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MORPHOLOGICAL, DENSITOMETRIC AND BIOMECHANICAL EVALUATIONS OF THE BONE STRENGTH IN BROILERS

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Ph.D. THESIS

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The thesis entitled "Morphological, Densitometric and Biomechanical Evaluation of the Bone Strength in Broilers" written by Ms. Komal KHAN within the framework of graduate program of Aydin Adnan Menderes University, Institute of Health Sciences, Department of Anatomy, was accepted as PhD thesis by the following jury.

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ABBREVIATIONS

BMD	: Bone Mineral Density
BMC	: Bone Mineral Content
Ca	: Calcium
Cd	: Caudal
CDCT	: Caudal Cortical Thickness
CI	: Confidence Interval
CMI	: Corticomedullary Index
CMI_{CRCD}	: Cranio-caudal Corticomedullay Index
CMI_{ML}	: Medio-lateral Corticomedullay Index
CO_2	: Carbondioxide
Cr	: Cranial
CRCT	: Cranial Cortical Thickness
CT	: Computed Tomography
CT	: Computerized Tomography
CV	: Coefficient of Variation
D	: Dorsal
DCT	: Dorsal Cortical Thickness
DEA	: Dual Energy Absorptiometry
DEXA	: Dual Energy X-Ray Absorbiometry
E	: Elastic Modulus
ExtCrCdD	: External Craniocaudal Diameter

ExtDVD	: External Dorsoventral Diameter
ExtMLD	: External Mediolateral Diameter
F	: Force
Fapc	: Femur Anteroposterior Curvature
Fedα	: Femur Distal Angulation
F_{max}	: Maximum Force
g	: Gram
G	: Shear Modulus
g/g	: Gram Per Gram
IntCrCdD	: Internal Craniocaudal Diameter
IntDVD	: Internal Dorsoventral Diameter
IntMLD	: Internal Mediolateral Diameter
I_x/I_y	: Moment of Inertia
i.e.	: That is
kN	: Kilo Newton
L/D	: Length To Diameter Ratio
LCT	: Lateral Cortical Thickness
MCT	: Medial Cortical Thickness
MRI	: Magnetic Resonance Imaging
mSv/hour	: Millisieverts
P	: Phosphorus
PMMA	: Polymethylmethacrylate
pQCT	: Peripheral Quantitative Computed Tomography

QCT	**:** Quantitative Computed Tomography
S	**:** Stiffness
SD	**:** Standard Deviation
Tapc	**:** Tibial Anteroposterior Curvature
TD	**:** Tibial Dyschondroplasis
Tidα	**:** Tibia Distal Angulation
Tidβ	**:** Tibia Distal Angulation
Tipβ	**:** Tibia Proximal Bending
vBMD	**:** Volumetric Bone Mineral Density
VCT	**:** Ventral Cortical Thickness
VV	**:** Varus-Valgus Deformity
ε	**:** Strain
°C	**:** Degree Celcius
τ	**:** Shear Stress
%	**:** Percentage
σ	**:** Stress

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ÖZET

ETLİK PİLİÇLERDE KEMİK DAYANIMININ MORFOLOJİK, DANSİTOMETRİK VE BİYOMEKANİK YÖNTEMLERLE DEĞERLENDİRİLMESİ

Khan K. Aydın Adnan Menderes Üniversitesi, Sağlık Bilimleri Enstitüsü, Veteriner Anatomi, Doktora Tezi, Aydın, 2021.

Amaç: Bu çalışmanın temel amacı etlik piliç uzun kemiklerinin morfolojik, dansitometrik ve biyomekanik özelliklerini analiz etmektir.

Gereç ve Yöntemler: Toplam 32 adet etlik pilice ait kanat ve bacak uzun kemikleri (humerus, radius, ulna, femur ve tibia) diseke edilip, dondurucuda saklandı. Bilgisayarlı tomografi görüntüleri kullanılarak kemiğin genel ve kesitsel geometrisini gösterir morfometrik ölçümler alındı. Radius ve ulna dışındaki kemikler için kemik yoğunlukları DEXA ile ölçüldü. Sağ taraf kemiklerinde üç nokta eğme testi sol taraf kemiklerinde kesme testi ile biyomekanik testler uygulandı.

Bulgular: Sağ ve sol taraf kemiklerinin morfometrik özellikleri arasında istatistiksel olarak farklılık görülmedi. Kortikal kalınlık indeksleri en yüksek olarak radius'da, en düşük femur'da görüldü. Maksimum eğme dayanımı, elastik modulus ve maksimum kesme dayanımı %95 güven aralığı değeri en yüksek olarak radius'da (84.23-97.68 Mpa; 3.64-4.36 GPa; 14.94-17.6 MPa) en düşük olarak femur'da (25.30-30.43Mpa; 0.46-0.56 GPa; 6.09-7.65 MPa) hesaplandı. Farklı biyomekanik test hesaplamalarının varyasyon katsayıları incelendiğinde genel olarak üç nokta eğme testinin varyasyon katsayılarının kesme testine göre daha düşük olduğu görüldü.

Sonuç: Bu çalışma sonuçları etlik piliçlerin tüm uzun kemiklerinin kesit geometrisi, kemik yoğunluğu ve farklı test yöntemleriyle elde edilen biyomekanik özellikleri hakkında bilgi vermektedir. Bu veriler etlik piliçlere ilişkin, yetiştiricilik ve besleme araştırmalarında, kemik dayanımının değerlendirilmesi aşamasında, araştırıcılara uygun değerlendirme metodunun kullanılması ve normal değerlerin bilinmesi yönünden destek olabilecek niteliktedir.

Anahtar Kelimeler: Biyomekanik, Dansite, Etlik piliç, Kemik, Morfometri.

ABSTRACT

MORPHOLOGICAL, DENSITOMETRIC AND BIOMECHANICAL EVALUATIONS OF THE BONE STRENGTH IN BROILERS

Khan K. Aydın Adnan Menderes University, Institute of Health Sciences, Veterinary Anatomy, Doctorate Thesis, Aydın, 2021.

Objective: The main aim of this study was to determine the morphological, densitometric and biomechanical properties of the long bones of the broiler.

Material and Methods: The long bones (humerus, radius, ulna, femur, and tibia) of wings and legs of 32 broiler chickens were dissected out and stored in the freezer. The bone morphometric measurements showing general and cross-sectional geometry were obtained using computed tomography images. Except for the radius and ulna, bone densities were measured by the DEXA. For biomechanical testing, three point bending was performed on right side and shear testing was conducted on the left side.

Results: There was no statistically significant difference between the morphometric features of the right and left side bones. Cortical thickness indices were seen highest in the radius and lowest in the femur. The 95% confidence interval value of maximum bending strength, elastic modulus and maximum shear strength was highest for the radius (84.23-97.68 MPa, 3.64-4.36 GPa and 14.94-17.6 MPa) and lowest for the femur (25.30-30.43 MPa, 0.46-0.56 GPa and 6.09-7.65 MPa), respectively. The coefficients of variation for different biomechanical test calculations showed that generally, the variability of the three-point bending test was lower than the shear test.

Conclusion: This study gives information about the cross-sectional geometry, mineral density and biomechanical properties of all the long bones of broiler obtained by different techniques. These data can be used in broilers' breeding and feeding studies for evaluation of the bone strength, by applying the most suitable method and comparing with the provided normal values.

Keywords: Biomechanical, Bone, Broiler, Density, Morphometry.

1. INTRODUCTION

Now-a-days, poultry industry is becoming substantial with every passing day as it is playing a vibrant role in the economy of any country. Poultry meat is responsible for overall growth of meat production, which accounted for 12% in 1961, 35% in 2013 (OECD/FAO, 2021) and it is predictable to grow up to 11% during this decade (Roser, 2017). Poultry meat is a rich and quickly available source of animal protein for humans (Almeida et al., 2018), ranging from 15 to 35% (of all animal protein sources) (Marangoni et al., 2015). This protein percentage is expected to increase by 17.8% and poultry meat is going to contribute for 41% of animal protein source by 2030; highest among all sources (OECD/FAO, 2021). The chicken meat production and consumption per capita have grown remarkably during the last decades. Chickens are most commonly raised as a source of nutrition as 20% protein is offered by the chicken meat (Soriano-Santos, 2010).

As poultry sector is growing gradually and steadily, studies are going on all over the world especially focusing on management of the production houses using cheap and easily available sources to get good quality meat, eggs and other by-products. For this purpose, researchers are emphasizing on welfare management and biosecurity measures to get healthy birds (Athrey, 2020). Growth rates in densely-grown industrial broiler chickens have been progressively accelerated in the last 60 years. Earlier in 1994, 2.273 kg live weight was attained in 49 days but now the same weight is achievable in just 35-42 days of age (Griffin and Goddard, 1994; Havenstein et al., 2003; Maharjan et al., 2021).

Chicken bones also play important role in their production, such as protecting internal organs and providing calcium for the egg shell formation (Korver et al., 2004c). But the readily available broiler's bones are negatively impacted by the fast growth-rate. The studies have shown that rapid and slow growing broilers have difference in bone composition and strength (Yalcin et al., 2001). The genetic selection on the basis of high production has resulted in change in the mechanical properties of the broiler bones as depicted by reduced strength due to scanty inorganic matter and profuse porosity (Shim et al., 2012).

It has been observed that altered ex-anatomical and biomechanical characteristics of bones of broiler (Paxton et al., 2014; Tickle et al., 2014) and turkeys (Abourachid, 1993) have given rise to changed gait pattern (Abourachid, 1991; Caplen et al., 2012; Paxton et al., 2013). It was important to determine if these specific kinetic and kinematic parameters of gait are heritable or not? The study showed that these gait patterns are significantly correlated with bone geometrical and mechanical properties in turkeys and can be improved through genetic selection. Although, weight gain increases in short time period but bones become unable to fully mature and get stronger within this small duration. That's why the up gradation of broiler chickens for meat could have potential for abnormal skeletal development (Korver et al., 2004b), as bones respond to an increase in body weight by constantly increasing remodeling rates (Turner and Robling, 2004; Warden, 2006). The heavy muscles in turn find slighter support from undeveloped bones both in broilers (Corr et al., 2003a, b) and turkeys (Ferket et al., 2008; Zhong et al., 2012).

With the increase in awareness, broiler's dealers also understand worth of getting better skeletal properties along with heavy weight broiler as it is seen that healthy bones are part and parcel of getting valuable meat and more profit. Since, active broilers can move rapidly towards feeders and drinkers to feed and gain weight. Contrarily, immobility can increase incidence of leg problems and abnormalities along with low quality meat production. The vast research has been done and is still continued to provide proper lightening, diet with balanced nutrients, good quality litter material for easy walking and environmental enrichment with material for exercise of the broiler birds (Kestin et al., 1992; Estevez, 2002; Bilgili et al., 2009; Xavier et al., 2010; Gholap, 2012; Garcês et al., 2013; Ramadan et al., 2013; Shao et al., 2015). The availability of perches has been proved good choice for bone development and strength as bones undergo mechanical loading and more activity while using perches. Moreover, faster growth rate of modern broilers is also responsible for decreased skeletal mineralization which is ultimately impacting bone strength (Tablante et al., 2003). Although light intensity (lux) was unable to affect bone properties (Chew et al., 2021) but its duration (hours) improved the leg condition and decreased pathologies (Renden et al., 1996).

In this scenario, selection/prediction of only good strains having better bone features seems to be a better option to get healthy profitable birds. There are various skeletal abnormalities, contributing to production loss (Harash et al., 2020) through affecting the mobility of chicken (Kestin et al., 1992a, 1999b; Bradshaw et al., 2002; Knowles et al., 2008), such as deformities, tibial dyschondroplasia, rickets, spondylolisthesis, caput femoris degeneration, chondrodystrophy, osteoporosis, *Mycoplasma synoviae* infection, viral arthritis and soles dermatitis, especially in broilers (Bradshaw et al., 2002). In another study, (Abrahamsson and Tauson, 1995) found bumble foot, distal toe pad hyperkeratosis and keel bone lesions as common problems especially in layers (Harlander-Matauschek et al., 2015). For decades researchers strive to make contribution for the diagnosis of these problems effectively and give suggestions for the improvement in skeletal deficiencies.

The perception of different bone parameters (morphological, densitometric, and geometric) is crucial to avoid infections resulting from bone fractures as oblique or spiral type breakage of bone can enter muscle tissue, bringing about wound and infection. This can damage quality of meat. The mechanical testing of bones is generally used for the prediction of their properties and strength. Imaging techniques (radiographic tools) like radiography, fluoroscopy, DEXA (Dual energy X-ray absorptiometry), CT (Computerized Tomography), MRI (Magnetic Resonance Imaging) etc. are also preferable tools because of their non-invasiveness.

So, the objective of this study was firstly, to provide information regarding structural features, BMD and mechanical properties of the broiler's (Ross 308) long bones, using CT scans, DEXA images and two different biomechanical testing methods. Secondly, to recognize the suitable fore/hindlimb bone for assessment of broiler wing/leg strength, using different mechanical testing techniques.

2. GENERAL INFORMATION

2.1. Bone Tissue

The bone is a tissue having very complex type of nature as it consists of cortical bone, cancellous bone and marrow space (Rath et al., 2000). Cortical bone has compactly arranged fibers while cancellous bone is comparatively more porous. Bone structure varies with location in the body, age sex and function. Bone is performing both mechanical and metabolic functions. The bony tissue is made up of organic (30-40%) and inorganic components (60-70%) (An and Draughn, 2000). Organic part (collagen type I) gives tensile strength (Riggs, 1993) while inorganic component is associated with stiffness of the bone. Therefore, the bone mineralization status is important indicator of bone health because it bounces its strength (Reichmann and Connor, 1977; Rath et al., 2000; Štofaníková et al., 2012). Bone strength, elasticity and biophysical properties are defined by inorganic part (An and Draughn, 2000). So, not only bone volume but also its mineralization strengthens the bone (Boivin and Meunier, 2002; Shim et al., 2012).

Moreover, bone quality is dependent on both its geometric and mechanical features (Zhang and Coon, 1997; Casinos and Cubo, 2001; Vitorović et al., 2009). Bone ash fraction (g/g dry weight), bone mineral content (BMC), bone mineral density (BMD) and bone mechanical properties are often used as indicators of bone mass and bone strength. Bone density could be either apparent (including pores and water) or material/true (without pores) density. BMD (g/cm^2) is bone mineral mass per unit bone volume while BMC (g or %) is unit bone mineral mass per unit bone weight (An and Draughn, 2000). Bone ash analysis and densitometric techniques are often used to evaluate these parameters. Regional differences in bones can be easily observed through their mechanical testing. There is a positive correlation between density and mechanical properties of bone. To accurately predict the fracture behavior of bone tissue, biomechanics seems a better option as it involves a critical analytical step of calculating internal stresses in the whole bone. This incriminates both the information about the geometry of anatomic site and its structural behavior. Histological methods are necessary to assess bone cell activity on specific bone surfaces and compartments, and this can be critical in determining whether changes in bone formation and bone resorption are the mechanism underlying bone mass or strength (Bonser and Casinos, 2003). The properties of the bone are not only dependent on its mineralization but are also subjective to its sponginess and matrix structuring (Shim et al., 2012).

2.2. Features of Birds' Bones and their Fundamental Discrepancies to Mammalian Bones

Bones have some structural differences among species depending on their functional variations. Unlike mammals, birds need support for flying through light weight skeleton. Humerus, coracoid, sternum, skull, pelvic girdle, lumbar and sacral vertebrae are pneumatic bones of birds. In addition, absence of true epiphyseal plate and primitive skeletal maturity help them to fly at younger age. In general, bone formation begins at primary and secondary ossification centers during development, but secondary ossification center is absent in most of the bird's bones (König et al., 2007; Jacob et al., 2013). Only tibia (proximal (Watanabe, 2017) and distal end) and metatarsus (proximal) have true bony epiphyses (Church and Johnson, 1964).

Besides, poultry have three types of bone tissues i.e., compact, spongy and medullary bone (only in layers). Medullary bone fulfils extra calcium (Ca) requirement of hens for the eggshell formation. Its location is endosteal surface of the bone. The main purpose of this is to prevent skeletal defects in layers during growth (Korver et al., 2004a) which otherwise can occur due to reduction in Ca supply. It provides 47% Ca out of total required for shell formation while rest of Ca is obtained from feed (Jacob et al., 2013). This bone extends as spikes within marrow cavity. For overall bone strength, rather than older belief of reduction in structural bone formation (Whitehead and Fleming, 2000), medullary bone prevents bones from breakage and osteoporosis (Bishop et al., 2000).

2.3. Skeletal Deformities in Chicken

There are a lot of skeletal problems in modern broiler birds arising from controlled environment, restricted feeding and exercise, fast growth rate and heavy weight. Both infectious and non-infectious musculoskeletal disorders can be seen. Most commonly legs are affected which result in development of conditions like osteomyelitis, femoral head necrosis, arthritis, *Mycoplasma* infection, foot pad dermatitis, hock burns, varus valgus deformity (VVD), tibial dyschondroplasia (TD), rickets and chondrodystrophy. Femoral head disintegration is most common and can be mostly infectious due to osteomyelitis and chondritis (by *Staphylococcus aureus*) or non- infectious known as epiphyseolosis. Arthritis in broilers is mostly inflammation of joint between tibia and tarso-metatarsus. *Mycoplasma synoviae* is also another common cause of swollen joints and lameness in broilers (Bradshaw et al., 2002). TD is seen as development of cartilaginous growth in the proximal extremity of tibia and its incidence could be as high as 40% in broilers (Shim et al., 2012). The VV deformity is related to angulation of femur and tibial distal extremity and broilers are mostly affected with valgus (lateral deviation) than varus (medial deviation (Hunter et al., 2008). Broilers suffering from TD or VV can cause economic losses due to increased susceptibility to fractures and lameness resulting in inability to move to feeders and drinkers.

All of these problems have variety of causal agents i.e., it could be related to either genetics, nutrition, or management practices. Although, a weak genetic correlation exists between growth and leg bone disorders (r=0.01-0.08) (González-Cerón et al., 2015). Yet, there is a possibility to assess skeletal properties of broilers and try to improve them either by using any possible management practice or cull them beforehand to avoid production and economic loss. This is why, earlier assessment of skeletal integrity of chickens can prove useful in the prevention of bone disorders.

2.4. Bone Strength

The bone strength can be measured by calculating the geometrical indices, radiographic or dual-energy X-ray absorptiometric density measurements, ash content measurements or the assays of bone turnover biomarkers. The biomechanical tests can also measure bone strength with reliable indicators. Bone ashing, bone mineral concentration and bone density etc. are invasive while digitized fluoroscopy, dual energy X-ray absorptiometry (DEXA), and computed tomography (CT) are commonly used non-invasive techniques.

2.5. Description of the Invasive Methods

2.5.1. Bone Ashing

Bone ashing means "the total inorganic matter present in a bone, remained after burning off all the organic material". Ash percentage is a strong predictive of the bone mineral content. It is good technique as it gives exact information about mineralization of the bone tissue. Other densitometry techniques for bone mineral density (BMD) measurement have a disadvantage that they may also include marrow spaces and porosities of the bone. However, bone ash has advantage of simplicity and easiness. It has also strong correlation with the bone breaking strength (r=0.77) (Hester et al., 2004a). Contrarily, this method has a negative side that is its inability to give information about cortical or cancellous bone separately.

It requires weight and volume of the bone. Bone volume can be obtained by either weight change in water method (Zhang and Coon, 1997), water volume change method (Cheng and Coon, 1990) or by water displacement method of overflow (Garlich et al., 1982).

Then the bone (fresh, dry, fat-free dry) is ashed at 600°C for 24 hr (Zhang and Coon, 1997); (Kim et al., 2004) or at 750°C for 22 hr (Rath et al., 1999) or at 520°C for 12 hr (McDevitt et al., 2006) or at 600ºC for 16 hr (Maji, 2012) (Onyango et al., 2003) or at 550ºC for 4 hr (Hossain et al., 2013), cooled in a desiccator, and weighed. Ash concentration is calculated by dividing the ash weight of each bone by its volume.

2.5.2. Histomorphometry

Although micro-computed tomography (μCT) can provide an assessment of bone microarchitecture but this is an expensive technique. That's why histological analyses of bone micro-architecture and bone cell activity is valuable method. Histomorphometry remains the only method by which bone formation can be assessed at a particular skeletal site. It involves florescent imaging or histological staining of bone tissue.

Fluorochrome labels (e.g. calcein, oxytetracycline) are injected to the experimental bird on two separate days before sacrifice (Hudson et al., 1993). These fluorochromes bind to circulating calcium for 24-48 hours after the injection. This labelled Ca is captured by the bone surface undergoing mineralization during this period which is expressed as a bright label under fluorescent microscope. Inter-label width is assessed between the two sites. But this labelling procedure seems less useful in very young chicks because the matrix formation and mineralization are developing so rapidly that labelling shows a diffuse band (Parfitt, 1983). But this analysis can also be used to quantify bone growth and development by measuring growth plate area and height, hypertrophic chondrocyte zone height and number (Ohashi et al., 2002), particularly to see impact of any management practice on growth and development of young poultry (Kim et al., 2012).

For histology, bones are fixed in 10% neutral buffered formalin, decalcified with 10% trichloroacetic acid (TCA) (Rath et al., 1999) or 10% formic acid solution (Maji, 2012), pass through ascending concentrations of alcohol and embed finally in paraffin. 4-5 µm sections are cut with microtome and are stained with either hematoxylin and eosin or toluidine blue (Rath et al., 1999) or modified Goldner's trichrome (Aguado et al., 2015). The histological parameters (widths of the proliferative zone, hypertrophic zone, mineralized zone, cortical thickness, bone volume (BV), trabecular volume (TV), osteoid thickness and volume) are then measured under a microscope (Rath et al., 1999). Increased %BV/TV means increase in trabecular number and thickness while decline in trabecular spacing. This technique is useful for both medullary and cancellous bones (Wilson et al., 1998; Kim et al., 2004).

2.5.3. Biochemistry

Atomic spectroscopy is the most common chemical method (Casinos and Cubo, 2001; Massé et al., 2003a) using blood samples. Blood serum can be analyzed for Ca (Ca-ocresolphthalein complex) and P (ammonium phosphomolybdate complex) (Shastak et al., 2012). Plasma can also be analyzed for Ca, inorganic P, iron, magnesium, glucose, triglyceride, cholesterol, protein, alkaline phosphatase (Kuyubaşı et al.), lactate dehydrogenase (LDH), and creatine kinase using an automated clinical chemistry analyzer (Rath et al., 1999). Moreover, the calcium content can also be determined as mmoles of calcium per mole of collagen using the colorimetric assay (Sparke et al., 2002). The ash analysis using plasma atomic emission spectrometry is another method to assess the mineral concentrations (calcium, phosphorus, magnesium, manganese, iron, copper and zinc) (Hossain et al., 2013).

TRAP histochemistry i.e., tartrate resistant acid phosphatase (TRAP) activity to identify and count osteoclasts along with toluidine blue counterstain is a good technique to see bone resorption. Another method is bone collagen biochemistry in which cortical bone samples are defatted in chloroform/methanol for 48 h at 4ºC, washed in methanol and distilled water. Samples are then converted into powder after cooling in liquid nitrogen. Then immature crosslinks (dehydrohydroxylysinonorleucine as hydroxylysinonorleucine (HLNL) and hydroxylysino-keto norleucine as dihydroxylysinonorleucine (DHLNL)), and the mature cross-links (HL-pyr and L-pyr) are determined using a modified gradient on an amino acid analyser (Sims et al., 2000). Besides, the total collagen content can also be assessed by hydroxyproline assay using a continuous flow autoanalyser (Sparke et al., 2002). Or serum PYD (pyridinoline) concentration can be quantified using ELISA tests (Van Wyhe et al., 2014). Increased circulating pyridinoline suggests an increase in bone resorption (Sparke et al., 2002). Another histochemical method is bone matrix extraction using a dye binding assay to measure proteoglycan and transforming growth factor-β (Chandrasekhar and Harvey, 1988). Moreover, collagen and pyridinium crosslink can be determined using HPLC (fluorescence detector) or the fluorescence of the collagenase extract can be evaluated using fluorometer (Rath et al., 1999). Plasma calcium, phosphate and bone alkaline phosphatase (Kuyubaşı et al. 2016) are also sometimes assayed on an automated COBAS-BIO autoanalyser (Massé et al., 2003b).

