In ovo feeding with minerals and vitamin D₃ improves bone properties in hatchlings and mature broilers

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ABSTRACT The objective of this study was to examine the effect of in ovo feeding (IOF) with inorganic minerals or organic minerals and vitamin D₃ on bone properties and mineral consumption. Eggs were incubated and divided into 4 groups: IOF with organic minerals, phosphate, and vitamin D₃ (IOF-OMD); IOF with inorganic minerals and phosphate (IOF-IM); sham; and non-treated controls (NTC). IOF was performed on embryonic day (E) 17; tibiae and yolk samples were taken on E19 and E21. Post-hatch, only chicks from the IOF-OMD, sham, and NTC were raised, and tibiae were taken on d 10 and 38. Yolk mineral content was examined by inductively coupled plasma spectroscopy. Tibiae were tested for their whole-bone mechanical properties, and mid-diaphysis bone sections were indented in a micro-indenter to determine bone material stiffness (Young’s modulus). Micro-computed tomography (μCT) was used to examine cortical and trabecular bone structure. Ash content analysis was used to examine bone mineralization. A latency-to-lie (LTL) test was used to measure standing ability of the d 38 broilers. The results showed that embryos from both IOF-OMD and IOF-IM treatments had elevated Cu, Mn, and Zn amounts in the yolk on E19 and E21 and consumed more of these minerals (between E19 and E21) in comparison to the sham and NTC. On E21, these hatchlings had higher whole-bone stiffness in comparison to the NTC. On d 38, the IOF-OMD had higher ash content, elevated whole-bone stiffness, and elevated Young’s modulus (in males) in comparison to the sham and NTC; however, no differences in standing ability were found. Very few structural differences were seen during the whole experiment. This study demonstrates that mineral supplementation by in ovo feeding is sufficient to induce higher mineral consumption from the yolk, regardless of its chemical form or the presence of vitamin D₃. Additionally, IOF with organic minerals and vitamin D₃ can increase bone ash content, as well as stiffness of the whole bone and bone material in the mature broiler, but does not lead to longer LTL.

Key words: in ovo feeding, bone, broiler, mineral, vitamin D₃.

INTRODUCTION

Embryonic nutrition has a pronounced effect on progeny performance. Nutritional insufficiencies during the embryonic period and early life can induce adaptive responses with long-lasting adverse consequences (Tygesen et al., 2008; Matthiesen et al., 2010; Smith et al., 2010; Langley-Evans, 2015). This phenomenon was previously termed “programming” (Lucas, 1991).

Unlike mammals, the broiler embryo develops independent from the mother, and therefore, the deposition of the finite nutrient storages to the egg by the hen is crucial to enable optimal embryonic development (Richards, 1997; Moran, 2007; Uni et al., 2012).

© 2015 Poultry Science Association Inc.
Received January 20, 2015.
Accepted July 15, 2015.
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The deposition of minerals to the different egg compartments is crucial for optimal embryonic development because minerals are important for the development of the skeletal, muscular immune, and cardiovascular systems of the broiler embryo (Caskey et al., 1944; Wilson, 1997; Favero et al., 2013; Oviedo-Rondón et al., 2013). The hen deposits minerals to the egg via two routes: through the ovary to the yolk or through the oviduct to the albumen, shell, and shell membrane (Richards and Packard, 1996). Each of these compartments contains a different variety of minerals; the yolk is the major mineral source for the embryo during incubation, containing most of the P, Zn, Cu, Mn, and Fe, while the albumen is the major source of Na and K in the egg and contains very low levels of P, Fe, Cu, Mn, and Zn (Richards and Packard, 1996; Richards, 1997; Yair and Uni, 2011). The shell contains high amounts of Ca and low amounts of Fe, Mg, Mn, P, and Zn; however, only high amounts of Ca, a much lower amount of Mg, and negligible amounts of Fe, Mn, and P are released from...
the shell and made available for the embryo (Packard
and Packard, 1991; Richards and Packard, 1996; Yair
and Uni, 2011).

Modern broilers underwent genetic selection for high-
growth and metabolic rates, resulting in annual im-
provements in BW gain (due to increased muscle mass),
feed efficiency, and meat yields (Havenstein et al., 2003;
Tona et al., 2004; Vieira and Angel, 2012); however,
with these improvements it became evident that some
systems, such as the skeletal system, were not keep-
ing up with the increase in muscle mass (Dibner et al.,
2007). As a result, modern-day broilers suffer from nu-
merous bone problems that were found to be closely
associated with their fast growth rate (Thorp, 1994; Ju-
lian, 1998; Angel, 2007; Dibner et al., 2007; Shim et al.,
2012; Prisby et al., 2014).

Leg problems are among the major causes for eco-

domic losses in the chicken house (Sullivan, 1994). These
problems were estimated to cause economic losses of
80 to 120 million dollars annually in the United States
(Sullivan, 1994). To our knowledge, no recent estima-
tions were published; however, in a survey of commer-
cial broiler flocks, Knowles et al. (2008) found that more
than 27.6% of the birds suffered from poor locomotion,
and 3.3% were almost unable to walk. Such impaired
walking ability is expected to reduce production due
to reduced feed consumption, increased frequency of
downgrades and condemnations, and elevated mortality
(Sullivan, 1994). Accordingly, it is reasonable to assume
that leg problems are still a cause for major economic
losses.

In addition, leg problems can dramatically affect
broiler welfare by causing lameness and impaired walk-
ing ability, inducing acute and chronic pain, reducing
access to feed and water, and even causing mortality
(Angel, 2007; Dibner et al., 2007; Nääs et al., 2009;
Ruiz-Feria et al., 2014). Due to these economic and
welfare issues, it is very important to minimize the in-
cidence of leg problems in broilers.

