ANIMAL WELL-BEING AND BEHAVIOR

Changes with age (from 0 to 37 D) in tibiae bone mineralization, chemical composition and structural organization in broiler chickens

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ABSTRACT Broiler chickens have an extreme physiology (rapid growth rates) that challenges the correct bone mineralization, being an interesting animal model for studying the development of bone pathologies. This work studies in detail how the mineralization, chemistry, and structural organization of tibiae bone in broiler chickens change with age during the first 5 wk (37 D) from hatching until acquiring the final weight for slaughter. During the early growth phase (first 2 wk), the rapid addition of bone tissue does not allow for bone organic matrix to fully mineralize and mature, and seems to be a critical period for bone development at which bone mineralization cannot keep pace with the rapid growth of bones. The low degree of bone mineralization and large porosity of cortical bone at this period might be responsible of leg deformation and/or other skeletal abnormalities commonly observed in these birds. Later, cortical bone porosity gradually decreases and the cortical bone became fully mineralized (65%) at 37 D of age. At the same time, bone mineral acquires the composition of mature bone tissue (decreased amount of carbonate, higher crystallinity, Ca/P = 1.68). However, the mineral part was still poorly organized even at 37 D. The oriented fraction was about 0.45 which means that more than half of apatite crystals within the mineral are randomly oriented. Mineral organization (crystal orientation) had an important contribution to bone-breaking strength. Nevertheless, locally determined (at tibia mid-shaft) bone properties (i.e., cortical thickness, crystal orientation) has only a moderate correlation (R^2 = 0.33) with bone breaking strength probably due to large and highly heterogeneous porosity of bone that acts as structural defects. On the other hand, the total amount of mineral (a global property) measured by total ash content was the best predictor for breaking strength (R^2 = 0.49). Knowledge acquired in this study could help in designing strategies to improve bone quality and reduce the incidence of skeletal problems in broiler chickens that have important welfare and economic implications.

Key words: broiler, tibiae, bone quality, osteoporosis, growth disorders

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INTRODUCTION

Meat-type (broiler) chickens have been selected for rapid growth and feed efficiency since the 1950s (Julian, 1998; Havenstein et al., 2003; Zuidhof et al., 2014). The extremely high growth rates in broilers poses many challenges particularly those related to bone health (Rawlinson et al., 2009). Modern broiler lines commonly shows skeletal abnormalities (i.e., rickets, leg bending) and suffer a high incidence of bone fractures and increased mortality (Julian, 1998; Williams et al., 2000; Talaty et al., 2010). Poor bone quality in broilers is mainly due to their low calcification and high porosity, making bones weak and prone to deformation and/or fracture (Williams et al., 2004; Rawlinson et al., 2009). Skeletal problems and poor bone quality in these birds seem to be associated with their very rapid early growth rates as bone development cannot keep pace with such a fast rate of increase in body weight. Bone quality can be improved by slowing the growth

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of chickens, for instance by feed restriction (Leterrier et al., 1998; Williams et al., 2004). Slower growing chickens produce stronger bones with a higher mineralization and/or reduced porosity.

Bone is a complex composite material composed of an inorganic phase (nanocrystalline carbonated apatite) mineralizing an organic matrix (mainly type I collagen) and water. The mechanical properties of bone are not only determined by the total bone mass but also by its geometrical distribution, degree of mineralization, porosity, its structural organization at different scales (i.e., osteons, collagen fibers, apatite nanocrystals), and the properties of the bone-constituting materials (i.e., mineral crystallinity, collagen cross-linking) (Martin and Ishida, 1989; Nakano et al., 2002; Fratzl et al., 2004; Boskey and Mendelsohn, 2005; Gupta et al., 2006; Gourion-Arsiquaud et al., 2009; Zimmermann et al., 2011; Rodriguez-Navarro et al., 2018). Bone is also a living tissue that is constantly changing and adapting during growth by bone cell remodeling in response to external stimuli like changing body weight, physical exercise, or calcium demand (Glimcher, 1998). Also bone development and properties are affected by multiple factors: genetic, sex, nutrition, and environment (housing, lightning program) (Rose et al., 1996; Leterrier et al., 1998; Rath et al., 2000; Fleming, 2008; Talaty et al., 2009).

There are a number of studies that have explored changes in bone morphology and mineralization during the development of broiler chicken skeleton (Williams et al., 2000, 2004; Applegate and Lilburn, 2002; Rawlinson et al., 2009; Talaty et al., 2009; Shim et al., 2012). However, there is no detailed work about how the chemistry and structural organization of bone-constituting materials changes during broiler growth. Tibiae bone is one of the most mineralized bones in the skeleton and also a good indicator of overall skeletal mineralization (Skinner and Waldroup, 1995; Angel, 2007). This work uses complementary analytical techniques such as electron microscopy (**SEM**), optical emission spectroscopy (**ICP-OES**), infrared spectroscopy (Fourier-transform infrared spectroscopy, **FTIR**), thermogravimetry (**TGA**), and X-ray diffraction (**XRD**) to analyze changes in tibiae bones of broiler chickens during the first 5 wk of age, from hatching (0 D) until attaining the final market weight (37 D). The techniques used provide detailed information about tibiae bone structural organization, chemical composition, mineral crystallinity, and collagen maturity (cross-linking), which have important contributions to bone properties (i.e., mechanical properties) and are useful to define bone quality (Fratzl et al., 2004; Boskey and Mendelsohn, 2005; Gupta et al., 2006; Donnelly et al., 2009). This study can help to better understand how bone calcification and properties are affected by fast growth rates during the early life of chickens and to identify critical stages of bone development. This knowledge can be useful to design new strategies for reducing skeletal problems in these animals which have important welfare implications and are a major source of economic losses for the poultry industry (at least 3.2%) (Cook, 2000).

