INFLUENCE OF HOUSING SYSTEMS ON BONE PROPERTIES OF LAYING HENS

By

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ABSTRACT

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Osteoporosis in caged hens is one driving factor for the U. S. egg industry to explore options regarding alternative housing systems for laying hens. The aim of this dissertation was to determine the housing system effects on bone quality of laying hens. The first study looked at tibiae and humeri of White Leghorn pullets reared in conventional cages (CC) and a cage-free aviary system (AV). At 16 wk, 120 birds were randomly sampled from each housing system for bone property analysis. Humeri and distal tibiae cortical density was greater in AV pullets compared to CC pullets ($P < 0.05$). Tibiae and humeri of AV pullets had a thicker cortex than the CC pullets ($P < 0.05$). Additionally, the tibiae and humeri of AV pullets had greater ($P < 0.05$) second moment of areas than the CC pullets. The aim of the second experiment was to study the influence of housing systems on 77 wk White Leghorn hens. Pullets raised in an aviary system were either continued in aviary hen systems (AV) or conventional cages (AC) whereas pullets reared in conventional cages continued in conventional hen cages (CC) or enriched colony cages (EN) at 19 wk. From each group, 120 hens were sampled at random for bone property analysis. Aviary (AV) hens had greater cortical thickness and density but similar outer dimensions to AC hens ($P < 0.05$). Hens in EN system had humeri with similar cortical thickness and density but wider outer dimensions than humeri of CC hens ($P < 0.05$). The follow-up study aimed at analyzing age-related changes in bone properties in different commercial housing systems. Pullets reared in conventional cages (CC) were continued in CC or moved to enriched colony cages (EN) at 19 wk whereas those reared in cage-free aviary (AV) were moved to AV hen

houses. Bone samples were collected from 60 hens at 18 and 72 wk and 30 hens at 26 and 56 wk from each housing system. AV pullets had 41% greater humeri and 19% greater tibiae cortical area than CC pullets ($P < 0.05$). Humeri and tibiae of AV pullets had greater stiffness (31% and 7% respectively). The geometrical and biomechanical differences between bones of AV and CC hens persisted throughout the laying cycle. Moving CC pullets to EN resulted in decreased endosteal resorption in humeri evident by 7.5% greater cortical area of EN hens ($P < 0.05$). Stiffness increased with age in both tibiae and humeri while energy to failure decreased. The final study was aimed at determining the housing system and strain effects on bone quality parameters. Tibia, femur, and keel of Hy-Line Brown (HB), Hy-Line Silver Brown (SB) and Barred Plymouth Rock (BR) hens housed in conventional cages (CC), cage-free (CF) and cage-free with range access (R) were studied. Bone samples were collected from sixty hens from each strain and housing combination for analysis. Tibia cortical thickness was greater ($P < 0.01$) in BR than HB and SB. Between housing systems, thickness was greater ($P < 0.05$) for mid and distal tibia for R and CF compared to CC. Tibiae and femoral cortex were denser ($P < 0.05$) in BR compared to HB and SB. Keel bone density was greater ($P < 0.05$) in CF and R birds compared to CC birds. Each housing system was associated with high prevalence (> 90%) of keel deformities and the housing and genotype influenced the type of deformity. These findings indicate that range and cage-free housing may have beneficial impact on tibia and keel bone integrity compared to conventional cages but the improvement may not be sufficient to prevent fractures or deformities, particularly of keel.

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KEY TO ABBREVIATIONS

AV	Cage-free Aviary
BMC	Bone Mineral Content
BMD	Bone Mineral Density
BR	Barred Plymouth Rock
CBD	Cortical Bone Density
CBT	Cortical Bone Thickness
CC	Conventional Cage
CF	Cage-free Litter and Slat
EN	Enriched Colony Cage
FR	Free Range
HB	Hyline Brown
OC	Osteocalcin
PYD	Pyridinoline
QCT	Quantitative Computed Tomography
SB	Hyline Silver Brown

CHAPTER 1.

General Introduction

Egg production systems have evolved over the past six decades with a recent wave of change concerning laying hen housing systems. In the U. S., egg producers are exploring other housing options to conventional cages as a response to legislative changes in certain states within the U. S. and throughout the European Union (EU). A great deal of interest has arisen to study health, management, and economical sustainability of the newer housing systems (enriched colony cages, single and multi-tier aviary systems). One of the important aspects of laying hen health is the skeletal integrity as this system serves the dual purpose of supporting the body mass as well as a calcium reservoir used for eggshell production. Skeletal reserves alone supplies 20 to 40% of the calcium required for eggshell formation (Edelstein et al., 1975) making the bone metabolism very dynamic and, thus, monitoring of skeletal health very important in commercial housing systems. Conventional cages, currently the most used in the U. S., have been associated with disuse-osteoporosis since its introduction. The provision of greater space and perches in the newer housing systems allow hens to perform activities like running, jumping and wing flapping presumably providing greater loading to their bones and strengthening them. At the time this dissertation work started, there was very little information on mechanical and structural properties of laying hens' bones in enriched colony cages and in commercial aviary systems but the aptness of the information is even more apparent now especially with reports of high incidences of keel bone fracture in these systems.