2.6. Description of Non-invasive Methods

2.6.1. Computed Tomography (CT) Scanning Technique

The quantitative computed tomography (QCT) scans offer remarkable improvements over DEXA in providing information about compartment-specific BMD and cross-sectional geometry. The data is collected in two dimensions by the rotation of an X-ray source and detectors around the sample/body. This data is then converted to three dimensional after accounting for scan slice thickness (0.5 to 1.0 mm). The specific region of interest can also be scanned by the user and consecutive slices can be obtained.

Due to minimal exposure of radiations to the vital organs, pQCT has achieved more widespread use in small animal research over the last decade. It is mostly limited to only limb bones (hence, the ‘peripheral’) and the radiation dose is less than 0.1 mSv/hour (Gasser, 2003). During scans, detectors rotate around the animal's limb only. Although an entire bone can be scanned but it requires anesthesia induction which is time-consuming. Variety of models with different resolutions are available and can be as low as 70 microns. For a bone scan having both cortical and cancellous bone, it is better to take average value from 3-4 consecutive slices to minimize error.

The BMC or BMD for the whole bone or for a region of interest specified by the user can also be measured as a true volumetric value (vBMD, in mg/cm^3). In addition, cross-sectional geometric (cross-sectional area and cross-sectional (polar) moment of inertia) can also be computed. These parameters have a critical impact on a bone's mechanical properties and are used for detecting material properties of bones after their mechanical testing. Further, these parameters can also be calculated separately for the cortical and cancellous bone compartments within the same scan slice. This can be particularly useful for medullary bone in laying hens (Kim et al., 2004). For example, recent data demonstrate a selective reduction in BMD of medullary and metaphyseal cancellous bone during a nine-day molting period, whereas there are no significant differences in cortical bone density over the same periods (Kim et al., 2012).

2.6.2. Micro Computed Tomography (μCT)

The microCT is an X-ray imaging in 3D, the same method used in CT scans, but on a small scale with massively increased resolution. This method provides 3D microscopy, where the very fine-scale structure of objects is imaged non-destructively, both in vivo and ex vivo. All bone samples are micro-CT imaged and the raw data are converted into a DICOM image format using software to crop and align the bone diaphysis with the image axes. The bone is positioned longitudinal to the scanner to get axial image slices. The obtained μCT images are measured. Area of cross-section, major and minor principal second moment of areas, major and minor section moduli, and average CT density can be assessed for each slice. Measurements from each slice are then averaged over the length of the test section. For volumetric CT density, the average CT density from each slice is weighed with the cross-sectional area of that slice (Vaughan et al., 2016).

The BMD values reflect a true volumetric value (vBMD, in mg/cm^3). Cross-sectional area and cross-sectional (polar) moment of inertia have a critical impact on a bone's mechanical properties and resistance to fracture. The cross-sectional areas and BMC/vBMD variables can be computed separately for the cortical and cancellous (or medullary) bone compartments within the same scan slice (Kim et al., 2004). But this method is an expensive and not easy to perform to evaluate bone strength.

2.6.3. Dual Energy X-ray Absorptiometry (DEXA) Technique

It is commonly known by the short form DEXA, and is the gold standard for clinical use in diagnosing osteoporosis in human patients. Most absorptiometry manufacturers offer software that is marketed for use in small animals. DEXA can be used to assess both body composition (distinguishing lean and fat mass from bone mass) (Mitchell et al., 1997) and bone mineral content/bone mineral density (BMC/BMD) in small animals and may be performed on unanesthetized animals as well (Hester et al., 2004b).

The use of photons of two different energy levels allows user to perform on bone surrounded by even large amounts of soft tissue, which is not possible by using single photon absorptiometry. Unlike the QCT methods, the two-dimensional bone area of interest can be selected for scanning. The BMD obtained is expressed as mg/cm^2 which called as ‘areal’ BMD instead of true volumetric density measures. In DEXA scanning, opportunity to select smaller regions of interest can help in assessment of density of mixed bone sites (e.g. metaphyses, diaphyses etc). For laying hens, a region of interest can be specified for exclusion of changes in BMD due to medullary bone (e.g. for humerus).

Evaluation of the skeleton by absorptiometry methods can also be expressed as indexes (for humans especially) and the information is entered into the software of the devices. These indices are known as Z score and T score (Kimmel, 2002; Elçi, 2004). Both T-scores and Z-scores are a standard deviation. The T-score is a bone mass of adult healthy person, while the Z-score represents bone mass of a person of matching age for reference (Carey and Delaney, 2010).

The radiation exposure involved is minimal, and whole bones can be imaged; both of these factors represent distinct advantages over CT techniques. Although full-size DEXA scanners are rather expensive, smaller versions designed to measure BMD in the human wrist are an appropriate size for many birds (pDEXA, Norland Medical Systems, Fort Atkinson, WI).

Other side to achieve acceptable reproducibility for research purposes, it is critical to standardize positioning of the bird's limb, because DEXA cannot correct for varying bone thickness within the designated region of interest. This latter challenge also limits the use of DEXA in rapidly growing animals, as absolute bone size will be dramatically different (Carter et al., 1992). The resolution is also much lower than that achieved with computed tomography.

The DEXA is a reliable and accurate tool for assessing the mineralization of bones both in vivo and ex vivo (Onyango et al., 2003; Schreiweis et al., 2003; Schreiweis et al., 2004, 2005) however, conducting scans, especially in live birds, is labour-intensive (Hester et al., 2004b). Using the small sized bones like phalanges instead of larger bones such as the tibia would reduce the time involved in scanning.

2.6.4. Digitized Radiographs and Fluoroscopy

Digital radiographs are usually taken using the X-ray generator. The non-anaesthetized birds may be used for this purpose. Birds are placed on a digital flat panel detector by safely fixing them. The bone is kept at right/left angles to the x-rays. The radiation field is located immediately above the center of the sample. Images are taken with 50.0 kV and at 2 mAs for each bird separately. Then, these radiographs are evaluated using the image processing system (Eusemann et al., 2018).

Digitized fluoroscopy is also a radiographic absorptiometry technique in which bone sample is exposed to single low dose X-ray. The aluminium wedge is kept along with the bone sample and video output is captured. The radiographic density (in mm Aluminium equivalent) can be derived later on using image software (Fleming et al., 2000). It is useful because live unanesthesized birds can be used as well. Also it has low cost, low radiation exposure and greater accessibility (Fleming et al., 2000). But its low sensitivity and resolution and inability to separate out cortical, cancellous, and medullary bone compartments, make it less preferable technique.

2.7. Bone Indices

There are some indices which can also give indirect information about bone strength. These are Seedor index/bone index, cortico-medullary index/tibiotarsal index and robusticity index. The formula for Seedor index is bone weight/bone length and its higher value shows that the bone sample is having higher density (Almeida et al., 2018). Robusticity index is obtained by bone length/cubic root of bone weight (Azad et al., 2020).

$$\text{Robusticity Index} = \frac{\text{length of the bone}}{\sqrt[3]{\text{weight}}\ \text{of the bone}}$$

Its low value is reveals structural strength of the bone. Cortico-medullary index (which is mostly mentioned as tibiotarsal index for tibia) and if its value is higher it means that the bone is stronger due to its better mineralization. It is obtained as (Mabelebele et al., 2017):

$$\frac{\text{Periosteal diameter-endosteal diameter}}{\text{Periosteal diameter}} \times 100$$

or

$$\frac{\text{Diaphyseal diameter-Medullary canal diameter}}{\text{Diaphyseal diameter}} \times 100$$

2.8. Biomechanical Testing Techniques

Mechanics is a branch of physics and biomechanics is the study of biological tissues (bones, ligaments and tendons) to assess their mechanical behavior in response to loading. As this science is studied and explained mostly by the engineers, it is very important to use it for tissues with great care. It has an advantage over other techniques that the mechanical properties of biological tissues can be tested directly (Turner and Burr, 1993). Mechanics is subdivided into static and dynamic branches which involve resting and moving (kinematics and kinetics) bodies, respectively.

Bone is a viscoelastic material means that it has both viscous and elastic components and strain rate dependent on time plus dissipates energy when force is applied. The mechanical properties of a bone tissue are determined by its density, porosity and micro-architecture (Ammann and Rizzoli, 2003). So, approaching bone both at a structural and material level through mechanical testing is the suitable method. There are variations in bones of different species regarding shape, size and strength (Rath et al., 2000).

The force (F) or load is a vector quantity which could be either compressive, tensile or shear type. The newton (N) is the most commonly used unit of force. It is the amount of force required to accelerate 1 kg body mass to 1 m per sec^2. As a result of it, force-displacement curve is obtained which could be converted into stress-strain curve by giving dimensions of a particular specimen, to get information about material properties of a tissue. Stress (σ) is the opposition of specimen to the applied load (i.e., Force/Area) and strain (ε) is the change in shape of specimen in response to the applied force. Strength is the internal resistance of specimen to deformation and fracture. Elasticity is the ability of material to return to its previous shape after removal of applied load. Stiffness (S) is resistance to deformation within elastic limit obtained simply from division of force by displacement but elastic modulus (E) is equal to stress divided by strain. Toughness is the energy absorbed by the specimen on load application (An and Draughn, 2000).

2.8.1. General Considerations for Appropriate Biomechanical Test

Before applying any test, material nature and its mechanical parameters should be understood. There are a lot of testing methods available for determination of mechanical strength of bones. The biomechanical tests are performed to evaluate the mechanical properties of normal bones and to see deviations from these normal values in animals/birds undergone different nutritional trials to get better production and avoid skeletal abnormalities (Kleczek et al., 2012; Świątkiewicz and Arczewska-Wlosek, 2012).

The biomechanical features of bones are dependent on numerous in-vivo (age, sex, species, body weight, hormones) and ex-vivo (preservation, fixation, boiling, drying, freezing, testing technique) factors. With increasing age, mineralization of bones is increased up to maturity but afterwards increasing porosity especially in females, results in weaker and stiffer bones. Sex is also another crucial concern as males have heavier and bigger bones and different hormones. Weight-bearing and non- weight bearing bones show different mechanical behavior. So, location and usage of the bone is also of critical concern. In addition, consideration of the external factors is vital as well. Chemical preservation, drying, boiling etc. are harmful methods for storage of bones before testing mechanical features as bones become stiffer and weaker (Turner and Burr, 1993; An and Draughn, 2000).

As bone is a biological structure, application of appropriate biomechanical test is necessary for accurate and reliable results. A single whole bone or piece of a bone can be tested based on fundamental principles of mechanics. Mostly whole bones and less often irregular shaped bones are undergone these tests. Testing of whole bone is little difficult because of their irregular symmetry. Three- or four-point bending or torsional tests are mostly applied on long bones, whereas vertebral bone or cylindrical specimens are tested in compression method.

The literature review about mechanical testing of poultry bones showed that previous concept was that radius and femur were more suitable for bending test instead of tibia due to their symmetry. The cross-sectional area of humerus seems appropriate for shear test (Harner and Wilson, 1986). But now mostly tibia is studied to know mechanical strength of poultry bones as it is thought that this rapidly growing bone undergoes more mechanical loading than other bones (Massé et al., 2003a). In general, humerus and tibia are mostly used to see long bones bending properties while femur is for tensile testing (Askari et al., 2015).

2.8.2. Samples Harvesting and Preparation for Biomechanical Tests

First step is to get the bones as soon as possible without drying just after birds are euthanized (CO_2 asphyxiation/severing jugular vein/cervical dislocation or euthanasia solutions (embutramide at 200 mg/mL, mebezonium iodide at 50 mg/mL, and tetracaine at 5 mg/mL) (Pratt and Cooper, 2018; Karaarslan and Nazlıgül, 2018). The mechanical properties of the bone shows great discrepancy depending on the circumstances provided for a particular test. Tissue autolysis of bone starts within hours of its removal from body and changes the mechanical properties of the bone. It has been seen that the mechanical properties of the bones are also changed by storage in chemicals like formalin or glutaraldehyde etc. (Turner and Burr, 1993).

Contrarily, freezing the dissected bones immediately at -20 to -25°C proved an effective technique in preserving the bone quality (Bernardis and Ziv, 2000; Moran et al., 2000; Martin et al., 2004). The bones are wrapped in normal saline-soaked gauze and stored at -20°C for long-term preservation before mechanical testing (Turner and Burr, 1993). In this way, bone samples can be stored for a maximum of eight months (Roe et al., 1988). The bone's ability to change shape depends on the water content of the bone. The wet cortical bone shows elasticity and absorbs maximum energy on applying mechanical test while dry bone is harder and brittle (Wiesel and Delahay, 2001). That's why it is recommended to soak bones in normal saline before freezing.

Next important step is to thaw the frozen bones before testing. Thawing appeared harmless for biomechanical properties of bones and maintained wet condition (Linde and Sørensen, 1993; Massé et al., 2003a). Thawing should be done slowly (for at least 3 hours) and temperature must be increased up to 20°C (Štofaníková et al., 2012) or 25°C (room temperature) to 37°C. Since, bones normally bear mechanical loading at this temperature (Turner and Burr, 1993). After thawing, some general measurements including bone length (mm), external, internal mid-diaphyseal medio-lateral and cranio-caudal diameters (mm) are noted down before testing (An and Draughn, 2000).

As bones have variety of shapes and sizes, sometimes samples are potted before any mechanical loading to have a definite grip and increase reliability of testing setup. For this purpose, mostly polymethylmethacrylate (PMMA) is used. Specimens should be continuously rehydrated during this procedure to maintain their true mechanical properties (An and Draughn, 2000).

2.8.3. Standard Biomechanical Tests

Generally, bones are tested through either compression or by three or four point bending while torsional and impact loading used less frequently. Short description of mechanical testing methods of whole bones is as follows:

2.8.3.1. Bending

Bending test are particularly performed to know the mechanical strength of bone when it is subjected to bending (Rubin and Lanyon, 1985; Robling et al., 2001; Ammann and Rizzoli, 2003; Warden et al., 2005; Lane et al., 2006). There are two types of bending tests: three-point or four-point bending experiments.

In three-point bending, the tested bone is placed onto two prongs with a definite distance between them (span), and a single-pronged load cell is applied to the opposite surface at a point precisely in the middle between the two supports. In this method, bone is fractured at the load application as maximum force is endured by this point. The four-point bending method is similar to three-point bending except that the double pronged loading is applied on the mid-point. The main advantage of this method lies in the fact that the entire section of bone between these two load-applying prongs is subjected to a uniform moment.

2.8.3.1.1. Three-point Bending Method

The experimental procedure involves the application of force in the middle of a bone, yielding a force–deformation curve. Parameters obtained from this curve include bone stiffness, yield-load, maximal/ultimate load and fracture load. Load–deformation curves are generated for all tests. For bending tests, load is represented as force (N). Stiffness (N/mm) is calculated as the slope of the linear portion of the force-deformation curve. Maximal load/force is the point at which the bone fractures. The area under this curve gives information about elastic (before yield-point) and plastic (after yield-point up to fracture) regions of a sample plus work to failure in the form of energy absorbed.

Bone is anisotropic, heterogeneous, viscoelastic material. By giving information about its geometry like mediolateral and craniocaudal diameters, its material properties can also be calculated. It is important to note down that internal diameters can be obtained either after breakage of the bone samples or CT scanned images can be used to measure internal diameters before breaking the bones. Stress (N/mm^2) is defined as force per unit area. The moment of inertia (mm^4) accounts for differences in area and shape of the bone cross section through which the force is applied. For a whole bone in cranio-caudal bending the formula is:

$\mathbf{I_x}$ = {(π/64)×((Medio-lateral External Diameter×(Cranio-caudal external diameter)^3)-(Medio-lateral Internal Diameter×(Cranio-caudal Internal diameter)^3))}.

When bending is performed in medio-lateral direction it could be as

$\mathbf{I_y}$ = {(π/64)×((Medio-lateral External Diameter)^3×Cranio-caudal external diameter)-((Medio-lateral Internal Diameter)^3×Cranio-caudal Internal diameter)}.

For breaking strength (MPa/GPa)

BS = (F_{max}×L× Cranio-caudal external diameter)/ 8 I_x

Elastic modulus (MPa/GPa) gives rigidity of bone as related to stress and strain and can be calculated as

E = ($S \times L^3$)/ 48I_x, (An and Draughn, 2000).

Using 20 mm/s load rate, Rath et al. (1999) found higher tibial strength and stiffness in older birds. Kim et al. (2004) observed that with load rate 50 mm/min., bone breaking strength of the fresh bone was better and highly correlated with dried weight, ash weight, and ash concentration than that of the fat-free dried bone. McDevitt et al. (2006) found that the tibiotarsus bones of the genetically selected chickens were twice as strong as those of the unselected chickens at the same age. But ASABE standards have provided 10 mm/min load rate for three-point bending test of poultry bones (Standarts, 2007).

2.8.3.1.2. Four-point Bending Method

The actuator/load cell two arms are usually spaced such that the area of interest is located between them for uniform bending moment. It is important that the two points of load application must contact the bone at the same time. Due to the irregular surface shape of some bones, this might be difficult to achieve. Data collection is same as described for three- point bending test (An and Draughn, 2000). Formula for the calculations is given as:

For breaking strength (MPa/GPa)

$$\textbf{BS} = (F_{max} \times a \times \text{Cranio-caudal external diameter}) / 4\ I_x$$

where “a” is distance between support and load cell.

Elastic modulus (MPa/GPa) can be calculated as

$$\textbf{E} = (S \times a^2) / 12\ I_x\ (3L\text{-}4a)$$

The chicken humeral bones’ biomechanical rigidity was measured by four-point bending test by Pehlivan et al. (2003). Regmi et al. (2015) used four-point bending and found that aviary birds (AV) had stronger bones (tibia = 3.7%, humerus = 6.3%) than that of the cage (CC) birds.

2.8.3.2. Cantilever Testing Method

Cantilever test is also a type of bending in which one end of the specimen is fixed firmly while the other end is kept freely movable. The bending moment varies from a maximum at the fixed end to zero at the force application point. It can be applied to a whole bone/ part of a whole bone/ cancellous/ cortical specimens. Fixing of one end can be accomplished by potting it into a container (either PVC or steel). The force is usually applied at the point on the free end most distant from the fixed end of the bone so that the highest bending moment is obtained. The side of the bone to which the load is applied depends on which bending direction is desired. Angular orientation of specimens is also possible within this testing system. Cantilever bending also uses single load application and data collection is same as for the three-point bending. For this 5 mm/min load rate is recommended (An and Draughn, 2000).

2.8.3.3. Compressive Testing Method

Compression test is mostly used to understand the mechanical properties of both cortical and trabecular bone (An and Friedman, 2020). Its effect is opposite to that of the tensile load. It causes shortening of the object in length and an increase in the cross-sectional area. For trabecular bone, smaller specimens can be studied by this test. More accurate results are seen if this test is applied on samples with smooth surfaces. The strain in the bone specimen is the same at both ends and middle of the bone (Turner and Burr, 1993; An and Friedman, 2020). This test has advantage over tensile test that shorter and easily prepared samples with only little flat surface can be used (Turner and Burr, 1993; Wiesel and Delahay, 2001). Moreover, the length of the prepared sample should be double than its diameter (An and Friedman, 2020). Compression test is applicable on the vertebra as well (Hirano et al., 1999; Turner and Robling, 2004; Mashiba et al., 2005). Calculations are carried out as

$$\sigma = 4\ F / \pi\ d^2$$

where F is applied force while d is the diameter of the sample. Elastic modulus for compression test is calculated as

$E = SL/A$, where S is stiffness, L is length of the sample and A is the area of the specimen.

2.8.3.4. Tensile Testing Method

Tensile testing is the most valid method for determination of material properties of a bone (cortical and trabecular) sample (An and Friedman, 2020). The material is stretched along the long axis and the cross sectional area is reduced. 4-8 mm wide bone samples are considered suitable for this test. Measurements will be accurate if this test can be performed without causing a torque (Turner and Burr, 1993). This test is less frequently used than compression test due to difficulties in preparing specimens for testing, especially for trabecular bones, the problem of attaching smaller specimens to the jaw of the machine is encountered always.

The intrinsic stiffness for tensile test is equal to Elastic modulus but extrinsic stiffness is measured as EA/L, where E is Elastic modulus, A is area of cross-section while L is length of the specimen (An and Friedman, 2020).

2.8.3.5. Torsional Testing Method

Torsional testing of whole bone is accomplished by firmly embedding the ends of the tested bone in rectangular or cylindrical blocks of plastic material. Angular deformation is seen on twisting one of these ends while keeping other fix. The bones are broken at their weakest mode (shear or tensile). The torsional test is applied on bones with straight diaphysis and uniform cross sections. The force-deformation curves obtained here are used to determine the angle of twist, ultimate torque, shear stress, and shear modulus of the sample. Formula for shear stress (τ) due to torsion test is

$$\tau = T \times r / J$$

where 'T' is applied torque, 'r' is radius of the bone (external diameter/ 2). J is the polar moment of inertia calculated as $J = \pi \times r^4 / 2$ (Turner and Burr, 1993).

Harner and Wilson (1986) found this test appropriate to evaluate the fracture mechanism of poultry bone. The structural strength of the bones is based on failure torque as measured by the peak torque generated. Van Wyhe et al. (2014) used torsional test and found that shear strength improved at the end of study after protein and energy was restricted by 60%, but some variations occurred throughout the study. Kuyubaşı et al. (2016) also applied this test on chicken bones.

2.8.3.6. Impact Test

Though this type of mechanical loading is not experienced by bones under normal physiological conditions but it is very important to predict the bone behavior after trauma or accidents (i.e. falling). That's why this type of test is performed by a hammer of precisely known weight which is dropped from a known height and hits the sample with power. Ultimately, it gives information on the resistance of whole bones to impact loading (Sharir et al., 2008).

2.8.3.7. Shear Testing Method

Its setup is like three-point bending test with one loading actuator and two supports but the distance between the two sample supports and the shear loading bar shall not exceed 0.05 mm. another difference is that the load rate of 5 mm/min should be used for shear test but for the three point bending test, a speed of 10 mm/min is recommended in broilers (Standarts, 2007) while maintaining a length to diameter ratio (L/D) equal to or greater than ten. When L/D is less than 10, the effects of shear must be considered. Onyango et al. (2003) concluded that in broiler chicks, tibia ash, BMC, and BMD may be more sensitive than tibial shear force as indicators of dietary Ca and P concentrations. Harner and Wilson (1986) recommended shear test for poultry studies because of the varying bone geometry along the diaphysis of the different bones. Formulae for shear stress (τ) and shear modulus (G) calculation are given below:

$$\tau = F / 2A$$

where F is applied force and A is cross-sectional area which can be calculated for elliptical and elliptical quadrant through different formulae.

For elliptical shaped bone it is given as

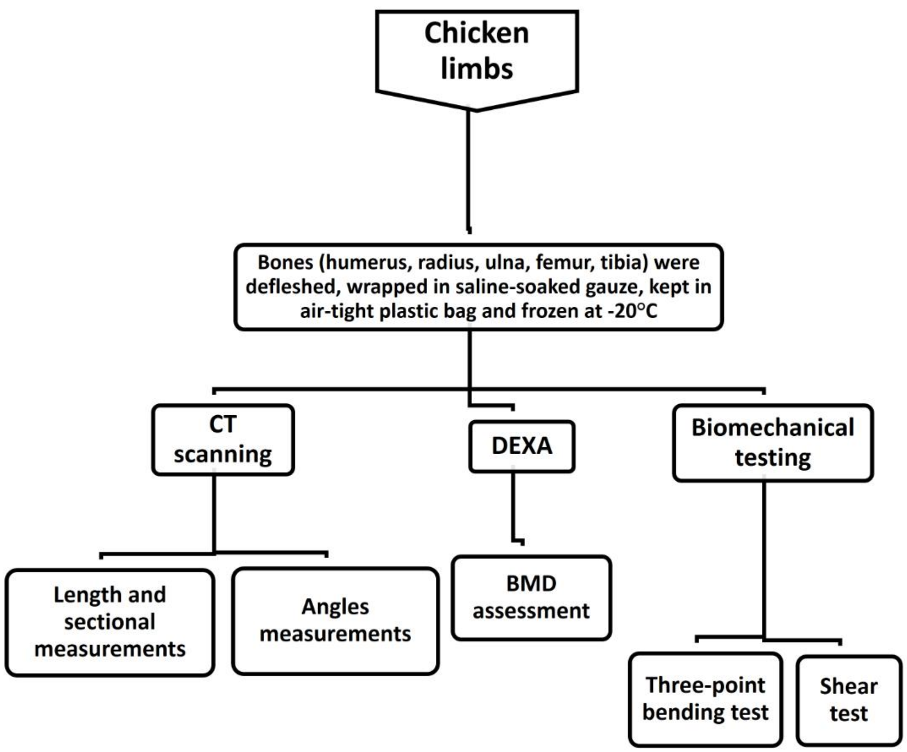
Area = ((π/4) × (ExtMLD × ExtCrCdD) - (IntMLD × IntCrCdD) while for elliptical quadrant it can be given as

Area = (π (external diameter)^2 - (external diameter - cortical wall thickness)^2)/ 4 + (2 × external diameter × cortical wall thickness - 3 × (cortical wall thickness)^2))). Where external diameter is ML and CRCD external diameters /2 and wall thickness is calculated as medial, lateral, cranial cortical thicknesses /3 (Combs et al., 1991).

