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Effects of Age, Vitamin D3 and Fructooligosaccharide on Correlation of Different Bone Parameters in Broiler Chickens

A. Regassa[1] and W.K. Kim[2]
[1]Department of Animal Science, University of Manitoba, Winnipeg, Manitoba, Canada
[2]Department of Poultry Science, University of Georgia, Athens, Georgia, USA

Abstract: Effects of age, vitamin D3 (VitD3) and Fructooligosaccharide (FOS) on the correlations of bone parameters were examined in broiler chickens. A total of 36 d-old male chicks were placed in battery brooders and fed a corn/soybean starter diet for 7 days and were randomly assigned to 12 pens each containing 3 birds. In group 1, birds were fed a corn/soybean starter diet and sacrificed on d 14 and 21. In groups 2 and 3, birds were fed a starter diet +250 µg VitD3/kg of diet and 4% FOS/kg of diet for 21 d, respectively. Birds were sacrificed to collect femur bones for measurement of bone parameters. Data were analyzed using Spearman's correlation analysis. Total mineral density (BMD) and trabecular density (r_{14} = 0.99, p_{14} = 0.0001; r_{21} = 0.90, p_{21} = 0.0001), total bone area and trabecular area (r_{14} = 0.99, p_{14} = 0.0001; r_{21} = 1.00, p_{21}<0.0001) and trabecular mineral content and cortical mineral content (r_{14} = 0.99, p_{14}<0.0001; r_{21} = 0.96, p_{21}<0.0001) were strongly correlated both on d 14 and 21. In birds treated with VitD3 and FOS, bone mineral content (BMC) and cortical bone mineral content (r = 0.95, p<0.0001) and BMD and trabecular mineral content (r = 0.92, p = 0.0004), were strongly correlated but most of the parameters were moderately or weakly correlated. In conclusion, results of this study show that age of birds, VitD3 and FOS treatments influence the relationships among the bone parameters.

Key words: Broiler, bone parameters, correlation

INTRODUCTION

In broilers, fast growth rates are generally correlated with musculoskeletal weakness because the development of bone in these animals is not well organized, less dense and more porous than slow growing ones (Williams et al., 2004; Bennett, 2008). This is attributed to the imbalance between meat production and skeletal growth caused by genetic selection resulting in abnormal skeletal development (Oviedo-Rondon et al., 2006). Such skeletal abnormalities are among the problems facing the poultry industry. For instance, leg problems and skeletal disorders, such as lameness, twisted legs, tibial dyschondroplasia and crooked toes, are the common causes of culling and mortality in broiler chicks (Bradshaw et al., 2002; Oviedo-Rondon et al., 2006).

Vitamin D3 (VitD3) can be synthesized under skin through ultraviolet irradiation of 7-dehydrocholesterol or absorbed from the diet in the intestinal tract (DeLuca, 2004). It is biologically inactive in this form and needs to be converted to 25-hydroxy vitamin D3 in the liver and subsequently to 1,25-dihydroxy vitamin D3 in the kidney (DeLuca, 2004). The hormone form of VitD3, 1,25-dihydroxy vitamin D3 acts through a nuclear receptor to express its numerous functions, including bone homeostasis, calcium and phosphate absorption in the small intestine, calcium mobilization in bone and calcium reabsorption in the kidney (Leeson and Summers, 2001; DeLuca et al., 2004). High levels of VitD3 reduces incidence of leg problem, including tibial dyschondroplasia and rickets in broilers (Whitehead et al., 2004; Atencio et al., 2005; Driver et al., 2006) and increasing dietary vitamin D3 improves the walking ability and welfare status of broiler chickens reared at high stocking densities (Sun et al., 2013). Additionally, high levels of VitD3 have been suggested to increase bone growth and mineral deposition in broiler chicks (Kim et al., 2011).