BONE BIOLOGY DURING EARLY STAGES AND THROUGHOUT THE PULLET PHASE

The dynamics of bone metabolism in laying hen is different compared to mammals in general. Laying hens use calcium from bones to meet the demands of egg production and the impact of hormones of egg production on bone modeling and remodeling becomes important to understand as well. Bone development in poultry starts *in ovo* with a hyaline cartilage model being laid down (Gilbert, 1997). The cartilage model is eventually replaced by fully mineralized skeleton by endochondral ossification. Onset and progression of ossification has been observed as early as 13th day of incubation in long bones and spine region (Wurbach et al., 2012) and continues after hatch. Long bones grow in length by endochondral ossification of cartilage and widen by intramembranous ossification in young pullets. Endochondral ossification is marked by differentiation of resting chondrocytes into proliferative chondrocytes in the epiphyseal growth plate at the end of long bones. These chondrocytes undergo further maturation into hypertrophic chondrocytes, which ultimately undergo apoptosis leading to vascular invasion and recruitment of the osteoblasts into the area (Whitehead, 2004). Osteoblasts lay down the matrix for mineralization and two major types of bone tissue are formed during this growth, cortical and trabecular lamellar bone (Figure 1), which provide the major structural integrity to the skeleton (Whitehead 2004). Cortical bone forms the outer structural shell of the bone and trabecular bone forms within the interior cavity as struts. Bone modeling is predominant during early stages of pullet growth and is characterized by lack of local coupling on bone modeling surfaces between osteoblasts and osteoclasts unlike bone remodeling (Bain and Watkins, 1993). Bone modeling allows longitudinal growth and periosteal expansion. Then, towards the end of the growing period remodeling increases with the formation of secondary osteons and subsequent interplay of

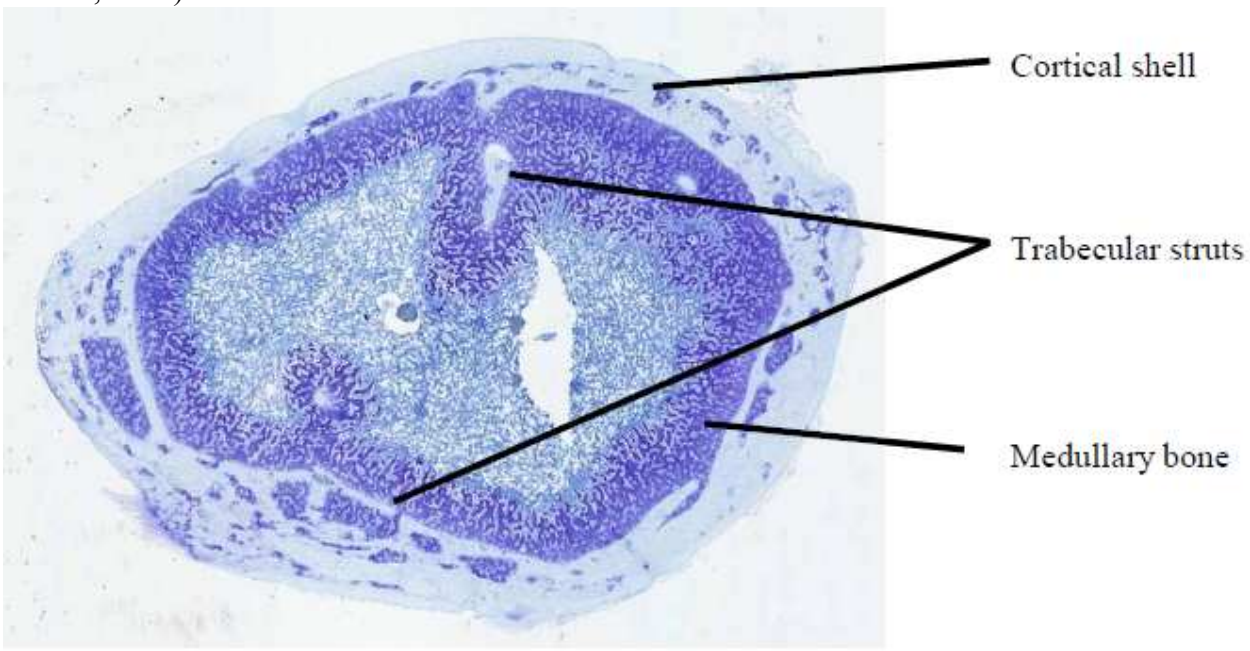
osteoclast-mediated resorption as well as osteoblast-mediated formation (Whitehead, 2004). The epiphyseal growth in long bones is closed and mineralized by the time pullets reach sexual maturity and long bones cease to grow in length (Whitehead, 2004). However, cross-sectional changes in geometry of humeri and tibiae might occur towards the end of the pullet phase. Pullets greatly increase the diameter of long bones (by about 20%) and increase bone quantity before the onset of lay in anticipation of calcium demand of egg-laying (Riddell, 1992). Bone growth during the pullet phase does not seem to be improved by nutritional intervention of Vitamin K, particulate limestone, and ascorbic acid (Fleming et al., 1998). Changes in architecture, composition and mechanical properties of pullets as a result of greater physical activity, or lack of, have not been studied in detail to date.