In order to reduce the incidence of leg problems, in
the last 25 years attempts were made to select broilers
for improved skeletal integrity (Williams et al., 2000;
Kapell et al., 2012). Some progress was reported by
Kapell et al. (2012), who showed that selection by ac-
curately scoring selection candidates and using a strin-
gent culling policy of discarding any selection candidate
with clinical leg defects has led to a reduction in the
incidence of some leg defects, such as tibial dyschon-
droplasias (TD) and crooked toes, but not of hock burn.
Despite this selection effort, recent works still have
shown that fast-growing broilers have a high incidence
of leg problems: Dinev et al. (2012) found that 24.22
to 27.70% of broilers from 3 commercial lines suffer from
some degree of TD. Wideman et al. (2013) found that
faster-growing commercial broilers are more susceptible
to lameness. Additionally, leg problems are affected by
nutrition and management, not only by growth rate.
For example, the content and chemical form of Ca, P,
Cu, Mn, Zn, and Vitamin D₃ in the diet can influence
bone development and leg problems (Richards, 1997;
Angel, 2007; Dibner et al., 2007; Favero et al., 2013),
while incubation conditions have an important effect
on bone properties, leg problems, and standing ability
of modern broilers (Oviedo-Rondón et al., 2008; Groves
and Muir, 2014; Van der Pol et al., 2014). Accordingly,
methods other than selection should also be used in or-
der to reduce the incidence of leg problems in modern
broilers.

It was previously shown that a shortage in Cu, Mn,
P, and Zn during the embryonic period and post-hatch
leads to impaired bone development (Caskey et al.,
1944; Dibner et al., 2007). We previously showed that
during the last days of incubation, the amount of Cu,
Mn, P, and Zn in the yolk is low, and therefore, the em-
broyo consumes little if any of those minerals during that
period (Yair and Uni, 2011). Correspondingly, most me-
chanical and geometric properties of the tibia and femur
remain unchanged or even deteriorate during that pe-
riod (Yair et al., 2012). Accordingly, it was previously
suggested that the limited availability of minerals dur-
ing the embryonic period and the first weeks post-hatch
limits skeletal development during its rapid-growth pe-
riod, thus increasing the incidence of leg problems (Dib-
nen et al., 2007; Yair et al., 2012).

Previous work has shown that embryonic enrichment
with organic Cu, Fe, Mn, and Zn, phosphate, vitamin
D₃, and carbohydrates using the in ovo feeding (IOF)
methodology (Uni and Ferket, 2003; Uni and Ferket,
2004) increased the content of these minerals in the
yolk and their consumption by the embryo pre-hatch
(Yair and Uni, 2011).

However, it is unclear if this effect is due to the en-
richment with minerals per se, or due to the organic
form of the added minerals, vitamin D₃, carbohydrates,
or their combination. Additionally, similar IOF led to
higher tibial stiffness, but only until 14 d post-hatch
(Yair et al., 2013).

Consequently, the objectives of this work were: 1) to
determine whether embryonic enrichment with minerals
(in inorganic form) is sufficient to induce higher embry-
onic mineral consumption, and 2) to examine the effect
of IOF with a solution of organic trace minerals (OTM),
phosphate, and vitamin D₃, which was prepared based
on our conclusions from the previous trials (Yair and
Uni, 2011; Yair et al., 2013) on the content and uptake
of minerals from the yolk, and to evaluate if it could
have a longer lasting effect on the mechanical proper-
ties of broiler bones (i.e., in the mature broiler, and not
only until d 14).

**MATERIALS AND METHODS**

**Overview**

The experiment was carried out in 2 sequential and
identical studies (treated as blocks). In each study,
150 fertile eggs were collected from Cobb 500 hens (35
and 37 wk of age, respectively, in the first and second
IN OVO FEEDING IMPROVES BONE PROPERTIES

Table 1. Formulation of the breeder and broiler diets.

<table>
<thead>
<tr>
<th></th>
<th>Breeder diet (pre-starter)</th>
<th>Breeder diet (starter)</th>
<th>Breeder diet (grower)</th>
<th>Breeder diet (finisher)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Proximate constituents</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Protein %</td>
<td>15.5</td>
<td>22.0</td>
<td>21.0</td>
<td>20.0</td>
</tr>
<tr>
<td>Fat %</td>
<td>4.0</td>
<td>6.0</td>
<td>7.0</td>
<td>7.5</td>
</tr>
<tr>
<td>Ash %</td>
<td>11.0</td>
<td>5.6</td>
<td>5.1</td>
<td>4.8</td>
</tr>
<tr>
<td>Sodium %</td>
<td>0.35</td>
<td>0.18</td>
<td>0.15</td>
<td>0.15</td>
</tr>
<tr>
<td>Available P %</td>
<td>0.35</td>
<td>0.18</td>
<td>0.15</td>
<td>0.15</td>
</tr>
<tr>
<td>Total P %</td>
<td>0.52</td>
<td>0.71</td>
<td>0.61</td>
<td>0.60</td>
</tr>
<tr>
<td>Ca %</td>
<td>3.30</td>
<td>0.95</td>
<td>0.85</td>
<td>0.8</td>
</tr>
<tr>
<td>Metabolic energy kcal/kg</td>
<td>2,750</td>
<td>3,225</td>
<td>3,150</td>
<td>3,100</td>
</tr>
<tr>
<td>Premix</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mn ppm</td>
<td>80</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>Zn ppm</td>
<td>100</td>
<td>90</td>
<td>90</td>
<td>90</td>
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<tr>
<td>Fe ppm</td>
<td>60</td>
<td>30</td>
<td>30</td>
<td>30</td>
</tr>
<tr>
<td>Cu ppm</td>
<td>10</td>
<td>50</td>
<td>50</td>
<td>50</td>
</tr>
<tr>
<td>Vitamin D₃ IU/kg</td>
<td>3,000</td>
<td>4,000</td>
<td>4,000</td>
<td>4,000</td>
</tr>
</tbody>
</table>

Table 2. Content of the IOF solutions.

