Revista Brasileira de Ciência Avícola
ISSN: 1516-635X
revista@facta.org.br
Fundação APINCO de Ciência e Tecnologia Avícolas
Brasil

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Revista Brasileira de Ciência Avícola, vol. 19, núm. 1, enero-marzo, 2017, pp. 159-169
Fundação APINCO de Ciência e Tecnologia Avícolas
Campinas, Brasil

Brazilian Journal of Poultry Science
Revista Brasileira de Ciência Avícola
ISSN 1516-635X Jan - Mar 2017 / v.19 / n.1 / 159-170
http://dx.doi.org/10.1590/1806-9061-2016-0285

Effect of Zinc Level and Source (Zinc Oxide Vs. Zinc Glycine) on Bone Mechanical and Geometric Parameters, and Histomorphology in Male Ross 308 Broiler Chicken

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■Keywords

Bone, chelate, chicken, histomorphometry, zinc.

Submitted: April/2016
Approved: August/2016

ABSTRACT

The aim of this study was to evaluate the effect of adding zinc (Zn) mineral supplements from organic (ZnGly) and inorganic (ZnO) sources to growing male Ross 308 chickens on the mechanical, geometric, and histomorphometrical parameters of long bones. A corn-soybean meal basal diet was supplemented with Zn at 50 or 100 mg·kg^{-1} of a premix, except the control group (0 suppl). The serum concentration of insulin-like growth factor 1 was higher in the ZnGly50 group compared with the control group. Positive influence of Zn on the femur is highlighted when considering the significant increase in parameters such as mean relative wall thickness, and maximum elastic and ultimate strengths after Zn administration. Zinc supplementation did not affect tibial parameters. The histomorphometric analysis showed a positive impact of Zn supplementation (irrespective of source and level) on femoral trabecular thickness. Rapid loss in actual bone volume in tibial metaphyseal trabeculae was observed with ZnGly at 50 mg·kg^{-1}. In conclusion, this study showed that dietary Zn supplementation positively influences bone mechanical properties, confirming its beneficial effect on the development of the skeletal system and bone tissue of broilers tested for a nutritional-osteoporotic factor. Adverse health effects in trabecular bone as a result of the use of Zn at 50 mg·kg^{-1} of the premix show that supplementing Zn at the recommended dose (100 mg·kg^{-1}) is essential.

INTRODUCTION

There has been an increasing demand for poultry meat, and therefore, rearing fast-growing and well-muscled broiler breeds is more profitable. However, the genetic selection of broilers for muscle deposition and growth rate have caused growth and bone mineralization abnormalities. During the short life of broilers their skeletal system undergoes intensive growth (Cook, 2000). The proper function of the skeletal system plays an essential role in poultry production, because it not only provides structural support for the bird but it is also an important mineral source for metabolic needs (Sahraei *et al.,* 2012). Moreover, limb bones are essential for proper locomotor function. Bone deformities or even fractures resulting from insufficient adaptation of the skeleton to high body weight are observed. There are several causal factors that often operate in parallel or cyclically, exacerbating the effects of a negative impact on bone development. These factors include unstable and unbalanced dietary energy, protein and minerals, poisoning, hormonal disorders, and genetic predisposition (Cook, 2000). Because male chickens have heavier body weights than females, the overloading or abnormal loading conditions of pelvic limb bones, resulting in structural damage and deformed lower legs, are more common in males than in females (Śliwa *et al.,* 1996; Tomaszewska *et al.,* 2017). According to Paz

(2013), femoral degeneration occurs in young birds, does not have defined etiology, and can affect one or both legs. Moreover, in due to their fast growth rate, disorders such as bone atrophy, tibial dyschondroplasia or chondrodystrophy are common in broilers (Śliwa *et al.*, 1996; Paz *et al.*, 2013).

Zinc (Zn) is an essential micronutrient for animals, and play specific physiological functions in all living systems, such as the maintenance of structural and functional integrity of biological membranes and facilitation of gene expression and protein synthesis (Cao *et al.*, 2002; Park *el al.*, 2004). Zn is critical for cell proliferation and differentiation, and it is required for the maintenance of immune functions, enzyme structure and function, and appetite regulation in all avian species. Nutritional Zn deficiency in experimental animals results in delayed sexual maturity and low testosterone levels (Root *et al.*, 1979). There is very little research available on the relationship between zinc and female sex hormones. However, some scientists found a link between zinc and prolactin (high blood levels of zinc inhibited prolactin secretion), the hormone that regulates a wide variety of biological processes in the ovary (Brandão-Neto *et al.*, 1995). Moreover, Zn is necessary for normal growth and bone development, especially in poultry for foot health (Cao *et al*., 2002; Park el al., 2004). It plays a crucial role as a catalyst of many enzymes that affect bone development, suggesting its role in bone disorders (Yamaguchi & Gao, 1998). Zn functions as a metal component of alkaline phosphatase, a metalloenzyme that plays a key function in the formation of new bone (Starcher *et al*., 1980). Moreover, Zn increases the synthesis of growth factors, such as insulin-like growth factor 1 (IGF-1), and influences the activity of calcium-regulating hormones (Lowe *et al*., 2002). Thus, Zn appears to have multiple important functions in bone development, formation, and metabolism (Seo *et al.,* 2010). Bones contain about 30% of total body Zn (Molokwu & Li, 2006). In poultry, Zn deficiency results in insufficient bone mineralization, skeletal malformation, and reduction of weight gain (Sahraei *et al.*, 2012). In addition, Zn-deficient diets reduce egg production and hatchability in layers and breeders (Kienholz *et al.*, 1961). In addition, zinc is essential for neurogenesis, synaptogenesis, neuronal growth, and neurotransmission; it is stored in specific synaptic vesicles by a class of glutaminergic neurons and released as a neuro-modulator in an activity-dependent manner (Maret & Sandstead, 2006).

