TIAGO FERREIRA BIRRO OLIVEIRA

EFEITO DA NUTRIÇÃO MINERAL *IN OVO* SOBRE O DESENVOLVIMENTO ÓSSEO E DESEMPENHO EM FRANGOS DE CORTE

LAVRAS – MG

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Tese apresentada à Universidade Federal de Lavras, como parte das exigências do Programa de Pós-Graduação em Zootecnia, área de concentração em Produção e Nutrição de Não Ruminantes, para a obtenção do título de Doutor.

Prof. Dr. Antônio Gilberto Bertechini

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RESUMO GERAL

O objetivo deste estudo foi avaliar os efeitos da injeção *in ovo* com diluente comercial contendo microminerais suplementares (Zn, Mn, e Cu) em associação com o tempo de retenção pós-eclosão, tempo de retenção (HT) na percentagem de cinzas ósseas (PBA) e concentração de minerais na tíbia de frangos de corte da linhagem Ross 708. Os ovos foram submetidos a 4 tratamentos usando um injector multi-ovo comercial no 17º dia de incubação. Os tratamentos incluíram não injectada (tratamento 1) e diluente (tratamento 2) como grupos de controle. As aves do tratamento 3 receberam diluente contendo 0,181, 0,087 e 0,010 mg/ml de Zn, Mn e Cu, respectivamente, e as aves do tratamento 4 receberam diluente contendo 0,544, 0,260 e 0,030 mg / ml de Zn, Mn e Cu, respectivamente. As aves dos 4 tratamentos, após a fase de incubação, foram, em seguida, sub-divididas em 2 grupos pós eclosão. Quinze aves foram alocados aleatoriamente para cada uma das 6 repetições, em cada um dos 8 TRT. O primeiro grupo HT teve acesso imediato à água e alimentação, e o segundo grupo HT foi constituído por aves que foram mantidos em cestas de transporte durante 24 h antes de serem liberadas. A eclodibilidade dos ovos férteis (HF) foi determinada em 20,5 e 21,5 dias de incubação. Em 21,5 dias de incubação, a HF e a eclosão peso do pinto (MHW) foram determinados. O peso fresco, peso seco, comprimento, largura das tibias, resistência óssea à ruptura (BBS) e percentagem de cinzas ósseas (PBA) foram também determinados. O efeito de tratamento sobre a injecção de HF em 21,5 dias de incubção foi significativo. A HF em 21,5 dias de incubação do tratamento 4 foi significativamente mais baixa do que a do grupo controle não-injetado, sendo o tratamento 3 intermediário. Os embriões de ovos que receberam tratamento 4 tiveram um PBA significativamente maior em comparação com todos os outros tratamentos. A nutrição *in ovo* destes minerais orgânicos influenciou positivamente a mineralização óssea.

Palavras-chave: Cinza. Qualidade óssea. Suplementação de ovo. Mineralização. Pós-nascimento.

GENERAL ABSTRACT

Effects of the *in ovo* injection of commercial diluent containing supplemental microminerals (Zn, Mn, and Cu) on hatchability, hatching chick quality variables and the *in ovo* injection of organic Zn, Mn and Cu in association with post-hatch (poh) holding time (HT; feed and water restriction) on percentage of bone ash (PBA) and the concentration of minerals in the tibia of broilersin Ross × Ross 708 broilers were examined. On 17 d of incubation (doi) eggs were subjected to 1 of 4 treatments using a commercial multi-egg injector. Treatments included non-injected(treatment 1) and diluent-injected (treatment 2) control groups. Those in treatment 3 received diluent containing 0.181, 0.087 and 0.010 mg/ml of Zn, Mn and Cu, respectively, and those in treatment 4 received diluent containing 0.544, 0.260 and 0.030 mg/ml of Zn, Mn and Cu, respectively. The4 TRT groups from the incubation phase were then sub-divided into 2poh HT groups. Fifteen birds were randomly allocated to each of 6 replicate mini pens in each of the 8 (4x2) TRT. 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.Hatchability of fertile eggs set (HF) was determined on 20.5 and 21.5 doi. On 21.5 doi,HF and mean hatching chick weight (MHW) were determined. The tibiae fresh and dry weight, length, width, bone breaking strength (BBS) and percentage of bone ash (PBA) were determined. There was a significant injection treatment effect on HF at 21.5 doi. The HF of eggs at 21.5 doi in treatment 4 was significantly lower than that of the non-injected control group, with treatment3 being intermediate. However, embryos from eggs that received treatment 4 had a significantly higher PBA in comparison to all other treatment. The *in ovo* injection of these organic minerals had a positive influence on bone mineralization.

Key words: Ash. Bone quality. *In ovo* supplementation. Mineralization. Posthatch.

SUMÁRIO

PRIMEIRA PARTE

1 INTRODUÇÃO

Problemas de pernas em frangos de corte têm causado perdas significativas para a indústria avícola, apesar de todo o investimento em pesquisa a respeito do desenvolvimento ósseo nos últimos tempos. Os problemas como a capacidade limitada de andar tem causado queda na produção devido à redução do consumo de água e de ração, aumento da frequência de condenações no abate e mortalidade elevada. Assim, é razoável supor que os problemas de perna ainda são motivo de grandes prejuízos econômicos. Além disso, os problemas nas pernas dos frangos de corte podem afetar drasticamente o seu bem-estar, induzindo dores aguda e crônica, e influenciando a resposta produtiva da ave. Esses problemas mostram a real necessidade de se entender a formação óssea do frango de corte. Desenvolver técnicas que aumentem a qualidade óssea torna-se importante visando diminuir os problemas causados pela sua má formação.

A maior parte dos minerais presentes no ovo são consumidos até ao 17º dia de incubação, deixando baixos níveis na gema residual. A gema residual é a maior fonte de energia e nutrientes durante o período de transição da fase embrionária e pós-eclosão. Além disso, de acordo com os técnicos de campo, a maior parte dos pintainhos produzidos somente tem acesso à alimentação após 36 a 48 horas pós-eclosão. Durante esse período, essas aves passam por uma demanda metabólica extremamente elevada, e a baixa concentração de minerais na gema residual pode prejudicar o desenvolvimento de órgãos e sistemas vitais durante este período. O baixo consumo de minerais até o dia da eclosão pode ser manipulado através da suplementação *in ovo* de minerais e outros nutrientes específicos como a vitamina D.

Os problemas locomotores e a má formação óssea são também causados pelo crescimento acelerado das linhagens modernas e pelo elevado peso do peito do frango que pode causar desajuste no centro de gravidade da ave. A seleção genética para o consumo de ração e deposição de carne prejudicou a mineralização dos ossos, tornando-os mais porosos, finos e frágeis, sendo mais susceptíveis a quebras ou outros problemas locomotores que podem prejudicar as aves no acesso à água e ração. As aves com tais problemas passam a maior parte do tempo sentadas, e às vezes não conseguem se levantar para alcançar o bebedouro ou o comedouro, pois, o seu crescimento não acompanhou o das outras aves. A condenação destas aves pode ocorrer no estágio avançado de produção, no qual aumenta ainda mais o prejuízo, pois estas aves podem ter consumido ração durante várias semanas. A ração na avicultura moderna é responsável por mais de 70% do custo de produção, sendo então importante evitar estas situações.

Assim, a presente pesquisa foi desenvolvida com o objetivo de avaliar os efeitos da injeção in ovo de minerais durante a fase final de incubação sobre o desenvolvimento embrionário e qualidade e desempenho e qualidade óssea das aves na fase pós-eclosão.

2 REFERENCIAL TEÓRICO

2.1 Desenvolvimento embrionário

O desenvolvimento embrionário é a base para a qualidade dos pintos de um dia. Segundo Moran Junior (2007), o desenvolvimento embrionário apresenta as fases de criação do germe, a conclusão da formação embrionária e a preparação para a emergência.

Durante o estabelecimento do germe, o embrião e as suas estruturas de sustentação retomam a proliferação de células dos 40.000 a 60.000 células já presentes na oviposição (FASENKO, 2007). A virada do ovo durante este período é crucial para permitir a formação adequada dos compartimentos do ovo e dar ao embrião acesso à glicose presente na membrana exterior. A membrana do saco vitelino seleciona os nutrientes sendo até retirados de suas reservas, o que inclui lipídios, proteínas, minerais e vitaminas. A membrana do saco vitelino pode também modificar esses nutrientes e servir como armazenamento de curto prazo.

O segundo terço de incubação caracteriza-se por um sistema vascular plenamente desenvolvido, com o *chorioalantois* capaz de assegurar o intercâmbio de O_2-CO_2. O embrião cresce muito rapidamente em tamanho durante essa fase. Os ácidos graxos essenciais são preservados para a síntese da membrana celular enquanto os ácidos graxos saturados são consumidos para sustentar as crescentes necessidades calóricas de tecidos formados. O embrião passa então por um outro período crítico, o da transição para a emergência.