3. MATERIAL AND METHODS

3.1. Sampling and Preservation of Bones

A total of 32 broiler chicken (Ross 308) limbs were taken from a butcher house (Can Tavukculuk, Aydın, Turkey) on 42nd days of age and with average carcass weights 1545.78 ± 56.57 g. The sex of the broilers was not confirmed. The bones were checked for normal healthy condition using gross observations and CT scans. There were no gross pathological signs but only one left radius and ulna were broken during slaughtering process. The flowchart for the methodology is given below in the Figure 1.



*VV deformity was determined using angular measurements of femur and tibia. TD was evaluated at the end of the study.

Figure 1. Flow diagram presenting testing techniques used in the experimental research.

The soft tissues i.e. muscles, ligaments etc were cleaned off gently from the forelimb (humerus, radius, ulna), and hind limb bones (femur, tibia/tibiotarsus). To preserve biomechanical properties, all the bones were immediately draped in sterile saline-soaked gauze, and stored in plastic bags at -20°C (Figure 2) until further analysis (An and Draughn, 2000). Before further testing and exploration, all the bones were slowly thawed by firstly keeping them at 4°C. For mechanical testing, one additional step was performed by placing samples in 20 degrees sterile saline to minimize moisture loss (Figure 3).

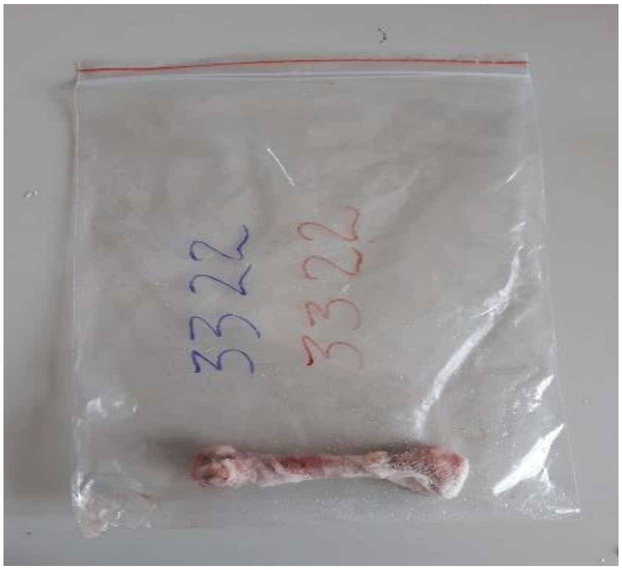


Figure 2. Storage of samples in air-tight bag after wrapping in a saline-soaked gauze

Figure 3. Thawing of samples before mechanical analysis

3.2. Computed Tomographic (CT) Scanning of Bones for Geometrical Measurements

In the first part of the study, CT scanning of bones was performed by TOSHIBA Aquilion Premium 140 Casette Multidetector Helical, facility present in Medical Faculty, Adnan Menderes University, Aydın. After thawing, the bone were subjected to CT analysis, in which all right and left side limb bones (femur, tibia, humerus, radius and ulna) were scanned. During analysis, all the bones were positioned in cranio-caudal direction (Figure 4) and were scanned as 1mm thick section with 1mm interval between the sections (120 kV, 200 mA, 500 msec).

Figure 4. Represents the CT apparatus and position of all the bones of one chicken in a scanogram (right and left), arranged on CT table for scanning.

After scanning, the images were accessed through PACS (Probel Picture Archiving and Communication System of ADU), and direct morphometric measurements were performed on all right and left side bones (Figure 5).

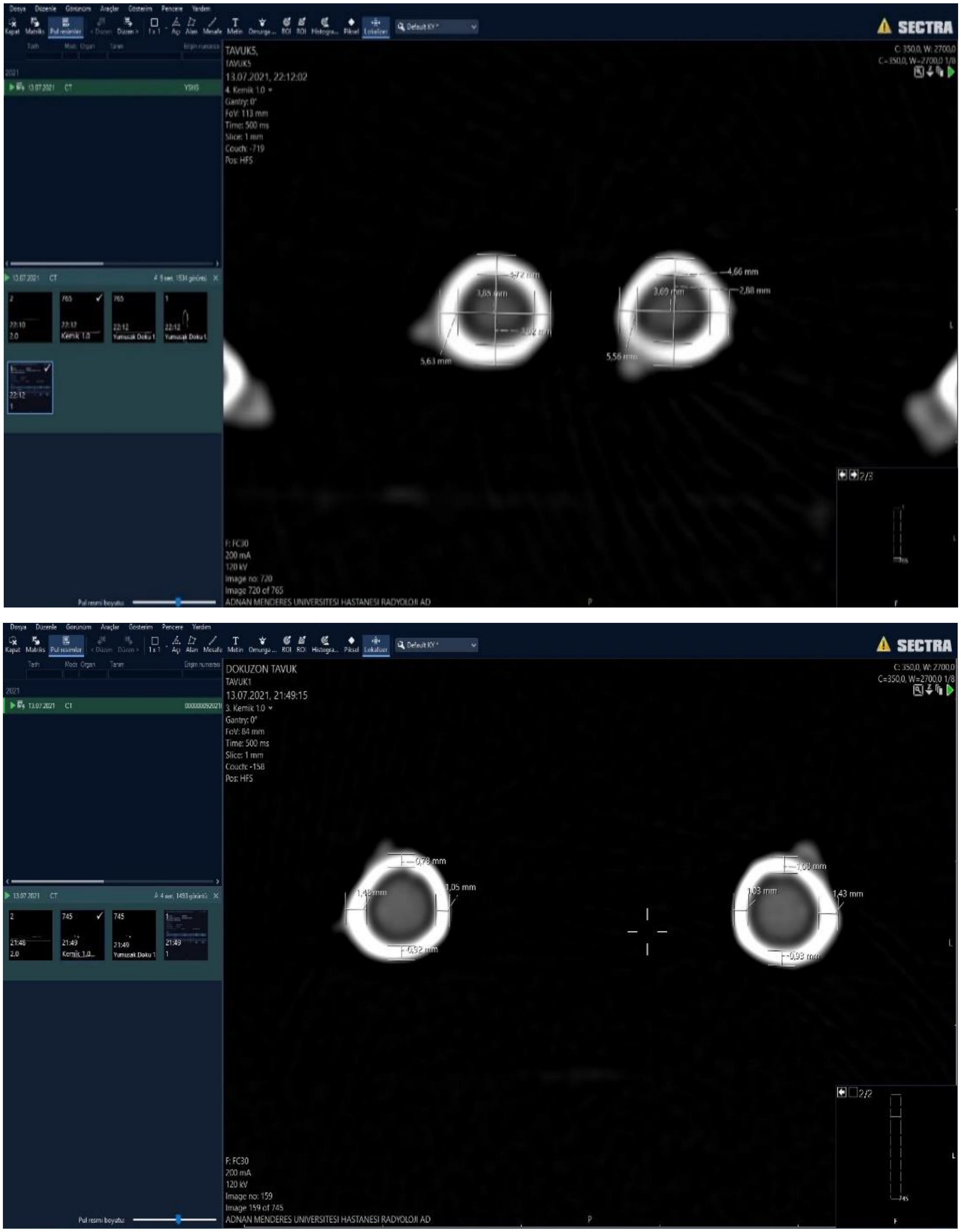


Figure 5. PACS system showing CT scanned images for measurement.

It is especially important to notice that as forelimbs/wings of birds are used for flying, their bones have dorso-ventral terminology unlike hindlimb bones of birds which have medio-lateral surfaces (Baumel, 1993). All the abbreviations related to CT measurements are presented in the Table 1.

Table 1. The description of the measurements obtained from the CT scans.

Parameter	**Abbreviation**	**Description**
External mediolateral diameter	ExtMLD	The distance from medial periosteal surface to lateral periosteal surface in the mid-shaft of the bone.
Internal mediolateral diameter	IntMLD	The distance from medial endosteal surface to lateral endosteal surface in the mid-shaft of the bone.
External craniocaudal diameter	ExtCrCdD	The distance from cranial periosteal surface to caudal periosteal surface in the mid-shaft of the bone.
Internal craniocaudal diameter	IntCrCdD	The distance from cranial endosteal surface to caudal endosteal surface in the mid-shaft of the bone.
External dorsoventral diameter	ExtDVD	The distance from dorsal periosteal surface to ventral periosteal surface in the mid-shaft of the bone (for forelimb bones only).
Internal dorsoventral diameter	IntDVD	The distance from dorsal endosteal surface to ventral endosteal surface in the mid-shaft of the bone (for forelimb bones only).
Medial cortical thickness	MCT	The medial wall thickness in the diaphyseal region of the bone
Lateral cortical thickness	LCT	The lateral wall thickness in the diaphyseal region of the bone.
Cranial cortical thickness	CRCT	The cranial wall thickness in the diaphyseal region of the bone.
Caudal cortical thickness	CDCT	The caudal wall thickness in the diaphyseal region of the bone.
Dorsal cortical thickness	DCT	The dorsal wall thickness in the diaphyseal region of the bone (for forelimb bones only).
Ventral cortical thickness	VCT	The ventral wall thickness in the diaphyseal region of the bone (for forelimb bones only).
Femur distal angulation	Fedα	Angle measured on the distal extremity of the femur in a cranial view.
Femur anteroposterior curvature	Fapc	The degree of femur being curved and it is measured on the lateral view of femur by drawing line from the centre of the caudal diaphysis.

Tibia distal angulation	Tidα	Angle measured on the distal epiphysis of the tibia in a cranial view.
Tibial proximal bending	Tipβ	Angle measured at the proximal end and lateral view of tibia.
Tibial distal bending	Tidβ	Angle measured at the distal end of the tiba in a lateral view.
Tibial anteroposterior curvature	Tapc	The degree of tibia being curved and it is measured on the lateral view of tibia by drawing line from the centre of the caudal diaphysis.

Bone mid-diaphyseal morphometry parameters including: cranio-caudal external and internal diameters; medio-lateral (dorso-ventral for forelimb) external and internal diameters; cranial, caudal, lateral (dorsal for forelimb), medial (ventral for forelimb) cortical thicknesses were calculated. The mediolateral (CMI_{ML}) and craniocaudal (CMI_{CRCD}) corticomedullary indices were also calculated using external and internal diameters (Mabelebele et al., 2017). The formula is given below

CMI_{ML} = {(ExtMLD-IntMLD)/ ExtMLD} ×100

CMI_{CRCD} = {(ExtCrCdD-IntCrCdD)/ ExtCrCdD} ×100

Bone length (L (mm); Figure 6A, 6B, 6C, 6D) and the angles of femur (distal angulation, anterioposterior curvature) and tibia (proximal and distal angulation and anterioposterior curvature) were calculated from these scans as well for VV deformity. Namely, femur distal angulation (Fedα), femur anteroposterior curvature (Fapc), tibia distal angulation (Tidα), proximal bending (Tipβ), distal bending (Tidβ) and anteroposterior curvature (Tapc) were evaluated for this purpose (Leterrier and Nys, 1992; Guo et al., 2019) (Figure 7 and 8).

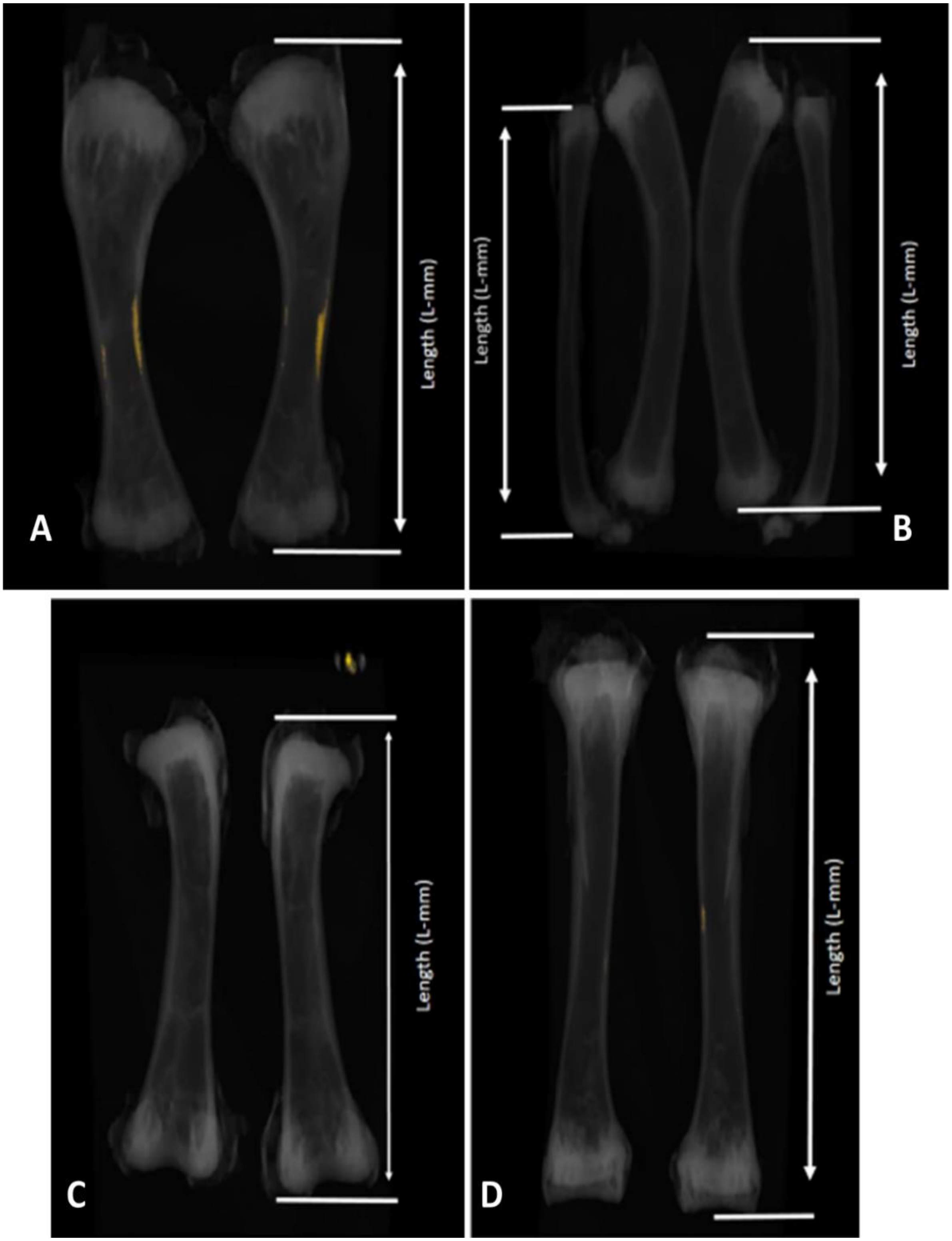


Figure 6. The bone length (mm) measurements for humerus (A), radius (B), ulna (B), femur (C) and tibia (D).

Figure 7. The measurements of femur distal angulation (Fedα, A) and femur anterioposterior curvature (Fapc, B) on the cranial (A) and lateral (B) view of a right femur.

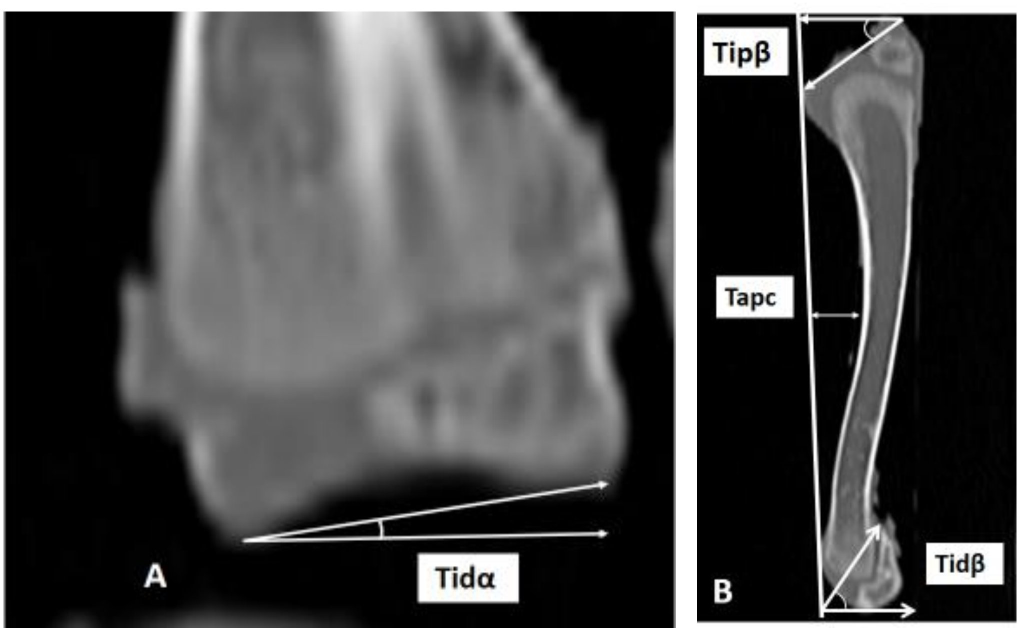


Figure 8. The measurements of tibial distal angulation (Tidα, A), proximal bending (Tipβ, B), distal bending (Tidβ, B) and anteroposterior curvature (Tapc, B) on the cranial (A) and lateral (B) view of a right tibia.

Before taking the sectional measurements, firstly an extra broiler bones (from the osteometry laboratory of Anatomy Department) were cut at the centre with the help of an electric saw, perpendicular to their length to get two equal parts. These bones were photographed to use them as reference for cross-sectional measurements (cranial (Cr), caudal (Cd), medial (M), lateral (L_o), dorsal (D), ventral (V)) of CT images (Figure 9-13). Then, all the CT scanned bones were measured at their respective central section which was making mid-point of the bone. External medio-lateral diameter (ExtMLD/ExtDVD for forelimb), internal mediolateral diameter (IntMLD/IntDVD for forelimb), ExtCrCdD (external craniocaudal diameter), IntCrCdD (internal craniocaudal diameter), cranial cortical thickness (CRCT), caudal cortical thickness (CDCT), medial cortical thickness (MCT/DCT for forelimb) and lateral cortical thickness (LCT/VCT for forelimb) were assessed (Figure 14, 15, 16 and 17). The cross-sectional measurements were performed on three consecutive sections on the mid-shaft. The averages of three measurements were used as cross-sectional geometric data.

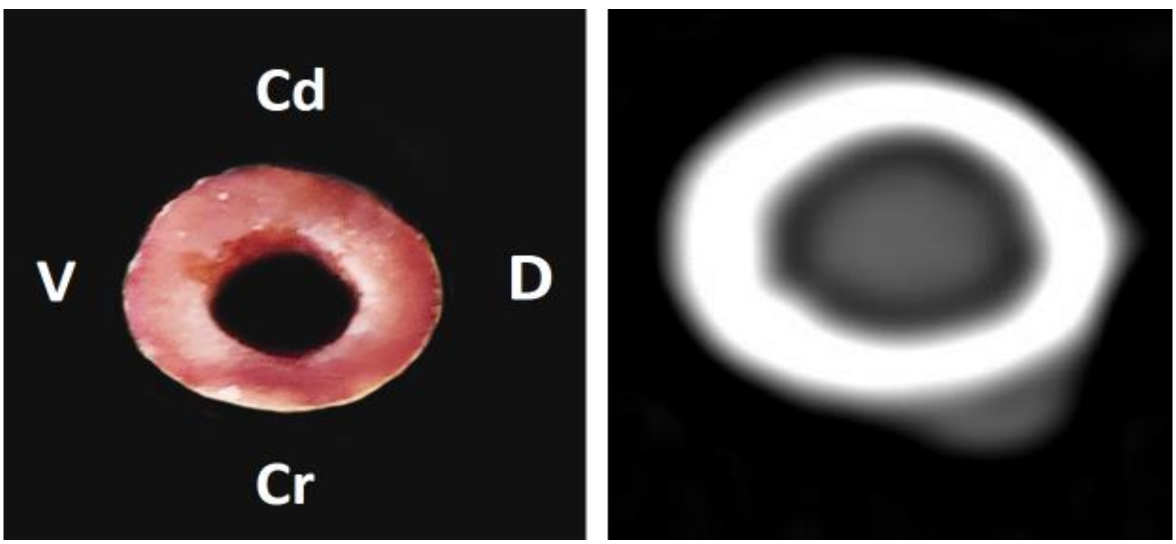


Figure 9. The cross-sectional view of right humerus (photograph and CT scan)

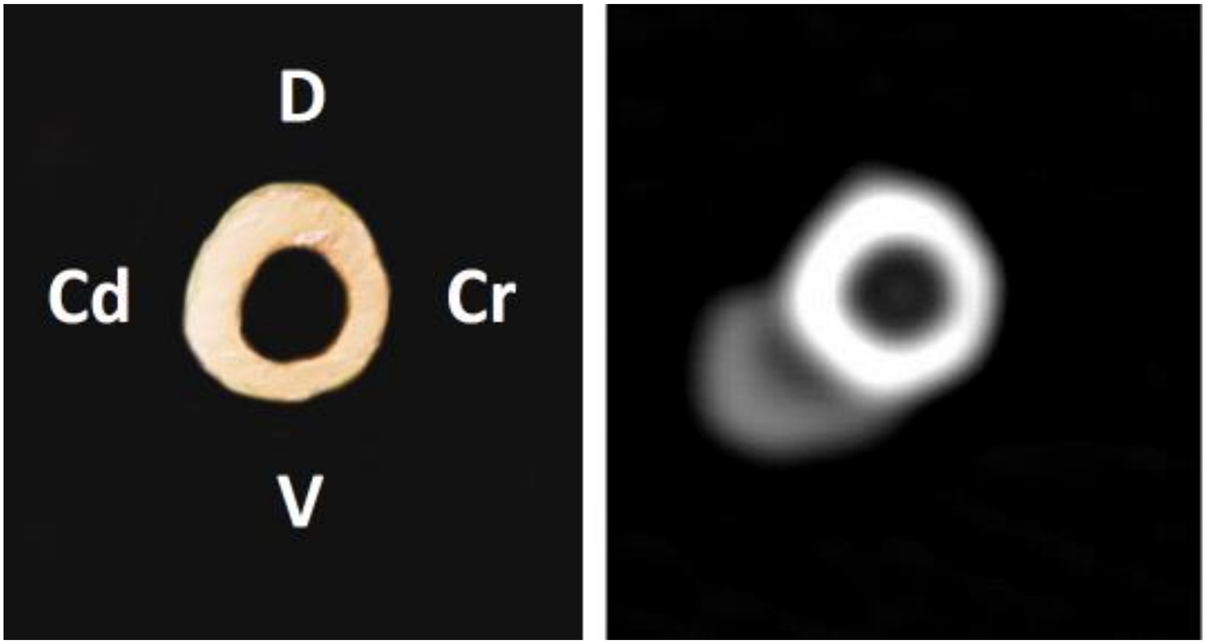


Figure 10. The cross-sectional view of right radius (photograph and CT scan)

