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Influence of maternal flock age, maternal trace mineral nutrition and incubation temperature on bone development of embryos and chicks

by

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DEDICATION

To my parents, my sisters and grandma for the unconditional love and support

(Para os meus pais, irmãs e avó por todo o amor, carinho e suporte)

ABSTRACT

At hatch, the skeleton of the chick is a well formed miniature of that in the adult bird; this is the end result of 21 days of development in the egg. In order to build a strong and healthy skeletal frame, the embryo relies on trace minerals (TM) deposited in the egg by the hen. The temperature at which the embryo grows is also a key factor influencing skeletal development. The overall purpose of this research was to understand the effects of maternal trace mineral nutrition on embryonic and post-hatch bone development and to investigate the relationship between temperature and bone characteristics. Mineral content in the egg and embryonic and post-hatch bone characteristics of embryos and chicks from Young (32 week), Mid (45 week) and Old (59 week) hens were not influenced when hens were supplemented with low levels of organic (OTM) copper, zinc and manganese relative to TM sulfates (ITM) at industry levels (Control). High ITM levels increased bone strength at hatch relative to Control but not relative to OTM; at hatch OTM widen bones from Young hens relative to all diets. Therefore, an opportunity exists for industry to reduce TM levels by supplementing OTM. As hens aged, the yolk Zn and Cu content increased and embryos from Young hens had reduced proportion of calcified tibia and femur relative to those from Older hens at day 20^{th} of incubation and weaker bones at hatch. In another study, an incubator temperature of 36.0^{0}C applied from the 15^{th} day of incubation until hatch increased bone strength relative to 37^{0}C. High eggshell temperature is negatively associated with bone calcification and strength. If the stronger bones at placement in the barn increased chick mobility

then water consumption and access to nutrients important for post-hatch bone growth could be increased and this might decrease future bone problems. In summary, considerable maternal age and incubator temperature variation existed on skeletal growth of the progeny, demonstrating that there may be opportunities to use maternal nutrition and hatchery management to increase skeletal health in chicks at hatch, especially those from young flocks.

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^0C	Celsius temperature
AA	Amino acid
AAFCO	Association of American Feed Control Officials
BW	Body weight
Ca	Calcium
CAM	Chorioallantoic membrane
CP	Crude protein
Cu	Copper
CuSO4	Copper sulfate
DMT1	Divalent metal transporter 1
EHP(15-19)	Cumulative embryonic heat production
EST	Eggshell temperature
EST(15-19))	Average eggshell temperature
EW	Egg weight
Fe	Iron
g	Grams
HMTBa	2-hydroxy-4-(methylthio) butanoic acid
ITM	Inorganic trace mineral
M	Metal ion
ME	Metabolizable energy

CHAPTER 1. LITERATURE REVIEW

1. INTRODUCTION

Broiler chicks (*Gallus gallus*) grow extremely rapidly. In 35 days the bird's body weight increases from approximately 36.4 g at hatch to 1,800 g (Schmidt et al., 2009). Although growth-selected and unselected birds have similar periods of rapid bone formation (4 to 18 days) and mineralization (4 to 11 days) in the post-hatch period, the cortical bone of selected birds are less mineralized (Williams et al., 2004). Furthermore, selected embryos (Rawlinson et al., 2009) and chicks (Williams et al., 2004) have more porous bones when compared to unselected birds; this may lead to bone deformities. Therefore, the skeleton of modern chicks might not be adequately calcified in the pre and post-hatch period to support body growth.

Bone quality of broilers has both welfare and economic implications (Oviedo-Rondón et al., 2006). A large-scale study in the United Kingdom reported that about 27% of broilers assessed close to market age showed poor locomotion, and about 3.3% were incapable of walking (Knowles et al., 2008). In 2011, Canada produced 620,964,000 chickens (statcan.gc.ca); assuming that the prevalence of leg disorders in Canada was similar to that in the United Kingdom, about 167,660,000 chickens might have experienced leg problems within a few days of being processed.

Considering that 37.5% of the chick's lifespan occurs within the egg (21 days of incubation in addition to 35 days post-hatch), it is important to understand factors that influence bone growth and have implications for bone quality at hatch. A strong, well-formed skeleton within the egg might indirectly facilitate the hatching process and might increase the chick's healthiness in the first days post-hatch. If increased healthfulness of bones at hatch translate into increased mobility, then feed and water consumption at placement could be increased. Therefore, this could increase access to macro and micro minerals present in the pre-starter diet which are essential for post-hatch bone development.

Shaw et al. 2010 reported that chicks from old breeder flocks (65 weeks) had stronger bones relative to those from young flocks (25 weeks) at hatch. An investigation whether strong bones at hatch result from differences in bone development among embryos throughout incubation will provide a better understanding of the role of maternal age on the developing chick both pre and post-hatch. Furthermore, investigating the influence of maternal trace mineral nutrition throughout the laying hen cycle will provide knowledge about 1) age related changes in egg mineral composition, and 2) whether maternal nutrition could be used as a tool to increase skeletal growth within the egg. Finally, understanding the association between incubator and eggshell temperature during the time when bone growth is highest will not only strengthen our knowledge on bone biology but it would also provide basic information to understand the factors influencing skeletal development.

1.2. OVERVIEW OF TRACE MINERALS: COPPER, ZINC AND MANGANESE

The trace minerals (TM) Cu, Zn and Mn are present and required in very small amounts (µg/kg) within chicken tissues (Angel, 2007). Despite their low abundance within the body, trace minerals play major role in metabolism as they function primarily as catalysts in enzyme systems or as components of enzymes (Richards, 1997).

Livestock diets are commonly supplemented with Cu, Zn and Mn as inorganic mineral salts (ITM), organic minerals (OTM) or a combination of the two forms. Organic minerals are reported to be more bioavailable than inorganic forms (Guo et al., 2001; Bao et al., 2007; Huang et al., 2009). Bioavailability is defined as the degree to which an ingested nutrient (mineral from a particular source, for instance) is absorbed in a form that can be utilized by animal (Ammerman et al., 1995). Increased bioavailability may relate to minerals bound to a carbon-based chelate or ligand (i.e. amino acids [AA] or other acids) being less susceptible to interact with anti-nutritional factors such as phytate (Yu et al., 2010). Phytate is the form in which phosphorus (P) is present but unavailable for absorption in the majority of plant feed ingredients for poultry. This anti-nutritional factor is also a chelate which binds tightly to trace minerals within the gut making them unavailable for absorption (Tahir et al., 2012).

Minerals in ITM are bound by weak ionic bonds to a metal-binding agent such as oxide or sulfate (Echigo and Kimata, 2010), whereas OTM consist of

minerals bound through covalent bonds to a carbon-based ligand (Leeson, 2005). Covalent bonds have a higher degree of chelation between the mineral and the ligand (Bai et al., 2011). High chelation strength confers stability of the trace mineral-ligand in the acid conditions of the upper gastrointestinal tract, thereby preventing mineral dissociation from its chelate at low pH (Predieri et al., 2003; Predieri et al., 2005; Predieri et al., 2009). Once the trace mineral dissociation is reduced, the likelihood of free ionized trace mineral interacting with a dietary antagonist (Tahir et al., 2012) or competing with other dietary minerals for the same absorption uptake mechanism is decreased (Iskandar et al., 2005). This can reduce mineral losses to the environment (Nollet et al., 2007; Leeson and Caston, 2008) and potentially increase trace mineral bioavailability.

However, not all chelation results in increased TM bioavailability for absorption; minerals including Cu, Zn, and Ca are all "stable" in the phytate molecule (Bohn et al., 2008) yet not as available as minerals chelated to AA or acids (Yu et al., 2010). This is because the phosphate groups present in the phytate molecule can form strong and insoluble complexes with minerals (Liem et al., 2009; Tahir et al., 2012). In common use, the term "organic mineral" is used to denote minerals chelated to an organic molecule, with the intention of increasing mineral bioavailability in animal diets.

1.2.1. Types of Organic Trace Minerals.

Several commercial organic Cu, Zn and Mn forms, including AA complexes, chelates to either AA or organic acids and proteinates have been

developed as supplements to animal feeds (Cao et al., 2000). The type of ligand to which trace minerals are chelated determines the category in which organic trace minerals are classified, as defined by the Association of American Feed Control Officials (AAFCO, 1998). Therefore, chelation combining a soluble metal salt with a single amino acid, multiple or from hydrolyzed protein is defined as an amino acid chelate, amino acid complexes or metal proteinates, respectively (AAFCO, 1998). Likewise, trace minerals can be chelated to a lipophilic organic acid such as 2-hydroxy-4-(methylthio) butanoic acid (HMTBa), a precursor of the amino acid methionine (Yi et al., 2007). The mineral in this organic form is bound via two covalent bonds to each of two molecules of HMTBa (Figure 1-1) to create chelates containing 16% Zn, 15% Cu, and 13% Mn, with 80, 78, and 76 % (by weight) HMTBa, respectively (Dibner, 2003; Yi et al., 2007).

1.2.2. Trace Mineral Absorption.

Because organic mineral supplements are widely reported to be more bioavailable than inorganic minerals (Huang et al., 2009; Zhao et al., 2010), it is of interest to review briefly the physiology of digestion and absorption of trace minerals.

Absorption of Cu, Zn and Mn takes place mainly in the ileum of the chick (Bai et al., 2008; Yu et al., 2008; Mondal et al., 2010; Yu et al., 2010) through a non-saturable diffusion process (Yu et al., 2008; Bai et al., 2008); and in the duodenum and jejunum through an energy dependent process (Yu et al., 2008; Bai et al., 2008). In general, digestion and absorption mechanisms involve several

steps: (1) initial dissociation of the inorganic trace mineral in low pH, (2) binding of the mineral to the mucosa, (3) movement of the mineral across the brush-border membrane into the epithelial cells, and (4) intracellular trafficking of trace minerals. Step 2 involves electrostatic binding of metals by endogenous soluble ligands (mucin) and surface ligands on the mucosa (Whitehead, 1996). Luminal mucins are proteins secreted throughout the gastrointestinal tract which has high affinity for metals ions (M) in the order of M^{3+}> M^{2+}> M^{+} (Whitehead et al., 1996; Powell et al., 1999). Binding of ionized minerals to mucins prevents the formation of insoluble mineral precipitates (Whitehead et al., 1996). Because mucins have no specificity for metals, competition may exist between different minerals for binding sites on mucin (Powell et al., 1999). The ability of ions to penetrate the gelatinous layer (or mucus) and reach the absorptive epithelium is inversely related with their strengths of binding to the mucus gel (M^{+} >M^{2+} >M^{3+}; Whitehead et al., 1996; Powell et al., 1999). Step 3 occurs via diffusion (ileum), and active processes (duodenum and jejunum) with the latter involving metal-binding protein transporters such as the divalent metal transporter 1 (DMT1; Bai et al., 2011) and Zn metal transporter protein (ZIP; Wang et al., 2004; Hill and Link, 2009). These proteins are located on the enterocyte surface and are involved in the mechanism that transports divalent cations across the brush border into the enterocytes (Bai et al., 2011). In step 4, the trace mineral binds to chaperones such as ceruloplasmin (Cu, Mn) and methallothionein (Zn) which will be transported through the blood to tissues (Sandrock et al., 1983).

Enhanced mineral bioavailability of OTM appears to be related to different digestion and absorption mechanisms versus inorganic supplements. However, the mechanisms by which organic mineral chelates are absorbed and whether (and how) such processes differ relative to inorganic minerals are not completely understood. Trace minerals chelated to HMTBa might be hydrolysed from the ligand at the site of absorption and transported as free ions by DMT1 and ZIP co-transporters into the intestinal cell (Yi et al., 2007) whereas the ligand might be absorbed separately by diffusion or by a carrier mediated system (Knight and Dibner, 1984; Dibner, 2003). Alternatively, it has been speculated that other types of organic minerals, such as those chelated to AA, might be transported intact across the absorptive epithelium (Ashmed, 1993).

1.2.3. Factors Influencing OTM Mineral Bioavailability.

The type of ligand and its chelating strength properties are important factors influencing differences in absorption between organic and inorganic trace mineral forms (Cao et al., 2000, Guo et al. 2001, Yu et al., 2010; Bai et al., 2011). For instance, Zn chelated to AA with a weak complex strength was found to exhibit similar bioavailability as the sulfate form when pancreas metallothionein mRNA level was used as an indicator of bioavailability. Zinc in a moderate complex strength, on the other hand, was found to be more available relative to Zn in a strong complex strength (Ao et al., 2009; Huang et al., 2009; Yu et al., 2010). Furthermore, Yu et al. (2010), using the in situ ligated intestinal loop technique, reported that Zn absorption in the duodenum, jejunum and ileum was greater for organic forms (two forms of Zn chelates, and three Zn-amino acid

complexes differing in degree of mineral-chelate strength) relative to $ZnSO_4$ or a combination of $ZnSO_4$ with either glycine or methionine. A similar study also indicated that intestinal uptake and transport of Mn was greater for MnAA with strong chelation relative to $MnSO_4$ (Bai et al., 2011).

Several investigators have looked at the bioavailability of organic minerals relative to inorganic sources. Li et al. (2004) tested a variety of organic Mn sources (AA chelates or proteinates) in an attempt to predict Mn bioavailability from physical characteristics such as solubility. Solubility of organic Mn sources at pH 5 varied from 24.5 to as high as 99.1 %, and was not related with bioavailability when concentration of Mn in the heart was used as bioavailability criteria. In fact, high trace mineral solubility was inversely related to bioavailability in both poultry and ruminants (Cao et al., 2000; Guo et al., 2001). The lack of correlation between solubility and bioavailability may relate to trace mineral sulfates being highly soluble in water and at low pH and therefore more likely to bind to other dietary antagonists in the gastrointestinal tract (Li et al., 2004).

1.3. MATERNAL TRACE MINERAL NUTRITION AS A FACTOR INFLUENCING BONE GROWTH

1.3.1. Maternal Trace Mineral Nutrition: Organic Versus Inorganic Forms.

In order to grow properly, the avian embryo is dependent on trace minerals that are pre-packaged in the egg at the time of laying (Romanoff, 1967). The importance of maternal mineral nutrition and transfer of TM to the egg has long

been recognized; a severe and prolonged Cu, Zn or Mn deficiency imposed on the hen increases the incidence of embryonic anomalies and mortality (Caskey and Norris, 1940; Kienholz et al., 1961; Simpson et al., 1967). Hens fed a Zn deficient diet containing 10 mg Zn/kg laid eggs with lower yolk Zn content and their embryos had impaired skeletal development relative to hens fed a supplemented diet as Zn carbonate at 55 mg/kg (Kienholz et al., 1961).

Previous research has investigated TM forms and levels in the hen's diet (Tables 1-1 and 1-2). Contradictory results among studies are likely due to levels of TM in the control diets, the levels of TM supplementation and the form of OTM studied. Furthermore, nutritional state of the hen (deficient or adequate) might also influence responses to OTM supplementation. For example, hens previously fed a Cu-, Zn- and Mn- deficient diet for 2 weeks and then supplemented from 39 to 52 weeks with Cu-HMTBa had increased Cu content in liver and yolk relative to a control diet at 43 weeks but not at 48 and 52 weeks (Sun et al., 2012a). The hen's mineral metabolism could be up-regulated which might have increased the response of OTM supplementation at least in the first days or weeks after beginning of dietary treatment.

Hudson et al. (2004a) reported that ZnAA supplemented at levels well above the requirements increased egg specific gravity, shell quality, and percentage of settable eggs in broiler breeder hens but did not influence egg weight or eggshell breaking strength relative to hens fed similar levels of Zn as Zn sulfate. ZnAA supplementation increased egg Zn content by about 12%, but

embryonic mortality was increased in early stages of development relative to hens fed similar levels of Zn as sulfate. The authors further reported that progeny body weight at hatch, organ weight and hepatic Zn concentration was not influenced by supplementing high levels of ZnAA, $ZnSO_4$ or a combination of trace mineral forms (Hudson et al., 2004b).

Other researchers have also reported variable effects on laying performance and progeny quality when supplementing high levels of OTM in addition of a sufficient trace mineral diet. Kidd et al. (1992), reported that supplementation of ZnAA increased dry bone weight of the progeny at hatch compared to Zn oxide but no response was observed for tibia ash. Accordingly, combination of ZnAA and MnAA increased livability of the progeny at days 0 to 17 and days 0 to 34, and increased some parameters of immune response (Virden et al., 2003; Virden et al., 2004). Sun et al. (2012b) reported that Cu, Zn and Mn chelated to HMTBa increased Cu content in the albumen, but reduced Cu yolk content, and enhanced progeny growth performance compared to similar levels of TM as sulfates (Sun et al., 2012b). Likewise, retention efficiency of Mn and Zn was higher in the eggshell from hens supplemented with Cu, Zn and Mn chelated to methionine relative to those fed similar levels as sulfates or oxides (Gheisari et al., 2011). The former studies indicate that addition of OTM in a sufficient diet can potentially increase TM deposition in the egg, benefit laying performance, and potentially benefit offspring relative to ITM supplementation.

One of the questions regarding TM supplementation in the hen's diet is whether replacement of ITM partially or completely with organic forms would sustain or increase egg production and mineral retention in the hen's body. Swiatkiewicz and Koreleski, (2008) reported that partial or complete substitution of inorganic Zn and Mn oxide with metal-amino acid complexes had no effect on laying performance parameters or tibia mineral retention but increased eggshell breaking strength in the late stages of egg production. Similarly, egg quality and TM retention efficiency was comparable between groups of hens supplemented at levels close to NRC requirements as either sulfates or metal AA complexes (Gheisari et al., 2011).

Mabe et al. (2003) reported that mineral form (metal chelated to AA complexes or as sulfates) and level (5 mg/kg Cu, 30 mg/kg Zn, 30 mg/kg Mn or 10 mg/kgCu, 60 mg/kg Zn, 60 mg/kg Mn) did not influence eggshell breaking strength, stiffness, elastic modulus (which influences the stiffness characteristic of the eggshell) and egg mineral content.

In industry, new OTM forms commercially available for dietary supplementation are often supplemented in addition to, rather than in place of, the traditional inorganic mineral form supplemented J.D. Richards (Novus International, St. Charles, MO, personal communication). This represents an opportunity to study 1) whether there is an opportunity to reduce total TM levels by supplementing OTM, 2) whether extra mineral would result in increased mineral retention, and 3) whether industry levels are already high enough and

adding more trace minerals would have no effect or negative effects on the hen or progeny. In fact, Mabe et al. (2003) reported that when compared with a basal diet at NRC levels, high levels of Cu, Zn and Mn (60 Cu mg/kg, 60 mg/kg Zn and 10 mg/kg Cu) increased Zn and Cu yolk content regardless of mineral form; but when diets were supplemented with low levels (30 Cu mg/kg, 30 mg/kg Zn and 5 mg/kg Cu) only the organic form increased Zn yolk content relative to the control diet. This indicates that levels of supplementation of organic trace minerals might be lowered without compromising egg mineral retention. Therefore an investigation examining low maternal OTM levels in the diet versus TM at or beyond industry levels and their effects on egg mineral composition would provide a better understanding about trace mineral nutrition in broiler breeders.

Replacing Zn and Mn oxides by 50% as Zn and MnAA did not influence egg quality; but when replaced as 100% TM-AA**,** the egg shell breaking strength was increased in a group of hens at 62 and 70 weeks of age (Swiatkiewicz and Koreleski, 2008). Furthermore substitution of Zn and Mn oxides and Cu sulfate by their organic form at low or marginal levels (50 to 70% of the NRC recommendation) was sufficient to maintain laying hen performance and eggshell quality in relation to hens fed higher levels of TM as either sulfates of chelated to AA complexes (Gheisari et al., 2011). From the previous research it appears that there is an opportunity to reduce TM levels in the hen diet without decreasing performance. It is important to consider that the hen's mineral requirement varies according to the bird's physiological state. For example, 25 mg/kg Mn in the laying hen diet was sufficient to meet maximum egg production and egg weight,

however, requirements for optimal shell quality were much higher than this dietary concentration (between 50 and 100 mg/kg; Inal et al., 2001). There is a gap in our knowledge of the impact on egg composition of supplementing low levels of OTM with regards to form and level on egg composition. It is possible that reduced levels of OTM supplemented in the previous studies may have been sufficient for egg production however, the requirements in terms of trace mineral transfer, and therefore embryonic bone development, might be comparable or higher.

1.3.2. Trace Minerals Chelated to HMTBa in the Maternal Diet.

Eusebio-Balcazar et al. (2010) reported that progeny of breeder hens supplemented with Cu, Zn and Mn HMTBa chelates to partially (30% of the total) replace ITM had increased walking ability compared to broilers from hens fed solely ITM. Furthermore, Oviedo et al. (2008) reported that, relative to hens fed ITM, supplementation of TM chelated to HMTBa in the hen diet did not influence tibia mineral density and bone mineral content at 49 days but reduced relative asymmetry of the shank in progeny at day 40; greater leg asymmetry may indicate a decrease walking ability (Møller and Manning, 2003). Sun et al. (2012b) reported that supplementing Cu, Zn and Mn-HMTBa in the maternal diet increased growth performance of the progeny at 42 days relative to sulfate supplementation.

Feeding Cu, Zn and Mn chelated to HMTBa can potentially enhance broiler skeletal development. In broilers, replacing mineral sulfates by organic

minerals chelated to HMTBa reduced the incidence of tibial dyschondroplasia (Dibner et al., 2007), varus and valgus abnormalities (at 15 weeks; Ferket et al., 2009). This represents an opportunity to investigate whether using this organic trace mineral in broiler breeder diet would influence the embryo and post-hatch growth. This might bridge the gaps in our knowledge about nutritional factors that influence egg nutrient content and perhaps benefit skeletal growth of progeny.

1.3.3. Trace Minerals in the Egg.

In laying hens eggs, Cu levels in egg yolk ranged from 0.54 to 3.32 µg/kg, from 10.7 to 70.4 mg/kg for Zn, and from 0.14 to 0.86 mg/kg for Mn (Kirkpatrick and Coffin, 1975; Fakayode and Olu-Owolabi, 2003; Skrivan et al., 2005; Nisianakis et al., 2009; Uluozlu et al., 2009). In meat-type hens, values reported for mineral content in the egg yolk were 31.8 µg Cu per egg, 0.99 mg Zn, and 21.62 µg Mn whereas albumen contained 9.82 µg Cu per egg, 0.59 mg Zn and 0.78 µg Mn (Yair and Uni, 2011).

Minerals are transported to the egg yolk via vitellogenin, a liver protein whose synthesis is induced by estrogen (Gruber et al., 1976). This protein has high affinity for bivalent metals and transports trace minerals to the ovary and ultimately to the egg yolk (Gruber et al., 1976; Richards and Packard, 1996; Richards, 1997). Once deposited in the egg yolk, vitellogenin along with the trace minerals being carried, are processed into the yolk proteins lipovitellin and phosvitin which are packed together in the granule fraction of the yolk (Richards, 1991; Salvante and Williams, 2002). Phosvitin has high affinity for Ca, Fe and

Cu; whereas lipovitellin binds P, Zn, Cu and Fe (Richards, 1997). Yolk granules also store Mn (Grau et al., 1979).

1.3.4. Egg Trace Mineral Content and Parental Flock Age.

Research on the long-term effects of dietary trace mineral supplements on egg mineral content is limited. Revell and Hughes, (2005) reported that the concentrations of Zn, Cu, Ca and K in egg yolk were relatively constant at 27, 37 and 57 weeks in eggs from hens fed a similar commercial layer diet). Sun et al. (2012a), however, reported that 43 week old hens laid eggs with greater Zn and Mn yolk content (by 7.3 and 19%, respectively), compared to 52 week old hens. These hens were previously fed a Cu-, Zn- and Mn- deficient diet from 37 to 39 weeks; this might have up-regulated the hen's mineral metabolism and perhaps influenced mineral transfer to eggs. Furthermore, the hen age effect was independent of trace mineral form supplemented (Cu, Zn and Mn chelated to HMTBa or as sulfates). Contrarily, Hudson et al. (2004a) reported a reduction of about 17% of Zn mineral content in the eggs from hens at 62 weeks relative to those at 32 weeks old. This effect was independent of trace mineral form in the hen's diet (sulfates or ZnAA).

Older hens in production lay larger but fewer eggs than younger hen and the proportion of yolk to whole egg is increased whereas proportion of albumen is reduced (Vieira and Moran, 1998; Revell and Hughes, 2005; Ulmer-Franco et al., 2010; Nangsuay et al., 2011). Because the yolk is the main source of nutrients used by the developing embryo (Romanoff, 1967) and the main Cu, Zn and Mn

storage site (Yair and Uni, 2011), a reduction in the yolk proportion could be a disadvantage for embryos developing in eggs from younger flocks. However, not much is known regarding trace minerals levels in the egg as hen ages. Aged hens might be less efficient in transferring trace minerals to the egg; hence their embryos may still have less trace minerals even if their egg yolks are bigger. However, definitive data has not been reported in the literature.

1.3.5. Importance of Cu, Zn and Mn for Bone Growth.

Copper, Zn and Mn are involved in several enzymes activities important for metabolic functions related with embryonic growth and post-hatch development (Angel, 2007; Dibner et al., 2007). Manganese is a cofactor of polymerase and galactotransferase which are enzymes involved in the synthesis of the mucopolysaccharide chondroitin sulfate (Leach et al., 1969), one of the main components of the bone hyaline cartilage model (Eyre, 2004). In fact, deficiency of Mn in the diet can reduce chick bone size (Caskey et al., 1939) possibly due to reduced mucopolysaccharide content of the bone organic matrix (Caskey et al., 1939; Leach et al., 1969).

Copper is important for crosslinking of collagen and elastin which are proteins that give bone its tensile strength and elasticity (Rucker et al., 1998; Dibner et al., 2007). Bones from chicks fed a Cu-deficient diet are brittle and distorted (Carlton and Henderson, 1964), possibly as a result of reduced activity of the copper-dependant enzyme lysyl oxidase (Rucker et al., 1969; Rucker et al.,

1998). Long-term deficiency of both Cu and Mn reduce osteogenesis and decrease bone resorption by reducing osteoclast activity in rats (Strause et al., 1987).

Zinc is a cofactor of collagenase and bone alkaline phosphatase enzymes (Starcher et al., 1980; Seo et al., 2010). This mineral increases osteogenesis by stimulating osteoblast proliferation and osteoprotegerin activity, an important regulator of bone density (Liang et al., 2012). Additionally, Zn plays regulatory roles in bone development by mechanisms that include changes in gene transcription that occurs during longitudinal bone growth at the growth plate (Starcher et al., 1980; Oviedo-Rondón et al., 2006). Chicks fed a diet deficient in Zn have reduced collagenase activity and thus reduced bone collagen synthesis (Starcher et al., 1980).

The former studies demonstrate the importance of Cu, Zn and Mn for bone formation. While the impact of a mineral deficient diet on skeletal health of the embryo has been well reported, there is less information available on the effects of trace mineral supplementation at current industry levels. Estimates of trace mineral requirements of broiler breeders are usually based on the values given for white egg layers by the Nation Research Council (NRC, 1994). However, these estimates (4 mg/kg Cu, 35 mg/kg Zn and 30 mg/kg Mn) are much different to values currently used commercially. For example, in the U.S. broiler industry it is common practice to formulate diets containing 100 to 120 mg/kg supplemental Zn; the major reason for this practice is that trace minerals in the diet represent

less than 0.2% of total diet costs (Leeson and Caston, 2008), and therefore over-supplementation in the diet occurs, in order to ensure a safety factor.

Concern for the environment, particularly the level of trace minerals in manure, has challenged nutritionists to reconsider levels of minerals supplemented in livestock diets. In this regard, supplementation of broiler breeder diets with more bioavailable trace minerals at levels close to NRC requirements can potentially reduce mineral waste without affecting animal performance.

1.4. OVERVIEW ON BONE COMPOSITION

Bone is a highly specialized form of mineralized connective tissue consisting of an organic matrix in which collagen and minerals work together to form a living active tissue (Roach, 1997; Rath et al., 2000). About 95% of this organic matrix is composed of collagen (Knott and Bailey, 1999; Rath et al., 2000), a specialized type of protein whose fibers have high tensile strength and thus are able to resist stretching (Rath et al., 2000; Saito and Marumo, 2010). Bone with high collagen content tends to be elastic with very high tensile strength (Rath et al., 2000; Williams et al., 2004). The matrix that contributes rigidity to bone consists of the mineral hydroxyapatite ($Ca_{10}(PO_4)_6(OH)_2$; Rath et al., 2000). Bones with a high mineral content tend to have high compressive strength but are brittle to tension and torsion (Turner, 2006). The combined properties of collagen content and minerals plus the linkage between the collagen fibers (Knott and Bailey, 1999; Rath et al., 1999), result in a bone structure that has both extreme

tensile and compressional strength (Rath et al., 2000; Viguet-Carrin et al., 2006; Foolen et al., 2008).

Bones also contain a varying amount of water that is an important determinant of their mechanical properties, for instance, wet bones tend to bend to a greater extent than dry bones (Crenshaw et al., 1981).

1.4.1. Bone Formation in the Chicken Embryo.

Femur and tibia development starts at 3.5 days of incubation, yet calcification beginning at 10^{th} day of incubation (Pechak et al., 1986a). Figure 1-2 displays an overview of the process of bone formation within the egg. In the very early stages of development embryonic mesenchymal cells are committed to an osteogenic cellular lineage (Pechak et al., 1986a; Pechak et al., 1986b; Figure 1-2.A), which are precursors of osteoblasts and chondrocytes (Roach, 1997). Osteoblasts are cells that build bone whereas osteoclasts are cells that break it down (Marks and Popoff, 1988). Chondrocytes lay down a cartilage model that has similar shape to that of the long bones of the legs of the mature skeleton (Osdoby and Caplan, 1981; Pechak et al., 1986a; Figure 1-2.B). This is the scaffold on which bone development will occur throughout incubation and after hatch. Outside the mid diaphysis, osteoblasts lay down layers of collagen type I that becomes mineralized at around the 11^{th} day of incubation (Pechak et al., 1986a; Figure 1-2.C and E10). In the meantime, vasculature invades the cartilage core region and starts to resorb the first mineral layer (Pechak et al., 1986a; Pechak et al., 1986b; Figure 1-2. E10-V). Longitudinal growth, on the other hand,

occurs through expansion of both proximal and distal epiphyses and diaphysis. In the chicken embryo, the thickness of the growth plate increases considerably as the bone grows because expansion of the proliferative zone occurs more rapidly than bone resorption at the distal area of the growth plate (Roach, 1997; (Figure 1-2: E10, E15 and E20).