Poultry diets are commonly supplemented with zinc, because many natural feed ingredients are marginally Zn-deficient (Fudge & Speer, 2001). Therefore, poultry diets require supplementation to ensure adequate intake of trace minerals. In the feed industry, organic trace mineral supplements have been increasingly used. Zinc oxide is one of the inorganic feed-grade zinc sources commercially used by the poultry feed industry; however, it is less bioavailable than other inorganic compounds, because of its possible interactions with other feed compounds in the chime (inhibitory effects of phytic acid) (Cao *et al*., 2002; Park *et al*., 2004). Moreover, Zn may interact with calcium and iron (Hambride *et al.*, 1986). For this reason, inorganic zinc is used at concentrations higher than those recommended, which may result in environmental pollution (Lesson, 2003; Aviagan, 2014; Park *et al.,* 2004). The recommended level of dietary Zn used to be 14-57 $mg \cdot kg^{-1}$ for different commercial broiler strains, except for growing male Ross 308 chickens (NRC, 1994). Currently, dietary Zn supplementation level is based on the nutritional recommendations for Ross 308 broilers (Aviagen, 2014) as 100 $mg \cdot kg^{-1}$ of Zn, irrespective of its content in the feedstuffs.

Amino-acid chelates are reported to have significantly higher absorption rates in the intestine compared with soluble inorganic metal salts (Ashmead *et al.,* 1985). Furthermore, due to their unique chemical structure, these chelates do not appear to ionize as readily as inorganic trace mineral sources under a variety of conditions, which results in reduction or elimination of their reactivity with various feed components, like phosphate or other natural factors (Vieira, 2008; Huang *et al.,* 2009). However, conflicting results have been reported regarding the bioavailability of Zn chelates and traditional inorganic forms (Cao et al., 2002). The application of the various chelates is regulated by EU Directive 1334/2003 (EC, 2003).

Although severe zinc deficiency is uncommon in avian populations, marginal deficiency is likely to be much more prevalent and associated with immune dysfunction or restricted physical development. Relative to the role of Zn, it is hypothesized that the supplementation of a more available form of Zn compared with an inorganic form may improve the development of the skeletal system of broilers.

The objective of this study was to evaluate the effect of the addition Zn supplements from an organic (ZnGly) or an inorganic (ZnO) source in growing male Ross 308 broiler diets on the mechanical, geometric, and histomorphometrical parameters of the long bones. The effect of different (recommended-100 $mg \cdot kg^{-1}$ and limited to 50 $mg \cdot kg^{-1}$ of the premix) levels of supplementation was also evaluated in this investigation.

2. MATERIALS AND METHODS

The experimental procedures used throughout this study were approved by the Local Ethics Committee on Animal Experimentation of the University of Life Sciences in Lublin, Poland.

2.1. Bird management and experimental design

A total of 250 one-day-old male Ross 308 broilers were randomly allocated to five Zn treatments with five replicates (cages) of 10 birds each, totaling 50 birds per treatment. All cages were located in the same room.

Birds were kept under standard rearing conditions, and air temperature was set at the optimal level according to age, as established in the European Council Directive 2007/43/EC (EC, 2007). During the first week, room temperature was maintained at 33 °C, and gradually reduced in 2 °C weekly until reaching 24 °C. Birds had constant access to fresh water, and feed was supplied *ad libitum*. The diets were formulated in accordance with the stage of the production cycle (Table 1): starter (crumbles, 1-21 days), grower (pellets, 22-35 days), and finisher (pellets, 36-42 days).

At the end of the experiment, when birds were 42 days old, 10 birds (two birds per replicate x five replicates) per treatment group were randomly selected, and sacrificed. Before sacrifice, birds were feed-fasted, but were allowed free access to water. Birds were weighed before placement in the experimental facility and before slaughter.

2.2. Basal diet and feed analyses

The basal diet was based on corn and soybean meal, and formulated to contain adequate levels of all nutrients, as recommended (NRC, 1994), except for Zn. The nutrient content of the diets was calculated based on the chemical composition and metabolizable energy values of the feedstuffs, according to the equations of the European Tables (Kwiecień *et al.*, 2014).

Dry matter, crude ash, crude protein, crude fat, and crude fiber contents of the experimental diets were determined by standard AOAC (2000) methods. Dietary Cu, Fe, Zn and Ca contents in feed samples were determined, after ashing at 550 °C, by the AAS flame technique in a spectrometer (Unicam 939 AA, Shimadzu Corp., Tokyo, Japan), according to the methods of AOAC (2000). Total P content in the feeds was colorimetrically determined (PN-ISO 6491:2000) in a Helios Alpha UV-VIS apparatus (Spectronic Unicam, Leeds, United Kingdom) (Kwiecień *et al.*, 2014).

Table 1 – Composition and nutritive value of the experimental diets

Ingredient (%)	Starter (1-21day)	Grower (22-35 day)	Finisher (36-42 day)
Corn	24.44	40.00	40.00
Wheat	42.99	27.84	28.84
Soybean meal *	25.0	24.97	22.87
Soybean oil	2.50	3.69	3.98
Phosphate 1-calcium	0.90	0,90	0.81
Fodder chalk	1.40	1.13	1.09
Sodium bicarbonate	0.08	0.08	0.08
Sodium chloride	0.29	0.25	0.26
Premix vita-min (no Zn)	0.50[a]	0.50[b]	0.50[c]
Concentrate protein-fat **	1.00	1.00	1.00
DL-methionine 99%	0.30	0.23	0.23
L-lysine HCl	0.42	0.28	0.27
L-threonine 99%	0.18	0.13	0.07
The nutritional value of 1 kg diet:			
[e] ME, MJ kg^{-1}	12.8	13.2	13.3
[d] Total protein,%	21.23	20.43	19.91
[d] Crude fiber,%	1.64	1.59	1.73
[d] Crude fat,%	4.57	5.42	5.53
[d] Lysine,%	1.28	1.14	1.08
[d]Methionine + Cystine,%	0.92	0.81	0.82
[d] Threonine,%	0.83	0.75	0.70
Thr:Lys ratio	0.65	0.66	0.64
[d] Total calcium,%	0.87	0.79	0.76
[d] Total phosphorus,%	0.65	0.66	0.64
[e] Available phosphorus,%	0.42	0.41	0.39
[e] Total available calcium/ phosphorus	2.12	1.90	1.92
[d] Zn from plants in basal diet, mg	28.32	25.87	24.99
[d] Iron, mg	40.22	39.92	39.68
[d]Copper, mg	14.54	14.72	13.59