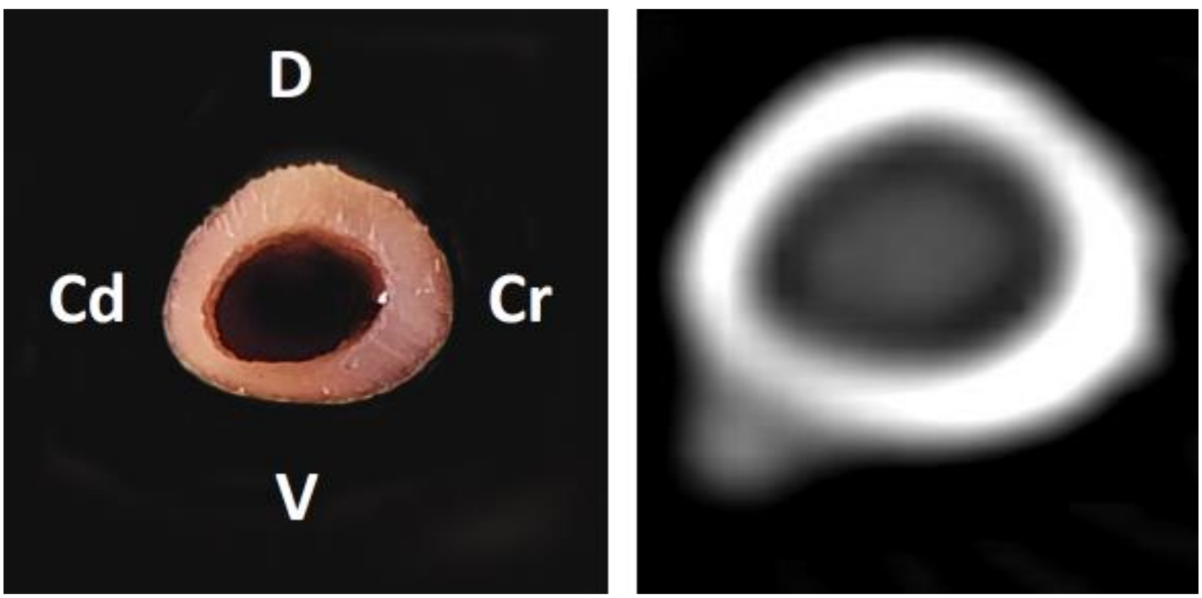


Figure 11. The cross-sectional view of right ulna (photograph and CT scan)

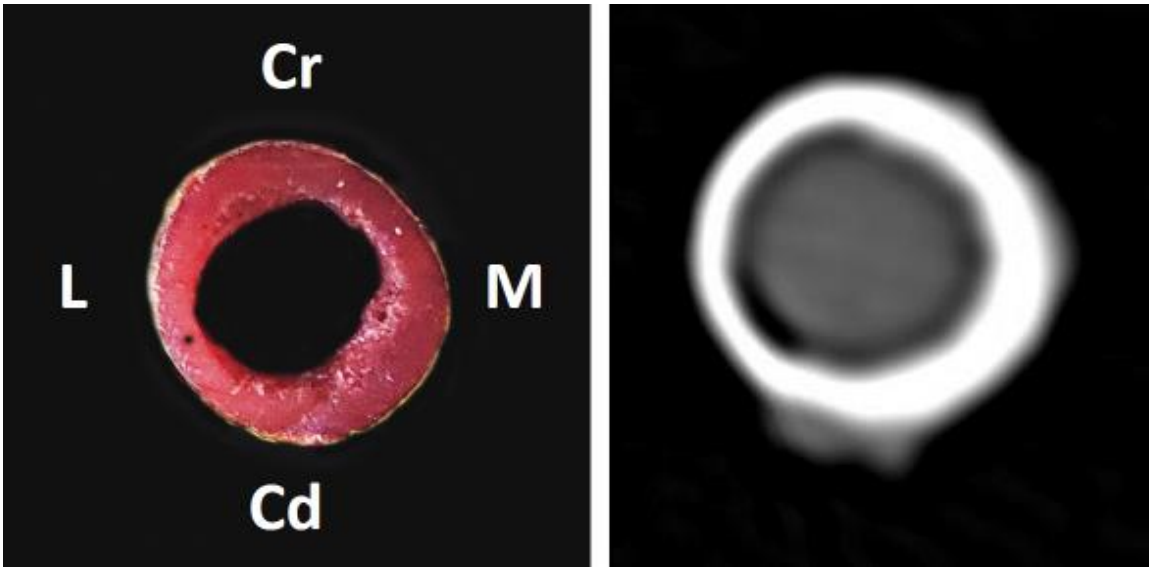


Figure 12. The cross-sectional view of right femur (photograph and CT scan)

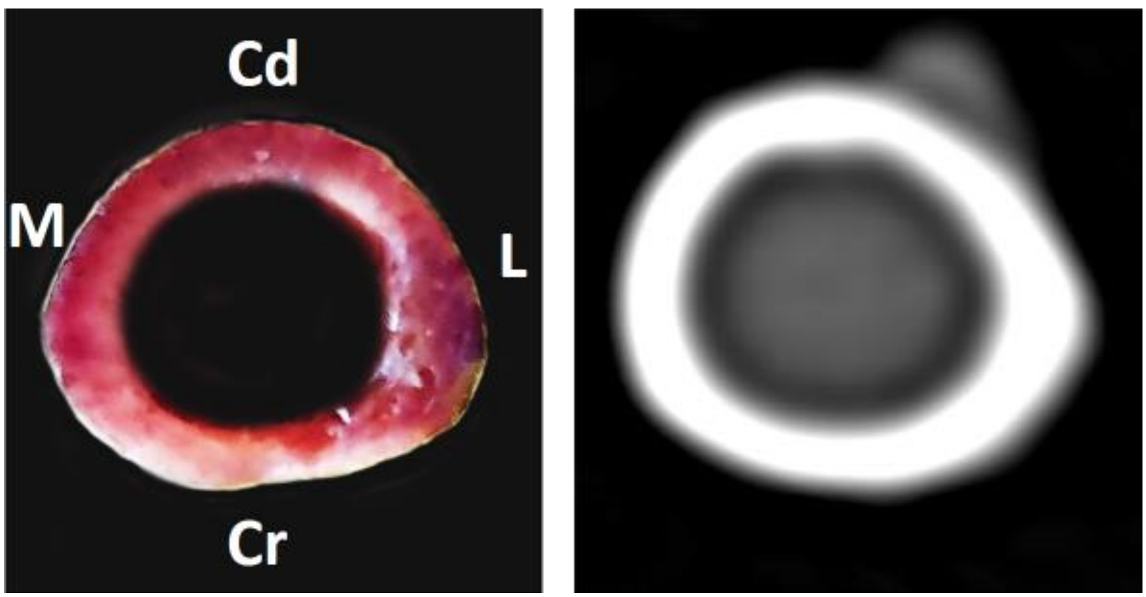


Figure 13. The cross-sectional view of right tibia (photograph and CT scan)

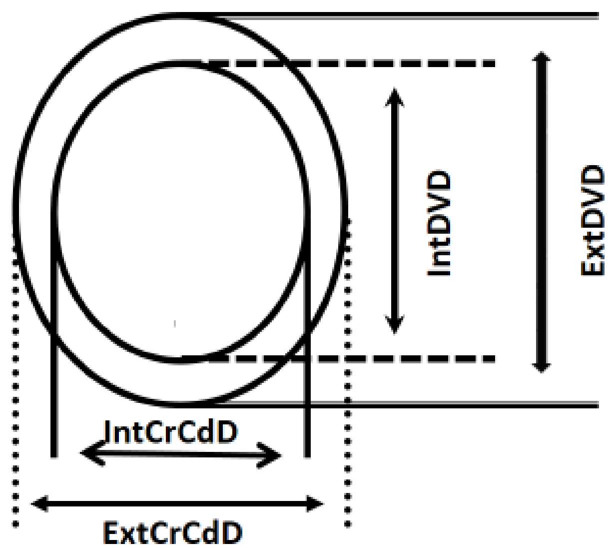


Figure 14. Sketch showing external and internal craniocaudal and dorsoventral diameters of forelimb bones (humerus, radius, ulna).

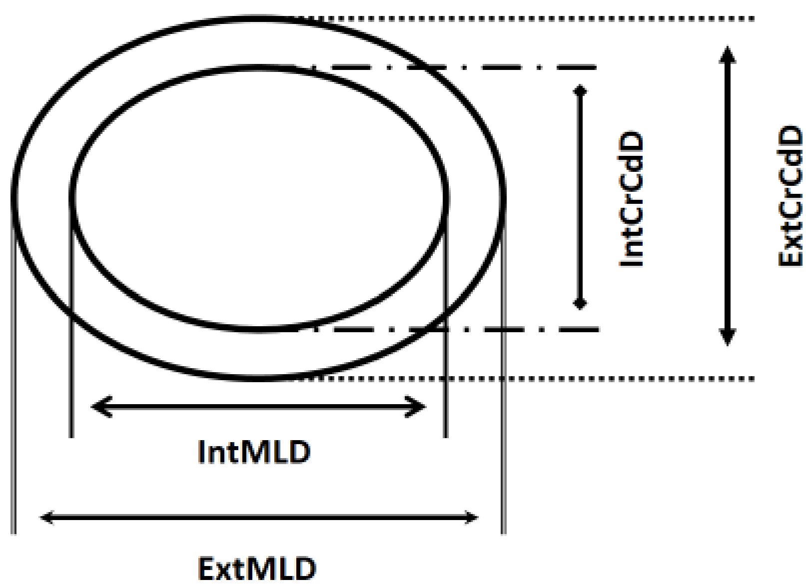


Figure 15. Sketch showing external and internal craniocaudal and mediolateral diameters of hindlimb bones (femur, tibia).

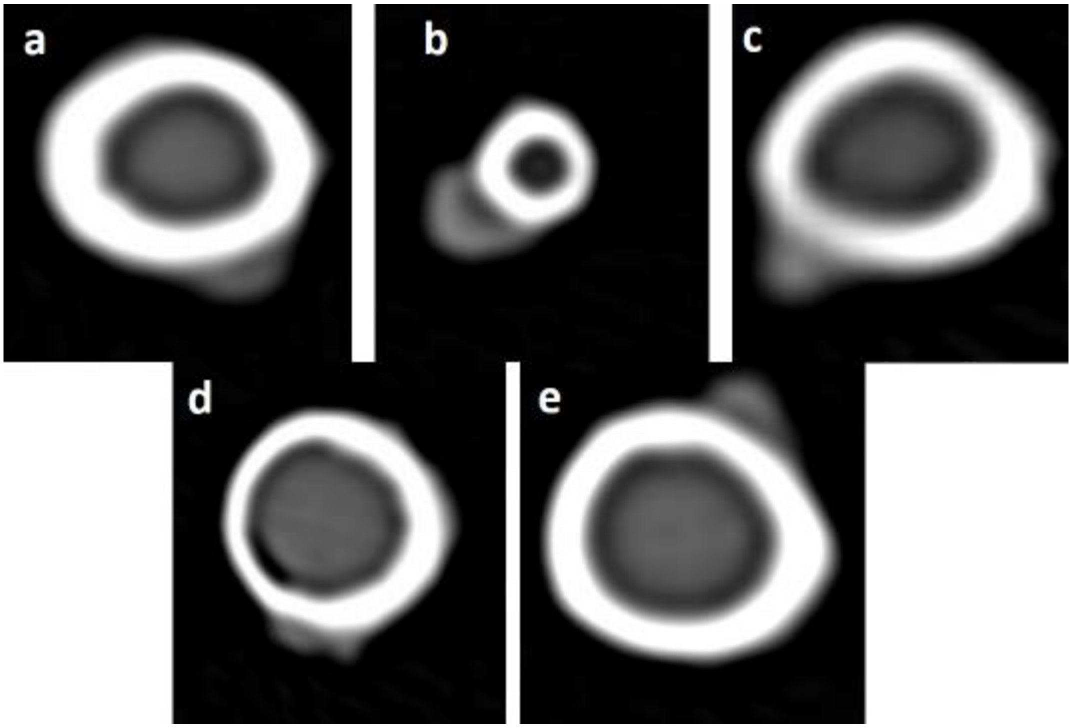


Figure 16. Showing cross-sections of right forelimb (humerus, a; radius, b; ulna, c) and hindlimb bones (femur, d; tibia, e) obtained from CT scans.

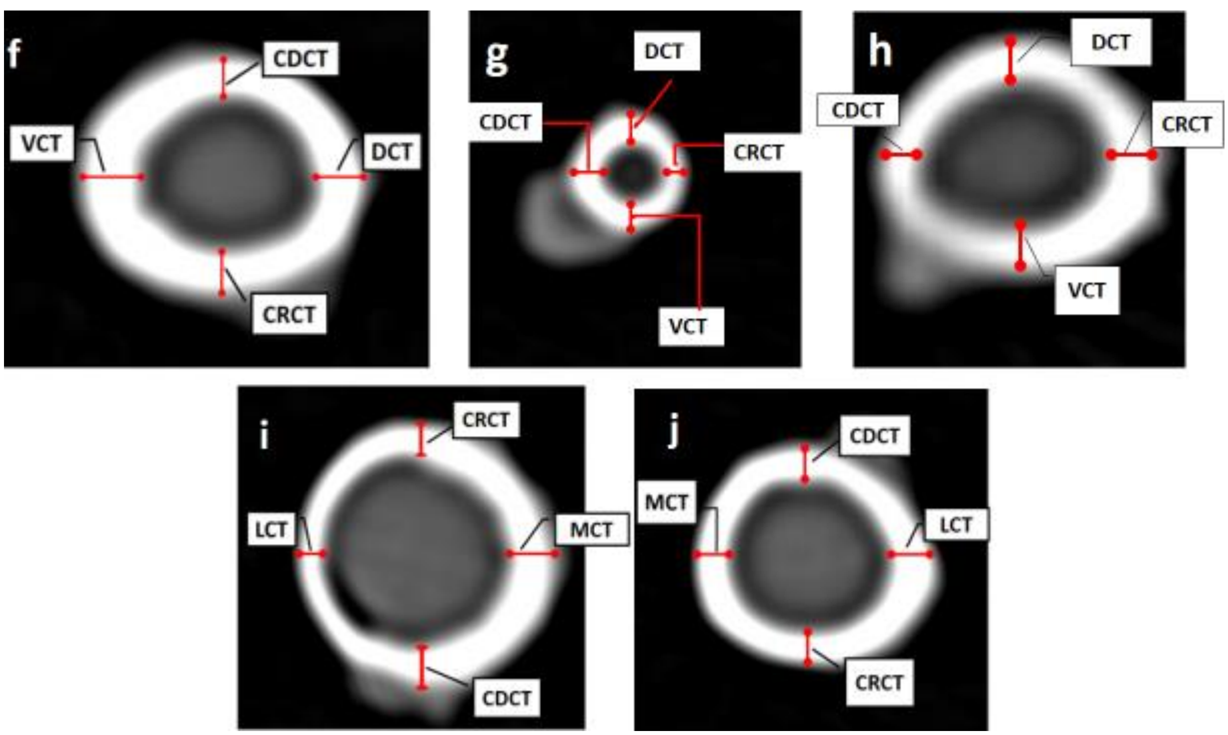


Figure 17. Showing sectional thicknesses of right forelimb (humerus, f; radius, g; ulna, h) and hindlimb bones (femur, i; tibia, j).

Before application of DEXA and mechanical tests, the 3D printed models of all the bones of a chicken were also produced. They were just used for easier understanding and more authenticity in orientation of all the bones for these analyses. For this purpose 3D slicer 4.10.2 software (www.slicer.org) was used to create models and then print them through 3D printer. The sequence of creation of models is given in the figures below (Figure 18 and 19).

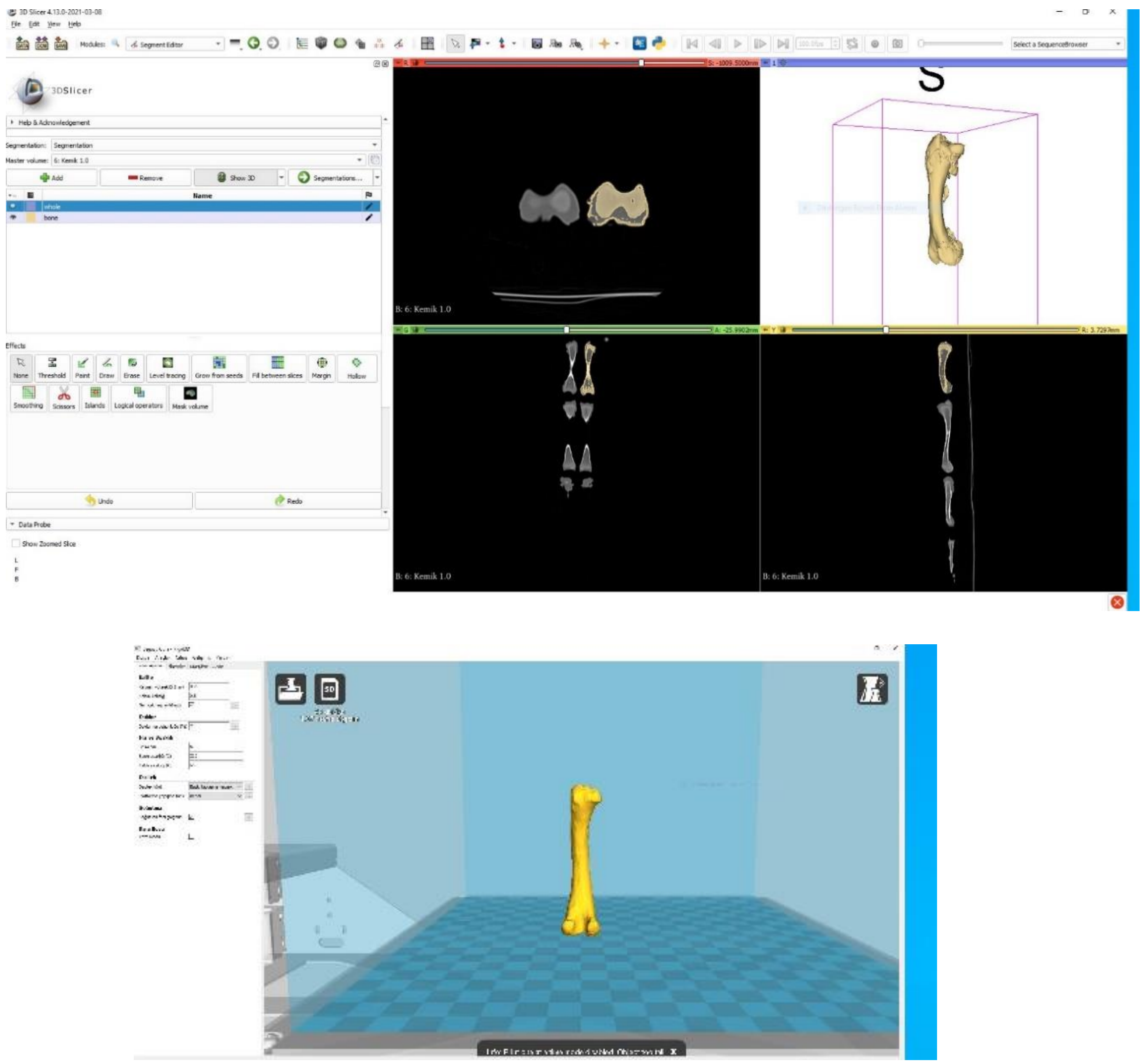


Figure 18. Showing images of 3D slicer and 3D printing of bones.

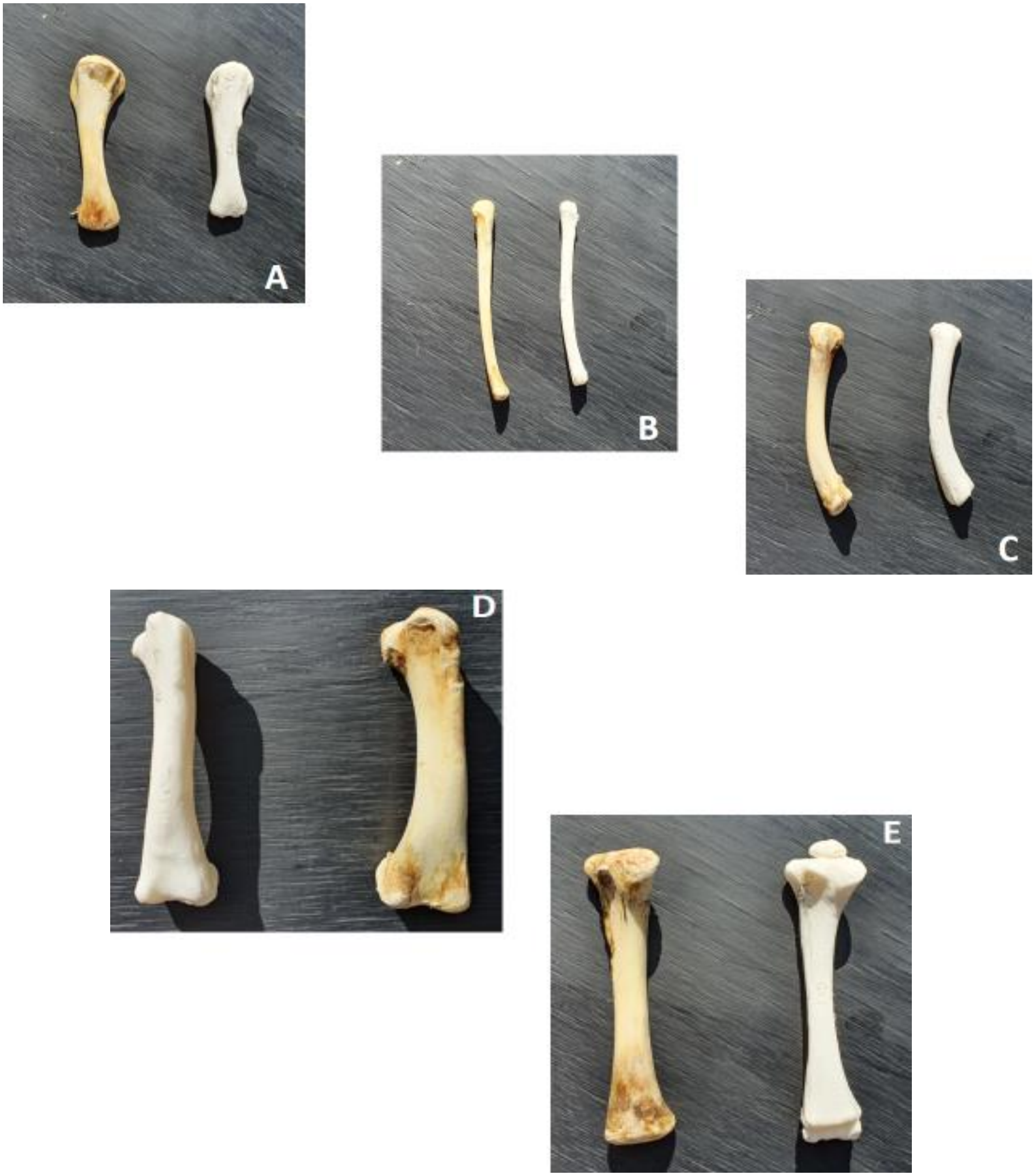


Figure 19. Showing forelimb and hindlimb bones and their models (humerus (A), radius (B), ulna (C), femur (D) and tibia (E)).

3.3. Dual Energy X-Ray Absorptiometry (DEXA) for Calculation of BMD (g/cm^2)

In the second part of the study, DEXA was performed to calculate BMD (g/cm^2). After CT scanning, the bone samples (n=10; right of femur, tibia, humerus, radius and ulna) were scanned using the dual energy X-ray absorptiometry (DEXA) method given in a study by (Karaarslan et al., 2021). Whole bone mineral density (BMD) and bone mineral content (BMC) were assessed. The bones were scanned using HOLOGIC Explorer QDR series (ASY-01250) (Figure 20) and measured through attached software (Medical Faculty, Adnan Menderes University, Aydın) (Figure 21). The humerus, femur and tibia were placed on their cranial surface and scanned in a cranio-caudal direction while radius and ulna were positioned in dorso-ventral direction for a trial (Damaziak et al., 2019). Bone density and mineral content were obtained for whole bone samples of humerus, femur and tibia (Figure 22).

Figure 20. DEXA apparatus with tibia sample ready for scanning.