Fructooligosaccharides (FOS) are low-molecular-weight non-digestible carbohydrates that have the potential to increase bone formation and reduce bone resorption (Ohta et al., 1995; Takahara et al., 2000). Studies in rats have demonstrated that FOS enhances true Ca, Mg, Fe and P absorptions in the intestine (Ohta et al., 1995; Kashimura et al., 1996; Morohashi et al., 1998), can increase femoral bone volume and mineral concentrations (Takasaki et al., 2000), femoral calcium content and bone mineral density (Zafar et al., 2004) and bone mass and biomechanical properties (Lobo et al., 2006). Studies have shown that FOS have the potential to maintain bone strength during molting of white leghorn hens (Kim et al., 2006) but do not have any beneficial effects on bone growth and skeletal integrity in growing male broiler chicks (Kim et al., 2011).

Corresponding Author: Woo Kyun Kim, Department of Poultry Science, University of Georgia, Athens, Georgia, USA

Studies have been conducted to examine bone mineralization rates and growth in different chicken types. For instance, differences in mineralization rates and growth characteristics among different skeletal structures have been reported in commercial broiler lines (Applegate and Lilburn, 2002). Lower epiphyseal ash values at younger ages and smaller body weights in 2010 commercial strains of Pekin ducks suggest the need to critically monitor the factors that influence bone mineralization (van Wyhe *et al.*, 2012). The results of a study in commercial male and female broilers have indicated that life cycle changes do not significantly affect bone mineralization and bone size traits (Talaty *et al.*, 2009). Large differences in bone traits have been reported in several purebred lines of meat-type chickens suggesting the potential to genetically select birds for increased bone mineral density (BMD) (Korver *et al.*, 2004). The study also showed changes in BMD of individual birds can be measured over time and BMD at specific time points can be correlated with production parameters and eggshell quality traits (Korver *et al.*, 2004).

Studies have also been conducted to establish the importance of some bone parameters in predicting the health of bone. Assessment of cortical bone strength has been suggested to predict the risk of bone fracture or as a choice of suitable therapeutic strategies in orthopedic surgery (Augat *et al.*, 1996, 1998). Similarly, BMD has been reported to be helpful for the estimation of bone strength and prediction of intracortical porosity and parameters related to porous structures of cortical bone (Wachter *et al.*, 2002).

The degrees of associations among bone parameters and production traits have also been examined in chicken. High correlation coefficients have been found for BMD and bone mineral content (BMC) between live and excised tibial bones in White Leghorn hens (Schreiweis *et al.*, 2005). Correlation between third-toe-amplitude dependent speed of sound measurement, shear strength and radiographic density values have been reported in caged and free-range hens (Fleming *et al.*, 2004). However, absence of correlation between egg production or egg quality traits and bone quality measures have been reported in White Leghorns housed in conventional, modified and commercially available colony battery cages (Jendral *et al.*, 2008).

These works have focused on the estimation of the degree of correlation between bone parameters and production traits or the effects of dietary treatments on the magnitude of bone parameter itself but not on the degree of correlation among different bone parameters. Thus the degree of association among different bone parameters due to age difference and dietary treatments, such as vitD3 and FOS, on the correlation of these parameters are largely unknown. Therefore, this study was conducted to determine the effects of age (Days 14 and 21), dietary treatments (VitD3 and FOS) on the degree of correlation among several bone parameters in broilers.

MATERIALS AND METHODS

General procedures: A total of 36 day-old male broiler chicks were placed in heated Petersime battery brooders and fed a corn/soybean broiler starter diet for seven days. Then, the chicks were individually weighed and randomly assigned to 12 pens of three birds each. The starter diet contained 23% crude protein, 3,200 kcal/kg metabolic energy, 1% calcium, 0.45% available phosphorus and 90 µg/kg VitD3. In treatment one, where we examined how bone parameters correlate in a normal corn/soybean dietary condition, birds were sacrificed on Days 14 and 21. In treatments two and three, birds were fed a control corn/soybean meal diet with 250 µg VitD3/kg of diet and 4% FOS for 21 days, respectively. At the end of each time points, birds were sacrificed by cervical dislocation to collect femur bones for peripheral quantitative computed tomography (pQCT) and mechanical testing. In order to determine how age affects the correlation among bone parameters in chicks fed a normal corn/soybean meal diet, the data of different bone parameters on Days 14 and 21 were analyzed separately for their respective correlation coefficients. To examine the effects of VitD3 and FOS on the correlation of bone parameters, data of each group were analyzed separately for their respective correlation coefficients. All animal procedures were done according to the University committee guideline for Animal Care.