MATERIALS AND METHODS

Animals, Sample Preparation, and Collection

Tibias from broiler chickens at 0 days (n = 30), 3 days (n = 50), 7 days (n = 50), 10 days (n = 8), 21 days (n = 8), and 37 days (n = 50) old chick were used for this study. Briefly, a total of 196 Ross male broiler chicks (Trouw Nutrition, S.A, Toledo, Spain) weighing 43 g in average were allocated in floor pens (2.38×1.68 m^2) with wood shavings litter and 6/18 h of light/dark periods. Each pen provided 0.10 m^2/bird. They were kept from the day of hatching (0-day-old) until 37 D of age (when they acquired the required weight for marketing). Birds were feed ad libitum a pelleted corn–wheat–soybean-based diet in 3 phases, with phase 1 being from day 0 to 10, phase 2 from day 10 to 28 and phase 3 from day 28 to 37, according to the NRC requirements of the species (National Research Council, 1994). The protein, Ca, and P in the feed varied for phase 1, 2, and 3 as follows: protein content was 22.5, 19.9, and 18.3%, respectively; Ca content was 0.86, 0.59, and 0.52%, respectively; P content was 0.54, 0.45, and 0.36%, respectively. At the preselected ages, broiler chickens were weighted (except 0-day-old) and killed by vertebra dislocation. All experiments followed the guidelines of the Ethics Committee of the Poultry Research Centre of Trouw Nutrition for the humane care and use of animals in research. Then tibia bones were cleaned with a scalpel and their main characteristics such as length, weight, and cortical thickness (**CT**) were determined. Samples were kept in a freezer at –20°C for further analyses. No chemical fixatives were used to preserve samples.

Microscopy

A selection of samples, from each age group, was prepared for optical microscopy (**OM**). Specifically, tibiae bones were embedded in Epothin epoxy resin (Buehler, Illinois, USA) and a cross-section at mid-diaphyses were prepared. Then, sections were hydrated in water during 1 h, and stained with hematoxylin/eosin and Masson's trichrome to differentiate between unmineralized and mineralized bone tissue. The morphology of bone samples was examined using OM (Eclipse LV100POL Nikon, Japan). Polished cross-sections of the tibiae mid-diaphyses were coated with carbon (Hitachi UHS evaporator, Hitachi Ltd., Tokyo, Japan) and observed with an SEM (Hitachi S-510, Hitachi Ltd.) using backscattering electron mode at an accelerating voltage of 20 keV.

Infrared Spectrometry

Cortical bone samples from mid-diaphyses were powdered to analyze the chemical composition by FTIR with a spectrometer JASCO 6200 equipped with a diamond attenuated total reflectance accessory (ATR Pro ONE, JASCO, Japan). The infrared spectra were recorded at a 2 cm^{-1} resolution over 40 scans. The relative amounts of water, proteins (collagen), lipids, phosphate, and carbonate in the bone samples were determined from the peak area of the absorption bands associated with the characteristic molecular groups of each component (e.g., OH: water; CH: lipids; amide I: collagen; v2, v3 CO_3: carbonates; v1, v3 PO_4: phosphates) (Boskey and Mendelsohn, 2005; Rodriguez-Navarro et al., 2006). Overlapping peaks were resolved and their integrated areas measured using specifically designed curve fitting software that uses a Gaussian peak fit from previously established peak positions, and it allowed to vary freely peak position, width, and height. From the peak areas, the following compositional parameters were determined to define bone material properties: (1) The relative amount of mineral to organic matrix (PO_4/Amide I) determined as the ratio of the main phosphate (v1, v3 PO_4; 900 to 1,200 cm^{-1}) to Amide I (1590 to 1710 cm^{-1}) band area ratio. (2) The amount of carbonate substituted in the mineral ($MinCO_3$_870) as the ratio of v2 CO_3 (850 to 890 cm^{-1}) to the main phosphate (900 to 1,200 cm^{-1}) band area, that mainly represents carbonate ions substituting for phosphate ions in the apatite crystalline structure (type B carbonate). This ratio increases as the bone mineral matures (Rey et al., 1989; Donnelly et al., 2009). (3) Total carbonate relative to mineral content ($MinCO_3$_1415) determined as the ratio of the main carbonate band (v3 CO_3; 1,390 to 1,440 cm^{-1}) to the main phosphate (900 to 1,200 cm^{-1}) band area ratio which decreases as bone mineral matures (Ou-Yang et al., 2001; Rodriguez-Navarro et al., 2006). Additionally, we evaluate the total amount of carbonate relative to organic matrix (CO_3 1415/Amide I) as the peak area ratio of the main carbonate band to the amide I band. (4) The mineral crystallinity index (**CI**) determined as the area ratio between phosphate sub-bands 1,030 and 1,020 cm^{-1} which increases as the mineral crystal size and perfection increases (Donnelly et al., 2009). (5) The amount of collagen cross-links was evaluated as the area ratio between amide I sub-bands at 1,660 and 1,690 cm^{-1} which increases with collagen maturity (Paschalis et al., 2001; Donnelly et al., 2009).

Optical Emission Spectroscopy

For the elemental analyses, 100 mg of powdered bone was dissolved in a 0.5 mL solution of 30% H_2O_2 and 0.5 mL 70% HNO_3. After digestion for 22 h, 4 mL of distilled water was added and the sample was filtered. Calcium, phosphorus, magnesium, and sodium concentrations were measured using an Optima 8300 ICP-OES spectrometer (Perkin Elmer, Massachusetts, USA). Concentrations were given in mg/kg dry weight. The precision of chemical analyses was higher than 1 mg/kg.

Two-dimensional X-ray Diffraction

Tibiae cortical bones (about 1 × 1 cm) cut from the midshaft of the diaphysis were analyzed in transmission mode with an X-ray single crystal diffractometer (D8 Venture, Bruker, Massachusetts, USA) equipped with a PHOTON area detector and Mo radiation. Crystallinity of bone mineral was determined by measuring the full width at half maximum (**FWHM**) of the main apatite peaks (e.g., 002, 211, 310) displayed in 2Theta scan, calculated by radially integrating intensities from two-dimensional (**2D**) XRD patterns. The sharper the peaks and smaller the FWHM, the greater is the crystallinity. A quantitative estimation of the degree of alignment of the c-axis of apatite crystals in the cortical bone was determined from the angular breadth of bands displayed in the intensity profile along the Debye-Scherrer ring associated with the 002 reflection of apatite mineral (002 Gamma scan; Rodriguez-Navarro et al., 2018). Additionally, we calculated the percentage of apatite crystals that are aligned or randomly oriented within the bone mineral. The intensity of peaks in the gamma scan accounts for the crystals that are preferentially oriented while the background intensity accounts for randomly oriented crystals. By calculating the ratio of intensities (Rho), Int_Oriented/(Int_Random + Int_Oriented), the relative amount of oriented crystals (oriented fraction) was quantified (Dominguez-Gasca et al., 2019). XRD2DScan v7.0 software (Malvern-PANalytical, The Netherlands) was used to analyze the collected 2D XRD patterns.