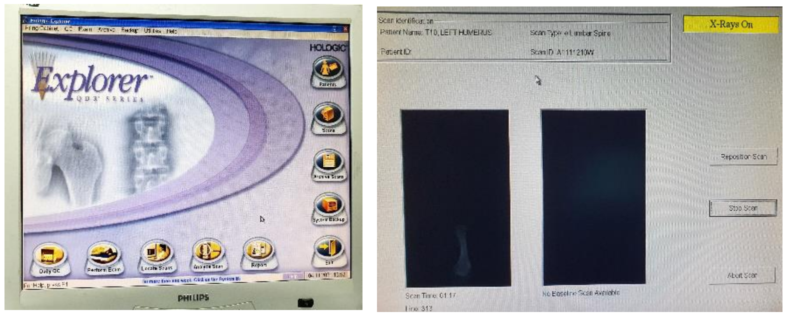


Figure 21. Represents DEXA processing for generating a scanned image.

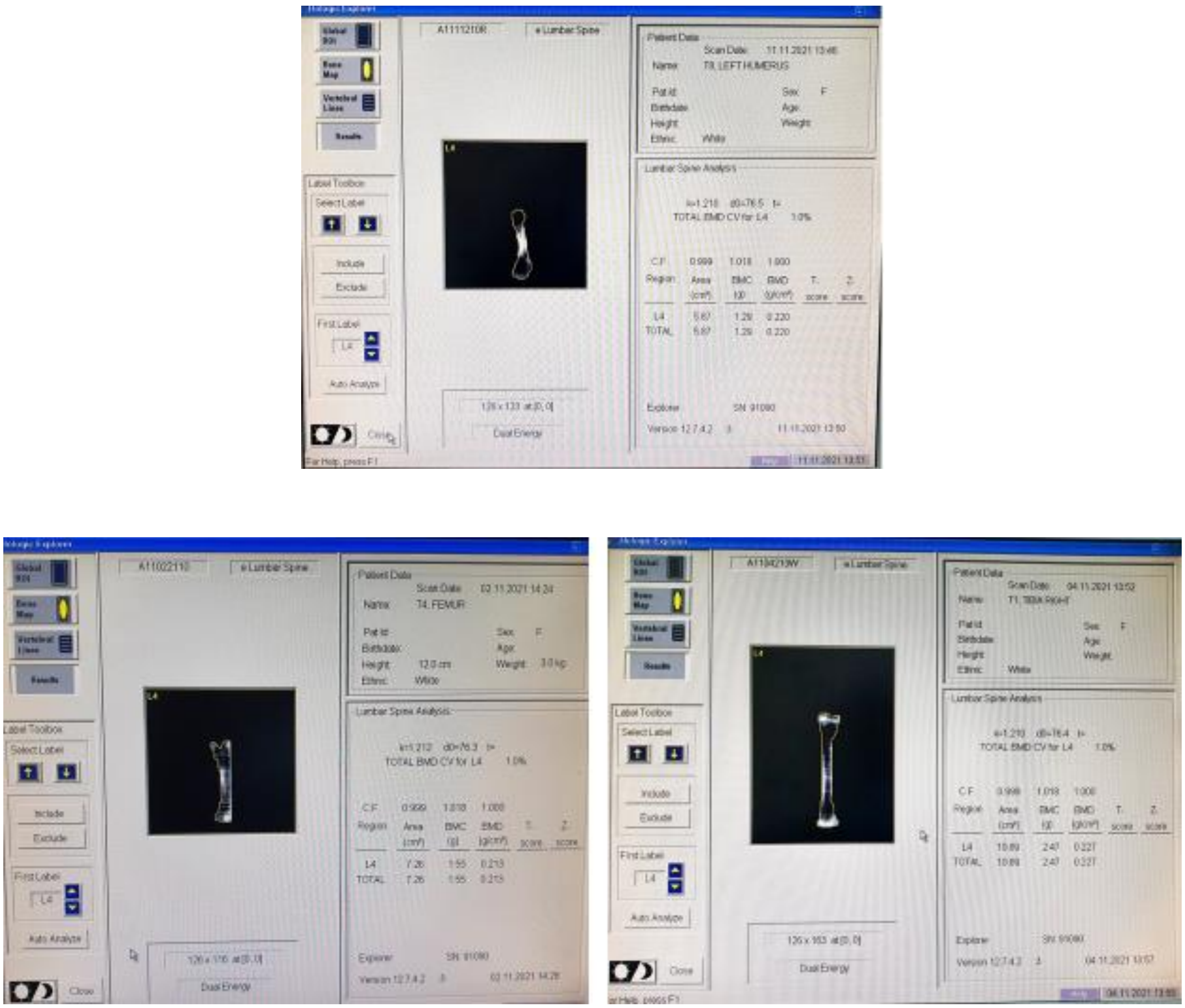


Figure 22. Showing DEXA scans of humerus, femur and tibia.

3.4. Biomechanical Testing of the Bones

In the third step of the study, the biomechanical testing was performed. Three-point bending testing of all the right bones and shear testing on the left bones (n=30; both forelimb and hindlimb) were conducted on a MTS Criterion™ Model 45 (LPS .204, 20 kN, 2.306 mV/V) mechanical testing machine at the Central Research Laboratory, Izmir Katip Çelebi University, Turkey, with 20kN load cell (Figure 22). All the procedures and calculations were performed according to ANISE/ASABE Standards (Standarts, 2007).

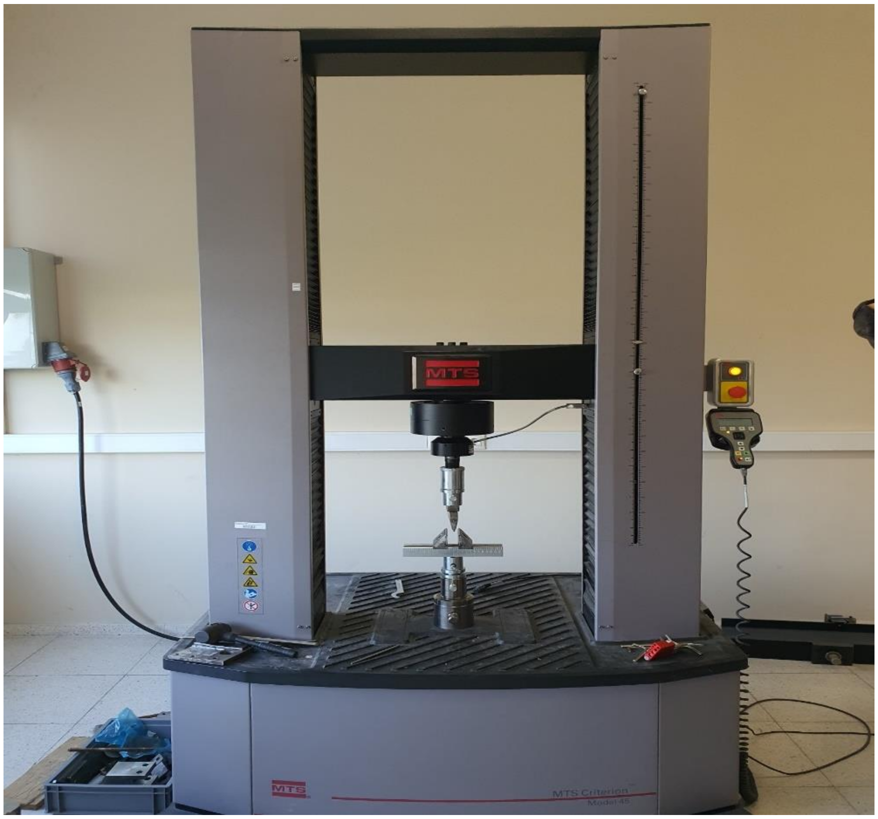


Figure 23. MTS Criterion™ Model 45 apparatus.

Length, external and internal diameters were already measured on the CT scans. The bones were placed with the cranial loading of humerus while caudal cortex was placed under compression for femur and tibia. The ulna and radius were loaded on the ventral surface. For the three point bending test, an inner span between two supports was kept 30 mm, 35mm, 35mm, 60 mm and 40 mm for humerus, radius, ulna, tibia and femur, respectively. A pre-load of 2 N was applied and a speed of 10 mm/min was used in case of three-point bending while 5 N and 5mm/min was kept for the shear testing with the constant span of 10.01 mm for all the bones (Standarts, 2007) (Figure 23 & 24).

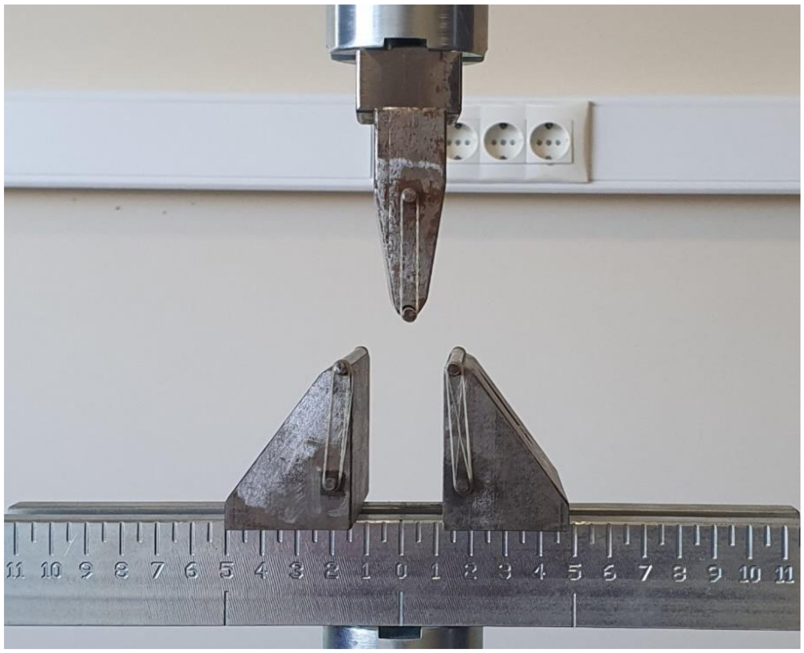


Figure 24. Three-point bending setup.

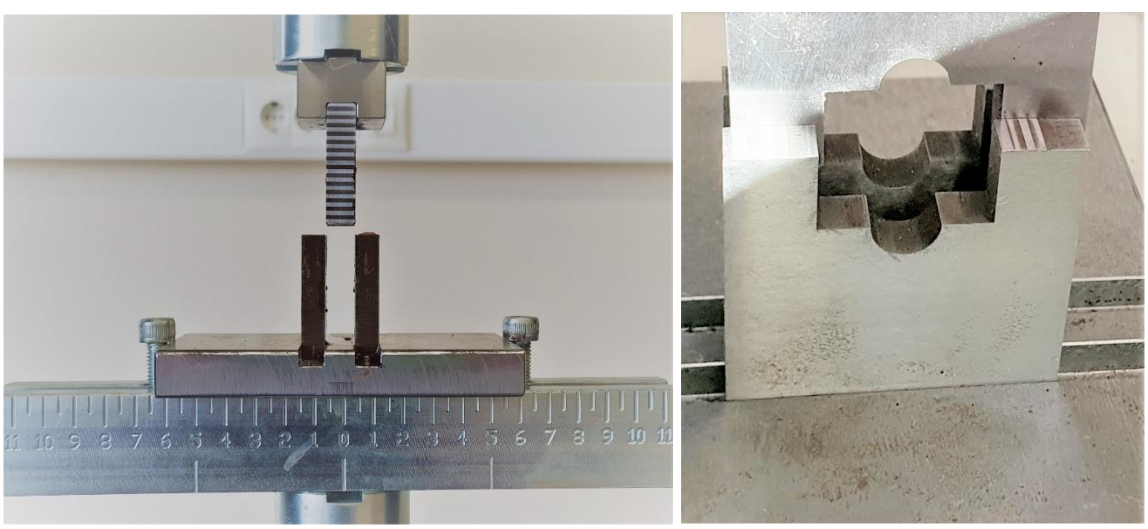


Figure 25. Shear testing set-up.

The bending test was performed on the right side bones (n=30) while shear testing was done on the left side bones (n=30). The measured data were recorded through MTS TestSuite TW Elite (twe) software (Figure 25 to 33).

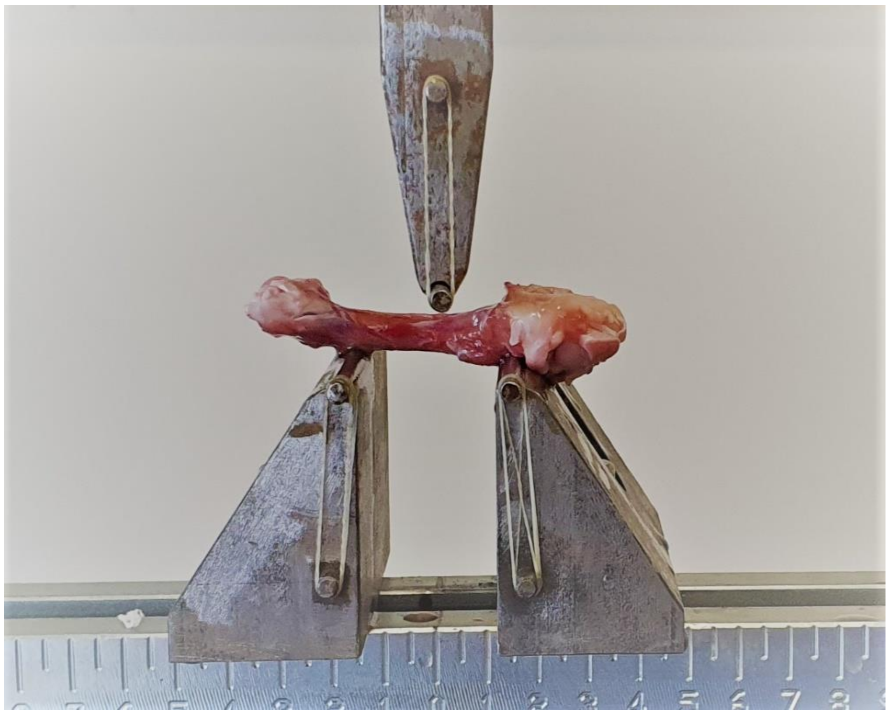


Figure 26. Three-point bending test of humerus.

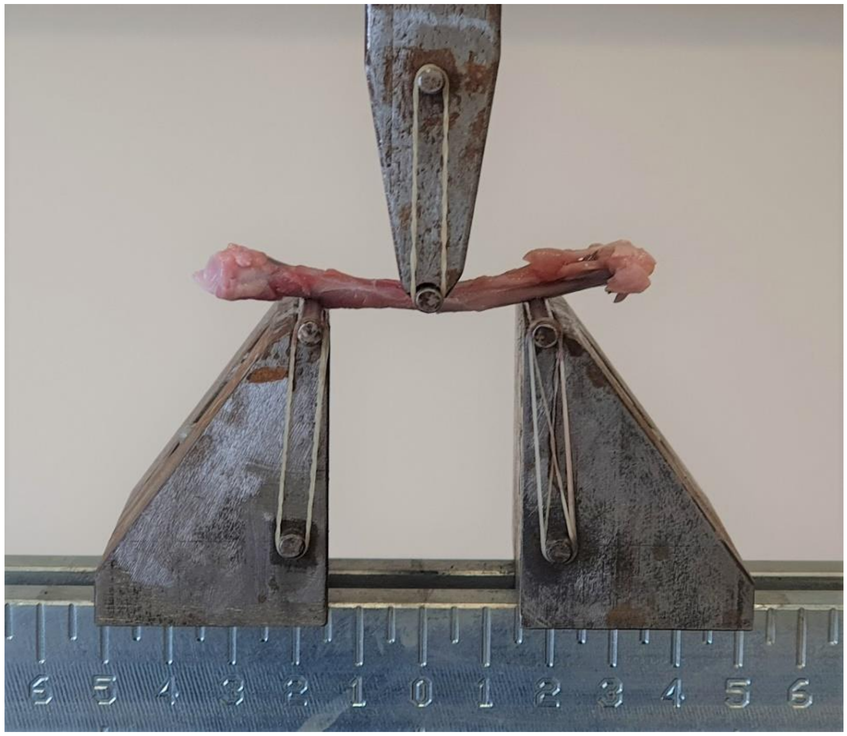


Figure 27. Three-point bending test of radius.

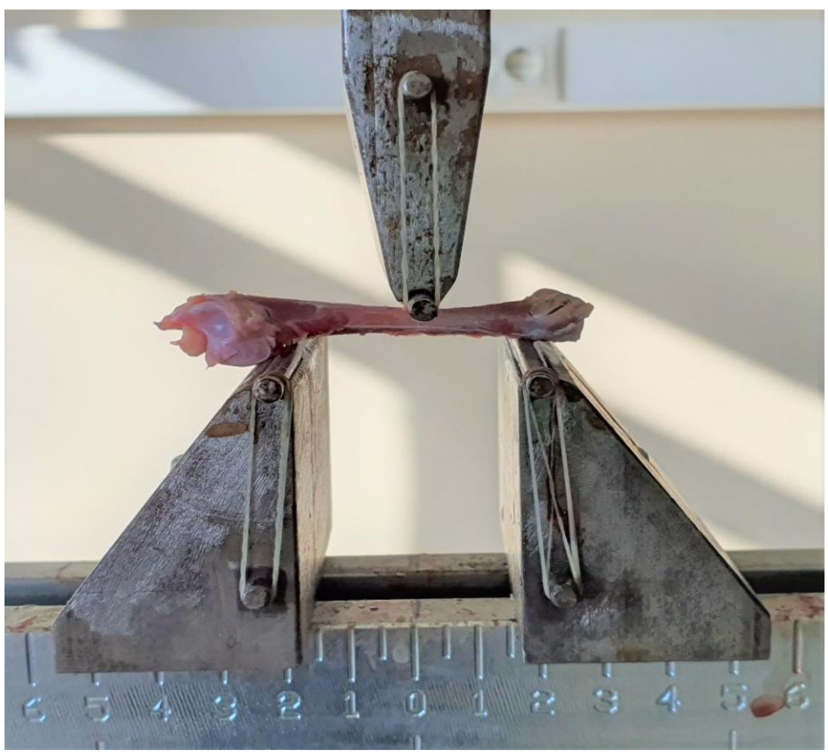


Figure 28. Three-point bending test of ulna.

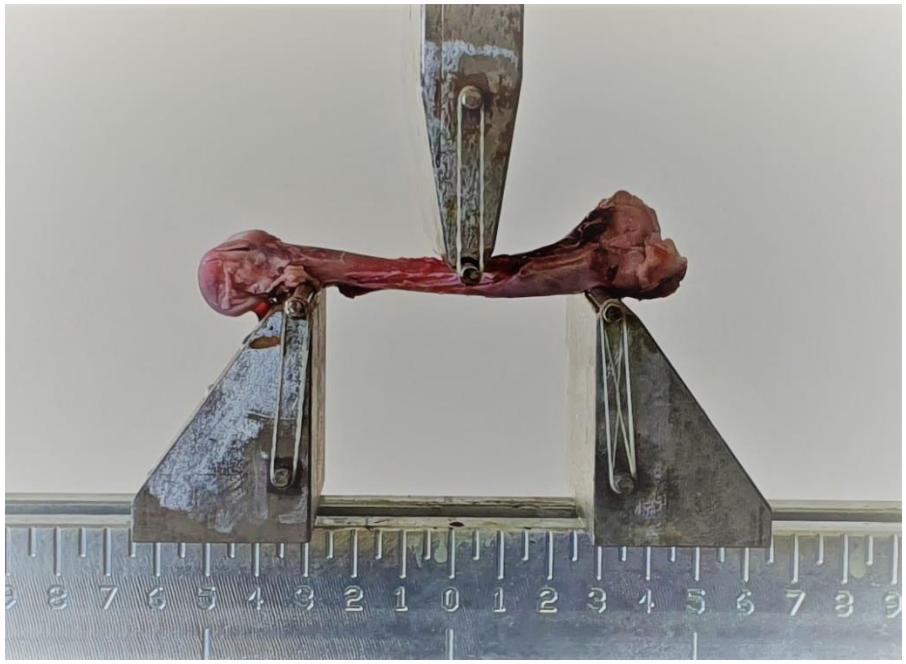


Figure 29. Three-point bending test of tibia.

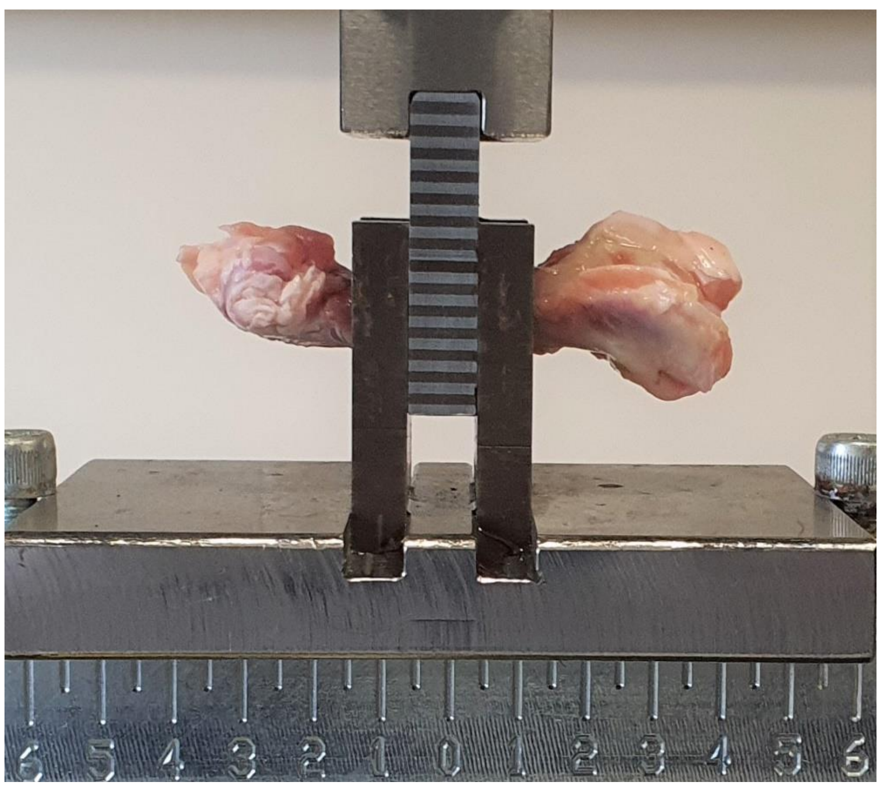


Figure 30. Shear test of humerus.

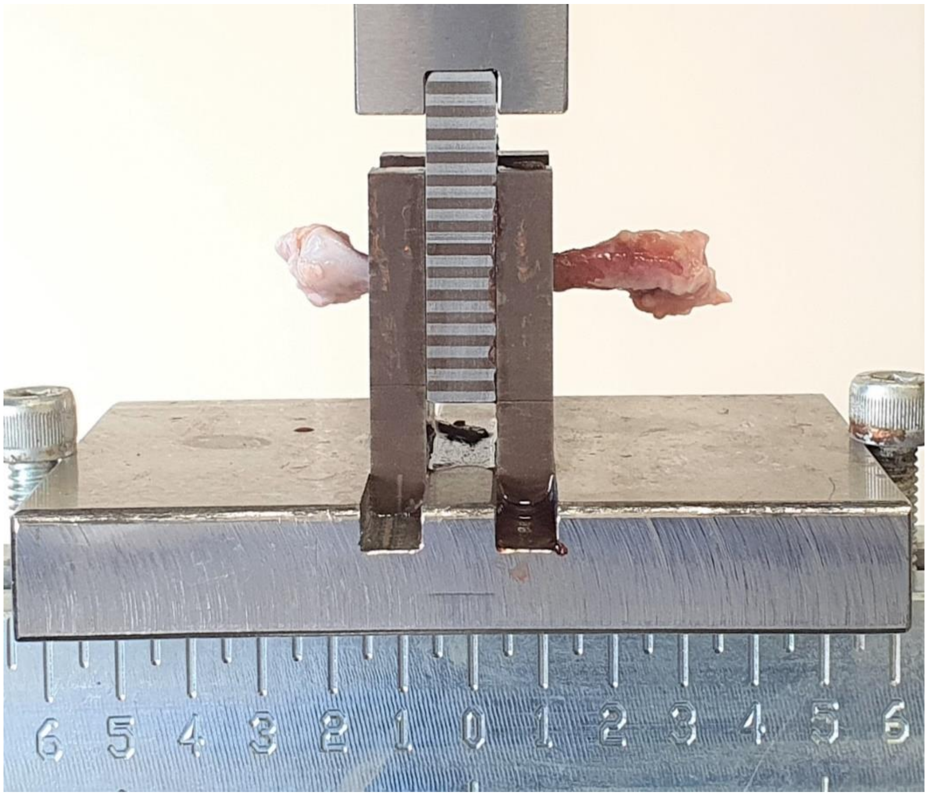


Figure 31. Shear test of radius.