<table>
<thead>
<tr>
<th></th>
<th>IOF-OMD</th>
<th>Amount per embryo (mg)</th>
<th>IOF-IM</th>
<th>Amount per embryo (mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chemical form</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Zn</td>
<td>3Organic</td>
<td>1.80</td>
<td>2ZnSO₄</td>
<td>1.45</td>
</tr>
<tr>
<td>Mn</td>
<td>3Organic</td>
<td>0.057</td>
<td>2MnSO₄</td>
<td>0.055</td>
</tr>
<tr>
<td>Cu</td>
<td>3Organic</td>
<td>0.088</td>
<td>2CuSO₄</td>
<td>0.08</td>
</tr>
<tr>
<td>P</td>
<td>3KH₂PO₄</td>
<td>2.03</td>
<td>3KH₂PO₄</td>
<td>2.08</td>
</tr>
<tr>
<td>K</td>
<td>3KH₂PO₄</td>
<td>1.22</td>
<td>3KH₂PO₄</td>
<td>1.25</td>
</tr>
<tr>
<td>Na</td>
<td>4NaH₂PO₄</td>
<td>0.68</td>
<td>4NaH₂PO₄</td>
<td>0.75</td>
</tr>
<tr>
<td>Vitamin D₃ Liquid</td>
<td>400 IU/mL</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

1Both solutions were injected to the amniotic fluid on E17.
2Organic minerals were supplemented as Glycine chelates (MAAC®, Novus International, Inc. (St. Charles, MO)).
3JT Baker (Phillipsburg, NJ).
4Merck (Darmstadt, Germany).
5Dor.Ky (Nes Tziona, Israel).

studies), which were housed in the Faculty of Agriculture (Rehovot, Israel) and fed according to Cobb’s protocols (Table 1). The eggs were divided into 4 groups, with each group consisting of similar egg weight distribution. The 4 groups were: (1) IOF with OTM, phosphate, and vitamin D₃ (IOF-OMD); (2) IOF with inorganic trace minerals and phosphate (IOF-IM); (3) sham (IOF with the diluent alone); and (4) non-treated controls (NTC). The eggs were incubated (Petersime, Zulte, Belgium) according to routine procedures (37.8°C and 56% relative humidity). On embryonic day 17 (E17) the amniotic fluids of the IOF-OMD, IOF-IM, and sham groups were enriched with solutions (0.6 mL per egg) by the IOF technique, as described by Uni and Ferket (2003; 2004). The content of the IOF solutions given to each embryo was planned to be as follows: 2.0 mg of P (in NaH₂PO₄ and KH₂PO₄ forms), 1.0, 0.05, and 0.03 mg of Zn, Cu, and Mn as Glycine chelates (MAAC®, Novus International, Inc. (St. Charles, MO)), and 240 IU of solubilize vitamin D₃ for the IOF-OMD. The IOF-IM were planned to have similar amount of P (in similar forms) than the IOF-OMD, similar amount of Zn, Cu, and Mn as sulfates, and no vitamin D₃. The mineral content of the solutions as analyzed by an inductively coupled plasma atomic emission spectroscopy (ICP-AES; Spectro Arcos, Kleve, Germany) was 1.92, 1.08, 0.053, and 0.034 mg of P, Zn, Cu, and Mn, respectively, for the IOF-OMD, and 2.12, 0.87, 0.048, and 0.033 mg of P, Zn, Cu, and Mn, respectively, for the IOF-IM (Table 2). The sham group solution contained only the diluent of the IOF solutions (DDW with Tween 20, which was used to solubilize the vitamin D₃).

Yolk and Bone Samples On E0, day of set, and E17, 4 eggs (in each study) were randomly selected and their yolk sacs taken for mineral analysis. On E19 and E21 (day of hatch), 4 eggs from each group (in each study) were randomly selected, and the yolk sacs and embryonic tibiae were removed. Upon hatching of the remaining eggs, hatchlings from the NTC, IOF-OMD, and sham groups were identified by a neck tag number and moved to a pen, and the floor was covered with soft pine wood shavings. As the IOF-IM group was used to see if embryonic enrichment with inorganic minerals without other nutrients (not organic, since they might influence consumption) is sufficient to increase mineral consumption pre-hatch, the IOF-IM hatchlings were not raised post-hatch. The chicks were housed in
one pen (stocking density of 8 to 10 chicks/m²) and given ad libitum access to feed (Table 1) and water. On d 10 post-hatch, 4 randomly selected broilers from each group (in each study) were selected, while on d 38 post-hatch (marketing age), 10 broilers from each group (in each study) were randomly selected. The selected broilers were sacrificed by cervical dislocation, their sex was determined by identifying the testis or the ovaries, and tibiae from both legs were removed. The selected bones at all time points were cleaned of all soft tissues, externally measured (weight and length), wrapped in saline-soaked gauze, and stored at –20°C. The selected yolk sacs were weighed, homogenized using a T-25 Ultra-Turrax homogenizer (IKA, Staufen, Germany), and stored at –20°C.

Mineral Analysis Samples (100 to 150 mg) from each yolk sac or albumen homogenate were digested with a mixture of 2 mL 30% H₂O₂ and 4 mL 70% HNO₃ inside a 50-mL plastic tube for 6 h in a 95°C bath. The digested samples were analyzed for their mineral content using ICP-AES (Spectro Arcos, Kleve, Germany).

Structural Analysis The left tibia of each embryo and chick was scanned using a high-resolution microcomputed tomography (μCT) scanner (SkyScan 1174, Belgium). The X-ray source was set at 50 kV and 800 μA. A total of 225 projections were acquired over an angular range of 180°. An aluminum filter of 0.25 mm thickness for samples from E19 and E21, and 0.5 mm for samples from d 10 and d 38 was used to decrease beam-hardening effects. The image slices were reconstructed using customized software (NRecon, SkyScan, Belgium). Scans were performed at the highest resolution possible, depending on bone size (7.9, 10.2, 16.1, and 30.1 μm, for E19, E21, d 10, and d 38, respectively). In addition, for each set of scanning parameters, 2 phantoms of known mineral density (0.25 g/cm³ and 0.75 g/cm³) also were scanned in order to allow calibration of the attenuation levels directly to bone mineral density (BMD) values.