The growth of the mid-diaphysis is seen in Figure 1-3: at day 15 of incubation new bone is deposited at the periosteum, in a radial direction, as the bone increases in girth. At this stage, there are great numbers of mineral trabeculae (not seen in the Figure 1-3) and each one is separated by channels (highly porous cortical bone). As the embryo grows, the expansion of the mid-diaphysis diameter occurs through the synthesis and deposition of trabecular bone in the periosteal bone followed by resorption of endosteum by osteoclasts on its inner surface (Roach and Shearer, 1989; Roach, 1997; Chen et al., 2008). This process results in expansion of the medullary cavity between day 19 of incubation and day 7 after hatch (Yair et al., 2012, Figure 1-3).

Around the 10^{th} of incubation, calcium from the egg shell is transported to the embryo by the chorioallantoic membrane (CAM; Gabrielli, 2004). The onset of calcium transport by the CAM and calcium accumulation in the embryonic bone is coincident with increased activity of acid phosphatase and alkaline phosphatase (Kubota et al., 1981; Alfonso-Torres et al., 2009). Both enzyme activities increase linearly after day 12 (Alfonso-Torres, et al., 2009, Kubota et al., 1980) to a peak at the 19^{th} day of incubation (Kubota et al., 1981). Therefore

as bone length and width increases significantly throughout incubation (Chen et al., 2008), bone ash content also increases from day 14 to 17 th and day 19 th until hatch but decreased between day 17 and 19 (Yair et al., 2012). Furthermore, bone calcium content also increases sharply from day 14 of incubation and starts to plateau at day 19 of incubation (Kubota et al., 1981) and is related to the degree of ossification (Johnston and Comar, 1955). The reason that bone ash decreases from the 17 to 19 days of incubation is not understood. It might be related to reallocation, and perhaps loss, of minerals as the medullary cavity expands approaching the last days of incubation.

Parallel to the increased bone elongation that occur from days 11 to 19, the embryo mobilizes about 90% of the Cu, Zn and Mn stored in the egg yolk between days 11 and 17 and hatches with minimal amounts of these minerals in the yolk sac (Yair and Uni, 2011). However, when supplemental minerals were added in the amniotic fluid of the developing embryo (by *in ovo* feeding) at day 17 of incubation, the yolk content and uptake of Zn, Cu and Mn significantly increased from day 18 to hatch relative to the Control non-enriched group (Yair and Uni, 2011). The authors suggested that embryos might lack these minerals in the last days of incubation, which might have implications on skeletal formation in the last days of incubation.

1.4.2. Bone Formation in the Post Hatch Period.

As happens in the egg, chick bone grows longitudinally by expansion of the epiphyses (Dibner et al., 2007) and in width by apposition of bone beneath the

periosteum accompanied by resorption of endosteum by osteclasts on its inner surface (Williams et al., 2004). Bone growth increases proportionally faster in the first 2 weeks after hatch: at 15 days of age, the tibia was 1.8 times longer and 2.1 mm wider relative to hatch; whereas tibia was about 1.9 to 2.0 times longer and 2.4 to 2.6 mm wider at 42 days relative to 14 days of age (Applegate and Lilburn, 2002; Angel, 2007). Figure 1-3 shows the pattern of bone cortical growth as the embryo and chick develop; bone diameter increases greatly in the first week post-hatch (Williams et al., 2004, Yair et al. 2012).

As bone grows, there is an increased accumulation of Ca and P; and thus bone mineral ash increases with age (Williams et al., 2004; Yair et al., 2012). There is a lack in knowledge regarding Cu, Zn and Mn accumulation in bone after hatch. Furthermore, information about macro- and micromineral requirements for bone growth in the post-hatch period is still lacking. As noted, Yair and Uni (2011) suggested that embryos might have limited mineral reserves in the yolk to support growth in the last days of incubation. In this context, having the knowledge of whether maternal mineral nutrition could affect bone growth within the egg and potentially after hatch will bridge one of the gaps in our knowledge about whether maternal nutrition could increase the egg mineral reserves and, in that way, meet mineral requirements for the developing embryo throughout incubation and enhance skeletal development in embryos and young broilers.

1.5. MATERNAL AGE AS A FACTOR INFLUENCING BONE GROWTH

As the hen ages fewer but heavier eggs are laid which in turn leads to heavier chicks at hatch (Tona et al., 2004; Willemsen et al., 2008). Yalçin et al. (2001) reported that day old chicks from older flocks (56 to 58 week old) had increased tibia density relative to 32 to 35 week old flocks. Shaw et al. (2010) reported increased breaking strength in chicks from 65 week old flocks relative to chicks from 25 week old flocks. Whether increased bone quality at hatch in chicks from old hens results from differences in bone growth and metabolism that already started during incubation is a research subject that warrants further investigation.

Chicks from older hens (62 week) had greater reserves of P in the yolk sac reserves at hatch than those from younger hens (27 weeks; Vieira and Moran, 1998). Furthermore, throughout incubation embryos from 53 week old hens were more efficient in terms of solubilizing egg yolk relative to embryos from hens at 29 weeks old (Nangsuay et al., 2011). Therefore, increased yolk availability might be an advantage for embryos from older hens to support embryonic growth rate in the last week of incubation. Yadgary et al. (2010) reported that in addition to having higher fat content in the egg yolk, the embryos from 50 week old hens also mobilized more fat at 13 and 15 days of incubation, but not afterwards, relative to embryos from 30 week old hens. Because embryonic bone development is dependent on minerals stored in the egg yolk, including P, Zn and Cu (Richards, 1997), it is possible that, as it is the case of fat uptake, embryos from young hens might have higher mineral uptake from the egg yolk in a period of greatest

embryonic bone development that occurs from the 15th to 19th days of incubation (Kubota et al., 1981).

Increased skeletal mineralization during embryonic bone development might indirectly facilitate the hatching process because a well-formed healthy skeleton might translate into increased mobility, thus the hatching process might be facilitated and hatchability increased. Furthermore, a healthy skeleton might increase mobility and therefore feed and water consumption in the post-hatch growth could be increased which might benefit post-hatch bone growth. Therefore, understanding embryonic skeletal growth among embryos from diverse hen ages combined with knowledge on whether maternal mineral nutrition could be used as a tool to increase embryonic skeletal development will provide a better understanding of embryonic bone biology and understand the mechanisms influencing skeletal development.

1.6. INCUBATION TEMPERATURE AS A FACTOR INFLUENCING BONE GROWTH

In artificial incubation, the temperature is maintained between 37.5ºC to 37.8ºC throughout incubation (Meijerhof and Beek, 1993). The actual embryonic temperature, however, differs from the incubator temperature (Joseph and Moran, 2005; Lourens et al., 2005; Lourens et al., 2011). The reasons behind this relates to the heat produced by the embryo (Lourens et al., 2011), and the subsequent heat transfer from the embryo to the surrounding environment (Meijerhof and Beek, 1993). Eggshell temperature (EST) is often used as an indicator of

embryonic temperature (Lourens et al., 2005; Joseph et al., 2006) and different studies reported that an EST of 37.5 to 37.8ºC during incubation results in the highest hatchability and chick development as measured by chick body weight, residual yolk weight, chick length and heart weight at hatch relative to eggs set at higher EST of ≥ 38.9 (Yildirim and Yetisir, 2004; Joseph and Moran, 2005; Lourens et al., 2005; Leksrisompong et al., 2007; Molenaar et al., 2011).

At similar egg weights, embryos from old hens (45, 55 and 59 wk of age) have increased embryonic heat production relative to those from young hens (34 and 40 wk; Hamidu et al., 2007). Additionally, for proper embryonic development, eggs from 60 week old hens required lower incubator temperatures relative to 28 week old hens to obtain an EST of 37.8ºC (Lourens et al., 2005). At standard incubation temperatures (37.5ºC to 37.8ºC; Meijerhof and Beek, 1993) embryos from old hens may have difficulty dissipating the heat produced by their own metabolism in conditions with insufficient air velocity or cooling capacity in the incubator (French, 1997; Hulet et al., 2007; Elibol and Brake, 2008).

The combined effects of embryonic heat production and incubator temperature may cause overheating in embryos, particularly those from old hens and towards the last days of incubation. This is coincident with the period of greatest embryonic bone development (Kubota et al., 1981). Indeed, incubator temperatures of 36.9 or 39.6ºC (Yalcin and Siegel, 2003; Yalçin et al., 2007), 38.5ºC (Hammond et al., 2007) and 36, 38 or 39ºC (Oviedo-Rondón et al., 2008) at early or late stages of development can impair bone development at hatch and

post-hatch (Oviedo-Rondón et al., 2009). Hammond et al. (2007) reported that increasing the incubator temperature from a constant incubator temperature of 37.5^{0}C to 38.5^{0}C at early stages of incubation (4th to 7th day) increased the length of tibia and tarsus of Leghorn embryos without alteration in bone mineralization. Furthermore, Yalçin and Siegel (2003) reported increased relative asymmetry of long bones when eggs were incubated at extreme temperatures (36.9 or 39.6^{0}C vs 37.8^{0}C) in early stages of incubation; such asymmetry, however, decreased towards hatching. Asymmetry and growth rate are inversely related; asymmetry in the legs could decrease walking ability (Møller et al., 1999; Møller and Manning, 2003). Therefore, bone asymmetry at hatch might have implications for bone development in the post-hatch period (Møller and Manning, 2003).

Chicks from eggs set at intermittent (6 h/day) high (39ºC) or low (36.9ºC) incubator temperatures from day 10 to day 18 of incubation had reduced tibia weights at day 14 and at hatch relative to chicks from eggs set at 37.8ºC (Yalçin et al., 2007). Oviedo-Rondón et al. (2008) reported that extreme incubator temperatures of 36ºC or 39ºC in the last 4 days of incubation reduced bone size at hatch relative to chicks from eggs set at 37ºC. Taken together, these studies provide evidence that incubator temperature plays a role on embryonic bone development and set the stage for investigation of the relationships between hen age and incubation temperature and their implications for embryonic bone development.

1.7. RESEARCH APPLICATION

In order to grow properly, chicken embryos rely on trace minerals deposited in the egg by the hen. Intensive bone growth occurs during incubation (Yair and Uni, 2011) yet bones are very porous (Pechak et al., 1986b) and not well mineralized at hatch (Angel, 2007). The first few days post-hatch are critical for bone development because trace mineral reserves in the yolk sac are low (Yair and Uni, 2011); hence the chicken depends on minerals from the diet to continue mineralization in the post-hatch period. These factors, combined with the importance of incubation temperature for bone formation, support the necessity of understanding factors that could increase skeletal quality at hatch.

The overall objective of this PhD thesis was divided into three main area: 1) investigating the effects of maternal age on bone development of the progeny; 2) whether Cu, Zn and Mn form and the level supplemented in the diet affects trace mineral content in the egg and thus bone skeletal formation; 3) the influence of incubator temperature on bone characteristics at hatch in chicks from diverse flock ages.

The overall goal of the research in Chapters 2 and 3 were to evaluate whether supplementing broiler breeder hens with Cu, Zn and Mn chelated to HMTBa would increase trace mineral deposition into the egg, potentially enhancing skeletal development of the embryo and perhaps having carry over effects in the post-hatch period.

The organic mineral form studied were Cu, Zn and Mn chelates commercially available for use in poultry diets under the trade name Mintrex® (Mintrex® Novus International Inc., St. Charles, MO).

In Chapter 4, the effect of incubator temperature and broiler breeder flock age on bone length, width, breaking strength and proportion of calcified bone at hatch was investigated.

The research questions investigated were as follow:

Hypothesis 1. It was hypothesized that supplementing low levels of Cu, Zn and Mn chelated to HMTBa would increase Cu, Zn and Mn content in the egg yolk relative to eggs from hens fed mineral sulfates at industry (i.e. higher) levels. Increased egg trace mineral content would thus enhance bone characteristics in the embryos and chicks; this effect would increase in the progeny of older hens.

In order to test this hypothesis, Chapter 2 and 3 investigated the effect of supplementing hens with trace mineral chelated to HMTBa at low levels (i.e NRC (1994) recommended levels) relative to TM levels commonly used in industry as sulfates. It was also investigated whether increasing trace minerals levels beyond the current industry levels in combination or singly would influence mineral levels in eggs and bone growth of the progeny.

Hypothesis 2. It was hypothesized that incubator temperatures of 36^0C or 36.5^0C from the day 15 of incubation until hatch would increase bone development of day old chicks from older hens relative to high incubator

temperature of 37^0C or 37.5^0C. It was also hypothesized that bone characteristics at hatch would be negatively affected by high heat production by embryos and high eggshell temperature.

These hypotheses were addressed in Chapter 4 by investigating the effect of incubator temperature on bone development in embryos from various broiler breeders flock ages.

Table 1-1. Summary of studies that investigated Cu, Zn and Mn trace minerals (TM) as inorganic (ITM) or organic (OTM) form and level (mg/kg) in the hen diet on egg shell characteristics and trace mineral content in tissues

TM basal diet (mg/kg)	Amount of supplemented TM (mg/kg)	OTM form	ITM form	Age of hen (weeks)	----------Response---------- Egg shell quality and TM content in egg and tissue	Ref
4.95 Cu 32.6 Zn 24.7 Mn	5 Cu + 30 Zn + 30 Mn; 10 Cu + 60 Zn + 60 Mn	TMAA[a]	$CuSO_4$ $ZnSO_4$ $MnSO_4$	30,60 or 69	Relative to basal diet: regardless of TM form the high TM levels increased both eggshell breaking strength and fracture toughness in group of hens at 60 or 69 week; High OTM level increased egg Zn and Mn content; low OTM level increased Zn yolk; TM supplementation did not influence % eggshell, eggshell index (shell weight per unit surface area), and eggshell stiffness	1
52 Zn 30 Mn	82 Zn + 80 Mn as ITM or OTM at 0, 50 or 100%	TMAA	ZNO MnO	25 to 70	100% OTM increased egg shell breaking strength at 62 and 70 weeks of age; egg production, egg weight, eggshell %, eggshell thickness and eggshell density were not influenced; No effect on tibia ash	2
4.2 Cu 30.2 Zn 19.8 Mn	ITM at: 7 Cu, 40 Zn, 40Mn; or 7 Cu, 65 Zn, 75Mn as ITM OTM at:	TMAA	$CuSO_4$ $ZnSO_4$ $MnSO_4$	38 to 53	No effect on TM content in egg shell; No effects on eggshell quality when high levels of ITM were replaced by OTM; Low OTM level maintained average egg production and reduced % high levels relative to high ITM level;	3

	3.5 Cu, 20 Zn, 20 Mn; 7.5 Cu, 40 Zn, 40 Mn; or 10.5 Cu, 60 Zn, 60 Mn	Low OTM level increased eggshell thickness and reduced % broken eggs relative to low ITM

[a] TMAA: Amino acid-mineral chelate,
[1]Mabe et al., 2003,
[2]Swiatkiewicz and Koreleski, 2008,
[3]Gheisari et al., 2010

Table 1-2. Summary of studies that investigated Cu, Zn and Mn trace minerals (TM) as inorganic (ITM) or organic (OTM) form and level in the hen diet on egg mineral content and progeny characteristics

TM in basal diet (mg/kg)	Amount of supplemented TM (mg/kg)	OTM form	ITM form	Age of hen (weeks)	Response			Ref
					Egg TM content	Tissue TM content	Progeny effect	
37 Zn	160 80 OTM +ITM	ZnAA [a]	$ZnSO_4$	22 to 65	ZnAA increased Zn relative to $ZnSO_4$	No effect on tibia ash	-	1
37 Zn	160 80 as OTM + ITM	ZnAA	$ZnSO_4$	22 to 65	-	Similar Zn content in liver at hatch	Similar body and organ weight; Similar intestine growth at hatch	2
72 Zn	80	ZnAA	ZnO	41 to 56	-	No effect of Zn in tibia ash	Increased dry tibia weight hatch; No effect body weight or ash content, immune cellular response	3
4.7 Cu 46 Zn 35.3 Mn	8 Cu + 50 Zn + 35.3 Mn or 50 Zn as OTM	HMTBa [b]	$CuSO_4$ $ZnSO_4$ $MnSO_4$	31 to 39	TM as HMTBa reduced yolk Cu and increased Cu albumen	No effect TM liver progeny	Increased progeny growth at 42 days	4

[a] ZnAA: Amino acid-mineral chelate
[b]HMTBa: 2-hydroxy-4-(methylthio) butanoic acid
[1]Hudson 2004
[2]Hudson 2004
[3]Kidd et al., 1992
[4]Sun et al.2012
(-): not investigated

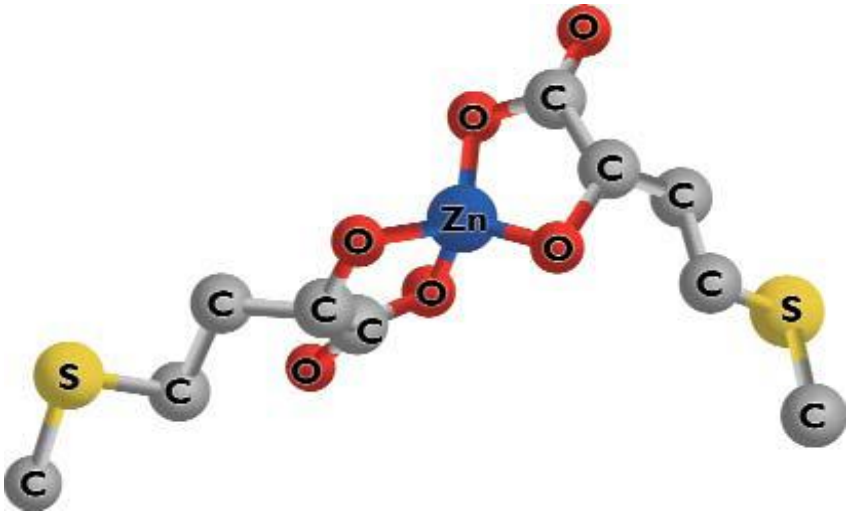

Figure 1-1. Zinc chelated to 2-hydroxy-4-(methylthio) butanoic acid (HMTBa).

Zinc in this organic form is bound via two covalent bonds to each of two molecules of HMTBa.

Source: Novus International, Inc.

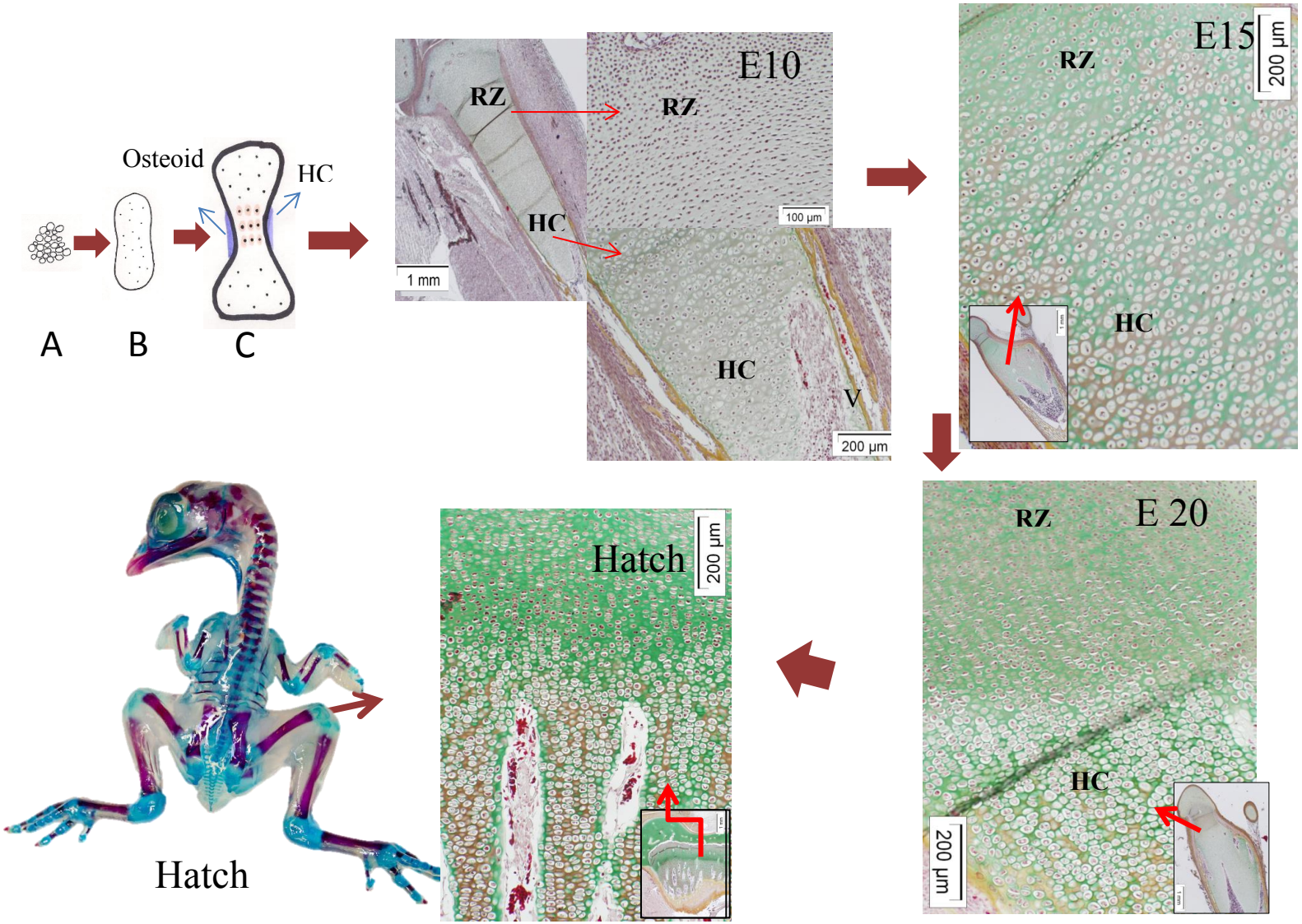
Osteoid
HC
A
B
C
RZ
RZ
E10
HC
100 µm
1 mm
HC
V
200 µm
RZ
E15
200 µm
HC
RZ
E 20
HC
200 µm
Hatch
200 µm
Hatch

Figure 1-2. Overview of the process of bone formation of the chicken embryo. Figures of embryonic day 10 (E10), E15, E20 show the longitudinal sections of the growth plate of femurs stained with Movat stain. The small figures show the approximate locations of the regions shown in higher magnification. RZ = resting zone of growth plate, HP = HP: hypertrophic zone, V = vasculature . (A): At around 3.5 days of incubation, a group of mesenchymal cells differentiate into an osteogenic cellular lineage which is precursors of osteoblasts and chondrocytes. (B): Chondrocytes lay down an organic matrix that leads to the formation of the bone cartilage model. C: Cartilage core expands in girth and length by synthesis of extracellular matrix by chondrocytes in the mid of the cartilage core; osteoblasts outside the cartilage core synthesize osteoid (purple). (E10, E15 and E20): as the embryo develops the thickness of the HP and the growth plate increases considerably. Osteoblasts continue to secrete osteoid so the mineralization expands proximally, distally as well as in a radial manner around the cartilage model. (Hatch): the skeleton is a well formed miniature of that in the adult bird.

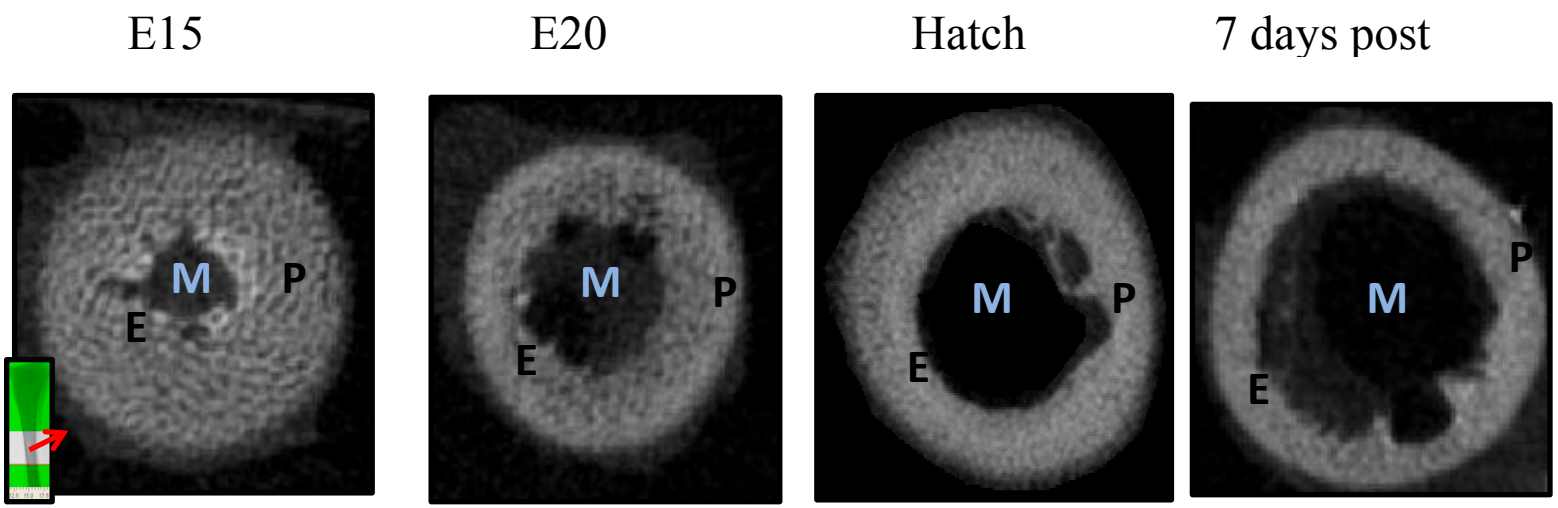

Figure 1-3. Micro computed tomography image of mid diaphysis cross sections of embryo and post-hatch chick femurs. The small figure shows the approximate locations of the regions shown in higher magnification. P = periosteum, E = endosteum, M = medulary cavity. As the embryo grows, the expansion of the mid-diaphysis diameter occurs through the synthesis and deposition of trabecular bone in the periosteal bone followed by resorption of endosteum by osteoclasts on its inner surface. This process results in expansion of the medullary cavity between day 19 of incubation and day 7 after hatch.

1.8. REFERENCES

American Feed Control Officials (AAFCO). 1998. Official Publication. Assoc. Amer. Feed Control Offic. Oxford, IN.

Alfonso-Torres, K. A., L. H. Gargaglioni, J. M. Pizauro, D. E. Faria Filho, R. L. Furlan, and M. Macari. 2009. Breeder age and bone development in broiler chicken embryos. Arq. Bras. Med. Vet. Zootec. 61:219-226.

Ammerman, C.B. 1995. Methods for estimation of mineral bioavailability. Pages 83-94 in Bioavailability of Nutrients for Animals: Amino Acids, Minerals, and Vitamins. C.B. Ammerman, D. H. Baker, and A.J. Lewis, ed. Acad. Press, New York, NY.

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.

Ao, T., J. L. Pierce, R. Power, A. J. Pescatore, A. H. Cantor, K. A. Dawson, and M. J. Ford. 2009. Effects of feeding different forms of zinc and copper on the performance and tissue mineral content of chick. Poult. Sci. 88:2171-2175.

Applegate, T., and M. Lilburn. 2002. Growth of the femur and tibia of a commercial broiler line. Poult. Sci. 81:1289-1294.

Ashmed, H. D. 1993. Comparative intestinal absorption and subsequent metabolism of metal amino acid chelates and inorganic metal salts. Pages 32-57 in The Roles of Amino Acid Chelates in Animal Nutrition. Park Ridge (NJ): Noyes Publications ed. C. B. Ammerman, Baker, D. H., and Lewis, A. J., eds.

Bai, S. P., L. Lu, R. L. Wang, L. Xi, L. Y. Zhang, and X. G. Luo. 2011. Manganese source affects manganese transport and gene expression of divalent metal transporter 1 in the small intestine of broilers. Br. J. Nutr. 108:1-10.

Bai, S. P., L. Lu, X. G. Luo, and B. Liu. 2008. Kinetics of manganese absorption in ligated small intestinal segments of broilers. Poult. Sci. 87:2596-2604.

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.

Bohn, L., A. Meyer, and S. Rasmussen. 2008. Phytate: impact on environment and human nutrition. J. Zhejiang Univ. Sci. B. 9:165-191.

Cao, J., P. R. Henry, R. Guo, R. A. Holwerda, J. P. Toth, R. C. Littell, R. D. Miles, and C. B. Ammerman. 2000. Chemical characteristics and relative bioavailability of supplemental organic zinc sources for poultry and ruminants. J. Anim. Sci. 78:2039-2054.

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

Caskey, C. D., W. D. Gallup, and L. C. Norris. 1939. The need for manganese in the bone development of the chick. J. Nutr. 17:407-417.

Chen, J. C., B. Zhao, M. T. Longaker, J. A. Helms, and D. R. Carter. 2008. Periosteal biaxial residual strains correlate with bone specific growth rates in chick embryos. Comput. Methods Biomech. Biomed. Engin. 11:453-461.

Dibner, J. J. 2003. Review of the metabolism of 2-hydroxy-4-(methylthio) butanoic acid. World's Poult. Sci. J. 59:99-110.

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.

Echigo, T., and M. Kimata. 2010. Crystal chemistry and genesis of organic minerals: A review of oxalate and polycyclic aromatic hydrocarbon minerals. Can. Mineral. 48:1329-1357.

Elibol, O., and J. Brake. 2008. Effect of egg weight and position relative to incubator fan on broiler hatchability and chick quality. Poult. Sci. 87:1913-1918.

Eusebio-Balcazar, P. E., E. O. Oviedo-Rondón, A. Mitchell, B. Brake, M. J. Wineland, V. Moraes, and N. Leandro. 2010. Effects of breeder feeding

and trace mineral source on leg health and bone traits of broiler progeny. Poult. Sci. 89 (Suppl. 1):223. (Abstr.)

Eyre, D. R. 2004. Collagens and cartilage matrix homeostasis. Clin. Orthop. Relat. Res. 427:118.

Fakayode, S. O., and I. B. Olu-Owolabi. 2003. Trace metal content and estimated daily human intake from chicken eggs in Ibadan, Nigeria. Arch. Environ. Occup. Health. 58:245-251.

Foolen, J., C. van Donkelaar, N. Nowlan, P. Murphy, R. Huiskes, and K. Ito. 2008. Collagen orientation in periosteum and perichondrium is aligned with preferential directions of tissue growth. J. Orthop. Res. 26:1263-1268.

