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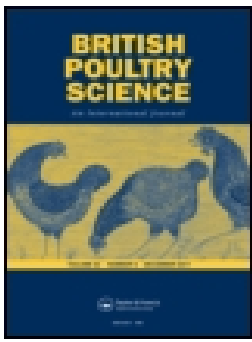
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Comparative analysis of the morphology, chemistry and structure of the tibiotarsus, humerus and keel bones in laying hens

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ABSTRACT

1. Bone properties are adapted to their specific functions in the animal, so various types of bones develop different characteristics depending on their location in the skeleton.

2. The aim of this research was to compare the chemical composition, crystalline characteristics and structural organisation in tibiotarsus, humerus and keel bones as representatives of hen skeletal mineralisation. Complementary analytical techniques, such as X-ray radiography, optical and electron microscopy, thermogravimetry and 2D X-ray diffraction, were used for characterisation.

3. The humerus had a thinner cortex and cortical bone mineral had higher crystallinity and a greater degree of crystal orientation than the tibiotarsus. The humerus generally lacks medullary bone although, when present, it has a more mineral content than seen in the tibiotarsus. These differences were attributed to the different forces that stimulate bone formation and remodelling.

4. The keel cortical bone had a lower degree of mineralisation than the tibiotarsus or humerus. Its degree of mineralisation decreased from the cranial to the distal end of the bone. This gradient may affect keel mechanical properties, making it more prone to deformation and fractures.

5. Data from studying different bones in laying hens can help to understand mineralisation as well as finding solutions to prevent osteoporosis-related fractures.

INTRODUCTION

Bone is a composite material composed of an inorganic phase (nanocrystalline carbonated apatite), organic matrix (mainly type I collagen) and water, in proportions varying according to age and location within the skeleton (Weiner and Wagner 1998; Fratzl *et al.,* 2004; Stock 2015). It is a living tissue that is constantly accreting and being remodelled by bone cells so that it can adapt to mechanical loads and supply minerals (i.e., Ca, P, Mg) needed for basic cellular functions (Glimcher 1998; Bonucci 2013).

The skeleton is designed to support the body weight and is scaled depending on body size (Schmidt-Nielsen 1984). Bone composition and structural changes occur in different parts of the skeleton, depending on their function. For instance, the skeleton of birds has unique characteristics as it is specially adapted for flying, walking on two legs and egg laying. Birds have pneumatic bones (such as the humerus) that are hollow and light to facilitate flight. The medullary bone, a type of woven tissue, fills the marrow cavities of the long bones and serves as a calcium reservoir for the rapid calcification of the eggshell (Dacke *et al.,* 1993; Whitehead 2004; Nys and Le Roy 2018; Kerschnitzki *et al.,* 2014). This is part of the specific adaptations that female birds have developed for egg laying as they need to store and mobilise large amounts of calcium for eggshell formation (Nys and Le Roy 2018), coming from both the diet and the skeleton.

During rearing, the hen's skeleton grows rapidly until reaching sexual maturity after 16 weeks of age, ceasing further development at about 20 weeks (Whitehead 2004; Fleming *et al.,* 2006; Nys and Le Roy 2018). When hens reach

sexual maturity, ~2 weeks before laying their first egg, there is a dramatic change in calcium and bone metabolism. Hormones stimulate the production of the active form of vitamin D in the kidneys, which greatly increases intestinal and uterine calcium absorption capacity to allow an adequate supply of calcium for eggshell formation (Wu *et al.,* 1995). At the same time, oestrogen diverts the function of osteoblasts from forming structural (cortical and trabecular) to medullary bone to store calcium for eggshell calcification (Dacke *et al.,* 1993; Van De Velde *et al.,* 1985; Whitehead 2004). Oestrogen induces differentiation of osteoblasts and decreases the number of osteoclasts on the endosteal surface (Kusuhara and Schraer 1982; Ohashi *et al.,* 1987). The formation and resorption of medullary bone is synchronised with the daily cycle of egg laying so that it supplies the calcium needed during night when the eggshell is mineralising and dietary calcium in the gut may be exhausted (Nys and Le Roy 2018). However, during egg laying, medullary bone is formed at the expense of cortical bone, which can result in a progressive loss of structural bone and the development of a severe form of osteoporosis if the balance of calcium supply and demand are unbalanced (Rodriguez-Navarro *et al.,* 2018; Fleming *et al.,* 2006). This problem may be aggravated in the sternum, since, at the onset of lay, the keel bone is poorly mineralised and the caudal part of it is entirely cartilaginous (Riber *et al.,* 2018). Keel mineralisation, which continues until hens are about 40 weeks old or more, may compete with eggshell formation, preventing the keel from receiving an adequate amount of calcium to become fully mineralised. This may be one of the reasons why keel bone has the highest incidence of fractures and deviations in hens, especially at the end of the laying cycle (Riber *et al.,* 2018; Eusemann *et al.,* 2018; Heerkens *et al.,* 2016; Petrik *et al.,* 2014; Richards *et al.,* 2012; Wilkins *et*