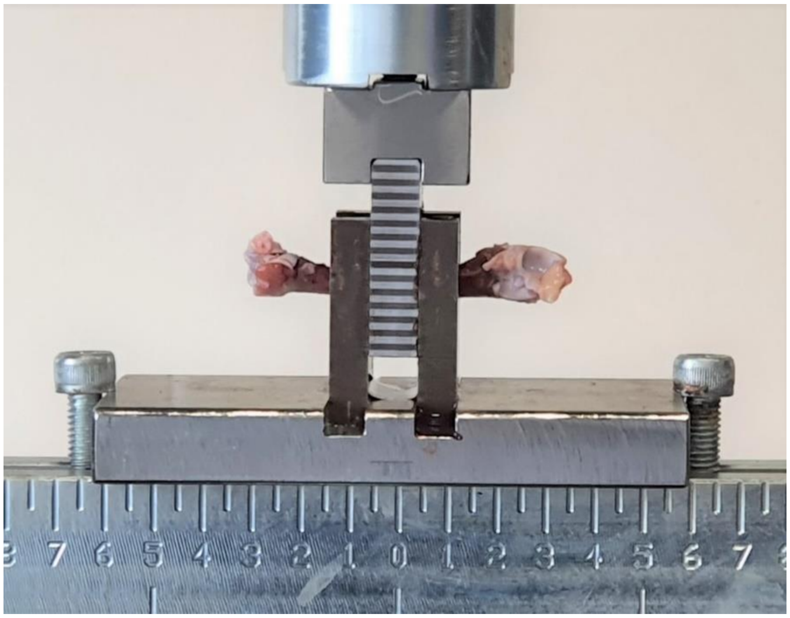


Figure 32. Shear test of ulna.

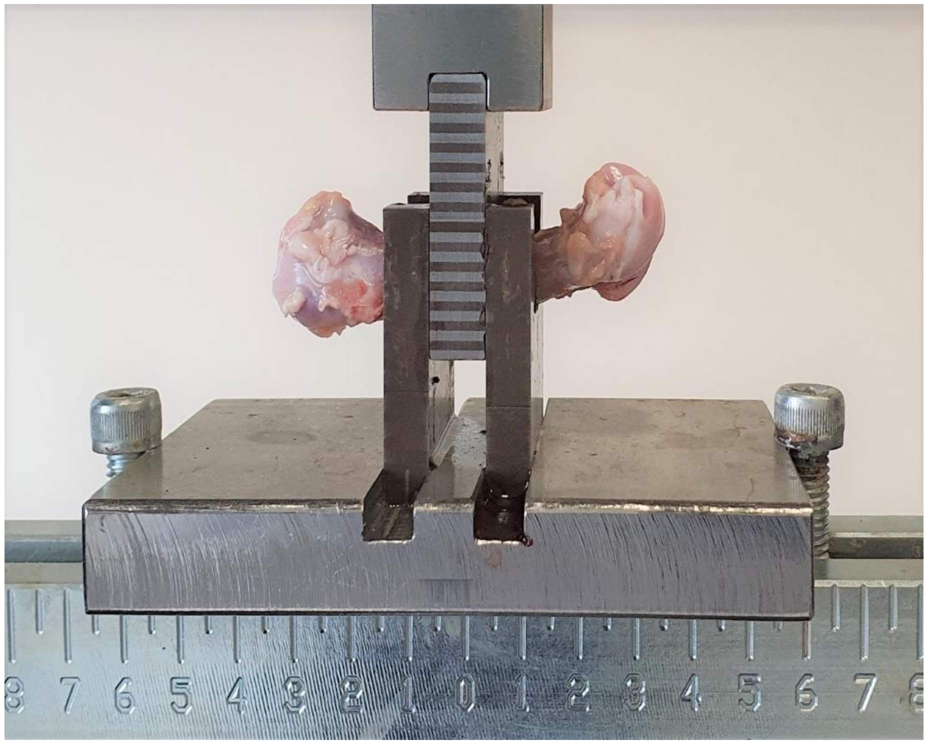


Figure 33. Shear test of femur.

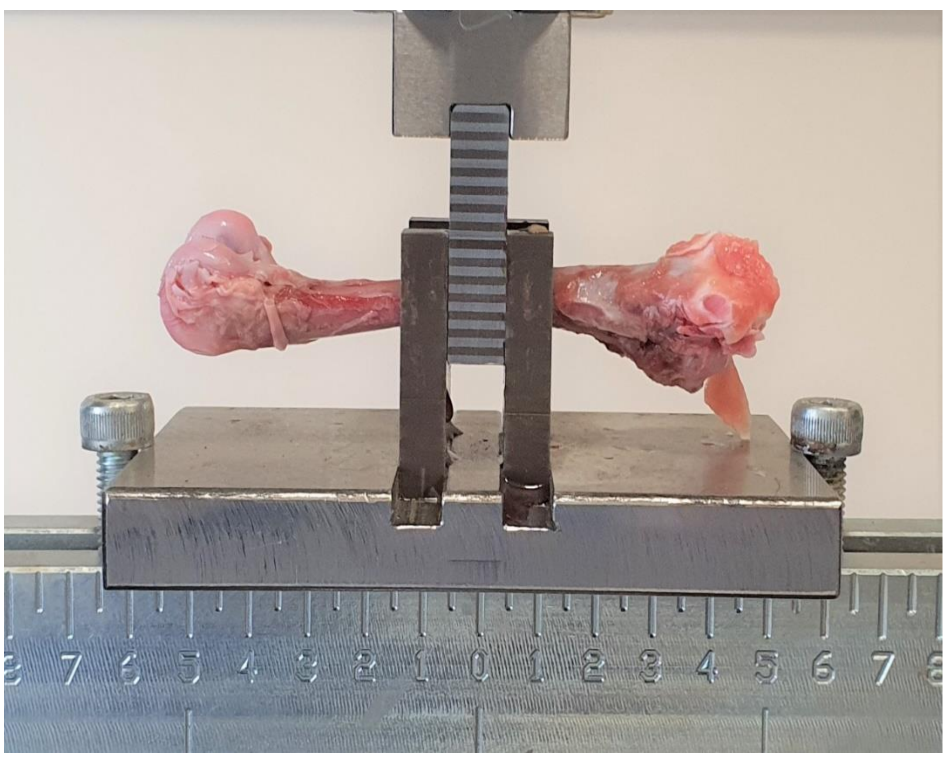


Figure 34. Shear test of tibia.

Figure 35. MTS TestSuite TW Elite (twe) software.

For both bending and shear, maximum force and deformation were obtained from the load-displacement curve (from excel file). This data was used to calculate slope, moment of inertia, strength and elastic modulus of all the bones.

The bending strength (MPa) was calculated as

BS = Fmax×L× Cranio-caudal external diameter/ 8×Ix, and Elastic modulus (GPa) as E = S×L3/ 48×Ix, where Fmax was maximum force, L was span, S was stiffness and Ix was moment of inertia.

For shear strength (τ, GPa), calculation was performed using the formula given below:

τ = Fmax / 2×A, where A was cross-sectional area.

3.5. Tibial Dyschondroplasia (TD)

After mechanical testing, tibial bones were cut longitudinally into two parts at the proximal epiphysis to see presence of cartilaginous growth in the metaphyseal area. The scoring method of 0, 1, 2 and 3 was used as described earlier in a study by Karaarslan and Nazlıgül (2018).

3.6. Statistical Analysis

All analyses were performed using SPSS (IBM Corporation, New York, USA) (Statistical Package for the Social Sciences). The coefficient of variation (CV) was calculated for one bone by taking five repeated measures just for angle measurements (Kara et al., 2018). The descriptive statistics were used to characterise bone properties. All measurements were presented as the mean ± Standard Deviation (Mean±SD). The 95% CI (confidence interval) of values were also presented in tables. The normal distribution of all the data was checked by the Shapiro-Wilk test. Right and left side bones were compared using paired sample T-test while the Wilcoxon test was used for non-parametric data. Analysis of variance (ANOVA) was used for statistical comparisons of the data obtained from different bones. The homogeneity of variances was also explored with Levene's test. For the homogeneous data, results of oneway-ANOVA and post-hoc Bonferroni tests were used while for the non-homogeneous, results of the Welch test and post-hoc Tamhane's T2 test were used. In case of non-parametric data, Kruskal-Wallis test was applied. The values having $P < 0.05$ were considered statistically significant.

Coefficients of variation were also calculated for each test group in the normalized mechanical test data.

4. RESULTS

4.1. Morphological Measurements (CT scans)

For CT angle measurements, coefficients of variation (CV) were 3.77% (Fedα), 1.76% (Fapc), 4.81% (Tidα), 0.97% (Tipβ), 5.06% (Tidβ) and 4.91% (Tapc). The results of morphometric parameters of right and left fore and hind limbs like, length, mediolateral (dorsoventral) and craniocaudal cross-sectional diameters, sectional thicknesses (medial (dorsal), lateral (ventral), cranial and caudal) etc., were presented in Table 2 and 3. There was no statistical difference for morphometric measurements between right and left bones. The mean±SD values along with *P*-value were given for the right and left bones.

Table 2. The cross-sectional measurements of right and left forelimb bones (mm).

Bones	Parameters	n	Right	Left	*P*
Humerus	ExtDVD	32	7.74±0.41	7.81±0.31	0.224
	IntDVD	32	5.13±0.54	5.19±0.50	0.345
	ExtCrCdD	32	6.64±0.35	6.73±0.35	0.085
	IntCrCdD	32	4.35±0.43	4.42±0.39	0.146
	VCT	32	1.28±0.21	1.26±0.20	0.305
	DCT	32	1.22±0.12	1.22±0.14	0.924
	CRCT	32	1.17±0.14	1.2±0.15	0.112
	CDCT	32	1.01±0.11	0.99±0.09	0.330
Radius	ExtCrCdD	32	3.32±0.2	3.28±0.21	0.131
	IntCrCdD	32	1.86±0.17	1.87±0.18	0.612
	ExtDVD	32	3.35±0.18	3.29±0.20	0.084
	IntDVD	32	1.83±0.20	1.87±0.17	0.072
	CDCT	32	0.84±0.15	0.8±0.10	0.235
	CRCT	32	0.56±0.10	0.59±0.10	0.352
	VCT	32	0.66±0.10	0.62±0.12	0.095
	DCT	32	0.75±0.11	0.72±0.13	0.112
Ulna	ExtCrCdD	32	6.04±0.39	6.12±0.34	0.118
	IntCrCdD	32	4.24±0.41	4.24±0.33	0.958
	ExtDVD	32	4.8±0.21	4.85±0.27	0.111
	IntDVD	32	3.17±0.27	3.21±0.26	0.188
	CDCT	32	0.77±0.10	0.74±0.11	0.257
	CRCT	32	1.00±0.09	0.97±0.08	0.151
	VCT	32	0.79±0.11	0.80±0.12	0.388
	DCT	32	0.72±0.11	0.74±0.09	0.595

The data was expressed as Mean±SD.

$P < 0.05$ was considered significant.

Table 3. The cross-sectional measurements of right and left hindlimb bones (mm).

Bones	**Parameters**	**n**	**Right**	**Left**	***P***
Femur	ExtMLD	32	8.46±0.42	8.41±0.42	0.208
	IntMLD	32	5.90±0.48	5.94±0.40	0.273
	ExtCrCdD	32	8.62±0.51	8.65±0.51	0.109
	IntCrCdD	32	5.97±0.51	5.98±0.51	0.667
	MCT	32	1.47±0.19	1.41±0.23	0.193
	LCT	32	0.93±0.11	0.91±0.14	0.378
	CRCT	32	1.15±0.13	1.13±0.15	0.137
	CDCT	32	1.14±0.14	1.14±0.19	0.868
Tibia	ExtMLD	32	7.93±0.50	7.90±0.50	0.551
	IntMLD	32	5.18±0.47	5.17±0.43	0.656
	ExtCrCdD	32	6.89±0.38	6.86±0.35	0.106
	IntCrCdD	32	4.65±0.37	4.63±0.34	0.386
	MCT	32	1.17±0.17	1.14±0.14	0.236
	LCT	32	1.49±0.26	1.51±0.24	0.600
	CRCT	32	0.94±0.11	0.90±0.09	0.070
	CDCT	32	1.16±0.13	1.14±0.14	0.251

The data was expressed as Mean±SD.

$P < 0.05$ was considered significant.

The average values of all the right and left sections and CI were provided in Table 4 and Table 5. According to the findings of this study, ExtCrCdD and IntCrCdD of humerus (6.69±0.32; 4.39±0.39) and ulna (6.08±0.34; 4.25±0.35), respectively were showing similar values. The ExtDVD and IntDVD of both these bones were obtained as 7.78±0.33 and 5.16±0.48 for humerus while 4.83±0.23 and 3.19±0.25 values were observed for ulna, respectively. The ExtCrCdD = 3.30±0.19, IntCrCdD = 1.87±0.17, ExtDVD = 3.32±0.18 and IntDVD = 1.85±0.17 were quantified for radius (Table 4). The caudal section of humerus showed higher value (1.19±0.14) and ulna showed lower value (0.75±0.08) among three bones of the forelimb. Although radius was thin bone on a gross view (unlike mammalian radius), but its caudal cortical section was thicker than that of the ulna (0.82±0.10). The CRCT of humerus and ulna showed CI of (0.97-1.03) and (0.96-1.01), respectively. The ventral section of humerus (1.27±0.20) was thicker than ulna (0.8±0.10) (Table 4).

Table 4. The average sectional measurements data for forelimb bones (mm), n=32.

Parameters	Humerus	Radius	Ulna
ExtCrCdD	6.69±0.32 (6.57-6.80)	3.30±0.19 (3.23-3.37)	6.08±0.34 (5.96-6.20)
IntCrCdD	4.39±0.39 (4.25-4.53)	1.87±0.17 (1.81-1.93)	4.25±0.35 (4.12-4.37)
ExtDVD	7.78±0.33 (7.66-7.90)	3.32±0.18 (3.26-3.39)	4.83±0.23 (4.74-4.91)
IntDVD	5.16±0.48 (4.99-5.33)	1.85±0.17 (1.79-1.91)	3.19±0.25 (3.10-3.28)
CDCT	1.19±0.14 (1.14-1.24)	0.82±0.10 (0.78-0.86)	0.75±0.08 (0.73-0.78)
CRCT	1.00±0.08 (0.97-1.03)	0.58±0.07 (0.56-0.60)	0.99±0.07 (0.96-1.01)
VCT	1.27±0.20 (1.20-1.34)	0.64±0.08 (0.61-0.67)	0.80±0.10 (0.76-0.83)
DCT	1.22±0.12 (1.18-1.27)	0.74±0.10 (0.70-0.78)	0.73±0.08 (0.70-0.76)
Area of cross-section (mm^2)	23.21±2.04 (22.45-23.98)	5.80±0.76 (5.51-6.09)	12.58±1.29 (12.10-13.06)

The data was expressed as Mean±SD along with 95% confidence interval.

The results of mean values of the diameters and sectional measurements of the hindlimb bones were given in the Table 5. In hindlimb, femur external and internal diameter in mediolateral direction were 8.44±0.41 and 5.92±0.43 while in craniocaudal direction were 8.64±0.51 and 5.98±0.50, respectively. The cortical wall thicknesses i.e., MCT, LCT, CRCT and CDCT values for the femur were 1.44±0.16, 0.93±0.10, 1.14±0.13 and 1.14±0.16, respectively. Similarly, MCT, LCT, CRCT, CDCT for tibia were 1.15±0.14, 1.50±0.24, 0.92±0.08, 1.15±0.13, respectively.

Table 5. The average sectional measurements data for hindlimb bones (mm), n=32.

Parameters	Femur	Tibia
ExtMLD	8.44±0.41 (8.29-8.58)	7.92±0.49 (7.74-8.09)
IntMLD	5.92±0.43 (5.77-6.08)	5.18±0.44 (5.02-5.34)
ExtCrCdD	8.64±0.51 (8.45-8.82)	6.88±0.36 (6.75-7.01)
IntCrCdD	5.98±0.50 (5.80-6.16)	4.64±0.35 (4.52-4.77)
MCT	1.44±0.16 (1.39-1.50)	1.15±0.14 (1.10-1.20)
LCT	0.93±0.10 (0.89-0.96)	1.50±0.24 (1.42-1.59)
CRCT	1.14±0.13 (1.10-1.19)	0.92±0.08 (0.89-0.95)
CDCT	1.14±0.16 (1.09-1.20)	1.15±0.13 (1.11-1.20)
Area of cross-section (mm^2)	29.35±4.12 (27.81-30.88)	23.76±2.90 (22.67-24.84)

The data was expressed as Mean±SD along with 95% confidence interval.

The calculated CMI data was presented in the Table 6. The results of CMI revealed that the cortex of broiler's radius was found thicker in both mediolateral and craniocaudal directions than the rest of the bones, measured as 43.46% and 44.19%, respectively.

Table 6. Medio-lateral (CMI_{ML}) and cranio-caudal (CMI_{CRCD}) cortico-medullay index (%) of forelimb and hindlimb bones.

Bones	**n**	**CMI_{ML}**	**CMI_{CRCD}**
Humerus	32	33.73 ± 4.33^{bc} (32.17-35.29)	34.41 ± 3.74^{b} (33.06-35.76)
Radius	32	43.46 ± 3.27^{a} (42.29-44.64)	44.19 ± 4.03^{a} (42.74-45.65)
Ulna	32	30.21 ± 4.05^{c} (28.75-31.67)	33.93 ± 3.84^{bc} (32.54-35.31)
Femur	32	29.83 ± 3.028^{c} (28.73-30.92)	30.78 ± 4.19^{c} (29.27-32.29)
Tibia	32	34.59 ± 3.58^{b} (33.29-35.88)	32.54 ± 2.99^{bc} (31.46-33.61)
P		**0.000**	**0.000**

$^{a, b, c, d}$ Means within the same column with different superscripts are significantly different ($P < 0.05$).
The data was expressed as Mean±SD along with 95% confidence interval.

The estimation of lengths of the forelimb bones showed that humerus was longest (71.84±3.25) bone followed by the ulna (67.68±3.17) then by the radius (62.93±2.69). Moreover, femur bone was shorter in length (78.38±2.88) than the tibia (106.78±4.61). The angles and curvature measurements of femur and tibia for prediction of varus-valgus deformity were presented in table 7. Femur distal angle measurements for right and left bone were 8.41±1.80 and 8.25±1.93, respectively. There was no statistical difference between right and left femur bones angles (P > 0.05). Furthermore, curvature measurements showed 9.09±0.85 value for right femur and 9.14±0.83 for left femur bone with no significant difference (P > 0.05).

The measurement for tibial bone were also performed namely, Tidα, Tipβ, Tidβ, and Tapc measurement of curvature. Tibial distal angle value for left side of tibia was 4.34±2.66 and for right tibia bone 4.24±2.45, Tipβ value of left tibia was 32.08±2.78 and for right tibia 31.98±3.34. Tibial distal bending for left side was 49.63±3.21, and for right side tibia was 48.73±2.52. There was no statistical difference between right and left tibia curvature measurements (P > 0.05). Tapc (mm) measurement for curvature of right tibial bone was 8.83±1.00, and for tibial bone was 8.82±0.89 (Table 7).

Table 2. The measured femoral and tibial length, angle and curvature measurements for right and left sides.

Parameters	n	Right	Left	*P*
Humerus Length (mm)	32	71.93±3.16 (70.79-73.07)	71.74±3.37 (70.52-72.96)	0.111
Radius Length (mm)	31	62.99±2.76 (61.99-63.98)	62.75±2.61 (61.79-63.71)	0.301
Ulna Length (mm)	31	67.74±3.24 (66.58-68.91)	67.46±3.06 (66.34-68.58)	0.343
Femur Length (mm)	32	78.39±2.93 (77.33-79.45)	78.37±2.88 (77.33-79.40)	0.845
Tibial Length (mm)	32	106.85±4.67 (105.16-108.52)	106.72±4.58 (105.07-108.37)	0.370
Fedα	32	8.41±1.80 (7.76-9.06)	8.25±1.93 (7.55-8.94)	0.528
Fapc (mm)	32	9.09±0.85 (8.79-9.40)	9.14±0.83 (8.84-9.44)	0.716
Tidα	32	4.34±2.66 (3.38-5.29)	4.24±2.45 (3.36-5.13)	0.742
Tipβ	32	32.08 ± 2.78 (31.08-33.08)	31.98±3.34 (30.77-33.18)	0.838
Tidβ	32	49.63±3.21 (48.48-50.79)	48.73±2.52 (47.82-49.64)	0.179
Tapc (mm)	32	8.83±1.00 (8.47-9.19)	8.82±0.89 (8.49-9.14)	0.938

The data was expressed as Mean±SD along with 95% confidence interval.

$P < 0.05$ was considered significant.

4.2. Bone Mineral Density Measurements

The measurements obtained from DEXA as area (cm^2), BMC (g) and BMD (g/cm^2) were given in the Table 8. The tibial bone showed higher area and BMC than femur and humerus bone and both values were statistically significant. When BMD values of both limbs were compared it was found that femur, tibia and humerus has similar numeric values without any significant difference ($P > 0.05$). The BMD of humerus was found as 0.22±0.017. In the hind limb, the values for femur and tibial BMD were 0.22±0.02 and 0.22±0.017, respectively. Radius and ulna were difficult to scan using the same resolution as that for other bones that's why they were excluded from the experiment.

Table 3. The DEXA measurements of humerus, femur and tibia.

Bone	**n**	**Area (cm^2)**	**BMC (g)**	**BMD (g/cm^2)**
Humerus	10	6.67±0.70$^{\mathbf{b}}$ (6.16-7.17)	1.44±0.17$^{\mathbf{c}}$ (1.31-1.57)	0.22±0.02 (0.20-0.22)
Femur	10	7.39±0.61$^{\mathbf{b}}$ (6.95-7.82)	1.65±0.15$^{\mathbf{b}}$ (1.54-1.76)	0.22±0.02 (0.21-0.24)
Tibia	10	9.86±1.35$^{\mathbf{a}}$ (8.89-10.82)	2.28±0.17$^{\mathbf{a}}$ (2.16-2.39)	0.22±0.02 (0.21-0.24)
P		**0.000**	**0.000**	0.561

$^{a, b, c, d}$ Means within the same column with different superscripts are significantly different ($P < 0.05$).
The data was expressed as Mean±SD along with 95% confidence interval.

4.3. Bone Biomechanical Testing

The three-point bending test results and calculations were presented in the Table 9. According to three-point bending test, the maximum force (N) applied on the humerus, radius, ulna, femur and tibia was 290.19±54.04, 36.54±5.30, 105.90±20.23, 190.72±45.43, and 187.60±36.34, resulting in the deformation (mm) of 2.83±0.34, 2.97±0.58, 2.45±0.39, 3.39±0.43, 3.79±0.63, respectively. The maximum force was highest for humerus and lowest was seen for radius. On calculations, the radius was observed as the strongest bone among forelimb and hindlimb bones of broiler with strength 90.96 MPa. The femur was the weakest among all with 27.87±6.74 MPa. The stiffness (N/mm) value was highest for the humerus (130.31±16.90) and lowest for the radius (26.06±3.47). The modulus of elasticity (GPa) was seen highest for the radius (4.00±0.9) followed by tibia and then by ulna, humerus and femur. All the parameters obtained after three-point bending of the bones were significantly different from one another ($P < 0.05$). The moment of inertia of femur (209.89±44.49) was higher as compared to all other bones. Tibia and humerus showed similar moment of inertia (104.75±19.65 and 103.46±17.30, respectively).

Table 4. The three-point bending test data of fore and hindlimb bones.