French, N. A. 1997. Modeling incubation temperature: the effects of incubator design, embryonic development, and egg size. Poult. Sci. 76:124-133.

Gabrielli, M. G. 2004. Carbonic anhydrases in chick extra-embryonic structures: a role for Ca in bicarbonate reabsorption through the chorioallantoic membrane. J. Enzyme Inhib. Med. Chem. 19:283-286.

Gheisari, A., A. Sanei, A. Samie, M. Gheisari, and M. Toghyani. 2011. Effect of diets supplemented with different levels of manganese, zinc, and copper from their organic or inorganic sources on egg production and quality characteristics in laying hens. Biol. Trace Elem. Res. 142:557-571.

Grau, C. R., T. E. Roudybush, and W. H. McGibbon. 1979. Mineral composition of yolk fractions and whole yolk from eggs of restricted ovulator hens. Poult. Sci. 58:1143-1148.

Gruber, M., E. S. Bos, and A.B. Geert. 1976. Hormonal control of vitellogenin synthesis in avian liver. Mol. Cell. Endocrinol. 5:41-50.

Guo, R., P. R. Henry, R. A. Holwerda, J. Cao, R. C. Littell, R. D. Miles, and C. B. Ammerman. 2001. Chemical characteristics and relative bioavailability of supplemental organic copper sources for poultry. J. Anim. Sci. 79:1132-1141.

Hamidu, J. A., G. M. Fasenko, J. J. R. Feddes, E. E. O'Dea, C. A. Ouellette, M. J. Wineland, and V. L. Christensen. 2007. The effect of broiler breeder genetic strain and parent flock age on eggshell conductance and embryonic metabolism. Poult. Sci. 86:2420-2432.

Hammond, C. L., B. H. Simbi, and N. C. Stickland. 2007. In ovo temperature manipulation influences embryonic motility and growth of limb tissues in the chick (*Gallus gallus*). J. Exp. Biol. 210:2667-2675.

Hill, G. M., and J. E. Link. 2009. Transporters in the absorption and utilization of zinc and copper. J. Anim. Sci. 87:85-89.

Huang, Y. L., L. Lu, S. F. Li, X. G. Luo, and B. Liu. 2009. Relative bio availabilities of organic zinc sources with different chelation strengths for

broilers fed a conventional corn-soybean meal diet. J. Anim. Sci. 87:2038-2046.

Hudson, B. P., W. A. Dozier III, J. L. Wilson, J. E. Sander, and T. L. Ward. 2004a. Reproductive performance and immune status of caged broiler breeder hens provided diets supplemented with either inorganic or organic sources of zinc from hatching to 65 wk of age. J. Appl. Poult. Res. 13:349-359.

Hudson, B. P., B. D. Fairchild, J. L. Wilson, W. A. Dozier, and R. J. Buhr. 2004b. Breeder age and zinc source in broiler breeder hen diets on progeny characteristics at hatching. J. Appl. Poult. Res. 13:55-64.

Hulet, R., G. Gladys, D. Hill, R. Meijerhof, and T. El-Shiekh. 2007. Influence of egg shell embryonic incubation temperature and broiler breeder flock age on posthatch growth performance and carcass characteristics. Poult. Sci. 86:408-412.

Inal, F., B. CoŞkun, N. GülŞen, and V. KurtoĞlu. 2001. The effects of withdrawal of vitamin and trace mineral supplements from layer diets on egg yield and trace mineral composition. Br. Poult. Sci. 42:77.

Iskandar, M., E. Swist, K. Trick, B. Wang, M. L'Abbé, and J. Bertinato. 2005. Copper Chaperone for Cu/Zn superoxide dismutase is a sensitive biomarker of mild copper deficiency induced by moderately high intakes of zinc. Nutr. J. 4:1-10.

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

Joseph, N. S., and E. T. Moran. 2005. Effect of flock age and post emergent holding in the hatcher on broiler live performance and further-processing yield. J. Appl. Poult. Res. 14:512-520.

Joseph, N., A. Lourens, and E. Moran. 2006. The effects of suboptimal eggshell temperature during incubation on broiler chick quality, live performance, and further processing yield. Poult. Sci. 85:932-938.

Kienholz, E. W., D. E. Turk, M. L. Sunde, and W. G. Hoekstra. 1961. Effect of zinc deficiency in the diets of hens. J. Nutr. 75:211-221.

Kirkpatrick, D. C., and D. E. Coffin. 1975. Trace metal content of chicken eggs. J. Sci. Food Agric. 26:99-103.

Knight, C. D., and J. J. Dibner. 1984. Comparative absorption of 2-hydroxy-4-(methylthio)-butanoic acid and l-methionine in the broiler chick. J. Nutr. 114:2179-2186.

Knott, L., and A. J. Bailey. 1999. Collagen biochemistry of avian bone: Comparison of bone type and skeletal site. Br. Poult. Sci. 40:371.

Knowles, T. G., S. C. Kestin, S. M. Haslam, S. N. Brown, L. E. Green, A. Butterworth, S. J. Pope, D. Pfeiffer, and C. J. Nicol. 2008. Leg disorders in

broiler chickens: prevalence, risk factors and prevention. PloS ONE 3:e1545.

Kubota, M., E. Abe, T. Shinki, and T. Suda. 1981. Vitamin D metabolism and its possible role in the developing chick embryo. Biochem. J. 194:103-109.

Leach, J. R. M., A. Muenster, and E. M. Wien. 1969. Studies on the role of manganese in bone formation: II. Effect upon chondroitin sulfate synthesis in chick epiphyseal cartilage. Arch. Biochem. Biophys. 133:22-28.

Leeson, S. 2005. Trace mineral requirements of poultry- validity of the NRC recommendations. Pages 107-117 In: Re-Defining Mineral Nutrition. L. A. Tucker, and Taylor-Pickard, J. A., eds. Nottingham University Press, England.

Leeson, S., and L. Caston. 2008. Using minimal supplements of trace minerals as a method of reducing trace mineral content of poultry manure. Anim. Feed Sci. Technol. 142:339-347.

Leksrisompong, N., H. Romero-Sanchez, P. W. Plumstead, K. E. Brannan, and J. Brake. 2007. Broiler incubation. 1. Effect of elevated temperature during late incubation on body weight and organs of chicks. Poult. Sci. 86:2685-269.

Li, S., X. Luo, B. Liu, T. D. Crenshaw, X. Kuang, G. Shao, and S. Yu. 2004. Use of chemical characteristics to predict the relative bioavailability of

supplemental organic manganese sources for broilers. J. Anim. Sci. 82:2352-2363.

Liang, D., M. Yang, B. Guo, J. Cao, L. Yang, and X. Guo. 2012. Zinc up regulates the expression of osteoprotegerin in mouse osteoblasts MC3T3-E1 through PKC/MAPK pathways. Biol. Trace Elem. Res. 146:340-348.

Liem, A., G. M. Pesti, A. Atencio, and H. M. Edwards. 2009. Experimental approach to optimize phytate phosphorus utilization by broiler chickens by addition of supplements. Poult. Sci. 88:1655-1665.

Lourens, A., R. Meijerhof, B. Kemp, and H. van den Brand. 2011. Energy partitioning during incubation and consequences for embryo temperature: A theoretical approach. Poult. Sci. 90:516-523.

Lourens, A., H. van den Brand, R. Meijerhof, and B. Kemp. 2005. Effect of eggshell temperature during incubation on embryo development, hatchability, and post-hatch development. Poult. Sci. 84:914-920.

Mabe, I., C. Rapp, M. Bain, and Y. Nys. 2003. Supplementation of a corn-soybean meal diet with manganese, copper, and zinc from organic or inorganic sources improves eggshell quality in aged laying hens. Poult. Sci. 82:1903-1913.

Marks, S. C., and S. N. Popoff. 1988. Bone cell biology: The regulation of development, structure, and function in the skeleton. Am. J. Anat. 183:1–44.

Meijerhof, R., and G. V. Beek. 1993. Mathematical modelling of temperature and moisture loss of hatching eggs. J. Theor. Biol. 165:27-41.

Molenaar, R., R. Hulet, R. Meijerhof, C. M. Maatjens, B. Kemp, and H. van den Brand. 2011. High eggshell temperatures during incubation decrease growth performance and increase the incidence of ascites in broiler chickens. Poult. Sci. 90:624-632.

Møller, A. P., G. S. Sanotra, and K. S. Vestergaard. 1999. Developmental instability and light regime in chickens (*Gallus gallus*). Appl. Anim. Behav. Sci. 62:57-71.

Mondal, S., S. Haldar, P. Saha, and T. Ghosh. 2010. Metabolism and tissue distribution of trace elements in broiler chickens' fed diets containing deficient and plethoric levels of copper, manganese, and zinc. Biol. Trace Elem. Res. 137:190-205.

Nangsuay, A., Y. Ruangpanit, R. Meijerhof, and S. Attamangkune. 2011. Yolk absorption and embryo development of small and large eggs originating from young and old breeder hens. Poult. Sci. 90:2648-2655.

National Research Council. 1994. Nutrient Requirements of Poultry. 9th rev. ed. Natl. Acad. Press, Washington, DC.

Nisianakis, P., I. Giannenas, A. Gavriil, G. Kontopidis, and I. Kyriazakis. 2009. Variation in trace element contents among chicken, turkey, duck, goose, and pigeon eggs analyzed by inductively coupled plasma mass spectrometry (ICP-MS). Biol. Trace Elem. Res. 128:62-71.

Nollet, L., J. D. van der Klis, M. Lensing, and P. Spring. 2007. The effect of replacing inorganic with organic trace minerals in broiler diets on productive performance and mineral excretion. J. Appl. Poult. Res. 16:592-597.

Osdoby, P., and A. I. Caplan. 1981. First bone formation in the developing chick limb. Dev. Biol. 86:147-156.

Oviedo-Rondón, E. O., L. Mejia-Sanchez, J. Small, K. E. Brannan, N. Leksrisompong, A. Mitchell, T. A. York, and J. Brake. 2008. Organic minerals (Mintrex® P) in broiler breeder diets and pre-incubation conditions on bone development and leg health of broilers. Poult. Sci. 87 (Supl. 1):162. (Abstr.).

Oviedo-Rondón, E. O., P. R. Ferket, and G.B. Havestein. 2006. Understanding long bone development in broilers and turkeys. Avian Poult. Biol. Rev. 17:77-88.

Oviedo-Rondón, E. O., M. J. Wineland, J. Small, H. Cutchin, A. McElroy, A. Barri, and S. Martin. 2009. Effect of incubation temperatures and chick transportation conditions on bone development and leg health. J. Appl. Poult. Res. 18:671-678.

Oviedo-Rondón, E. O., J. Small, M. J. Wineland, V. L. Christensen, P. S. Mozdziak, M. D. Koci, S. V. L. Funderburk, D. T. Ort, and K. M. Mann. 2008. Broiler embryo bone development is influenced by incubator temperature, oxygen concentration and eggshell conductance at the plateau stage in oxygen consumption. Br. Poult. Sci. 49:666-676.

Møller, A.P., and J. Manning. 2003. Growth and developmental instability. Vet. J. 166:19-27.

Pechak, D. G., M. J. Kujawa, and A. I. Caplan. 1986a. Morphological and histochemical events during first bone formation in embryonic chick limbs. Bone. 7:441-458.

Pechak, D. G., M. J. Kujawa, and A. I. Caplan. 1986b. Morphology of bone development and bone remodeling in embryonic chick limbs. Bone. 7:459-472.

Powell, J. J., M. W. Whitehead, C. C. Ainley, M. D. Kendall, J. K. Nicholson, and R. P. H. Thompson. 1999. Dietary minerals in the gastrointestinal tract: hydroxypolymerisation of aluminium is regulated by luminal mucins. J. Inorg. Biochem. 75:167-180.

Predieri, G., D. Beltrami, R. Pattacini, M. L. Parisi, A. Sinicropi, D. Valensin, and R. Basosi. 2009. Structural studies in solution and in the solid state on the zinc chelate of 2-hydroxy-(4-methylthio) butanoic acid, an effective mineral supplement in animal feeding. Inorg. Chim. Acta. 362:1115-1121.

Predieri, G., L. Elviri, M. Tegoni, I. Zagnoni, E. Cinti, G. Biagi, S. Ferruzza, and G. Leonardi. 2005. Metal chelates of 2-hydroxy-4-methylthiobutanoic acid in animal feeding: Part 2: further characterizations, in vitro and in vivo investigations. J. Inorg. Biochem. 99:627-636.

Predieri, G., M. Tegoni, E. Cinti, G. Leonardi, and S. Ferruzza. 2003. Metal chelates of 2-hydroxy-4-methylthiobutanoic acid in animal feeding: preliminary investigations on stability and bioavailability. J. Inorg. Biochem. 95:221-224.

Rath, N., G. Huff, W. Huff, and J. Balog. 2000. Factors regulating bone maturity and strength in poultry. Poult. Sci. 79:1024-1032.

Rath, N., J. Balog, W. Huff, G. Huff, G. Kulkarni, and J. Tierce. 1999. Comparative differences in the composition and biomechanical properties of tibiae of seven- and seventy-two-week-old male and female broiler breeder chickens. Poult. Sci. 78:1232-1239.

Rawlinson, S. C. F., D. Murray, J. R. Mosley, C. D. P. Wright, J. C. Bredl, L. K. Saxon, N. Loveridge, C. Leterrier, P. Constantin, C. Farquharson, and A. A. Pitsillides. 2009. Genetic selection for fast growth generates bone

architecture characterised by enhanced periosteal expansion and limited consolidation of the cortices but a diminution in the early responses to mechanical loading. Bone 45:357-366.

Revell, D. K., and R. J. Hughes. 2005. Between-bird variation in the concentrations of iron and other minerals in egg yolk across a laying cycle. Annual Australian Poultry Science Symposium (17th : 2005 : Sydney, Australia):1034-6260.

Richards, M. P., and M. J. Packard. 1997. Mineral metabolism in avian embryos. Poult. Avian Biol. Rev. 7:143-161.

Richards, M. P. 1991. Mineral metabolism in the developing turkey embryo - I. The effects of developmental age and shell-less culture on trace element contents of selected tissues. Comp. Biochem. Physiol. 100:1009-1016.

Richards, M. 1997. Trace mineral metabolism in the avian embryo. Poult. Sci. 76:152-164.

Roach, H. I. 1997. New aspects of endochondral ossification in the chick: chondrocyte apoptosis, bone formation by former chondrocytes, and acid phosphatase activity in the endochondral bone matrix. J. Bone Miner. Res. 12:795-805.

Roach, H. I., and J. R. Shearer. 1989. Cartilage resorption and endochondral bone formation during the development of long bones in chick embryos. Bone Miner. 6:289-309.

Romanoff, A. L. 1967. The avian embryo: Structure and functional development. MacMillan, New. York.

Rucker, R. B., T. Kosonen, M. S. Clegg, A. E. Mitchell, B. R. Rucker, J. Y. Uriu-Hare, and C. L. Keen. 1998. Copper, lysyl oxidase, and extracellular matrix protein cross-linking. Am J Clin Nutr. 67:996-1002.

Rucker, R. B., H. E. Parker, and J. C. Rogler. 1969. Effect of copper deficiency on chick bone collagen and selected bone enzymes. J. Nutr. 98:57-63.

Saito, M., and K. Marumo. 2010. Collagen cross-links as a determinant of bone quality: a possible explanation for bone fragility in aging, osteoporosis, and diabetes mellitus. Osteoporos. Int. 21:195-214.

Salvante, K. G., and T. D. Williams. 2002. Vitellogenin dynamics during egg-laying: daily variation, repeatability and relationship with egg size. J. Avian Biol. 33:391–398.

Sandrock, B., S. Kern, and S. Bryan. 1983. The movement of zinc and copper from the fertilized egg into metallothionein-like proteins in developing chick hepatic tissue. Biol. Trace Elem. Res. 5:503-515.

Schmidt, C. J., M. E. Persia, E. Feierstein, B. Kingham, and W. W. Saylor. 2009. Comparison of a modern broiler line and a heritage line unselected since the 1950s. Poult. Sci. 88:2610-2619.

Seo, H., Y. Cho, T. Kim, H. Shin, and I. Kwun. 2010. Zinc may increase bone formation through stimulating cell proliferation, alkaline phosphatase activity and collagen synthesis in osteoblastic MC3T3-E1 cells. Nutr. Res. Pract. 4:356-361.

Shaw, A. L., J. P. Blake, and E. T. Moran. 2010. Effects of flesh attachment on bone breaking and of phosphorus concentration on performance of broilers hatched from young and old flocks. Poult. Sci. 89:295-302.

Simpson, C. F., J. E. Jones, and R. H. Harms. 1967. Ultrastructure of aortic tissue in copper-deficient and control chick embryos. J. Nutr. 91:283-291.

Skrivan, M., V. Skrivanova, and M. Marounek. 2005. Effects of dietary zinc, iron, and copper in layer feed on distribution of these elements in eggs, liver, excreta, soil, and herbage. Poult. Sci. 84:1570-1575.

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

Statcan.gc.ca http://www.statcan.gc.ca/pub/23-015-x/2012002/t001-eng.htm accessed Oct.2012.

Strause, L., P. Saltman, and J. Glowacki. 1987. The effect of deficiencies of manganese and copper on osteo induction and on resorption of bone particles in rats. Calcif. Tissue Int. 41:145-150.

Sun, Q., Y. Guo, J. Li, T. Zhang, and J. Wen. 2012a. Effects of methionine 54ydroxyl analog chelated Cu/Mn/Zn on laying performance, egg quality, enzyme activity and mineral retention of laying hens. J. Poult. Sci. 49:20-25.

Sun, Q., Y. Guo, S. Ma, J. Yuan, S. An, and J. Li. 2012b. Dietary mineral sources altered lipid and antioxidant profiles in broiler breeders and posthatch growth of their offsprings. Biol Trace Elem Res 145:318-324.

Swiatkiewicz, S., and J. Koreleski. 2008. The effect of zinc and manganese source in the diet for laying hens on eggshell and bones quality. Vet. Med. 53:555-563.

Tahir, M., M. Y. Shim, N. E. Ward, C. Smith, E. Foster, A. C. Guney, and G. M. Pesti. 2012. Phytate and other nutrient components of feed ingredients for poultry. Poult. Sci. 91:928-935.

Tona, K., O. Onagbesan, B. De Ketelaere, E. Decuypere, and V. Bruggeman. 2004. Effects of age of broiler breeders and egg storage on egg quality, hatchability, chick quality, chick weight, and chick post-hatch growth to forty-two days. J. Appl. Poult. Res. 13:10-18.

Turner, C. H. 2006. Bone Strength: Current Concepts. Ann. N. Y. Acad. Sci. 1068:429–446.

Ulmer-Franco, A. M., G. M. Fasenko, and E. E. O'Dea Christopher. 2010. Hatching egg characteristics, chick quality, and broiler performance at 2 breeder flock ages and from 3 egg weights. Poult. Sci. 89:2735-2742.

Uluozlu, O. D., M. Tuzen, D. Mendil, and M. Soylak. 2009. Assessment of trace element contents of chicken products from Turkey. J. Hazard. Mater. 163:982-987.

Vieira, S. L., and E. T. Moran. 1998. Eggs and chicks from broiler breeders of extremely different age. J. Appl. Poult. Res. 7:372-376.

Viguet-Carrin, S., P. Garnero, and P. D. Delmas. 2006. The role of collagen in bone strength. Osteoporos. Int. 17:319-36.

Virden, W. S., J. B. Yeatman, S. J. Barber, C. D. Zumwalt, T. L. Ward, A. B. Johnson, and M. T. Kidd. 2003. Hen mineral nutrition impacts progeny livability. J. Appl. Poult. Res. 12:411-416.

Virden, W., J. Yeatman, S. Barber, K. Willeford, T. Ward, T. Fakler, R. Wideman, and M. Kidd. 2004. Immune system and cardiac functions of progeny chicks from dams fed diets differing in zinc and manganese level and source. Poult. Sci. 83:344-351.

Wang, F., B. Kim, M. J. Petris, and D. J. Eide. 2004. The mammalian Zip5 Protein is a zinc transporter that localizes to the basolateral surface of polarized cells. J. Biol. Chem. 279:51433-51441.

Whitehead, M. W., R. P. Thompson, and J. J. Powell. 1996. Regulation of metal absorption in the gastrointestinal tract. Gut 39:625–628.

Willemsen, H., N. Everaert, A. Witters, L. De Smit, M. Debonne, F. Verschuere, P. Garain, D. Berckmans, E. Decuypere, and V. Bruggeman. 2008. Critical assessment of chick quality measurements as an indicator of post-hatch performance. Poult. Sci. 87:2358-2366.

Williams, B., D. Waddington, D. H. Murray, and C. Farquharson. 2004. Bone strength during growth: influence of growth rate on cortical porosity and mineralization. Calcif. Tissue Int. 74:236-245.

Yadgary, L., A. Cahaner, O. Kedar, and Z. Uni. 2010. Yolk sac nutrient composition and fat uptake in late-term embryos in eggs from young and old broiler breeder hens. Poult. Sci. 89:2441-2452.

Yair, R., Z. Uni, and R. Shahar. 2012. Bone characteristics of late-term embryonic and hatchling broilers: Bone development under extreme growth rate. Poult. Sci. 91:2614-2620.

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.

Yalcin, S., and P. Siegel. 2003. Exposure to cold or heat during incubation on developmental stability of broiler embryos. Poult. Sci. 82:1388-1392.

Yalçin, S., H. B. Molayoğlu, M. Baka, O. Genin, and M. Pines. 2007. Effect of temperature during the incubation period on tibial growth plate chondrocyte differentiation and the incidence of tibial dyschondroplasia. Poult. Sci. 86:1772-1783.

Yalçin, S., S. Özkan, E. Coskuner, S. Özkan, Y. Delen, Y. Kurtulmus, and T. Tanyalçin. 2001. Effects of strain, maternal age and sex on morphological characteristics and composition of tibial bone in broilers. Br. Poult. Sci. 42:184.

Yi, G. F., C. A. Atwell, J. A. Hume, J. J. Dibner, C. D. Knight, and J. D. Richards. 2007. Determining the methionine activity of Mintrex organic trace minerals in broiler chicks by using radiolabel tracing or growth assay. Poult. Sci. 86:877-887.

Yildirim, I., and R. Yetisir. 2004. Effects of different hatcher temperatures on hatching traits of broiler embryos during the last five days of incubation. S. Afr. J. Anim. Sci. 34:211-216.

Yu, Y., L. Lu, R. L. Wang, L. Xi, X. G. Luo, and B. Liu. 2010. Effects of zinc source and phytate on zinc absorption by in situ ligated intestinal loops of broilers. Poult. Sci. 89:2157-2165.

Yu, Y., L. Lu, X. G. Luo, and B. Liu. 2008. Kinetics of Zinc Absorption by In Situ Ligated Intestinal Loops of Broilers Involved in Zinc Transporters. Poult. Sci. 87:1146-1155.

Yuan, J., Z. Xu, C. Huang, S. Zhou, and Y. Guo. 2011. Effect of dietary Mintrex-Zn/Mn on performance, gene expression of Zn transfer proteins, activities of Zn/Mn related enzymes and fecal mineral excretion in broiler chickens. Anim. Feed Sci. Technol. 168:72-79.

Zhao, J., R. B. Shirley, M. Vazquez-Anon, J. J. Dibner, J. D. Richards, P. Fisher, T. Hampton, K. D. Christensen, J. P. Allard, and A. F. Giesen. 2010. Effects of chelated trace minerals on growth performance, breast meat yield, and footpad health in commercial meat broilers. J. Appl. Poult. Res. 19:365-372.

CHAPTER 2: THE INFLUENCE OF MATERNAL DIETARY CU, ZN AND MN ON BONE DEVELOPMENT OF BROILER CHICKEN EMBRYOS FROM THREE HEN AGES

2.1. INTRODUCTION

Copper, Zn and Mn are micro nutrients essential for proper embryonic bone development. A severe and prolonged Zn deficiency imposed in a hen's diet, for example, increased the incidence of skeletal anomalies and embryonic mortality in her progeny (Kienholz et al., 1961). However, trace mineral I deficiencies in commercial poultry production are not commonly reported because the maternal diet is commonly supplemented with inorganic mineral (ITM) levels significantly above the minimum TM level recommended by the National Research Council (Leeson and Caston, 2008).

Copper, Zn and Mn are involved in several enzymatic activities important for skeletal metabolism (Angel, 2007; Dibner et al., 2007). Early bone growth depends on Mn for synthesis of the bone cartilage model (Leach et al., 1969; Eyre, 2004); whereas, Cu is important for bone strength and elasticity (Rucker et al., 1998; Dibner et al., 2007). Zinc is an important regulator of bone density by stimulating osteoblast proliferation and osteoprotegerin activity (Liang et al., 2012). Increases in skeletal development in the offspring might be accomplished through supplementation of a combination of Cu, Zn and Mn organic trace

minerals (OTM) in the hen's diet. In fact, chicks from hens fed a basal diet supplemented with 80 mg/kg of Zn methionine had increased dry bone weight at hatch relative to those from hens fed the same levels as Zn oxide (Kidd et al., 1992).

Concerns regarding the impact of trace minerals levels in manure have challenged nutritionists to reduce supplemented TM levels in poultry diets (Leeson and Caston, 2008). In this context there is an opportunity to lower TM levels by supplementing with OTM. Organic minerals are widely reported to be more bioavailable then ITM forms (Huang et al., 2009; Zhao et al., 2010) and this may relate to minerals chelated to an organic chelate (amino acid [AA] or organic acid) being less susceptible to binding by anti-nutritional factors that adversely affect the uptake of trace minerals ions in the gastrointestinal tract (Yi et al., 2007). In fact, progeny of broiler breeder hens fed a diet supplemented with Cu-, Zn- and Mn-HMTBa (2-hydroxy-4-(methylthio) butanoic acid) chelates to replace 30% of the supplemental ITM had increased walking ability compared to progeny from hens fed only ITM (Eusebio-Balcazar et al., 2010). Furthermore, TM chelated to HMTBa in a hen's diet did not influence tibia mineral density and bone mineral content of the progeny at 49 days relative to those from hens fed ITM but reduced relative shank asymmetry in the progeny at 40 days (Oviedo-Rondón et al., 2008a). Bone asymmetries can decrease chicken growth and worsen tibial dyschondroplasia, which is a pathological leg disorder in chickens (Møller et al., 1999).

Swiatkiewicz and Koreleski, (2008) reported that replacing 50% of the Zn and Mn oxides with Zn and Mn AA maintained egg quality in laying hens. Likewise, low or marginal levels of TM chelated to AA were sufficient to maintain laying hen performance and eggshell quality in relation to a group of hens fed higher levels of TM as sulfates (Gheisari et al., 2011). One of the pertinent questions in breeder trace mineral nutrition is whether feeding reduced levels of OTM would increase trace mineral content in the egg relative to those from hens fed inorganic salt supplementation. Supplementation of more available forms of Cu, Zn and Mn in the maternal diet might benefit embryonic development within the egg. Likewise, the age-related changes in egg yolk trace mineral content in eggs from hens fed various mineral forms has been investigated by relatively few researchers. The purpose of the current study was to investigate whether TM form and level in the hen diet would influence trace minerals in the egg yolk of hens of diverse ages. Increased TM content in the egg might increase skeletal development of embryos from hens of diverse ages. This information could contribute to a better understanding of the factors and mechanisms that might affect skeletal development that starts within the egg.

2.2 MATERIALS AND METHODS

2.2.1. Broiler Breeder Management and Diets

The experimental protocol was approved by the University of Alberta Animal Care and Use Committee for Livestock. Ross 308 broiler breeder pullets were managed according to the primary breeder management guide (Aviagen,

2006). A total of 360 pullets and 90 males were reared in floor pens in a light-tight facility (12.5m^2 each, 4 pens with 61 birds and 2 pens with 60 hens; 2 pens of males with 36 and 37 birds each). Birds received 23 h of light/d (80 to100 lux in brooding area) for the first 2 d post-hatch. At day 3, the lighting program was reduced daily to 8 h of light/d (30-60 lux in brooding area) until day 10 and kept constant until 22 weeks of age. From hatch to 3 weeks of age, the pullets received a standard starter diet (2.90 Mcal ME/kg; 19.00% CP) fed ad libitum. At 4 weeks of age, pullets were fed a grower diet (2.86 Mcal ME/kg; 15.00% CP) to 21 weeks; pullets were weighed on a weekly basis and feed allocation was based on the mean weekly body weight (BW) per pen.

At 22 weeks of age 18 hens per treatment (n=72 in total) and 60 males were transferred to another light-tight facility where they were randomly placed individually into laying cages with sloped floors (48 cm width x 46 cm depth x 42 cm height) and Specht rooster cages (34 cm x 57 x 42 with sloped floors), respectively. Photo-stimulation occurred at 22 weeks when the photoperiod was increased to 11h of light/d. Photo-period was increased weekly until 15h of light/d at 27 weeks and was then kept constant up to 60 weeks. All hens were individually weighed (BW-2050 version 2.20+, Weltech Int., Cambridgeshire, England) on a weekly basis from 22 to 60 weeks of age whereas males were individually weighed every other week. The feed management program followed the management guide of the broiler breeders (Aviagen, 2006) considering the average body weight within each treatment and weekly egg production.

At 22 weeks of age, each broiler breeder was assigned to one of four dietary treatments and fed the experimental diets up to 60 weeks of age. Dietary treatments consisted of a basal corn-wheat-soy ration low in Cu, Zn and Mn to which trace minerals as inorganic (sulfates) or organic were added (Table 2-1). The Control diet (ITM) contained Cu, Zn and Mn supplemented at industry-relevant levels, i.e., mineral as sulfates at 100 mg Zn, 120 mg Mn and 10 mg Cu/kg diet. In Diet 2 (OTM) trace minerals were supplemented following an approximation of the National Research Council (NRC; 1994) recommended levels for laying hens (50 mg Zn, 60 mg Mn, 10 mg Cu/kg diet chelated HMTBa as Mintrex® Zn, Mintrex Mn and Mintrex Cu, respectively; Novus International Inc., St. Charles, MO). Dietary treatments 3 and 4 consisted of increased supplementation of TM as either OTM or ITM. Diet 3 included OTM + ITM (Diet 1 plus an additional 40 mg Zn, 40 mg Mn and 20 mg Cu/kg diet as Mintrex P (Novus International Inc., St. Charles, MO); and Diet 4 included high levels of ITM (Diet 1 plus 40 mg Zn, 40 mg Mn and 20 mg Cu/kg diet as sulfates). To ensure that the diets contained equal amounts of methionine activity, HMTBa as a methionine source (Alimet; Novus international, Inc. St. Louis, MO) was decreased accordingly in diets containing Mintrex P or Mintrex Cu, Mintrex Zn and Mintrex Mn. Therefore, all diets had similar nutrient composition with the exception of the source and level of supplemental Cu, Mn and Zn. Inductively coupled plasma analyses were conducted to determine the trace mineral content of the experimental diets at Novus International Inc., St. Charles, MO.