Cortical bone analysis was performed at mid-diaphysis of the examined bone, selecting a 100-slice region of interest. The following cortical properties were measured: bone volume fraction as % of total tissue volume (BV/TV, %), BMD (g/cm³), cortical area (mm²), medullary area (the area of the medullary cavity surrounded by the cortex) (mm²), polar moment of inertia (mm⁴), and mean cortical thickness (μm).

Cancellous bone analysis was performed at the distal epiphysial area of each examined bone over a 100-slice region of interest. The first slice of the selected region of interest was set at the most proximal area where the cross-section was filled with trabeculae, and the region of interest consisted of the next 100 slices in the distal direction. A customized software (CTan, SkyScan, Belgium) was used to manually specify the limits of the trabecular area on each slice in order to separate it from the cortical shell surrounding it; measurements were taken only from within these limits. The following trabecular properties were measured: bone volume fraction as % of total tissue volume (BV/TV, %), mean trabecular thickness (μm), and mean trabecular separation (μm). Cancellous bone analysis was performed only on d 10 and d 38, due to insufficient amounts of trabecular struts at younger ages.

Mechanical Testing After completion of the structural analysis, tibiae were biomechanically tested. Testing of the smaller bones (E19 to d 10) was performed with a custom-built micromechanical testing device; an axial-motion DC motor (PI M-235.2 DG, Physik Instrumente, GmbH, Germany) moved a metal shaft into the testing chamber in small sub-micron steps while being able to apply substantial force (>100 N). The metal shaft was connected in series to a 120 N load cell (AL311, Sensotec, Honeywell, United States, ±0.4 N), to which was attached in turn a movable anvil that was designed to contact the bone sample at its mid-point. The movement of the anvil was measured using an optic encoder with 50 nm resolution (Model RGHI2H30D63, Renishaw, United Kingdom). In order to test the bones in an environment that approximates their physiological state, they were tested while immersed in saline. The load and displacement values were monitored through an in-house written program (Labview, National Instruments, Austin, TX).

Biomechanical testing of the bones from d 38 was similarly performed using an Instron 3345 materials testing machine (Instron, Norwood, MA). The bones were tested in a plastic container that allowed them to be immersed in saline during testing. Force and displacement data were collected at 20 Hz by Instron software (BlueHill, Buckinghamshire, United Kingdom).

All bones were tested by the 3-point bending method. The bones were placed on 2 supports having rounded profiles (0.5 mm radius) to limit stress concentration at the point of load application, such that the supports were equidistant from the ends of the bone, and both contacted the posterior aspect of the diaphysis. The distance between the supports was adjusted to be the maximal distance possible in the cylindrical part of the diaphysis. Each bone was loaded on its anterior aspect by a moving prong with rounded profile at the point of load between the bottom supports and at the mid-point along its length. Loading was conducted at a constant rate of 2 mm/min up to fracture point, as identified by a sudden decrease in load.

The resulting load-displacement curves were used to calculate whole-bone stiffness (slope of the linear portion of the load-displacement curve), maximal load, and work to fracture (WTF) (Lanyon et al., 1982).

In order to determine the stiffness of the material of the cortical bone of mature broilers, tibiae (n = 5) from d 38 obtained from the second study were tested by a micro-indentor using a Vickers hardness tip (Evans et al., 1990; Bonser and Casinos, 2003); 5 mm-thick transverse bone sections were cut from the mid diaphysis using a slow water-cooled diamond saw (Buehler Isomet, Lake Bluff, IL). The sections were then hand-polished from both sides using emery paper (800, 1,200,
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Figure 1. A sample of an indentation done on a mature broiler bone (d 38). The length of the indentation diagonals are noted as d1 and d2. Tibiae cross sections were indented using a Future-Tech FM-300 micro hardness tester.

2,400, and 4,000 grit) and a polishing cloth with 3 μm diamond suspension (Buehler Minimet Polisher, Lake Bluff, IL). Vickers hardness was measured using a Future-Tech FM-300 micro hardness tester with a load of 25 g and a dwell time of 10 sec. Each bone section was indented in the center of its anterior part; 10 indentations per sample were performed in 2 parallel lines of 5 indentations. The distance between each two adjacent indentations was 200 μm. Micro-hardness at each indentation site was determined by using the following formula:

\[ VHN = \frac{1.8544P}{d^2} \]

VHN is Vickers hardness number (kg/mm²), P is the test load (kg), and d is the average indentation diagonal length (mm) (Figure 1). The Young’s modulus (E) was calculated by using the conversion formula from hardness to stiffness published by Evans et al. (1990):

\[ E = \sqrt{\frac{(VHN - 17.5)}{0.104}} \]

Ash Content After the tibiae were mechanically tested, a small section from the mid diaphysis of each bone (about 10 to 20% of the bone’s length) was removed and processed for ash quantification, following a previously described protocol (Yair et al., 2013).

Standing Ability Test In order to assess differences in standing ability and leg weakness of chicks from the various treatment groups, a latency-to-lie (LTL) test (Weeks, 2001; Berg and Sanotra, 2003) was performed in the second study. On d 35, 12 chicks per group were subjected to a modified LTL test as described by Berg and Sanotra (2003). Briefly, each chick was put in a small plastic tub filled with 3 cm of water (∼32°C), surrounded by disposable cardboard sheets to limit the chick’s ability to see and jump outside the test area. The time until the chick could not continue standing and attempted to lie was measured by a stopwatch. If the bird was still standing after 600 sec, the test was interrupted.

Ethical Approval Embryos and chicks were sacrificed at predetermined time points as described. The experiment was approved by the Ethics Committee for Animal Experimentation, Faculty of Agricultural, Food and Environmental Sciences, the Hebrew University of Jerusalem.

Statistical Analysis Data from the sampling day were subjected to 3-way full-factorial ANOVA with 4 groups for E19 and E21 (NTC, IOF-OMD, IOF-IM, and sham) or 3 groups for d 10 and d 38 (NTC, IOF-OMD, and sham), 2 studies, 2 sexes, and all of their interactions. The group means from E19 and E21 were compared by the Tukey Kramer HSD test, while results from bones of chicks aged 10 and 38 d were compared by Student’s t-test. All statistical analyses were conducted using the JMP software (SAS Institute Inc., Cary, NC), and differences were considered statistically significant at P < 0.05.