Na preparação para a emergência, o tamanho e os movimentos embrionários causam a ruptura da membrana que separa o albúmen e o fluido amniótico. Em seguida, o embrião consome o fluido amniótico por via oral, que passa através do sistema gastrointestinal. Nessa fase de desenvolvimento intestinal, enterócitos do duodeno e o jejuno são capazes de absorver

macromoléculas de proteína, num processo semelhante à absorção do colostro de mamíferos. Tal consumo continua até que o líquido amniótico com albumina desapareça e a bicagem interna comece. Dessa forma, o desenvolvimento de tecido esquelético embrionário é completado nesse ponto, os nutrientes absorvidos são usados para os órgãos viscerais e a maior parte é armazenada como glicogénio.

A emergência começa quando o embrião quebra alantoide e a parte interna da membrana perto do saco aéreo, o que é chamado de bicagem interna.

Neste ponto, o embrião deve iniciar a respiração pulmonar, uma vez que a membrana da casca exterior perde contato com o reservatório. Este é um período crítico porque a oferta limitada de oxigênio suprime a utilização contínua de lipídios como fonte de energia, de modo que o metabolismo muda novamente para o catabolismo anaeróbico da glicose a partir de reservas de glicogênio produtoras de lactato. O saco vitelino restante é retraído para dentro da cavidade abdominal, e o sangue periférico é recuperado para o embrião. A relativa grande quantidade de energia é usada para sustentar movimentos de bicagem do embrião para quebrar a casca e girar o corpo. O acesso ao ar externo, neste momento, fornece oxigênio suficiente para a oxidação de ácidos graxos e recuperação de lactato no fígado. O embrião continua a quebrar a casca, girar e, usando os pés, empurrar até que esteja livre da concha.

2.2 Nutrição *in ovo*

A vacinação *in ovo*, iniciada nos anos 80 contra a doença de Marek (SHARMA; BURMESTER, 1982), provou ser eficaz contra a exposição pós-eclosão das aves ao vírus. A vacinação *in ovo* é considerada uma das técnicas que mais contribuíram para a criação de aves e, ainda hoje, quase quatro décadas depois, esse tema ainda ocupa um espaço ativo nos principais periódicos do mundo.

Em 2003, Uni e Ferket introduziram o conceito de administração de alto volume (0,4 - 1,2 ml) de nutrientes inseridos no líquido amniótico dos ovos, com o objetivo de enriquecer o conteúdo disponível ao embrião, que consome o líquido amniótico antes de eclodir (UNI; FERKET, 2003). Seus estudos, focados na nutrição *in ovo*, visavam obter vantagens comparativas, entre as quais a reduzida mortalidade e mobilidade pós-eclosão, melhoria da resposta imune, redução da incidência de distúrbios do esqueleto de desenvolvimento, maior desenvolvimento muscular e rendimento de carne de peito. Uni e Ferket destacaram vantagens provenientes da técnica de nutrição *in ovo*, a saber, desenvolvimento intestinal melhorada e capacidade digestiva (BOHORQUEZ; SANTOS JUNIOR; FERKET, 2007; SMIRNOV et al., 2006), aumento da taxa de crescimento, melhoria da eficiência alimentar (KORNASIO et al., 2011), melhoria da mineralização óssea (YAIR; SHAHAR; UNI, 2013) e melhoria do rendimento de carne de peito (KORNASIO et al., 2011).

Os efeitos positivos foram observados como soluções de nutrição *in ovo*, contendo NaCl, sacarose, butirato de maltose, dextrina e beta-hidroxi-beta-metil, arginina, proteína de clara de ovo, e Zn-metionina. Muitos outros grupos de pesquisa do Brasil, EUA, China e outros países estão utilizando esta metodologia e apontam para as mesmas vantagens.

Os frangos de corte modernos estão sendo submetidosà seleção genética para altas taxas de crescimento ao longo do tempo, resultando em melhorias anuais no ganho de peso vivo (devido ao aumento da massa muscular), na eficiência alimentar e nos rendimentos de carne. No entanto, com essas melhorias, tornou-se evidente que alguns sistemas, como o esquelético, não acompanharam o aumento da massa muscular (DIBNER et al., 2007). As linhagens atuais de frangos de corte comerciais são capazes de quadruplicar o seu peso de eclosão até ao final da primeira semana de vida e ganho de peso diário de cerca de 70 g até 40 dias de idade. Apesar dessa taxa de crescimento

pós-eclosão rápida alcançada por meio da seleção ao longo dos últimos 50 anos, o período de tempo que passa um pintinho dentro do ovo durante a incubação manteve-se essencialmente o mesmo.

O segmento referente a incubação é relativamente grande em relação a fase de criação do frango. Assim, torna-se importante conhecer como o desenvolvimento embrionário pode afetar o desempenho da ave no período pós-eclosão. Como uma espécie ovípara, os embriões de galinha dependem exclusivamente dos fosfolipídios e nutrientes à base de proteínas embutidas na gema de ovo como seu reservatório de nutrientes. A nutrição embrionária pode ter um efeito pronunciado no desempenho da progênie. As insuficiências nutricionais durante o período embrionário e início da vida podem induzir respostas adaptativas com consequências adversas de longa duração.

Além da energia, aminoácidos e vitaminas, os minerais podem contribuir com a nutrição do embrião e influenciar na sua boa formação óssea inicial. Os frangos de corte têm apresentado vários problemas ósseos estreitamente associado à sua taxa de crescimento rápido (ANGEL, 2007; DIBNER et al., 2007; SHIM et al., 2012). Os problemas ósseos têm causado perdas econômicas importantes, além de afetar o bem-estar das aves. Afim de reduzir essa incidência, foram feitas tentativas em selecionar frangos para o melhor desenvolvimento do esqueleto nos últimos anos (WILLIAMS; MURRAY; BRAKER, 2000). Algum progresso foi relatado por Kapell et al. (2012), que mostraram que a seleção rigorosa das aves com base em estratégias de abate com avaliação clínica, tem conduzido a uma redução na incidência de alguns defeitos nas pernas, tais como discondroplasia tibial (DT) e dedos dos pés curvados.

Apesar desse esforço no processo de melhoramento genético, estudos ainda têm mostrado que os frangos de corte de crescimento rápido têm alta incidência de problemas de pernas. Dinev et al. (2012) verificaram que 24,22 -

27,70% de frangos de corte de três linhas comerciais sofrem de algum grau de TD. Além disso, problemas nas pernas podem ser afetados pela alimentação e manejo, não apenas pela taxa de crescimento.

Ao contrário dos mamíferos, o embrião de pintainhos de corte desenvolve independentemente da galinha. Consequentemente, a deposição dos nutrientes nos armazenamentos são limitados ao ovo e, por isso, é crucial para o bom desenvolvimento embrionário. Desta forma, a deposição de minerais para os diferentes compartimentos do ovo é fundamental para o desenvolvimento embrionário devido à participação destes no desenvolvimento do esqueleto, sistema imunológico, muscular, e sistemas cardiovascular do embrião (FAVERO et al., 2013; OVIEDO-RONDÓN et al., 2013). A deposiçãode minerais no ovo acontece por duas vias: do ovário para a gema ou através do oviduto ao albúmen, casca e membrana da casca (RICHARDS; PACKARD, 1997). Cada um desses compartimentos contém uma variedade de diferentes minerais. A casca contém quantidades elevadas de Ca e baixas quantidades de Fe, Mg, Mn, P, e Zn. No entanto, apenas grandes quantidades de Ca, uma quantidade muito menor de Mg, e quantidades insignificantes de Fe, Mn, e P são liberados a partir da casca e disponibilizados para o embrião. A gema é a principal fonte de minerais para o embrião durante a incubação, contendo a maior parte do P, Zn, Cu, Mn, e Fe, enquanto que o albúmen é a principal fonte de Na e K (YAI; UNI, 2011). Dibner et al. (2007) demonstraram que a falta de Cu, Mn, P e Zn durante o período embrionário e pós-eclosão prejudica o desenvolvimento do osso. Do mesmo modo, a maioria das propriedades mecânicas e geométricas da tíbia e do fêmur permanecem inalterados ou até mesmo deterioram-se durante esse período (YAIR; SHAHAR; UNI, 2013). Em conformidade, foi sugerido anteriormente que a limitada disponibilidade de minerais durante o período embrionário e nas primeiras semanas após a eclosão limita o desenvolvimento do esqueleto durante o seu período de rápido

crescimento, aumentando assim a incidência de problemas de pernas (DIBNER et al., 2007; YAIR; SHAHAR; UNI, 2013).