Individual daily egg production was recorded for determination of weekly and total egg production per hen. The total egg production period was calculated from 23 to 60 weeks of hen age. Eggs were collected at the same time once per day and an egg quality code was assigned. An egg with an intact shell and a single yolk was defined as a hatching egg. A cracked, broken, soft-shelled or double-yolked egg was defined as defective. Total number of hatching eggs was calculated from the total number of eggs less the total number of defective eggs produced throughout the production period.

A total of 8 hatching eggs per treatment (n=32/ hen age) of hens at 32 week old (Young) , 45 week (Mid) and 59 week (Old) were saved for egg trait measurements. Whole egg, yolk and albumen were weighed; egg yolk and albumen were expressed as a percentage of the whole egg weight. Egg yolks were stored at -20^{0}C until analysis for Cu, Zn and Mn content. Trace mineral contents were measured at The Natural Resources Analytical Laboratory at the University of Alberta. Yolk samples which had been previously freeze dried were weighed and digested with 10 mL of concentrated HNO_3 and then 30% H_2O_2 (Campbell and Plank, 1998; Purpose, 2011). The digested samples were analyzed for their Cu, Zn and Mn trace mineral content using a Varian 880 Atomic Absorption spectrometer (AAS), and were expressed as concentration in egg yolk (µg/g of egg yolk as dry matter basis) and total amount in egg yolk (µg/egg yolk; which is a function of the trace mineral concentration of egg yolk [dry matter basis] and the weight of the yolk [wet matter basis]). Because about 40% of the yolk samples were shown to contain Mn levels below the minimum reported limits (< 1 per

µg/g) data were not analyzed statistically. The certified reference material in bovine liver standard was obtained from the National Institute of Standards and Technology (standard reference material 1577b bovine liver). Reference recovery of the nitric acid digestion ranged from 105.7% for Zn and 105.3% for Cu, these values were close to the quality control recoveries, which ranged from 103.8% for Zn and 104.2% for Cu.

Embryonic bone development evaluation was conducted in eggs from hens at Young (28 to 29 weeks), Mid (42 to 43 weeks) and Old (56 to 57 weeks of age). Two weeks prior the beginning of egg collection, broiler breeder hens were artificially inseminated for 2 consecutive days in the first week and once per week at the same week day in the following two weeks. Females were inseminated with 0.05 mL/day of pooled semen from a population of 60 males. Fertile eggs were individually labeled by hen, saved for incubation and stored at 17°C and 75 to 80% RH for no longer than 10 days. At placement, eggs from the same hen were set together into trays of 18 eggs. Each tray had at least one group of eggs from the same hen of each treatment. Trays were placed within a 5,000 egg capacity Jamesway single-stage setter (Jamesway Incubator Company, Cambridge, Ontario, Canada) and incubated until 20 days at a dry bulb temperature of 37.5°C and 56% RH.

With the exception of embryos at 15 days of incubation (E15) from Young hen which were not sampled due to technical error, at each breeder flock age embryos at E15, E17 and E20 were sampled for bone evaluation. At each

embryonic age, one embryo per hen was removed from the shell and immediately killed by cervical dislocation. The stage of tibia and femur development was investigated using the differential staining technique in which bones were stained with Alcian Blue and Alizarin Red (Kelly and Bryden, 1983; Blom and Lilja, 2004) for analysis of non-calcified and calcified tissue, respectively. Digital images were taken using an Olympus Stylus Tough – 6020 digital camera mounted on a photo station. Bones were positioned in a similar orientation along a ruler marked in millimetres (scale bar); and pictures were taken from the antero-posterior face of each bone. The length, width and % calcification ([calcified area/ bone area]*100) were measured from digital images and calculated with ImageJ (version 1.43q, available online at: http://rsb.info.nih. Gov/nih-image/). The scale bar printed on the digital images was used to calibrate the program. Femur length was determined from the proximal edge of the trochanter to the distal edge of the condyle. Tibia length was measured from the proximal end at the intercondylar eminence to the end of the distal epiphysis. Bone medial-lateral width was measured at the midpoint (50% of length). Measurements were taken by the same investigator to reduce variability.

2.2.2. Statistical Analysis

Individual hens, eggs, embryos and chicks were considered experimental units. The total number of eggs and hatching eggs produced throughout 23 to 60 weeks were analyzed as a 1-way analysis of variance with dietary treatment (Control, OTM, OTM + ITM or High ITM) as the main effect using the Mixed Model analysis in SAS (SAS Institute Inc., Cary, NC). Repeated measures

analysis using the Mixed Model analysis in SAS (SAS Institute Inc., Cary, NC) was performed on hen body weight, egg and bone trait data. The appropriate covariance structure was selected to account for correlations between the observations made on the same experimental unit (subject) and heterogeneous variances among observations on the same subject over time (Wang and Goonewardene, 2004). The following model was used to determine differences among the treatment groups: $Yijk = \mu + \alpha i + \beta j + (\alpha\beta)ij + \varepsilon ijk$, where Yijk was the associated constant to all observations during the experiment (variable measured), μ was the general variable mean, αi was the effect of hen age (where I = {Young, Mid or Old}), βj was the effect of mineral form where j = {Control, OTM, OTM + ITM or High ITM}, $(\alpha\beta)ij$ was the interaction effect between the I and j factors, and εijk was the error associated with each observation. Eggs or bones of embryos from the experimental unit (hen) were the repeated term in the overall model, and hen age was the time factor. When the model was significant at $P < 0.05$, the least significant difference method was used to identify statistically different means.

2.3. RESULTS AND DISCUSSION

2.3.1. Broiler Breeder Performance

As expected, average body weight increased as the hen aged ($P < 0.001$, Figure 2-1); the average BW at 23 weeks of age was 2,344 ± 0.06 kg whereas BW at 60 weeks was 3,579 ± 0.06 kg which represents about 7.7 and 5.5% under the target BW recommended by the broiler breeder manual (Aviagen, 2006). Dietary treatment did not influence breeder body weights (Table 2-2), this was expected

because breeder hens were managed to reach the body weight determined by the breeder's guide.

There was no influence of dietary treatment on either total number of eggs produced or total number of hatching eggs produced from 23 to 60 weeks (Table 2-3). Although not significant, hens from the High ITM diet numerically laid about 33 eggs less than hens fed the Control diet. The small sample size in the study may have limited the statistical significance of the results; this trend warrants further investigation. Gheisari et al. (2011) reported that supplementation of trace mineral sulfates at 7 mg Cu, 65 mg Zn and 75 mg Mn/kg of diet did not influence hen day egg production and percentage of broken eggs relative to minerals chelated to AA at 10.5 mg Cu, 60 mg Zn and 60 mg Mn/kg of diet. Furthermore, partial or complete substitution of ITM with OTM had no effect on egg production (Swiatkiewicz and Koreleski, 2008; Gheisari et al.; 2011). Therefore, it appears that supplementing the hen's diet with low OTM levels maintained egg production performance; however, further studies with larger number of hens are required.

Regardless of dietary treatment, eggs from Old hens were heavier and had a larger proportion of yolk and decreased proportion of albumen compared with eggs from Mid (except proportion of albumen) or Young hens (Table 2-3). These results agree with the findings of Suarez et al. (1997), Shaw et al. (2010) and Ulmer-Franco et al. (2010) who also reported that as hens aged the egg weight and proportion of yolk increased whereas albumen decreased.

Egg yolk Cu concentration (µg/g of dry yolk) was not affected by dietary treatment or hen age (Table 2-4). Total amount of Cu in the egg yolk (µg/egg yolk) increased in eggs from Old hens compared to Young and Mid hens. Egg yolk was used as a covariate in the statistical analysis, which indicates that the increase in Cu and Zn content in the eggs from Old hens was caused by a mechanism other than more yolk being deposited in the egg. Sun et al. (2012b) reported that supplementation of minerals at 8 mg/kg Cu, 50 mg/kg Zn and 60 mg/kg Mn chelated to HMTBa reduced Cu concentration in the egg yolk of 39 week hens by about 19% but increased Cu by about 20% in the albumen relative to those from hens fed same mineral levels as sulfates. Although the majority of Cu is stored in the egg yolk (Yair and Uni 2011) the albumen and eggshell also contain some Cu (Richards, 1997). Albumen TM content was not investigated in the present study therefore conclusions cannot be made. It is possible that OTM supplementation might increase Cu transfer to the egg via the oviduct route to the albumen and egg shell; however this is a proposed requires further investigation.

Sun et al. (2012a) found that complete replacement of Cu sulfate (10 mg/kg) as Cu-HMTBa increased yolk Cu concentration from laying hens at 43 weeks but not at 48 or 52 weeks of age. However, the present study differed by providing diets supplemented with trace minerals from the beginning of the study (23 weeks) whereas hens from the previous research were supplemented with trace mineral deficient diets for two weeks before the beginning of that trial. Therefore it is possible that TM deficient hens might have greater responses to TM supplementation relative to non-deficient hens.

Zinc concentration (µg/g of dry yolk) in the yolk from hens supplemented with Cu, Zn and Mn at industry levels (Control) were not significantly different from hens fed low levels of OTM at any age (Figure 2-2). Increasing level of TM as ITM increased Zn concentration in the egg yolk relative to Control in eggs from Mid hens, but was not significantly different from OTM or OTM + ITM (Figure 2-2). In general, increasing mineral levels as OTM or ITM in the hen diet did not remarkably influence Cu or Zn concentration in the egg yolk. It is possible that trace minerals supplemented at industry levels are already high enough and therefore adding more would have no extra effect. Previously, Mabe et al. (2003) also reported that increasing trace mineral levels (30 mg/kg, 30 mg/kg, 5 mg/kg or 60 mg/kg, 60 mg/kg, 10 mg/kg of Zn, Mn and Cu, respectively) as either organic (minerals chelated to amino acid) or as $ZnSO_4$, MnO and $CuSO_4$ did not increase Cu, Zn and Mn content in the egg yolk of 69 week old hens. Furthermore, hens fed a diet supplemented with similar mineral levels as in the present study, i.e. Zn at 160 mg/kg as Zn+AA or a combination of Zn+AA and $ZnSO_4$, also laid eggs (albumen + yolk) with similar Zn content; however Zn+AA eggs had increased Zn content relative to eggs from hens fed $ZnSO_4$ (Hudson et al., 2004). The data reported here indicate that a 50% reduction in Zn was sufficient to meet requirements for Zn content in the egg yolk when supplied as OTM.

Total amount of Zn in the egg yolk (µg/egg yolk) increased in eggs from Old hens compared to Young and Mid hens; which were not significantly different from each other. This result indicates that increased Zn content in the yolk from older hens yolk was likely not related with their larger yolks compared

to younger hens because the effect of egg yolk weight was used as a covariate in the statistical model ($P < 0.0001$, Table 2-4). Hudson et al., (2004) reported that eggs from 62 week old hens had about 17% less Zn content relative to 32 week old hens; however, Zn content was measured from a blend of yolk + albumen whereas the present study investigated Zn content in the egg yolk.

2.3.2. Embryonic Bone Characteristics

At E15, embryos from Mid hens had greater bone length and proportion of calcified tissue compared to embryos from Old hens (Tables 2-5 and 2-6). Furthermore, regardless of hen age, maternal dietary supplementation of OTM + ITM increased femur length relative to embryos from hens fed Control or High ITM diets (Table 2-5). At E17, embryos from Young hens had tibias and femurs with greater length and width in comparison with bones of embryos from Mid (except tibia width) and Old hens. Embryos from Mid hens had the highest proportion of calcified tissue, whereas embryos from Old hens had the least. Embryos from Young hens were intermediate and significantly different from both other hen ages (Tables 2-5 and 2-6). At E17, embryos from hens fed OTM tended to have longer femurs relative to embryos from hens fed Control diet ($P=0.053$, Table 2-5). There were no other effects of dietary treatment on bone traits at days 15, 17 or 20. Results from this study indicated that OTM supplementation in the maternal diet increased femur length of chicken embryos at E15 and E17 but not at E20; the small sample size in the study may have limited the statistical significance of the results; these trends warrant further investigation. Because there were no significant effects of diet on mineral

concentration in the egg yolk, the mechanism causing the difference in bone growth between embryos from hens fed OTM or ITM are not clear and requires further investigation.

At E20, embryos from Mid hens had longer and wider femurs and tibia relative to Young (except tibia length) and Old hens' embryos. Moreover, Mid hens' embryos had the highest proportion of calcified tissue compared to Young (tibia and femur) and Old (tibia) hens. Collectively, data from the current study indicated that embryos from Young hens have reduced bone development relative to embryos from Old hens towards the end of incubation.

Embryos from 60 week old Cobb broiler breeders had wider and heavier tibia and femur than those from 30 week old hens; however, the effect disappeared when bone weights were expressed as a proportion of body weight (Alfonso-Torres et al., 2009). Bone alkaline phosphatase (ALP) activity, used as an indicator of endochondral ossification, was also not significantly different between embryo from young and old hens (Alfonso-Torres et al., 2009). Bone alkaline phosphatase releases inorganic phosphate for formation of hydroxyapatite crystal in the bone matrix during endochondral ossification. Although this enzyme activity is correlated with the rate of skeletal mineralization in Great Tits *(Parus major*), no correlation was observed with elongation of long bones (Tilgar et al., 2008). This indicates that using ALP enzymatic activity as the only measurement to compare skeletal development among embryos from different ages might not tell the whole story. In the current study, the distribution of mineralized tissue (i.e

pattern of calcified and non-calcified tissue) was used as a measure to assess bone calcification. Therefore, it is possible that variations in mineralization among embryos from diverse hen ages might be caused by a mechanism other than ALP activity; however, this possibility requires further investigation. It is also important to note that conflicting findings between studies could result from the study of different strains of birds studied. The present study investigated bone growth in embryos from Ross 308, whereas Alfonso-Torres et al. (2009) studied bone growth from Cobb embryos. In fact, embryonic metabolism, growth (Hamidu et al., 2007; Tona et al., 2010) and bone development (Oviedo-Rondón et al., 2008b) are known to differ among embryos from different genetic strains (Hamidu et al., 2007; Tona et al., 2010). Whether the pattern of skeletal mineralization and ALP activity varies among embryos from different strains and hen ages warrants further investigation.

Concomitant with the time of the highest rate of bone development within the egg, minerals important for bone growth (i.e. P, Fe, Zn, Cu and Mn) are absorbed to the greatest extent from the egg yolk from day 11 to day 17 of incubation in embryos from hens at 50 week old (Yair and Uni., 2011). Whether differences exist regarding the pattern of egg yolk mineral absorption by embryos from diverse hen ages throughout incubation has yet to be investigated. Because Zn and Cu are important for bone development (Seo et al., 2010; Liang et al., 2012), it is possible that the increased Cu and Zn minerals in the yolk observed in this study might be associated with differences in bone quality among embryos from Young versus Mid and Old hens. Speier et al. (2012) reported that certain

genes related with carbohydrate and protein metabolism were up-regulated in the yolk sac membrane of embryos from 22 to 30 week-old relative to embryos from 45 to 50 week-old flocks. Furthermore, Yadgary et al. (2010) reported that in addition to having more fat in the egg yolk, the embryos from a 50 week-old flock mobilized fat more efficiently at days 13 and 15 relative to embryos from a 30 week-old flock. Collectively, it is possible that, as it is the case of fat uptake, embryos from Young hens might have sufficient mineral (perhaps, Ca, P, Zn, Mn) from the egg yolk in a period of greatest embryonic bone development from days 15 to 17, but limited mineral resources for the last days of development because those embryos come from eggs with lesser Cu and Zn in the egg yolk compared to eggs from older hens. Embryos from younger hens might lack nutrients to support bone growth towards the last days of incubation; this proposed is supported by the fact that at day 20 the bones of embryos from Young hens were less mineralized relative to embryos from Old hens. Increased of yolk minerals (Cu and Zn) and efficiency of nutrient assimilation might be an advantage for embryos to support growth, especially in the last two days of incubation; however, this might not be the case for embryos from young flocks.;

2.4. CONCLUSIONS

Dietary mineral form and level supplemented to hens from 22 to 60 weeks of age did not consistently influence the concentration nor total amount of Cu or Zn in the yolk and embryonic bone characteristics and this lack of effect was independent of hen age. Maternal supplementation with low levels of Cu, Zn and

Mn as OTM generally did not influence egg mineral concentration and embryonic skeletal characteristics relative to TM levels currently used in industrial diets, although some significant or near significant effects of diet were seen with femur length. Because trace mineral transport to the egg could already be maximized, it is not possible to conclude that low levels of TM as OTM were more available than mineral sulfates supplemented at current industry levels. Nevertheless, the data reported here demonstrate that reduced levels of TM supplied as OTM supported comparable egg yolk mineral levels and bone development as did industry levels of ITM. Furthermore, increasing mineral levels as either a combination of OTM + ITM or as ITM solely had no effect on egg mineral content likely because minerals supplemented at industry levels might be already high enough and therefore adding more had no added effect.

As hens aged, Zn and Cu content in the egg yolk was increased. Furthermore, embryos from Young hens had inferior bone development in the last days of incubation than embryos from Mid or Old hens. Additional research is needed to investigate if different patterns of embryonic bone growth impact bone quality at hatch.

Table 2-1. Composition of experimental diets fed to broiler breeder hens from 22 to 60 weeks of age

Item	Control[1]	OTM[2]	OTM + ITM[3]	High ITM[4]
Ingredient	---------- (% of diet)----------			
Basal breeder diet[5]	90.0	90.0	90.0	90.0
Wheat	8.43	8.51	8.39	8.34
Canola oil	0.49	0.50	0.49	0.53
Alimet[6]	0.17	0.10	0.11	0.18
Vitamin E premix[7]	0.80	0.80	0.80	0.80
Breeder mineral premix[8]	0.10		0.10	0.13
Total, kg	100	100	100	100
Nutrient composition				
ME, Kcal/kg	2,882	2,884	2,881	2,883
Crude Protein, %	15.49	15.48	15.48	15.48
Crude Fat,%	3.82	3.83	3.82	3.86
Crude Fibre,%	2.19	2.19	2.19	2.19
Calcium, %	3.37	3.36	3.36	3.37
Available phosphorus, %	0.37	0.37	0.37	0.37
Met + Cys, %	0.69	0.69	0.68	0.69
Methionine, %	0.40	0.41	0.40	0.42
Lysine, %	0.72	0.73	0.72	0.72
Tryptophan, %	0.19	0.19	0.19	0.19
Threonine, %	0.54	0.54	0.54	0.54
Arginine, %	0.95	0.95	0.95	0.95
Sodium, %	0.18	0.18	0.18	0.18

[1]Control: Mineral sulfates at 100 mg/kg Zn, 120 mg/kg Mn and 10 mg/kg Cu. Diet contained Cu 23.67, Zn 159.40 and Mn 174.67 mg /kg of diet based on actual analysis.

[2]OTM: Mintrex Cu, Zn and Mn added at 0.06, 0.04 and 0.03% respectively to provide 50 mg/kg Zn, 60 mg/kg Mn and 10 mg/kg Cu. Diet contained Cu 24.27, Zn 119.75 and Mn 114.87 mg /kg of diet based on actual analysis.

[3]OTM + ITM: Mintrex P added at 0.1 % to provide an additional 40 mg/kg Zn, 40 mg/kg Mn and 20 mg/kg Cu. Diet contained Cu 43.07, Zn 185.85 and Mn 186.65 mg /kg of diet based on actual analysis.

[4]High ITM: Control plus an additional of 40 mg/kg Zn, 40 mg/kg Mn and 20 mg/kg (or 0.0067% of Cu and 0.0017% of Zn as sulfates). Diet contained Cu 44.37, Zn 176.25 and Mn 192.80 mg /kg of diet based on actual analysis.

[5]Basal breeder diet contained a vitamin-premix (0.1% inclusion) which contained: Mg 87.86 mg, Zn 99.93 mg, Fe 80.12 mg, Cu 15.02 mg, Se 0.3 mg, Vitamin A 12.5 UI, Vitamin D 3.1 UI, Vitamin K 2.49 mg, Thiamin 2.58 mg, Riboflavin 7.52 mg, Niacin 37.53 mg, Pyridoxine 4.97 mg, pantothenate acid 12.49 mg,

choline 2.70 mg, vitamin B12 0.019 mg, Folic acid 0.90 mg, biotin 0.15 mg per kg of diet

[6] Alimet feed supplement (Novus International Inc., St. Louis, MO) provided 88% methionine activity as 2-hydroxy-4-(methylthio) butanoic acid (HMTBa).

[7]Vitamin E premix provided 40 mg UI per kg of diet as natural vitamin E.

[8]Breeder mineral premix provided 100 mg of Zn, 119.7 mg of Mn and 10mg of Cu per kilogram of mixed feed.

Table 2-2. Effect of dietary Cu, Mn and Zn form and level on hen body weight (23 to 60 weeks old) total and settable egg production from 22 to 60 weeks old

Item	Hen body weight[1]	Egg production[2]	
		----------Total-------	--------Settable-----
Dietary treatment			
Control[3]	3.272	174	173
OTM [4]	3.294	164	159
OTM+ITM[5]	3.291	171	167
High ITM[6]	3.282	147	140
SEM	0.053	10	10
P-value			
Diet	0.9730	0.2133	0.1012
Hen age	< 0.0001	-	-
Diet × Hen age	0.9998	-	-

[1]Average body weight of hens from 23 to 60 weeks.

[2]Total egg production: total number of eggs laid between 23 and 60 weeks of egg production. Settable eggs: total number of eggs laid per hen that were not cracked, soft shell, shell less or double yolk.

[3]Control: mineral sulfates at 100 mg/kg Zn, 120 mg/kg Mn and 10 mg/kg Cu.

[4]OTM: Mintrex P (Zn, Mn and Cu chelated by HMTBA) at 50 mg/kg Zn, 60 mg/kg Mn, 10 mg/kg Cu.

[5]OTM + ITM: Control plus an additional 40 mg/kg Zn, 40 mg/kg Mn and 20 mg/kg Cu as OTM.

[6]High ITM: Control plus 40 mg/kg Zn, 40 mg/kg Mn and 20 mg/kg Cu as sulfates.

Table 2-3. Effect of dietary Cu, Zn and Mn trace mineral form and maternal flock age on egg characteristics

Item	Egg weight (g)	Yolk (%)	Albumen (%)
Dietary treatment			
Control[1]	64.16	32.54	54.00
OTM[2]	63.95	31.79	53.53
OTM+ITM[3]	62.32	32.82	52.51
High ITM[4]	65.03	32.64	53.06
SEM	0.89	0.49	0.49
Hen age[5]			
Young, Y	57.08[c]	29.77[c]	54.22[a]
Mid, M	64.85[b]	33.00[b]	52.20[b]
Old, O	69.97[a]	34.57[a]	52.67[b]
SEM	0.81	0.44	0.42
Diet × Hen age			
Control × Y	57.75	29.43	54.28
Control × M	64.73	33.38	53.00
Control × O	70.21	34.82	51.71
OTM × Y	56.57	29.54	54.20
OTM × M	65.13	31.60	53.37
OTM × O	70.16	34.21	53.03
OTM+ITM × Y	56.97	29.95	54.14
OTM+ITM × M	62.73	34.38	50.10
OTM+ITM × O	67.27	34.11	53.30
High ITM × Y	57.23	30.16	54.25
High ITM × M	66.81	32.64	52.31
High ITM × O	71.05	35.12	52.63
SEM	1.33	0.84	0.85
P-value			
Diet	0.0577	0.4731	0.5286
Hen age	<0.0001	<0.0001	0.0021
Diet × Hen age	0.6729	0.7025	0.2260

[a-c] Means within a column lacking a common superscript differ significantly ($P < 0.05$).

[1]Control: mineral sulfates at 100 mg/kg Zn, 120 mg/kg Mn and 10 mg/kg Cu.

[2]OTM: Mintrex Cu, Mintrex Zn and Mintrex Mn (Zn, Mn and Cu chelated by HMTBA) at 50 mg/kg Zn, 60 mg/kg Mn, 10 mg/kg Cu.

[3]OTM + ITM: Control plus an additional 40 mg/kg Zn, 40 mg/kg Mn and 20 mg/kg Cu as Mintrex P.

[4]High ITM: Control plus 40 mg/kg Zn, 40 mg/kg Mn and 20 mg/kg Cu as sulfates.

[5]Hen age: Young (32 weeks of age), Mid (45 weeks) and Old (59 weeks).

Table 2-4. Effect of dietary Cu, Zn and Mn trace mineral forms on Cu, Zn and Mn concentration in the egg yolk from Young Mid and Old hens

	---------- Cu ----------		---------- Zn ----------	
Item	µg/g[1]	µg/egg yolk[2]	µg/g	µg/egg yolk
Dietary treatment				
Control[3]	4.90	41.17	94.76	783.1
OTM[4]	5.60	46.99	94.05	806.4
OTM+ITM[5]	6.61	59.18	96.09	810.4
High ITM[6]	6.34	58.53	98.10	874.5
SEM	0.71	7.25	3.09	27.5
Hen age[7]				
Young, Y	6.12	39.85^{b}	97.80	736.0^{b}
Mid, M	5.53	48.59^{b}	95.73	807.6^{b}
Old, O	5.94	65.97^{a}	93.72	912.2^{a}
SEM	0.50	4.95	2.12	31.40
Diet × Hen age[8]				
Control × Y	5.34	33.45	101.68^{abc}	771.0
Control × M	3.97	33.01	90.58^{bc}	728.2
Control × O	5.39	57.04	92.04^{bc}	850.1
OTM × Y	6.13	38.64	95.22^{abc}	716.0
OTM × M	5.36	42.03	96.95^{ab}	812.5
OTM × O	5.30	60.30	89.99^{c}	890.5
OTM+ITM × Y	6.21	37.32	98.08^{abc}	705.2
OTM+ITM × M	6.60	60.47	92.33^{abc}	769.6
OTM+ITM × O	7.02	79.77	97.85^{ab}	956.4
High ITM × Y	6.81	49.98	96.23^{abc}	751.7
High ITM × M	6.17	58.83	103.07^{a}	920.2
High ITM × O	6.05	66.78	95.00^{bc}	951.5
SEM	1.01	9.84	4.25	52.01
P-value				
Diet	0.3298	0.2288	0.8094	0.1689
Hen Age	0.6038	0.0129	0.3108	0.0128
Diet × Hen age	0.9006	0.5969	0.0301	0.2733
EY[9]	NS	NS	NS	<0.0001

[a-c] Means within a column lacking a common superscript differ significantly ($P < 0.05$).

[1]µg/g: trace mineral per milligrams per gram of egg yolk as dry matter basis.

[2]µg/egg yolk: calculated based on egg yolk weight (wet matter), egg yolk dry matter content and trace mineral content per µg/g (dry mater basis).

[3] Control: mineral sulfates at 100 mg/kg Zn, 120 mg/kg Mn and 10 mg/kg Cu.

[4] OTM: Mintrex Cu, Mintrex Zn and Mintrex Mn (Zn, Mn and Cu chelated by HMTBA) at 50 mg/kg Zn, 60 mg/kg Mn, 10 mg/kg Cu.

[5]OTM + ITM: Control plus an additional 40 mg/kg Zn, 40 mg/kg Mn and 20 mg/kg Cu as Mintrex P.

[6] High ITM: Control plus 40 mg/kg Zn, 40 mg/kg Mn and 20 mg/kg Cu as sulfates.

[7] Hen age: Young (32 week of age), Mid (45 week) and Old (59week).
[8] n = 8 egg per maternal dietary treatment and hen age.
[9]EY: P value shows the effect of egg yolk weight on mineral concentration in the egg yolk, which was then used as a covariate for the analysis.

Table 2-5. Effect of dietary Cu, Zn and Mn trace mineral form and maternal flock age on femur length, width and calcified tissue in chick embryos

	----------Length (mm) ----------			---------- Width (mm) --------			---------- Calcified tissue (%) -------		
Item	E15	E17	E20	E15	E17	E20	E15	E17	E20
Dietary treatment									
Control[1]	14.77[bc]	19.07	22.51	1.53	2.00	2.30	37.53	54.18	60.54
OTM[2]	14.86[ab]	19.45	22.74	1.55	2.03	2.25	38.87	53.52	59.65
OTM+ITM[3]	15.07[a]	19.27	22.31	1.54	2.00	2.19	37.70	53.91	59.03
High ITM[4]	14.59[c]	19.05	22.26	1.55	2.01	2.27	37.90	54.23	59.36
SEM	0.09	0.11	0.18	0.02	0.02	0.03	0.90	0.64	0.86
Hen age[5]									
Young, Y	-	20.59[a]	22.32[b]	-	2.08[a]	2.09[c]	-	54.23[b]	52.55[b]
Mid, M	14.94[a]	18.83[b]	22.98[a]	1.54	2.00[b]	2.38[a]	44.03[a]	56.37[a]	63.30[a]
Old, O	14.70[b]	18.21[c]	22.07[b]	1.55	1.96[b]	2.28[b]	31.97[b]	51.28[c]	63.07[a]
SEM	0.06	0.12	0.18	0.01	0.02	0.03	0.64	0.63	0.61
Diet × Hen age[6]									
Control × Y	-	20.65	22.37	-	2.06	2.18	-	54.53	54.69
Control × M	14.87	18.71	23.04	1.50	1.97	2.37	45.08	56.34	63.54
Control × O	14.68	17.87	22.12	1.55	1.99	2.33	29.96	51.66	63.36
OTM × Y	-	20.75	22.53	-	2.09	2.12	-	52.54	51.74
OTM × M	15.17	18.92	23.52	1.56	2.03	2.40	44.54	56.98	64.48
OTM × O	14.55	18.68	22.18	1.54	1.99	2.23	33.21	51.03	62.71
OTM+ITM × Y	-	20.64	22.01	-	2.08	1.99	-	55.50	50.65
OTM+ITM × M	15.19	19.04	22.65	1.53	2.00	2.32	42.94	56.81	62.34
OTM+ITM × O	14.94	18.14	22.28	1.56	1.92	2.23	32.46	49.42	64.09
High ITM × Y	-	20.33	22.40	-	2.08	2.05	-	54.34	53.14
High ITM × M	14.54	18.66	22.68	1.55	2.01	2.41	43.54	55.34	62.82
High ITM × O	14.64	18.16	21.70	1.55	1.93	22.34	32.27	53.01	62.13
SEM	0.13	0.17	0.31	0.03	0.03	0.06	1.28	1.26	1.23

P-value									
Diet	0.0077	0.0532	0.2626	0.8558	0.6552	0.1785	0.7098	0.8605	0.6387
Hen age	0.0117	<0.0001	0.0090	0.4986	0.0063	<0.0001	<0.0001	0.0004	<0.0001
Diet × Hen age	0.0642	0.2792	0.3933	0.7235	0.8259	0.5707	0.2755	0.4093	0.2534
BW[7]	0.0001	<0.0001	0.0491	0.0001	0.0139	NS	NS	NS	NS

[a-c] Means within a column lacking a common superscript differ significantly ($P < 0.05$).