RESULTS

Yolk Mineral Levels

The amounts of Cu, Mn, P, and Zn in the yolk during incubation are presented in Figure 2. On E19, the IOF-OMD and IOF-IM groups had 5.6 to 6.8 fold higher Cu, 3.3 to 4.5 fold higher Mn, and 2.8 to 3.1 fold higher Zn in comparison to the NTC and sham control groups. On E21, the IOF-OMD and IOF-IM groups had 2.4 to 5.5 fold higher Cu, 2.3 to 3.2 fold higher Mn, and 3.3 to 4.7 fold higher Zn in comparison to the NTC and sham control groups. No differences in P content were observed among the groups on E19 and E21.

The consumption of Cu, Mn, P, and Zn from the yolk between E19 and E21 (measured for each group by subtracting the average mineral level on E21 from its level on E19) is presented in Figure 3. Since we cannot measure the individual consumption value for each embryo, only the average consumption of each group was estimated. Therefore, no statistical analysis was performed and the groups were compared numerically. Generally, the IOF-OMD and IOF-IM had similar consumption of Cu, Mn, and Zn, which was higher than the consumption of the sham and NTC groups. For example, the IOF-OMD consumed 27.9 and 58.3% more Zn than the sham and NTC, respectively, while the IOF-OM had 50.5 and 86.4% more Zn than the sham and NTC, respectively.

Body and Tibia Morphometric Parameters Body weight, tibia weight, and tibia length are presented in...
Figure 2. Content of A) Cu, B) Mn, C) P, and D) Zn in the yolk of embryos from the NTC, sham, IOF-OMD, and IOF-IM groups during incubation (n = 8). a-b Significant differences among groups are marked with different superscripts (P < 0.05). 1 Values are means ± SE of males and females that did not differ. 2 At E17 the amniotic fluids of the IOF-OMD, IOF-IM, and sham groups were injected with a solution of organic minerals, phosphate, and vitamin D3 (IOF-OMD); inorganic minerals and phosphate (IOF-IM); or the diluent of the IOF-OMD and IOF-IM (sham). The NTC were non-treated controls.

Tables 3 and 4. There were no statistical differences among the groups in those parameters.

Bone Mineralization Tibia ash weight percent and BMD are presented in Figure 4. Mineralization differences between the groups were seen only on d 38; the IOF-OMD group had 1.2 and 1.6% higher ash content compared to the sham and NTC groups, respectively.

Mechanical Properties Tibial whole-bone stiffness, maximal load, and WTF, as well as cortical bone material Young’s modulus are presented in Figure 5. Generally, the IOF-OMD group showed elevated whole-bone stiffness in comparison to the NTC and sham groups during the compared sampled days (other than d 10 and E19 for the sham group). On E19, the IOF-OMD had 40% higher stiffness than the NTC (but not than the IOF-IM and sham). Interestingly, between E19 and E21, the stiffness values of all the groups decreased by 20 to 45%. On E21, the IOF-OMD group (which had the smallest decrease between E19 and E21) still had 90 and 52% higher stiffness than the NTC and sham, respectively. Additionally, the IOF-IM tibiae were 55% stiffer than the NTC. On d 38 the IOF-OMD tibiae were 20% and 12% stiffer than the NTC and the sham, respectively. These observations were coupled with the measurement of Young’s modulus (on d 38) that was 26 to 27% higher than the NTC and sham (in the males). No differences between the groups were seen in the maximal load and WTF.

Structural Properties The BV/TV, cortical area, medullary area, polar moment of inertia, and cross-sectional thickness of the tibial cortices are presented in Figure 6. Generally there were only small differences between the groups; however, between E19 and E21, the medullary area of the NTC, sham, and IOF-IM grew
IN OVO FEEDING IMPROVES BONE PROPERTIES

Figure 3. Consumption of Cu, Mn, Zn (μg/day), and P (mg/day) from the yolk of the NTC, sham, IOF-OMD, and IOF-IM groups between E19 and E21. *These are numeric values calculated from the means of each group on E19 and E21, so no statistical testing was done. †At E17 the amniotic fluids of the IOF-OMD, IOF-IM, and sham groups were injected with a solution of organic minerals, phosphate, and vitamin D₃ (IOF-OMD); inorganic minerals and phosphate (IOF-IM); or the diluent of these solutions (sham). The NTC were non-treated controls.

DISCUSSION

This study demonstrates that embryonic enrichment with minerals and vitamin D₃ by IOF has the potential to improve bone quality as seen by increased ash content, whole-bone stiffness, and Young’s modulus.

Inserting minerals into the amniotic fluid elevates yolk mineral content 36 h post IOF (E19). This shows a transfer of minerals from the amniotic fluid to the yolk sac as was previously described (Yair and Uni, 2011), and can be explained by transfer of amniotic fluid to the yolk sac via the gastrointestinal tract and the vitelline diverticulum (Esteban et al., 1991).

Following this mineral enrichment by IOF methodology, the higher levels of Cu, Mn, and Zn in the yolks of the IOF-OMD and IOF-IM groups on E19 led to elevated consumption of those minerals from the yolk between E19 and E21. The negative consumption of Cu and Mn seen in the NTC and sham could be a result of an entrance of Cu and Mn to the yolk from the other egg compartments, such as the shell and amniotic fluid, which contain low amounts of Cu and Mn (Richards, 1997; Yair and Uni, 2011).

On the other hand, no significant differences in the levels and consumption of P were found. This might be explained by the fact that the P amount in the in ovo solutions was only 2 to 2.2% of the initial yolk P amount (at E0), while the amounts of Cu, Mn, and Zn in the IOF solutions were more than 150% of their amounts in the yolk at E0.