Trabalhos publicados anteriormente demonstraram que o enriquecimento embrionário com Cu, Fe, Mn, e Zn, fosfato, a vitamina D_3 e carboidratos, utilizando a metodologia de nutrição in ovo (UNI; FERKET, 2003, 2004) aumentou o teor destes minerais na gema e seu consumo pelo embrião pré-eclosão (YAIR; UNI, 2011). No entanto, não deixa claro se este efeito é devido ao enriquecimento com minerais *per se* ou devido à forma biológica dos minerais adicionados, a vitamina D_3, carboidratos, ou se realmente somente a combinação.

2.3 Incubação e pós eclosão

É comum na avicultura em todo o mundo, manter pintainhos sem alimento e água por muitas horas após a eclosão. Os primeiros pintainhos que nascem, podem permanecer por até 36 horas após a eclosão, antes de serem retirados do nascedouro e, então, a ave pode levar um adicional de 24-36 horas antes de ter acesso à alimentação e água.

Foram realizados vários estudos para avaliar o impacto do jejum no início do desenvolvimento, comparando pintainhos que foram alojadas durante 24 horas com aqueles que tiveram acesso *ad libitum* aos alimentos e água imediatamente depois de terem sido retirados do nascedouro. Careghi et al. (2005) observaram que pintainhos alimentados imediatamente após o nascimento apresentaram maior ganho de peso, em comparação com o lote que obteve acesso a ração prontamente após a saída do nascedouro.

A restrição alimentar no início da vida pode mais tarde causar um estado de obesidade na vida das aves (ZHAN et al., 2007), alterando permanentemente a produção de enzimas relacionadas com a energia e suas funções. Velleman e

Mozdziak (2005) obtiveram o crescimento muscular reduzido entre os pintos que experimentaram 72 horas de jejum após o nascimento.

2.4 Janela de nascimento

Janela de nascimento é definido como o tempo que leva a partir da primeira eclosão até o momento da retirada do lote. A janela de nascimento ampla pode exceder 36 a 48 horas

O tempo ótimo para retirar os pintainhos do nascedouro é muitas vezes difícil de determinar, pois, considerando uma janela de nascimento ampla, os embriões não nascem todos ao mesmo tempo. Se os pintainhos são retirados do nascedouro muito cedo, muitos embriões em estado final para eclosão são desnecessariamente eliminados. Mas, se retirados muito tarde, muitos dos nascidos primeiros sofrerão de desidratação e empobrecimento das suas reservas de energia, comprometendo assim o desempenho e o peso final. A determinação do momento ideal de retirada é muito mais fácil com uma janela de nascimento pequena, e a menos problemas de qualidade. A duração da janela de nascimento pode ser afetada por vários fatores como a idade da matriz, temperatura de incubação, tempo de armazenamento de ovo e localização na incubadora. Wyatt, Weaver Junior e Beane (1985) relataram que pintos de corte de aves mais velhas começaram a nascer 6 horas mais cedo do que pintainhos de matrizes mais jovens.

A posição na incubadora afeta a temperatura do ovo devido a diferenças no fluxo de ar. A temperatura da casca do ovo pode ser mais elevada do que a temperatura da incubadora, especialmente após a primeira metade do período de incubação, quando o embrião já produz calor, sujeitando assim os embriões a estresse térmico.

Alguns especialistas em gerenciamento de incubatório estão sugerindo uma maneira revolucionária para incubar e lidar com pintos para minimizar o

atraso na alimentação. Os ovos são transferidos para unidades especiais onde possam nascer com a alimentação e água disponíveis, e onde permanecem por um dia antes de serem enviados para as granjas.

2.5 Incubação e microminerais

A maior parte dos minerais presentes no ovo estão localizados na gema e alguns podem ter suas concentrações manipuladas por meio da dieta materna (KIDD, 2003). A nutrição mineral das matrizes vem sendo pesquisada com algun sucesso. Alternativamente, alguns pesquisadores averiguaram que fornecendo dietas com níveis elevados de níquel, cobre e ferro não eleva a concentração mineral dos ovos (STAHL; COOK; GREGER, 1988). Dessa forma, torna-se interessante o desenvolvimento de novas tecnologias e estratégias que possibilitem elevar mais eficientemente a concentração desses minerais no ovo, e a dos minerais que tiveram resultados por meio da nutrição materna, podendo melhorar os índices de produção.

O cálcio que o embrião da ave requer é sucessivamente mobilizado a partir da gema e, em seguida, a partir da casca do ovo por meio do saco vitelino e membranas corioalantóicas, respectivamente (ONO; TUAN, 1991). Além disso, a suplementação *in ovo* no reservatório de nutrientes do embrião com suplementos tais como a vitamina D_3 (BELLO et al., 2013), carboidratos (ZHAI et al., 2008) e aminoácidos (OHTA; KIDD; ISHIBASHI, 1999), foi relatado ser benéfico para a eclodibilidade e desenvolvimento durante os períodos de incubação e pós-eclosão.

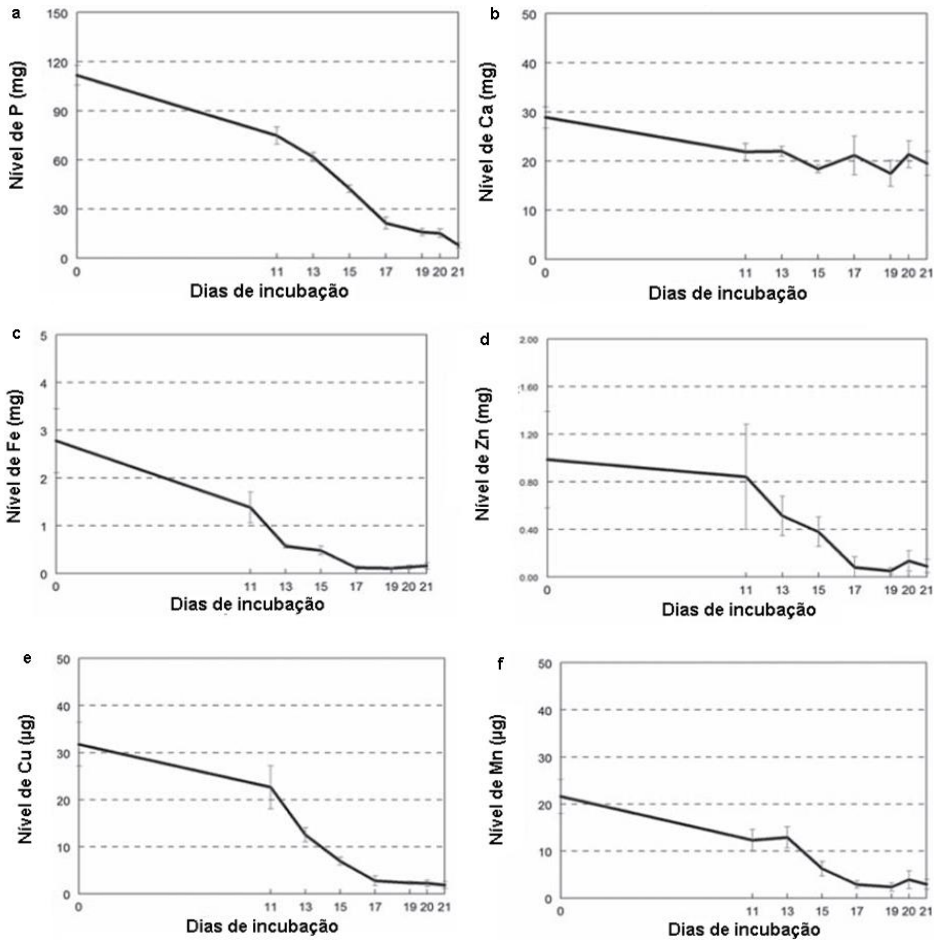
Yair, Shahar e Uni (2013) mostraram que a injeção de uma solução de enriquecimento contendo microminerais orgânicos e vitaminas, incluindo a vitamina D_3, no âmnio de ovos de frango no 17º dia de incubação, melhorou as propriedades físicas dos ossos do lote que recebeu a solução.

Bello et al. (2013) relataram que as concentrações séricas de 25-hidroxicolecalciferol [25(OH)D_3], um precursor estável do metabólito 1,25- di-hidroxicolecalciferol, no 19º dia de incubação foram aumentadas em três vezes devido a injeção in ovo de 0,60 g de 25 (OH) D_3 no âmnio no 18º dia de incubação. No mesmo estudo, demonstrou-se que a injeção *in ovo* de 0,60 ug 25 (OH) D_3 no 18º dia de incubação mostrou-se capaz de minimizar atrasos na taxa de eclosão de pintos de corte, quando comparado com a injeção *in ovo* de diluente comercial. A utilização da dosagem de 0,60 ug de 25 (OH) D3 em diluente comercial não demonstraram efeitos negativos sobre a embriogenese, desenvolvimento ósseo ou sobrevivência do embrião.