[a] content of vitamins and minerals per 1 kg of Starter diet: Mn 100 mg, I 1 mg, Fe 40 mg, Cu 16 mg, Se 0.15 mg, vitamin A 15 000 UI, vitamin D_3 5 000 UI, vitamin E 75 mg, vitamin K_3 4 mg, vitamin B_1 3 mg, vitamin B_2 8 mg, vitamin B_6 5 mg, vitamin B_{12} 0,016 mg, biotin 0.2 mg, folic acid 2 mg, nicotinic acid 60 mg, pantothenic acid 18 mg, choline 1 800 mg;

[b] content of vitamins and minerals per 1 kg of Grower diet: Mn 100 mg, I 1 mg, Fe 40 mg, Cu 16 mg, Se 0,15 mg, vitamin A 12 000 UI, vitamin D_3 5 000 UI, vitamin E 50 mg, vitamin K_3 3 mg, vitamin B_1 2 mg, vitamin B_2 6 mg, vitamin B_6 4 mg, vitamin B_{12} 0.016 µg, biotin 0.2 mg, folic acid 1.75 mg, nicotinic acid 60 mg, pantothenic acid 18 mg, choline 1 600 mg;

[c] content of vitamins and minerals per 1 kg of Finisher diet: Mn 100 mg, I 1 mg, Fe 40 mg, Cu 16 mg, Se 0.15 mg, vitamin A 12 000 UI, vitamin D_3 5 000 UI, vitamin E 50 mg, vitamin K_3 2 mg, vitamin B_1 2 mg, vitamin B_2 5 mg, vitamin B_6 3 mg, vitamin B_{12} 0.011 µg, biotin 0,05 mg, folic acid 1.5 mg, nicotinic acid 35 mg, pantothenic acid 18 mg, choline 1 600 mg;

[d] analysed values;

[e] calculated values;

* 46% Total protein in dry matter;

** 1 kg concentrate protein-fat contains: 2% crude fat, 39% crude protein, 10,8 MJ EM

The amino acid composition in the diets was determined by ion-exchange chromatography using an INGOS AAA 400 amino acid analyzer with post-column derivatization of ninhydrin and spectrophotometric detection, according to the standard manufacturer's

procedure, and MCMiAŻ/PB-03 test procedure, as previously described (Kwiecień *et al.*, 2014). Cysteine and methionine (sulfur amino acids) were determined in a separate analysis, as previously described (Kwiecień *et al.*, 2014). Available lysine was determined based on the difference between total lysine and the so-called residual lysine, which did not react with DNFB (dinitrofluorobenzene) (Kwiecień *et al.*, 2014). Following this reaction, the tested samples were again subjected to acid hydrolysis (Žilic *et al.*, 2006).

The birds also were weighed at 10, 21, 35 days of age (data not shown) to determine feed intake (FI) and feed conversion ratio (FCR).

2.3. Supplementation of Zn amino acid chelate and Zn oxide

Birds were distributed into a control group (CONT=0 Suppl), which diet contained a premix devoid of Zn additive, and groups supplemented with two different Zn sources (Zn oxide as standard Zn inorganic source or Zn-glycine chelate as Zn organic form) and two Zn levels (100% or 50% of the recommended Zn level). Therefore, the following treatment groups were established: ZnGly50 – Zn-glycine at 50% of the recommendations (50 mg·kg^{-1} of diet or 313 mg of chelate/kg premix); ZnGly100 - Zn-glycine at 100% of the recommendations (100 mg·kg^{-1} diet or 625 mg of chelate/kg premix); ZnO50 – Zn oxide at 50% of the recommendations (50 mg·kg^{-1} diet or 64 mg of ZnO/kg premix); and ZnO100 – Zn oxide at 100% of the recommendations (100 mg·kg^{-1} diet or 128 mg of ZnO/kg premix). The Zn-glycine chelate contained 16% Zn, and zinc oxide contained 78% Zn, and were added to the premix which did not contain Zn (Arkop Sp. Z o.o., Bukowno, Poland). The analyzed Zn contents of the basal diets (Table 1) was 24.99-28.32 mg·kg^{-1} of Zn on as-fed basis.

The amount of Zn in the diet was based on the nutritional recommendations for Ross 308 broilers (Aviagen, 2014), i.e., 100 mg·kg^{-1} of Zn, irrespective of its content in the basal diet. According to those recommendations, diets should contain the same Zn levels during the entire rearing period, which was taken into account in this study.

2.4. Bone measurements

After slaughter, the right and left tibiae and femora were collected, and their weight and length were measured after the removal of soft tissues. Each limb bone was wrapped with a gauze soaked in isotonic saline, and stored at -25 °C until further analysis. Bone weight/length index was calculated as weight (mg) to the length (mm) ratio. This is a simple index of bone density introduced for the first time by Seedor and co-workers. In general, the higher the bone weight/length index, the denser is the bone (Seedor *et al.*, 1991; Ziaie *et al*, 2011b), and indicates changes in bone mineralization (Ziaie *et al*. 2011a; Ziaie *et al*. 2011b).