Bone Mechanical Properties

The main morphological properties of tibiae such as length, weight, diameter, and CT were measured. Biomechanical properties (i.e., tibiae breaking strength and stiffness) were determined by 3-point bending test using a material testing machine (Stable Micro Systems TA. XT plus 100) with a 100 kg loading cell and speed of 3 mm/s. These properties were only determined for 37 D old age group.

Thermogravimetry Analysis

The absolute water, organic matter, carbonate, and phosphate content was determined by TGA in bone samples of broiler chickens at 37 D of age. For these analyses, about 25 mg of the powdered bone was introduced into a crucible and analyzed using a TGA system from METTLER-TOLEDO (mod. TGA/DSC1). A heating rate of 20°C/min was used for registering the TGA curves from room temperature to 950°C. Total bone mineral content in whole tibia was determined as ash weight after heating in Carbolite oven (model RWF 1100) at 550°C for 12 h. Ash weight was measured in the left tibiae that were not used for the other analyses.

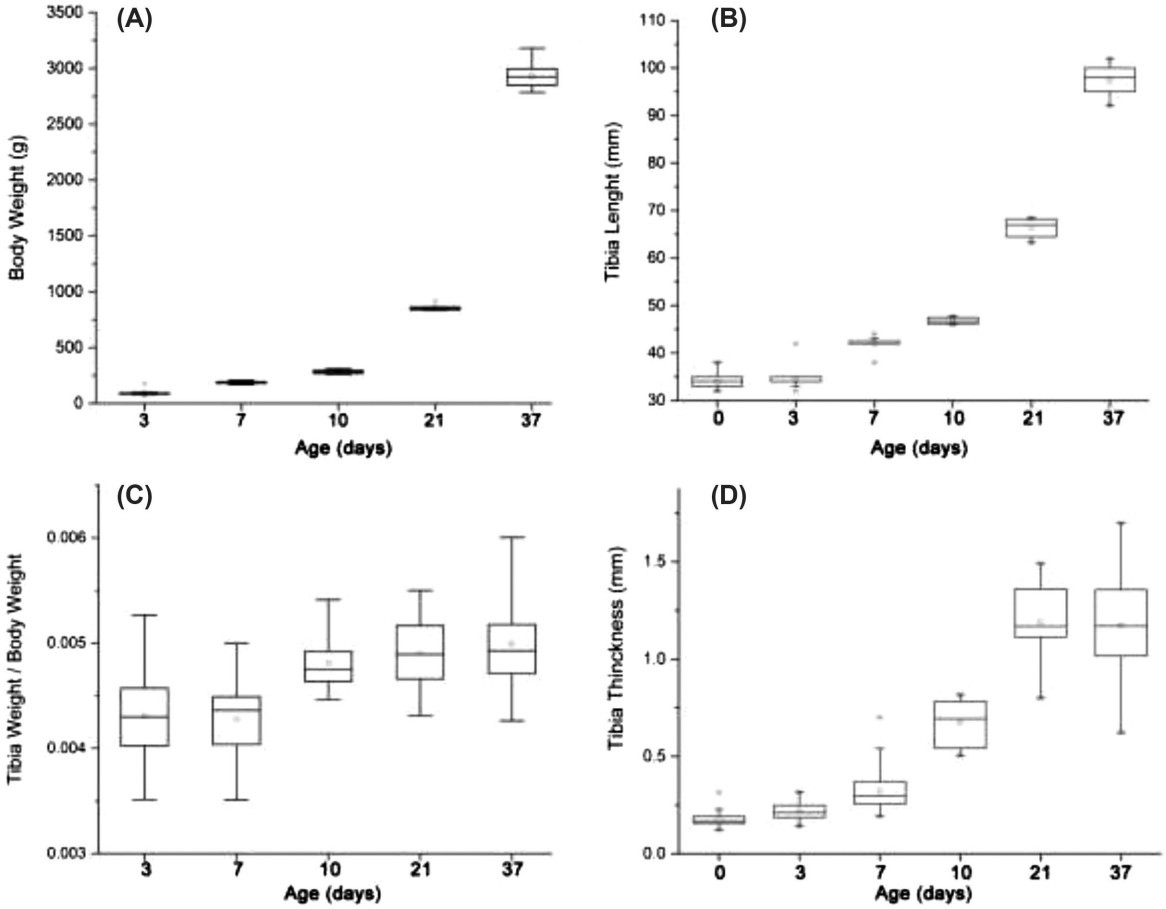

Figure 1. Body weight and bone properties as a function of chicken age. (A) Body weight, (B) tibia length, (C) tibia weight/body weight ratio, and (D) cortical bone thickness.

Statistical Analysis

Basic descriptive statistics were used to characterize bone properties. One-factor ANOVA was used to determine differences for all variables among groups. Pearson's correlation analysis and multivariate linear regression models were used to study the relationships between the different properties of bone and bone mechanical properties. Differences were considered significant at $P < 0.05$. All statistical analyses were performed using Origin Pro (OriginLab Corporation, Massachusetts, USA) software package.

