sources of Zn, Cu, and Mn in the feed of broiler breeders. This substitution resulted in improvements in bone mineralization in the progeny and had no effect on hatchability or hatchling weight. The provision of feed to progeny immediately after hatching has also been used to further improve bone development. In the United States, the transport of hatching chicks from the nearest commercial hatchery to the farm can take up to 8 h. However, according to reports of field professionals, this period can be significantly longer in other countries. Making feed available to chicks during their transport from the hatchery to the farm, or even inside the hatcher unit, has likewise been tested by researchers (Bigot et al., 2003; Kidd et al., 2007; and Rada et al., 2013). There are a number of ways to technically provide early nutrition; however, in ovo nutrition is the earliest and most advanced method.

The use of in ovo vaccination to prevent diseases such as Marek's disease and Newcastle disease, is a methodology well established and widely used worldwide. This method has also been studied as a means to deliver amino acids (Ohta et al., 1999), vitamins (Bello et al., 2013; Bello et al. 2014a,b, Bello et al., 2015), carbohydrates (Zhai et al., 2011a), and other nutrients (Keralapurath et al., 2010; McGruder et al., 2011) to embryos during the late incubation period. The administration of 25-hidroxy cholecalciferol [**25(OH)D_3**] by in ovo injection was shown by Bello et al. (2013) to improve the hatchability of fertilized broiler hatching eggs without having any detrimental effects on hatchling quality. In a later related study, the same research group (Bello et al., 2014b) showed that the in ovo injection of up to 1.20 μg of 25(OH)D_3 had no detrimental effects on the survival or overall POH performance (including BW gain) of broilers. Yair et al. (2013) injected P, Ca, Zn, Mn, and Cu, along with carbohydrates and vitamins, into eggs and reported a higher rate of mineralization and better mechanical properties of bones in broiler embryos and POH chicks. The yolk, as mentioned previously, has limited concentrations of Zn, Cu, and Mn, and these microminerals are important for bone development (Liu et al., 1994; Rath et al., 2000; Angel, 2007; Dibner et al., 2007; Bao et al., 2007). These minerals also contribute to enzyme activity along metabolic pathways that are related to the formation of the skeletal system (Bao et al., 2007). Zinc participates in important regulatory pathways for bone and cartilage formation (Starcher et al., 1980; Sauer et al., 1997), copper is part of the linkage between elastin and collagen, which gives the bone its tensile strength (Carlton and Henderson, 1964), and manganese insufficiencies can lead to the malformation of the epiphyseal plate of the tibia (Liu et al., 1994). Therefore, the objectives of this study were to investigate effects of the in ovo injection of organic Mn, Zn, and Cu in association with POH feed and water restriction, on the performance and the physical and chemical bone parameters of broilers.

MATERIALS AND METHODS

Eggs and Incubation

The protocols for the current study were approved by the Institutional Animal Care and Use Committee of Mississippi State University. Hatching eggs of approximately similar weight (64.6 ± 0.15 g) were obtained from a breeder flock (Ross 708) at 32 wk of age (n = 1,872) and then stored under commercial conditions for a maximum of 2 d. Eggs were subsequently weighed, and those that weighed within 10% of the mean weight of all 1,872 eggs were set for incubation. Eggs were randomly set for incubation (Zhai et al., 2011a,b,c) on each of 6 trays in 3 Natureform incubators (Model 2,340 Natureform, Jacksonville, FL). Initially, the eggs were equally and randomly distributed among the 3 incubators, with 26 eggs assigned to each of 4 pre-specified treatment groups on each of 6 replicate tray levels in each incubator. Eggs were incubated under standard commercial conditions. At 12 days of incubation (**doi**), all eggs were candled, and those eggs with shells that were cracked, or that were unfertilized or contained dead embryos, were discarded (Ernst et al., 2004). The trial ultimately included 8 experimental treatments that were arranged in a 4 × 2 factorial design, with each experimental treatment replicated 6 times.

Injection Solutions

Four in ovo injection treatment (**TRT**) groups were designated at 17 doi. The first was non-injected control group (**Noninjected**) containing eggs that were not injected, but were subjected to the same handling procedures as the following TRT groups. The second were, fertile eggs injected with 150 μL of commercial diluent (Poulvac® Sterile Diluent; Pfizer, Exton, PA) that were designated as diluent-injected controls (**Diluent**). The third and fourth were those injected with 150 μL of diluent containing added organic microminerals, and were designated as enrichment solution TRT. Those eggs receiving solutions containing low and high mineral doses were respectively designated more specifically as **LMD** and **HMD** TRT groups. The added organic microminerals, which included organic Zn, Cu, and Mn (Mintrex Zn, Cu, and Mn; Novus, Saint Louis, MO), were used to promote bone development. The chelated trace minerals combine HMTBa (hydroxy analog of methionine) with an essential trace mineral in a two-to-one chelated molecule. The advantage of organic compared to inorganic trace minerals is that the binding of the mineral to the organic ligand provides

Table 1. Composition of the enrichment solutions containing organic microminerals.