BONE BIOLOGY DURING THE LAYING PHASE

With the onset of lay, however, structural bone formation ceases and a new type of bone called medullary bone (Figure 1) formation starts, which acts as a labile source of calcium for eggshell formation (Whitehead 2004). Formation of medullary bone is related with the surge of estrogen levels in blood as the hen reaches sexual maturity (Miller, 1992). Estrogen concentration increases markedly from 16 to 20 wks of age and remain high for several weeks in coordination with LH and progesterone surge (Johnson and van Tienhoven, 1980). Along with regulating gut and renal factors to maintain calcium homeostasis during egg production (Tanaka et al., 1978; Elaroussi et al., 1993), estrogen concentration drives the change in function of osteoblast from lamellar bone formation to medullary bone formation (Whitehead, 2004). However, the initial accumulation of medullary bone in the marrow cavity might occur at the expense of cancellous bone. Osteoclast activity is independent of estrogen level and bone resorption continues into the laying cycle. Structural bone may also be resorbed along with medullary bone typically at exposed surfaces (Whitehead, 2004). It is very unlikely for the lost structural bone to be replenished during lay, leading to progressive weakening of bones. Bone loss occurs at varying rates and at varying ages for the epiphyseal and diaphyseal region, with epiphyseal bone loss more pronounced during first 10 weeks of the onset of lay and bone loss from the midshaft region occurring after 25 weeks of age (Whitehead and Fleming, 2000). Fleming et al., (1998) reported a rapid loss of cancellous bone in proximal tarsometatarsus (PTM) and as free thoracic vertebra during the first 10 weeks of sexual maturity whereas a marked accumulation of medullary bone occurred in PTM at the same time. The rate of decline in cancellous bone volume decreased after 25 wks but the total bone volume kept increasing until 70 wks (Fleming et al., 1998). Cessation of structural bone formation in femur width during the

laying cycle was also confirmed by lack of incorporation of fluorochrome labels in the bone (Hudson et al., 1993). The same may not be true for other bones like humeri and particularly the keel bone. Keel bone has been reported to have significant amount of cartilage tissue even at 22 wks of age (Breugelmans et al., 2007).

Figure 1: Different bone tissue types of an adult laying hen (reproduced with permission from Korver, 2012).



Medullary bone is characterized by random orientation of collagen fibers (Whitehead and Fleming, 2000) and the hydroxyapatite crystals are randomly distributed throughout the matrix as well (Dacke et al., 1993). Medullary bone has higher mineral to collagen ratio (Taylor et al., 1971) and are not different in radiographic density compared to cortical bone (Fleming et al., 2006). Medullary bone is also characterized by good vascularization, large surface area and high number of osteoclasts (Hurwitz, 1965; van de Velde et al., 1984). These characteristics indicate that medullary bone is very active in remodeling and supplies as much as 40% of the calcium for eggshell formation (Mueller et al., 1969). Interestingly, medullary bone content and osteoclastic activity of medullary bone were unaffected whereas cortical bones were depleted when hens

were fed calcium-deficient diets for 7 days (Taylor and Moore, 1954). Modern strains of laying hens produce almost an egg per day for more than a year and are presumably under constant negative calcium balance and if the hens preserve their medullary bone volume at the expense of structural bone, the result is thinning of cortices observed at the later stages of the laying cycle (Hudson et al., 1993; Whitehead, 2004). The thinning of cortices, however, may not be accompanied by change in bone mineral content or density. In a longitudinal study of laying hens, DEXA measured radiographic density of humeri and tibiae increased with age and while tibiae plateaued at 55 wks, the density of humeri kept on increasing (Schreiweis et al., 2004). DEXA scans are unable to differentiate between cortical and trabecular/medullary bone and hence the overall increase in bone density might have been due to greater amounts of highly mineralized medullary bone in older hens. Medullary bone can provide minimal structural support by connecting the trabeculae and imparting stiffness to the bone (Whitehead and Fleming, 2000) but stiffness comes at the expense of toughness and in older hens denser bones with poor collagen crosslinks might be more brittle and prone to fracture. These studies also underline the importance of trabecular tissue in bone metabolism. Osteoporosis is characterized by loss of trabecular bone up to 50% in the epiphysis of long bone (Seeman, 2003) and in laying hens, a loss of over 50 grams or around 70% of the total weight fraction of trabecular bone can reduce the energy required to fracture by 85% (Passi and Gefen, 2005). Trabecular tissues have not been studied extensively in laying hens (some stereological histomorphometry have been conducted) but recent advances in macro and micro quantitative computed tomography scans should be able to explain age related changes in trabecular structure.