Table 3. Body weights of the NTC, sham, IOF-OMD, and IOF-IM groups.¹ ²

<table>
<thead>
<tr>
<th>Sample day</th>
<th>Males</th>
<th></th>
<th>Females</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>NTC</td>
<td>Sham</td>
<td>IOF-OMD</td>
<td>IOF-IM</td>
</tr>
<tr>
<td>Body weight (g)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>E17 Mean</td>
<td>25.08</td>
<td>23.99</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SE</td>
<td>0.87</td>
<td>0.87</td>
<td></td>
<td></td>
</tr>
<tr>
<td>E19 Mean</td>
<td>34.00</td>
<td>33.05</td>
<td>32.53</td>
<td>33.93</td>
</tr>
<tr>
<td>SE</td>
<td>1.09</td>
<td>0.81</td>
<td>0.81</td>
<td>1.03</td>
</tr>
<tr>
<td>E21 Mean</td>
<td>44.59</td>
<td>45.09</td>
<td>45.40</td>
<td>44.44</td>
</tr>
<tr>
<td>SE</td>
<td>0.56</td>
<td>0.61</td>
<td>0.99</td>
<td>0.81</td>
</tr>
<tr>
<td>*d 7 Mean</td>
<td>167.7</td>
<td>161.8</td>
<td>160.0</td>
<td>160.0</td>
</tr>
<tr>
<td>SE</td>
<td>2.1</td>
<td>2.4</td>
<td>2.4</td>
<td>2.4</td>
</tr>
<tr>
<td>*d 14 Mean</td>
<td>436.5</td>
<td>431.9</td>
<td>435.3</td>
<td>435.3</td>
</tr>
<tr>
<td>SE</td>
<td>5.6</td>
<td>6.6</td>
<td>6.3</td>
<td>6.3</td>
</tr>
<tr>
<td>*d 21 Mean</td>
<td>905.4</td>
<td>892.1</td>
<td>894.9</td>
<td>894.9</td>
</tr>
<tr>
<td>SE</td>
<td>11.6</td>
<td>13.8</td>
<td>13.2</td>
<td>13.2</td>
</tr>
<tr>
<td>*d 28 Mean</td>
<td>1544</td>
<td>1547</td>
<td>1547</td>
<td>1547</td>
</tr>
<tr>
<td>SE</td>
<td>18</td>
<td>21</td>
<td>20</td>
<td>20</td>
</tr>
<tr>
<td>*d 38 Mean</td>
<td>2418</td>
<td>2447</td>
<td>2416</td>
<td>2416</td>
</tr>
<tr>
<td>SE</td>
<td>29</td>
<td>35</td>
<td>33</td>
<td>33</td>
</tr>
</tbody>
</table>

¹No statistical differences between the groups were found (P > 0.05.)
²At E17 the amniotic fluids of the IOF-OMD, IOF-IM, and sham groups were injected with a solution of organic minerals, phosphate, and vitamin D₃ (IOF-OMD); inorganic minerals and phosphate (IOF-IM); or the diluent of these solutions (sham). The NTC were non-treated controls. The IOF-IM group was not raised after hatch (E21).
³n = 8; ¹55 < n < 65 for the NTC, sham, and IOF-OMD groups; n = 15 for the IOF-IM group; *55 < n < 65.

Standing Ability

The average standing time of each group in the LTL test is presented in Figure 7. No statistical differences were seen among the groups.

by 60 to 70%, while in the IOF-OMD group it grew by only 35%, leading to a smaller medullary area of the IOF-OMD on E21 in comparison to the NTC group.

The BV/TV, trabecular thickness and trabecular separation of the trabecular bone are presented in Table 5. Differences were seen in BV/TV only on d 38, but they were inconsistent; the sham showed reduced BV/TV in females (in comparison to the NTC and IOF-OMD groups) and increased BV/TV in males (in comparison to the NTC group).
Table 4. Tibia weight and length of the NTC, sham, IOF-OMD, and IOF-IM groups.1,2

<table>
<thead>
<tr>
<th>Sample day</th>
<th>Males</th>
<th>Females</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>NTC</td>
<td>Sham</td>
</tr>
<tr>
<td>Bone weight (g)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>*E17 Mean</td>
<td>0.17</td>
<td>0.13</td>
</tr>
<tr>
<td>SE</td>
<td>0.01</td>
<td>0.01</td>
</tr>
<tr>
<td>*E19 Mean</td>
<td>0.27</td>
<td>0.27</td>
</tr>
<tr>
<td>SE</td>
<td>0.02</td>
<td>0.02</td>
</tr>
<tr>
<td>*E21 Mean</td>
<td>0.45</td>
<td>0.43</td>
</tr>
<tr>
<td>SE</td>
<td>0.03</td>
<td>0.04</td>
</tr>
<tr>
<td>*d 10 Mean</td>
<td>2.26</td>
<td>2.30</td>
</tr>
<tr>
<td>SE</td>
<td>0.14</td>
<td>0.15</td>
</tr>
<tr>
<td>$d 38 Mean</td>
<td>19.08</td>
<td>18.97</td>
</tr>
<tr>
<td>SE</td>
<td>0.51</td>
<td>0.41</td>
</tr>
</tbody>
</table>

| Bone length (cm) |       |         |         |        |       |         |         |        |
| *E17 Mean  | 2.32  | 2.26    | 2.30    | 2.30   | 2.88  | 2.88    | 2.80    | 2.88   |
| SE         | 0.07  | 0.07    | 0.07    | 0.07   | 0.06  | 0.06    | 0.07    | 0.07   |
| *E19 Mean  | 2.86  | 2.82    | 2.82    | 2.82   | 3.06  | 3.15    | 3.13    | 3.18   |
| SE         | 0.07  | 0.07    | 0.07    | 0.07   | 0.06  | 0.06    | 0.07    | 0.07   |
| *E21 Mean  | 3.16  | 3.16    | 3.16    | 3.16   | 3.06  | 3.06    | 3.03    | 3.03   |
| SE         | 0.04  | 0.04    | 0.04    | 0.04   | 0.03  | 0.03    | 0.03    | 0.03   |
| *d 10 Mean | 4.95  | 4.93    | 4.93    |        | 4.95  | 4.95    | 4.93    | 4.93   |
| SE         | 0.07  | 0.08    | 0.08    |        | 0.07  | 0.07    | 0.08    | 0.08   |
| $d 38 Mean | 10.55 | 10.21   | 10.21   | 10.21  | 10.05 | 10.21   | 10.21   | 10.21  |
| SE         | 0.09  | 0.07    | 0.07    | 0.07   | 0.06  | 0.06    | 0.06    | 0.06   |