[1]Control: mineral sulfates at 100 mg/kg Zn, 120 mg/kg Mn and 10 mg/kg Cu.

[2]OTM: Mintrex Cu, Mintrex Zn and Mintrex Mn (Zn, Mn and Cu chelated by HMTBA) at 50 mg/kg Zn, 60 mg/kg Mn, 10 mg/kg Cu.

[3]OTM + ITM: Control plus an additional 40 mg/kg Zn, 40 mg/kg Mn and 20 mg/kg Cu as Mintrex P.

[4]High ITM: Control plus 40 mg/kg Zn, 40 mg/kg Mn and 20 mg/kg Cu as sulfates.

[5] P value shows the effect of chick embryo body weight on bone traits, which was then used as a covariate for the analysis.

Hen age: Young (32 week of age), Mid (45 week) and Old (59week).

[6]n = 18 embryos per maternal dietary treatment and hen age

[7]BW: P value shows the effect of chick embryo body weight on bone traits, which was then used as a covariate for the analysis.

Table 2-6. Effect of dietary Cu, Zn and Mn trace mineral form and maternal flock age on tibia length, width and calcified tissue in chick embryos

	----------Length (mm) ----------			--------- Width (mm) ------			--------- Calcified tissue (%) -------		
Item	E15	E17	E20	E15	E17	E20	E15	E17	E20
Dietary treatment									
Control[1]	18.31	25.58	29.99	1.54	2.00	2.19	38.14	53.01	56.85
OTM[2]	18.27	26.08	30.12	1.53	2.00	2.22	37.73	53.55	57.19
OTM+ITM[3]	17.73	25.52	30.02	1.54	2.04	2.16	38.72	53.50	56.07
High ITM[4]	17.34	25.40	29.92	1.50	2.03	2.16	39.19	54.08	54.88
SEM	0.39	0.20	0.14	0.03	0.03	0.04	1.00	0.84	0.63
Hen age[5]									
Young, Y	-	27.37^{a}	30.06^{ab}	-	2.10^{a}	2.11^{b}	-	53.59^{b}	50.63^{c}
Mid, M	19.86^{a}	25.33^{b}	30.40^{a}	1.55	2.02^{a}	2.28^{a}	44.08^{a}	55.73^{a}	60.72^{a}
Old, O	15.97^{b}	24.24^{c}	29.57^{b}	1.51	1.93^{b}	2.15^{b}	32.81^{b}	51.27^{c}	57.39^{b}
SEM	0.24	0.16	0.15	0.02	0.03	0.03	0.70	0.73	0.54
Diet × Hen age[6]									
Control × Y	-	27.71	30.07	-	2.11	2.14	-	54.07	51.06
Control × M	20.16	25.08	30.34	1.55	1.93	2.23	44.52	54.34	61.57
Control × O	16.47	23.93	29.54	1.54	1.96	2.21	31.77	51.11	57.92
OTM × Y	-	27.61	30.17	-	2.02	2.15	-	53.49	51.26
OTM × M	20.20	25.72	30.55	1.58	2.03	2.39	42.16	55.95	61.57
OTM × O	16.34	24.92	29.62	1.49	1.94	2.13	33.30	50.62	58.75
OTM+ITM × Y	-	26.87	29.92	-	2.18	2.06	-	54.91	49.83
OTM+ITM× M	20.04	25.52	30.39	1.56	2.08	2.25	44.69	55.64	59.54
OTM+ITM × O	15.42	24.17	29.75	1.51	1.86	2.18	32.75	49.96	58.85
High ITM × Y	-	27.28	30.09	-	2.08	2.10	-	54.34	50.37
High ITM × M	19.03	25.00	30.31	1.51	2.06	2.27	44.95	55.47	60.19
High ITM × O	16.65	23.94	29.38	1.50	1.96	2.10	33.42	51.92	54.06
SEM	0.48	0.32	0.26	0.04	0.05	0.06	1.41	1.43	1.07

P-value									
Diet	0.2759	0.1237	0.8231	0.7978	0.6451	0.6874	0.7487	0.8616	0.0656
Hen age	<0.0001	<.0001	<0.0001	0.2170	<0.0001	0.0011	<0.0001	0.0003	<0.0001
Diet × Hen age	0.7280	0.2720	0.9706	0.6674	0.1012	0.6001	0.5479	0.9158	0.0634
BW[7]	0.0192	<0.0001	0.0075	NS	NS	NS	NS	NS	NS

[a-c] Means within a column lacking a common superscript differ significantly (P < 0.05).
[1]Control: mineral sulfates at 100 mg/kg Zn, 120 mg/kg Mn and 10 mg/kg Cu.
[2]OTM: Mintrex Cu, Mintrex Zn and Mintrex Mn (Zn, Mn and Cu chelated by HMTBA) at 50 mg/kg Zn, 60 mg/kg Mn, 10 mg/kg Cu.
[3]OTM + ITM: Control plus an additional 40 mg/kg Zn, 40 mg/kg Mn and 20 mg/kg Cu as Mintrex P.
[4]High ITM: Control plus 40 mg/kg Zn, 40 mg/kg Mn and 20 mg/kg Cu as sulfates.
[5]Hen age: Young (32 week of age), Mid (45 week) and Old (59week).
[6]n = 18 embryos per maternal dietary treatment and hen age
[7]BW: P value shows the effect of chick embryo body weight on bone traits, which was then used as a covariate for the analysis.

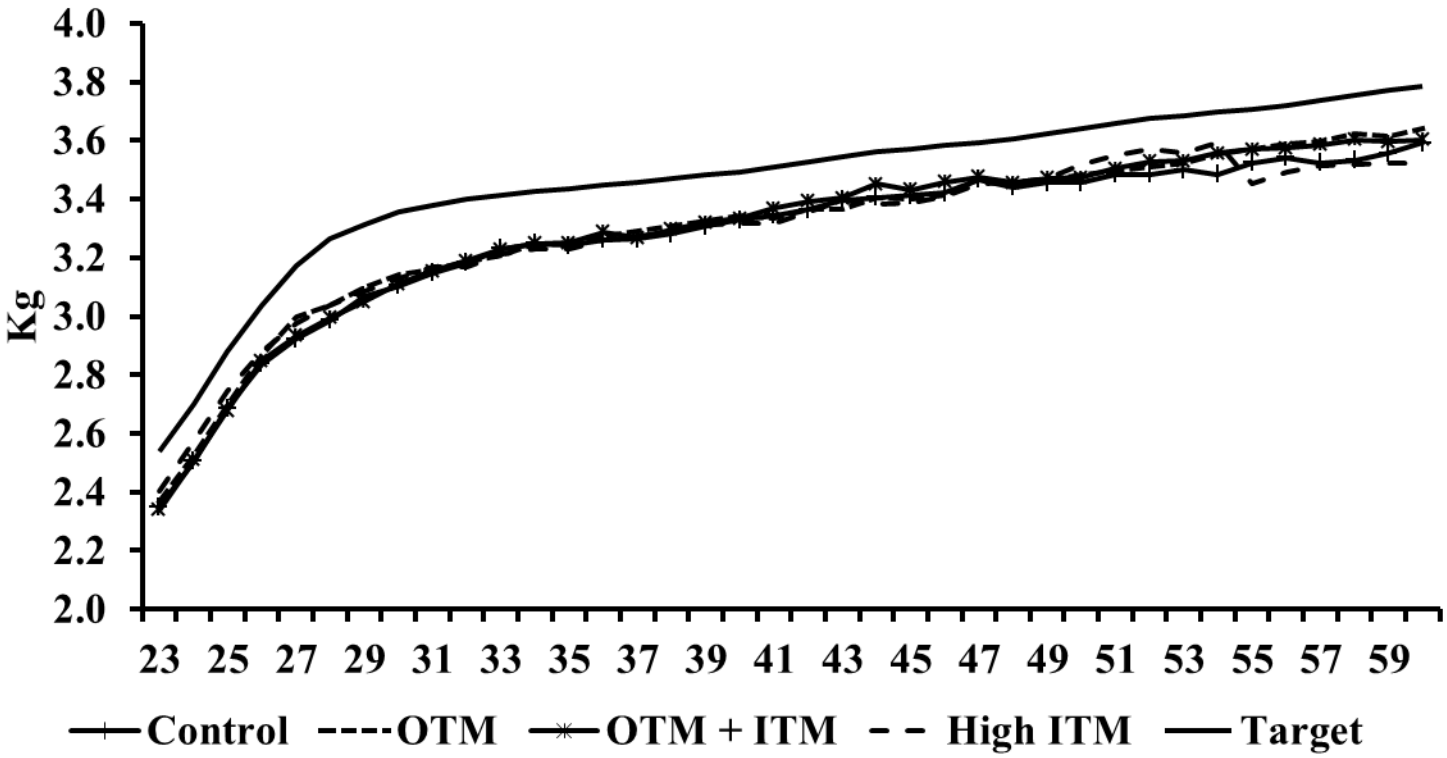

Figure 2-1. Effect of dietary Cu, Zn and Mn trace mineral forms and level of broiler breeder body weight from 23 to 60 weeks.

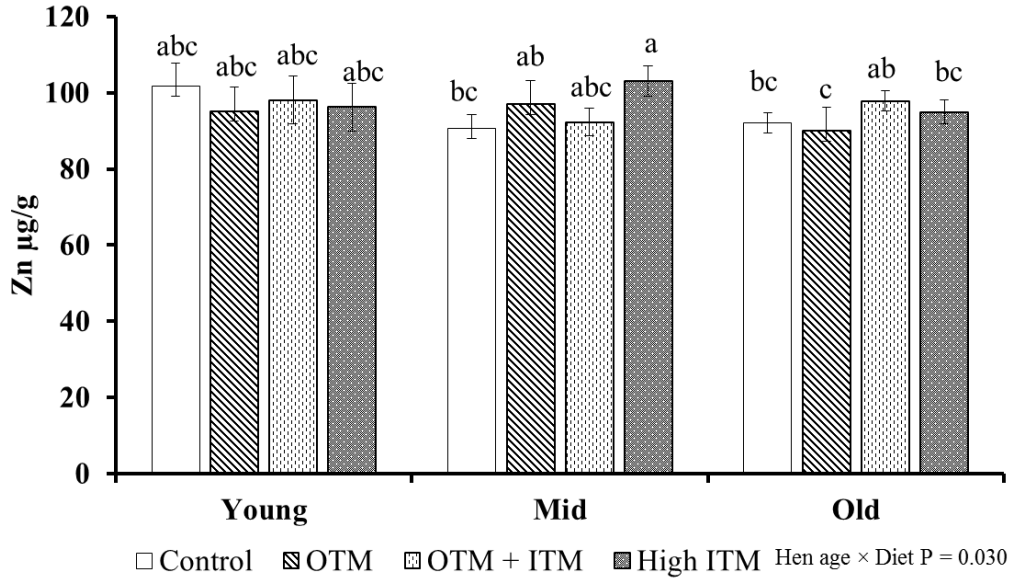

Figure 2-2. Effect of dietary Cu, Zn and Mn trace mineral forms on Zn concentration in the egg yolk (µg/g) from Young Mid and Old hens. The Control treatment was broiler breeders fed mineral sulfates at 100 mg/kg Zn, 120 mg/kg Mn and 10 mg/kg Cu. The OTM was broiler breeders fed Zn, Mn and Cu chelated by HMTBa at 50 mg/kg Zn, 60 mg/kg Mn, 10 mg/kg Cu. The OTM + ITM was the Control plus an additional 40 mg/kg Zn, 40 mg/kg Mn and 20 mg/kg Cu as OTM; and High ITM was the Control plus 40 mg/kg Zn, 40 mg/kg Mn and 20 mg/kg Cu as sulfates; Hen age: Young (32 week of age), Mid (45 week) and Old (59week). n= 36 eggs per treatment per hen age. $^{a-c}$ Means with differing letters are significant different ($P < 0.05$) within effect or interaction.

2.5. REFERENCES

Alfonso-Torres, K. A., L. H. Gargaglioni, J. M. Pizauro, D. E. Faria Filho, R. L. Furlan, and M. Macari. 2009. Breeder age and bone development in broiler chicken embryos. Arq. Bras. Med. Vet. Zootec. 61:219-226.

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.

Aviagen. 2006. Ross broiler management manual.

Blom, J., and C. Lilja. 2004. A comparative study of growth, skeletal development and eggshell composition in some species of birds. J. Zool. 262:361-369.

Campbell, C. R., and C. O. Plank. 1998. Preparation of plant tissue for laboratory analysis. Pages 44-45 in Handbook and Reference Methods for Plant Analysis. Y .P. Kalra ed. CRC Press.

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.

Eusebio-Balcazar, P. E., E. O. Oviedo-RondónOviedo-Rondón, A. Mitchell, B. Brake, M. J. Wineland, V. Moraes, and N. Leandro. 2010. Effects of breeder feeding and trace mineral source on leg health and bone traits of broiler progeny. Poult. Sci. 89 (Suppl. 1):223(Abstr.)

Eyre, D. R. 2004. Collagens and cartilage matrix homeostasis. Clin. Orthop. Relat. Res. 427:118-122.

Gheisari, A., A. Sanei, A. Samie, M. Gheisari, and M. Toghyani. 2011. Effect of diets supplemented with different levels of manganese, zinc, and copper from their organic or inorganic sources on egg production and quality characteristics in laying hens. Biol. Trace Elem. Res. 142:557-571.

Hamidu, J. A., G. M, Fasenko, J. J. R, Feddes, E. E, O'Dea, C. A., Ouellette, M. J., Wineland, and V. L. Christensen. 2007. The effect of broiler breeder genetic strain and parent flock age on eggshell conductance and embryonic metabolism. Poult. Sci. 86:2420-2432.

Huang, Y. L., L. Lu, S. F. Li, X. G. Luo, and B. Liu. 2009. Relative bio availabilities of organic zinc sources with different chelation strengths for broilers fed a conventional corn-soybean meal diet. J. Anim. Sci. 87:2038-2046.

Hudson, B. P., W. A. Dozier III, J. L. Wilson, J. E. Sander, and T. L. Ward. 2004. Reproductive performance and immune status of caged broiler breeder hens provided diets supplemented with either inorganic or organic sources of zinc from hatching to 65 wk of age. J. Appl. Poult. Res. 13:349-359.

Kelly, W. L., and M. M. Bryden. 1983. A modified differential stain for cartilage and bone in whole mount preparations of mammalian fetuses and small vertebrates. Biotech. Histochem. 58:131-134.

Kidd, M. T., N. B. Anthony, and S. R. Lee. 1992. Progeny performance when dams and chicks are fed supplemental zinc. Poult Sci. 71:1201-1206.

Kienholz, E. W., D. E. Turk, M. L. Sunde, and W. G. Hoekstra. 1961. Effect of zinc deficiency in the diets of hens. J. Nutr. 75:211-221.

Leach, J. R. M., A. Muenster, and E. M. Wien. 1969. Studies on the role of manganese in bone formation: II. Effect upon chondroitin sulfate synthesis in chick epiphyseal cartilage. Arch. Biochem. Biophys. 133:22-28.

Leeson, S., and L. Caston. 2008. Using minimal supplements of trace minerals as a method of reducing trace mineral content of poultry manure. Anim. Feed Sci. Technol. 142:339-347.

Liang, D., M. Yang, B. Guo, J. Cao, L. Yang, and X. Guo. 2012. Zinc up regulates the expression of osteo protegerin in mouse osteoblasts MC3T3-E1 through PKC/MAPK pathways. Biol. Trace Elem. Res. 146:340-348.

Oviedo-Rondón, E. O., L. Mejia-Sanchez, J. Small, K. E. Brannan, N. Leksrisompong, A. Mitchell, T. A. York, and J. Brake. 2008a. Organic minerals (Mintrex® P) in broiler breeder diets and pre-incubation conditions on bone development and leg health of broilers. Poult Sci. 87(Supl. 1):162 (Abstr.).

Oviedo-Rondón, E. O., J. Small, M. J. Wineland, V. L. Christensen, J. L. Grimes, S. V. L. Funderburk, D. T. Ort, and K. M. Mann. 2008b. Effects of

incubator temperature and oxygen concentration during the plateau stage of oxygen consumption on turkey embryo long bone development. Poult. Sci. 87:1484-1492.

Purpose, A. 2011. Plant Analysis. Page 206 in Laboratory Guide for Conducting Soil Test and Plant Analysis. J. B. J. Jones, ed. CRC press.

Richards, M. 1997. Trace mineral metabolism in the avian embryo. Poult. Sci. 76:152-164.

Rucker, R. B., T. Kosonen, M. S. Clegg, A. E. Mitchell, B. R. Rucker, J. Y. Uriu-Hare, and C. L. Keen. 1998. Copper, lysyl oxidase, and extracellular matrix protein cross-linking. Am. J. Clin. Nutr. 67:996-1002.

Seo, H., Y. Cho, T. Kim, H. Shin, and I. Kwun. 2010. Zinc may increase bone formation through stimulating cell proliferation, alkaline phosphatase activity and collagen synthesis in osteoblastic MC3T3-E1 cells. Nutr. Res. Pract. 4:356-361.

Speier, J.S., Yadgary, L., Uni, Z., and Wong, E. A. 2012. Gene expression of nutrient transporters and digestive enzymes in the yolk sac membrane and small intestine of the developing embryonic chick. Poult. Sci. 91:1941-1949.

Sun, Q., Y. Guo, J. Li, T. Zhang, and J. Wen. 2012a. Effects of methionine hydroxy analog chelated Cu/Mn/Zn on laying performance, egg quality,

enzyme activity and mineral retention of laying hens. J. Poult. Sci. 49:20-25.

Sun, Q., Y. Guo, S. Ma, J. Yuan, S. An, and J. Li. 2012b. Dietary mineral sources altered lipid and antioxidant profiles in broiler breeders and post-hatch growth of their offspring. Biol. Trace. Elem. Res. 145:318-324.

Swiatkiewicz, S., and J. Koreleski. 2008. The effect of zinc and manganese source in the diet for laying hens on eggshell and bones quality. Vet. Med. 53:555-563.

Tilgar, V., P. Kilgas, A. Viitak, and S. J. Reynolds. 2008. The rate of bone mineralization in birds is directly related to alkaline phosphatase activity. Physiol. Biochem. Zool. 81:106-111.

Tona, K., O. M. Onagbesan, B. Kamers, N. Everaert, V. Bruggeman, and E. Decuypere. 2010. Comparison of Cobb and Ross strains in embryo physiology and chick juvenile growth. Poult. Sci. 89:1677-1683.

Wang, A., and L. A. Goonewardene. 2004. The use of MIXED models in the analysis of animal experiments with repeated measures data. Can. J. Anim. Sci. 84:1-11.

Yadgary, L., A. Cahaner, O. Kedar, and Z. Uni. 2010. Yolk sac nutrient composition and fat uptake in late-term embryos in eggs from young and old broiler breeder hens. Poult. Sci. 89:2441-2452.

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.

Yi, G. F., C. A. Atwell, J. A. Hume, J. J. Dibner, C. D. Knight, and J. D. Richards. 2007. Determining the methionine activity of Mintrex organic trace minerals in broiler chicks by using radiolabel tracing or growth assay. Poult. Sci. 86:877-887.

Zhao, J., R. B. Shirley, M. Vazquez-Anon, J. J. Dibner, J. D. Richards, P. Fisher, T. Hampton, K. D. Christensen, J. P. Allard, and A. F. Giesen. 2010. Effects of chelated trace minerals on growth performance, breast meat yield, and footpad health in commercial meat broilers. J. Appl. Poult. Res.19:365-372.

CHAPTER 3. THE INFLUENCE OF BROILER BREEDER AGE AND DIETARY CU, ZN AND MN FORM AND LEVEL ON BONE DEVELOPMENT IN BROILER CHICKS AT HATCH AND POST-HATCH

3.1. INTRODUCTION

Trace minerals are important for optimal growth and development of broiler hens or chickens. Despite their importance for many metabolic processes in birds, including bone metabolism, the effects of supplementing highly bioavailable forms of trace minerals Cu, Zn and Mn in the hen's diet during embryonic and post-hatch bone development are not yet fully understood.

Commercial organic Cu, Zn and Mn forms chelated to either AA (amino acid) or organic acids are available for livestock supplementation (Cao et al., 2000) and are widely reported to be more bioavailable than inorganic minerals (Huang et al., 2009; Zhao et al., 2010). Supplementing broilers with Cu, Zn and Mn chelated to HMTBa (2-hydroxy-4-(methylthio) butanoic acid) reduced the incidence of tibial dyschondroplasia (Dibner et al., 2007), varus and valgus abnormalities (at 15 weeks; Ferket et al., 2009) relative to sulfate forms. Likewise, progeny of breeder hens supplemented with 30% of Cu, Zn and Mn HMTBa chelates to replace inorganic trace mineral (ITM) had increased walking ability (Eusebio-Balcazar et al., 2010) and progeny had reduced relative shank asymmetry at day 40 relative to progeny of hens fed ITM (Oviedo-Rondón et al., 2008). Sun et al. (2012) reported that supplementing Cu, Zn and Mn-HMTBa

increased growth performance of progeny at 42 days relative to sulfate supplementation. Therefore, supplementing Cu, Zn and Mn chelated to HMTBa can potentially enhance broiler skeletal development. This represents an opportunity to investigate whether using this organic trace mineral (OTM) on broiler breeder diet would influence the embryo and post-hatch growth.

Research on long-term effects of dietary trace minerals in broiler breeder diets and the effect on bone development of the progeny is limited. The purpose of the current study was to investigate the influence of maternal dietary supplementation of Cu, Zn and Mn chelated to HMTBa on skeletal characteristics of progeny, from hens of different ages, at hatch, 7 and 14 days post-hatch. It was hypothesized that bone quality of chicks from hens fed trace minerals chelated to HMTBa, at an approximation of the National Research Council (NRC; 1994) requirements, would be increased in chicks of hens supplemented with mineral sulfates at industry levels, and that effect would be stronger in the progeny of older hens.

3.2. MATERIALS AND METHODS

3.2.1. Broiler Breeders Management and Diets

The experimental protocol was approved by the University of Alberta Animal Care and Use Committee for Livestock under the Canadian Council on Animal Care guidelines (Canadian Council on Animal Care, 1993). A total of 360 day old Ross 308 broiler breeder pullets were reared in environmentally controlled rooms until 22 weeks of age and managed as previously described in

Chapter 2. At 22 weeks of age, each hen was assigned to one of four dietary treatments to 60 weeks of age. Experimental diets consisted of a basal corn-wheat-soy ration low in Cu, Zn and Mn to which trace minerals in inorganic (sulfates) or organic form were added (Table 2-1). The Control diet (ITM) contained Cu, Zn and Mn supplemented at industry-relevant levels, i.e., mineral as sulfates at 100 mg Zn, 120 mg Mn and 10 mg Cu/kg of diet. The OTM diet contained Mintrex Zn, Mintrex Mn and Mintrex Cu, respectively (Zn, Mn and Cu chelated by (HMTBa; Novus International Inc., St. Charles, MO) supplemented at values approximating the NRC mineral recommended levels for laying hens (50 Zn, 60 Mn, 10 Cu/kg of diet). The OTM + ITM and High ITM treatments consisted of increased supplementation of ITM as either OTM or ITM. The OTM + ITM diet included both mineral forms and consisted of the Control diet plus an additional 40 mg Zn, 40 mg Mn and 20 mg Cu/kg of diet as Mintrex P®. The High ITM diet included high levels of inorganic minerals (Control diet plus 40 mg Zn, 40 mg Mn and 20 mg Cu/kg of diet as sulfates). To ensure that diets contained equal amounts of methionine activity, HMTBa as a methionine source (Alimet; Novus international, Inc. St. Louis, MO) was decreased accordingly in any diet containing a Mintrex trace mineral product. Therefore, all diets had similar nutrient composition with the exception of the source and level of supplemental Cu, Mn and Zn. Levels of dietary Cu, Zn and Mn were analyzed through inductively coupled plasma analyses as described in Chapter 2.

Progeny evaluation was conducted in fertile eggs from hens when Young (32 to 33), Mid (45 to 46) and Old (59 to 60) weeks of age. Hens were artificially

inseminated and eggs were managed as previously described in Chapter 2. At set, hatching eggs from each hen were randomly set into trays of 18 eggs. Each tray was placed within a 5,000 egg capacity Jamesway single-stage setter (Jamesway Incubator Company, Cambridge, Ontario, Canada). All eggs were incubated for 18 d at a dry bulb temperature of 37.5°C and 56% RH. At 18 days of incubation, all eggs were removed from the incubator and transferred to a 5,000 egg capacity Jamesway hatcher (Jamesway Incubator Company, Cambridge, ON, Canada) at 37.5°C until hatch (21.5 days). Eggs were placed in pedigree hatch baskets (dimensions = 8 x 8 cm) so that individual eggs and chicks could be traced back to the individual hen.

After 21.5 days of incubation, all hatched chicks were removed and counted. Chicks that had physical abnormalities, were weak, or had unhealed navels were not considered normal and were culled. Hatchability of total eggs set was calculated based on normal healthy chicks only. All normal chicks were feather-sexed, individually weighed and neck tagged (Mark III Swiftack Tagging Gun, Avery Dennison, Pasadena, CA).

At each breeder flock age, between 5 and 8 chicks per hen were placed in Specht pullet-rearing cages (53 x 119 cm, between 5 to 6 cages per treatment depending upon the number of chicks) with plastic-covered wire flooring. Temperature, humidity, and ventilation were standardized in all pens. The broilers were reared to 14 days of age with a photoperiod of 23L:1D. At placement, chicks were fed a starter diet containing 23% CP and 3,025 ME Kcal/kg and

supplemented with inorganic minerals at industry-recommended levels (22 Cu, 80 Zn and 96 of Mn/kg of diet, all as sulfates). Feed and water were provided *ad libitum* throughout the experiment.

3.2.2. Progeny Femur and Tibia Morphological Characteristics

At hatch, 7 and 14 days of age, one chick per hen was randomly chosen for tibia and femur bone sampling (n=72/maternal age). Individual chick body weight was recorded, chicks were euthanized by cervical dislocation, and both legs were removed at the coxo-femoral joint. The left tibia and femur were dissected and cleaned of all adhering muscle tissue. Bone morphological measurements (length and width) were performed with the use of a digital caliper (0.01 resolution mm; ± 0.02 mm accuracy, Absolute Digimatic Mitutoyo) as previously described in Chapter 2. After measurements were taken, bone samples were stored in plastic bags at -20°C until analysis for breaking strength was conducted.

3.2.3. Progeny Femur and Tibia Mechanical Properties

On the day of testing, the left tibia and femur were thawed to room temperature inside plastic bags. At the time of breaking strength analysis, each bone sample was removed from the bag and kept moist with cotton imbibed with distilled water until being placed in the instrument. To avoid introducing additional variation in the strength measurement, each bone was oriented to ensure that bending occurred around the midpoint of the antero-posterior face. Bone breaking strength was measured using the 3-point bending test using an

Instron Materials Tester (Model 4411, Instron Corp., Canton, MA) with BlueHill2 software. The bone was cradled on two support points measuring 5, 10 and 18 mm apart, at hatch, 7 and 14 days, respectively. A probe with a round base was attached to a 5-kg load cell (hatch and 7 days) and 100-kg (14 days) and the force was applied to the midpoint of the antero-posterior face of each bone with a crosshead speed of 1mm/min. Bone breaking strength was defined as the maximum compressive load (kilograms) applied in which the bone sample was no longer resilient (Turner, 2006).

3.2.4. Statistical Analysis

Bone data were analyzed using repeated measures analysis performed using the Proc Mixed procedure of SAS (SAS Institute Inc., Cary, NC) with hen age and dietary treatment as the main effects. The appropriate covariance structure was selected according with Wang and Goonewardene, (2004). The following model was used to determine differences between the treatment groups: $Yijk = \mu + \alpha i + \beta j + (\alpha\beta)ij + \varepsilon ijk$, where Yijk was the associated constant to all observations during the experiment (variable measured), μ was the general variable mean, αi was the effect of hen age where i = {Young, Mid or Old}, βj was the effect of mineral form where j = {Control, OTM, OTM+ITM or High ITM}, (αβ)ij was the interaction effect between the i and j factors, and εijk was the error associated with each observation. Chicks from the experimental unit (hen) were the repeated term in the overall model, and hen age was the time factor. Sex of the chick at hatch, 7 and 14 days post-hatch was included as a random term in the statistical model. Chick body weight at hatch was tested as a

covariate and it was later removed from the model when it did not reveal significance. The least significant difference method was used to identify difference among means. Significance level was set at $P<0.05$.

3.3. RESULTS AND DISCUSSION

3.3.1. Progeny body weight

At hatch, chicks from eggs of Old hens were heavier than chicks from Young hens; chicks from Mid hens were intermediate and significantly different from both other hen ages (Table 3-1). A similar pattern was observed at day 7. At 14 days of age, however, chicks from Mid hens were the heaviest whereas chicks from Young and Old hens were lighter. Increased body weight in chicks from Old hens was expected because, as previously described in Chapter 2, egg weight increased as breeders aged (Table 2-3), and a positive relationship exists between egg size and chick weight at hatch (Decuypere et al., 2001; Tona et al., 2004; Willemsen et al., 2008). Our results are similar to those of Hamidu et al. (2007) who reported that eggs from 59 week old Ross 308 hens hatched heavier chicks than eggs from 34, 40 and 45 week old hens, even when egg weight was held constant. Similar results were also reported for chick body weight at hatch and through the following two weeks post-hatch for chicks from two extreme flock ages of 29 vs 59 weeks old in Cobb 500 flocks (Ulmer-Franco et al., 2010) and 25 vs 65 weeks old in Ross 708 flocks (Shaw et al., 2010). Dietary treatment did not influence chick body weight at hatch, 7 or 14 days among progeny from Young,

Mid and Old hens (Table 3-1). Dietary treatment and hen age did not interact with body weight at any chick age.