BONE BIOLOGY AFTER MOLTING

Estrogen concentration gradually decreases towards the end of the laying cycle together with decrease in estrogen receptor populations in both kidney and duodenum and is very low at 70 wks compared to 29 wks (Hansen et al., 2003). The effect of estrogen decline corresponds with gradual disappearance of medullary bone and formation of structural bone. Structural bone formation has been reported to occur over the layer of medullary bone that previously coated the structural bone (Whitehead, 2004). Hens subjected to induced molt by 9d feed withdrawal had no medullary bone in the marrow cavity (Kim et al., 2007). In the same study, density of cancellous and medullary bone measured by peripheral quantitative computed tomography was higher in un-molted controls compared to the molted hens. An interaction between bone type and molting was observed for bone density (BMD) and mineral content (BMC) measured by DEXA (Mazzuco and Hester, 2005). Tibia bone density decreased significantly compared to pre-molt (67 wks) after 7d of fasting (at 77 wks) but BMD decline in humerus was only noted at 87 wks age (Mazzuco and Hester, 2005). The reason for this phenomenon might be the higher medullary bone content in tibia, which is lost rapidly following molting, compared to that of humeri, which is generally a pneumatic bone. *In vivo* scans of live hens during pre-molt, molt and post-molt reported BMD values of humeri significantly lower than at pre-molt stage after molting and even until the end of second cycle whereas BMD of tibia recovered late into the second laying cycle when egg production started to decline (Mazzuco and Hester, 2005). Decline in bone ash weight was also reported in tibia immediately following feed withdrawal (Kim et al., 2007; Mazzuco and Hester, 2005).

Studies involving nutritional interventions to maintain skeletal integrity suggest nutritional factors like Vitamin D and C, calcium and phosphorus sources (like particulate

limestone) and trace minerals like copper, zinc and manganese are important but not sufficient for modern laying hen strains. The compositional and structural properties of bones are largely defined by genetic make-up of an animal, but various external and environmental factors like stresses and strains created by muscles or external loads constantly modify these features throughout life. Housing systems currently used by the egg industry is one such factor that can have significant effects on the overall bone biology because of the wide variation in the hen's accessibility to perform physical activities. In paragraphs to follow, a review of published literature has been presented about the effects of different housing types on bone properties in laying hens.

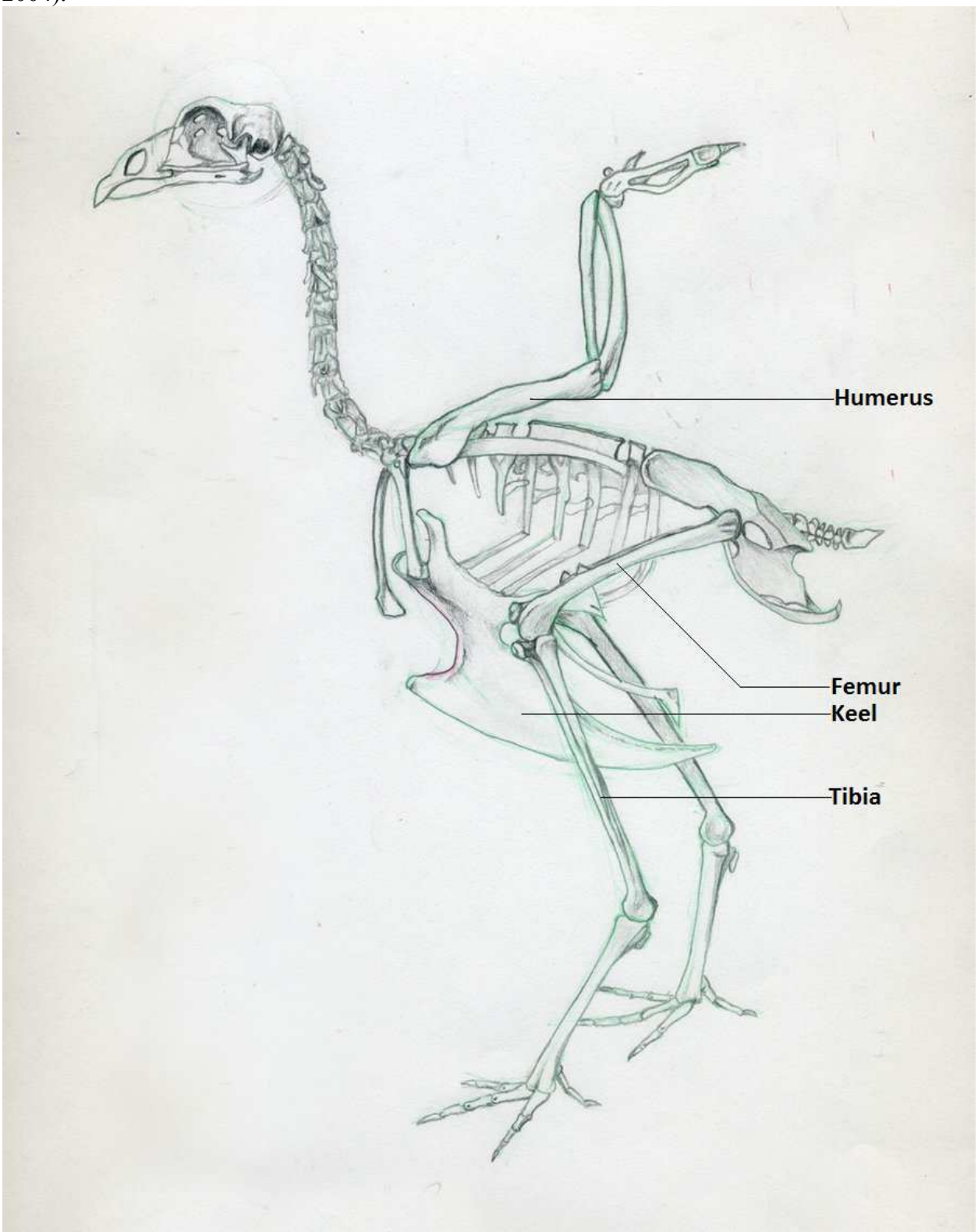
EFFECT OF HOUSING SYSTEMS ON BONE BIOLOGY