2.5. Analysis of bone mechanical and geometric properties

The mechanical properties of the collected bones were determined after 3-hour thawing at room temperature by applying the three-point bending test. A Zwick Z010 universal testing machine (Zwick GmbH & Company KG, Ulm, Germany), equipped with a measuring head (Zwick GmbH & Company KG, Ulm, Germany) with an operation range up to 10 kN, linked to a computer with test TestXpert II 3.1 software (Zwick GmbH & Company KG, Ulm, Germany) was used, as previously described (Tomaszewska *et al*., 2013; Tomaszewska *et al*., 2015). Maximum elastic strength and ultimate strength were determined as previously described (Ferretti *et al*., 1993; Tomaszewska *et al.*, 2012).

Based on the measurements of the horizontal and vertical diameters of the mid-diaphyseal cross-section of the bones (data not shown), the cross-sectional area and mean relative wall thickness were derived (Ferretti *et al*., 1993). The cortical index of the bone was estimated as previously described (Tomaszewska *et al*., 2015).

2.6. Histomorphometry

Cylindrical samples (cartilage and bone) with 20-mm thickness were taken from the same anatomical position of the knee joint, i.e., from the middle of the lateral femoral and tibial condyle. Sagittal bone sections were cut perpendicular to the articular surface. The tissue samples were subjected to histology and microscopy procedures, as previously described (Tomaszewska *et al.*, 2013).

Microscopic bright-field images were collected using a confocal microscope (Axiovert 200M, Carl Zeiss, Jena, Germany) equipped with a camera (AxioCam HRc, Carl Zeiss, Jena, Germany) and a fluorescent lamp (450-490 nm excitation wavelength).

Bone volume and tissue volume (data not shown) were measured in the photographs of the bone tissue sections using pixel count to determine relative bone volume (BV/TV%), as previously described (Tomaszewska *et al.*, 2015). Other trabecular bone

parameters examined were mean and maximum trabecular thickness (Tb.Th), and trabecular separation (Tb.Sp), defined as the distance between the edges of adjacent trabeculae (directly measured) (Tomaszewska *et al.*, 2012).

2.7. Growth hormone and bone turnover markers

Each chicken was fasted for 12 hours before blood collection by standard venipuncture from brachial vein. After clotting at room temperature, blood was centrifuged and frozen at -80 °C until further analysis. All the samples were determined in duplicate.

Serum concentrations of chicken growth hormone, insulin-like growth factor 1, osteocalcin, and leptin were determined using an enzyme-linked immunosorbent assay kit (ELISA; Uscn Life Science Inc. Wuhan, China) with minimum detectable concentrations of 0.056 ng/mL, 7.4 pg/mL, 0.67 pg/mL, and 14.8 pg/mL, respectively.

2.8. Selected macro- and trace minerals in the serum

The serum concentrations of zinc, calcium, copper, iron, manganese, and phosphorus were determined by colorimetric method using a Metrolab 2300 GL unit (Metrolab SA, Argentina) and sets of biochemical reagents produced by BioMaxima (Lublin, Poland).

2.9. Statistical analysis

The results of the experiment are expressed as LSMEANS ± SEM (standard error of the mean). As our aim was to evaluate the effect of an addition of organic and inorganic zinc mineral supplements, as well as different levels of Zn supplementation on the mechanical and histomorphometric parameters and the geometry of long bones, the following statistical model was applied:

$$x_{ij} = \mu + \alpha_i + \varepsilon_{ij}$$

where: x_{ij} = an observation (bone parameter value); i = concentration factor (ZnO50, ZnO100, ZnGly50, ZnGly100, CONT); j = number of observations; μ = constant; α_i = the main effect of the i^{th} level; ε_{ij} = random error of the i^{th} level of the j^{th} observation. Because both factors were controlled, the considered model is a fixed-effect model. One-way analysis of variance was applied.

Contrast analysis was used to compare the results obtained in Zn-supplemented groups *vs*. the control group: (1) the control diet (CONT- 0 Suppl) *vs.* the diets containing inorganic Zn (ZnO50 and ZnO100); and (2) CONT *vs*. the diets containing organic Zn (ZnGly50 and ZnGly100), and to compare Zn supplementation level (50 or 100 mg) vs. no Zn supplementation (control group). Significance was declared at $P<0.05$. All statistical analyses were carried out using Statistica 10.0 software (StatSoft Inc., Tulsa, OK, USA).

3. RESULTS

3.1. Body weight, feed intake (FI) and feed conversion ratio (FCR)

Birds had approximately the same initial body weight and presented similar final body weight (Table 2). Feed intake and FCR (feed intake per kg body weight gain) at 10, 21, 35, and 42 days of age were not influenced by Zn source or level (data not shown).

3.2. Bone morphology, geometry, and mechanical parameters

No differences in femoral length, mass, and weight/length index were detected between the control and Zn-supplemented birds (Table 3), irrespective of Zn source or level. However, the relative bone weight of birds fed 50 mg·kg^{-1} ZnGly was significantly higher compared with the controls. Higher femoral strength, mean relative wall thickness, and cortical index were determined in the ZnGly group compared with the

Table 2 – The initial body weight after hatching and the effect of inorganic or organic zinc dietary supplementation on the final body weight of 42-day-old Ross 308 broilers

	Diet[a]					SEM[b]	p-Value[c]					
	CONT= 0 Supp	ZnO100	ZnO50	ZnGly100	ZnGly50		CONT* ZnO	CONT* ZnGly	CONT* ZnO100	CONT* ZnO50	CONT* ZnGly100	CONT* ZnGly50
Initial weight [g]	48	48	48	48	48	0.6	0.68	0.54	0.72	0.72	0.72	0.16
Finish weight [g]	2658	2508	2692	2543	2632	89	0.60	0.52	0.24	0.79	0.37	0.84