Treatment	Nutrient	Organic micromineral concentration in diluent (mg/ml)	Total amount of organic micromineral injected into each egg (mg)
Noninjected	Zn	–	–
	Mn	–	–
	Cu	–	–
Diluent	Zn	–	–
	Mn	–	–
	Cu	–	–
LMD	Zn	0.181	0.0272
	Mn	0.087	0.0130
	Cu	0.010	0.0015
HMD	Zn	0.544	0.0816
	Mn	0.260	0.0390
	Cu	0.030	0.0045

stability of the complex in the upper gastrointestinal system. The compositions of the enrichment solutions used are presented in Table 1. The injection procedure was as previously described by Oliveira et al. (2015). After injection, the eggs were transferred to a Jamesway model PS 500 hatcher unit (Jamesway Incubator Company Inc. Cambridge, Ontario, Canada) and were incubated under standard commercial conditions. Egg injection and handling prior to transfer required a maximum of 5 min. The positions of the TRT replicate groups in the hatcher corresponded to their positions in the setter.

Grow-Out Phase

At hatch, chicks belonging to a common TRT replicate group from each incubator were pooled together, and were subsequently sexed and weighed. Each of the 4 TRT groups from the incubation phase were then subdivided into another 2 POH holding time (**HT**) groups, which resulted in a total of 8 treatments (4 TRT × 2 POH HT). Fifteen male birds were randomly allocated to each of 6 replicate mini-pens (0.914 m × 1.219 m) within each of the 8 treatment groups. Initial bird density in each mini-pen was approximately 0.074 m^2 per bird. The first HT group, designated as having a 0 h HT (**0HT**), had immediate access to water and feed, and the second HT group, designated as having a 24 h HT (**24HT**), contained birds that were kept in transport baskets for 24 h before being placed inside their respective treatment-replicate pen. After the HT period, but before the birds were released, the feeders in each pen were weighed. For birds in the 0HT treatment group, standard brooding conditions and ad libitum feed and water were provided from 0 to 42 d POH. Birds in the 24HT treatment group were likewise provided the same conditions and had ad libitum access to feed and water after the HT period.

Data Collection

In each pen, mortality was recorded daily and total bird BW, bird numbers, and the weight of unconsumed and added feed were recorded on d 7, 14, 21, 35, and 42 POH. Mean BW gain, feed consumption, and feed conversion were calculated for each replicate pen between 0 and 7, 0 and 14, 0 and 21, 0 and 35, and 0 and 42 d POH. Feed consumption over the entire grow-out period (0 to 42 d) was calculated by totaling feed consumption in each time interval and correcting for loss of birds due to mortality and sampling. Feed conversion was calculated by dividing total feed consumption by total BW gain in each pen. On d 1 POH (immediately before releasing birds belonging to the 24HT group), one bird that weighed within 5% of the mean BW of the birds in each of the respective 48 pens was randomly selected, weighed, and its length (from the tip of the beak to the tip of the middle toe, excluding the nail) was measured (Molenaar et al., 2010). Subsequently, the selected birds were necropsied to confirm their sex and for the extraction of their left and right tibiae. On d 1, 7, 14, and 21 POH, the same sampling procedure was performed for extraction of the left and right tibiae from one bird randomly selected from each pen. Muscle was removed from the left tibiae and then then weighed to determine fresh bone weight. Subsequently, the bones were oven-dried until no further weight loss was observed. The bones were then allowed to equilibrate to room temperature before their dry weight (**BDW**) was determined (Zhai et al., 2011b). Fresh and dry bone weights were calculated as percentages of BW. With the use of an Instron Universal Testing Instrument (Table Model 5544, Instron, Norwood, MA), dried left tibiae were subjected to breaking strength analysis using the method described by Shim et al. (2012). The cradle and plunger of the Instron Instrument were adjusted to accommodate size differences of the bone samples collected. The broken bones were weighed and ashed in a muffle furnace (Iso-temp D3714, Fisher Scientific, Pittsburgh, PA) for determination of percentage of bone ash **(PBA)** using AOAC (1990) methods. For bone mineral concentration analysis, bone ash samples from one bird from each pen was selected. Using methods specified by the US-EPA (1986), the samples were dissolved and digested (method 3051), and the concentrations of Ca, P, K, Mg, Zn, Mn, and Cu in each ash sample were

analyzed by inductively coupled plasma optical emission spectrometry (method 6010B).

The frozen right tibiae were transferred to the Department of Animal Science at Purdue University, where they were thawed and then scanned using a model 476D014 dual-energy x-ray absorptiometry (DEXA, Norland Medical Systems, Fort Atkinson, WI) analyzer to determine bone mineral density (**BMD**) and bone mineral content (**BMC**). Description of the DEXA analyzer and the procedures of its operation were as described by Hester et al. (2004).

Statistical Description

A randomized complete block design was used in the arrangement of eggs in the setter and hatcher units and in the placement of chicks in floor pens. The 4 TRT were equally represented within each setter tray and hatching basket level. Each of the 6 groups of floor pens (blocks) represented a replicate unit. TRT and HT were designated as fixed effects and block as a random effect. Data on d 1, 7, 14, and 21 POH were analyzed separately. All variables in this study were analyzed by ANOVA using the MIXED procedure of SAS software 9.2 (SAS Institute, 2010). Least squares means were compared in the event of significant global effects. Global effects and least square means differences were considered significant at $P \leq 0.05$.