Laying-hen housing system has constantly evolved since the egg industry came into existence in the early 1900s. Until 1950s, the farming system was mainly centered on free-range and semi-intensive systems. Free-range hens during that period were kept on well-drained pastures at low stocking densities (about 250 birds/hectare) in mobile coups or slatted cages. The semi-intensive systems had a higher stocking density (about 750 birds/hectare) and included fixed houses with alternate grassed pens used in rotation (Elson, 2011). In the early 1950s, with the expansion of the egg industry, laying hens were housed either in battery cages or the deep litter system. By 1980 almost 95% of the hens were in conventional cages (Elson, 2002). The intensification in farming system with increased use of cages were accompanied by laying hens genetically selected for egg production and skeletal problems were reported as early as 1955. Couch (1955) first reported a condition called caged layer fatigue in laying hens kept in battery cages. Caged layer fatigue was a severe form of osteoporosis characterized by structural bone loss in the vertebrae and the subsequent spinal paralysis (Whitehead, 2004). Fracture (single or multiple) prevalence of 29% has been reported in end-of-lay caged hens, which soared to a staggering 98% by the time the carcasses reached the evisceration line (Gregory and Wilkins, 1989). Fractures of tibia, humerus, and keel (Figure 2) among others are most common. Conventional cages were received with criticism over the ethical concerns of hens kept in them (Brambell, 1965) and eventually were banned in E. U. at the start of 2012 (CEC, 1999) making way for enriched or furnished cages and non-cage systems (single and multi-tier aviaries, free-range).

In recent years, studies involving the newer housing systems have provided some evidences that providing opportunities for loading exercises can help increase bone mass in

pullets and decrease bone resorption in adult hens. On the contrary, induced inactivity has been reported to promote osteoporosis in birds (Nightingale et al., 1972). Some key laying hens bone researches prior to the beginning of this dissertation work have been summarized in Table 1. Breaking strength of the tibia was significantly higher in floor-reared birds than caged birds and birds put through exercise machines (Meyer and Sunde, 1974). This indicates that the nature of exercise also influences the bone quality and that more vigorous exercise may be needed for optimal strength development. The nature of load bearing exercise also seems to dictate the response of a particular to mechanical loading.

Figure 2: Anatomical representation of skeletal system of poultry (adapted from Goldfinger 2004).



Provision of perches in the housing system has been associated with improved bone strength (Sandilands et al., 2009). Cages provided with perches resulted in improved tarsometatarsus bone volume but no such effect was observed in tibia (Hughes et al., 1993). Similarly, bones were stronger in birds kept in extensive system with perches compared to those kept in cages with perches, indicating the extent of movement allowed in the husbandry system to be an important factor in addition to the provision of perches (Knowles and Broom, 1990; Leyendecker et al., 2005).