É importante lembrar que o embrião possui recursos minerais limitados para o desenvolvimento esquelético e esses recursos são também requisitados para outras funções fisiológicas e do desenvolvimento embrionário. A disponibilidade de minerais, particularmente a combinação de Zn, Cu e Mn, possui um papel crítico no desenvolvimento prematuro devido às suas funções integradas a metaloenzimas que participam na formação de tecidos estruturais conectivos (DIBNER et al., 2007).

O consumo relativo total de minerais durante a incubação (figura 1) foi calculada por Yair e Uni (2011) dividindo a quantidade de mineral consumida pelo embrião no dia da amostragem pela quantidade mineral total no dia da oviposição. Os gráficos demonstram um consumo acelerado dos mineras entre o 11º e o 17º dias de incubação. Nos últimos dias de incubação, a quantidade de P, Fe, Zn, Cu e Mn na gema é bastante reduzida, cessando o consumo pelo embrião. Enriquecer o ovo com nutrientes a partir do 17º dia de incubação, possivelmente permitirá o aumento do consumo desses minerais pelo embrião.

Figura 1 - Conteúdo da gema para P (a), Ca (b), Fe (c), Zn (d), Cu (e), e Mn (f) durante a incubação



Fonte: Yair e Uni (2011).

A habilidade de modificar a quantidade de minerais pode estar mais relacionada com a proporção dos componentes do ovo (aumentar o tamanho da gema) do que a concentração mineral propriamente dita (ANGEL, 2007). Entretanto, o que se tem mostrado promissor é modificar a forma química dos minerais, melhorando a sua utilização pelo embrião e a nutrição *in ovo*; fornecendo suplementação nutritiva durante a incubação. Nutrientes e outros

componentes metabólicos utilizados na injeção *in ovo*, como aminoácidos, carboidratos e vitaminas, têm sido pesquisados por diversos grupos de pesquisadores (FOYE; UNI; FERKET, 2006; KADAM et al., 2008; KERALAPURATH et al., 2010; TAKO; FERKET; UNI, 2004; et al., 2004; UNI et al., 2005; ZHAI et al., 2008). Tem sido mostrado também que a injeção desses suplementos pode beneficiar o crescimento pós-eclosão e aumentar o ganho de peso das aves.

O âmnion embrionário das aves tem se mostrado ser um local eficiente para a injeção in ovo (JOCHEMSEN; JEURISSEN, 2002; KERALAPURATH et al., 2010; ZHAI et al., 2008). Durante a embriogênese, soluções injetadas no líquido amniótico são subsequentemente deglutidos, digeridos e absorvidos pelo embrião (UNI et al., 2005). A suplementação de nutrientes *in ovo* pode ajudar o desenvolvimento embrionário na fase mais avançada do seu desenvolvimento, quando a concentração nutritiva do ovo já se encontra reduzida.

Minerais como Cu, Mn e Zn são essenciais para o desenvolvimento normal de frangos de corte, pois estão envolvidos em inúmeros processos digestivos e fisiológicos no corpo. Esses minerais fazem parte da estrutura de enzimas que participam em processos metabólicos importantes e fazem parte de proteínas que envolvem o metabolismo, secreção de hormônios e funcionamento do sistema imune (BAO et al., 2007).

2.5.1 Zinco

O Zn participa de importantes vias de regulação da cristalização da hidroxiapatita (SAUER et al., 1997), síntese de colágeno (STARCHER; HILL; MADARAS, 1980), e invasão celular da matriz cartilaginosa pelos osteoblastos (NIE et al., 1998). Essa invasão requer a atividade de moléculas chamadas de metaloproteinase da matriz, principalmente a colagenase-3, a qual contém o Zn na sua estrutura (ORTH, 1999). O fato de o fluxo intracelular de Zn ser

associado a apoptose nos condrócitos do disco epifisário sugere que o Zn possui um importante papel na ossificação endocondral (SAUER et al., 2003).

O Zn é necessário para a proliferação e diferenciação dos condrócitos. Durante a proliferação em especial, a necessidade de Zn pode ser elevada (OVIEDO-RONDÓN; FERKET; HAVENSTEIRN, 2006). A deficiência de Zn em um curto período de tempo pode inibir a proliferação de condrócitos, diferenciação celular e induzir a apoptose celular durante o crescimento do disco epifisário (WANG et al., 2002). A biodisponibilidade do micromineral é importante e varia de acordo com a fonte (CAO et al., 2000), e o nível depositado no osso aumenta conforme se eleva a concentração dietética. Minerais orgânicos e quelatados tem se mostrado mais eficientes que a forma inorgânica, melhorando o desempenho e a saúde animal, independente do nível suplementado (KIDD et al., 1994).

A formação óssea insuficiente observada durante os primeiros dias pós-eclosão é comumente ligada à nutrição materna imprópria ou problemas de absorção durante o processo embrionário (OVIEDO-RONDÓN; FERKET; HAVENSTEIN, 2006). Kidd, Anthony e Lee (1992) constataram que a progênie de matrizes alimentadas com dietas suplementadas com zinco-metionina obtiveram um aumento no conteúdo mineral da tíbia, quando comparado a fonte inorgânica do mineral. Entretanto esse aumento foi limitado.

2.5.2 Cobre

O Cu é um mineral amorfo da matriz óssea, que possui a característica de prevenir a sua cristalização prematura. Possui um papel importante na ligação da elastina com o colágeno, o que confere capacidade elástica e tensil ao osso (CARLTON; HENDERSON, 1964).

Mesmo o Zn sendo importante na síntese de colágeno, a menos que haja suficiente Cu presente, as fibrilas não serão devidamente formadas e o resultado

são estruturas enfraquecidas que podem ser rompidas (RATH; HUFF; BALOG, 2000). O desenvolvimento apropriado de tecidos conectivos é importante e necessário não somente nos ossos, mas em órgãos como o intestino que possui a capacidade de adaptar a mudanças no volume da digesta (DIBNER et al., 2007).

Fontes orgânicas de Cu, como complexados a aminoácidos ou quelatados têm sido desenvolvidas para serem utilizadas na nutrição animal. A biodisponibilidade dessas fontes orgânicas de Cu varia de 88 a 147%, em resposta ao sulfato de cobre. Uma das características consideradas mais importantes destes minerais complexados na função fisiológica é o nível com o qual essas ligações se mantêm intactas durante a digestão e absorção.

A disponibilidade de Cu pode ser significativamente reduzida pela presença de elementos antagonistas na dieta, incluindo o Zn e o Fe (ABDEL-MAGEED; OEHME, 1991). Para reduzir esses efeitos adversos na disponibilidade de Cu, a sua suplementação é necessária. A deficiencia em Cu causa má formação do colágeno e diminui a mineralização (OSPHAL et al., 1982). Banks et al. (2004) observaram que, mesmo não achando diferença significativa para ganho de peso quando as dietas de frangos de corte são suplementadas com fontes orgânica e inorgânica de Cu, a fonte orgânica resultou em maior porcentagem de cinzas do que a fonte inorgânica.

2.5.3 Manganês

O Mn é um mineral essencial para a formação de mucopolissacarídeos, substâncias que compõem o modelo de cartilagem do osso. A sua deficiência causa anormalidades embrionárias e reduz a eficiência de eclosão (BAIN; WATKINS, 1993). Pintos com níveis inadequados de Mn possuem menos proteoglicanos na cartilagem do disco epifisário da tíbia do que pintos recebendo níveis adequados do mineral (LIU et al., 1995).

A suplementação de Mn tornou-se uma crescente preocupação devido ao aumento extremamente rápido da taxa de crescimento das linhagens modernas de frango de corte, o qual adiciona estresse na estrutura do osso (JI et al., 2006). Já foi comprovado que a fonte orgânica de Mn é mais biodisponível que a fonte inorgânica (LU et al., 2006). Metionina é o primeiro aminoácido limitante para frangos de corte, por isso, é a forma mais comumente utilizada de metal-aminoácido na produção avícola, que é capaz de ser absorvido pelas células da mucosa intestinal e conduzido através da parede intestinal, mantendo a sua estrutura intacta (JI et al., 2006).