RESULTS

All performance parameters evaluated are presented in Table 2. There were no significant TRT × HT interactions for any parameters evaluated in this study. Furthermore, there were no significant main effects due to TRT on mean POH BWG, FI, or FCR. However, there were significant main effects due to HT on 0 to 7 d ($P < 0.0001$) and 0 to 14 d ($P < 0.0001$) BWG; 0 to 7 d ($P < 0.0001$), 0 to 14 d ($P < 0.0001$), and 0 to 21 d ($P < 0.0001$) FI; and on 0 to 7 d FCR ($P < 0.02$). Birds in the 0HT group had a higher BWG through d 7 and 14 than did the 24HT group. The birds belonging to the 0HT group also had a higher FI through d 7, 14, and 21 than did birds belonging to the 24HT group. Furthermore, birds in the 0HT group had a lower or more improved FCR than did those in the 24HT group. Due to commensurate increases in both the FI and BWG of birds, no significant differences were observed for FCR past d 7 POH.

The bone breaking strength (BBS) results (Table 3) indicate that bone strength was not improved by the in ovo injection of diluent containing either supplemental mineral dosage at any of the ages evaluated. Delayed access to feed and water also had no negative effect on bone strength until 21 d POH. The bones of the birds in all of the treatment groups on d 1, 7, 14, and 21 POH were scanned for determination of BMD. However, only bones from birds at 14 and 21 d POH were successfully

Table 2. Body weight gain (BWG), feed intake (FI) and feed conversion rate (FCR) on d 7, 14, 21, 35, and 42 posthatch in Noninjected, diluent-injected, low mineral dose (LMD), and high mineral dose (HMD) injected treatment groups, and at 0 h (0HT) and 24 h (24HT) holding times.

	BWG (g)					FI (g)					FCR				
	Posthatch Days of Age														
Item	7	14	21	35	42	7	14	21	35	42	7	14	21	35	42
Noninjected	129.1	433.9	918.0	2365.9	3113.5	166.6	557.7	1063.9	3400.3	5063.9	1.296	1.286	1.11	1.435	1.599
Diluent	125.0	430.8	894.0	2346.4	3120.2	168.9	550.3	990.4	3293.9	4899.2	1.363	1.277	1.05	1.413	1.595
LMD	125.0	430.0	913.8	2342.2	3148.2	169.7	556.9	1059.0	3424.9	5200.0	1.364	1.308	1.15	1.499	1.633
HMD	129.0	441.7	922.9	2447.7	3153.8	172.5	581.6	1096.9	3469.7	5064.5	1.339	1.292	1.19	1.444	1.620
SEM	2.9	7.2	17.2	37.6	37.7	3.9	8.683	37.1	47.9	75.8	0.031	0.011	0.04	0.025	0.019
P-value	0.58	0.71	0.65	0.18	0.83	0.77	0.07	0.24	0.07	0.06	0.40	0.25	0.13	0.11	0.48
0HT	137.1[a]	449.7[a]	913.24	2417.9	3168.0	178.2[a]	588.5[a]	1087.6[a]	3425.8	5122.0	1.304[a]	1.297	1.166	1.448	1.623
24HT	116.9[b]	418.5[b]	911.13	2333.1	3099.9	160.6[b]	534.7[b]	1017.5[b]	3368.6	4986.9	1.377[b]	1.284	1.088	1.448	1.599
SEM	2.03	4.7	12.18	26.58	26.6	2.8	8.7	26.4	35.1	53.7	0.022	0.008	0.031	0.0178	0.013
P-value	<0.0001	<0.0001	0.90	0.99	0.43	<0.0001	<0.0001	<0.0001	0.25	0.08	0.03	0.25	0.08	0.99	0.22

[a,b]Means within a parameter with no common superscript differ ($P \leq 0.05$).

Table 3. Bone breaking strength (kg of force) on d 1, 7, 14, and 21 posthatch in Noninjected, diluent-injected, low mineral dose (LMD), and high mineral dose (HMD) injected treatment groups, and at 0 h (0HT) and 24 h (24HT) holding times.

	Posthatch Days of Age			
Item	1	7	14	21
Noninjected	1.191	3.370	7.995	25.723
Diluent	1.161	3.079	7.454	22.606
LMD	1.119	3.324	7.780	24.115
HMD	1.216	3.036	8.187	21.723
SEM	0.597	0.183	0.364	1.949
P-value	0.73	0.48	0.53	0.49
0HT	1.149	3.392	8.028	23.368
24HT	1.195	3.013	7.680	23.715
SEM	0.042	0.135	0.258	1.385
P-value	0.45	0.50	0.63	0.56

Table 4. Bone mineral density (g/cm^2) and bone mineral content (g) on d 1, 14, and 21 posthatch in Noninjected, diluent-injected, low mineral dose (LMD), and high mineral dose (HMD) injected treatment groups, and at 0 h (0HT) and 24 h (24HT) holding times.

	BMD		BMC	
	Posthatch Days of Age			
Item	14	21	14	21
Noninjected	0.0763	0.1256	0.224	1.135
Diluent	0.0756	0.1231	0.237	1.183
LMD	0.0746	0.1254	0.210	1.112
HMD	0.0758	0.1260	0.225	1.125
SEM	0.0009	0.0034	0.017	0.047
P-value	0.59	0.92	0.74	0.72
0HT	0.0757	0.1244	0.222	1.159
24HT	0.0754	0.1256	0.226	1.119
SEM	0.0006	0.0024	0.011	0.331
P-value	0.77	0.72	0.81	0.39

scanned. The scanner was not able to precisely determine the mineralization of the bones from d 1 and 7 POH. Nevertheless, no significant main or interactive effects involving treatment for BMD or BMC on d 14 and 21 were noted (Table 4).