al., 2011; Käppeli *et al.,* 2011; Rodenburg *et al.,* 2008; Fleming *et al.,* 2004). Keel bone fractures are likely to be painful and impair animal welfare (Nasr *et al.,* 2012, 2013; Riber *et al.,* 2018). Furthermore, in commercial farms, keel bone fractures are less likely to be detected than long bone fractures, potentially leading to prolonged suffering (Fleming *et al.,* 2004). The development of avian osteoporosis and the high incidence related bone fractures, particularly at the end of the laying cycle, is one of the most relevant welfare problems facing the egg industry nowadays (Whitehead 2004; Webster 2004; Mazzuco and Hester 2005; Fleming *et al.,* 2006). This has important economic implications, as birds affected by bone fractures have a decreased egg production and increased food intake and mortality (Nasr *et al.,* 2012, 2013; Riber *et al.,* 2018).

The following work was a detailed study of the morphology, structure and composition of representative bones of the skeleton of laying hens. The tibiotarsus, a fusion of tibia and some of the tarsal bones, and the humerus and keel bone were assessed. The work particularly focussed on the keel, since this bone is the most frequently affected by fractures and deformations with incidence of up to 62 - 82% or higher, in birds at the end of the laying cycle (Riber *et al.,* 2018; Eusemann *et al.,* 2018; Heerkens *et al.,* 2016; Petrik *et al.,* 2014; Richards *et al.,* 2012; Wilkins *et al.,* 2011; Käppeli *et al.,* 2011; Rodenburg *et al.,* 2008; Fleming *et al.,* 2004). The keel is part of the sternum and the most prominent frontal part of the skeleton where the pectoral muscles attach (Zheng *et al.,* 2012). It is a flat bone and has a different structure and properties compared to long bones (*i.e.*, tibia, humerus). The tibia, as part of the lower limb, is a weight bearing bone. It has been widely used as an indicator of skeletal growth and mineralisation in chickens, due to its high growth rate and is one of the most

mineralised bones in the skeleton (Skinner and Waldroup 1995; Angel 2007; Shim *et al.,* 2012). The humerus, although a non-weight bearing bone, undergoes loading from the attached muscles involved in flight. To date, little is known about the differences in bone structure and composition between these types of bones. The selected bones are good indicators of skeletal mineralisation and have been used to determine an index based on measurements of breaking strength and radiographic density for the selection of hens with better bone quality (Bishop *et al.,* 2000; I. C. Dunn *et al.,* 2007). A closer look at such differences can help to understand skeletal structure in laying hens and why specific bones, especially the keel bone, are more prone to fractures. The information gathered in this research can help define strategies to reduce the incidence of skeletal problems in commercial poultry farms.

MATERIALS AND METHODS

Bone samples.

Bone material was collected from Rhode Island Red (RIR) laying hens at the end of the breeding cycle (at 62 weeks old) after euthanasia with pentobarbitone. All birds were culled between 08:00 and 12:00 hours when the hens would be in the process of early shell formation (Lights off 17:00, 16L:8D). Tibia (n =30), humerus (n =30) and keel (n =30) bones were selected at random from 307 birds used in a previous study (Dunn *et al.,* 2021) using the 'Generate Random Sample' command without replacement in Genstat v18 (https://www.vsni.co.uk/software/genstat). No further selection was applied to the samples. The body weight at cull was 1901 ± 38 g and egg production until cull

was 274.2 ± 2.9. The cortical and medullary bone from the tibia and humerus mid-diaphyses and from the keel were manually separated using a scaler after sectioning with a saw. Cortex thickness was measured in the same region of each sample with a digital micrometer (four measurements per sample). In keel bones, three different locations (cranial or proximal, middle and distal end or leading edge) were sampled.