Peripheral quantitative computed tomography: Bone samples were scanned using a XCT Research instrument (Stratec, Norland, Fort Atkinson, WI). This model has a minimum voxel size of 0.07 mm and a scanning beam thickness of 0.50 mm. Scan sites included the mid-shaft femurs (3 slices of each bone located at one-half the total bone length ±2 mm) for volumetric bone mineral density (vBMD) of the cortical bones the proximal femurs were scanned to assess metaphyseal trabecular vBMD (3 slices located 8.0, 8.5 and 9.0 mm from the proximal end of the bone). All scans were obtained at a scan speed of 2.5 mm/sec, with a voxel resolution of 0.07x0.07x0.50 mm. In addition, the mid diaphyseal cross-sectional moment of inertia (CSMI), a measure of bone material in a cross section area and indicator of its distribution around the neutral bending axis to resist bending (Bennett, 2008), was obtained with respect to the neutral bending axis of the femur bone shaft during 3-point bending (mechanical property testing).

Mechanical testing: After pQCT scans were completed, mechanical properties of the mid shaft femurs were determined by 3-point bending to failure with an Instron

1125 servo-controlled testing machine (Instron Corp., Canton, MA) according to previously published procedures (Allen and Bloomfield, 2003). The femurs were thawed at room temperature and placed posterior side down on metal pin supports located ±10 mm (femurs) from the mid diaphysis testing site. With a 1,000 Lb (455 kg) load cell, quasi-static loading (2.5 mm/min) was applied to the anterior surface of the femurs until fractures occurred. All specimens were sprayed with PBS just before testing to maintain hydration. Displacements were monitored by a linear variable differential transformer interfaced with a personal computer. Raw data, collected at 10 Hz as load versus displacement curves, were analyzed with Table-Curve 2.0 software (Jandel, San Rafael, CA). Structural variables (ultimate load and stiffness) were obtained directly from the load: displacement curves. The maximum load obtained was defined as the ultimate load (UL, in N) and the slope of the elastic portion of the curve was defined as stiffness (S, in N/mm). Bone tissue material properties were calculated by normalizing structural properties for cross-sectional bone geometry at the site of testing by using CSMI (in mm^4 from pQCT), bone diameter (D, in mm) as measured by calipers and the appropriate bottom support span distance (L, which was 20 or 60 mm). The appropriate formulas for elastic modulus (E, in GPa) and ultimate stress (US, in MPa) were as follows:

$$E = SxL3/(48,000xCSMI) \text{ and } US = UlxLxD/(8xCSMI)$$

Statistical analysis: Bone parameter data were analyzed using Spearman's correlation analysis (SAS Institute, 2008). Correlations among the parameters were considered significant at p<0.05. Strongly correlated parameters were identified using r = 0.90.

RESULTS AND DISCUSSION

Correlation analysis of bone parameters in chicks on Days 14 and 21: Age based correlation analysis of bone parameters in birds fed a normal corn/soybean meal diet revealed that most of the parameters were strongly correlated with each other both on Day 14 and 21 with slightly declined or weaker correlations on Day 21 (Fig. 1 and Table 1 and 2). Parameters that were strongly correlated both on Day 14 and 21 include: total mineral density with trabecular density ($r14 = 0.98$, $p_{14}<0.0001$; $r_{21} = 0.91$, $p_{21} = 0.0001$), total bone area with trabecular area ($r_{14} = 0.99$, $p_{14} = 0.0001$; $r_{21} = 1.00$, $p_{21}<0.0001$), trabecular mineral content with cortical mineral content ($r_{14} = 0.99$, $p_{14}<0.0001$; $r_{21} = 0.96$, $p_{21}<0.0001$), total mineral content with trabecular mineral content ($r_{14} = 0.99$, $p_{14}<0.0001$; $r_{21} = 0.96$, $p_{21}<0.0001$), total bone area with cortical area ($r_{14} = 0.97$, $p_{14} = 0.0001$; $r_{21} = 0.97$, $p_{21}<0.0001$) and cortical mineral content with cortical area ($r_{14} = 0.97$, $p_{14} = 0.0001$; $r_{21} = 0.97$, $p_{21}<0.0001$).