RESULTS

Bone Morphology

Figure 1 shows the evolution of chicken body weight and bone macroscopic properties during growth. Body weight of broilers increased very rapidly with age following a power law (Figure 1A). Body weight was significantly different between consecutive age groups ($P < 0.001$). Tibia bone weight and length increased also very rapidly with age mirroring the changes in body weight and also showing significantly differences among consecutive age intervals ($P < 0.05$), except between 0- and 3-day-old chicks ($P = 0.787$). The thickness of cortical bone also increased rapidly with age until 21 D and remained constant afterward (Figure 1D). The changes in CT are significant between consecutive age interval ($P < 0.02$), except between 0- and 3-day-old ($P = 0.850$) and between 21- and 37-day-old ($P = 0.999$). The ratio between tibiae weight and body weight was kept nearly constant until 7 D, increased at 10 D ($P = 0.005$), staying nearly constant then after (Figure 1C).

The cortical bone of chickens from 0 to 10 D is highly porous and has a spongy appearance, consisting of primary osteons with poorly mineralized walls and large canals (Figure 2A–D). The thickness of the active spongy zone (**AZ**) in the periosteal area of the cortical bone decreases with age as osteoblasts gradually deposit new bone tissue filling the canals and reducing their inner diameter (Figure 2E and F). At 21 D and after, the cortex became denser and at 37 D is formed by mature osteons with very small canals, with the exception of the periosteal surface layer which still shows the deposition of new bone tissue. This metabolically active periosteal surface of cortical bone as well as the inner surface of osteons are stained in red, indicating the bone organic matrix in these regions is not mineralized yet and corresponds to newly deposited primary

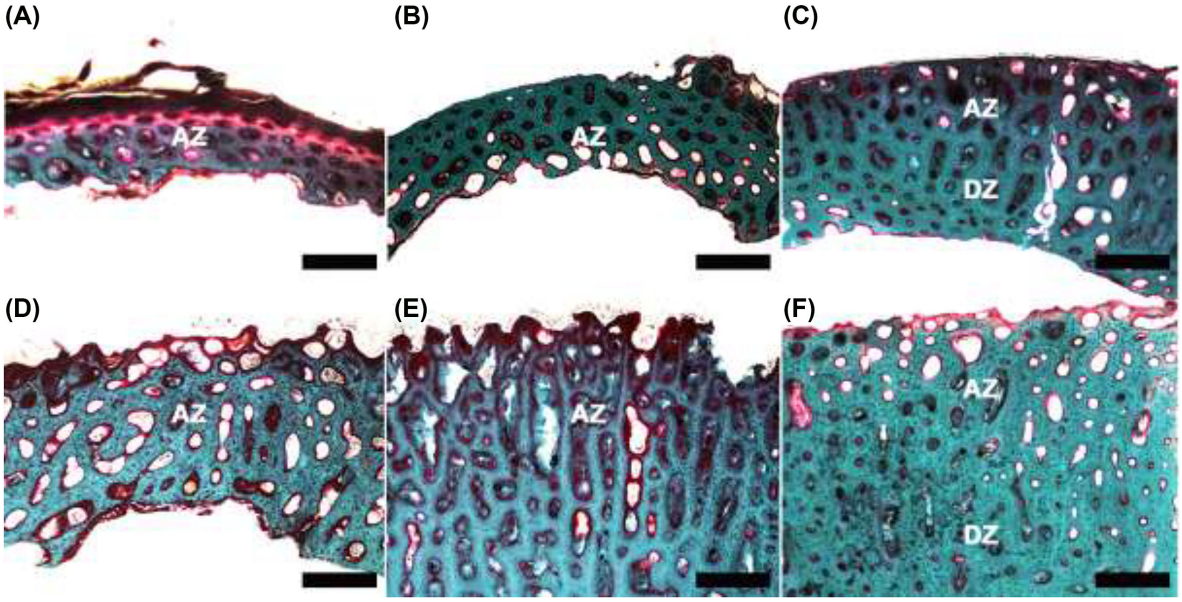

Figure 2. Images of cross-section of tibiae bone of broiler chickens of different ages as seen by optical microscopy and Masson's trichrome that stains newly formed bone tissue in red and mineralized bone in green. Tibiae bone at (A) 0 D, showing a large proportion of unmineralized bone organic matrix stained in red; (B) 3 , (C) 7 and (D) 10 D, and (E) 21 and (F) 37 D. Scale bar: 250 μm.

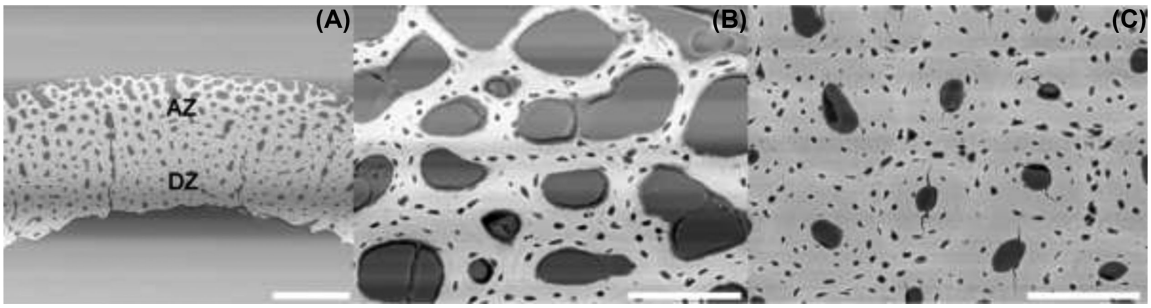

Figure 3. Images of cross-section of tibiae bone of a 37-day-old broiler chicken as seen by electron scanning microscopy (BSE-SEM). (A) Tibiae bone, (B) active periosteal area (AZ), and (C) dense cortical area (DZ). Scale bar: (A) 500 μm and (B and C) 100 μm.

bone tissue (Figure 2F). On the other hand, mature bone areas showed a green staining, as osteon are fully mineralized.

Figure 3 shows the mid-shaft cross-section of tibia from a 37-D broiler chicken as seen by scanning electron microscopy. Two distinct zones can be differentiated in the cortex: a less dense AZ at the periosteal surface consisting of primary osteons with thin mineralized walls and large oval-shaped canals, elongated toward the outer bone surface. Also, there is an inner denser zone where osteons have thicker walls and smaller canals. The osteons show also the concentric disposition of osteocytes as microporosity.