X-ray radiography

The whole bird and individual bones were radiographed in a Faxitron 405 soft X-ray apparatus (Faxitron, Tucson, AZ, USA). Exposure was 15 s at 28 kV. Each exposed plate included an aluminium step wedge for calibration.

Optical microscopy.

A random selection of bone samples (n=5 each bone) were prepared for histological analyses. The samples were fixed in neutral buffered formalin and then decalcified in 10% EDTA (pH 7.4). Afterwards, samples were dehydrated, cleared in xylene, and embedded in paraffin wax. Serial histological sections, 5 μm thick, were cut with a Leica RM 2235 microtome and stained with haematoxylin and eosin. Stained thin-sections of transversally cut bone were viewed with an optical microscopy (Leica DMRB, Germany).

Electron microscopy.

Scanning electron microscopy (SEM) was carried out on polished cross-sections of the tibia and humerus mid-diaphyses, and from keel sagittal sections. The bone samples were fixed in 4% glutaraldehyde, embedded in epothin epoxy resin (Buehler, Lake Bluff, IL, USA), cut, polished, coated with carbon (Hitachi UHS evaporator, Tokyo, Japan) and observed with a variable pressure SEM (Leo 1430-

VP, USA) using a backscattering electron (BSE) detector and an accelerating voltage of 30 keV.

Thermogravimetry.

Different compositional parameters were determined by thermogravimetry (TGA).to describe the chemical composition of cortical and medullary bone from tibia, humerus, and keel bone. The percentage of water, organic matter, carbonate and mineral content in the bone samples were determined by TGA. Powdered bones were treated by heating for 1 h at different temperatures (200, 600, and 900 C) in a RWF 1100 furnace (Carbolite, UK) and weighed to determine the percentage of each component.

Two-dimensional (2D) X-ray diffraction.

Tibia and humerus cortical bone (~ 1× 0.5 cm) cut from the mid shaft of the diaphysis and similar size pieces from the keel bone tip were analysed in the transmission mode of a single crystal diffractometer equipped with an PHOTON area detector (D8 Venture, Bruker, Germany) and Mo radiation. Crystallinity of bone minerals 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 2D X-ray diffraction patterns. The sharper the peaks and smaller the FWHM, the greater 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 (Gamma scan; Rodriguez-Navarro *et al*., 2018). The wider the band, the greater the scattering in the orientation of the c-axis of apatite

crystals. XRD2DScan 7.0 software was used to analyse the collected 2D X-ray diffraction patterns (PANAlytical, The Netherlands).

Statistical analyses

Basic descriptive statistics were used to characterise bone properties. Analysis of variance (one-way ANOVA) and the *post-hoc* Tukey test were used to compare properties between different types of bones. P values <0.05 were considered statistically significant. All analyses were performed using Origin Pro (Microcal) or SPSS (IBM) software packages.

RESULTS

Bone morphology.

Figure 1 shows a lateral view of the laying hen skeleton with a fully formed egg and individual bones (tibia, humerus and keel). These X-ray images clearly showed the morphology and size of different bones from the lower and upper limbs as well as the vertebrae and rib cage with the keel and sternum. Long bones are filled by low density material that weakly absorbs X-rays and are delineated by a lighter outer rim, produced by the denser cortical bone that more strongly absorb the X-rays (Figure 1A). The X-ray density of different bones was markedly different (P<0.001) and decreased following the sequence tibia > humerus > keel (Figure 1B and Table 1).