These results suggest that there is a trend where total bone parameters, such as total bone area, total BMD, total BMC, are strongly correlated with their respective regional parameters, such as trabecular and cortical area, trabecular mineral density, trabecular and cortical mineral content (Table 1 and 2). This can be attributed to the fact that regional bone parameters are the components of the total parameters (Franck and Munz, 2000). Bone mineral density measurements have been suggested to be combined with trabecular micro-architecture to predict the fracture risk and the severity of osteoporosis in middle-aged men showing the association between the two parameters (Legrand *et al.* 2000). Additionally, significant correlation between cortical and trabecular bone mineral content has previously been reported in women (Bohr and Schaadt, 1985).

Mechanical strength is highly correlated with stiffness, particularly on Day 14 (r = 0.90, p = 0.0009) (Fig. 2). Mechanical strength of a material is its ability to withstand an applied stress without forming a fracture (Duane, 2003; WuBian *et al.*, 2010) and stiffness is the resistance of an elastic body to deformation (Gopalakrishnan and Zukoski, 2007). Their strong correlation in this study can be explained by the fact that both of them are the factors influencing bone strength and measuring one of these parameters could provide an indirect value for the other.

The correlations between mechanical strength and stiffness ($r_{14} = 0.90$, $p_{14}<0.0009$; $r_{21} = 0.74$, $p_{21} = 0.022$) (Fig. 2), total bone area with cortical thickness ($r_{14} = 0.88$, $p_{14} = 0.0038$; $r_{21} = 0.80$, $p_{21} = 0.0095$), trabecular mineral content ($r_{14} = 0.87$, $p_{14} = 0.0051$; $r_{21} = 0.79$, $p_{21} = 0.01$), trabecular mineral content with trabecular area ($r_{14} = 0.87$, $p_{14} = 0.0051$; $r_{21} = 0.79$, $p_{21} = 0.01$), trabecular density with cortical thickness ($r_{14} = 0.89$, $p_{14} = 0.0024$; $r_{21} = 0.71$, $p_{21} = 0.03$) and trabecular mineral content ($r_{14} = 0.94$, $p_{14} = 0.0005$; $r_{21} = 0.75$, $p_{21} = 0.02$) showed declining tendencies with age (Table 1 and 2). Previously, an age-related decline in trabecular density has been reported in a human study (Singer and Breidahl, 1990). This age related declining correlations between the parameters in the present study might indicate that the parameters are not changing proportionally as age increases and this requires further investigation.

Although most of the parameters showed strong to moderate correlation at both stages, some of them were poorly correlated on day 21 (Fig. 3 and Table 2). Parameters that were poorly correlated on Day 21 include BMD with cortical area ($r_{21} = 0.1$, $p_{21} = 0.79$), cortical thickness ($r_{21} = 0.46$, $p_{21} = 0.2$), cortical mineral content ($r_{21} = 0.3$, $p_{21} = 0.42$) and trabecular mineral content ($r_{21} = 0.49$, $p_{21} = 0.17$) (Table 2). These data are consistent with those from human studies.

Table 1: Correlation coefficients and their respective probabilities of bone parameters obtained from birds fed starter diet for 14 days

	Trabecular area (mm²)	Cortical area (mm²)	Cortical thickness (mm)	Total mineral content (g/cm)	Trabecular mineral content (g/cm)	Cortical mineral content (g/cm)
Total area (mm²)	r = 0.99, p = 0.0001	r = 0.97, p = 0.0001	r = 0.88, p = 0.0038	r = 0.91, p= 0.0016	r = 0.87, p = 0.0051	r = 0.91, p = 0.0016
Cortical area (mm²)	r = 0.97, p = 0.0001	1.0000	r = 0.97, p= 0.0001	r = 0.97, p= 0.0001	r = 0.95, p = 0.0003	r = 0.97, p = 0.0001
Trabecular area (mm²)	1.0000	r = 0.97, p<0.0001	r = 0.88, p = 0.0039	r = 0.91, p= 0.0016	r = 0.87, p= 0.0051	r = 0.91, p = 0.0016
Total mineral content (g/cm)	r = 0.87, p = 0.0051	r = 0.95, p = 0.0003	r = 0.97, p = 0.0001	r = 0.99, p<0.0001	1.0000	r = 0.99, p<0.0001
Total mineral density (mg/cm³)	r = 0.1, p = 0.79	r = 0.76, p = 0.028	r = 0.86, p = 0.0064	r = 0.88, p = 0.0038	r = 0.92, p = 0.0013	r = 0.87, p = 0.004
Trabecular density (mg/cm³)	r = 0.2, p = 0.62	r = 0.80, p = 0.016	r = 0.89, p = 0.0024	r = 0.89, p = 0.0028	r = 0.94, p = 0.0005	r = 0.89, p = 0.003