2.6 Desenvolvimento ósseo e nutrição in ovo

Recentemente, a alimentação *in ovo* com minerais, vitaminas e carboidratos foi pesquisada; mostrando-se capaz de elevar os níveis de minerais e o seu consumo a partir da gema durante o período de pré-eclosão (Yair et al., 2011). Esta suplementação de nutrientes para os embriões de frangos de corte mostrou um efeito significativo no seu desenvolvimento esquelético, uma vez que os minerais e vitaminas incluídos no ovo em solução são importantes para o desenvolvimento ósseo.

O presente estudo foi desenvolvido visando examinar o efeito do enriquecimento *in ovo* com minerais sobre as propriedades estruturais, mecânicas e de composição dos ossos longos do período embrionário até a maturidade. Os resultados mostraram que, em geral, houve um efeito positivo sobre os ossos de frangos que receberam a alimentação *in ovo*. O trabalho demonstra também a potencial influência da nutrição embrionária sobre o desempenho, tanto a curto prazo, pré-eclosão e a longo prazo. Além disso, otimizando o teor de solução de alimentação in ovo, poderá reforçar o efeito da alimentação in-ovo no desenvolvimento ósseo e suas propriedades.

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SEGUNDA PARTE - ARTIGOS

ARTIGO 1 - EFFECTS OF *IN OVO* INJECTION OF ORGANIC ZINC, MANGANESE, AND COPPER ON THE HATCHABILITY AND BONE PARAMETERS OF BROILER HATCHLINGS

Formatado de acordo com a norma do periódico Poultry Science e adaptado a formatação da UFLA.

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ABSTRACT Effects of the *in ovo* injection of commercial diluent containing supplemental microminerals (Zn, Mn, and Cu) on hatchability and hatching chick quality variables in Ross × Ross 708 broilers were examined. On 17 d of incubation (doi) eggs were subjected to 1 of 4 treatments using a commercial multi-egg injector. Treatments included non-injected(treatment 1) and diluent-injected (treatment 2) control groups. Those in treatment 3 received diluent containing 0.181, 0.087 and 0.010 mg/ml of Zn, Mn and Cu, respectively, and those in treatment 4 received diluent containing 0.544, 0.260 and 0.030 mg/ml of Zn, Mn and Cu, respectively. A total of 1,872 eggs were distributed among 4 treatment groups on each of 6 replicate tray levels. Hatchability of fertile eggs set (HF) was determined on 20.5 and 21.5 doi. On 21.5 doi,HF and mean hatching chick weight (MHW) were determined. One bird from each treatment replicate group was randomly selected, weighed and necropsied for the extraction of their livers and tibiae. The tibiae fresh and dry weight, length, width, bone breaking strength (BBS) and percentage of bone ash (PBA) were determined. The dry livers were weighed and ashed. Injection treatment had no significant effect on HF at 20.5 doi. However, there was a significant injection treatment effect on HF at 21.5 doi. The HF of eggs at 21.5 doi in treatment 4 was significantly lower than that of the non-injected control group, with treatment3 being intermediate. Furthermore, There were no significant treatment effects noted for MHW fresh and dry tibia weights, tibia length and width, tibia length to weight ratio, BBS, liver ash content, or percentage of minerals (Ca, P, Mg, Mn and Zn) in the tibia ash. However, embryos from eggs that received treatment 4 had a significantly higher PBA in comparison to all other treatment. In conclusion, although treatment4 negativelyaffectedHF, the injection of diluent containing the high micromineral concentration has the potential to improve bone mineralization.

INTRODUCTION

Production losses as a result of leg problems are a major concern of broiler companies throughout the world. Because of this, intervention strategies involving prehatch and posthatch nutrient supplementation have been developed to reduce these losses. *In ovo* vaccination has been widely used in the poultry industry as a way to control the incidence of diseases. More recently, however, research groups have used the technology of automated *in ovo* injection to deliver nutrients such as amino acids (Ohta et al., 1999), vitamins (Bello et al., 2013), carbohydrates (Zhai et al, 2011a) and other nutrients (Keralapurath et al., 2010; McGruder et al., 2011) that may be of limited availability to broiler embryos and hatchlings. Improvements that are anticipated in response to this type of supplementation include immunity, hatchability, posthatch performance, and bone development.

The bone conditions and compositions of broilers have been a subject of increased study in the past few decades due to the increasing incidence of leg problems associated with various metabolic disorders (Angel, 2007). These bone problems have primarily arisen in association with genetic selection for fast muscle deposition. The rapid growth rate of the bird is also highly related to an acceleration of bone deposition at the periosteal surface, which increases the porosity of the cortical bone, subsequently causing poorer biomechanical properties of the bone (Williams et al., 2004). Microminerals that are important to bone formation and strength include Cu, Zn and Mn, which are greatly reduced in concentration in the egg by the 17^{th} d of incubation (**doi**)(Yair and Uni, 2011). These minerals also participate through their contribution 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, such as collagen synthesis (Starcher

et al., 1980), and hydroxyapatite crystallization (Sauer et al., 1997). Copper is part of the linkage between elastin and collagen, which gives the bone its tensile strength (Carton & Henderson, 1964). Although Zn is important for collagen synthesis, Cu concentrations must be concomitantly sufficient so that fibrils are not weakened and become susceptible to breakage (Rath et al., 2000). Manganese is also an essential part of mucopolissacarides, which constitute bone cartilage. Manganese insufficiencies can lead to the malformation of the epiphyseal plate of the tibia (Liu et al., 1994).

Residual yolk is the main source of nutrients during the transitional period between the hatch and grow-out phases (Gonzales et al., 2003; Henderson et al., 2008). Therefore bone development can be further compromised by a reduction in the amount of minerals stored in the yolk sac. The fastest development phase of the skeleton of the chick occurs during the first 2 wk of posthatch age, and primarily during the first few d of age, when the bone is not completely formed. Micromineral consumption in the first few d of grow-out may be insufficient to meet the demand for cartilage ossification. Furthermore, a low mineral absorptive capacity of the intestine during this period may exacerbate this insufficiency. A low consumption of nutrients during incubation can be alleviated by the *in ovo* injection of nutrients. Bello et al. (2014) tested the *in ovo* injection of different levels of 25-hydroxycholecalciferol, and reported that high dosages have the potential to increase bone mineralization. Upon injecting P, Ca, Zn, Mn and Cu along with carbohydrates and vitamins into eggs, Yair and Uni (2011) increased the concentrations of Fe, Zn, Mn, and Cu in the egg and also the consumption of these minerals by the embryo. Limited concentrations of minerals in the egg may limit bone development in the broiler embryo and posthatch chick. In addition, when Yair et al. (2013) injected the same solution that was used in the previous work by Yair and Uni (2011), they found improvements in the mineralization and mechanical

properties of the bones of embryos and posthatch chicks. The objectives of this research were to investigate effects of the *in ovo* injection of the organic forms of Zn, Mn, and Cu on the hatchability and bone parameters of broiler chicks.

MATERIALS AND METHODS

Incubation

The current study was approved by the Institutional Animal Care and Use Committee of Mississippi State University. Eggs were collected from a commercial broiler breeder flock (Ross x Ross 708) at 32 wk of age and transported to the Poultry Research Unit of Mississippi State University. The collected eggs were stored under commercial conditions for 2 d before weighing and setting. All eggs were weighed individually, and those that were normal in appearance and within 10 % of the mean weight of all eggs weighed were randomly set on each of 6 trays in 3 Natureform incubators (Model 2,340 Natureform, Jacksonville, FL). A total of 1,872 eggs were distributed amongthe 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. On 12 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).

Treatments: Injection Solutions

A non-injected control group (treatment 1) containing eggs that were not injected, but were subjected to the same handling procedures as the following *in ovo* diluent-inject control andenrichmenttreatment groups, was included. At 17 doi, fertile eggs that were injected with 150 μL of commercial diluent (Poulvac®Sterile Diluent; Pfizer, Exton, PA)were designated as diluent-injected

controls (treatment 2). Those injected with150 μL of diluent containing added organic microminerals at 17 doi, were designated as enrichment solution treatments (treatments 3 and 4). 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 compositions of the enrichmentsolutions used are presented in Table 1.

Injection Procedure

The treatment solutions were injected in the eggs using an Embrex Inovoject M (Zoetis; Florham Park, NJ) multi-egg injector capable of simultaneously injecting 56 eggs. Embryonated eggs were injected through the air cell with a blunt tip injector needle [1.27-mm bore width (i.d.)] to target the amnion. The needle provided an approximate 2.54 cm injection depth from the top of the large end of the egg (Keralapurath et al., 2010). The eggs from the non-injected control group remained outside the setter forthe same length of time as those eggs that were injected.After injection, the eggs from each of the 3 incubators were transferred according to treatment replicate group 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 position of the treatment replicate groups in the hatcher corresponded to their positions in the setter.