Fig 1 and Table 1 here

All different analysed bones (tibiae, humeri and keels), even though being very different morphologically, had a thin outer shell of cortical bone and a cavity partially filled with medullary bone (Figure 2). The keel is a slender flat bone with a triangular shape. The keel sample of cortical bone at the surface and medullary bone filled only the central part between the thin lateral plates (about 170 μm thick; Figure 2A). Humerus and tibia are long bones, but the former is shorter and wider and has a thinner cortex (see Fig 1 and Table 1). The humerus had a hollow and empty marrow cavity (pneumatised bone) but, in some hens (about 1/3), this cavity may be partially or even completely filled with medullary bone (Figure 2E). Tibiae bone are generally filled with medullary bone, mostly near the endosteal surfaces (Figure 2A). Cortical and medullary bone had very different organic matrix composition and stained differentially. For instance, haematoxylin and eosin staining produced a characteristic lighter pink colour in structural cortical bone, whereas for woven medullary bone it produced a darker purple/blue colour (Figure 2). The bone structure of tibia and humerus had a similar appearance at the microscopic level when viewed using either optical or electron (BSE-SEM) microscopy observation (Figure 2 B-D). Cortical bone was remodelled by osteons, characterised by the concentric distribution of osteocytes around a central canal (*e.g.*, Haversian channels) where blood vessels and nerves run (Figure 2B). The medullary bone consisted of isolated trabeculae elements which were generally surrounded by a large number of osteoclasts and/or osteoblasts. The medullary bone trabeculae could incorporate osteocytes but did not have osteons or Haversian channels (Fig 2C). The tibia of hens with severe osteoporosis showed cortical bone with large resorption cavities in which the

cortical bone was partially replaced by medullary bone, making them fragile and prone to fracture (Figure 2D).

Fig 2 here

Bone chemistry

The chemical composition of bone was analysed by TGA and the percentage of the main chemical components of bone tissue: water, organic matter (collagen), carbonate and phosphate (apatite), were determined. The percentage of these components were used to determine different bone compositional parameters: %Mineral, %CO_3.

Table 1 summarises the main properties determined for the different types of bone. The mineral content of cortical bone in tibia and humerus was similar (about 66-67%). In the keel, cortical bone had a slightly lower mineral content which decreased toward the distal end, or keel leading edge (cranial: 63.1%; middle: 62.3%; distal end: 62.2%). However, cortical bone in keel bone had slightly lower amounts of carbonate (6.5 %) than seen in the tibia and humerus (7-8%). Regarding medullary bone, mineral level, as determined by TGA, showed slightly greater values for humerus than for tibia (38 *vs*. 31 %) which indicated that, when medullary bone was present in the humerus, it was better mineralised. In keel samples, the mineral content in medullary bone, as determined by TGA, was greater (up to 61%) than in the other types, but decreased toward the distal end (57%). In the keel, medullary bone has a significantly lower amount of carbonate (6%) than in tibiae or humerus (13 and 11%, respectively). It was noted

that medullary bone particles in the humerus, tibia and keel showed similar brightness when observed under SEM (in the black scattered electron (BSE) mode), which suggested that medullary bone may have the same mineral content in all bones. Thus, the lower mineral content of medullary bone in tibiae and humerus bone was due to the fact that mineral particles were mixed with marrow organic matter. Thus, keel should have a lower amount of organic matter in the medullary space.

Bone mineralogy

The crystallinity and organisation of bone mineral in the tibia, humerus and keel samples were analysed by X-ray diffraction. The 2D X-ray diffraction patterns from the tibia and humerus cortical bone showed that 002 and 310 reflections were concentrated in arcs as apatite crystals and were preferentially oriented with their c-axis, parallel to the long axis of bone (Figure 3A, B). In contrast, for keel bone (Figure 3C), the intensity of rings was homogeneous as apatite crystals were randomly rotated within the keel wall.

Fig 3 here

Similarly, the intensity profile along 002 ring (002 Gamma scan) showed a broad peak for both tibia and humerus, and there was a nearly constant intensity in keel bone, due to the preferential orientation of apatite crystals in bone mineral in the former bone, and random orientation in keel (Figure 3E). On the other hand, the 2Theta scans, calculated by radially integrating the 2D patterns, show highly anisotropic peak which broadened as apatite crystals elongated along the c-axis,

producing sharper peaks for the 002 reflections than for the 310 reflections (Figure 3D). Generally, cortical bone mineral in the tibia had a slightly lower crystallinity than in the humerus (smaller crystallite size; 191 *vs*. 244 Å) and lower degree of crystal orientation (higher AS values; 49 *vs*. 41 deg.). Keel bone mineral had lower crystallinity (crystallite size 179Å) than the other bones and produces a 2Theta scan with broader peaks. It showed a broad band at lower angles (around 10°) due to the diffuse scattering of the organic matrix.