Bone Chemistry

Figure 4 illustrates the changes of bone composition with chicken age determined by FTIR spectroscopy data. The degree of mineralization (PO_4/Amide I) of cortical bone increases with age, most notably from 21 to 37 D ($P < 0.049$). In contrast, the amount of total carbonate in bone mineral ($MinCO_3$ 1415) decreases steadily from 0 to 7 D and stays nearly constant then after. On the other hand, the amount of carbonate substituted in the mineral ($MinCO_3$ 870) is maintained nearly constant during chicken growth except at 7 and 37 D in which the values are greater ($P = 0.001$). The degree of cross-linking in collagen (LNK 1660/1690) shows large variations and was not possible to identify a clear trend with age. The values reached a minimum value at 3 D and a maximum at 7 D.

Figure 5 shows the evolution of tibiae bone composition with age as determined by TGA and ICP-OES. The degree of mineralization (PO_4/OM) of cortical bone in new born chicks (0 to 3 D) is low increases notably at 7 D, remaining nearly constant until 21 D to increase again at 37 D ($P < 0.001$; Figure 4A). At this age, bone tissue reached its maximum degree of mineralization (65% mineral content). On the other hand, the amount of carbonate in the mineral (CO_3/PO_4) decreased initially from 0 to 7 D and stays nearly constant afterward (Figure 5B), following the same trend of total carbonate substituted

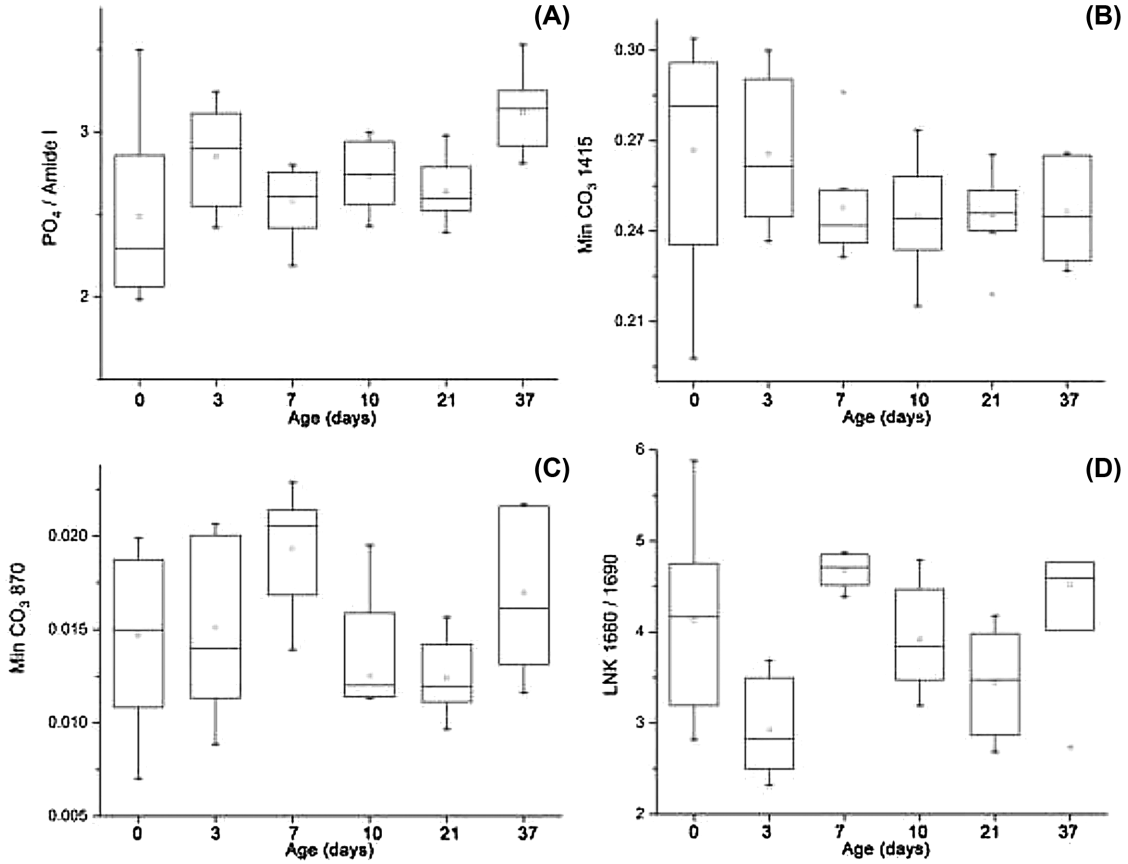

Figure 4. Composition of cortical bone determined by FTIR. (A) Degree of mineralization (PO_4/Amide I), (B) total amount of carbonate in bone mineral ($MinCO_3$ 1415), (C) degree of carbonate substitution in biomineral ($MinCO_3$ 870), and (D) cross-linking (LNK 1660/1690).

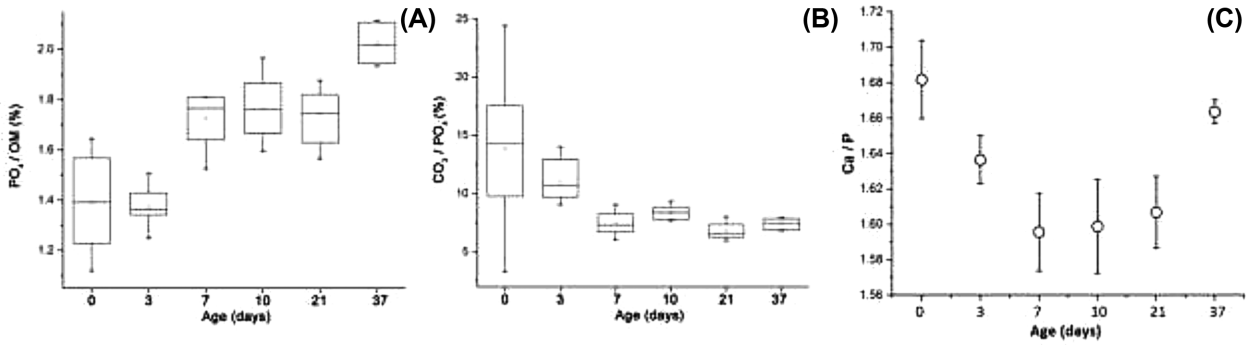

Figure 5. Composition of cortical bone determined by thermogravimetry and ICP-OES. (A) Degree of mineralization (PO_4/OM), (B) total amount of carbonate in the mineral ($MinCO_3$), and (C) Ca/P ratio in the mineral.