Bone	n	Force (N)	Deformation (mm)	Moment of Inertia (mm^4)	Strength (MPa)	Stiffness (N/mm)	Elastic modulus (GPa)
Humerus	30	290.19±54.04^{a} (270.01-310.37)	2.83±0.34^{cd} (2.70-2.96)	103.46±17.30^{bc} (97.00-109.92)	70.03±11.50^{bc} (65.74-74.33)	130.31±16.90^{a} (124.00-136.62)	0.72±0.11^{d} (0.68-0.76)
Radius	30	36.54±5.30^{d} (34.56-38.51)	2.97±0.58^{bc} (2.76-3.19)	6.04±1.19^{d} (5.59-6.48)	90.96±18.01^{a} (84.23-97.68)	26.06±3.47^{c} (24.76-27.36)	4.00±0.90^{a} (3.64-4.36)
Ulna	30	105.90±20.23^{c} (98.34-113.45)	2.45±0.39^{d} (2.31-2.60)	30.94±5.63^{d} (28.84-33.04)	73.06±14.80^{b} (67.54-78.59)	66.28±10.39^{b} (62.40-70.16)	1.96±0.40^{c} (1.81-2.11)
Femur	29	190.72±45.43^{b} (173.44-207.99)	3.39±0.43^{ab} (3.22-3.55)	209.89±44.49^{a} (192.97-226.81)	27.87±6.74^{d} (25.30-30.43)	77.90±14.38^{b} (72.43-83.37)	0.51±0.13^{e} (0.46-0.56)
Tibia	30	187.60±36.34^{b} (174.02-201.17)	3.79±0.63^{a} (3.55-4.02)	104.75±19.65^{b} (97.42-112.09)	63.27±7.96^{c} (60.29-66.24)	66.07±8.61^{b} (62.86-69.29)	2.92±0.59^{b} (2.70-3.14)
P		**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**

[a, b, c, d] Means within the same column with different superscripts are significantly different ($P < 0.05$).
The data was expressed as Mean±SD along with 95% confidence interval.

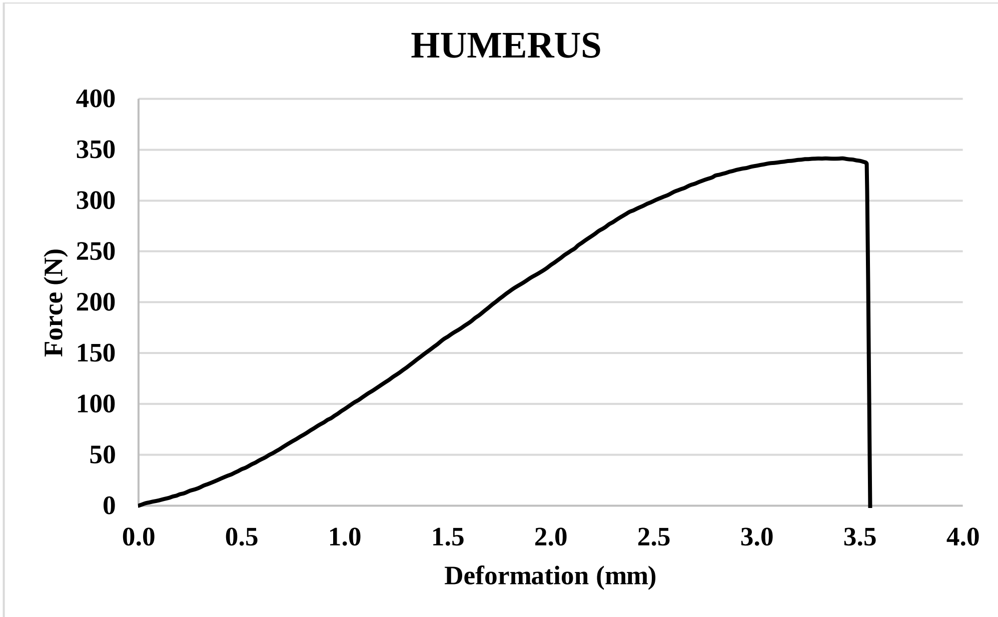


Figure 36. Force-deformation curve for the three-point bending test of humerus.

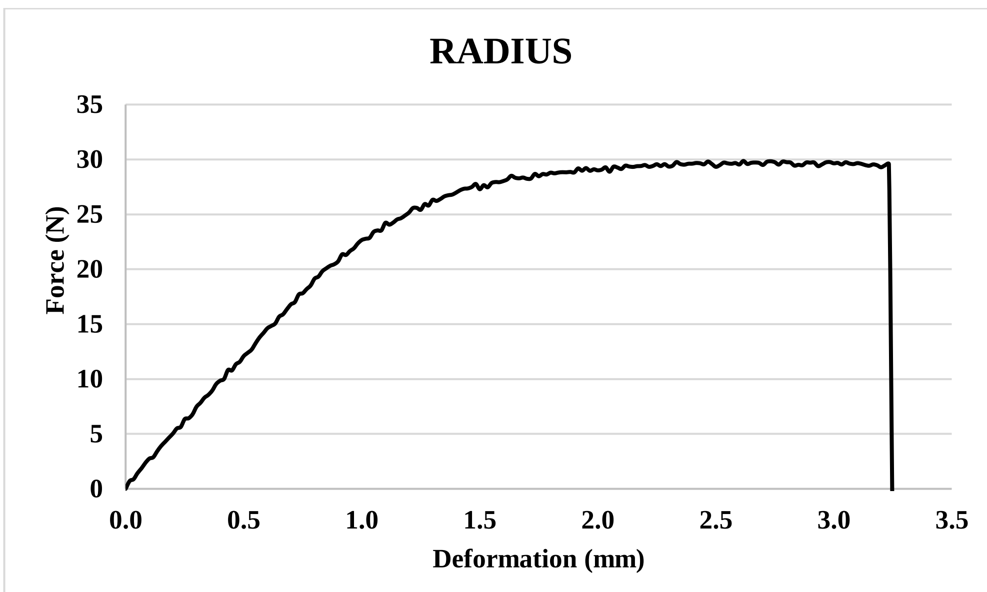


Figure 37. Force-deformation curve for the three-point bending test of radius.

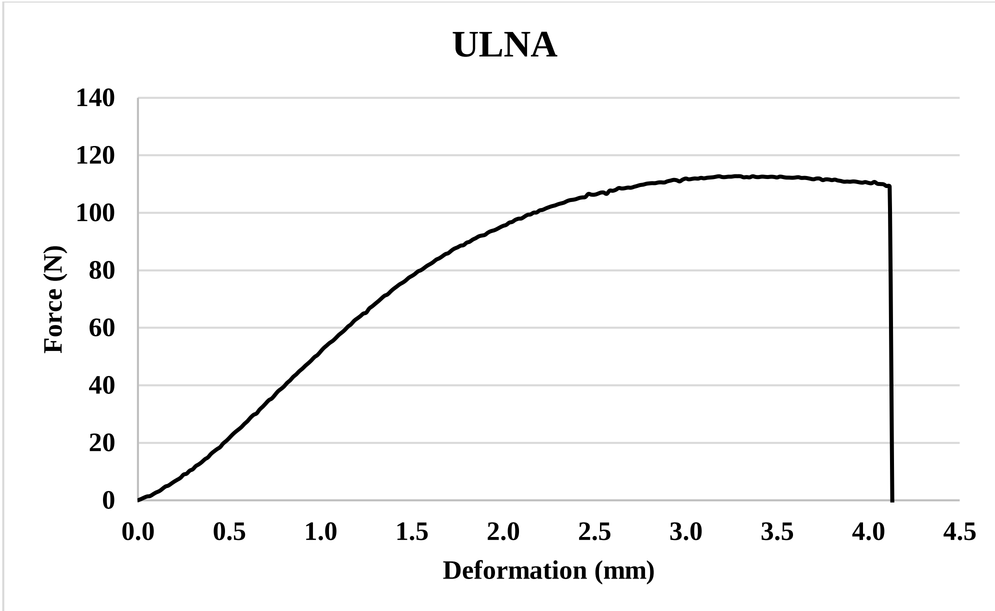


Figure 38. Force-deformation curve for the three-point bending test of ulna.

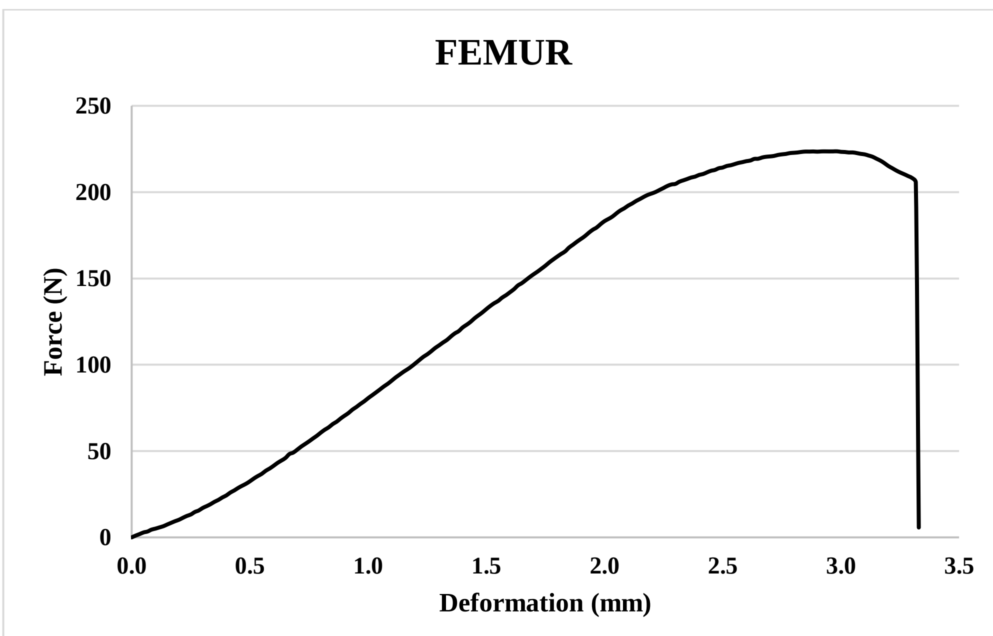


Figure 39. Force-deformation curve for the three-point bending test of femur.

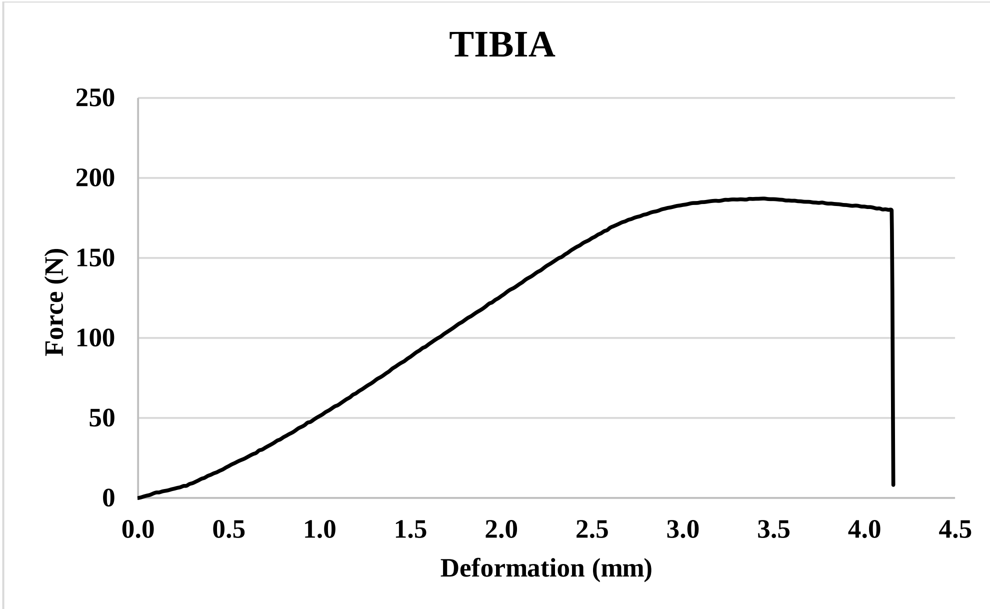


Figure 40. Force-deformation curve for the three-point bending test of tibia.

The shear test results and calculations were presented in the Table 10. Shear testing of the bones of forelimb and hindlimb also showed significantly different ($P < 0.05$) values for shear force, resulting deformation and the shear stress. The shear force (N) for humerus (496.94±82.08) and tibia (498.68±173.83) was similar but the humerus was undergone more deformation (2.07 mm) than the tibia bone. The shear strength (N) was highest for the radius (16.27±3.5).

Table 5. The shear testing data of fore and hindlimb bones.

Bones	**n**	**Area (mm²)**	**Force (N)**	**Deformation (mm)**	**Strength (MPa)**
Humerus	30	23.21±2.04^{b} (22.45-23.98)	496.94±82.08^{a} (466.29-527.59)	2.07±0.93^{a} (1.72-2.42)	10.77±1.88^{b} (10.06-11.47)
Radius	29	5.80±0.76^{d} (5.51-6.09)	187.25±40.22^{b} (171.95-202.55)	1.11±0.32^{c} (0.99-1.23)	16.27±3.50^{a} (14.94-17.60)
Ulna	30	12.58±1.29^{c} (12.10-13.06)	202.7±38.79^{b} (188.21-217.18)	1.20±0.26^{bc} (1.10-1.30)	8.1±1.60^{cd} (7.51-8.70)
Femur	30	29.35±4.12^{a} (27.81-30.88)	397.63±111.71^{a} (355.92-439.35)	1.73±0.40^{b} (1.58-1.88)	6.87±2.10^{d} (6.09-7.65)
Tibia	30	23.76±2.90^{b} (22.67-24.84)	498.68±173.83^{a} (433.77-563.59)	1.33±0.40^{c} (1.18-1.48)	10.53±3.65^{bc} (9.17-11.90)
P		**0.000**	**0.000**	**0.000**	**0.000**

$^{a, b, c, d}$ Means within the same column with different superscripts are significantly different ($P < 0.05$).
The data was expressed as Mean±SD along with 95% confidence interval.

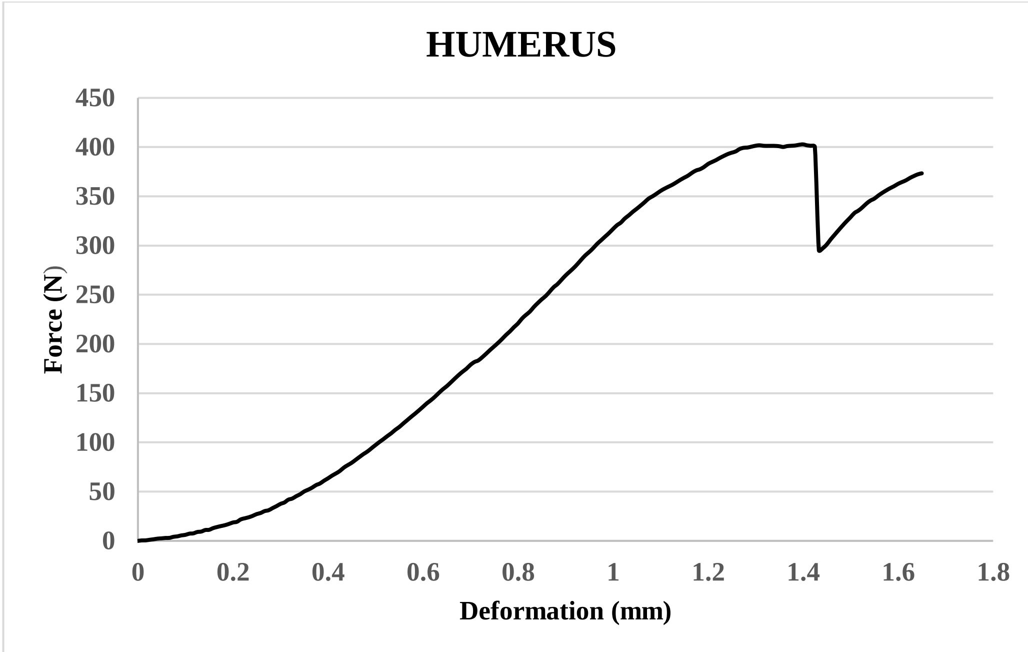


Figure 41. Force-deformation curve for the shear test of humerus.

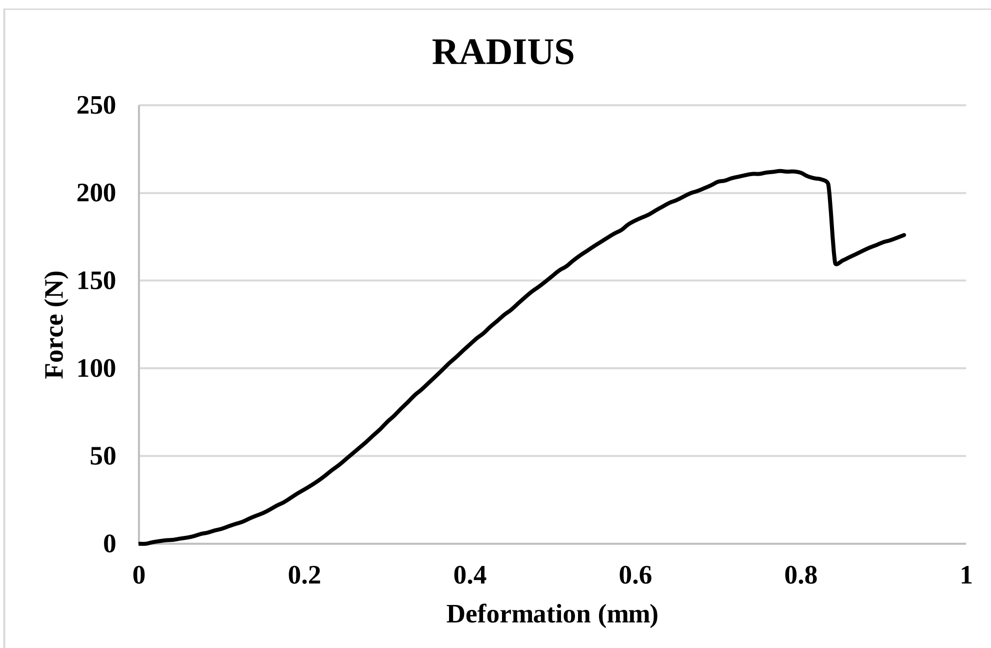


Figure 42. Force-deformation curve for the shear test of radius.

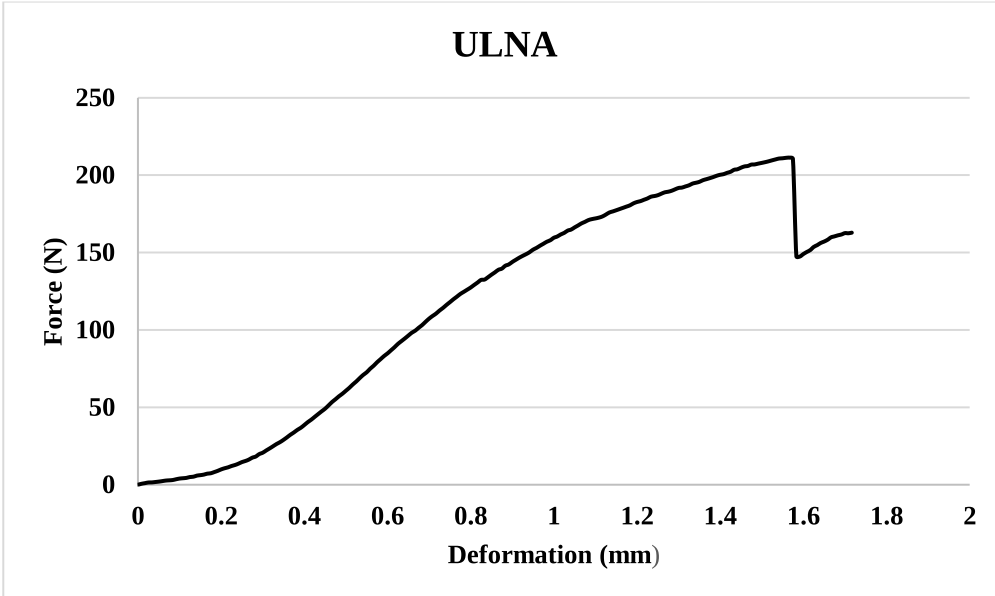


Figure 43. Force-deformation curve for the shear test of ulna.

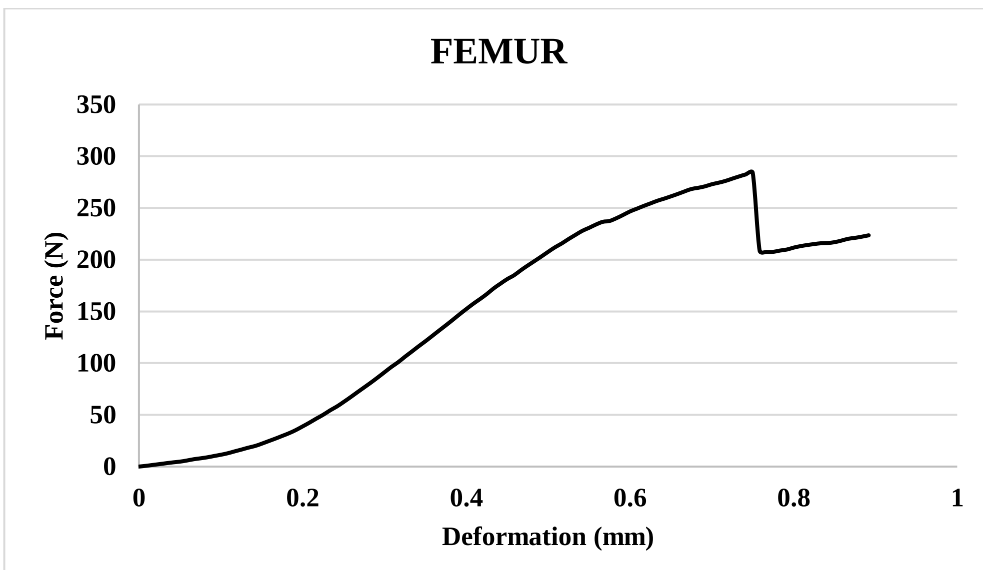


Figure 44. Force-deformation curve for the shear test of femur.

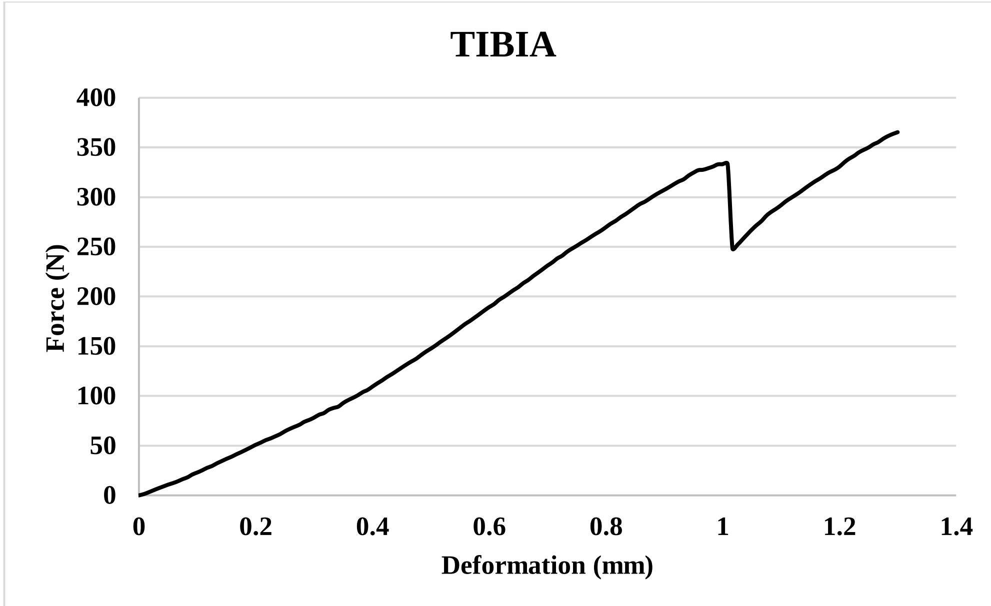


Figure 45. Force-deformation curve for the shear test of tibia.

CV was calculated for the both biomechanical tests and it was seen that humerus showed least coefficient of variations for all the measurements obtained from both three-point bending and shear tests (Table 11). Besides, overall CV was found higher for shear testing technique than three-point bending test.

Table 6. The CV (coefficient of variation, %) calculation for three-point bending and shear tests.