Fresh bone weight was not affected by TRT or HT (Table 5). The BDW, which was calculated as a percentage of BW, was also not affected by TRT. However, there was a significant effect of HT on d 1 ($P \leq 0.001$) and 14 ($P \leq 0.004$) POH. On d 1 POH, the BDW of the birds from the 0HT group was lower than that of the 24HT group. The opposite was observed on d 14, in which the birds from the 0HT group had a higher BDW than did those from the 24HT group. No significant difference between HT treatments for bone ash was observed. The percentage of ash in the bones of the birds belonging to the HMD group was significantly higher ($P \leq 0.01$) on d 1 in comparison to the other TRT. The TRT did not affect bone ash concentration on d 14. However, on d 21, mean PBA of the birds from the HMD treatment group was significantly ($P \leq 0.04$) higher than those from the Noninjected group.

There were no significant interactive effects involving TRT and HT for bone Ca, P, and Mg concentrations on d 1, 7, and 21 POH (Table 6). Furthermore, there were no main effects due to TRT or HT for bone Mg concentration on d 21 POH or for Ca and P on d 1, 7, and 21 POH (Table 6). However, on d 1, the concentration of Mg in the bones of birds belonging to the TRT groups that received the supplemental minerals by in ovo injection was significantly ($P \leq 0.04$) higher than those of the other TRT groups. On d 7, the ash of the bones from the Noninjected group had a lower ($P \leq 0.011$) Mg concentration than the other TRT. Curiously, the bones of birds belonging to the Diluent group had a higher Mg concentration than did the Noninjected control birds. In addition, on d 1, bones from the birds belonging to the 0HT treatment group had a significantly ($P< 0.0001$) higher Mg concentration than did those from the 24HT treatment group.

The microminerals (Mn, Zn, and Cu) used in the injection solutions were analyzed in the ash of the bones of all selected birds at 1, 7, and 21 d POH (Figure 1). Due to undetectable concentrations of Cu in the ash of these bones, the data for this mineral is not presented. Nevertheless, there were TRT effects on bone Mn concentrations on d 1 and 7 POH. On d 1, the birds that received any of the mineral supplements (LMD or HMD) by in ovo injection had a higher concentration of Mn than did either control group. On d 7, the HMD group had a significantly higher concentration of Mn than did the Noninjected group. Although the bone concentration of Zn exhibited a numerical change that was similar to that of Mn in response to the injection of Zn, no significant TRT effect was observed on d 1 and 7 POH. The opposite was observed on d 21, when no significant change in Mn concentration was observed among TRT, whereas the concentration of Zn did change significantly. The concentration of Zn in the bones of the birds from the HMD group was higher than that of birds in both control groups. Furthermore, no significant difference was observed for the concentration of these minerals between the 2 HT treatment groups.

DISCUSSION

The objective of the present study was to examine the effects of in ovo TRT in conjunction with HT on the bone development of broilers. In spite of our expectations, no TRT × HT interaction was observed for any of the bone parameters evaluated. As reported by Yair and Uni (2011) and Yair et al. (2013), bone development and their subsequent properties in broilers are affected by nutrient availability during the embryonic and POH periods. In those reports, it was observed that in ovo enrichment using several nutrients (Fe, Zn, Mn, Ca, Cu, P, Maltodextrin, Vitamin A, Vitamin D_3, and Vitamin E) resulted in numerous structural changes in the bones of birds during the incubational and POH periods. Bello et al. (2014a) investigated the in ovo injection of 25(OH)D_3, and found that it had various

Table 5. Fresh bone as percentage of BW, bone dry weight as percentage (BDW) of BW, bone ash as percentage (PBA) of BDM on d 1, 14, and 21 posthatch in Noninjected, diluent-injected, low mineral dose (LMD), and high mineral dose (HMD) injected treatment groups, and at (0HT) and 24 h (24HT) holding times.

	Fresh Bone			BDW			PBA		
	Posthatch Days of Age								
	1	14	21	1	14	21	1	14	21
Noninjected	0.94	0.90	0.91	0.27	0.29	0.32	24.54^{b}	34.75	36.68^{b}
Diluent	1.08	0.86	0.88	0.29	0.27	0.29	24.53^{b}	34.04	40.26^{a,b}
LMD	1.04	0.94	0.88	0.28	0.30	0.30	23.37^{b}	36.32	40.13^{a,b}
HMD	1.07	0.81	0.85	0.28	0.28	0.30	26.69^{a}	36.37	42.82^{a}
SEM	0.04	0.04	0.05	0.01	0.01	0.15	0.89	1.91	1.40
P-value	0.12	0.27	0.89	0.39	0.23	0.50	0.01	0.55	0.04
0 HT	1.01	0.87	0.85	0.27^{b}	0.27^{b}	0.30	25.16	35.85	39.51
24 HT	1.06	0.89	0.91	0.30^{a}	0.30^{a}	0.30	24.40	34.89	40.43
SEM	0.03	0.03	0.04	0.01	0.01	0.10	0.44	1.35	1.00
P-value	0.23	0.30	0.08	0.001	0.004	0.85	0.24	0.48	0.52

[a,b]Means within a parameter with no common superscript differ ($P \leq 0.05$).