in the mineral (MinCO3_1415) determined by infrared spectroscopy (Figure 4B). Figure 5C shows the evolution of Ca/P ratio in bone mineral as a function of age. The Ca/P ratio steadily decreased from 1.68 (±0.04) at 0 D to 1.60 (±0.03) at 7 D, stayed nearly constant until 21 D to increase again until 1.67 (±0.01) at 37 D. These values indicate that bone mineral of chicks at hatch has a composition slightly above stochiometric apatite (1.68) and that progressively acquire a composition of increasingly calcium deficient apatite (1.60) to acquire finally a composition of stoichometric apatite (1.67). The behavior until 21 D mirrors the evolution of total carbonate in bone mineral. During the early growth phase (from 0 to 10 D), the rapid addition of bone tissue makes cortical bone acquire the composition of newly deposited bone (lower degree of mineralization, higher amount of carbonate, lower Ca/P ratio). During a later slower growth phase, there is time for bone mineralization to progress and bone tissue acquires the composition of more mature bone (higher degree of mineralization, lower amount of carbonate, higher Ca/P ratio).

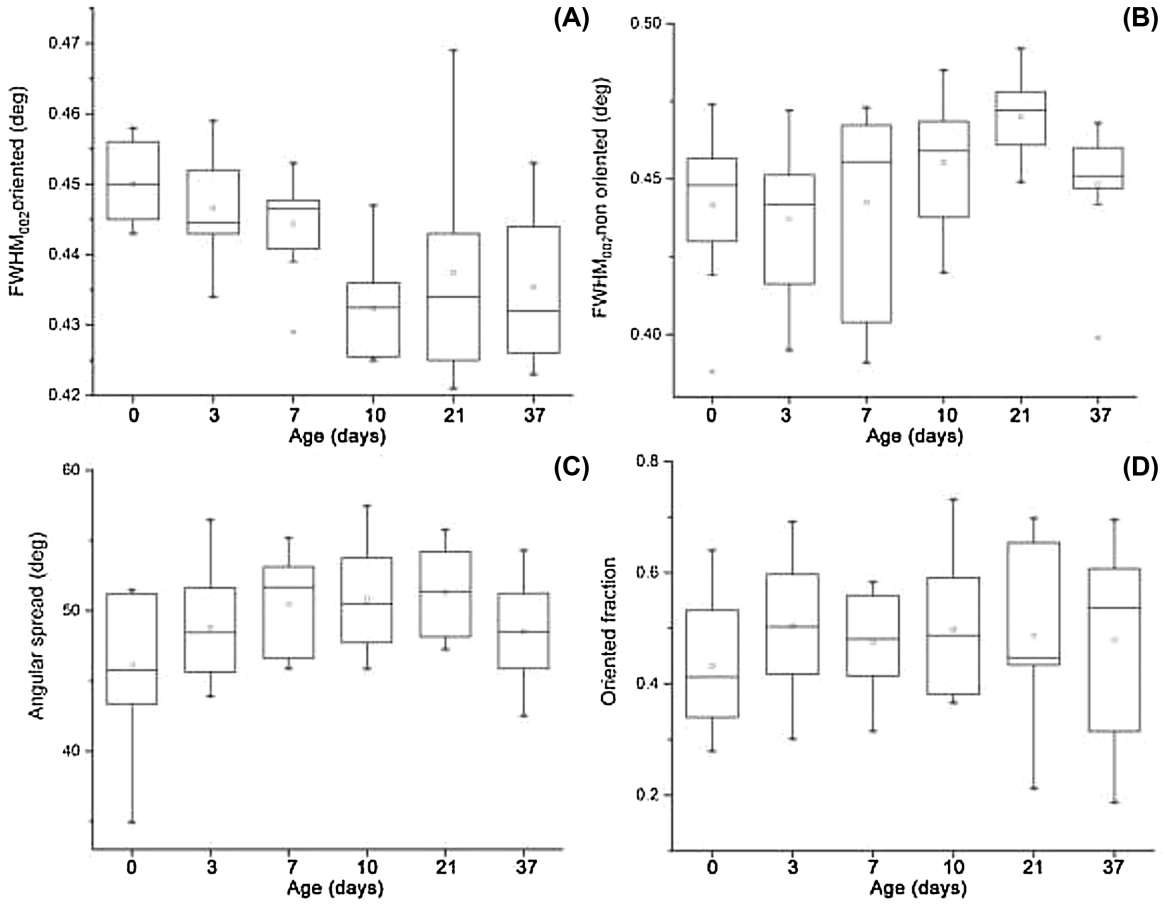

Figure 6. XRD determined parameters for cortical bone mineral and their evolution with age. (A) FWHM of 002 for the oriented apatite crystals fraction, (B) FWHM of 002 for the non-oriented apatite crystals fraction, (C) angular spread of crystal orientation calculated from 002 Gamma scans, and (D) oriented fraction Rho.