Data given are LSMEANS ±SEM

[a] CONT = 0 Supp diet - without added Zn; 100 or 50 = 100 or 50 mg of Zn /kg of Premix added to basal diet;

[b] SEM = standard error of the mean (n = 10 birds for each diet);

[c] CONT*ZnO = control diet vs. diets containing inorganic Zn (CONT *vs.* both 100 and 50); CONT* ZnGly = control diet *vs*. diets containing organic Zn (CONT *vs*. Both 100 and 50); CONT* ZnO100= control diet *vs*. ZnO100; CONT* ZnO50=control diet *vs*. ZnO50; CONT* ZnGly100= control diet *vs*. ZnGly100; CONT* ZnGly50= control diet *vs*. ZnGly50

Table 3 – The effect of inorganic or organic zinc dietary supplementation on the mechanical and geometric properties of the femur of 42-day-old Ross 308 broilers.

	Diet[a]					SEM[b]	P-Value[c]					
	CONT	ZnO100	ZnO50	ZnGly100	ZnGly50		CONT* ZnO	CONT* ZnGly	CONT* ZnO100	CONT* ZnO50	CONT* ZnGly100	CONT* ZnGly50
Bone weight [g]	9.85	10.82	11.33	10.58	10.96	0.65	0.14	0.27	0.31	0.13	0.44	0.25
Length [mm]	74.2	74.0	76.2	75.2	75.0	1.78	0.69	0.69	0.94	0.44	0.70	0.76
Weight/length index [mg/mm]	132.7	146.2	148.7	140.7	146.1	15.1	0.06	0.06	0.21	0.19	0.33	0.25
Bone weight (g/100g of BW)	0.372	0.436	0.423	0.418	0.423	0.009	0.16	0.06	0.41	0.11	0.29	0.03
Ultimate strength [N]	206	250	313	272	312	31	0.06	0.04	0.34	0.03	0.17	0.03
Max. elastic strength [N]	123	164	180	166	202	21	0.08	0.03	0.19	0.70	0.17	0.02
Midshaft Volume [cm^3]	1.12	1.47	1.61	1.29	1.70	0.05	<0.01	<0.01	0.02	<0.01	0.28	<0.01
Cross-sectional area [mm^2]	37.9	49.9	53.0	42.6	56.2	5.1	0.05	0.08	0.11	0.05	0.52	0.02
Mean relative wall thickness	0.46	0.54	0.67	0.55	0.70	0.06	0.07	0.04	0.37	0.03	0.31	0.01
Cortical index [%]	31.1	34.2	39.4	35.0	40.9	2.3	0.07	0.03	0.38	0.03	0.28	0.01

Data given are LSMEANS ±SEM

[a] CONT = 0 Supp diet - without added Zn; 100 or 50 = 100 or 50 mg of Zn /kg of Premix added to basal diet;

[b] SEM = standard error of the mean (n = 10 birds for each diet);

[c] CONT*ZnO = control diet vs. diets containing inorganic Zn (CONT *vs*. Both 100 and 50); CONT* ZnGly = control diet *vs*. diets containing organic Zn (CONT *vs*. Both 100 and 50); CONT* ZnO100= control diet *vs*. ZnO100; CONT* ZnO50=control diet *vs*. ZnO50; CONT* ZnGly100= control diet *vs*. ZnGly100; CONT* ZnGly50= control diet *vs*. ZnGly50

BW – body weight

control group (0 Suppl), with higher values observed for the ZnGly50 group (Table 3). Significantly higher ultimate strength and mean relative wall thickness values were obtained in the ZnO50 group compared with the control group. Similarly, the ZnGly50 group presented larger cross-sectional area compared with the control group. On the other hand, ZnO treatment influenced significantly the cross section of area and also with higher values noted for the ZnO50 group.

The diet containing Zn, irrespective of its form and concentration, did not influence tibia development (except of relative bone mass) compared with the control, non-supplemented birds (Table 4).

3.3. Bone histomorphometry

Histomorphometric analysis revealed that the supplementation of Zn as ZnO influenced only femoral trabecular thickness, with higher values for the ZnO100 group (Table 5). On the other hand, tibial relative bone volume decreased in the both diets (containing ZnO or ZnGly) compared with the control group (0 Suppl). Moreover, trabecular thickness was reduced and

Table 4 – The effect of inorganic or organic zinc dietary supplementation on the mechanical and geometric properties of the tibia of 42-day-old Ross 308 broilers

	Diet[a]					SEM[b]	p-Value[c]					
	CONT= 0 Supp	ZnO100	ZnO50	ZnGly100	ZnGly50		CONT* ZnO	CONT* ZnGly	CONT* ZnO100	CONT* ZnO50	CONT* ZnGly100	CONT* ZnGly50
Bone weight [g]	13.53	13.52	15.50	14.03	15.63	0.93	0.40	0.27	0.99	0.15	0.71	0.13
Length [mm]	102.4	99.8	103.6	102.2	104.0	1.97	0.78	0.78	0.36	0.67	0.94	0.57
Weight/length index [mg/mm]	132.1	135.5	149.6	137.3	150.3	21.2	0.06	0.06	0.23	0.25	0.33	0.12
Bone weight (g/100g of BW)	0.511	0.545	0.578	0.555	0.606	0.013	0.16	0.06	0.41	0.11	0.29	0.03
Ultimate strength [N]	291	289	322	277	368	30	0.70	0.40	0.97	0.48	0.77	0.09
Max. elastic strength [N]	196	197	232	139	185	28	0.60	0.33	0.98	0.37	0.17	0.78
Midshaft Volume [cm^3]	1.68	1.69	1.78	1.50	1.86	0.03	0.75	0.98	0.96	0.62	0.36	0.38
Cross-sectional area [mm^2]	41.1	42.4	43.5	36.5	44.7	5.1	0.78	0.93	0.87	0.75	0.53	0.62
Mean relative wall thickness	0.78	0.70	0.68	0.69	0.63	0.12	0.55	0.43	0.65	0.56	0.60	0.39
Cortical index [%]	43.4	41.2	37.6	38.6	37.7	3.6	0.43	0.30	0.71	0.32	0.41	0.33