1No statistical differences between the groups were found (P > 0.05.)
2At E17 the amniotic fluids of the IOF-OMD, IOF-IM, and sham groups were injected with a solution of organic minerals, phosphate, and vitamin D3 (IOF-OMD); inorganic minerals and phosphate (IOF-IM); or the diluent of these solutions (sham). The NTC were non-treated controls. The IOF-IM group was not raised after hatch (E21).

The amounts of most minerals in the egg cannot be increased by increasing their concentration (in inorganic forms) in the maternal diet (Naber, 1979). Therefore, in recent years, work has focused on changing the chemical form of the minerals to organic forms due to their ease of mobilization and use by the embryo, rather than increasing their concentrations in the hen diet (Kidd et al., 1992; Hudson et al., 2004; Dobrzanski et al., 2008; Favero et al., 2013; Oviedo-Rondón et al., 2013). It was previously shown that IOF with organic minerals, vitamin D3, and carbohydrates elevated mineral levels and consumption from the yolk (Yair and Uni, 2011). However, it is still unclear whether supplementing the embryo with inorganic minerals also will...
Figure 5. Bone mechanical properties of the NTC, sham, IOF-OMD, and IOF-IM groups. A) Stiffness, B) maximal load, and C) WTF of the whole bone between E17 and d 38 (n = 8 for E17 to d 10, and n = 20 for d 38). D) Young’s modulus of the males and females on d 38 (n = 5). Significant differences among groups are marked with different superscripts ($P < 0.05$). For the Young’s modulus, significant interaction was found between group and sex factors ($P = 0.012$). For A, B, and C, values are means ± SE of males and females.

At E17 the amniotic fluids of the IOF-OMD, IOF-IM, and sham groups were injected with a solution of organic minerals, phosphate, and vitamin D$_3$ (IOF-OMD); inorganic minerals and phosphate (IOF-IM); or the diluent of these solutions (sham). The NTC were non-treated controls. The IOF-IM group was not raised after hatch (E21). Stiffness, maximal load, and WTF were measured using a custom-built micro-mechanical testing device on E17 to d 10, while for d 38 they were measured using an Instron 3345 materials testing machine (Instron, Norwood, MA). Young’s modulus was calculated using data obtained from micro indentations of mid diaphysis tibiae cross sections (by Future-Tech FM-300 micro hardness tester).

result in higher mineral content and utilization. In this work we also examined an IOF solution with just inorganic minerals (no vitamin D$_3$ and carbohydrates) in order to determine if inorganic minerals also can elevate yolk mineral content and consumption (regardless of the chemical form of the added minerals or the addition of vitamin D$_3$). Our results show that mineral content and consumption of the IOF-OMD and IOF-IM groups at E19 and E21 did not differ, which suggests that both organic and inorganic minerals have a similar effect on elevation of yolk mineral content. As the goal of the IOF-IM group was to determine if IOF with inorganic minerals can elevate yolk mineral content and consumption, there was no need to grow the IOF-IM chicks beyond the incubation period and, therefore, this treatment group was not raised post-hatch.

The IOF-OMD treatment led to increased ash content of mature broiler bones at d 38. As the mechanical performance of bones is determined by their structure and composition (Weiner and Wagner, 1998; Sharir et al., 2008), this increase in mineral density can explain the increased stiffness and Young’s modulus (only in the males) of the mature broilers from the IOF-OMD group.

On the other hand, with the exception of the changes in the medullary area at E21, there were very few differences in geometry between the groups in this study and in a similar previous experiment (Yair et al., 2013); this
observation suggests that bone geometry is conserved and almost unaffected by nutritional manipulations.

Interestingly, differences in Young’s modulus between the IOF-OMD and the controls on d 38 were found in the males, but not in the females. As broiler males are much heavier (about 23% higher BW on d 38 according to Table 3) and, therefore, their bones have to withstand higher loads, this elevated Young’s modulus is of high importance. Similar results were found by Bello et al. (2014), which showed that IOF of
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Table 5. Structural properties of the trabecular bone of the NTC, sham, IOF-OMD, and IOF-IM groups\(^1\) (n = 8). Bone volume fraction (BV/TV), trabecular thickness, and trabecular separation.\(^2\)

<table>
<thead>
<tr>
<th>Sample day</th>
<th>Males</th>
<th></th>
<th>Females</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>NTC</td>
<td>Sham</td>
<td>IOF-OMD</td>
<td>NTC</td>
</tr>
<tr>
<td>BV/TV (%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>D 10</td>
<td>Mean</td>
<td>37.48</td>
<td>37.70</td>
<td>37.91</td>
</tr>
<tr>
<td></td>
<td>SE</td>
<td>1.89</td>
<td>1.49</td>
<td>1.89</td>
</tr>
<tr>
<td>&gt; d 38</td>
<td>Mean</td>
<td>41.45</td>
<td>50.61</td>
<td>55.11</td>
</tr>
<tr>
<td></td>
<td>SE</td>
<td>1.79</td>
<td>1.79</td>
<td>1.26</td>
</tr>
<tr>
<td>Trabecular thickness (μm)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>d 10</td>
<td>Mean</td>
<td>0.10</td>
<td>0.11</td>
<td>0.11</td>
</tr>
<tr>
<td></td>
<td>SE</td>
<td>0.003</td>
<td>0.003</td>
<td>0.003</td>
</tr>
<tr>
<td>d 38</td>
<td>Mean</td>
<td>0.21</td>
<td>0.22</td>
<td>0.21</td>
</tr>
<tr>
<td></td>
<td>SE</td>
<td>0.01</td>
<td>0.01</td>
<td>0.01</td>
</tr>
<tr>
<td>Trabecular separation (μm)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>d 10</td>
<td>Mean</td>
<td>0.15</td>
<td>0.17</td>
<td>0.16</td>
</tr>
<tr>
<td></td>
<td>SE</td>
<td>0.01</td>
<td>0.01</td>
<td>0.01</td>
</tr>
<tr>
<td>d 38</td>
<td>Mean</td>
<td>0.32</td>
<td>0.26</td>
<td>0.29</td>
</tr>
<tr>
<td></td>
<td>SE</td>
<td>0.03</td>
<td>0.02</td>
<td>0.03</td>
</tr>
</tbody>
</table>