3.3.2. Trace Mineral Form and Level

Young hens supplemented with OTM hatched chicks that had wider femurs at hatch relative to chicks from hens fed Control and High ITM; and wider tibia relative to all other treatments (Tables 3-2 and 3-3; Figures 3-1 and 3-2). However, chicks from Mid hens fed Control, OTM or High ITM had wider femurs relative to chicks from OTM + ITM hens (Table 3-2; Figure 3-1). There was no dietary treatment effect on tibia or femur width for chicks from Old hens; in general these chicks had thinner bones than chicks from Young hens. Reasons for inconsistent dietary treatment effects on bone width in chicks from diverse hen ages are not fully understood. This result combined with the non-significance effect of diet on Cu, Zn and Mn content in egg yolk (Chapter 2) suggest that trace mineral form influences bone characteristics by a mechanism other than increasing trace mineral content in the egg. Sun et al (2012) reported that broiler breeder hens supplemented with 8 mg/kg of Cu, 50 mg/kg Zn and 60 mg/kg Mn chelated to HMTBa laid eggs with greater triglyceride and Cu content (albumen) concentration in the egg relative to sulfate minerals. This result indicates that this OTM source can alter, not only trace mineral retention in the egg, but also other nutrients which might be associated with increased growth performance of the progeny.

Supplementing trace minerals in the hen's diet in the form of OTM resulted in similar bone length and strength relative to Control even though the former had trace minerals at substantially lower levels. In this context, reducing levels of inorganic trace minerals by supplementing Cu, Zn and Mn chelated to HMTBa might be enough to meet the hens' requirements. Future dose-response studies, starting at levels expected to be below the hen's requirement, and adding increasing levels of either OTM or ITM are advised.

Given the potential confounding effects of TM bioavailability between OTM and Control, the current study also examined whether adding more trace minerals to the diet resulted in changes in progeny bone characteristics. Although femur breaking strength was not significantly influenced by dietary treatment (Table 3-2), tibia of chicks from hens supplemented with High ITM were stronger at hatch relative to those from Control hens, and relative to all other dietary treatments at day 7 (Table 3-3). Tibia breaking strength at hatch was similar among chicks from hens supplemented with organic mineral forms (OTM vs OTM + ITM), and relative to the performance of chicks from hens fed Control or High ITM diets (Table 3-3). Mineral supplementation in the maternal diet did not influence bone size and strength at day 14 post-hatch in chicks from Young, Mid or Old hens.

Interestingly, high trace mineral level treatments either as OTM + ITM or High ITM in the maternal diet resulted in similar tibia breaking strength of the progeny at hatch; but at 7 days post-hatch the High ITM resulted in increased tibia

strength relative to OTM + ITM. The reasons behind these results are not completely understood. Furthermore, High ITM levels in the maternal diet increased tibia breaking strength of the progeny at hatch and day 7 relative to Control treatment. These results suggest that an opportunity exists to increase bone strength by supplementing trace minerals at levels above current industry levels. Regardless of the increase in breaking strength during post-hatch growth, it is important to note that high trace mineral levels (143 mg/kg Zn and 34 mg/kg Cu) as sulfates in a laying hen diet is correlated with increased mineral excretion into the environment (Skrivan et al., 2005). Furthermore, although not significant, the High ITM hens tended to lay about 21% and 19% less hatchable eggs than Control and OTM + ITM hens, respectively (P = 0.10, Chapter 2; Table 2-3). A trade-off exists in that high levels of inorganic Cu, Zn and Mn in the maternal diet might increase bone breaking strength in progeny but reduce the number of progeny hatching. The ability to detect significant differences in the egg production data was limited by sample size (72 hens per dietary treatment). Hens fed a diet supplemented with similar Zn mineral level as the high trace mineral treatment in the present study, i.e. Zn at 160 mg Zn/kg of diet (50% from ZnAA and $ZnSO_4$) or 100% ZnAA, laid eggs with increased percentage of settable eggs, reduced % of defective eggs and increased egg specific gravity relative to Zn sulfate (Hudson et al., 2004). Gheisari et al. (2011) demonstrated, by scanning electron microscopy, that 65 mg Zn, 75 mg Mn and 7 mg Cu/kg from oxide sources resulted in abnormal development of the mammillary layer of the eggshell of laying hens relative to eggs from hens fed similar mineral levels as sulfates or

chelated to AA. The High ITM treatment from the present study contained mineral levels at 20 mg Cu, 140 mg Zn and 160 mg Mn/kg of diet which were higher than those studied by Gheisari et al. (2011); therefore it is possible that even though High ITM hens laid the same number of total eggs as the Control and OTM + ITM hens, the excess TM levels in that treatment might have impaired matrix formation and calcification of the eggshell resulting in increased number of defective eggs and therefore fewer number of settable eggs.

In industry, new trace mineral supplements are often added to the diet in addition to, rather than in place, of the usual inorganic trace mineral supplementation J.D. Richards (Novus International Inc., St. Charles, MO, personal communication), therefore we hypothesized that adding additional trace mineral as a more bioavailable form in the control diet (OTM + ITM) would increase bone development of the progeny. Contrary to what was predicted, the OTM + ITM treatment reduced tibia width in chicks from Mid hens, and tibia strength at day 7 relative to the High ITM diet. Although the mechanisms of trace mineral absorption are not completely understood, the combination of mineral forms as OTM + ITM might engage more absorption sites or mineral transporters in the intestine of chicks relative to inorganic forms solely (Bai et al., 2008; Yu et al., 2008; Bai et al., 2011). Thus, if TM were limiting, OTM + ITM in the diet could increase mineral retention in the hen's tissue, and therefore enhance bone growth of the progeny. However, OTM + ITM combination did not increase chick quality in terms of bone size and strength relative to trace mineral supplementation at the industry standards contained in the Control diet. This lack

of response might be related to the fact that industry TM-levels could already exceed the minimum needed to maximize bone growth.

3.3.3 Hen Age Effect

At hatch, chicks from Mid hens had longer femurs relative to chicks from Young and Old hens; and stronger femurs relative to chicks from Young hens (Table 3-2). At day 7, chicks from Young hens had wider and shorter femurs relative to Mid and Old hens and at day 14 relative to chicks from Old hens. At day 14, femurs of chicks from Young hens were the weakest whereas femurs from Mid hens were the strongest. Femurs from Old hens were intermediate and significantly differed from both other hen ages.

At hatch chicks from Mid hens had longer tibias than chicks from Old hens; but similar breaking strength (Table 3-3). Chicks from Mid and Old hens had stronger tibia relative to chicks from Young hens. At 7 days, chicks from Young hens had longer tibias relative to those from Mid and Old hens, and stronger tibias than chicks from Mid hens. At 14 days chicks from Young hens had the widest tibias, whereas chicks from Old hens had the narrowest tibia; chicks from Young and Mid hens had stronger tibias relative to chicks from Old hens. Shaw et al. (2010) reported that Ross 708 chicks from an young flock (25 week) also had decreased tibia strength relative to those hatched from an old flock (65 week old; Shaw et al., 2010). Yalcin et al. (2001) reported that chicks hatched from 32 to 35 week old hens had reduced mineral content in the tibia relative to those from 56 to 58 weeks of age. It is important to highlight, that the former

studies of Yalcin et al. (2001) and Shaw et al. (2010) measured chicks from multiple breeder flocks; thus comparisons among studies should be interpreted with that in mind. Our study compared chicks produced within the same experimental broiler breeder flock which were fed the same basal diet and managed similarly throughout the entire study. Therefore, variations due to maternal diet content and nutrient composition, as well as breeder farm management which have been shown to influence chick quality (Yassin et al., 2009), were not sources of variation in the current study.

Bone mineral and collagen content are factors associated with bone strength (Rath et al., 2000); increased mineral, and perhaps, collagen content in bones of chicks from old hens may explain the increased breaking strength observed in chicks from Old vs Young hens observed in this study. Furthermore, because reduced mineralization impaired enzymatic crosslinking (Saito et al., 2010) it is possible that embryos from young hens (who had low calcification, Chapter 2) might also have reduced conversion of immature crosslink into their mature stable form (Saito et al. 2010) resulting in reduced breaking strength at hatch relative to chicks from old hens.

When bone traits were considered as parameters of chick quality, the results from the present study indicate that chicks from Mid hens had higher quality in terms of bone breaking strength at hatch and post-hatch relative to chicks from Young or Old hens. Furthermore, as previously described, chicks from Young hens had reduced bone breaking strength at hatch than those from

Old hens. These results are very interesting, especially considering that the effect of hen age on bone strength was corrected for variation associated with differences in chick body weight (chick body weight was used as a covariate). The exact mechanism by which bone growth changed in chicks from different hen ages is not fully understood. This finding, combined with results from Chapter 2, strongly suggests that reduced breaking strength at hatch in chicks from Young hens might result from differences in bone mineralization that started within the egg This is supported by the fact that embryos from Young hens had greater bone size and calcification at day 17 of incubation but less in the day 20 relative to embryos from Old hens. Yair et al. (2012) reported that, between days 19 and 20 of incubation, embryos from Cobb 500 have reduced mineralization relative to earlier stages of development; embryos from hens at 50 week old had reduced trace mineral reserves within the egg yolk after day 18 of incubation (Yair and Uni, 2011), this might physiologically limit bone growth towards the last days of incubation. Because there is a gap in our knowledge of the mechanisms which result in reduced bone development in embryos and chicks from Young hens, relative to those from Old hens, it is possible that embryos from Young hens, which come from eggs with reduced Cu and Zn content (Chapter 2), might be limited physiologically; chicks from Young hens might have sufficient minerals in their egg yolk reserves to support bone growth up to 15 to 17 of incubation, but not afterwards, relative to embryos from Old hens.

Hen age is a factor determining bone quality at hatch; if weaker bones at hatch are associated with reduced mobility during the hatching process, late

embryonic mortality could increase. Furthermore, if weaker bones at hatch translate into reduced mobility, reduced feed consumption and weight gain in the first days after hatch might result. In this case, chicks might have not access to minerals in the pre-starter diet to meet bone growth requirements in the post-hatch period. Therefore, chicks from young hens might start at the brooder house at a disadvantage relative to chicks from older flocks. As bone grows, there is an increased accumulation of calcium and phosphorus, and thus bone mineral ash, with age (Williams et al., 2004; Yair et al., 2012). Knowledge regarding Cu, Zn and Mn accumulation in bone after hatch and information about macro- and micro mineral requirements for bone growth in the post-hatch period are lacking. Further studies regarding mineral requirements for bone growth in the first days (and perhaps in the following two weeks after hatch) in chicks produced by hens of diverse ages could provide a better understanding of the role of minerals in bone development in broilers and perhaps formulate a pre-starter diet with adequate level of trace minerals.

Interestingly, as previously reported, the femurs and tibias of chicks from Old hens were stronger than those from Young hens at hatch. The same trend was observed in the femur of chicks from Old hens at day 14; however, tibias were weaker than those from Young hens (Tables 3-2 and 3-3). This indicates that femur characteristics might not be indicative of development of other bones such as the tibia. This lack of correlation was also proposed by Applegate and Lilburn (2002). The tibia and femur endure different stresses in supporting the weight of the avian body (Montes et al., 2005). When the bird moves or assumes an

anatomical position, both bones are subjected to longitudinal stresses (caused by compression, tension, or bending loads). The femur is also subjected to torsional loading (Paxton et al., 2010; Abourachid et al., 2011), which is caused by tension of the thigh muscles that twists and suspends the femur against the hip joint in an almost parallel position to the ground (Habib and Ruff, 2008; Paxton et al., 2010). It appears that hen age influenced bone growth pattern differently (e.g., while femur strength decreased; tibia breaking strength increased in chicks from Young hens relative to chicks from Old hens). Chicks from old flocks have increased body weight gain in the first 14 days post-hatch relative to young flocks (Shaw et al., 2010; Ulmer-Franco et al., 2010) a time coincident with intense bone formation after hatch (Applegate and Lilburn, 2002; Yair et al., 2012). Different stresses can influence bone growth, for example, weight-loading narrowed the growth plate and shortened the tibia and femur of young chicks (4 day old; Reich et al., 2005). It is interesting to note, that even though variance due to chick body weight was controlled by covariate analysis ($P < 0.0001$, Table 3-2) hen age still influenced femur and tibia strength at 14 days post-hatch. The mechanisms by which hen age influenced bone strength are not completely understood. Unfortunately, performance data in the post-hatch growth period was not investigated, and therefore it is not possible to speculate whether weight gain influenced bone growth.

3.4. CONCLUSIONS

Hen age and maternal trace mineral nutrition are factors influencing progeny bone development as expressed by bone length, width and breaking strength at hatch, 7 and 14 days. Mineral levels in the hen's diet currently used in industry, based on inorganic forms, could be reduced without decreasing bone quality of the progeny by adding minerals chelated to HMTBa at NRC recommended levels. The main effect of maternal mineral nutrition on bone quality of progeny was demonstrated by supplementing hens with high levels of Cu, Zn and Mn as mineral sulfates relative to industry level at hatch and 7 days. Despite a lower level of supplemental trace mineral, tibia development at hatch was increased in the OTM group relative to all other treatments for Young hens. OTM resulted in greater femur development of chicks relative to progeny of Young hens fed ITM and High ITM diets, and relative to OTM + ITM for progeny of Mid hens. Further studies investigating the dose-response of broiler breeders supplemented with either organic minerals chelated to HMTBa or mineral sulfates in terms of egg production parameters, hatchability and skeletal development of the progeny are advised.

As hens aged, their progeny had stronger bones at hatch. As the chick grew, the patterns of change were not consistent among chicks from different hen ages. In general chicks from Mid hens had the greatest bone quality, whereas chicks from Young hens had the poorest. Chicks from Young hens might start at the brooder house at a disadvantage relative to chicks from older flocks. Future studies regarding nutrient requirements and bone growth among chicks from hens of diverse hen ages in the days after hatch could help formulate strategies to

optimize skeletal growth, especially in chicks from hens, at a time when bone metabolism, growth and, perhaps, mineral requirements are high.

Table 3-1. Effect of 3 broiler breeder ages, Cu, Zn and Mn trace mineral form in the maternal diet and their interaction on chick body weight (BW) at hatch, 7 and 14 days post-hatch

	----------Body weight----------		
Item	Hatch	7 d	14 d
Dietary treatment			
Control[1]	46.0	154.5	432.8
OTM [2]	44.1	151.8	428.2
OTM+ITM[3]	44.9	149.9	434.3
High ITM[4]	44.6	148.3	434.0
SEM	0.8	3.4	41.1
Hen age[5]			
Young, Y	41.1[c]	134.4[c]	422.0[b]
Mid, M	45.7[b]	156.1[b]	451.3[a]
Old, O	47.9[a]	162.9[a]	423.6[b]
SEM	0.5	2.7	9.4
Treatment × Hen age			
Control × Y	41.5	134.4	410.4
Control × M	46.4	161.6	465.8
Control × O	50.1	167.5	422.2
OTM × Y	40.5	141.3	416.3
OTM × M	45.2	150.9	437.6
OTM × O	46.6	163.3	430.7
OTM+ITM × Y	41.6	136.7	429.5
OTM+ITM × M	46.1	152.6	456.9
OTM+ITM × O	47.1	160.4	416.5
High ITM × Y	40.9	125.2	431.9
High ITM × M	45.2	159.3	445.0
High ITM × O	47.8	160.2	425.2
SEM	0.9	5.0	15.5
P-value			
Treatment	0.3185	0.5561	0.9521
Hen age	<0.0001	<0.0001	0.0059
Treatment × Hen age	0.2229	0.1804	0.6468

[a-c] Means within a column lacking a common superscript differ significantly at $P < 0.05$.

[1]Control: mineral sulfates at 100 mg/kg Zn, 120 mg/kg Mn and 10 mg/kg Cu.

[2]OTM: Mintrex Cu, Mintrex Zn and Mintrex Mn (Zn, Mn and Cu chelated by HMTBA) at 50 mg/kg Zn, 60 mg/kg Mn, 10 mg/kg Cu.

[3]OTM + ITM: Control plus an additional 40 mg/kg Zn, 40 mg/kg Mn and 20 mg/kg Cu as Mintrex P.

[4]High ITM: Control plus 40 mg/kg Zn, 40 mg/kg Mn and 20 mg/kg Cu as sulfates.

[5]Hen age: Young (32 to 33 week of age), Mid (45 to 46 wk) and Old (59 to 60 wk).

Table 3-2. Effect of 3 broiler breeder flock ages, Cu, Zn and Mn trace mineral form in the maternal diet and their interaction on physical and mechanical properties of femur from chicks at hatch, 7 and 14 days

	----------Femur width----------			----------Femur length---------			----Femur breaking strength---		
Item	Hatch	7d	14d	Hatch	7d	14d	Hatch	7d	14d
Dietary treatment									
Control[1]	1.85	2.75	4.69	24.86	33.96	46.70	0.99	3.07	11.74
OTM[2]	1.88	2.75	4.67	24.77	34.07	46.99	0.99	3.11	11.85
OTM+ITM[3]	1.83	2.76	4.64	24.72	33.88	47.21	1.01	3.10	11.31
High ITM[4]	1.85	2.81	4.62	24.90	33.97	46.92	1.01	3.30	10.39
SEM	0.032	0.024	0.063	0.113	0.117	0.221	0.026	0.081	0.637
Hen age[5]									
Young, Y	1.92^{a}	2.94^{a}	4.74^{a}	24.53^{b}	33.69^{b}	47.22^{a}	0.94^{b}	3.15	9.95^{c}
Mid, M	1.89^{a}	2.68^{b}	4.70^{a}	25.17^{a}	33.96^{b}	46.92^{ab}	1.04^{a}	3.08	12.34^{a}
Old, O	1.75^{b}	2.68^{b}	4.53^{b}	24.74^{b}	34.26^{a}	46.72^{b}	1.03^{a}	3.21	11.68^{b}
SEM	0.031	0.023	0.058	0.110	0.109	0.160	0.025	0.069	0.439
Treatment × Hen age									
Control × Y	1.89^{bc}	2.92	4.86	24.62	33.52	46.90	0.91	3.03	11.76
Control × M	1.90^{b}	2.67	4.59	25.13	34.07	46.86	1.03	2.99	12.12
Control × O	1.77^{de}	2.67	4.61	24.85	34.30	46.34	1.04	3.18	11.33
OTM × Y	1.99^{a}	2.93	4.70	24.32	33.99	47.30	0.96	3.10	11.28
OTM × M	1.91^{b}	2.63	4.84	25.18	33.74	46.34	1.03	3.05	12.76
OTM × O	1.74^{e}	2.68	4.49	24.81	34.49	47.34	0.99	3.19	11.49
OTM+ITM × Y	1.93^{ab}	2.93	4.69	24.55	33.75	47.69	0.91	3.28	9.23
OTM+ITM × M	1.83^{cd}	2.65	4.70	25.06	33.80	47.26	1.08	2.89	12.85
OTM+ITM × O	1.74^{e}	2.70	4.53	24.54	34.10	46.68	1.04	3.14	11.84
High ITM × Y	1.87^{bc}	2.99	4.70	24.63	33.49	47.01	0.97	3.20	7.51
High ITM × M	1.93^{ab}	2.75	4.65	25.32	34.25	47.23	1.02	3.37	11.62
High ITM × O	1.75^{e}	2.68	4.50	24.75	34.17	46.52	1.04	3.32	12.04
SEM	0.038	0.044	0.085	0.195	0.202	0.318	0.04	0.132	0.873
P-value									
Treatment	0.2787	0.3289	0.6836	0.6232	0.7146	0.4020	0.9206	0.2095	0.4026
Hen age	<0.0001	<0.0001	<0.0001	<0.0001	0.0024	0.0418	0.0144	0.3346	0.0160

Treatment × Hen age	0.0054	0.8380	0.1595	0.8391	0.1974	0.0544	0.6531	0.2738	0.2240
BW[6]	0.0240	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001

[a-d]Means within a column lacking a common superscript differ significantly at $P < 0.05$.

[1]Control: mineral sulfates at 100 mg/kg Zn, 120 mg/kg Mn and 10 mg/kg Cu.

[2]OTM: Mintrex P (Zn, Mn and Cu chelated by HMTBA) at 50 mg/kg Zn, 60 mg/kg Mn, 10 mg/kg Cu.

[3]OTM + ITM: Control plus an additional 40 mg/kg Zn, 40 mg/kg Mn and 20 mg/kg Cu as OTM.

[4]High ITM: Control plus 40 mg/kg Zn, 40 mg/kg Mn and 20 mg/kg Cu as sulfates.

[5]Hen age: Young (32 to 33 week of age), Mid (45 to 46 wk) and Old (59 to 60 wk).

[6]P value shows the effect of chick body weight on bone traits, which was then used as a covariate for the analysis.

Table 3-3. Effect of 3 broiler breeder ages, Cu, Zn and Mn trace mineral form in the maternal diet and their interaction on physical and mechanical properties of tibia from chicks at hatch, 7 and 14 days

	----------Tibia width---------			----------Tibia length---------			----Tibia breaking strength---		
Item	Hatch	7d	14d	Hatch	7d	14d	Hatch	7d	14d
Dietary treatment									
Control[1]	1.87	2.72	4.59	33.61	45.28	62.22	0.84^{b}	2.46^{b}	8.38
OTM[2]	1.89	2.71	4.64	33.82	45.41	63.03	0.89^{ab}	2.48^{b}	8.72
OTM+ITM[3]	1.84	2.70	4.67	33.50	45.04	62.51	0.90^{ab}	2.45^{b}	8.79
High ITM[4]	1.87	2.78	4.70	33.81	45.14	62.60	0.96^{a}	2.68^{a}	9.07
SEM	0.026	0.027	0.102	0.136	0.159	0.313	0.039	0.055	0.235
Hen age[5]									
Young, Y	1.93	2.87^{a}	5.03^{a}	33.69^{ab}	45.04	62.60	0.77^{b}	2.57^{a}	8.86^{a}
Mid, M	1.87	2.64^{b}	4.59^{b}	33.96^{a}	45.11	62.91	0.97^{a}	2.42^{b}	9.09^{a}
Old, O	1.80	2.67^{b}	4.32^{c}	33.42^{b}	45.50	62.26	0.94^{a}	2.58^{a}	8.27^{b}
SEM	0.024	0.023	0.102	0.129	0.147	0.230	0.039	0.050	0.211
Treatment × Hen age									
Control × Y	1.91^{b}	2.84	5.10	33.53	45.11	62.15	0.64	2.44	8.43
Control × M	1.88^{bc}	2.69	4.37	33.82	45.17	62.61	0.97	2.43	8.64
Control × O	1.83^{cd}	2.65	4.29	33.50	45.55	61.91	0.89	2.52	8.09
OTM × Y	2.01^{a}	2.89	4.92	33.74	45.49	63.03	0.77	2.50	8.63
OTM × M	1.88^{bc}	2.57	4.72	33.99	45.08	62.65	0.95	2.37	9.68
OTM × O	1.78^{d}	2.66	4.27	33.74	45.65	63.41	0.94	2.58	7.85
OTM+ITM × Y	1.90^{b}	2.86	5.06	33.60	44.77	62.74	0.81	2.62	8.61
OTM+ITM × M	1.82^{cd}	2.59	4.63	33.81	44.92	63.06	0.97	2.24	9.36
OTM+ITM × O	1.80^{d}	2.65	4.31	33.09	45.44	61.72	0.93	2.49	8.38
High ITM × Y	1.90^{b}	2.89	50.5	33.88	44.79	62.47	0.88	2.70	9.75
High ITM × M	1.90^{b}	2.72	4.64	34.20	45.27	63.32	0.98	2.63	8.69
High ITM × O	1.81^{cd}	2.74	4.40	33.35	45.35	62.01	1.01	2.72	8.77
SEM	0.030	0.044	0.137	0.245	0.280	0.458	0.055	0.097	0.385
P-value									
Treatment	0.2137	0.1375	0.6025	0.2769	0.3929	0.3330	0.0135	0.0127	0.1604
Hen age	<0.0001	<0.0001	<0.0001	0.0097	0.0817	0.1024	<0.0001	0.0382	0.0072
Treatment × Hen	0.0436	0.3850	0.2581	0.8020	0.8244	0.1947	0.2594	0.5177	0.1140

age								
BW[6]	NS	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	0.0233	<0.0001	<0.0001

[a-d]Means within a column lacking a common superscript differ significantly at $P < 0.05$.
[1]Control: mineral sulfates at 100 mg/kg Zn, 120 mg/kg Mn and 10 mg/kg Cu.
[2]OTM: Mintrex P (Zn, Mn and Cu chelated by HMTBA) at 50 mg/kg Zn, 60 mg/kg Mn, 10 mg/kg Cu.
[3]OTM + ITM: Control plus an additional 40 mg/kg Zn, 40 mg/kg Mn and 20 mg/kg Cu as OTM.
[4]High ITM: Control plus 40 mg/kg Zn, 40 mg/kg Mn and 20 mg/kg Cu as sulfates.
[5]Hen age: Young (32 to 33 week of age), Mid (45 to 46 wk) and Old (59 to 60 wk).
[6]P value shows the effect of chick body weight on bone traits, which was then used as a covariate for the analysis.
NS: not significant.

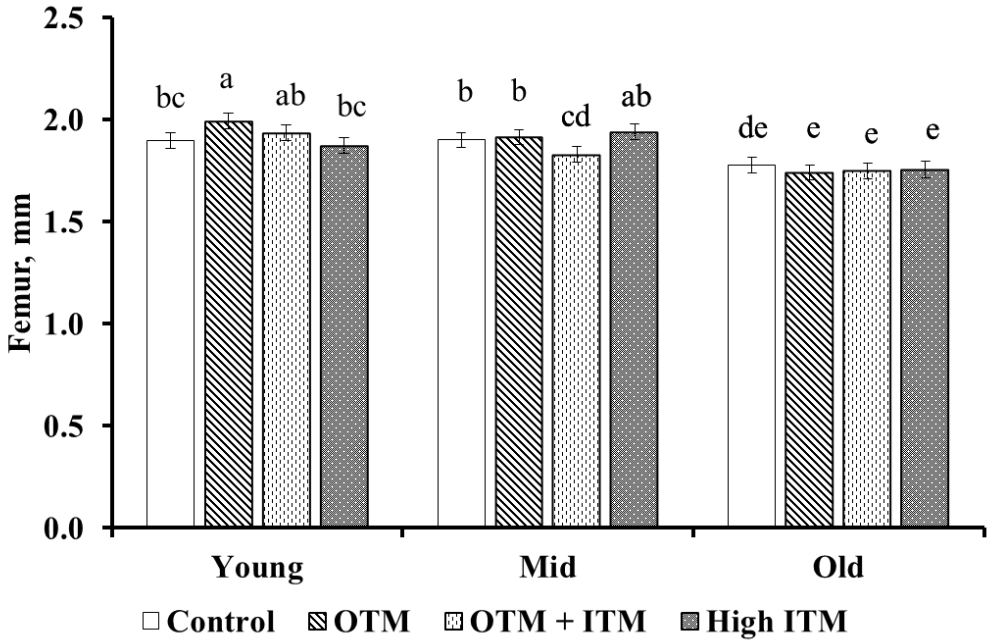

Figure 3-1. Effect of dietary Cu, Zn and Mn trace mineral forms on femur width at hatch in chicks from Young, Mid and Old hens. In the Control treatment broiler breeders were fed mineral sulfates at 100 mg/kg Zn, 120 mg/kg Mn and 10 mg/kg Cu. In the OTM treatment broiler breeders were fed Zn, Mn and Cu chelated by HMTBa at 50 mg/kg Zn, 60 mg/kg Mn, 10 mg/kg Cu. In the OTM + ITM treatment birds were fed the Control diet plus an additional 40 mg/kg Zn, 40 mg/kg Mn and 20 mg/kg Cu as OTM; and High ITM diet was the Control diet plus 40 mg/kg Zn, 40 mg/kg Mn and 20 mg/kg Cu as sulfates; Hen age: Young (32 to 33 week of age), Mid (45 to 46 wk) and Old (59 to 60 wk). $^{a\text{-}c}$Means with different letters are significantly different ($P < 0.05$).

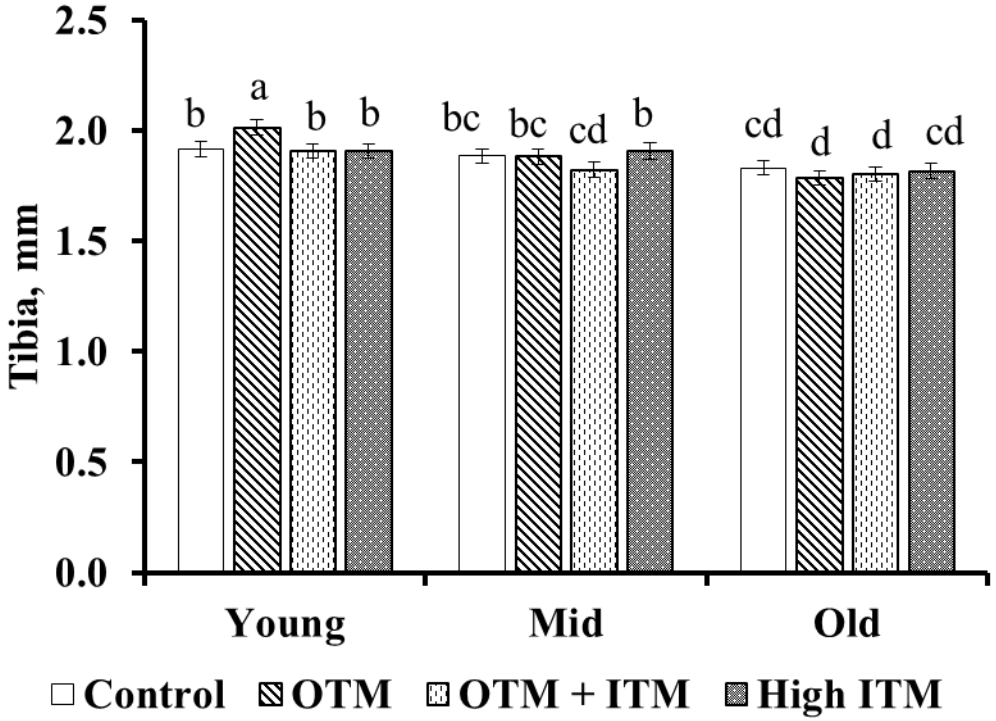

Figure 3-2. Effect of dietary Cu, Zn and Mn trace mineral forms on tibia width at hatch in chicks from Young, Mid and Old hens. In the Control treatment broiler breeders were fed mineral sulfates at 100 mg/kg Zn, 120 mg/kg Mn and 10 mg/kg Cu. In the OTM diet broiler breeders were fed Zn, Mn and Cu chelated by HMTBa at 50 mg/kg Zn, 60 mg/kg Mn, 10 mg/kg Cu. In the OTM + ITM treatment birds were fed the Control diet plus an additional 40 mg/kg Zn, 40 mg/kg Mn and 20 mg/kg Cu as OTM; and in the High ITM treatment birds were fed the Control diet plus 40 mg/kg Zn, 40 mg/kg Mn and 20 mg/kg Cu as sulfates; Hen age: Young (32 to 33 week of age), Mid (45 to 46 wk) and Old (59 to 60 wk). Means with different letters are significantly different ($P < 0.05$).