r = correlation coefficient, p = probability

Table 2: Correlation coefficients and their respective probabilities of bone parameters obtained from birds fed starter diet for 21 days

	Trabecular area (mm²)	Cortical area (mm²)	Cortical thickness (mm)	Total mineral content (g/cm)	Trabecular mineral content (g/cm)	Cortical mineral content (g/cm)
Total area (mm²)	r = 1.000, p<0.0001	r = 0.97, p = 0.0001	r = 0.80, p= 0.0095	r = 0.91, p = 0.0005	r = 0.79, p = 0.01	r = 0.91, p = 0.0006
Cortical area (mm²)	r = 0.96, p<0.0001	1.0000	r = 0.92, p = 0.0003	r = 0.98, p<0.0001	r = 0.91, p = 0.0005	r = 0.97, p<0.0001
Trabecular area (mm²)	1.0000	r = 0.96, p<0.0001	r = 0.80, p = 0.0095	r = 0.91, p = 0.0005	r = 0.79, p = 0.01	r = 0.91, p = 0.0006
Total mineral content (g/cm)	r = 0.79, p = 0.01	r = 0.91, p = 0.0005	r = 0.98, p = 0.0005	r = 0.96, p<0.0001	1.0000	r = 0.96, p<0.0001
Total mineral density (mg/cm³)	r = 0.1, p = 0.79	r = 0.14, p = 0.70	r = 0.46, p = 0.2	r = 0.3, p = 0.42	r = 0.49, p = 0.17	r = 0.30, p = 0.42
Trabecular density (mg/cm³)	r = 0.19, p = 0.62	r = 0.43, p = 0.24	r = 0.71, p = 0.03	r = 0.55, p = 0.12	r = 0.75, p = 0.02	r = 0.54, p = 0.13

r = correlation coefficient, p = probability

Table 3: Correlation coefficients and their respective probabilities of bone parameters obtained from birds treated with 250 µg of vitamin D3 per kilogram of diet for 21 days

	Ultimate strength (N/cm²)	Cortical area (mm²)	BMC (mm)	Trabecular mineral density (mg/cm³)	Cortical mineral content (mm)
Mechanical force (N/cm²)	r = 0.83, p = 0.005	r = 0.52, p = 0.14	r = 0.65, p = 0.05	r = 0.74, p = 0.02	r = 0.68, p = 0.04
BMD (mg/cm³)	r = 0.78, p = 0.01	r = 0.59, p = 0.08	r = 0.67, p = 0.04	r = 0.95, p<0.0001	r = 0.70, p = 0.03
Total bone area (mm²)	r = -0.88, p = 0.0016	r = 0.85, p = 0.0032	r = 0.83, p = 0.005	r = 0.13, p = 0.73	r = 0.80, p = 0.0087
Cortical area (mm²)	r = 0.06, p = 0.87	-	r = 0.97, p<0.0001	r = 0.60, p = 0.08	r = 0.97, p<0.0001
Trabecular area (mm²)	r = -0.30, p = 0.41	r = 0.85, p = 0.003	r = 0.83, p = 0.005	r = 0.13, p = 0.73	r = 0.8, p = 0.007

r = correlation coefficient, p = probability

Table 4: Correlation coefficients and their respective probabilities of bone parameters obtained from birds treated with 4% of fructo oligosaccharide per kilogram of diet for 21 days