Data given are LSMEANS ±SEM

[a] CONT = 0 Supp diet - without added Zn; 100 or 50 = 100 or 50 mg of Zn /kg of Premix added to basal diet;

[b] SEM = standard error of the mean (n = 10 birds for each diet);

[c] CONT*ZnO = control diet *vs*. diets containing inorganic Zn (CONT *vs*. Both 100 and 50); CONT* ZnGly = control diet *vs*. diets containing organic Zn (CONT *vs*. Both 100 and 50); CONT* ZnO100= control diet *vs*. ZnO100; CONT* ZnO50=control diet *vs*. ZnO50; CONT* ZnGly100= control diet *vs*. ZnGly100; CONT* ZnGly50= control diet *vs*. ZnGly50

BW – body weight

Table 5 – The effect of inorganic or organic zinc dietary supplementation on trabecular bone morphology of the metaphysis of long bones of 42-day-old Ross 308 broilers.

	Diet[a]					SEM[b]	P-Value[c]					
	CONT= 0 Supp	ZnO100	ZnO50	ZnGly100	ZnGly50		CONT* ZnO	CONT* ZnGly	CONT* ZnO100	CONT* ZnO50	CONT* ZnGly100	CONT* ZnGly50
Femur												
BV/TV [%]	23	31	31	28	18	5.0	0.22	0.78	0.30	0.25	0.77	0.48
Tb.Th mean [μm]	62	125	102	103	73	14.8	0.02	0.23	0.01	0.10	0.09	0.66
Tb.Th max [μm]	139	247	201	206	140	25	0.02	0.35	0.01	0.13	0.10	0.99
Tb.Sp [μm]	249	268	325	297	334	28	0.23	0.10	0.67	0.09	0.28	0.06
Tibia												
BV/TV [%]	57[a]	32	33	25	13	4.5	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01
Tb.Th mean [μm]	212	128	78	112	79	21	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01
Tb.Th max [μm]	341	258	252	232	137	45	<0.01	<0.01	0.01	<0.01	<0.01	<0.01
Tb.Sp [μm]	152	222	250	338	395	19	<0.01	<0.01	0.01	<0.01	<0.01	<0.01

Data given are LSMEANS ±SEM

[a] CONT = 0 Supp diet - without added Zn; 100 or 50 = 100 or 50 mg of Zn /kg of Premix added to basal diet;

[b] SEM = standard error of the mean (n = 10 birds for each diet);

[c] CONT*ZnO = control diet vs. diets containing inorganic Zn (CONT *vs*. Both 100 and 50); CONT* ZnGly = control diet *vs*. diets containing organic Zn (CONT *vs*. Both 100 and 50); CONT* ZnO100= control diet *vs*. ZnO100; CONT* ZnO50=control diet *vs*. ZnO50; CONT* ZnGly100= control diet *vs*. ZnGly100; CONT* ZnGly50= control diet vs. ZnGly50

BV/TV% - relative bone volume; Tb.Th mean – the mean trabecular thickness; Tb.Th max – the maximal trabecular thickness; Tb.Sp mean - the mean trabecular separation; Tb.Sp max - maximal trabecular separation

trabecular space was enhanced in the cancellous bone of tibia, irrespective of diets compared with the control group (0 Suppl) (Table 5).

3.4. Growth hormone, leptin, and bone turnover markers

The concentration of growth hormone, osteocalcin and leptin was similar among treatments. The concentration of insulin-like growth factor 1 was enhanced only in the ZnGly50 group compared to the 0 Suppl group (Table 6).

3.5. Selected micro- and macroelements in the serum

The birds fed diets containing Zn, irrespective of its concentration and form, presented similar serum levels of selected macro and trace elements (except for copper) to those receiving the control diet with no supplementation (Table 7), except for copper, which serum level was higher in the ZnGly50 compared with the control group.

4. DISCUSSION

Trace minerals, such as zinc, are important for a variety of physiological processes that are essential for optimal bird growth and development. The response of bone development to different dietary Zn forms and concentrations are not known, although physiological mechanisms of Zn action has been proposed. The results of presented study are novel and, to our knowledge, the effects of dietary Zn limited to half

Table 6 – The effect of inorganic or organic zinc dietary supplementation on the serum levels of growth hormone, insulin-like growth factor 1, osteocalcin and leptin of 42-day-old Ross 308 broilers

	Diet[a]					SEM[b]	p-Value[c]					
	CONT	ZnO100	ZnO50	ZnGly100	ZnGly50		CONT* ZnO	CONT* ZnGly	CONT* ZnO100	CONT* ZnO50	CONT* ZnGly100	CONT* ZnGly50
Growth hormone [ng/mL]	3.62	3.45	2.89	3.11	2.92	0.5	0.47	0.42	0.81	0.32	0.48	0.49
Insulin-like growth factor 1 [pg/mL]	122	123	193	178	280	21	0.12	<0.01	0.65	0.03	0.08	<0.01
Osteocalcin [pg/mL]	53.85	43.68	54.60	51.46	51.50	5.21	0.63	0.75	0.37	0.95	0.91	0.66
Leptin [pg/mL]	111	94	130	126	121	13	0.79	0.36	0.39	0.19	0.44	0.42

Data given are LSMEANS ±SEM

[a] CONT = 0 Supp diet - without added Zn; 100 or 50 = 100 or 50 mg of Zn /kg of Premix added to basal diet;

[b] SEM = standard error of the mean (n = 10 birds for each diet);

[c] CONT*ZnO = control diet *vs*. diets containing inorganic Zn (CONT *vs*. Both 100 and 50); CONT* ZnGly = control diet *vs*. diets containing organic Zn (CONT *vs*. Both 100 and 50); CONT* ZnO100= control diet *vs*. ZnO100; CONT* ZnO50=control diet *vs*. ZnO50; CONT* ZnGly100= control diet *vs*. ZnGly100; CONT* ZnGly50= control diet *vs*. ZnGly50

Table 7 – The effect of inorganic or organic zinc dietary supplementation on the serum levels of selected minerals of 42-day-old Ross 308 broilers.