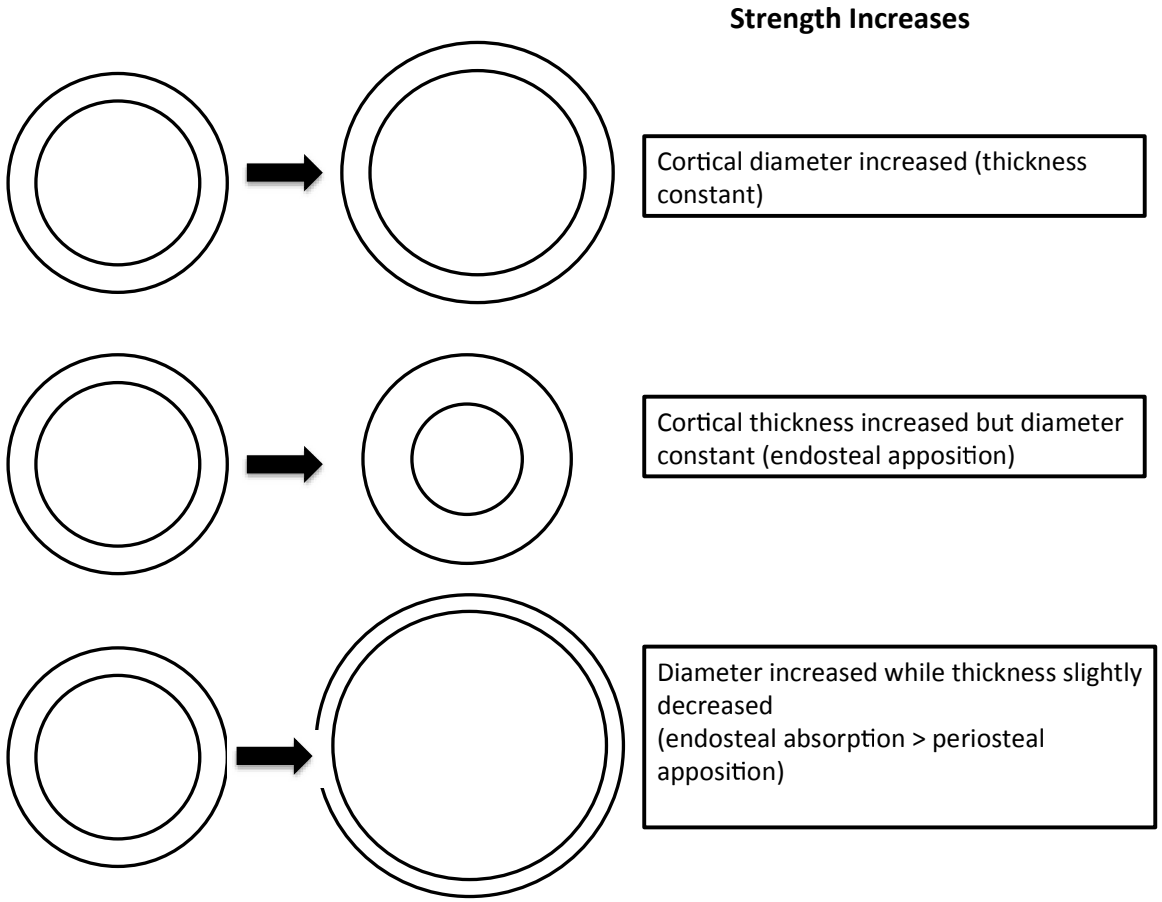
Bone mechanical property can be attributed to its structure, composition, or to a combination of both (Sharir et al., 2008). Compositional parameters often studied as marker of bone health are bone mineral density, amount of collagen fibers and its orientation while thickness and cross-sectional area of cortices, trabeculae and medullary bones, periosteal and endosteal radius, total length of bone etc. are parameters used to study the structural integrity of bone. Freedom of movement associated with extensive housing system can alter one or more of these properties. In a study comparing caged and aviary birds, birds housed in aviary had significantly higher tibio-tarsal cortical area but similar cross-sectional external area compared to the birds housed in cages (Fleming et al., 2006). Proportion of mineralized bone mass and bone mineral density of the tibia and humerus were also significantly higher in aviary birds compared to caged birds. This improvement in material and structural properties of the bones was reflected in the mechanical properties with higher breaking strength for those bones in the aviary birds (Fleming et al., 2006). In the same study, the number of osteoclasts was lower in aviary birds at 25 weeks compared to caged birds of same age but there was no difference at 50 weeks. The results of this study indicate that load bearing exercise in adult hens improves bone strength by prevention of resorption rather than stimulating structural bone formation. The relationship of

changes in cortical width and bone strength is shown below in Figure 3. Recently, Shipov et al., (2010) measured cortical and medullary bone area using micro-computed tomography and reported a similar total cross-sectional areas (sum of cortical and marrow area) of the humeri and tibiae of birds housed in conventional cages and free range systems while the marrow area was significantly higher in the caged birds. These results support the finding that the lower cortical area in caged birds was due to higher endosteal resorption rather than additional bone formation in the free-range birds. Computed tomographic studies of laying hens' bone has revealed that the cortical and trabecular tissues had similar densities between the housing systems despite having significant differences in cortical areas (Jendral et al., 2008; Shipov et al., 2010) suggesting that exercise improves bone quality by chiefly altering its structural property rather than mineral composition. Although the freedom of movement that the hens are allowed in current housing systems are beneficial to protect the structural integrity and prevent the incidence of osteoporosis, whereas the extent of improvement is still not sufficient to bring about compositional changes in the bone. Similarly, exercise seems to have very little impact on stimulating bone growth during the rearing period of hens (Whitehead, 2004). Enneking et al., (2012) found no difference in bone mineral density, bone length and width in birds that were housed in cages with perches during the pullet stage compared to the birds kept in cages without perches. However, the bone mineral content of tibia and humerus were significantly different at 12 weeks age between the groups.

Although it is fairly well established that the freedom of movement and provision of load bearing exercise improves the bone quality in laying hens, the incidence of old fractures, particularly of keel and furculum, is alarmingly high in the extensive housing system. Gregory et al., (1990) reported that the incidence of old fractures was 25% in birds housed in percheries and

12% in free-range systems. The statistics are significantly higher to the 5% seen in conventional cages.

Figure 3: The impact of changing cortical diameter and width in bone strength (adapted from Davison et al., 2006).



Various other authors have reported similar results with the incidence of old fractures ranging from 49 to 74% in a variety of extensive housing systems (Freire et al., 2003; Nicol et al., 2006; Moinard et al., 2004). Fractures of the furculum and the keel bone account for nearly 90% of the observed breaks in laying hens.