\(^{a}\)Significant differences between groups within the males are marked with different letters (\(P < 0.05\)).

\(^{b}\)Significant differences between groups within the females are marked with different letters (\(P < 0.05\)).

\(^{c}\)Significant interaction between group and sex factors (\(P = 0.001\)).

\(^1\)At E17 the amniotic fluids of the IOF-OMD and sham groups were injected with a solution of organic minerals, phosphate, and vitamin D\(_3\) (IOF-OMD) or the diluent of this solution (sham). The NTC were non-treated controls. The IOF-IM were not raised after hatch (E21).

\(^2\)Structural properties of the trabecular bone were measured by micro-computed tomography of the distal epiphyseal area.

25-hydroxycholecalciferol (25-OH vitamin D\(_3\)) led to elevated bone-breaking strength on d 28 post-hatch in males but not in females. Yalcin et al. (2001) showed that between d 16 and 48 post-hatch (but not earlier) males exhibit higher bone weight, width, and volume than females, hinting at accelerated bone growth. Accordingly, it can be hypothesized that the benefits of the IOF procedure are more substantial for males only during that increased bone growth rate period.

On E19, E21, and d 10, no differences in ash content were observed among the groups, and therefore, the elevated bone stiffness of both IOF groups at hatch cannot be explained by differences in mineral content. Similar results showing increased bone mechanical properties at hatch were reported previously in embryos that had undergone IOF with minerals, vitamins, and carbohydrates and explained by a reduced medullary area (Yair et al., 2013). Such reduction in the medullary area at hatch might explain the elevated stiffness of the IOF-OMD group, but not of the IOF-IM group whose medullary area did not differ from the control groups.

Changes in the medullary area are probably due to differences in endosteal bone resorption rate; between E19 and E21, the medullary area of the NTC group increased at a much higher rate than the IOF-OMD (but not the IOF-IM), hinting at a higher resorption rate of the NTC. A similar phenomenon of a higher endosteal resorption rate towards birth was found in mice, and it led to reduced bone mineral density (Sharir et al., 2013); however, such reduction was not seen in the current work. The difference in the medullary area (and bone resorption rate) seen in the chicks in this study probably should be attributed to one of the ingredients of the IOF-OMD solution, e.g., vitamin D\(_3\), phosphate, or the mineral-amino acid complexes of the organic minerals. The authors are not aware of any evidence to an effect of phosphate and organic minerals on bone resorption rate. Vitamin D\(_3\) was found to affect bone properties of broiler embryos: IOF of 25-hydroxycholecalciferol led to higher bone-breaking strength in male birds on d 28 post-hatch (Bello et al., 2014), while maternal supplementation with vitamin D\(_3\) or 25-hydroxycholecalciferol increased tibia ash percent of progenies at hatch (Atencio et al., 2005).

Although vitamin D also was found to have the ability to suppress bone resorption (Ikeda and Ogata, 1999),
there are contradictory reports, e.g., the addition of the active form of vitamin D, calcitriol, to vitamin D deficient embryos on E14 elevated bone resorption between E14 and E17 (Narbaitz and Tsang, 1989). Thus, although it is possible that vitamin D$_3$ is responsible for the changes in the medullary area (and bone resorption rate), more research is needed to conclude which compound is responsible for the changes in the medullary area on E21.

Similar to what was previously reported (Yair et al., 2013), in this work the effect of IOF with minerals and vitamin D$_3$ can be seen in 2 periods: A rapid response (elevated stiffness and reduced medullary area) can be seen as early as E19 (36 h after the enrichment procedure) and E21, and a longer lasting effect (exhibited by the increased ash content, stiffness, and Young’s modulus) in the mature broiler (d 38). Between these 2 periods, on d 10 the control groups (NTC and sham) caught up with all parameters, possibly due to compensatory response (catch-up growth) as was previously seen (Yair et al., 2013).

Although the IOF treatments led to changes in mineral metabolism and bone mechanical, structural, and compositional (ash content) properties, there were no significant differences in BW, as well as bone length and weight. Previous work with the IOF method has shown that IOF can lead to elevated BW of the young and mature broilers (Tako et al., 2004; Kornasio et al., 2011); however, these IOF solutions contained carbohydrates and β-hydroxy-β-methylbutyrate (a leucine metabolite), which were directed to support intestinal development and breast muscle growth, while in this work the IOF solutions were aimed at supporting the skeleton and, therefore, contained minerals and vitamin D$_3$, but no carbohydrates.

Although there was an increase in some bone mechanical properties as a result of IOF, it was insufficient to induce changes in broiler standing ability, suggesting that the selected IOF-OMD solution does not have a significant positive effect on leg problems in broilers. It should be noted that, although walking ability and lameness of broilers are influenced by their bone properties, they also are influenced by the muscles, cartilages, joints, and tendons (Berg and Sanotra, 2003; Ruiz-Feria et al., 2014). Therefore, an IOF solution that aims to improve the cartilage, joints, and tendons of broilers might more effectively improve their walking ability.

In summary, here we show that IOF leads to increased mineral content of the yolk and embryonic consumption of minerals, as well as changes in the composition and mechanical properties of the skeleton, but does not have positive functional effects, such as improved standing ability.

REFERENCES