Data Collection

On 20.5 and 21.5 doi the number of chicks that hatched were counted. Hatchabilty of fertile eggs (HF) was determined at these 2 time periods for the evaluation of hatch rate. On 21.5 doi, HF and mean hatching chick weight (MHW) were measured for each treatment replicate group. After hatch, the

respective treatment replicate groups from the 3 incubators were pooled prior to sampling and then one bird that weighed within 5% of the mean BW of the birds in each of the respective 24 replicate treatmentgroups was randomly selected for further evaluation. The selected birds were weighed, and their 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 livers and tibiae (left and right).

The legs of each chick were removed at the hip and cleaned of soft tissue. The right tibiae were stored a ±20 ^{o}C for future analyses. The left tibiae were weighed (g) to 4 decimals, and their lengths and widths (epiphyseal and diaphyseal sections) were measured in millimeters to 2 decimal places using a digital caliper (Venier Caliper 530-118, Mitutoyo, Houston, TX). Subsequently,the bones were oven-dried until no further weight loss was observed.They werethen allowed to equilibrate to room temperature before their dry weight 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 tibias 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 liversand broken boneswere weighed and ashed in a muffle furnace (Iso-temp D3714, Fisher Scientific, Pittsburgh, PA) for determination of percentages of bone **(PBA)**and liver ash using AOAC (1990) methods.

For bone mineral concentration analysis, one bone ash sample from each treatment replicate group was selected. Using methods specified by the Environmental Protection Agency (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).

Statistical Description

A randomized complete block experimental design was employed for the incubational component of the study. Incubator tray levels were treated as blocks, with all 4 treatments equally represented on each of the 6 tray levels.Incubator was taken into consideration as a random effect.After hatch, birds that were equally selected from each treatment replicate group, were sexed and their tibiae sampled for further tibia analyses. All variables in this study were analyzed using the MIXED procedure of SAS software 9.2 (SAS Institute, 2010). All parameters were analyzed using ANOVA, with treatment viewed as a fixed effect and block as a random effect. Least squares means were compared in the event of significant global effects. Global effects and least squares mean differences were considered significant at $P \leq 0.05$.

RESULTS AND DISCUSSION

Mean set egg weight ± SEM across all treatment groups was 64.6 ± 0.15 g. Injection treatment had no significant effect (P = 0.56) on HF at 20.5 doi (Fig. 1). However, there was a significant injection treatment effect (P = 0.04) on HF at 21.5 doi (Fig. 2). The HF of eggs at 21.5 doi in treatment 4 was significantly lower than that of the non-injected control group, with the diluent-injected control group and treatment 3 being intermediate (Fig 2). Several papers evaluating the injection of various nutrients [carbohydrates (Zhai et al., 2011); $25(OH)D_3$ (Bello et al., 2013)] reported that these nutrients at various concentrations delayed hatch when compared to non-injected control eggs. In the current study, the injection of higher mineral concentrations into the amnion interfered with embryogenesis during late incubation.This effect may have been

188 due to the creation of a mineral imbalance associated with the relative
189 insolubility of the minerals. Ebrahimi et al. (2012) evaluated the *in ovo* injection
190 of L-carnitine, vitamin E, and vitamin C, and reported that the injection of these
191 nutrients was associated with a decrease in hatchability. Bello et al. (2013) also
192 observed negative effects of high dosages (1.80 and 5.40 μg) of
193 25(OH)D_3whencompared to non-injected and diluent-injected controls and to the
194 injection of lower dosages of 25(OH)D_3(0.2 and 0.6μg). Dżugan et al. (2014)
195 evaluated effects of the injection of Cd and Zn, individually and in combination,
196 on chicken egg hatchability.They reported that both minerals, when injected
197 separately, negatively affected hatchability, but had no effect when injected
198 together. However there is no report in the literature regarding effects of the *in*
199 *ovo* injection of Zn, Cu, or Mn on the hatchability parameters of broiler
200 chickens.

201 Furthermore, in this study, there was no significant treatment effect on
202 MHW (Fig. 3). Substituting organic for inorganic sources of Zn, Cu, and Mn in
203 the feed of broiler breeders, Favero et al. (2013) observed no effect on
204 hatchability or hatchling weight. Changing the source of minerals used in the
205 feed of breeders is also a strategy that can be used in an attempt to improve the
206 embryonic growth and hatchability of broilers. The lack of significant
207 differences between the non-injected and diluent-injected treatments for the
208 parameters investigated in the current study are in accordance with results
209 reported in the study by Yair and Uni (2013). In that study, a diluent-injected
210 treatment was not incorporated into the experimental design because previous
211 reports indicated that there were no differences in the effects of these 2
212 treatments.

213 There were no significant treatment effects noted for fresh and dry tibia
214 weights, tibia length and width, tibia length to weight ratio **(L/W)**, breaking

bone strength **(BBS),** or liver ash content in the current study. Nevertheless, the treatment means for the above parameters are provided in Table 2 for observation. However, a significant treatment effect ($P = 0.004$) was found for PBA (Fig. 4). Embryos from eggs that received treatment 4 (highest concentration of microminerals) had a significantly higher level of tibia ash in comparison to all other treatments. However, an increase in tibia ash in response to the treatment containing the highest micromineral concentration was not associated with an increase in BBS. Bello et al. (2014) did not observe differencesin the tibia ash concentrations of hatchlings in response to the *in ovo* injectionof different levels of $25(OH)D_3$(0.15, 0.30, 0.60, and 1.2 μg). Yair and Uni (2013) injected eggs on 17 doi with a solution containing several nutrients including those in the present study. It was observed that bone ash on 19 doi was increased, but that the non-injected control group also had a higher concentration of ash in their tibiae and femurs on d 3 posthatch. Star et al. (2012) fed broilers with feed containing different sources and levels of Zn, but did not observe any significant treatment effects on tibia ash. Nevertheless, they did observe that the level of Zn in the tibia increased when an organic source was used. In order to achieve proper bone mineralization during the embryonic phase, the concentration of the minerals used as a substrate for ossification by osteocytes must be at appropriate levels. Yair and Uni (2011) showed that the bone concentrations of Ca and P are not reduced as are the concentrations of Zn, Cu, and Mn between 17 and 21 doi. Reduced concentrations of these minerals may restrict the ossification process of cartilage during the last days of incubation and during the first few days posthatch. Improvements in the concentrations and sources (organic) of available trace minerals (i.e. Zn, Cu, and Mn) may be related to an increase in tibia ash, particularly as these minerals are used as components of metalloenzymes necessary for connective tissue synthesis. The mineral enrichment provided by treatment 3, which had a 3 fold lower

concentration of minerals than treatment 4, apparently had no negative effect on hatchability or tibia ash concentration. Although the injection treatments used affected the concentration of ash, the percentages of Ca, P, Mg, Mn and Zn in the ash was not significantly affected (Table 3). At this age, it was not possible to determine the concentration of Cu in the ash.It was expected that the higher ash content of the tibia would have been associated with a higher BBS. Nevertheless, the mechanical function of the bone is not only determined byits composition, but also byits structure and confirmation (Sharir et al., 2008). These findings are in accordance with those of Yair and Uni (2013), who observed an increase in the ash content, but did not find a change in the mechanical properties of bones from 19 doi through 3 d posthatch. Bone mineralization is not complete at hatch; therefore, although the mineral content of the bones may have increased, because mineralization is not entirely complete at that time, the bone may still not be entirely resistant to higher compression pressures.

Among its many functions, the liver of chicken embryos must store and homeostatically regulate trace mineral metabolism. The concentration of trace minerals in the liver is relevant because of the capacity of the liver to export minerals from its reserves to other tissues. In situations in which minerals are lacking, such as the early posthatch period, this reserve may be essential for proper organ development (Richards, 1997). However, based on these current results the mineral enriched solutions used in the current study apparently did not increase the overall concentration of mineral in the liver.The injection of diluent with the highest micromineral concentration has the potential to improve bone mineralization.Further research to determine effects of *in ovo*-injected mineral solutions on post-hatchperformance, bone development, and bone mineralization in broilers should be considered.