KEEL BONE FRACTURES AND DEFORMITIES IN LAYING HENS

Welfare concerns over laying hen housing and skeletal health led to banning of conventional and furnished cages in the E. U. since 2012. At the same time, keel breaks and deformities observed in non-cage systems put 90% of the 350 million chickens at risk of keel fractures (Tarlton et al., 2013) and potentially similar damage can be expected in the U. S. with the egg industry moving away from conventional cages. However, there is a thorough lack of understanding of the factors influencing the occurrence of keel injuries and deformities. Gregory et al., (1990) reported the incidence of fracture at the end-of-lay in birds reared in houses with perches (25%) and in free range systems (12%) are significantly higher compared to birds from battery cages (5%). Other researchers have reported similar results with the incidence of old fractures ranging from 49 to 74% in a variety of extensive housing systems around farms within E. U. (Freire et al., 2003; Nicol et al., 2006; Wilkins et al., 2004). Almost 90% of the breaks sustained by laying hens are to the furculum and the keel.

Keel bone deformities and fractures have been associated with the exposed anatomical location of the keel bone making it vulnerable to fractures upon collision within the poultry houses. Keel deformity also seems to have a genetic component (Warren, 1937). Differences between lines selected for bone quality have been reported (Bishop et al., 2000; Fleming et al., 2004; Vits et al., 2005; Clark et al., 2008; Kappeli et al., 2011). Contrastingly, Wilkins et al., (2011) did not find significant differences among commercial lines such as Lohmann and Hy-Line on an extensive study using alternative housing systems. The study was done with 67 flocks housed in eight broad subcategories where the birds were assessed at the end of the production period. All systems were associated with certain level of keel damage with flocks housed in furnished cages having the lowest prevalence (36%) despite having significantly weaker bones

compared to flocks raised in houses equipped with multilevel perches (over 80%). This study suggests that keel deformities in terms of both, prevalence and severity, are strongly associated with the design of housing system and perches used.

Table 1: Effect of housing systems on properties of different bone types in laying hens.

Article	Age (weeks)	[1]Housing type	Bone type	Variables	
Meyer and Sunde (1974)	44	Litter vs. CC	Tibia	Breaking strength (lbs.) 55.9 vs. 46.9 (*)	
Hughes and Wilson (1993)	72	$CC_{(P)}$ vs. CC	Tibia	Breaking strength (N) 161.5 vs. 157.1	Trabecular bone volume 13.9 vs. 11.36*
Leyendecker et al. (2005)	42, 54, and 62	CC vs. FC vs. AV	Tibia Humerus	Breaking strength (N) 116.7 vs. 121.6 vs. 175.4 (CC-FC, CC-AV*, FC-AV*) 104.5 vs. 129.6 vs. 247.0 (CC-FC*, CC-AV*, FC-AV*)	
Jendral et al. (2008)	65	CC vs. FC	Femur Tibia Humerus	Cortical area (mm^2) 22.9 vs. 28.0* 19.4 vs. 24.4* 9.3 vs. 10.8*	Breaking strength $(kgf)^2$ 21.9 vs. 29.6* 22.0 vs. 28.6* 9.7 vs. 13.7*
Shipov et al. (2010)	96	CC vs. FR	Tibia Humerus	Ultimate load (N) 250.4 vs. 193.1* 163.1 vs. 108.9*	Stiffness (N/mm) 873.6 vs. 486.6* 601.4 vs. 219.6*
Silversides et al. (2012)	50	CC vs. Litter	Tibia Radius	Cortical density (mg/cm^3) 953 vs. 1012 1013 vs. 1057	Cortical area (mm^2) 23.3 vs. 25.7 4.6 vs. 6.0*

*Values are statistically different between the housing types compared in each article

[1]Litter (Litter/floor system); CC (Conventional cage); $CC_{(P)}$ (Conventional cage with perches); FC (Furnished cage); AV (Cage-free aviary system); FR (Free-range)

Aerial perches have been considered one of the major risk factors for keel bone damage (Abrahamsson and Tauson, 1993; Sandilands et al., 2009; Wilkins et al., 2011). There are other studies that indicate perches cannot be directly responsible for keel bone damage in commercial free-range systems (Sandilands et al., 2010; Nicholson and O'Connell, 2010). Fleming et al. (2004) did not find any differences in the caged and free-range (litter and slats and no perches) hens in terms of proportion of normal, twisted, and severely deformed keels. Donaldson et al. (2012) reported that average palpated keel score increased with age but not significantly affected by perch treatment. Recent studies with pressure load on keel bone with different design and material of perches indicate rubber-coated metal perches were associated with significantly higher prevalence of keel bone deformities compared to the plastic perches (Pickel et al., 2011). Although, the incidences of keel damage and severity increases with age and is at maximum towards the end of lay, there is variation in the reports regarding when it starts. The prevalence of old broken bones in perchery hens increased from 0% at 19 weeks of age to 23% at 72 weeks and the breaks started to appear between 25 to 45 weeks of age (Gregory and Wilkins, 1996). Fleming et al., (2004) reported incidence of keel deformities increased with age and the incidence ranged from 0.8% at 15 weeks to 5.3% at 70 weeks or the end of lay. Ex-vivo fracture study in keel bone reported no effect of age on fracture occurrence when similar collision energies were applied to birds 31, 45 and 65 weeks of age (Toscano et al., 2013) which contradicts previous hypothesis that keel bones get progressively weaker with age making them more vulnerable to fracture. Bone biology in laying hen is different compared to mammals in general. Two major types of bone tissue are formed during growth, cortical and trabecular lamellar bone, which provide the major structural integrity to the skeleton (Whitehead 2004). Most long bones grow by endochondral ossification and the rate of ossification may vary with