Bone Mineral Crystallinity and Crystal Orientation

The evolution of bone mineral crystallinity and the preferential orientation of apatite crystals within bone mineral were studied by 2D XRD. Figure 5 shows that the peak width of 002 reflection of the oriented mineral fraction (measured as $FWHM_{002}$ oriented) decreases with age until 10 D remaining nearly constant after that. These results indicate that the crystallite size of bone apatite increases from a minimum value of 185 Å at 0 D to a value of 200 Å at 10 D and stays nearly constant thereafter. On the other hand, the peak width of the 002 reflection for the randomly oriented mineral fraction ($FWHM_{002}$ non-oriented) slightly increases from 0 to 21 D and decreases again at 37 D. However, the changes of this parameter with age was not statistically significant ($P > 0.05$). Overall, the peak width for the 002 for the non-oriented fraction was larger than that of the 002 oriented fraction, which indicates that the crystallinity of the non-oriented fraction was always lower than that of the oriented fraction. The calculated crystallite size for the non-oriented was 175 Å compared to 185 Å, for the oriented fraction as the former represents a more immature bone mineral. On the other hand, the angular spread (**AS**) of apatite crystals c-axis in bone mineral ranges from 45 to 50 degrees, increases initially with age up to 7 D, and then remains nearly constant and decreases again at 37 D which indicates that the degree of preferential orientation of apatite crystals within the mineral decreases initially to increase in fully grown birds (37 D). We also determined the oriented fraction in bone mineral that varies between 0.40 and 0.45, increasing from 0 to 3 D and remaining nearly constant after that age. These results indicate that most of bone mineral is poorly organized as most of apatite crystals are randomly oriented like in newly deposited immature bone tissue. Thus, rapid bone mineral deposition does not allow for sufficient time for bone to become well organized.

Relationship among Bone Chemical and Structural Parameters

Pearson correlation analysis shows well-defined relationships among different bone compositional and structural parameters. Specifically, the degree of mineralization (PO_4/Amide I) and the total carbonate in bone ($CO_3$1415/Amide I) were positively and highly

correlated (R = 0.714; $P < 0.001$), as carbonate and phosphate are the main components of bone mineral. In contrast, total carbonate in bone mineral ($MinCO_3$ 1415) is negatively correlated with the degree of mineralization for all ages (R = –0.753; $P < 0.001$). Additionally, there is a positive correlation between the degree of mineralization and mineral crystallinity (i.e., FWHM 211 values decrease; R = –0.385; $P = 0.006$) as well as with collagen cross-linking (a1660/a1690) (R = 0.395; $P = 0.005$). Thus, as bone mineralization progresses, bone mineral matures, increasing in crystallinity and incorporating more carbonate into its crystalline structure (type B carbonate), whereas the relative amount of labile carbonate from the outer hydrated layer of apatite crystals is reduced during crystal maturation, accounting for the reduction of the total amount of carbonate in the mineral ($MinCO_3$ 1415).

Bone Mechanical Properties

Tibia bone-breaking strength for 37-day-old chickens ranges from 300 to 500 N. A multivariate linear regression model was built to better understand the contribution of the determined bone characteristics to tibiae mechanical properties (breaking strength) (see Table 1). We considered in the model the influence of different macroscopic parameters (CT) as well as parameters related to bone composition (degree of mineralization; **CDM**) and the structural organization of bone mineral (intensities of 002 and 211, AS of crystal orientation). The contribution of CT was low and could explain only up to 16% of the variance in bone-breaking strength. After adding the degree of mineralization (CDM) in the model there was a slight improvement of the fitting, up to 20%. Adding I_{211} and I_{002} improved the fitting up to 24 and 29%, respectively. Finally, when the AS parameter was added to the model, the fitting improved further (up to 33%). Still there was a large percentage of variability that could not be explained by the model. Other factors such as porosity that was not accounted could have a very important effect on the mechanical properties. Also, these bone properties were determined locally at the tibiae midshaft which is more mineralized than other regions of bone (distal parts). More representative measurements such as bone ash weight of whole tibiae had a larger correlation to bone mechanical properties ($R^2 = 0.490$; $P < 0.001$) as it is also a global property.

Table 1. Multivariate linear regression model of bone mechanical properties and bone material properties.

Max. Load (n = 50)	R^2	P
Total bone ash	0.490	<0.001
CT	0.163	0.002
CT+CDM	0.197	0.002
CT+CDM+I_{211}	0.238	0.001
CT+CDM+I_{211}+I_{002}	0.292	<0.001
CT+CDM+I_{211}+I_{002}+AS	0.326	<0.001

AS, angular spread of crystal orientation in cortical bone; CDM, cortical bone degree of mineralization; CT, cortical bone thickness; I_{002} and I_{211} are the intensities of 002 and 211 apatite peaks.

DISCUSSION

The present work aimed to study in detail changes with age of bone mineralization, composition, and structural organization in meat-type (broiler) chickens to better understand the development of skeletal abnormalities commonly observed in these birds and that seems to be associated to their very rapid early growth rates. This study and previous ones (Leterrier et al., 1998; Williams et al., 2000, 2004; Applegate and Lilburn, 2002; Shim et al., 2012) show that the rate at which leg bone growth in broiler chickens is coupled with increasing body weight to provide adequate support for the growing bird. Leg bones in broilers expand radially very rapidly with the deposition of primary osteons at the periosteal surface. The cortex of leg bones becomes highly porous as osteoblasts do not have enough time to fill osteons canals, at least during broiler early growth phase (first 2 to 3 wk of age) (Williams et al., 2000, 2004). Later on, with age, the bone tissue became more mineralized as osteoblasts progressively mineralize and close the inner canals. Tibiae bone thickness and its mineral density steadily increases with age reaching a maximum at 4 to 5 wk of age and staying constant afterward (Talaty et al., 2009). However, bone quality in broiler is always poorer (lower mineralization, higher porosity) than in slower growing chickens, making bones of broilers weaker and more susceptible to skeletal problems (i.e., leg deformation, rickets) (Williams et al., 2000, 2004; Angel, 2007; Dibner et al., 2007; Shim et al., 2012). Bone quality can be improved by slowing the growth of chickens by feed restriction as slower growing chickens produce stronger bones with a higher mineralization and/or reduced porosity (Leterrier et al., 1998; Williams et al., 2004). Nevertheless, leg bone size and weight in broilers, once corrected for body weight, are similar to slower growing chickens (Talaty et al., 2010; Shim et al., 2012). In any case, the first weeks of age is a critical period for bone development at which bone mineralization cannot keep pace with the rapid growth of bones that develop a large porosity and a low degree of mineralization. These bone characteristics (lower mineralization and high porosity) should negatively impact bone mechanical properties (i.e., bending, breaking strength) which are mainly determined by the amount of mineral (Williams et al., 2000, 2004; Fratzl et al., 2004) and could result in leg deformation and/or other skeletal abnormalities commonly observed in broiler chickens.