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

Treatment	Nutrient	Organic micromineral concentration in diluent (mg / ml)	Total amount of organic micromineral injected into each egg (mg)
1	Zn	-	-
	Mn	-	-
	Cu	-	-
2	Zn	-	-
	Mn	-	-
	Cu	-	-
3	Zn	0.181	0.0272
	Mn	0.087	0.0130
	Cu	0.010	0.0015
4	Zn	0.544	0.0816
	Mn	0.260	0.0390
	Cu	0.030	0.0045

Table 2. Mean fresh and dry tibia weights as percentages of BW; tibia length, width, and length to width ratios (L/W ratio); tibia breaking strength (BBS); and percentage of liver ash content of embryos from eggs belonging no non-injected (TRT1) and diluent-injected control groups (TRT2), and of those from eggs injected with diluent containing low (TRT3) and high (TRT4) levels of organic microminerals

	Fresh (%)	Dry (%)	Length (cm)	Width (cm)	L/W Ratio	BBS (kg)	Liver ash (%)
TRT1	0.9232	0.2538	3.250	0.422	7.768	1.212	4.951
TRT2	0.9918	0.2776	3.250	0.400	8.211	1.105	4.875
TRT3	1.0039	0.2600	3.525	0.400	8.310	1.194	4.713
TRT4	1.0206	0.2686	3.240	0.413	8.085	1.083	5.074
SEM	0.060	0.012	0.07	0.02	0.40	0.088	0.48
P-value	0.2241	0.3176	0.4251	0.7262	0.6999	0.6662	0.9535

392 **Table 3.**Percentage of Ca, P, Mg, Zn and Mn in the tibiae ash of 0d birds
393 belonging to non-injected (TRT1) and diluent-injected control groups (TRT2),
394 and of those from eggs injected with diluent containing low (TRT3) and high
395 (TRT4) levels of organic microminerals

	Ca	**P**	**Mg**	**Zn**	**Mn**
TRT1	32.23	17.28	0.77	0.053	0.0032
TRT2	30.69	16.82	0.83	0.048	0.0029
TRT3	33.48	17.95	0.84	0.049	0.0042
TRT4	33.40	17.73	0.80	0.059	0.0039
SEM	0.83	0.71	0.023	0.003	0.0003
P-value	0.08	0.68	0.16	0.11	0.10

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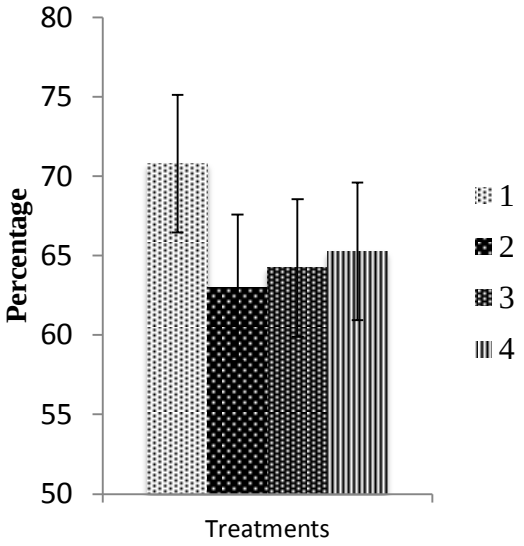


397

398 **Figure 1.**Percentage hatchability of fertilized eggs on 20.5 doi in non-injected
399 and diluent-injected (150 µL) controls, and in eggs injected with diluent (150
400 µL) containing low (Treatment 3) and high (Treatment 4)

401

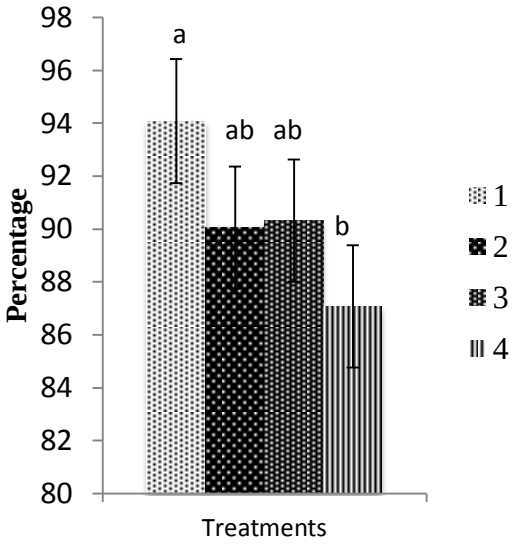
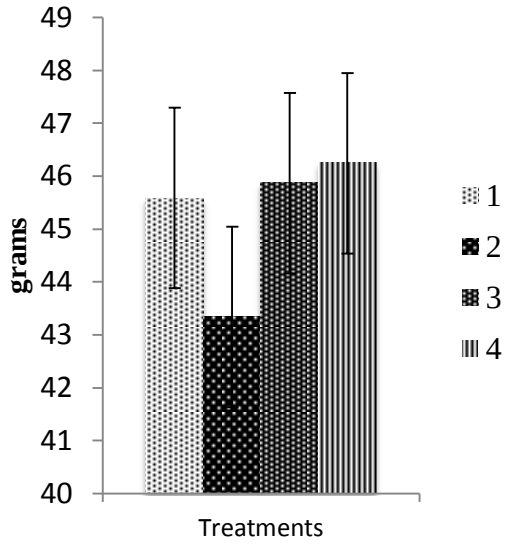


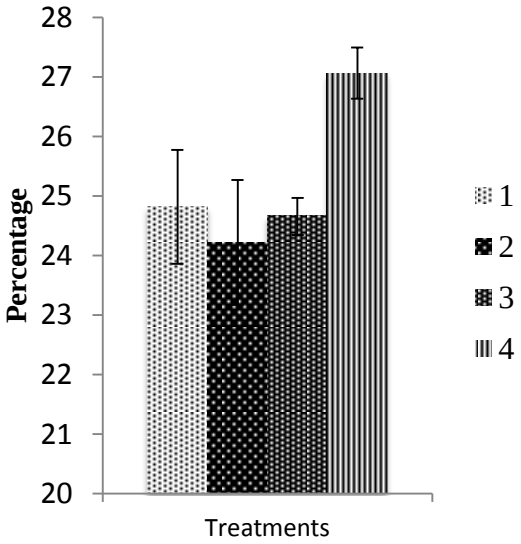
Figure 2. Percentage hatchability of fertilized eggs on 21.5 doi in non-injected and diluent-injected (150 µL) controls, and in eggs injected with diluent (150 µL) containing low (Treatment 3) and high (Treatment 4) levels of organic microminerals. $^{a-c}$ Treatment means with no common superscript differ ($P \leq 0.05$).



409

410 **Figure 3.** Mean hatch weight (g) of chicks in non-injected and diluent-injected
411 (150 µL) controls, and in eggs injected with diluent (150 µL) containing low
412 (Treatment 3) and high (Treatment 4) levels of organic minerals.

413



414

415 **Figure 4.**Percentage of bone ash of chicksin non-injected and diluent-injected
416 (150 µL) controls, and in eggs injected with diluent (150 µL) containing low
417 (Treatment 3) and high (Treatment 4) levels of organic microminerals.

418

ARTIGO 2 – EFFECTS OF *IN OVO* INJECTION OF ORGANIC TRACE MINERALS AND POST-HATCH HOLDING TIME ON BROILER PERFORMANCE AND BONE CHARACTERISTICS

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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 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**) included 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 were 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. Birds in the 0HT group had a higher BW gain and feed intake, and a lower FCR until 21 poh than did birds in the 24HT group. 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.

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 Johnson 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 20-25% of the BW of posthatch chicks and provides immediate nutrition for maintenance and growth (Romanoff, 1960; Sklan and Noy, 2000; Khan, 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 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 US, 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 like 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

participate through their contribution 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 (Carton & Henderson, 1964). 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 on 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 [(4 TRT groups and 2 poh holding time (**HT**)], 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 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 sub-divided into another 2 poh HT groups, which resulted in a total of 8 treatments (4 TRT x 2 poh HT). Fifteen birds were randomly allocated to each of 6 replicate mini-pens (0.914 m x 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 (g/bird), 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 (g of feed intake/bird) 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 (g of feed consumed/g of BW gain) 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 Environmental Protection Agency (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 x 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 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 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 the ones 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 x 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 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 observed that ADFI and ADG were not increased during the period in which they were

299 provided feed and water (22 to 63 d poh). Furthermore, early feed restriction has
300 been shown to significantly improve the FCR of broilers when compared with
301 full fed controls birds (Deaton, 1995). Our data showing that the birds from the
302 24HT group exhibited an improved FCR in comparison to those from the 0HT
303 group through 7 d poh, confirm this earlier finding. During the remainder of the
304 trial, no differences were observed for FCR besides the existence of numerical
305 differences.

306 In this study, the TRT and HT employed were noted to have no effect on
307 tibia BBS. It was expected that by increasing mineral availability to the embryo
308 through *in ovo* injection, that early bone development would be improved. It was
309 further expected that early bone development and its subsequent effects on their
310 mechanical properties would be enhanced when mineral injection was used in
311 conjuction with an imposed decrease in growth rate (24HT). Yair et al (2013)
312 reported that long bones (tibia and femur) from birds that received *in ovo*
313 supplementation of nutrients had superior mechanical properties at d 3 poh in
314 comparison to Noninjected controls. However, at d 7 poh, no differences were
315 observed between TRT in this study. In a study by Manangi et al. (2012), the
316 supplementation of broiler chick diets with inorganic or organic Cu, Mn, and Zn
317 did not exert different effects on BBS.