	Ultimate strength (N/m²)	Mechanical force (N/m²)	Total area (mm²)	Cortical thickness (mm)	Trabecular mineral content (g/cm³)	Cortical mineral content (g/cm)
Trabecular area (mm²)	r = -0.88, p = 0.0016	r = -0.87, p = 0.0022	r = 1.000, p<0.0001	r = 0.91, p = 0.0005	r = 0.7, p= 0.03	r = 0.84, p = 0.004
Cortical area (mm²)	r = 0.72, p = 0.02	r = -0.83, p = 0.0058	r = 0.91, p = 0.0005	r = 0.91, p = 0.0005	r = 0.89, p = 0.0012	r = 0.86, p = 0.0025
BMC (g/cm³)	r = - 0.54, p = 0.13	r = 0.39, p = 0.29	r = 0.86, p = 0.003	r = 0.86, p = 0.0031	r = 0.92, p = 0.0004	r = 0.99, p<0.0001
Cortical thickness (mm)	r = -0.38, p = 0.30	r = 0.36, p = 0.33	r = 0.63, p = 0.06	r = 1.000	r = 0.87, p = 0.0021	r =0.80, p = 0.0091

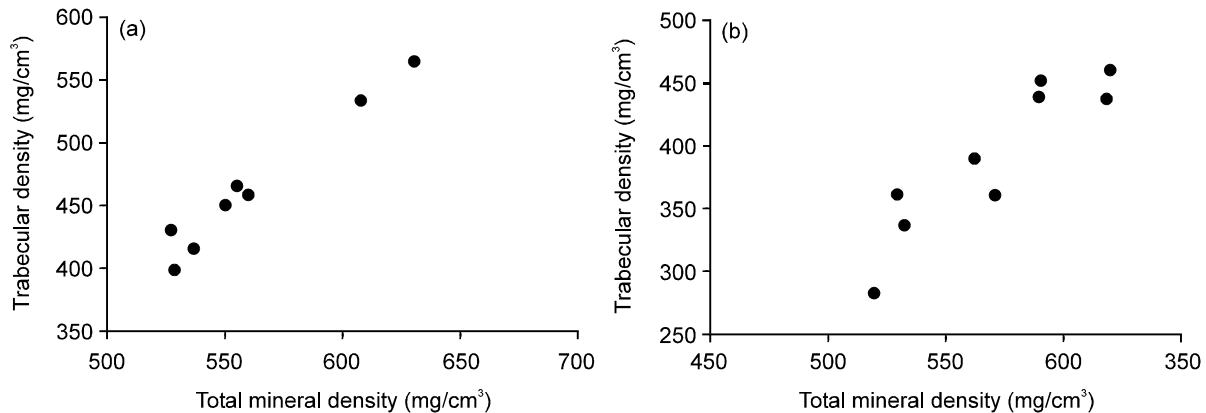r = correlation coefficient, p = probability

Fig. 1(a-b): Correlation between total mineral density and trabecular density on day 14 (a) and day 21 (b) of the experimental period. Although showing a declining tendency with age, the parameters were strongly correlated both on day 14 (r = 0.98092, p<0.0001) and day 21 (r = 0.90964, p = 0.0001)
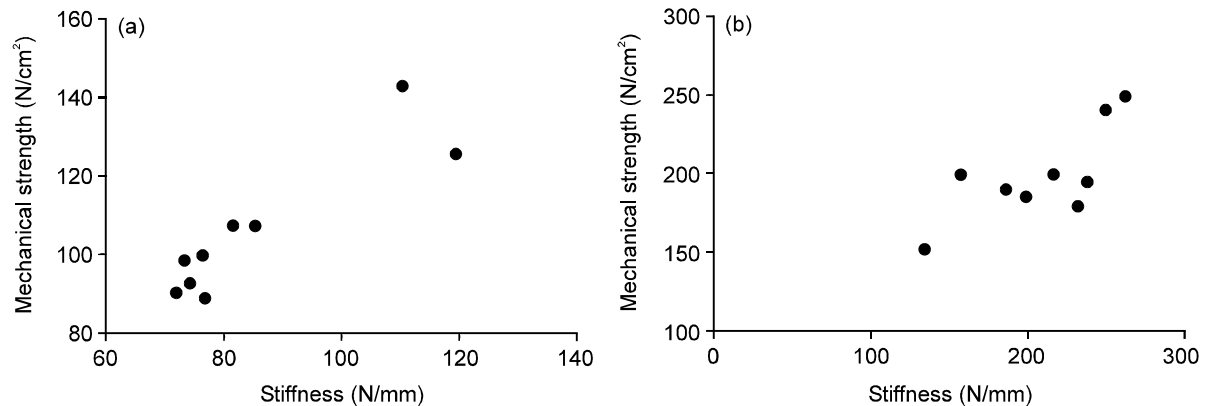