	Diet[a]					SEM[b]	p-Value[c]					
	CONT	ZnO100	ZnO50	ZnGly100	ZnGly50		CONT* ZnO	CONT* ZnGly	CONT* ZnO100	CONT* ZnO50	CONT* ZnGly100	CONT* ZnGly50
Zinc [µmol/L]	22.5	27.0	26.7	29.9	22.8	2.1	0.38	0.33	0.15	0.97	0.90	0.96
Copper [µmol/L]	1.5	1.1	1.7	1.7	2.0	0.3	0.19	0.20	0.27	0.27	0.77	0.05
Calcium [µmol/L]	2.26	2.35	2.27	2.28	2.19	0.03	0.58	0.14	0.45	0.84	0.48	0.06
Manganese [µmol/L]	0.87	0.90	0.90	0.80	0.86	0.07	0.86	0.49	0.34	0.98	0.28	0.92
Iron [µmol/L]	9.2	9.4	9.3	10.5	10.3	0.7	0.87	0.17	0.87	0.90	0.20	0.27
Phosphorus [µmol/L]	2.0	1.9	1.9	1.9	2.4	0,1	0.78	0.60	0.87	0.53	0.49	0.12

Data given are LSMEANS ±SEM

[a] CONT = 0 Supp diet - without added Zn; 100 or 50 = 100 or 50 mg of Zn /kg of Premix added to basal diet;

[b] SEM = standard error of the mean (n = 10 birds for each diet);

[c] CONT*ZnO = control diet vs. diets containing inorganic Zn (CONT *vs*. Both 100 and 50); CONT* ZnGly = control diet *vs*. diets containing organic Zn (CONT *vs*. Both 100 and 50); CONT* ZnO100= control diet *vs*. ZnO100; CONT* ZnO50=control diet *vs*. ZnO50; CONT* ZnGly100= control diet *vs*. ZnGly100; CONT* ZnGly50= control diet *vs*. ZnGly50

of the recommended level, irrespective of its source, have not been investigated yet in birds and any other animals on bone development, considering bone tissue histomorphometry, geometry, and mechanics, as well as growth hormone and bone turnover markers, have not been investigated yet in birds and any other animals.

Our study showed that the Zn, irrespective of source and concentration, did not influence broiler feed intake or body weight, neither the weight and length of leg bones. Rossi *et al*. (2007) did not observe any changes in broiler body weight or weight gain when Zn was added in organic form (0, 15, 30, 60 ppm). Our findings are also in agreement a study showing that the tibial weight of male Ross broilers did not change when the basal diet was supplemented with Zn at 100, 150, or 200 mg·kg^{-1} as oxide or an organic form (Sahraei *et al.*, 2012). However, in Sahraei *et al.* (2012), the supplements were fed from the 8th to 28th day of the grower phase, whereas in our study, Zn was supplemented during the entire rearing period and at 100 mg·kg^{-1}. Moreover, our supplementation period is typical for the 42-day rearing cycle and was consistent with the study of Ziaie *et al.* (2011a), but longer than that evaluated by El-Husseiny *et al.* (2012), of 11-35 days of age. The duration of the current study might explain the lack of response to dietary Zn supplementation as the experimental period was not long enough to cause a depletion of Zn in the birds fed with Zn limited to the concentration of 50 mg·kg^{-1}. Furthermore, our results are in agreement with the findings of Sahraei *et al.* (2012) who did not observe any differences in the biological value between the oxide and organic zinc supplementation to broilers.

On the other hand, some studies report greater bioefficacy of organic Zn sources compared with Zn oxide (Świątkiewicz *et al.*, 2001). Investigation of bone development in relation to trace minerals from inorganic and organic sources in poultry is very important because bone weakness and lesions lead to worse performance and poor carcass quality (Rath *et al.*, 2000; Sahraei *et al.*, 2012). Weak legs are associated with reduced feed intake, which finally affects weight gain. Therefore, leg bone quality is commonly used as an indicator of mineral adequacy in poultry diets (Sahraei *et al.*, 2012).

Although our diet did not influence the weight of both evaluated bones, Zn supplemented at 50 mg·kg^{-1} enhanced tibial relative weight. This may indicate that the increase of bone weight relative to carcass weight may be due to the reduced weight gain of those birds. In addition, irrespective of Zn form at the 50 or 100 mg·kg^{-1} level did not influence the mineral density of both bones, according to the bone weight/length index. Although Zn bone content was not evaluated, in an earlier study, the diet with 25 mg·kg^{-1}of organic Zn (25% of the recommended level) resulted in similar Zn bone content as that of the control group (Tomaszewska *et al.*, 2016). Moreover, the level of 50 mg·kg^{-1}, irrespective of Zn source, increased femoral mechanical parameter values, cross-sectional area, and wall thickness. It seems that the geometry of the femur showed earlier maturation, resulting in higher values of both ultimate and maximum elastic strength, especially in the group supplemented with ZnGly. It is worth underlining that these positive effects were not influenced by body weight or sex, since the birds were weight- and sex-matched at the beginning of this experiment. Furthermore, the final body weight values between the groups of chickens did not differ. Considering this positive effect of Zn supplemented at the dose of 50 mg·kg^{-1} of premix, irrespective of

source, on cortical bone mechanical properties, it can be hypothesized that, in addition to geometry, other minerals, such as calcium, were better utilized, and therefore, improved bone quality; however, this needs to be further investigated.