318 Conversely, the TRT and HT used in this study affected fresh bone
319 weight. On d 7 and 14 poh, HT significantly affected dry bone weight. The
320 observed effect of HT might be due to the lower BW of the birds belonging to
321 the 24HT group on d 1 and 14, rather than being due to differences in their bone
322 structure. On d 21, no difference due to TRT or HT was observed for BDW or
323 BWG, which supports this relationship. The HMD TRT had a positive effect on
324 PBA at d 1 poh when compared to all of the other TRT. The superiority of tibial
325 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 (2013) 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 concentration of Ca and P, as percentages of dry bone weight, was nearly 2 fold higher than that of a Noninjected group. However, they observed that on d 7 poh, the concentration of Ca and P in the bone was higher than that in the Noninjected group. Bello et al. (2014) 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 then 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 not 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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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

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

Table 5. Fresh bone as percentage of BW, bone dry weight as percentage (**BDW**) of BW, bone ash as percentage (**PBA**) of BDMon 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.

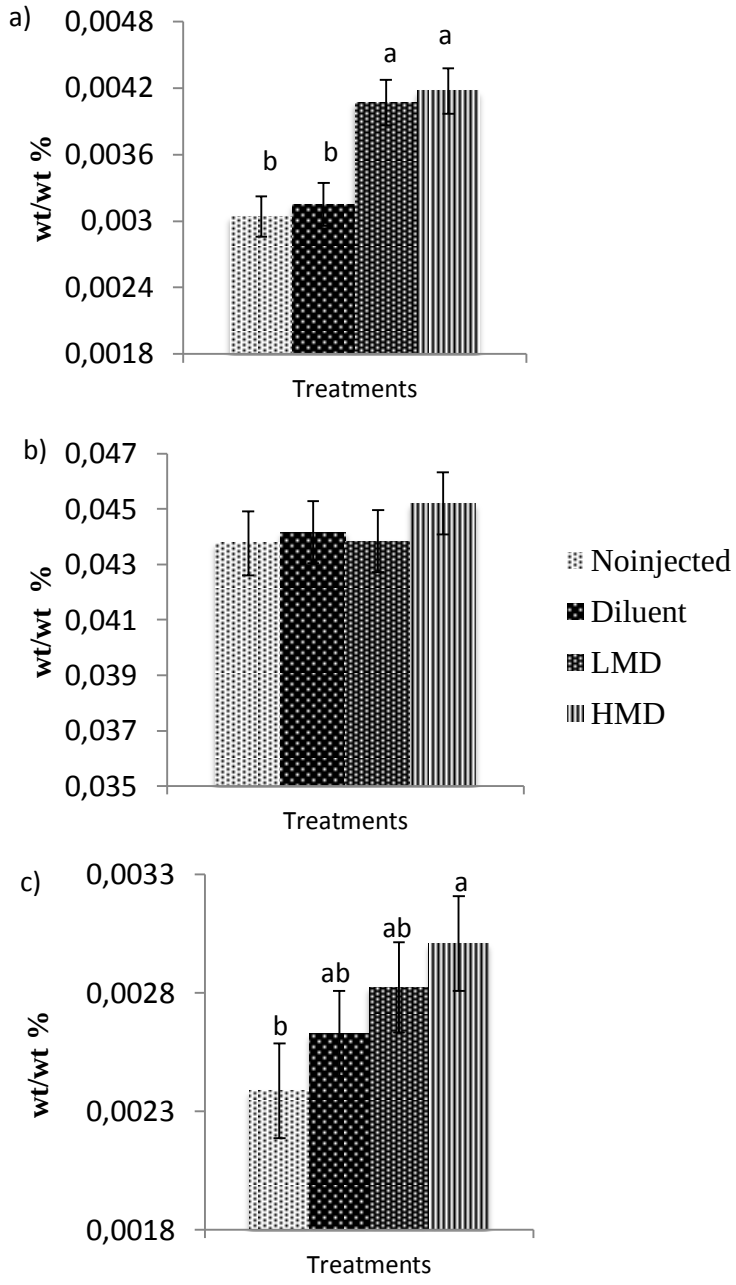
	Fresh Bone			BDW			PBA		
	Posthatch Days of Age								
	1	14	21	1	14	21	1	14	21
Noninjected	0.94	0.90	0.91	0.27	0.29	0.32	24.54[b]	34.75	36.68[b]
Diluent	1.08	0.86	0.88	0.29	0.27	0.29	24.53[b]	34.04	40.26[ab]
LMD	1.04	0.94	0.88	0.28	0.30	0.30	23.37[b]	36.32	40.13[ab]
HMD	1.07	0.81	0.85	0.28	0.28	0.30	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[a]	0.30	25.16	35.85	39.51
24 HT	1.06	0.89	0.91	0.30[a]	0.30[b]	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 ($P \leq 0.05$).


a)
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0,003
0,0024
0,0018
wt/wt %
b
b
a
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Treatments
b)
0,047
0,045
0,043
0,041
0,039
0,037
0,035
wt/wt %
Treatments
Noinjected
Diluent
LMD
HMD
c)
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0,0028
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0,0018
wt/wt %
b
ab
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Treatments

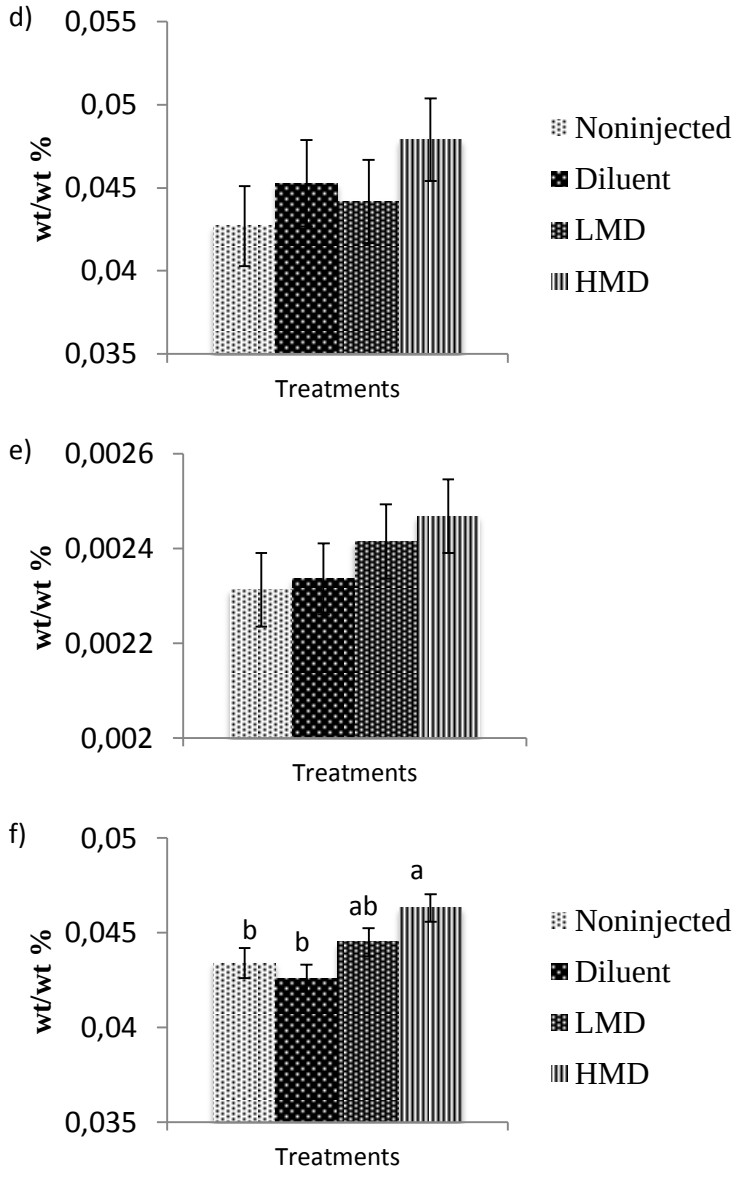

Figure 1. Percentage Mn (a, c, e) and Zn (b, d, f) on d 1, 7, and 21 posthatch in noninjected and diluent-injected control groups and low (**LMD**) and high (**HMD**) of Zn, Mn, and Cu concentration injection treatment groups.
[a-b]Means within a parameter with no common superscript differ ($P \leq 0.05$)