3.5. REFERENCES

Abourachid, A., R. Hackert, M. Herbin, P. A. Libourel, F. Lambert, H. Gioanni, P. Provini, P. Blazevic, and V. Hugel. 2011. Bird terrestrial locomotion as revealed by 3D kinematics. Zoology (Jena) 114:360-368.

Applegate, T., and M. Lilburn. 2002. Growth of the femur and tibia of a commercial broiler line. Poult. Sci. 81:1289-1294.

Bai, S. P., L. Lu, R. L. Wang, L. Xi, L. Y. Zhang, and X. G. Luo. 2011. Manganese source affects manganese transport and gene expression of divalent metal transporter 1 in the small intestine of broilers. Br. J. Nutr 108:1-10.

Bai, S. P., L. Lu, X. G. Luo, and B. Liu. 2008. Kinetics of manganese absorption in ligated small intestinal segments of broilers. Poult. Sci. 87:2596-2604.

Canadian Council on Animal Care. 1993. A guide to the care and use of experimental animals. 2nd ed. CCAC, Ottawa, ON, Canada.

Cao, J., P. R. Henry, R. Guo, R. A. Holwerda, J. P. Toth, R. C. Littell, R. D. Miles, and C. B. Ammerman. 2000. Chemical characteristics and relative bioavailability of supplemental organic zinc sources for poultry and ruminants. J. Anim. Sci. 78:2039-2054.

Decuypere, E., K. Tona, V. Bruggeman, and F. Bamelis. 2001. The day-old chick: a crucial hinge between breeders and broilers. Worlds Poult. Sci. J. 57:127-138.

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.

Eusebio-Balcazar, P. E., E. O. Oviedo-Rondón, A. Mitchell, B. Brake, M. J. Wineland, V. Moraes, and N. Leandro. 2010. Effects of breeder feeding and trace mineral source on leg health and bone traits of broiler progeny. Poult. Sci. 89 (Suppl. 1):223. (Abstr.)

Ferket, P.R., Oviedo-Rondón, E.O., Mente, P.L., Bohórquez, D.V., Santos Jr., A.A., Grimes, J.L., Richards, J.D., Dibner, J.J., Felts, V. 2009. Organic trace minerals and 25-hydroxycholechalciferol affect performance characteristics, leg abnormalities, and biomechanical properties of leg bones of turkeys. Poult. Sci. 88:118-131.

Gheisari, A., A. Sanei, A. Samie, M. Gheisari, and M. Toghyani. 2011. Effect of diets supplemented with different levels of manganese, zinc, and copper from their organic or inorganic sources on egg production and quality characteristics in laying hens. Biol. Trace Elem. Res. 142:557-571.

Habib, M. B., and C. B. Ruff. 2008. The effects of locomotion on the structural characteristics of avian limb bones. Zool. J. Linn. Soc. 153:601-624.

Hamidu, J. A., G. M. Fasenko, J. J. R. Feddes, E. E. O'Dea, C. A. Ouellette, M. J. Wineland, and V. L. Christensen. 2007. The effect of broiler breeder genetic strain and parent flock age on eggshell conductance and embryonic metabolism. Poult. Sci. 86:2420-2432.

Huang, Y. L., L. Lu, S. F. Li, X. G. Luo, and B. Liu. 2009. Relative bioavailabilities of organic zinc sources with different chelation strengths for broilers fed a conventional corn-soybean meal diet. J. Anim. Sci. 87:2038-2046.

Hudson, B. P., W. A. Dozier III, J. L. Wilson, J. E. Sander, and T. L. Ward. 2004. Reproductive performance and immune status of caged broiler breeder hens provided diets supplemented with either inorganic or organic sources of zinc from hatching to 65 wk of age. J. Appl. Poult. Res. 13:349-359.

National Research Council. 1994. Nutrient Requirements of Poultry. 9th rev. ed. Natl. Acad. Press, Washington, DC.

Montes, L., E. De Margerie, J. Castanet, A. De Ricqlès, and J. Cubo. 2005. Relationship between bone growth rate and the thickness of calcified cartilage in the long bones of the Galloanserae (Aves). J. Anat. 206:445–452.

Oviedo-Rondón, E. O., L. Mejia-Sanchez, J. Small, K. E. Brannan, N. Leksrisompong, A. Mitchell, T. A. York, and J. Brake. 2008. Organic minerals (Mintrex® P) in broiler breeder diets and pre-incubation

conditions on bone development and leg health of broilers. Poult. Sci. 87 (Supl. 1):162. (Abstr.).

Paxton, H., N. B. Anthony, S. A. Corr, and J. R. Hutchinson. 2010. The effects of selective breeding on the architectural properties of the pelvic limb in broiler chickens: a comparative study across modern and ancestral populations. J. Anat. 217:153-166.

Rath, N., G. Huff, W. Huff, and J. Balog. 2000. Factors regulating bone maturity and strength in poultry. Poult. Sci. 79:1024-1032.

Reich, A., N. Jaffe, A. Tong, I. Lavelin, O. Genina, M. Pines, D. Sklan, A. Nussinovitch, and E. Monsonego-Ornan. 2005. Weight loading young chicks inhibits bone elongation and promotes growth plate ossification and vascularization. J. Appl. Physiol. 98:2381-2389.

Saito, M., and K. Marumo. 2010. Collagen cross-links as a determinant of bone quality: a possible explanation for bone fragility in aging, osteoporosis, and diabetes mellitus. Osteoporos. Int. 21:195-214.

Shaw, A. L., J. P. Blake, and E. T. Moran. 2010. Effects of flesh attachment on bone breaking and of phosphorus concentration on performance of broilers hatched from young and old flocks. Poult. Sci. 89:295-302.

Skrivan, M., V. Skrivanova, and M. Marounek. 2005. Effects of dietary zinc, iron, and copper in layer feed on distribution of these elements in eggs, liver, excreta, soil, and herbage. Poult. Sci. 84:1570-1575.

Sun, Q., Y. Guo, S. Ma, J. Yuan, S. An, and J. Li. 2012. Dietary mineral sources altered lipid and antioxidant profiles in broiler breeders and post-hatch growth of their offsprings. Biol. Trace Elem. Res. 145:318-324.

Tona, K., O. Onagbesan, B. De Ketelaere, E. Decuypere, and V. Bruggeman. 2004. Effects of age of broiler breeders and egg storage on egg quality, hatchability, chick quality, chick weight, and chick post-hatch growth to forty-two days. J. Appl. Poult. Res 13:10-18.

Turner, C. H. 2006. Bone strength: current concepts. Ann. N. Y. Acad. Sci. 1068:429–446.

Ulmer-Franco, A. M., G. M. Fasenko, and E. E. O'Dea Christopher. 2010. Hatching egg characteristics, chick quality, and broiler performance at 2 breeder flock ages and from 3 egg weights. Poult. Sci. 89:2735-2742.

Wang, A., and L. A. Goonewardene. 2004. The use of MIXED models in the analysis of animal experiments with repeated measures data. Can. J. Anim. Sci. 84:1-11.

Willemsen, H., N. Everaert, A. Witters, L. De Smit, M. Debonne, F. Verschuere, P. Garain, D. Berckmans, E. Decuypere, and V. Bruggeman. 2008. Critical

assessment of chick quality measurements as an indicator of post-hatch performance. Poult. Sci. 87:2358-2366.

Williams, B., D. Waddington, D. H. Murray, and C. Farquharson. 2004. Bone strength during growth: influence of growth rate on cortical porosity and mineralization. Calcif. Tissue Int. 74:236-245.

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., Z. Uni, and R. Shahar. 2012. Bone characteristics of late-term embryonic and hatchling broilers: Bone development under extreme growth rate. Poult. Sci. 91:2614-2620.

Yalcin, S., S. Özkan, E. Coskuner, S. Özkan, Y. Delen, Y. Kurtulmus, and T. Tanyalçin. 2001. Effects of strain, maternal age and sex on morphological characteristics and composition of tibial bone in broilers. Br. Poult. Sci. 42:184.

Yassin, H., A. G. J. Velthuis, M. Boerjan, and J. van Riel. 2009. Field study on broilers' first-week mortality. Poult. Sci. 88:798-804.

Yu, Y., L. Lu, X. G. Luo, and B. Liu. 2008. Kinetics of zinc absorption by in situ ligated intestinal loops of broilers involved in zinc transporters. Poult. Sci. 87:1146-1155.

Zhao, J., R. B. Shirley, M. Vazquez-Anon, J. J. Dibner, J. D. Richards, P. Fisher, T. Hampton, K. D. Christensen, J. P. Allard, and A. F. Giesen. 2010. Effects of chelated trace minerals on growth performance, breast meat yield, and footpad health in commercial meat broilers. J. Appl. Poult. Res 19:365-372.

CHAPTER 4. THE EFFECT OF INCUBATOR TEMPERATURE PROFILES AND PARENT FLOCK AGE IN BONE CHARACTERISTICS OF ROSS 708 CHICKS AT HATCH

4.1. INTRODUCTION

The body weight of the chicken embryo increases linearly from the 8th day of incubation until hatch (Kubota et al., 1981; Yair et al., 2012). The growth rate of the tibia, as measured by its weight, starts to increase at around day 12 of incubation with a peak at day 19 (Kubota et al., 1981). This is concomitant with the highest uptake of calcium from the eggshell and its deposition in the tibia (Kubota et al., 1981; Chen et al., 2008). In Chapter 3, it was reported that day-old chicks from Mid (45 to 46 week old) and Old (59 to 60 week) Ross 308 broiler breeders had stronger tibiae relative to those from Young hens (32 to 33 week) when incubated at 37.5^{0}C. Peebles et al. (2001) reported that embryos from older hens (36 week old) have lower relative weight during the last week of incubation relative to embryos from young parents (27 week old). Furthermore, at similar egg weights, embryos from older flocks (45, 55 and 59 week of age) have increased metabolism heat production relative to those from younger flocks (34 and 40 week; Hamidu et al., 2007). Embryos from 60 week old flocks required lower incubator temperatures relative to those from young flocks (28 week old) to obtain an eggshell temperature (EST) of 37.8ºC (Lourens et al., 2005). Embryonic growth and hatchability were increased at an EST of 37.8^{0}C relative to 38.9^{0}C

(Lourens et al., 2005; Joseph et al., 2006; Lourens et al., 2006; Lourens et al., 2007) or 39.5ºC (Joseph et al., 2006).

Commercially, hatching eggs are incubated at initial incubation temperatures of 37.5^{0}C to 37.8^{0}C until day18, regardless of parent flock age (Meijerhof and Beek, 1993); and 36.4^{0}C afterwards is considered ideal in terms of navel quality of the hatched chick D. Hill (HatchTech Incubation Technology, Mountain Home, AR, personal communication). It is possible that differences in the growth pattern (Peebles et al., 2001) and metabolism (Hamidu et al., 2007) among embryos from different hen ages, combined with an incubator temperature of 37.5^{0}C in the last third of incubation, may influence embryonic bone development. Under these circumstances, the embryos from older flocks may experience heat stress during the time of greatest bone development within the egg (days 15 to 19 of incubation; Kubota et al., 1981) which might impair bone growth. The objectives of the current study were to investigate whether changes in incubator temperature would influence bone development at hatch in eggs from different hen ages. It was hypothesized that incubator temperature of 36^{0}C or 36.5^{0}C from day 15 of incubation until hatch would increase bone development in chicks from older hens relative to incubator temperatures of 37.5^{0}C. Furthermore, it was also hypothesized that a negative relationship would exist between bone characteristics at hatch in embryos with high embryonic heat production and EST from days 15 to 19 of incubation.

4.2. MATERIAL AND METHODS

4.2.1. Experimental design

The experimental protocol was approved by the University of Alberta Animal Care and Use Committee for Livestock under the Canadian Council on Animal Care guidelines (Canadian Council on Animal Care, 1993). Fertile hatching eggs from commercial Ross 708 broiler breeder flocks at 26 to 34 week (Young); 35 to 45 week (Mid); and 46 to 54 week (Old) were obtained from commercial flocks. On 8 separate occasions, for replication of each experimental temperature, 18 eggs were selected within ± 0.5 g of the average egg weight for each hen age and stored for 2 to 4 days at 18ºC and 73% RH. Eggs were incubated from day 1 to day 14 at 37.5ºC and 56% RH in a Jamesway AVN single-stage incubator (Jamesway Incubator Company, Inc., Cambridge, Ontario, Canada). At day 15, 6 eggs from each of the three hen ages (n =12 eggs for each hen age at each experimental temperature) were transferred to a modified Jamesway AVN single-stage incubator and individually placed in 1 of 24 identical one L air-tight metabolic chambers placed inside this incubator. The incubator temperature was set to one of four different temperatures treatments of 36.0, 36.5, 37 (Control), or 37.5ºC from day 15 of incubation until hatch (21.5). Each of the 4 temperature treatments was replicated twice in batches of 23 eggs in the first 4 set of trials, and 24 in the last 4 trials. In the first 4 trials, one egg per flock age was randomly removed in order to check the consistency between incubator and chamber temperatures; this procedure was rotated from trial to trial to make sure that not only one flock age was affected. The temperature of both incubator and metabolic

chambers was centrally controlled and monitored using a temperature probe as previously described by Hamidu et al. (2007).

In each trial, the O_2 consumption and CO_2 production were recorded daily for metabolic measurements taken for a concurrent study and the data were used to calculate embryonic heat production as an indicator of embryonic metabolism (Hamidu et al., 2011). Eggshell temperature was measured by a temperature probe that was held in place by a piece of foam attached to the incubator flat in direct contact with the eggshell. The EST, O_2 and CO_2 measurements from each metabolic chamber were taken 6 times per day as previously described by Hamidu et al. (2007).

At hatch, chick body weight was recorded, the chick was euthanized by cervical dislocation, and both tibias and femurs were removed and cleaned of adhering muscle and connective tissue. The left tibia and femur were dissected, cleaned of all adhering muscle tissue, and stored in plastic bags at -20°C until analysis for breaking strength was conducted. The right tibia and femur were stored in 10% neutral buffered formalin for a minimum of 7 days. The stage of tibia and femur development was investigated using the differential staining technique as described previously in Chapter 2. The length, width and % calcification ([calcified tissue area/whole bone area]*100) were measured from digital images and calculated with ImageJ (version 1.43q, available online at: http://rsb.info.nih. gov/nih-image/). Measurements of bone size (length and width) were investigated using the method previously described in Chapter 2.

Breaking strength analysis of the left tibia and femur was measured as previously described in Chapter 3. Briefly, bone breaking strength was measured using the 3-point bending test using an Instron Materials Tester (Model 4411, Instron Corp., Canton, MA) with BlueHill software. At the time of breaking strength analysis, each bone sample was removed from a plastic bag and kept moist with cotton imbibed with distilled water until being placed in the instrument. Each bone was oriented to ensure that bending occurred around the midpoint of the antero-posterior face. The femur or tibia was similarly positioned on two support points measuring 10 or 14 mm apart, respectively. A probe with a round base was attached to a 5-kg load cell and the force was applied with a crosshead speed of 1 mm/min.

The relationship between embryonic metabolism from days 15 to 19 of incubation ($EST_{(15-19)}$), cumulative embryonic heat production ($EHP_{(15-19)}$) and bone traits were calculated based on embryonic metabolism data. The $EST_{(15-19)}$ was determined by calculating the average of all six EST measurements recorded per day. Then the average EST per day was used to calculate $EST_{(15-19)}$). The $EHP_{(15-19)}$ was determined by summing the average heat production (mW) per day from day 15 through day 19.

4.2.2.Statistical analysis

All data obtained were analyzed as a two-way ANOVA using the mixed procedure of SAS (SAS Institute Inc., Cary, NC) with hen age and incubator temperatures as the main effects. The following model was used to determine

differences between the treatment groups: $Yijk = \mu + \alpha i + \beta j + (\alpha\beta)ij + \varepsilon ijk$, where Yijk was the associated constant to all observations during the experiment (variable measured), μ was the general variable mean, αi was the effect of parental flock age where i = {Young, Mid or Old}, βj was the effect of temperature where j = {36.0, 36.5, 37.0 or 37.5ºC}, (αβ)ij was the interaction effect between the i and j factors, and εijk was the error associated with each observation. The random term in the statistical model included the interaction between parental flock age and incubation temperature nested in farm to account for any differences due to variations in farm conditions. Chick body weight at hatch was tested as a covariate and it was removed from the model when it did not reveal significance. Differences between least square means (lsmeans) were considered significant at $P < 0.05$. Regression analysis was also performed on the continuous variables of maternal flock age and incubation temperature to determine if a multiple relationship existed between the main factors and bone traits at hatch.

Also, multiple regression analysis performed on the continuous variable of $EST_{(15\text{-}19)}$ and $EHP_{(15\text{-}19)}$ to determine if relationships existed between embryonic metabolism and EST on bone characteristics at hatch.

4.3. RESULTS AND DISCUSSION

4.3.1. Effect of parental flock age

As hen age increased there was an increase in the femur and tibia breaking strengths of the progeny (Table 4-1). Likewise, at a given body weight, day old

chicks from Ross 708 broiler breeders from a flock at 65 weeks old flock had stronger tibia relative to chicks from a different flock at 25 week flock (Shaw et al., 2010). As previously described in Chapter 3, chicks from Mid and Old Ross 308 hens also had stronger tibia and femurs relative to those from Young hens; but there was no significant difference in tibia breaking strength between Mid and Old hens. Contrarily, in the present study using Ross 708 breeders, we found that chicks from Old hens had significantly stronger femurs than chicks from Mid hens.

Regardless of incubator temperature, bone length, width and calcification at hatch were not significantly different among chicks from Young, Mid and Old hens. These results differ from our previous study comparing the influence of parent flock age for Ross 308 birds and maternal mineral nutrition on bone size at hatch (Chapter 3). In that study, after covariate analysis for body weight, chicks hatched from Mid hens had longer femurs and tibias relative to those from Old hens and longer femur relative to those from Young hens, whereas bone length was not significantly different between chicks from Old and Young hens. It is possible that the contradictory findings could be the result of the different strain of breeders studied. Ross 708 is a broiler strain that has been developed to grow at a slower initial growth rate than the Ross 308 strains (Persia and Saylor, 2006). In fact, Ross 308 chicks tend to be heavier and have increased total bone ash (but not when expressed as a percentage of bone weight) at 5 and 23 days relative to Ross 708 broilers (Persia and Saylor, 2006).

Data from the present study are in agreement with that of Yalcin et al. (2001) who reported that chicks from young (32 to 35 week old hens) and old hens (56 to 58 week) had similar tibia width and length. Alfonso-Torres et al. (2009), however, demonstrated that tibias of embryos and one day old chicks from Cobb hens at 60 weeks of age were wider and heavier on an absolute basis, but not when expressed as % of body weight relative to those from 38 week-old hens. Furthermore, Yalcin et al. (2001) reported that, after covariate adjustment for body weight, chicks from old hens had higher tibia mineral content than those from the young hens. In the present study, however, there was no effect of hen age on proportion of calcified bone at hatch (Table 4-1). Conflicting findings among studies could be the result of the different strain of birds studied, as previously stated, or due to different methods used to assess bone calcification. Yalcin et al. (2001) investigated tibia mineral density (bone ash weight per unit of bone volume). In the current study, bone calcification was studied by measuring the proportion at which non-calcified bone was replaced by calcified tissue. However, this method does not quantify the amount of calcium accumulated in bone. Further studies should investigate the pattern of calcification and the rate of calcium deposition in the embryonic bone throughout incubation among embryos from diverse hen ages. It would also be interesting to investigate whether skeletal development of embryos from diverse breeder strains is different at hatch.

As reported in Chapter 2, Young hens laid smaller eggs which in turn hatched smaller chicks with weaker bones relative to the offspring from Old hens. If weaker bones at hatch reduced chick mobility, feed and water consumption at

placement could be reduced. Chicks from young breeders had increased first week mortality (Yassin et al., 2009); it is not known whether this effect was related to early bone quality in chicks. Under commercial conditions, chicks do not have access to feed or water up to 24 to 72 hours after hatch because of variation in hatching time, hatchery management, and transport times (Pinchasov and Noy, 1993; Careghi et al., 2005). Therefore, a delay in food access may relate to lower performance in the post hatch period (Pinchasov and Noy, 1993); this effect might be worsened in chicks from young flocks.

4.3.2. Effect of incubator temperature

An incubator temperature of 37^{0}C increased chick bone size but reduced femur breaking strength compared to 36^{0}C (Table 4-1). It was previously hypothesized that reduced incubator temperature would increase bone development and this effect would be increased in chicks from older flocks. However, an incubator temperature of 36^{0}C enhanced bone strength and this effect was independent of hen age. Oviedo-Randón et al. (2008a) reported that incubator temperatures of 36^{0}C applied from day 17 of incubation to hatch (day 21) did not influence femur or tibia length but increased femur weight when expressed as a proportion of body weight, without yolk, relative to chicks from eggs set at 37^{0}C. In contrast with previous research, the present study differed by setting eggs at 36^{0}C 2 days earlier. This may have had greater impact on bone development, as intense bone metabolism occurs between days 15 to 19 of incubation (Kubota et al., 1981; Yair et al., 2012). Furthermore, incubator temperature of 36^{0}C from day 15 of incubation to hatch increased bone strength

of chicks relative to temperatures of 36.5 or 37^{0}C but did not influence calcification (Table 4-1). Yalçin et al. (2007) reported that an intermittent (6h/day) 36.9 or 39.0^{0}C incubator temperature from day 10 to day 18 of incubation did not influence tibia mineral composition, as well as growth plate differentiation, relative to chicks from eggs set at 37.8^{0}C; however the lightest tibia weights were observed in chicks exposed to 39.0^{0}C. Furthermore, Oviedo-Rondón et al. (2008a) reported that tibias of broilers were longest when incubated at 38^{0}C compared with chicks incubated at 36^{0}C or 39^{0}C, but similar to incubation at 37^{0}C; femur weight, as a proportion of body weight without residual yolk, was heavier in chicks from eggs set at 36^{0}C and 39^{0}C compared to those set at 37^{0}C or 38^{0}C. The results from other studies, combined with those from the present study, indicate that temperatures below 37^{0}C, in the last days of incubation, appear to increase bone health at hatch.

Although chicks from eggs set at 37.5^{0}C had reduced bone width (except for tibia) and length, femurs were stronger at hatch relative to chicks incubated at 37 and 36^{0}C (Table 4-1). This effect was unexpected and may relate to the fact that chicks from the 37.5^{0}C and 36^{0}C treatment hatched later than the Control chicks (Hamidu et al., unpublished). The latter chicks would have had a longer holding time in the hatcher which might have negatively affected bone strength. In fact, Shim and Pesti, (2011) reported that tibia length of chicks decreased with increasing time in the hatcher. The reasons for the unexpected increased femur breaking strength in chicks from eggs set at 37.5^{0}C relative to Control is not apparent. An increase in relative asymmetry of length of the right and left legs of

hatched poults was found when eggs were set at 38 or 39ºC relative to 36 or 37ºC during the last four days of embryo development (Oviedo-Rondón et al., 2008b). therefore, it was speculated that embryos incubated at standard incubation temperatures of 37.5^{0}C to 37.8^{0}C (Meijerhof and Beek, 1993) would result in impaired bone growth relative to chicks from eggs set at 37^{0}C, however, this was not the observed in the present study.

It was also expected that the incubator temperature of 37.5^{0}C would lessen bone calcification; however, incubator temperature did not influence proportion of calcified tissue (Table 4-1). Similarly, Hammond et al., (2007) reported that, although incubator temperature of 38.5^{0}C from days 4 to 7 of incubation increased the length of tibia and tarsus of Leghorn embryos, bone mineralization at hatch was not influenced relative to incubation at 37.5^{0}C. The present study differed from the study of Hammond et al. (2007) in that temperature treatments were applied in the last third of incubation, which is coincident with highest bone growth within the egg (Kubota et al., 1981; Yair et al., 2012). An incubator temperature of 40.6^{0}C during the last days of incubation reduced yolk utilisation and thyroid metabolism (Willemsen et al., 2010) and heat stress decreased chondrocyte proliferation and differentiation in the growth plate of chicken embryos (Yalçin et al., 2007). Therefore, it was expected that incubation at 37.5^{0}C would lessen bone calcification as reduced yolk utilisation (Willemsen et al., 2010) could limit access to macro- and micro-minerals important for bone growth that are mostly stored in the egg yolk (Yair and Uni, 2011) and reduced thyroid metabolism (Willemsen et al., 2010) could decrease bone development

because thyroid hormone is critical for chondrocyte differentiation in the growth plate (Oviedo-Rondón et al., 2006). Calcium deposition in the skeleton is a function of both growth in size and the increase in calcium concentration in the bone (Tilgar et al., 2008). It is important to note that bone calcification was assessed by measuring the replacement of cartilage (stained blue) into bone (stained red; Kelly and Bryden, 1983); however, the method does not quantify the amount of calcium accumulated in bone. Incubation temperature of 36.9^{0}C in the early stages of embryonic development increases proportion of ash and calcium content in the tibia at hatch relative to eggs set at 37.8^{0}C (Yalçin et al., 2007). Therefore, it is possible that incubator temperature might have affected calcium accumulation in later stages of bone growth; however, this process was not investigated in the current study and requires further investigation.

Technical challenges for commercial incubators include the fact that the temperature the embryo experiences is different from the air temperature (Lourens et al., 2005) and embryonic temperature is not uniform within the incubator (Lourens, 2001). Greatest chick quality at hatch, as defined by navel quality, are obtained when commercial incubators are set at 37.5^{0}C to 38.05^{0}C from day one to day 18 and the hatcher is set at 36.4^{0}C, D. Hill (HatchTech Incubation Technology, Mountain Home, AR, personal communication). However, variability in the airflow and temperature within the hatcher are the main reasons for managers to raise the hatcher temperature in order to increase uniformity of hatch (but not necessarily chick quality) D. Hill (HatchTech Incubation Technology, Mountain Home, AR, personal communication). In the present

research, eggs were individually incubated in metabolic chambers under strictly controlled environmental conditions. Therefore, this incubation procedure was not representative of what occurs in commercial hatcheries, nor was meant it to be. Because embryos from commercial incubators and hatchers are probably not experiencing an "ideal" embryonic temperature towards the last stages of development, this study sets the stage for further investigation in a commercial scale on the effect of reducing temperature, to 36^0C, on bone and chick quality at hatch.

4.3.3. Relationships between hen age, incubator temperature, embryonic metabolism and EST on bone traits

A multiple relationship existed between parent flock age and incubator temperature on bone characteristics (except tibia length) investigated in this study (Table 4-2). Relationships were weak, but indicated that incubator temperature and parent flock age contributed to about 27% of the variation in femur breaking strength. Interestingly, within this explained variation, 16% and 48% was explained by incubator temperature and flock age respectively ($R^2 = 0.272$, $P < 0.05$, Table 4-2). Furthermore, there was a negative relationship between incubator temperature on femur (but not for tibia) calcification and strength; whereas, a positive relationship between parent flock age and bone traits were observed for all bone characteristics except tibia length.

Furthermore, a weak multiple relationship between $EST_{(15\text{-}19)}$ and $HP_{(15\text{-}19)}$ and bone traits (Table 4-3) indicated that about 7%, 16%, 5% and 12% of the variation influencing femur thickness, length, calcification and strength,

respectively, were explained by $EST_{(15-19)}$ and the cumulative effect of $EHP_{(15-19)}$. A negative relationship was observed only between $EST_{(15-19)}$ and femur calcification and strength. Moreover, there was a positive multiple relationship of $EST_{(15-19)}$ and $EHP_{(15-19)}$ on tibia size; the relationship was weak but represented about 2% and 16% of the variation influencing tibia width and length, and appeared to not influence tibia calcification and strength.

Both incubator and eggshell temperatures had significant but weak relationships with bone development and may therefore be considered as factors influencing chick quality at hatch. Incubator temperature had a positive relationship with bone traits, whereas the opposite was observed for the average $EST_{(15-19)}$. In the current trial, $EST_{(15-19)}$ ranged between 37.6^0C and 40.4^0C. This was 2.6^0C above 37.8^0 EST reported to increased embryonic growth and hatchability relative to 38.9^0C (Lourens et al., 2005; Joseph et al., 2006; Lourens et al., 2006; Lourens et al., 2007) or 39.5ºC (Joseph et al., 2006). It is also interesting to note that from day 15 to 20 of incubation the eggshell temperature of the 37.5^0C and 37^0C experimental treatments were higher relative to that of 36^0C and 36.5^0C groups (Hamidu et al., unpublished). This might be associated with the negative relationship between $EST_{(15-19)}$ and the proportion of calcified tissue and femur breaking strength observed in the present study (Table 4-3).

These puzzling results indicate that incubator temperature and EST might influence bone growth by different mechanisms. For example, incubator temperature might have indirectly increased bone strength by decreasing hatching

time as mentioned above. Eggshell temperature, on the other hand, might have had a direct effect on calcium uptake from the eggshell by reducing chorioallantoic membrane activity (CAM; Gabrielli, 2004). The CAM adheres to the inner shell membrane inside the eggshell and calcium stored in the eggshell is released by activity of Ca^{2+} ATPase present in this membrane (Gabrielli, 2004). In vitro studies demonstrated that calcium uptake from Ca^{2+} ATPase in CAM preparations were temperature dependant: a temperature of 23^0C inhibited calcium uptake relative to 37^0C (Akins and Tuan, 1993). In the present study the highest EST was 40.4^0C; it is possible that such a high EST might had reduced calcium uptake and therefore might have reduced mineral deposition in the growing skeleton. Although evidence from the avian literature is lacking, embryos from the leatherback turtles (*Dermochelys coriacea*) have higher mobilization of calcium from the eggshell and increased accumulation of this mineral in their skeletons when eggs are incubated at sand temperatures of 28.5 or 29.5^0C relative to 31^0C (Bilinski et al., 2001). Whether the EST that the embryo experiences influences enzymatic activity in the CAM (i.e calcium release from eggshell) and mineral deposition in the chick skeleton has yet to be investigated in future research.