	Three-point Bending			Shear Test		
Bones	**Force**	**Deformation**	**Strength**	**Force**	**Deformation**	**Strength**
Humerus	18.62	12.13	16.42	16.52	44.95	17.45
Radius	14.50	19.46	19.80	21.48	28.76	21.52
Ulna	19.11	16.03	20.25	19.14	22.05	19.75
Femur	23.82	12.80	24.19	28.09	23.33	30.52
Tibia	19.37	16.61	12.59	34.86	29.93	34.64

4.5. Quantification of TD lesion

Both right and left tibias manifested healthy condition as 0 TD score was observed in all of them (Figure 36).

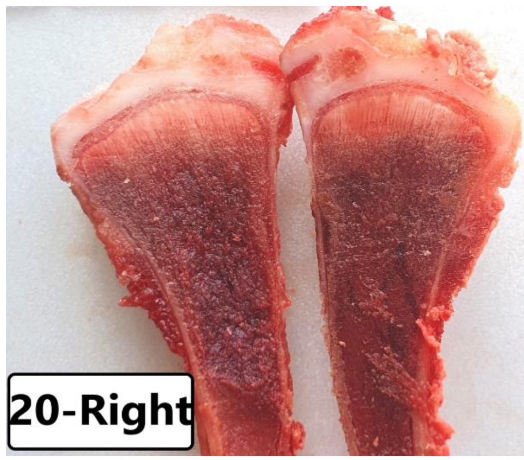


Figure 36. Right tibia with 0 TD score.

5. DISCUSSION

As different methodologies were used in this study, it was important to inspect their precision and reliability. The calculation of coefficient of variation (CV) is a method to measure the reliability by taking repeated measures of the same sample. The reliability of angular measurements was done by calculating CV using five repeated measures of one sample. It was found minimum for Tipβ (0.97%) and maximum for Tidβ (5.06%). So, the measurement method for angles was perceived correct and reliable (Kara et al., 2018). The average values of section measurements and thicknesses were used from three consecutive sections i.e., at the center of all the bones plus 1 section above and below the mid-point. So, CV was not calculated for them. The DEXA method was performed using same instrument and methodology followed by Karaarslan et al. (2021). Therefore, CV for the same apparatus was not repeatedly measured in this study. The biomechanical tests were carried out according to the ASABE standards available for broilers. According to these standards, at least 25 specimens should be used for an acceptable level of confidence in the results of biomechanical tests. As 30 specimens were used for each bone in the present study so, no need to check variation for these tests (Standarts, 2007). Just one bone (femur) was excluded related to inappropriate test. Moreover, the instrument used for mechanical testing was capable of load cell with sensitivity ±0.5% of reading (Prairie, 2018).

It was important to use healthy chickens for the present research because this anatomical study was planned to give standard values of the geometry of broiler forelimb and hindlimb bones. Thus, the bones were evaluated for healthy normal categorization by checking VV deformity using femoral and tibial angular measurements on the 3D images. For VV deformity, CT images of femur and tibia (right and left) were used and the angulation of the bones showed that there was no lateral or medial rotation in these bones (Leterrier and Nys, 1992). TD scoring was performed at the end of the study which also confirmed that all broiler chickens were in a normal healthy condition as 0 score was obtained for all the right and left tibias. Similarly, no pathological lesions were seen on the CT images for the confirmation of healthy status of all the bones.

The global demand for poultry has increased in past few decades. To fulfil the demands of growing population, poultry industry has adopted various methods of fast broiler production (Breugelmans et al., 2007). This huge pressure of demand and supply leads to genetic selection for high production traits on the expense of good skeletal health and welfare. Strategies including selection of broiler strains with high growth rate, increased weight gain and immunity lead to weak skeleton (Mabelebele et al., 2017). Skeletal integrity has become now paramount distress as bones respond to this increased weight by improving their weight and length (Nkukwana et al., 2014). In poultry research, studies are mostly conducted in order to understand the effect of new diets, treatments and management practices on the bone structure and function (Abrahamsson and Tauson, 1995; Massé et al., 2003a; McDevitt et al., 2006; Gholap, 2012; Marangoni et al., 2015; Almeida et al., 2018; Maharjan et al., 2021). Each broiler strain has different growth rate and response to feeding or treatment, therefore, the skeleton also shows different morphometric or biomechanical properties. Each bone of the skeletal system has its own importance for judging the health of musculoskeletal system. There are many techniques like ash analysis, radiography, mechanical testing or direct morphometric measurements to calculate indices for bone strength assessment. The correlation coefficient of bone ash and breaking strength was found as 0.98 (Rowland et al., 1967). However, no single biomechanical or radiographic test can accurately measure bone strength. It is expected that the scientific literature will grow further to identify a single technique for testing the bone strength in broilers and that will lead to deeper understanding of broiler skeletal structure and function. Therefore, in the present study, standard broiler bones of both fore and hind limbs were subjected to CT, DEXA and biomechanical tests to understand bone strength by their geometry, density and mechanical properties. The standard values were targeted at the end of growth period in order to develop deeper understanding of association of the biomechanical and imaging techniques. Besides, to the best of knowledge, there is very small information in the literature to compare and analyze the association of right and left side of both forelimb and hind limb bones of broilers. The birds generally stand on one leg during their resting phase, so whether broilers have differences in skeletal properties or not like other animals? The focus here was also on understanding how fore and hind limb of broiler were interrelated and how far, different measurements were obtained by different testing methodologies.

Earlier, CT methodology was used to predict abdominal fat and muscle weight in broilers (Andrássy-Baka et al., 2003) but now it is used for bone morphometry and density as well. It is well-established method to see bone structure and development in live birds (Dewez et al., 2018). Modern CT techniques (qCT and μCT) can be used to measure cortical thickness, BMD, cortical bone area and total cross-sectional area of the broiler's bone (Martin et al., 2004; Harash et al., 2020). It can also be used in the preliminary evaluation of the cause of bone degeneration (Olkowski et al., 2011). So, in the present study geometrical analysis of the bones was performed using CT method. These morphometric measurements can be done manually using digital calipers or images can be processed using software for calculations but here, CT images were used for this purpose to get more accurate bone dimensions. Before geometrical analysis, it was important to understand directional terminology related to forelimb/wing and hindlimb of birds' bones. Due to flying ability, dorsal and ventral term is used for humerus, radius and ulna bones instead of medial and lateral. The sectional measurements showed that both dorsoventral external and internal diameters were larger than craniocaudal diameters of the humerus. Radius was thicker in dorsal and caudal directions as compared to its ventral and cranial surfaces. In contrast to humerus, ulna was wider both externally and internally in craniocaudal direction than the dorsoventral direction. Additionally, its cranial cortex was thicker than the caudal cortex. It was depicted that humerus and ulna were having nearly similar outer and inner widths in their respective cranial and caudal directions. The radius was showing smaller dorsoventral and craniocaudal diameters as compared to other bones of the forelimb. In the hindlimb, femur measurements displayed medially thicker cortex than all other cortices. Tibia exposed larger external and internal mediolateral diameters and thicker lateral and caudal cortices than that of its medial and cranial sections, respectively. On comparison of forelimb and hindlimb bones, it was perceived that femur showed wider external and internal craniocaudal diameters than all other four bones (humerus, radius, ulna, and tibia). Radius possessed smallest craniocaudal periosteal and endosteal thicknesses among all of the studied bones. Moreover, tibia was observed as the longest among all the five bones while radius was the shortest bone. The mean values for the length of femur and tibia were 78.38±2.88 and 106.78±4.61 mm, respectively. These were lower than those reported by Mabelebele et al. (2017) who indicated that at 90 days of age, male Ross 308 broiler chickens' femur was 96.89 mm and tibia was 144.90 mm; even female Ross 308 had larger femur (92.38 mm) and tibia (126.06 mm). The difference in slaughtering age here could be the cause of dissimilarity. Moreover, there was only one study that suggested no difference between morphometry of right and left bones of broilers by measuring their widths and lengths (Genç, 2019). According to the outcomes of this study also, the geometrical measurements of right and left side bones showed no statistically significant variation. This was probably due to absence of dominant limb in broilers unlike other species.

The cross-sectional geometrical analysis, performed on the CT images, was used to measure cortico-medullary index (CMI) which can indirectly be used to judge bone strength in the absence of availability of other facilities like ash technique, mechanical testing or radiographic density techniques. It is indirect measurement of the bone mineralization (Kocabağli, 2001). The CMI of radius (95%CI=42.29-45.65) was highest among all the five bones both in mediolateral/dorsoventral and craniocaudal directions. Tibia (95%CI=33.30-33.61) and humerus (CI=32.17-35.76) showed nearly similar CMI in this study while femur displayed smallest index value (95%CI=28.73-32.29) among all the bones of forelimb and hindlimb. Interestingly, femur showed similar index values to humerus in mediolateral direction but craniocaudal CMI significantly differed (P = 0.001) for both of these bones. Generally, it was perceived from broiler studies that most of them used only tibial index to check cortical thicknesses of the broiler's bone. In a previous study, the tibial CMI was found smaller in male and female Ross 308 broilers (25.00% and 27.37%, respectively) but index data similar to the recent study (ML=34.59% and CRCD= 32.54%) were seen for Venda strain (male: 32.35%; female: 33.50%) by Mabelebele et al. (2017) and for Ross 308 (30.00-35.00%) by Muszyński et al. (2018). The reason for this could be longer and wider tibia bones of previous studied Ross 308 as compared to their Venda strain which was indigenous breed with smaller size and present Ross 308 which was slaughtered at 42 days (earlier age). The tibial CMI of Ross (308) of this study was higher than the index values observed in a study on comparison of bone integrity of different broiler strains (Marshal R, 26.67±3.92 and Marshal Y, 25.19±2.26) except for Arbor Acre (34.68±1.61) and Hubbard (30.24±2.10) (by Salaam et al. (2016). But, in an earlier work tibial index of Ross (308) was found higher (42.3%) in tibias of 45 days old chicken (Kocabağli, 2001) than the present study. In contrast to this study, the cortical index of tibia even after 35 days was found larger (39.75±8.17) in another study on broilers (Kleczek et al., 2012). Equally, Rehman et al. (2018) and Dereli Fidan et al. (2021) found larger tibial indices (41.38% and 39.19%) in broilers than presently observed index value. The reason could be that their bones were obtained from some research related to provision of the best dietary supplementation or management conditions. Or might be their manual measurement of the bone diameters as compared to this study methodology, had played some role in bringing about difference in the results.

The bone mineral density, an indicator of bone mineralization, by means of DEXA is positively correlated with bone breaking force and bone ash (Hester et al., 2004a). BMC is the amount of mineral in a total scanned region (g) while BMD is the amount of mineral in certain volume of a bone tissue (g/cm^2) (Foutz et al., 2007). The more value of BMD reflects the bone is strong and dense. In this trial, humerus, femur and tibia retained same mean density values with the 95% CI of 0.204-0.228, 0.209-0.238 and 0.212-0.237, respectively. As their area (g/cm^2) expressed similar 95% confidence intervals (22.45-23.98, 27.81-30.88 and 22.67-24.84), that's why they showed similar mineralization irrespective of their length. The BMD values of broiler's humerus and tibia, showing higher values just after 42 days of age, were in agreement with the previous findings of comparison of broilers with White Leghorn layers (Hester et al., 2004a). Besides, tibial mineral density was larger (0.224 g/cm^2) than that of found by Muszyński et al. (2018) in the broilers of same age. The density of femur and tibia of rapidly growing broiler 857K after day 53rd of rearing, was higher even in diaphysis (Aguado et al., 2015) than the whole bones of Ross 308 broilers of this study. It was might be caused by different strain type and more rapid growth rate of 857K strain or feeding differences. Furthermore, BMD of tibia of Ross 308 was larger than the control group (0.154 g/cm^2) of a study on immobilized tibias of Arbor-Ross females (Foutz et al., 2007). Importantly, BMD measurement results of radius and ulna were eliminated from this study as due to their thinner geometry, we were unable to correctly mark the borders of these bones for BMD calculations.

The three-point bending biomechanical test is mostly preferred for testing the bones of the poultry birds (Güz, 2022). For this test, there are standard protocols which must be followed (Standarts, 2007). Among these, specimen gentle dissection, storage, thawing, load rate, span, preload, direction of load application, length to diameter ratio of the specimen are some important steps which can affect the final results. Thawing of the specimen should be slow and must be by keeping it in the normal saline/phosphate buffer saline for at least 3 hours otherwise material properties can be compromised due to loss of water content. Secondly, the span is important to experience exact mechanical testing because it is used in the formula for calculations of the strength and elastic modulus so, it can influence the obtained values. Moreover, if span is less than the standard value, it can produce frictional forces and bone can undergo shear rather than bending (Turner and Burr, 1993; An and Draughn, 2000; Standarts, 2007). In this study, ideal span for each bone was determined according to the diaphyseal length of the bone and span for humerus, radius/ulna, femur and tibia was kept 30 mm, 35 mm, 40 mm and 60 mm, respectively. The span of 25-60 mm (Štofaníková et al., 2012), 40 mm (Lewis et al., 2009), 50 mm (Harash et al., 2020), 50 mm (Mirakzehi et al., 2013) were used in multiple studies for three-point bending test of broiler tibia and 30 mm for both femur and tibia in a study by Zhang et al. (2020). But, the distance between two supports was kept 10 cm in a study on tibia of broiler breeders (Rath et al., 1999). Thirdly, load rate is important as bone properties can be changed according to the applied load due to its viscoelastic nature. This may also change the results so standard protocols must be followed to compare the results with previous studies. The load rate of 1-100 mm/min. depicts normal physiological circumstance while faster speed (1-5 m/sec.) shows trauma state (Komal et al., 2021). Here recommended standard load rate of 10 mm/min. was used for three-point bending test of the bones (Standarts, 2007). The load rate in previous studies on poultry was observed as 2 mm/min. (Shipov et al., 2010), 10 mm/min. (Gebhardt-Henrich et al., 2017; Zhang et al., 2020), 20mm/min. (Hocking et al., 2003; Donkó et al., 2018), 30 mm/min. (Sparke et al., 2002; McDevitt et al., 2006; Maidin et al., 2021), 50 mm/min. (Cheng and Coon, 1990; Kim et al., 2004; Dunn et al., 2021), 100 mm/min. (Karásek et al., 2017).

The parameters of three-point bending test gives information about structural and material properties of a bone. The higher stiffness and elastic modulus values are indicative of bone rigidity (Turner, 2002) while low modulus tells about ductility of bone (Mutuş et al., 2006). The maximum bending force of humerus was significantly higher ($P < 0.05$) while radius showed higher bending strength and elastic modulus ($P < 0.05$) among all the bones. Generally, deformation was significantly higher ($P < 0.05$) in hindlimb bones as compared to the forelimb. The maximum force (N) for bending was larger for both femur and tibia while elastic modulus of femur was smaller and that of tibia was larger for Ross (308) chicken of this study as compared to Arbor Acre, Hubbard, Marshal R and Marshal Y strains of broiler (Salaam et al., 2016). This was may be due to higher growth rate of chickens of the present study. The maximum force and stiffness for femur and tibia of broiler were found larger in some recently published studies (Rubin et al., 2007; Karásek et al., 2017; Muszyński et al., 2018; Zhang et al., 2020) as compared to this study. The bone breaking force (N) mentioned wrongly as breaking strength, in some research works, about layers was higher for femur and tibia but lower for humerus (Sparke et al., 2002; Kim et al., 2004; Shipov et al., 2010; Maidin et al., 2021) when related to the results of present study. The tibial breaking strength (infect breaking force, N) of Cobb-500 broiler chicken was found higher (338.3 ± 22.86) after 35 days of age than the present Ross broiler (Nkukwana et al., 2014). Contrarily, Ross 308 chicken's tibial breaking force was seen higher than Cobb-500 in a previous study (Lewis et al., 2009). In another study about femur and tibia (Askari et al., 2015), breaking strength (N/m^2), was found higher for both (246.00 and 297.33) than the present study. Likewise, the modulus of elasticity of tibia of same strain was also seen larger in a study by Kocabağli (2001) than that of this study. But on three-point bending of tibia, Muszyński et al. (2018) found ultimate load and stiffness values similar to the measurements attained here. The bending force of broiler's humerus and tibia was higher while similar for radius, ulna and femur in comparison to layers (142.6-154.3 N, 169.3-171.5 N, 36.6-38.4 N, 96.2-100.9 N, 194.2-200.0 N, respectively) whereas bending strength (MPa) was greater for all these bones of layers (93.0-98.9, 188.8-190.3, 195.0-201.2, 187.5-190.0, 107.9-109.4, respectively) (Harner and Wilson, 1986) as compared to outcomes of this study. This difference was shown as a result of keeping layers for longer duration and presence of medullary bone. Overall, it can be summarized that the results obtained at 42 days, for all bending test parameters of the long bones like maximum force, deformation, stiffness, strength and modulus of elasticity revealed significant differences ($P < 0.05$) across all the bones. This inferred that on mechanical loading all the broiler bones behaved differently due to their different geometry which was responsible for their corresponding biomechanical characteristics. However, femur had the least bending strength and elastic modulus while higher value of bending strength was observed in the radius. This may likely be attributed to the shorter length of this bone and larger CMI as compared to other bones.

The shear test was performed on the bones of opposite side according to the ASABE standards. The testing apparatus was designed and 5 mm/min. load rate was used according to these standards (Standarts, 2007). For shear test, earlier work showed 2 mm/ min. (Ravindran et al., 1995; Liu et al., 2003) and 5 mm/min. (Onyango et al., 2003) loading rates were mostly used for the poultry birds The bone shear force, deformation and strength, when compared, the statistically significant variance were observed ($P < 0.05$). Shear force (N) for humerus (466.29-527.59), femur (355.92-439.35) and tibia (433.77-563.59) was similar but strength was smallest for femur. Tibia and humerus were having similar strength in response to the shear loading and ulna and femur were equally weaker when endured shear testing. Hence, it was determined from the results of shear test that radius was the strongest and femur was the weakest in response to the applied shear load. On comparison of the maximum shear force (N) and the deformation produced in tibia, it was observed that similar force was required to produce shear but resulting deformation was smaller than that of the observed by Kleczek et al. (2012). The possibility of smaller deformation here could be perchance due to the different bone size and load rate (5 mm/min.) as compared to 10 mm/min. used by them. The higher shear force (N) was observed for layers' humerus=269.9-299.8, radius=232- 242.6, ulna=292.1-316.3, femur=358-357 and tibia=404.8-407.5 and their shear strength (MPa) was calculated as humerus=10.6-11.4, radius=28.8-29.2, ulna=18.6-19.1, femur=11.9-11.3 and tibia=14.6-14.4 (Harner and Wilson, 1986) which was also higher except for humerus. The shear force of tibia was also measured as 395-483 N (Onyango et al., 2003) and 26.8-60.1 kg (Ravindran et al., 1995) in studies on broilers. The maximum shear force (N) and strength (GPa) of tibia were found lower in both the mobilized (285.4-348.6; 4.8-6.0) and immobilized (1-2 weeks) (207.9-274.6; 4.4-6.2) broiler chickens (Foutz et al., 2007) which was result of shorter and less denser tibia in comparison to the recent study. The loading of layer's bones in shear, showed greater shear force for humerus (269.9-299.8 N), radius (232.0-242.6 N), ulna (292.1-316.3 N) but almost similar force for femur (357.0-358.0 N) and lower for tibia (404.8-407.5 N) as compared to broiler chicken of this study. The layer's and broiler's humerus (10.6-11.4) were equally stronger to shear loading but all other long bones of layers expressed greater shear strength (MPa) than the broiler's long bones (28.8-29.2, 18.6-19.1, 11.3-11.9 and 14.4-14.6, respectively) (Harner and Wilson, 1986). The probability for these higher values could be difference in rearing period and presence of extra calcium reservoir in the bones of layers.

As ASABE standards offer two different biomechanical testing methods for the poultry bones, it was quite questionable which testing technique and which long bone of the poultry birds was most suitable for the estimation of the skeletal strength through these tests. So, the observations of CV for three-point bending parameters revealed that for femur CV was highest among all the bones, afterwards tibia, radius and ulna were seen. But humerus showed lowest variation coefficient for bending test which might be due to its medium length and elliptical geometry properly seized upon two supports of the testing set-up. Tibia and femur showed maximum CV also for shear force and stress which were followed by radius and ulna. Humerus turned up as more appropriate bone for this test as well due to its suitable elliptical geometry. Overall, on comparison of three-point bending and shear tests, it was seen that the former proved to be better because of smaller variability as identified from CV for both these tests. Also, it was easy to perform and information about this test was most abundantly available in the literature. Additionally, though humerus showed least variation among all the bones for both testing methods yet we cannot validate about the most proper bone for these tests. Because some limitations were revealed for testing techniques like periosteum and muscle remains could hinder in obtaining true results for the shear test. The direction of load application has also impact on the results of the shear test and in this study, the cortical thickness and direction of the applied load was different for all the bones. The span used for bending test could also affect manual calculations of the strength and elastic modulus as there were no constant values available for this in the given ASABE standards. These were the only available standards for three-point bending and shear testing of the broiler bones. There are some controversies about these tests. Thus, it can be assumed that observed differences in bones under the influence of external forces may be associated with other factors, such as the alterations in the structure of the organic matrix, bone geometry and dietary factors.

6. CONCLUSION AND RECOMMENDATIONS

In conclusion, this study has provided knowledge about cross-sectional geometry, BMD and mechanical testing data to understand the strength of all the long bones of broiler. The strength of broilers' bones was evaluated using different methodologies altogether in one study. The geometrical measurements of right and left side bones showed no variation. The cortical thicknesses were seen relatively thicker in the radius and thinner in the femur among all the broiler bones. The BMD data for humerus, tibia and femur bones showed no significant difference but the statistical comparison of BMD data was not enough and true as radius and ulna were excluded from the study due to inability to measure their exact BMD.

The femur appeared as weakest bone on application of bending load and also showed least elasticity but radius was strongest among all the long bones. Same outcomes were observed on application of shear force. This finding was also strengthened by its shorter size and larger CMI as compared to the other bones of this study.

Though, humerus displayed least CV for almost all the parameters of both the tests yet our judgment about the better broiler bone for any biomechanical test is not appropriate because there were some shortcomings in proper performance of these tests. In general, the three-point bending test was easy to perform; plentiful literature was available for this test; and showed smaller variability (CV) than the shear test for all the parameters. Therefore, it can be said that three-point bending test may be better for the broiler long bones than the shear test. But further studies may be needed for comparisons of these tests to find out ideal biomechanical testing methods for poultry long bones.

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T.C.

AYDIN ADNAN MENDERES ÜNİVERSİTESİ

SAĞLIK BİLİMLERİ ENSTİTÜSÜ

BİLİMSEL ETİK BEYANI

“Etlik Piliçlerde Kemik Dayaniminin Morfolojik, Dansitometrik ve Biyomekanik Yöntemlerle Değerlendirilmesi” başlıklı Doktora tezimdeki bütün bilgileri etik davranış ve akademik kurallar çerçevesinde elde ettiğimi, tez yazım kurallarına uygun olarak hazırlanan bu çalışmada, bana ait olmayan her türlü ifade ve bilginin kaynağına eksiz atıf yaptığımı bildiririm. İfade ettiklerimin aksi ortaya çıktığında ise her türlü yasal sonucu kabul ettiğimi beyan ederim.

…………………..

Komal KHAN

19 / 01 / 2022

REPUBLIC OF TURKEY

AYDIN ADNAN MENDERES UNIVERSITY

GRADUATE SCHOOL OF HEALTH SCIENCES

STATEMENT OF ETHICS IN SCIENCE

"Morphological, Densitometric and Biomechanical Evaluations of the Bone Strength in Broilers" entitled Ph.D. thesis, I have written within the framework of ethical behavior and academic rules. In the thesis, which has been prepared in accordance with the thesis writing rules, I declare that I have cited all kinds of sources and information that do not belong to me. I declare that I accept all kinds of legal consequences when the opposite of what I have expressed is revealed.

……………………..

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19 / 01 / 2022

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ACADEMIC PUBLICATIONS

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Ahmad, H. Y., Masood, S., Zaneb, H., Aslam, A., Sikandar, A., Adil, M., & Tamkeen, R. (2014). Comparative Effects of Organic Acids Fed Diet on Morphometry of Visceral Organs and Intestinal Histology of Two Broiler Strains. Global Veterinaria, 13(2), 221-226.

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