It is known that Zn effects on bone tissue metabolism are dose-dependent. It stimulates DNA synthesis in the osteoblasts, bone mass increase, and Ca content (Ma & Yamaguchi, 2000). Zinc excess or deficiency can cause body weight loss, including bone mass and density, which may result in bone deformities, low bone mineralization, reduced bone and serum Ca levels (Rath et al., 2000). Although severe zinc deficiency is uncommon in avian populations, marginal deficiency is likely to be much more prevalent, and associated with to immune dysfunctions or restricted physical development (Scrimgeour et al., 2007).

The positive changes in femoral midshaft in our study were not observed in the tibia. This finding is different from the results of El-Husseiny *et al.* (2012), who reported higher tibia strength in female broilers supplemented with Zn at 50 mg/kg diet. The effect of Zn supplementation on the bone development may be due to its direct impact on protein synthesis, positively affecting bone formation (Seo *et al.*, 2010). Another factor that may affect Zn requirements is sex. Males require higher nutrient levels than females at a similar age; however, when expressed as a percentage of the diet, there seems to be little difference in nutrient requirements between sexes (Salim *et al.*, 2012).

In the present experiment, no differences in the serum levels of Zn or other minerals were detected, irrespective of dose and source. It should be noted that high Zn intake result in copper, iron, or manganese deficiency (Skřivan *et al.*, 2005; Suttle, 2010). Currently, there are no sensitive and specific biomarkers to detect Zn deficiency in animals. Low plasma or serum Zn levels are typically used as a biomarkers of Zn status, although less than 0.1% of body Zn is present in the plasma and its concentration appears to be under strict homeostatic control (Towers *et al.*, 1981). On the other hand, the activities of Zn-dependent enzymes in the plasma may be useful as functional indices of Zn status in broilers or intervention studies as their analysis to faster and more accessible compared with bone Zn content. The study of Wedeking *et al.* (1990) shows that the tibial Zn content of chickens fed a corn-soybean meal diet was markedly increased by dietary Zn supplementation, but did not provide an estimate of the requirements (Wedekind & Baker, 1990).

As mentioned above, the three-point bending test on long bones demonstrated significant differences in the mechanical parameters especially of the femur. The effect of Zn supplementation on bone development may be due to its direct impact on hormonal growth mediators. The anabolic effect of insulin-like growth factor 1 on osteoblasts is enhanced by Zn (Wang *et al.*, 2002). Moreover, the higher bioavailability of ZnGly indicates that more Zn was absorbed, and not only deposited in bone tissue compared with ZnO, also influenced cell function through its effects on hormones and growth factors. It should be noted that the ZnGly50 group presented higher insulin-like growth factor 1 (IGF-1) levels compared with the control group. It is suggested that IGF-1 locally produced in the tissues plays a more important role in growth than that circulating IGF-1 and the produced by the liver (Wang *et al.*, 2002). Cellular changes, which are induced by reduced Zn available from blood supply, are locally observed in areas located away from blood vessels. For this reason, it is suggested that longitudinal growth might be very sensitive to Zn status (Wang *et al.*, 2002).

Among the parameters recommended by the American Society for Bone and Mineral Research, we assessed the volume of bone trabeculae and the mean thickness of bone trabeculae (Mocetti *et al.*, 2000). The histomorphometric analysis showed a positive impact of Zn supplementation, irrespective of source and level, on femoral trabecular thickness and rapid loss in actual bone volume in tibial metaphyseal trabeculae in the birds fed with both Zn sources, but with largest decline observed in those fed ZnGly at 50 $mg \cdot kg^{-1}$ of premix. This result suggests a predisposition to lesions of the joint, hindering movement. This influence of Zn on the skeletal system was also observed in the Zn-supplemented chickens relative to the control group. However, the mechanism responsible for these effects is difficult to explain and needs to be further studied. We hypothesize that diet with no Zn supplementation may have supported bone development due to possible maximum intestinal Zn absorption and adequate Ca utilization. In addition, the competition for nonspecific multivalent cation channels between low Zn levels and other divalent cations, like calcium, may have been balanced, resulting in sufficient mineralization of the cortical bone midshaft. However, reduced trabecular bone was observed. The positive influence of Zn on bone tissue is highlighted when considering the significant increase in mean relative wall thickness, maximum elastic strength and ultimate strength in broilers after Zn administration.

The discrepancy observed between femoral and tibial results in our study may be due to different biomechanical properties of these both bones. An earlier study showed that the mechanical strength of the femur and the tibia turkeys with leg deformities were 20.6% and 22.6%, respectively, lower compared with healthy controls (Tatara et al., 2004).

5. CONCLUSION

In conclusion, this study demonstrated that Zn dietary supplementation up to 50 mg·kg^{-1}, irrespective of its form, positively influences mechanical properties of the femur, confirming its beneficial effect on the development of the skeletal system and bone tissues of broilers tested for a nutritional-osteoporotic factor. However, histomorphometry showed greater changes in cancellous bone than in cortical bone. This osteoporotic effects on trabecular bone observed when the Zn-deficient diet was fed emphasizes the need to supplement zinc at the recommended dose (100 mg·kg^{-1}) to broilers.

CONFLICT OF INTEREST

There are no known conflicts. Financial support for this work does no influence its outcome.

The manuscript has been read and approved by all named authors and that there are no other persons who satisfied the criteria for authorship but are not listed. The order of authors listed in the manuscript has been approved by all authors.

ACKNOWLEDGMENT

This work was financially by the project NN 311543540 from the Ministry of Science and Higher Education, Poland.

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