Table 6. Broiler bone Ca, P, and Mg concentrations (wt/wt%) on d 1, 7, and 21 posthatch in Noninjected, diluent-injected, low mineral dose (LMD), and high mineral dose (HMD) injected treatment groups, and at 0 h (0HT) and 24 h (24HT) holding times.

	Posthatch Days of Age								
	1			7			21		
Item	Ca	P	Mg	Ca	P	Mg	Ca	P	Mg
Noninjected	31.92	17.38	0.7360^{c}	30.61	19.85	0.8418^{b}	32.09	17.22	0.7658
Diluent	31.94	16.79	0.7599^{b}	33.28	21.69	0.9393^{a}	31.83	17.02	0.7496
LMD	32.98	18.21	0.7804^{a}	33.63	20.96	0.9128^{a}	32.21	17.07	0.7550
HMD	33.52	17.95	0.7994^{a}	34.24	21.83	0.9562^{a}	32.51	17.17	0.7959
SEM	0.522	0.347	0.0163	1.046	0.552	0.023	0.731	0.202	0.026
P-value	0.087	0.066	0.044	0.123	0.072	0.011	0.936	0.903	0.595
0HT	32.65	17.27	0.8059^{a}	32.90	20.99	0.9177	32.51	17.16	0.7784
24HT	32.54	17.90	0.7319^{b}	32.97	21.18	0.9074	31.80	17.08	0.7548
SEM	0.360	0.267	0.0112	0.7405	0.390	0.016	0.531	0.143	0.018
P-value	0.826	0.111	<0.0001	0.949	0.732	0.657	0.345	0.685	0.369

[a–c]Means within a parameter with no common superscript differ ($P \leq 0.05$).

effects on the mechanical properties of the tibia. The TRT employed in this study had no effect on the performance of broilers. This finding is in accordance with those of Bello et al. (2014b), who evaluated effects of the in ovo injection of different levels (0.15, 0.30, 0.60, or 1.20 μg) of 25(OH)D_3 on broiler performance through 21 d POH. It was shown that 25(OH)D_3 at all the injection levels employed had no negative effects an broiler performance. Results of the current study showed that the broiler chicken has the ability to undergo compensatory BW gain. The birds were able to compensate by 21 d POH for a reduction in BW at 14 POH, which was caused by early feed and water deprivation. Feed restriction obviously decreased the BWG and FI of the chicks until a certain age. It is widely accepted that compensatory growth occurs so that birds eventually can reach a genetically programmed BW if provided the adequate nutrients at the right time (Pinheiro et al., 2004). This suggested compensatory growth was confirmed by the FI and BWG results that we observed in this study. Zhan et al. (2007) raised feed-restricted broilers that were deprived of feed for 4 h each d from 1 to 21 d of age, and it was observed that ADFI and ADG were not increased during the period in which they were provided feed and water (22 to 63 d POH). Furthermore, early feed restriction has been shown to significantly improve the FCR of broilers when compared with full fed controls birds (Deaton, 1995). Our data showing that the birds from the 24HT group exhibited an improved FCR in comparison to those from the 0HT group through 7 d POH, confirm this earlier finding. During the remainder of the trial, no differences were observed for FCR besides the existence of numerical differences.

In this study, the TRT and HT employed were noted to have no effect on tibia BBS. It was expected that by increasing mineral availability to the embryo through in ovo injection, that early bone development would be improved. It was further expected that early bone development and its subsequent effects on their mechanical properties would be enhanced when mineral injection was used in conjunction with an imposed decrease in growth rate (24HT). Yair et al. (2013) reported that long bones (tibia and femur) from birds