To study particular variations in bone mineral density, crystallinity and crystal orientation within bone mineral the tibia, humerus and keel bone samples were analysed at different points (1 cm apart) along the bone, starting from the distal (or leading edge of the keel) to the cranial end. Figure 4 shows the evolution of parameters determined by X-ray diffraction (intensity of the main reflection for apatite (211), crystallite size and angular spread or scattering in the orientation of bone apatite crystals using the 002 reflection) at different positions along the bone. For the tibia, these parameters did not change significantly with position, which indicated that bone mineral density, crystallinity and mineral organisation was quite homogenous in this bone. The humerus and keel, however, did show a gradual increase in the diffracting intensity and in crystallite size with position, which was most notable in the keel bone, whereby the crystallite size increased from 100 to 200 Å. In addition, for the humerus, there was a gradual decrease of the angular spread, which indicated that the organisation or degree of crystal orientation increased toward the midshaft. All in all, the keel data showed that the amount of mineral and its crystallinity increased from the leading edge to the base of the keel. In the humerus, the mineral component had increased crystallinity and became better organised, manifested as higher crystal orientation, toward the

midshaft, whereas the tibia showed a more constant arrangement. The minerals in the medullary bone consistently had a much lower crystallinity (crystallite size; 166 Å) than cortical bone in the tibia or humerus or even the keel bone.

DISCUSSION

This study examined bone structure, composition and mineralisation in laying hens using bones from different locations within the skeleton (tibia, humerus and keel). A special focus was put on explaining possible differences between these bones, especially the keel, which shows a high fracture prevalence and required a thorough assessment (Toscano *et al.,* 2020; Harlander-Matauschek *et al.,* 2015; Riber *et al.,* 2018; Eusemann *et al.,* 2018; Heerkens *et al.,* 2016; Petrik *et al.,* 2014; Richards *et al.,* 2012; Wilkins *et al.,* 2011; Käppeli *et al.,* 2011), the humerus, as a non-weight bearing long bone in the wing, and the tibiotarsus, as a weight bearing long bone of the leg. The keel bone is likely to be affected by the pressure caused by the perching while resting (Pickel *et al.,* 2011). One cause of the higher prevalence of fractures and deformation in the keel, compared to other bones, may be due to its prominent location within the hen's body and the relatively low breast muscle mass in laying hens, that lead to exposure and vulnerability (Fleming *et al.,* 2004). The data indicated that the high prevalence could also be related to the non-homogenous mineralisation and complex structure of this bone as described hereafter.

The structure and composition of the tibia, humerus and keel have been adapted to their specific function and the forces to which they are subjected in the skeleton.

The current analyses showed large differences in mineralisation, composition and structure. For instance, the humerus, compared to tibia, had a thinner cortex with a slightly higher degree of mineralisation and crystallinity and greater degree of crystal orientation. These differences in bone characteristics may be due to the different type of mechanical loading (Fleming *et al.,* 2006). The tibia supports the birds body weight and are continually subjected to the forces of walking. These forces are known to stimulate bone formation and can explain why the tibia developed a thicker cortex. In fact, when hens have greater physical activity (flying, perching), their bones have a greater cross-sectional area and a thicker cortex (Newman and Leeson 1998; Shipov *et al.,* 2010). Mechanical loading stimulates bone remodelling, changing its composition and organisation (Casey-Trott *et al.,* 2017; Fleming *et al.,* 2006; Rodriguez-Navarro *et al.,* 2018). Rapid bone turnover constantly renovates bone mineral turnover, preventing it acquiring the highly crystalline, organized composition of mature bone tissue which may explain the differences between the tibia and humerus (Donnelly *et al.,* 2010; Wang *et al.,* 2013; Rodriguez-Navarro *et al.,* 2018). These structural and compositional differences can be expected to have an effect on the mechanical properties of these bones (Martin and Ishida 1989; Nakano *et al.,* 2002; Fleming *et al.,* 2006; Ishimoto *et al.,* 2013).