Fig. 2(a-b): Correlation between mechanical strength and bone stiffness on day 14 (a) and day 21 (b) of the experimental period. The parameters were strongly correlated on day 14 (r= 0.90, p = 0.0009) but moderately correlated on day 21 (r = 0.74, p = 0.0225)

Age-dependent decline in cortical BMC and trabecular BMD at the distal radius, spinal BMD, trabecular bone volume fraction, trabecular thickness, trabecular number and fractal dimension have been reported in premenopausal women (Majumdar *et al.*, 1997). An increased growth rate is associated with lower mineral density in broiler chicken (Leterrier and Nys, 1992) and consistent with some of these data, the correlations of some of the parameters were weaker on day 21 than on day 14. On the other hand Lee *et al.* (2001) have reported a strong negative correlation between total and regional BMD in menopausal Japanese women showing the effect of interspecies differences on the correlation of bone parameters.

Correlation analysis of bone parameters in Vit D3 and FOS fed birds: As opposed to a normal corn/soybean meal dietary condition where most parameters were strongly correlated, VitD3 and FOS treatments affected the correlation of the parameters. Except for strong

correlation between BMC and cortical bone mineral content (r = 0.95, p<0.0001), BMD and trabecular mineral content (r = 0.92, p = 0.0004), mechanical strength and BMD (r = 0.89, p = 0.0012), trabecular area and cortical thickness (r = 0.91, p = 0.0005) and cortical area and total area (r = 0.91, p = 0.0005), most of the parameters were moderately or weakly correlated in birds treated with VitD3 and FOS (Table 3 and 4, Fig. 4). There were also parameters which were negatively correlated emphasizing the effects of these dietary treatments on the association of bone parameters (Table 3 and 4). Consistent with strong correlation between BMD and mechanical strength in the present study, high correlation coefficient between BMD and strength has been reported in female cadavers indicating the dependence of bone strength on BMD (Graham *et al.