This work also shows that the chemical composition of bone tissue changes in a well-defined way during broiler growth. At the earlier ages (up to 3 to 7 D), the degree of mineralization of bone tissue is low and bone mineral is enriched in labile carbonate, having a composition typical of newly deposited bone

during early stages of bone mineralization. Subsequently, at later ages (up to 37 D), the degree of mineralization steadily increases and the total amount of carbonate in bone mineral decreases while the amount of substituted carbonate in the apatite structure increases. Finally, the degree of mineralization increases reaching a maximum of 65% (mineral) at 37 D of age which are values comparable to 72-wk-old chickens and indicates that bone is fully mineralized (Rath et al., 1999). The chemical composition and structural organization of bone mineral in fully broiler broilers is similar to laying hens (Rodriguez-Navarro et al., 2018) though bone mineral is less organized. Specifically, apatite crystals have a slightly larger AS (48 vs. 45 deg, respectively), and there is a notably smaller oriented fraction (0.45 vs. 0.90, respectively). Multivariate statistical analysis shows that bone mechanical properties has only a moderate correlation with determined bone properties (CT, degree of mineralization, whole ash weight) presumably due to large and highly heterogeneous porosity that can act as structural defects facilitating bone fracture. Nevertheless, unlike in laying hens (Rodriguez-Navarro et al., 2018), crystal orientation in bone mineral has a notable influence on bone mechanical properties.

Bone organic matrix mineralization occurs in 2 phases. The primary mineralization phase is characterized by the deposition of woven bone which has randomly oriented collagen fibers mineralized with apatite crystals rich in labile carbonate and acid phosphate, making the mineral highly reactive and soluble (Glimcher, 1998; Cazalbou et al., 2004; Fratzl et al., 2004; Donnelly et al., 2009; Rey et al., 2009; Ambekar et al., 2012; Wang et al., 2013). During a secondary mineralization phase, woven bone is resorbed and lamellar bone, formed by highly aligned collagen fibrils and mineralized with apatite crystals oriented with the c-axis parallel to the fibrils, is deposited. Subsequently, with bone mineral maturation, bone mineral crystallinity as the amount of the carbonate ionic substitutions in the apatite crystal lattice increases while the amount of labile carbonate in the non-apatitic outer hydrated layer decreases (Ou-Yang et al., 2001; Cazalbou et al., 2004; Rey et al., 2009). Therefore, newly deposited bone is less mineralized and the mineral is poorly crystalline, disorganized, and rich in labile carbonate. In contrast, older mature bone tissue is more mineralized and the mineral has a higher crystallinity, is more organized (higher crystal orientation), and has a lesser amount of labile carbonate. Also, newly deposited bone mineral is characterized with a lower Ca/P ratio due to ionic substitutions (Glimcher, 1998). Thus, the observed changes with age in bone mineralization and composition in broilers can be explained with the normal evolution of bone characteristics during the normal progress of bone tissue mineralization and maturation (Glimcher, 1998; Donnelly et al., 2009; Wang et al., 2013; Rodriguez-Navarro et al., 2018). Interestingly, bone mineral in chicks at hatch has a higher Ca/P ratio (1.68) than expected for young bone tissue. Some authors have found in fetal and newborn mice bone even an excess of calcium (>1.67) (Lange et al., 2011). Nevertheless, the deposition of new bone tissue during the fastest growth phase (first 2 wk) lowered the Ca/P ratio and bone mineral acquired the composition of calcium deficient apatite (1.60). Subsequently, at 37 D bone mineral acquired the composition of stoichometric apatite (1.67) characteristic of mature bone mineral. The behavior until 21 D mirrors the evolution of total carbonate in bone mineral, suggesting that Ca and/or P ionic substitutions in the mineral are coupled with carbonate substitution in bone. Most probably, ionic substitution occurs mainly in the outer hydrated layer of bone mineral rich in labile carbonate which is more easily accessible and reactive, whereas apatitic carbonate type B substituted in the mineral stays nearly constant (Cazalbou et al., 2004; Rey et al., 2009).

Bone disorders have both genetic and nutritional factors controlling their incidence and severity. In fact, implementation of new breeding selection strategies since the early 1990s, and nutritional supplements have ameliorated the problem by improving skeletal quality in addition to growth performance (Williams et al., 2000). Also, increased physical activity could substantially improve bone quality in chicken as increased physical activity stimulates bone formation in chickens (Whitehead, 2004; Fleming and Tsokos, 2006; Shipov et al., 2010; Aguado et al., 2015, 2017; Rodriguez-Navarro et al., 2018). In the case of broilers, the increase in body weight can stimulate bone growth. On the other hand, the mechanical properties of bone are mainly determined not only by its mineral content but also of its geometrical distribution, and its structural organization at different scales (Martin and Ishida, 1989; Jäger and Fratzl, 2000; Nakano et al., 2002; Boskey and Mendelsohn, 2005; Gupta et al., 2006; Gourion-Arsiquaud et al., 2009; Zimmermann et al., 2011; Rodriguez-Navarro et al., 2018). In fact, though the total amount of mineral measured by total ash content and the CT were the best predictors for chicken tibiae breaking strength, parameters related to the mineral organization determined by XRD (i.e., I_{211}, I_{310} and AS) had a small but notable contribution to explain the variability in bone-breaking strength. Thus, crystal orientation in bone mineral has a notable influence on bone mechanical properties in broilers unlike in laying hens (Rodriguez-Navarro et al., 2018).

In conclusion, in this study we show that there are well-defined changes in the bone composition and its structural organization with chicken age. We have also identified that during the earliest stages of growth (first 2 wk), the correct mineralization and structuring of bone is compromised. Bone has a low degree of mineralization and is highly porous making bone weak. Specific actions (improved nutrition, genetic selection) need to be done at early age to correct for this problem. Thus, knowledge acquired in this study could help in designing strategies to improve bone quality and reduce the

incidence of skeletal problems in broiler chickens. This study also shows that broiler chickens are a very interesting and unique animal model for studying bone pathologies associated to rapid growth that challenges the correct mineralization of bone.

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