It is important to consider the contribution of medullary bone to the mechanical properties of bone. That may work in two ways, directly by providing strength (Fleming *et al.,* 1998; Rodriguez-Navarro *et al.,* 2018) or potentially by protecting the cortical bone from resorption, through ensuring an adequate supply of calcium for egg formation from medullary bone. The contribution of medullary bone may be more important in the tibia, as in general it contains a significantly larger

amount of this type of bone. It should be remembered that the amount and properties of medullary bone changes during the daily egg cycle (Van De Velde *et al.,* 1985; Kerschnitzki *et al.,* 2014). In the current study, all birds were sacrificed within a limited time frame (from 08:00 to 12:00) when they would be in the process of early shell formation. Thus, the daily variation in medullary bone should have been minimised.

Cortical keel bone had a lower degree of mineralisation and higher carbonate content compared with tibia and humerus. In addition, the mineral component was less crystalline and highly disorganized (apatite crystals are randomly oriented). Mineral content, bone mineral crystallinity and composition markedly change across the keel, increasing from the distal end to the middle-cranial region. The flat shape and spatial variation in the degree of mineralisation should greatly affect keel bone mechanical properties and make them more prone to deformation and fracture, particularly in regions with major changes in mineralisation. Keel bone mineral density increases with hen age and physical activity (Fleming *et al.,* 2006). In this study, the keel bone was almost fully mineralised as the hens used were older. However, in young hens the keel is poorly mineralised and should be more prone to deformation. Regarding physical activity and the keel, when the use of the pectoral muscles was encouraged by housing hens in an aviary system, as opposed to cages, the density of the keel increased as well as long bone strength (Fleming *et al.,* 2006). This seemed to be a direct effect of increasing loading that stimulates structural bone formation and/or reduces its resorption (Fleming *et al.,* 2006; Newman and Leeson 1998; Shipov *et al.,* 2010). Unfortunately, although greater physical activity improves bone quality, it increases collisions in extensive

systems and the incidence of keel bone fractures and deformations (Riber *et al.,* 2018).

The current study demonstrated how different parts of the hen's skeleton, such as the humerus, tibia and keel bone, have different characteristics at the morphological level and in the degree of bone mineralisation, chemistry, crystallinity and structural organisation of bone mineral. Selecting hens for early sexual maturity and high egg production may prevent them from building a well mineralised skeleton and accumulating sufficient amounts of medullary bone needed during lay to export an adequate amount of calcium for eggshell mineralisation (Riber *et al.,* 2018; Nys 2017; Dunn *et al.,* 2021). Thus, high calcium demand during lay challenges hen calcium homeostasis and skeletal mineralisation appears to be responsible for the observed skeletal problems, with a high incidence of bone fractures and deformation, especially in keel bone, that does not become fully mineralised until hens are 40 weeks or older (Riber *et al.,* 2018; Whitehead 2004). This problem could worsen as the industry aims to extend the laying cycle of commercial flocks from 65-70 to 100 weeks and produce 500 eggs per hen in a single cycle without moult (Bain *et al.,* 2016; Nys 2017). Therefore, strategies based on selection, husbandry and/or nutrition have to be implemented to maintain bone quality in general, and keel bone quality in particular, in older hens. The information gathered from the current study can help to better understand the properties of different bones and their susceptibility to fracture as well as the implications on bettering animal husbandry practices to improve animal welfare.

CONCLUSIONS

This study showed significant differences in mineral composition and structural organisation in bones from different anatomical locations in laying hens. The keel, tibia and humerus presented notable differences in the cortical and medullary bone properties, which were attributed to the different forces that act on them and their different physiological function within the skeleton. The variations in composition (*i.e.*, degree of mineralisation, carbonate content) and structural characteristics (*i.e.*, orientation degree, crystallinity) within each bone type should greatly affect its mechanical properties, and could be, in part, responsible of the higher incidence of deformation and fractures (in keel bone). This knowledge could help in designing strategies to reduce the incidence of skeletal problems (deformation and fractures) in laying hens. The current study provided relevant information about the implication of bone quality on animal welfare in poultry and can be used to inform efforts to improve bone quality by genetic selection.