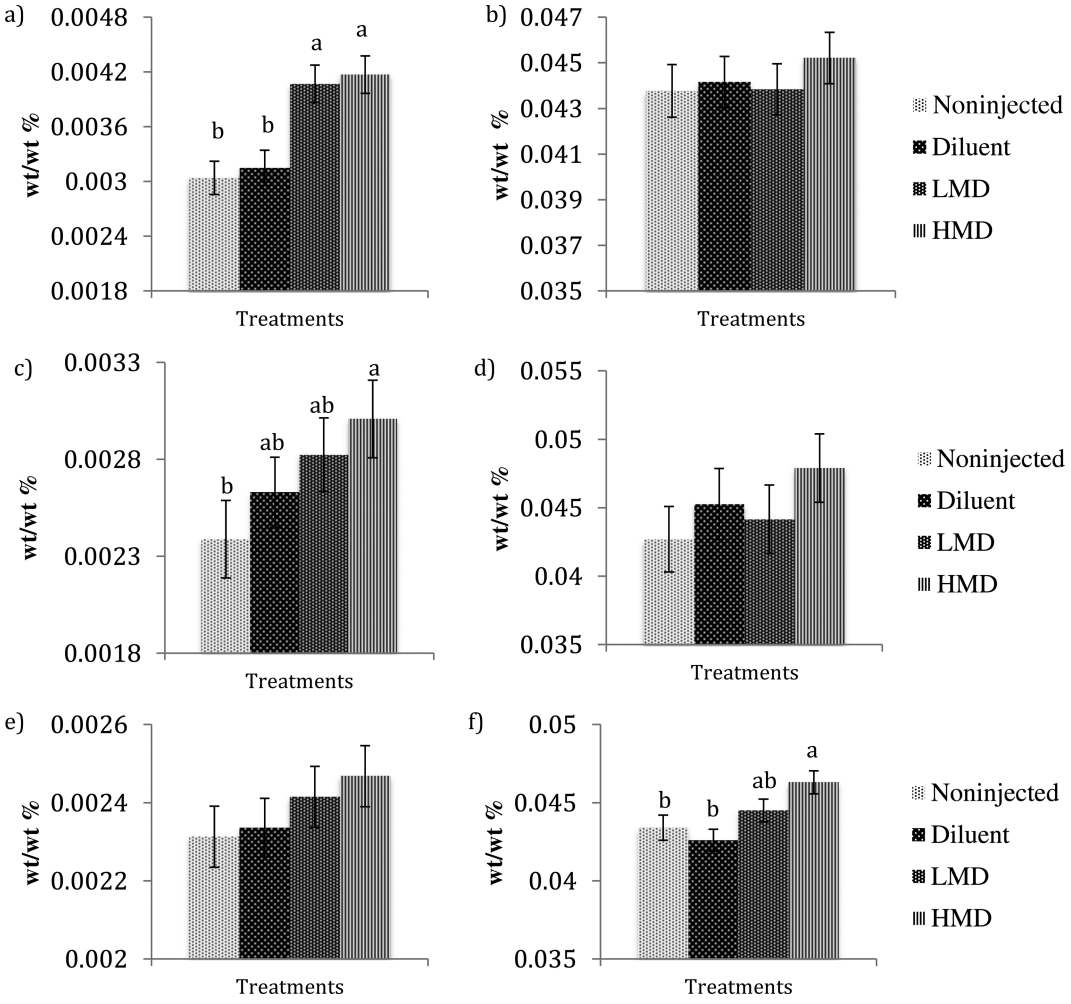

Figure 1. Percentage Mn (a, c, e) and Zn (b, d, f) on d 1, 7, and 21 posthatch in noninjected and diluent-injected control groups and low (**LMD**) and high (**HMD**) of Zn, Mn, and Cu concentration injection treatment groups. [a–b]Means within a parameter with no common superscript differ ($P \leq 0.05$).

that received in ovo supplementation of nutrients had superior mechanical properties at d 3 POH in comparison to Noninjected controls. However, at d 7 POH, no differences were observed between TRT in this study. In a study by Manangi et al. (2012), the supplementation of broiler chick diets with inorganic or organic Cu, Mn, and Zn did not exert different effects on BBS.

Conversely, the TRT and HT used in this study affected fresh bone weight. On d 7 and 14 POH, HT significantly affected dry bone weight. The observed effect of HT might be due to the lower BW of the birds belonging to the 24HT group on d 1 and 14, rather than being due to differences in their bone structure. On d 21, no difference due to TRT or HT was observed for BDW or BWG, which supports this relationship. The HMD TRT had a positive effect on PBA at d 1 POH when compared to all of the other TRT. The superiority of tibial PBA in the birds that received HMD when compared to those from the Noninjected group, shows that mineral injection has the potential to improve bone development even during the later stage of POH growth. Yair and Uni (2011) observed that broiler bone ash on 19 doi was increased due to in ovo nutrient injections, but that birds in the Noninjected group also had a higher PBA on d 3 POH. Star et al. (2012) used diets containing different forms and levels of Zn, but did not observe any significant treatment effects on tibia ash. Similar to BBS, TRT, and HT had no effect on BMD or BMC in this study. Oliveira et al. (2015) used the same TRT of the present study and reported positive effects of HMD on PBA at 1 d POH. The higher PBA had no correlation to the mechanical properties evaluated, which have been commonly observed in other reports (Yair and Uni, 2011).

There were no significant TRT or HT effects on Ca, P, or Mg in any of the samples analyzed, with the exception of TRT and HT effects on Mg on d 1 POH and a TRT effect on Mg on d 7 POH. Interestingly, the in ovo injection of LMD and HMD significantly increased the level of Mn in the bone ash of the birds. Numerical differences in Ca and P along with Mn in the tibial ash of birds that received an in ovo injection of organic minerals are suggestive of a potential for increased mineralization in the bones by 1 d POH. The same effect was not observed on d 7 or 21 POH in this study. Yair et al. (2013) reported that by 2 d after an in ovo injection

of nutrients, that the concentrations of Ca and P, as percentages of dry bone weight, were nearly 2-fold higher than those of a Noninjected group. However, they observed that on d 7 POH, the concentrations of Ca and P in the bone were higher than those in the Noninjected group. Bello et al. (2014a) reported that no significant changes in bone Ca, P, Mg, or K were caused when various levels of 25 $(OH)D_3$ were administered by in ovo injection. On d 1 and 7 POH, the injection of HMD or LMD had no current effect on bone Zn concentration. However, on d 1 POH, the in ovo injection of minerals resulted in higher concentrations of bone Mn when compared to those belonging to the control groups. Also, on d 7 POH, the mean concentration of Mn in the ash of the birds from the HMD TRT was higher than those belonging to the Noninjected control group. Bao et al. (2007) fed broilers with different sources and levels of organic Cu, Fe, Mn, and Zn, and observed that there were no differences in the concentration of these minerals in the bones of birds that received either inorganic minerals or high concentrations of organic minerals. On d 21 POH in the current study, the mean concentration of bone Zn of birds from the HMD group was higher than that of birds from the control groups. In a previous study by Yair et al. (2013), it was found that the injection of nutrients involved in bone development had positive effects on the concentration of Mn but not of Zn.