4.4. CONCLUSIONS

Parental flock age and incubator temperatures from day 15 of incubation to hatch are two factors that independently influenced bone growth at hatch. The main effect of parent flock age in this study was an increase in bone breaking strength at hatch as hens aged. Reduced breaking strength at hatch could be a disadvantage for chicks from young hens compared with chicks from older breeder hens when reared under the same management practices because feed consumption and post-hatch growth might be impaired. Providing high quality pre-starter diets with adequate levels of available minerals combined with feed management to boost feed consumption may increase skeletal health in chicks from younger flocks. These factors could decrease the high incidence of first week mortality commonly observed in chicks from young flocks (Yalçin et al., 2009) and perhaps increase performance.

Incubating eggs at a continuous incubator temperature of 37.5 or 36^{0}C from day 15 of incubation to hatch increased bone strength but reduced size. High EST can weaken bone quality by reducing calcification and strength. Future studies should investigate whether high EST reduces calcium uptake from the eggshell which might decrease bone quality of the embryo. The present research indicated that a temperature of 36 and 37.5^{0}C from 15 days of incubation to hatch increased bone strength.

Table 4-1. Femur and tibia characteristics of chicks from different Ross 708 flock ages and incubated at different machine temperatures

Item	Width[1]	Length[2]	Calcification[3]	Strength[4]	Width[1]	Length[2]	Calcification[3]	Strength[4]
	----------Femur----------				----------Tibia----------			
Flock age[5]								
Young, Y	2.29	23.23	66.57	0.73^{c}	2.22	31.06	62.98	0.74^{c}
Mid, M	2.33	23.28	67.72	0.81^{b}	2.27	31.21	64.25	0.80^{b}
Old, O	2.39	23.23	68.47	0.90^{a}	2.25	30.99	65.44	0.88^{a}
SEM	0.04	0.18	0.96	0.02	0.02	0.36	0.82	0.02
Inc.Temp.(°C)[6]								
36.0	2.22^{b}	22.72^{c}	68.15	0.88^{a}	2.18^{b}	30.37^{b}	64.93	0.79
36.5	2.40^{a}	23.40^{b}	68.49	0.80^{bc}	2.30^{a}	31.27^{ab}	63.09	0.84
37.0	2.47^{a}	23.98^{a}	66.38	0.74^{c}	2.28^{a}	32.07^{a}	63.85	0.79
37.5	2.25^{b}	22.88^{bc}	67.33	0.82^{ab}	2.22^{ab}	30.63^{b}	65.02	0.80
SEM	0.05	0.20	1.04	0.03	0.03	0.36	0.95	0.02
Flock age × Inc. Temp.(°C)[7]								
Y × 36.0	2.27	23.08	67.70	0.79	2.19	30.53	65.05	0.75
Y × 36.5	2.15	22.84	68.87	0.91	2.17	30.85	64.63	0.77
Y × 37.0	2.25	22.25	67.88	0.93	2.19	29.74	65.10	0.85
Y × 37.5	2.34	23.54	67.52	0.73	2.27	31.24	62.36	0.76
M × 36.0	2.45	23.91	68.52	0.74	2.33	31.68	63.04	0.84
M × 36.5	2.41	23.50	69.42	0.91	2.30	30.89	63.88	0.92
M × 37.0	2.35	23.72	65.52	0.65	2.20	31.72	62.24	0.72
M × 37.5	2.40	23.89	65.70	0.75	2.27	31.69	64.06	0.76
O × 36.0	2.66	24.50	67.92	0.82	2.37	32.81	66.26	0.88
O × 36.5	2.21	22.94	65.54	0.72	2.22	30.74	63.26	0.71
O × 37.0	2.30	22.96	67.79	0.82	2.30	30.62	65.28	0.85
O × 37.5	2.23	22.74	68.66	0.92	2.16	30.52	66.51	0.86
SEM	0.07	0.33	1.92	0.04	0.04	0.63	1.62	0.04
P-Value								
Flock age	0.3573	0.9598	0.3932	0.0004	0.4287	0.8811	0.1462	0.0007
Inc.Temp.	0.0031	0.0014	0.5762	0.0209	0.0367	0.0252	0.4281	0.4519
Interaction	0.3274	0.3889	0.9789	0.7625	0.3551	0.7732	0.8331	0.7232

BW[8]	0.0581	0.0044	NS	NS	0.0038	0.006	NS	NS

[a-c] Different superscript within the same column indicate significant differences among means ($P \leq 0.05$).

[1] Bone width: measured at the midpoint (50% of length).

[2] Bone length: determined from the proximal edge of the trochanter to the distal edge of the condyle.

[3] Bone calcification: (calcified tissue area/whole bone area)*100.

[4] bone strength: bone breaking force (Kg).

[5] Flock age: Young (26-34 week of age), Mid (35-45 week) and Old (46-55week).

[6] Inc. Temp.: Incubation temperature of 36.0, 36.5, 37 or 37.5^{0}C from day 15 to hatch.

[7] n = 12 chicks per hen age

[8] P value shows the effect of chick body weight (BW) on bone traits, which was then used as a covariate for the analysis. NS = not significant.

Table 4-2. Relationship between femur and tibia parameters as affected by maternal flock age and incubation temperature

Variable	Label	Parameter estimate	SE	Partial *P*-value	Model *P*-value	R^{2a}	Standardized estimate
Femur							
Width	Intercept	1.28	1.13	0.2601	0.0037	0.07	0
	Inc.Temp	0.02	0.03	0.4995			0.06
	Flock age	0.007	0.002	0.0009			0.30
Length	Intercept	17.92	6.11	0.0040	0.0231	0.06	0
	Inc.Temp	0.11	0.16	0.4996			0.06
	Flock age	0.03	0.01	0.0076			0.24
Calcification	Intercept	104.1	21.2	0.0001	0.0004	0.11	0
	Inc.Temp	-1.13	0.57	0.0489			-0.16
	Flock age	0.13	0.04	0.0012			0.28
Strength	Intercept	2.06	0.73	0.0059	0.0001	0.27	0
	Inc.Temp[1]	-0.04	0.02	0.0028			-0.16
	Flock age	0.0008	0.001	0.0001			0.48
Tibia							
Width	Intercept	1.17	0.891	0.1888	0.0011	0.10	0
	Inc.Temp	0.02	0.03	0.3492			0.01
	Flock age	0.006	0.001	0.0003			0.31
Length	Intercept	23.29	8.55	0.0074	0.1612	0.03	0
	Inc.Temp	0.18	0.23	0.4319			0.07
	Flock age	0.029	0.01	0.0705			0.16
Calcification	Intercept	58.7	23.0	0.0118	0.0118	0.06	0
	Inc.Temp	0.001	0.62	0.9976			0
	Flock age	0.13	0.04	0.0030			0.26
Tibia Strength	Intercept	0.38	0.68	0.5733	0.0001	0.20	0
	Inc.Temp	0.003	0.02	0.8559			0.01
	Flock age	0.007	0.001	0.0001			0.45

R^2: Regression coefficient

[1]Inc. Temp.: Incubation temperature of 36.0, 36.5, 37 or 37.5^{0}C from day 15 to hatch.

n = 12 eggs per hen age

Table 4-3. Relationship between femur and tibia parameters as affected by embryonic heat production ($EHP_{(15-19)}$) and eggshell temperature ($EST_{(15-19)}$)

Item	Label	Parameter estimate	SE	Partial *P*-value	Model *P*-value	R^{2a}	Standardized estimate
Femur							
Width	Intercept	-0.38	0.91	0.6780	0.0105	0.07	0
	$EST_{(15-19)}$[1]	0.07	0.02	0.0050			0.25
	$EHP_{(15-19)}$[2]	0.00008	0.0002	0.7130			0.03
Length	Intercept	10.6	4.59	0.0224	0.0001	0.16	0
	$EST_{(15-19)}$	0.27	0.12	0.0393			0.18
	$EHP_{(15-19)}$	0.01	0.00	0.0005			0.31
Calcification	Intercept	102.8	17.94	0.0001	0.0321	0.05	0
	$EST_{(15-19)}$	-1.08	0.48	0.0274			-0.20
	$EHP_{(15-19)}$	0.008	0.004	0.0455			0.18
Strength	Intercept	3.52	0.65	0.0001	0.0002	0.12	0
	$EST_{(15-19)}$	-0.01	0.01	0.0001			-0.37
	$EHP_{(15-19)}$	0.001	0.0001	0.1813			0.11
Tibia							
Width	Intercept	1.45	0.75	0.0565	0.1911	0.02	0
	$EST_{(15-19)}$	0.01	0.02	0.4302			0.07
	$EHP_{(15-19)}$	0.0002	0.0002	0.1831			0.12
Length	Intercept	9.66	6.43	0.1350	0.0001	0.16	0
	$EST_{(15-19)}$	0.47	0.17	0.0075			0.23
	$EHP_{(15-19)}$	0.004	0.001	0.0022			0.26
Calcification	Intercept	81.67	19.26	0.0001	0.2168	0.02	0
	$EST_{(15-19)}$	-0.59	0.52	0.2548			-0.10
	$EHP_{(15-19)}$	0.007	0.004	0.1082			0.15
Strength	Intercept	0.61	0.61	0.3180	0.4420	0.01	0
	$EST_{(15-19)}$	0.001	0.01	0.9289			0.00
	$EHP_{(15-19)}$	0.00001	0.0001	0.2350			0.11

[a] R^2: Regression coefficient.

[1] $EST_{(15-19)}$: Eggshell temperature (EST) in ^{0}C from days 15 to 19 of incubation.

[2] $EHP_{(15-19)}$: Embryonic heat production (EHP) in mW from days15 to 19 of incubation.

n = 12 eggs per hen age

4.5. REFERENCES

Akins, R. E., and R. S. Tuan. 1993. Trans epithelial calcium transport in the chick chorioallantoic membrane. I. Isolation and characterization of chorionic ectoderm cells. J. Cell. Sci. 105:369-379.

Alfonso-Torres, K. A., L. H. Gargaglioni, J. M. Pizauro, D. E. Faria Filho, R. L. Furlan, and M. Macari. 2009. Breeder age and bone development in broiler chicken embryos. Arq. Bras. Med. Vet. Zootec. 61:219-226.

Bilinski, J. J., R. D. Reina, J. R. Spotila, and F. V. Paladino. 2001. The effects of nest environment on calcium mobilization by leatherback turtle embryos (*Dermochelys coriacea*) during development. Comp. Biochem. Physiol. - Part A: Molec. Integ. Physiol. 130:151-162.

Canadian Council on Animal Care. 1993. A guide to the care and use of experimental animals. 2nd ed. CCAC, Ottawa, ON, Canada.

Careghi, C., K. Tona, O. Onagbesan, J. Buyse, E. Decuypere, and V. Bruggeman. 2005. The effects of the spread of hatch and interaction with delayed feed access after hatch on broiler performance until seven days of age. Poult. Sci. 84:1314-1320.

Chen, J. C., B. Zhao, M. T. Longaker, J. A. Helms, and D. R. Carter. 2008. Periosteal biaxial residual strains correlate with bone specific growth rates

in chick embryos. Comput. Methods. Biomech. Biomed. Engin. 11:453-461.

Gabrielli, M. G. 2004. Carbonic anhydrases in chick extra-embryonic structures: a role for Ca in bicarbonate reabsorption through the chorioallantoic membrane. J. Enzyme Inhib. Med. Chem. 19:283-286.

Hamidu, J. A., G. M. Fasenko, L. Guan, D. R. Barreda, and J. J. R. Feddes. 2011. Influence of parent flock age on embryonic metabolism in modern turkey strains. Poult. Sci. 90:426-434.

Hamidu, J. A., G. M. Fasenko, J. J. R. Feddes, E. E. O'Dea, C. A. Ouellette, M. J. Wineland, and V. L. Christensen. 2007. The effect of broiler breeder genetic strain and parent flock age on eggshell conductance and embryonic metabolism. Poult. Sci. 86:2420-2432.

Hammond, C. L., B. H. Simbi, and N. C. Stickland. 2007. In ovo temperature manipulation influences embryonic motility and growth of limb tissues in the chick (*Gallus gallus*). J. Exp. Biol. 210:2667-2675.

Joseph, N., A. Lourens, and E. Moran. 2006. The effects of suboptimal eggshell temperature during incubation on broiler chick quality, live performance, and further processing yield. Poult. Sci. 85:932-938.

Kelly, W. L., and M. M. Bryden. 1983. A modified differential stain for cartilage and bone in whole mount preparations of mammalian fetuses and small vertebrates. Biotech. Histochem. 58:131-134.

Kubota, M., E. Abe, T. Shinki, and T. Suda. 1981. Vitamin D metabolism and its possible role in the developing chick embryo. Biochem. J. 194:103-109.

Lourens, A. 2001. The importance of air velocity in incubation. World Poult. 17:29-30.

Lourens, A., H. van den Brand, M. J. W. Heetkamp, R. Meijerhof, and B. Kemp. 2007. Effects of eggshell temperature and oxygen concentration on embryo growth and metabolism during incubation. Poult. Sci. 86:2194-2199.

Lourens, A., R. Molenaar, H. van den Brand, M. Heetkamp, R. Meijerhof, and B. Kemp. 2006. Effect of egg size on heat production and the transition of energy from egg to hatchling. Poult. Sci. 85:770-776.

Lourens, A., H. van den Brand, R. Meijerhof, and B. Kemp. 2005. Effect of eggshell temperature during incubation on embryo development, hatchability, and post-hatch development. Poult. Sci. 84:914-920.

Meijerhof, R., and G. V. Beek. 1993. Mathematical modelling of temperature and moisture loss of hatching eggs. J. Theor. Biol. 165:27-41.

Oviedo-Rondón, E. O., P. R. Ferket, and G.B.Havestein. 2006. Understanding long bone development in broilers and turkeys. Avian Poult. Biol. Rev. 17:77-88.

Oviedo-Rondón, E. O., J. Small, M. J. Wineland, V. L. Christensen, P. S. Mozdziak, M. D. Koci, S. V. L. Funderburk, D. T. Ort, and K. M. Mann. 2008a. Broiler embryo bone development is influenced by incubator temperature, oxygen concentration and eggshell conductance at the plateau stage in oxygen consumption. Br. Poult. Sci. 49:666.

Oviedo-Rondón, E. O., J. Small, M. J. Wineland, V. L. Christensen, J. L. Grimes, S.V.L. Funderburk, and K. M. Mann. 2008b. Effects of incubator temperature and oxygen concentration during the plateau stage of oxygen consumption on turkey embryo long development. Poult. Sci. 87:1484-1492.

Peebles, E., S. Doyle, C. Zumwalt, P. Gerard, M. Latour, C. Boyle, and T. Smith. 2001. Breeder age influences embryogenesis in broiler hatching eggs. Poult. Sci. 80:272-277.

Persia, M. E., and W. W. Saylor. 2006. Effects of broiler strain, dietary non-phytate phosphorus, and phytase supplementation on chick performance and tibia ash. J. Appl. Poult. Res. 15:72-81.

Pinchasov, Y., and Y. Noy. 1993. Comparison of post-hatch holding time and subsequent early performance of broiler chicks and turkey poults. Br. Poult. Sci. 34:111-120.

Shaw, A. L., J. P. Blake, and E. T. Moran. 2010. Effects of flesh attachment on bone breaking and of phosphorus concentration on performance of broilers hatched from young and old flocks. Poult. Sci. 89:295-302.

Shim, M. Y., and G. M. Pesti. 2011. Effects of incubation temperature on the bone development of broilers. Poult. Sci. 90:1867-1877.

Tilgar, V., P. Kilgas, A. Viitak, and S. J. Reynolds. 2008. The rate of bone mineralization in birds is directly related to alkaline phosphatase activity. Physiol. Biochem. Zool. 81:106-111.

Willemsen, H., B. Kamers, F. Dahlke, H. Han, Z. Song, Z. Ansari Pirsaraei, K. Tona, E. Decuypere, and N. Everaert. 2010. High- and low-temperature manipulation during late incubation: Effects on embryonic development, the hatching process, and metabolism in broilers. Poult. Sci. 89:2678-2690.

Yair, R., Z. Uni, and R. Shahar. 2012. Bone characteristics of late-term embryonic and hatchling broilers: Bone development under extreme growth rate. Poult. Sci. 91:2614-2620.

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.

Yalcin, S., S. Özkan, E. Coskuner, S. Özkan, Y. Delen, Y. Kurtulmus, and T. Tanyalçin. 2001. Effects of strain, maternal age and sex on morphological characteristics and composition of tibial bone in broilers. Br. Poult. Sci. 42:184.

Yalçin, S., H. B. Molayoğlu, M. Baka, O. Genin, and M. Pines. 2007. Effect of temperature during the incubation period on tibial growth plate chondrocyte differentiation and the incidence of tibial dyschondroplasia. Poult. Sci. 86:1772-1783.

Yassin, H., A. G. J. Velthuis, M. Boerjan, and J. van Riel. 2009. Field study on broilers' first-week mortality. Poult. Sci. 88:798-804.

CHAPTER 5. GENERAL DISCUSSION AND CONCLUSIONS

5.1 Review of hypotheses and Conclusions

Hypothesis 1. It was hypothesized that supplementing low levels of Cu, Zn and Mn chelated to HMTBa would increase Cu, Zn and Mn content in the egg yolk relative to eggs from hens fed mineral sulfates at industry levels. Increased egg trace mineral content will thus enhance bone characteristics in the embryos and chicks; this effect will be increased in the progeny of older hens.

This hypothesis was rejected because Cu, Zn and Mn content in the egg yolk was not significantly different in eggs from the Control and OTM treatments. Therefore, the results on bone characteristics described in this research might not have been associated with trace mineral supply within the egg.

Hypothesis 2. It was hypothesized that incubator temperatures of 36^0C or 36.5^0C from the 15^{th} day of incubation until hatch would increase bone development of day old chicks, particularly chicks from older hens relative to high incubator temperature of 37^0C or 37.5^0C.

The hypothesis was rejected because there was no significant interaction between hen age and incubator temperature. Chicks from eggs set at 36^0C had stronger bones relative to Control even though bone size was reduced. Furthermore, chicks from eggs set at 36.5^0C had similar or inferior (shorter femur) bone characteristic relative to Control chicks.

Hypothesis 3. It was hypothesized that a negative relationship would exist between embryos with high heat production from day 15 to19th day and bone characteristics at hatch.

This hypothesis rejected because a weak positive relationship existed between incubator temperature in the last third of incubation and bone characteristics at hatch.

Hypothesis 4. It was hypothesized that a negative relationship would exists between high eggshell temperature from 15 to19th day of incubation and bone characteristics at hatch.

This hypothesis was supported because a negative relationship existed between femur calcification and strength and the high eggshell temperature from 15 to19th day of incubation.

5.2. DISCUSSION AND CONCLUSIONS

At hatch the skeletal frame of the chicks' body is assembled yet not fully calcified. This framework will grow the most in the first two weeks after hatch and is important as a scaffold to anchor the musculature in development. A strong, well-formed skeletal frame at hatch might increase healthiness in the first days post-hatch. This might in turn result in increased activity in the barn and increased feed and water consumption. Understanding the long term effects of maternal nutrition in the offspring, along with knowledge on the relationship between incubator temperature and bone development in eggs from hens of different ages will help us understand the factors influencing bone growth and perhaps help increase bone quality in chicks.

The influences of maternal nutrition and hen age on bone development of the offspring were investigated in Chapters 2 and 3. It was expected that bone development of the progeny would be enhanced due to the increased levels of Cu, Zn and Mn in the egg yolk when hens were supplemented with low levels of organic trace minerals (OTM) compared to hens fed mineral levels often supplemented in industry (as inorganic form; Control diet). Contrary to what was expected, there were no significant differences between these two treatments except that OTM at low levels increased tibia width in chicks from Young and Mid hens. This result, combined with the non-significance on Cu, Zn and Mn content in egg yolk, suggest that trace mineral form influenced bone characteristics by another mechanism. It is not possible to conclude whether OTM

were more available than minerals supplemented at industry-recommended levels because this effect may have been confounded if trace mineral transport to the egg was already maximized.

Given the potential confounding effects of TM bioavailability between minerals at NRC levels and those at industry levels, it was also investigated whether adding more trace minerals to the diet resulted in changes in egg or bone characteristics of the progeny. Dietary levels of Cu, Zn and Mn were increased by adding organic (OTM + ITM) or inorganic forms (High ITM) on top of mineral at industry levels (Control). Increasing mineral levels as OTM or ITM in the hen diet did not influence trace mineral concentration per µg/g yolk in the egg. This lack of response might indicate: 1) that increasing mineral levels as either OTM or ITM has no effect on egg mineral content; or 2) that trace minerals supplemented at industry levels are already high enough and therefore adding more would have no extra effect.

There were no significant differences between OTM + ITM and Control in most of the bone characteristics investigated. Surprisingly, the OTM + ITM reduced femur width relative to Control in chicks from Mid hens only. This might indicate that high levels of supplementation by adding a combination of OTM and ITM may reduce bioavailability; however, the mechanism is not completely understood.

High levels of mineral sulfates increased Zn µg/g yolk in the eggs from Mid hens relative to those from hens fed inorganic minerals at industry levels,

increased bone strength at hatch relative to industry inorganic levels and at 7 days relative to all diets. However the application of this result for industry is questionable; high levels of trace minerals might be related to increased excretion of trace minerals to the environment, an effect not investigated in this trial. Furthermore, hens fed high mineral levels as sulfates tended to lay low number of hatching eggs over the course of this 37 week trial relative to low and conventional industry mineral levels. It appears that a trade-off existed in that bone strength increased but the number of hatching eggs (and potentially hatchability) was reduced.

The lack of bone responses between the 2 different forms at high trace mineral levels (except at 7 days regarding tibia breaking strength) indicated that in general there were no differences in response due to the form of TM supplementation. Because High ITM increased bone quality relative to Control, these results suggest that there is an opportunity to increase bone characteristics through trace mineral supplementation beyond current industry practice. Further studies investigating the dose response of broiler breeders supplemented with either organic minerals chelated to HMTBa or mineral sulfates are warranted. In such studies, initial levels would be expected to be below the hen's requirement and supplemented with increasing levels of either OTM or ITM. Studies should also be designed to investigate the effects of feeding Cu, Zn or Mn independent of one another, in order to understand the biological effects of each mineral, as well as all a combination of Cu, Zn and Mn in order to understand the bioavailability and effectiveness of supplementing a combination of these minerals. Parameters

such as eggshell quality, egg mineral content, performance parameters (i.e. total number of eggs produced and chicks per hen housed, incidence of embryonic mortality), egg nutrient content, skeletal characteristics of the progeny, and mineral excretion are variables suggested to be measured in the future.

Interestingly, as the hen aged, Zn and Cu mineral content in the yolk (µg/egg) was increased (Chapter 2) Interestingly, bone strength increased in chicks hatched from older hens (Chapter 3 and 4), this suggest that increased Zn and Cu content in the egg might be a factor influencing bone growth in embryos and chicks from hens of diverse ages.

Results from Chapters 3 and 4 indicated that breaking strength was consistently increased as the hen aged (Young < Mid=Old, Chapter 3; Young < Mid < Old, Chapter 4). Other studies have shown that chicks from a very young flock (25 week old) also have weaker bones relative to chicks hatched from a very old flock (65 weeks; Shaw et al., 2010). Furthermore, regression analysis reported in Chapter 4 indicated that about 27% of the variation influencing femur breaking strength was explained by flock age and incubator temperature. Interestingly, within the 27%, about 48% was explained by flock age and 16% due to incubator temperature.

In addition to investigating bone development at hatch, the present study took one step back and investigated the influence of hen age on bone development while the embryo was growing within the egg. It was demonstrated that bone development followed a non-uniform pattern among embryos from hen of

different ages (Chapter 2). Embryos from Young hens, for example, had decreased proportion of calcified femur and tibia at 20 days of incubation relative to chicks from Mid and Older hens (Chapter 2). This finding, combined with results from Chapter 3, strongly suggests that reduced breaking strength at hatch observed in chicks from young hens might be directly influenced by the rate and pattern of bone mineralization that has previously started within the egg. In this study, bone calcification was assessed by measuring the replacement of cartilage (stained blue) into bone (stained red; Kelly and Bryden, 1983) at different stages of embryo development. However, the method does not quantify the amount of calcium accumulated in bone. Further studies should investigate the pattern and the rate of calcium deposition in the embryonic bone throughout incubation among embryos from diverse hen ages. Another factor that might be related with increased bone strength in the progeny as the hen ages is collagen content and crosslinking. Because reduced mineralization impairs enzymatic crosslinking (Saito et al., 2006) it is possible that embryos from young hens (that had low calcification, Chapter 2) might also have reduced conversion of immature crosslink into their mature stable form (Saito et al., 2006), resulting in reduced breaking strength at hatch relative to chicks from old hens. However, basic scientific knowledge regarding collagen content and crosslinking in the embryonic bone is still lacking.

This study sets the stage for further investigation of macro and trace mineral metabolism in chicken embryos from diverse hen ages. One of the questions that arose from this study is whether calcium metabolism (in terms of

eggshell calcium release through the chorioallantoic membrane and its deposition in the growing bone), as well as trace mineral metabolism throughout incubation, differ among embryos from hen of diverse ages. It is known that embryos from young and old hens absorb egg yolk and mobilize fat from the egg yolk differently throughout incubation (Yadgary et al., 2010; Nangsuay et al., 2011). It is also known that minerals stored in the egg yolk are mostly mobilized between day 11 and 17 in embryos from 59 week old hens (Yadgary et al., 2011). This is concomitant with increased gene expression of calcium nutrient transporter TRPV6 in the yolk sac membrane of embryos from 50 week old hens (Yadgary et al., 2011). Furthermore, some nutrient transporters in the yolk sac membrane (none of them related with macro and trace mineral transport) had greater expression in embryos from young (30 weeks) relative to those from old flocks (50 week; Speier et al., 2012). The results from the previous and current studies combined strongly indicate that mineral metabolism is different among embryos from hen of diverse ages and might impact chick quality at hatch.

Embryo development is also reliant on incubation conditions, one of the more important of these being temperature (Freeman and Vince, 1974). The influence of incubator temperature on bone development at hatch among embryos from different flock ages was investigated in Chapter 4. Interestingly, results showed that flock age and incubator temperature independently influenced bone growth at hatch. It was observed that calcification and bone size were not significantly different among embryos from diverse flock ages, however, as

described previously, bone breaking strength of the progeny increased with flock age.

Results from Chapter 4 indicated that regardless of incubator temperature, bone quality of the progeny increased as the hen aged; regardless of hen age, incubator temperature of 36^{0}C from day 15 of incubation to hatch increased bone strength relative to temperatures of 36.5 or 37^{0}C. Furthermore, results indicated that high eggshell temperature (EST) during the time when the most rapid bone growth occurs (15 to 19 days of incubation) is detrimental to bone formation. The mechanism by which EST impairs bone calcification is not completely understood. Further studies investigating the influence of EST on efficiency of calcium uptake from the eggshell and its accumulation in the embryonic bone might bridge the gaps in our knowledge and might explain the mechanism behind the influence of incubator temperature, EST and skeletal development.

Data from Chapter 4 provided basic information on the relationship between incubator temperature and EST on bone growth. Eggs were individually incubated in metabolic chambers under strictly controlled humidity and temperature conditions. Therefore it is acknowledged that this incubation procedure was not representative of what occurs in commercial hatcheries. In commercial incubators, an air temperature of 37.5 to 38.0^{0}C is considered optimum before piping (18 days of incubation), whereas a temperature of 36.4^{0}C in the hatcher is considered ideal for navel quality of the hatched chick D. Hill (HatchTech Incubation Technology, Mountain Home, AR, personal

communication). However, in multistage incubators, the temperature that the embryo experiences is different from the air temperature during incubation (Lourens et al., 2005) and EST can vary largely in different places within an incubator. Lourens (2001) reported that, although the EST was close to 37.5^{0}C, it varied between 36.2 and 40.2^{0}C depending on egg placement within the incubator, therefore bone growth is likely to be influenced to a similar extent. This incubator conditions might increase the variation in embryonic bone development and therefore might decrease uniformity at hatch.

5.3. REFERENCES

Freeman, B. M., and M. A. Vince. 1974. Development of the avian embryo. Chapman and Hall, London, UK.

Kelly, W. L., and M. M. Bryden. 1983. A modified differential stain for cartilage and bone in whole mount preparations of mammalian fetuses and small vertebrates. Biotech. Histochem. 58:131-134.

Lourens, A., H. van den Brand, R. Meijerhof, and B. Kemp. 2005. Effect of eggshell temperature during incubation on embryo development, hatchability, and post-hatch development. Poult. Sci. 84:914-920.

Lourens, A. 2001. The importance of air velocity in incubation. World Poult. 17:29-30.

Nangsuay, A., Y. Ruangpanit, R. Meijerhof, and S. Attamangkune. 2011. Yolk absorption and embryo development of small and large eggs originating from young and old breeder hens. Poult. Sci. 90:2648-2655.

Saito, M., K. Fujii, and K. Marumo. 2006. Degree of mineralization-related collagen crosslinking in the femoral neck cancellous bone in cases of hip fracture and controls. Calcif. Tissue Int. 79:160-168.

Speier, J.S., Yadgary, L., Uni, Z., and Wong, E. A. 2012. Gene expression of nutrient transporters and digestive enzymes in the yolk sac membrane and small intestine of the developing embryonic chick. Poult. Sci. 91:1941-1949.

Shaw, A. L., J. P. Blake, and E. T. Moran. 2010. Effects of flesh attachment on bone breaking and of phosphorus concentration on performance of broilers hatched from young and old flocks. Poult. Sci. 89:295-302.

Yadgary, L., R. Yair, and Z. Uni. 2011. The chick embryo yolk sac membrane expresses nutrient transporter and digestive enzyme genes. Poult. Sci. 90:410-416.

Yadgary, L., A. Cahaner, O. Kedar, and Z. Uni. 2010. Yolk sac nutrient composition and fat uptake in late-term embryos in eggs from young and old broiler breeder hens. Poult. Sci. 89:2441-2452.