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Table 1. Summary of determined bone properties for tibia, humerus and keel bone using different analytical techniques (X-ray density, XRD, TGA). XRD parameters: Crystallite size (D002) and AS_002. TGA parameters: Mineral and CO3/PO4.

	Tibia		Humerus		Keel		F	P value
	Mean	SD	Mean	SD	Mean	SD		
Cortical thickness (mm)	0.593^a	0.125	0.388^b	0.065	0.176^c	0.04 1	123.6	<0.001
X-ray density Al (mm)	2.43^a	0.26	1.58^b	0.39	0.82^c	0.09	263.8	<0.001
Cortical bone								
Mineral (%)	66.1^a	1.5	67.4^a	2.0	62.6^b	6.7	25.0	<0.001
CO3/PO4 (%)	8.1^a	1.4	7.6^a	0.8	6.5^b	0.8	13.4	<0.001
D002 (Å)	191.2^a	44.9	244.1^b	16.6	194.1^a	16.4	23.6	<0.001
AS_002 (deg.)	48.8^a	5.0	42.1^a	4.2	180.0^b	0.0	30.4	<0.001
Medullary bone								
Mineral (%)	31.5^a	8.1	38.2^b	10.9	61.4^c	5.4	55.6	<0.001
CO3/PO4 (%)	13.4^a	4.6	11.2^b	3.1	6.1^c	0.7	24.2	<0.001

Different letters in rows indicate significant differences between the types of bones in the Post-hoc Tukey test.

FIGURE LEGENDS

Figure 1. X-ray radiography. A) Laying hen skeleton with a fully formed egg. The aluminum step wedges used for density calibration are shown on the left bottom corner; B) Images of selected bones (tibia, humerus and keel) for this study. (Scale bar = 10 mm). C, M and D indicate the cranial, middle and distal end or leading edge, regions of the keel.

Figure 2. Bone morphology. A) Histological cross-section of a tibia at mid diaphysis (Hematoxylin and eosin, Scale Bar = 500μm). B) BSE image of tibiae cortical bone (Scale bar = 20 μm); C) BSE image of tibiae medullary bone (Scale bar = 20 μm); D) BSE of tibiae cortical bone with large resorption centers (RC) from a hen with severe osteoporosis (Scale bar = 100 μm); E) Detail of histological section of non-pneumatised humerus (Hematoxylin and Eosin, Scale bar = 500μm). F) Histological section of a keel bone cranial area cut from dorsal to ventral (frontal plane) (Hematoxylin and Eosin, Scale bar = 2 cm). G) Histological section of a keel bone through the carina sternii (anterior process). The leading edge of the keel bone is on the left and the base of the keel bone on the right. The two lateral plates with cortical bone and medullary bone filling the space between are clearly visible (Hematoxylin and Eosin, Scale bar = 2cm).

Figure 3. 2D X-ray diffraction patterns from cortical bone in tibia (A), and humerus (B), and keel bone (C). The white arrows indicate the direction of elongation of bones and the preferential orientation of apatite crystals. D) 2Theta scan of tibiae, humerus and keel bone calculated by radial integration of

intensities in 2D patterns. E) Gamma scan displaying the intensity variation along the 002 Debye ring for tibiae, humerus and keel bone. AS stands for angular scattering in the c-axis orientation of apatite crystals.

Figure 4. Changes in cortical bone properties determined by X-ray diffraction in different locations in a tibia, humerus and keel bone: A) Intensity of main 211 apatite reflection; B) Crystallite size; C) Angular scattering (AS) in the orientation of apatite crystals.

TABLES

Table 1. Summary of determined bone properties for tibia, humerus and keel bone using different analytical techniques (X-ray density, XRD, TGA). XRD parameters: Crystallite size_002 and AS_002. TGA parameters: Mineral and CO3/PO4.

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D002_CB (Å)	191.2	44.9	244.1	16.6	194.1	16.4	23.6	<0.001
AS_002_CB (deg.)	48.8	5.0	42.1	4.2	180.0	0.0	30.4	<0.001
Medullary bone								
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CO3/PO4 (%)	13.4	4.6	11.2	3.1	6.1	0.7	24.2	<0.001