Based on these current results, it can be concluded that a 24 h delay in placement has little or no effect on broiler bone development. However, the in ovo injection of organic minerals involved in bone mineralization may potentially benefit bone quality. Further research to determine the optimal dosages of various other organic minerals that may be administered by in ovo injection alone or in combination with those used in this study for improved bone development and mineralization in broilers, should be considered.

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REFERENCES

Angel, R. 2007. Metabolic Disorders: Limitations to Growth of and Mineral Deposition into the Broiler Skeleton after Hatch and Potential Implications for Leg Problems. J. Appl. Poult. Res. 16:138–149.

Association of Official Analytical Chemists. 1990. Official Methods of Analysis. 15th ed. AOAC Press, Gaithersburg, VA.

Bao, Y. M., M. Choct, P. A. Iji, and K. Bruerton. 2007. Effect of Organically Complexed Copper, Iron, Manganese, and Zinc on Broiler Performance, Mineral Excretion, and Accumulation in Tissues. J. Appl. Poult. Res. 16:448–455.

Bello, A., W. Zhai, P. D. Gerard, and E. D. Peebles. 2013. Effects of the commercial in ovo injection of 25-hydroxycholecalciferol on the hatchability and hatching chick quality of broilers. Poult. Sci. 92:2551–2559.

Bello, A., R. M. Bricka, P. D. Gerard, and E. D. Peebles. 2014a. Effects of commercial in ovo injection of 25-hydroxycholecalciferol on broiler bone development and mineralization on days 0 and 21 posthatch. Poult. Sci. 93:1053–1058.

Bello, A., W. Zhai, P. D. Gerard, and E. D. Peebles. 2014b. Effects of the commercial in ovo injection of 25-hydroxycholecalciferol on broiler posthatch performance and carcass characteristics. Poult. Sci. 93:155–162.

Bello, A., M. Nascimento, N. Pelici, S. K. Womack, W. Zhai, P. D. Gerard, and E. D. Peebles. 2015. Effects of the in ovo injection of 25-hydroxycholecalciferol on the yolk and serum characteristics of male and female broiler embryos. Poult. Sci. 94: 734–739.

Bigot, K., S. Mignon-Grasteau, M. Picard, and S. Tesseraud. 2003. Effects of delayed feed intake on body, intestine, and muscle development in neonate broilers. Poult. Sci. 82:781–788.

Deaton, J. W. 1995. The effect of early feed restriction on broiler performance. Poult. Sci. 74:1280–1286.

Dibner, J. J., J. D. Richards, M. L. Kitchell, and M. A. Quiroz. 2007. Metabolic challenges and early bone development. J. Appl. Poult. Res. 16:126–137.

Carlton, W. W., and W. Henderson. 1964. Skeletal lesions in experimental copper-deficiency in chickens. Avian Dis. 8:48–55.

Ernst, R. A., F. A. Bradley, U. K. Abbott, and R. M. Craig 2004. Egg candling and breakout analysis for hatchery quality assurance and analysis of poor hatches. University of California, Div. Agric. and Nat. Resourc. 8134.

Favero, A., S. L. Vieira, C. R. Angel, A. Bos-Mikich, N. Lothhammel, D. Taschetto, R. F. A. Cruz, and T. L. Wardum. 2013. Development of bone in chick embryos from Cobb 500 breeder hens fed diets supplemented with zinc, manganese, and copper from inorganic and amino acid-complexed sources. Poult. Res. 92: 402–411

Hester, P. Y., M. A. Schreiweis, J. I. Orban, H. Mazzuco, M. N. Kopka, M. C. Ledur, and D. E. Moody. 2004. Assessing bone mineral density in vivo: Dual energy X-ray absorptiometry. Poult. Sci. 83:215–221.

Johnston, P., and C. Comar. 1955. Distribution of calcium from the albumen, yolk and shell to the developing chick embryo. Am. J. Physiol. 183:365–370.

Khan, K. A., S. A. Khan, A. Aslam, M. Rabbani, and M. Y. Tipu. 2004. Factors contributing to yolk retention in poultry: A review. Pakistan Vet. J. 24:46–51.

Keralapurath, M. M., R. W. Keirs, A. Corzo, L. W. Bennett, R. Pulikanti, and E. D. Peebles. 2010. Effects of in ovo injection of L-carnitine on subsequent broiler chick tissue nutrient profiles. Poult. Sci. 89:335–341.

Kidd, M. T., J. W. Taylor, C. M. Page, B. D. Lott, and T. N. Chamblee et al., 2007. Hatchery feeding of starter diets to broiler chicks. J. Appl. Poult. Res. 16:234–239.

Rada, V., M. Foltyn, A. Musilová, and M. Lichovníková. 2013. The effect of feeding chicken during transport. 19th European Symposium on Poultry Nutrition.1. Potsdam: The German Branch of the WPSA. 105–114.