*, 2007). On the other hand, low correlation coefficients have been reported between shear force and BMD or BMC in three weeks old broiler chicks received adequate, medium and low dietary Ca and P levels
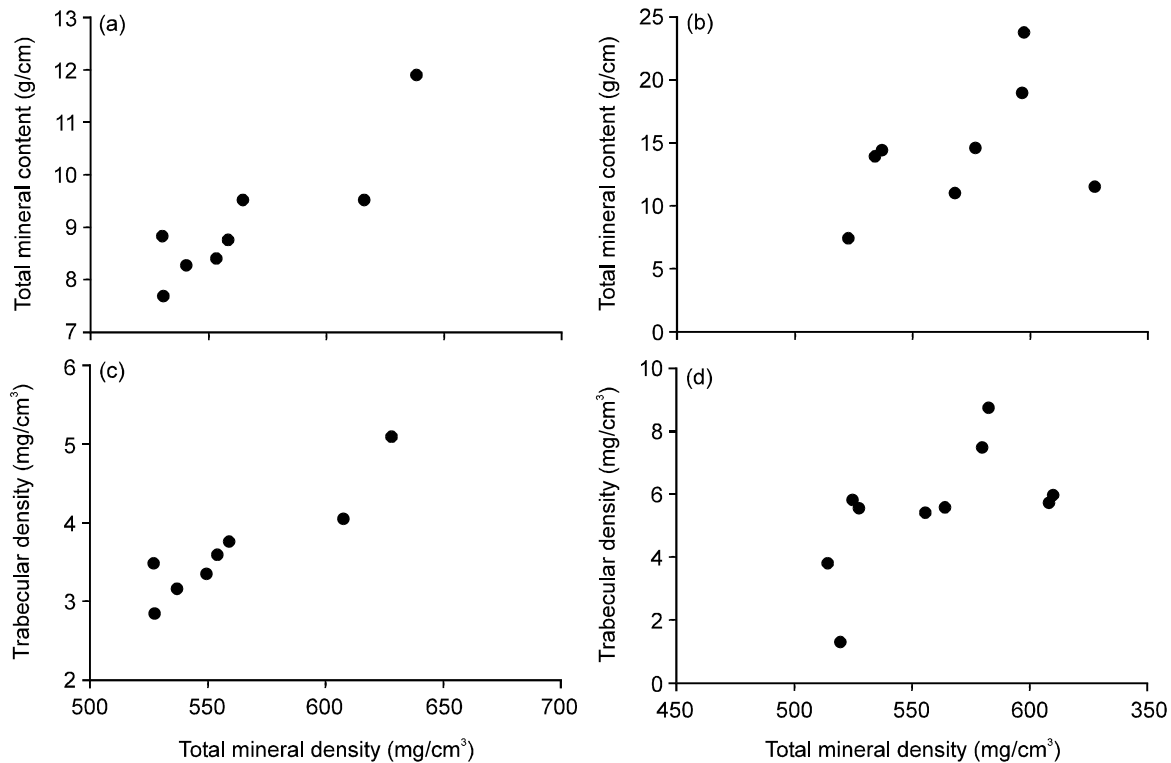
Fig. 3(a-d): Correlation between BMD and BMC and between BMD and trabecular mineral content on day 14 (a and c) and day 21 (b and d) of the experimental period. The parameters were strongly correlated on day 14 (r_a = 0.88, p_a = 0.0038, r_c = 0.91, p_c = 0.0013) but did not correlate on day 21 (r_b = 0.30, p_b= 0.42, r_d = 0.49, p_d = 0.1758)
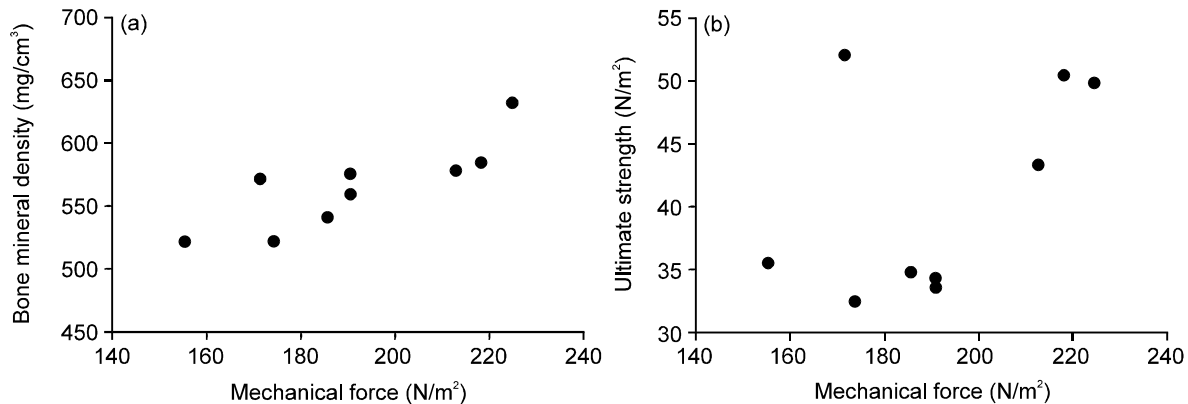

Fig. 4(a-b): Correlation between mechanical force and BMD in birds treated with 4% FOS (a) and between mechanical force and ultimate strength in birds treated with 250 μg of VitD3/Kg of diet (b) ra, rb, rc and rd stand for the coefficients of correlation between the parameters depicted on figure 3A, B, C and D, respectively and pa, pb, pc and pd stand for their respective probabilities

(Onyango *et al.*, 2003) suggesting a possible interspecies variation among different bone parameters.

Conclusion: In conclusion, the results of this study showed that most of the parameters in broiler chicks fed a normal corn/soybean meal diet are strongly and moderately correlated, and age of the birds appears to influence the correlation of bone parameters providing an insight into how the parameters are associated during the broiler growth period and dietary supplementation of VitD3 and FOS affects the direction and magnitude of correlation.

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