Liu, A. C. H., B. S. Heinrichs, and R. M. Leach. 1994. Influence of manganese deficiency on the characteristics of proteoglycans of avian epiphyseal growth plate cartilage. Poult. Sci. 73: 663–669.

Manangi, M. K., M. Vazquez-Anon, J. D. Richards, S. Carter, R. E. Buresh, and K. D. Christensen. 2012. Impact of feeding lower levels of chelated trace minerals vs. industry levels of inorganic trace minerals on broiler performance, yield, foot pad health and litter mineral concentration. J. Appl. Poult. Res. 21: 881–890.

McGruder, B. M., W. Zhai, M. M. Keralapurath, L. W. Bennett, P. D. Gerard, and E. D. Peebles. 2011. Effects of in ovo injection of electrolyte solutions on the pre- and posthatch physiological characteristics of broilers. Poult. Sci. 90:1058–1066.

Molenaar, R., I. A. M. Reijrink, R. Meijerhof, and H. Van den Brand. 2010. Meeting embryonic requirements of broilers throughout incubation: A review. Braz. J. Poult. Sci. 12:137–148.

Ohta, Y., N. Tsushima, K. Koide, M. K. Kidd, and T. Ishibashi. 1999. Effect of amino acid injection in broiler breeder eggs on embryonic growth and hatchability of chicks. Poult. Sci. 78:1493–1498.

Oliveira, T. F. B., A. G. Bertechini, R. M. Bricka, E. J. Kim, P. D. Gerard, and E. D. Peebles. 2015. Effects of in ovo injection of organic zinc, manganese, and cooper on the hatchability and bone parameters of broiler hatchlings. Poult. Sci. 0:00–00.

Pinheiro, D. F., V. C. Cruz, J. R. Sartori, and M. L. M. Vicentini Paulino. 2004. Effect of early feed restriction and enzyme supplementation on digestive enzyme activities in broilers. Poult. Sci. 83:1544–1550.

Rath, N. C., G. R. Huff, W. E. Huff, and J. M. Balog. 2000. Factors Regulating Bone Maturity and Strength in Poultry. Poult. Sci. 79:1024–1032.

Romanoff, A. L. 1960. The Avian Embryo: Structural and Functional Development. Macmillan Co., New York, NY.

SAS Institute Inc. 2010. SAS Proprietary Software Release 9.2. SAS Inst. Inc., Cary, NC.

Sauer, G. R., L. N. Wu, M. Iijima, and R. E. Wuthier. 1997. The influence of trace elements on calcium phosphate formation by matrix vesicles. J. Inorg. Biochem. 65:57–65.

Starcher, B. C., C. H. Hill, and J. G. Madaras. 1980. Effect of zinc deficiency of bone collagenase and collagen turnover. J. Nutr. 110:2095–2102.

Simkiss, K. 1961. Calcium metabolism and avian reproduction. Biol. Rev. 36:321–367.

Shim, M. Y., A. B. Karnuah, A. D. Mitchell, N. B. Anthony, G. M. Pesti, and S. E. Aggrey. 2012. The effects of growth rate on leg morphology and tibia breaking strength, mineral density, mineral content, and bone ash in broilers. Poult. Sci. 91:1790–1795.

Sklan, D., and Y. Noy. 2000. Hydrolysis and absorption in the small intestines of posthatch chicks. Poult. Sci. 79:1306–1310.

Star, L., J. D. Van der Klis, C. Rapp, and T. L. Ward. 2012. Bioavailability of organic and inorganic zinc sources in male broilers. Poult. Sci. 12:3115–3120.

Tuan, R. S., and T. Ono. 1986. Regulation of extraembryonic calcium mobilization by the developing chick embryo. J. Embryol. Exp. Morphol. 97:63–74.

US-EPA. 1986. Method 3051. Microwave assisted acid digestion of sediments, sludges, and soils, and oils. In: Test Methods for Evaluating Solid Waste. U.S. Environmental Protection Agency, Washington, DC.

Yair, R., and Z. Uni. 2011. Content and uptake of minerals in the yolk of broiler embryos during incubation and effect of nutrient enrichment. Poult. Sci. 90:1523–1531.

Yair, R., R. Shahar, and Z. Uni. 2013. Prenatal nutritional manipulation by in ovo enrichment influences bone structure, composition and mechanical properties. J. Anim. Sci. 91:2784–2793.

Zhai, W., L. W. Bennett, P. D. Gerard, R. Pulikanti, and E. D. Peebles. 2011a. Effects of in ovo injection of carbohydrates on somatic characteristics and liver nutrient profiles of broiler embryos and hatchlings. Poult. Sci. 90:2681–2688.

Zhai, W., P. D. Gerard, R. Pulikanti, and E. D. Peebles. 2011b. Effects of in ovo injection of carbohydrates on embryonic metabolism, hatchability, and subsequent somatic characteristics of broiler hatchlings. Poult. Sci. 90:2134–2143.

Zhai, W., D. E. Rowe, and E. D. Peebles. 2011c. Effects of commercial in ovo injection of carbohydrates on broiler embryogenesis. Poult. Sci. 90:1295–1301.

Zhan, X. A., M. Wang, H. Ren, R. Q. Zhao, J. X. Li, and Z. L. Tan. 2007. Effect of early feed restriction on Metabolic Programming and Compensatory Growth in broiler chickens. Poult. Sci. 86:654–660.