type and anatomical location of bones. Keel bone is believed to ossify at slower rate compared to other long bones. Limited published literatures on keel growth indicate the bone is not ossified completely in laying hens until 6 months of age (Karan-Durdic et al., 1980). Sternum of 22 weeks old Rhode Island Red laying hens still consisted 10.5 to 26% cartilage (Breugelmans et al., 2007). These findings suggest that birds enter laying cycle with a significant portion of the keel bone still to be ossified. With the onset of lay, however, structural bone formation ceases and medullary bone formation starts which may further slow-down the keel maturity. In vitro and in vivo studies have established the effects of mechanical loading in bone formation and load bearing. Extensive housing systems, which allow more load bearing exercises like wing flapping and walking or running, can alter the bone characteristics. The nature of load bearing exercise dictates whether or not a particular bone responds to mechanical loading. Provision of perches in the housing system has been associated with greater bone strength than controls (Sandilands et al., 2009). In context of the keel, we lack the knowledge if load-bearing activities, provided in extensive housing systems in particular, play any role in keel maturation or deformation. Severe keel deformation is considered to be pathological and traumatic in origin and fracture callus material (FCM) was found in the histological study of deformed keels (Fleming et al., 2004; Scholz et al., 2008). Traumatic fracture may be inflicting pain as chickens have a sensitive pain perception mechanism (Hocking et al., 2005). Chickens respond with behavioral and neuronal changes to painful procedures like beak trimming and feather removal (Jentle, 2011). Nociceptors that mediate pain have been identified in chicken legs (Jentle and Tilston, 2000). Periosteum of bone is richly innervated with peptide rich C fibers that mediate pain but the density of these fibers is lowest in cartilage (Sweet, 2012). Since a significant proportion of keel bone is cartilaginous until almost 6 months age (Breugelmans et al., 2007), perception of pain

might be inconsistent in keel bone until certain age. Birds with keel damage are reported to have restricted movement or flying abilities especially during jumping down or up the perch (Nasr et al., 2012; Richards et al., 2012). This problem may also have an economic facet because of the decreased egg production and reduced egg quality. Nasr et al. (2012, 2013) reported birds with suspected keel fractures to have decreased feed consumption, egg production and inferior shell weight when compared to birds without fracture.

A summary of previous studies relevant to the housing systems (Table 1) highlight the information we have gleaned so far as well as the knowledge gaps. Studies conducted so far have evaluated bone properties at single time point, most often at the end of the laying period. The type of housing systems used in the study has wide variation and the parameters studied are often lacking a complete architectural, compositional, and mechanical picture of the bones under study. The chapters in this thesis are directed towards filling these gaps with the following objectives:

1. Analyze the effects of rearing housing environment on properties of tibia and humerus of white leghorns at the end of the pullet phase. Quantify geometrical, compositional, and mechanical changes to discuss the mechanism with which the housing system brought changes in the bones.
2. Quantify the changes in bone properties at the end of the laying period as a result of changing housing system at the end of pullet phase. To examine if the bone mass gained as a result of increased activity during pullet phase is preserved when opportunities to perform such activities are continued or discontinued.

3. Analyze bone properties of tibia and humerus, as well as systemic markers of bone formation and resorption at different time points throughout hens' productive life in commercial conventional cages, enriched colony cages, and cage-free aviary systems.
4. Compare the prevalence and severity of keel bone fractures between contemporary strains of laying hen with a heritage breed housed in different housing systems. Analyze tibia and femur to confirm egg-laying capacity is directly related to bone properties.

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CHAPTER 2.

Effect of rearing environment on bone growth of pullets

INTRODUCTION

Egg production systems have changed in the past 6 decades, with 90% of laying hens being kept in deep litter in 1954 (Elson, 2011) to 95% of hens in the U. S. being housed in conventional cages in 2008 (UEP, 2009). Meanwhile, egg production per hen has also increased mainly due to highly intensive farming systems, optimized nutrition, and enhanced genetics. Conventional cages used in intensive layer production have been associated with disuse-osteoporosis and caged layer fatigue since their introduction. Osteoporosis was first reported by Couch (1955) as a condition characterized by high bone loss and has been defined as a net decrease in the amount of mineralized structural bone that over time makes it fragile and prone to fracture (Whitehead and Fleming, 2000).

Alternative housing systems with provision of perches and greater space are being explored to mitigate the issues of osteoporosis. In vitro and in vivo studies have established the effects of mechanical loading in bone formation and load bearing (Robling et al., 2008; Wan et al., 2013). Birds housed in alternative systems are allowed more area for load bearing exercises like wing flapping and walking or running, that in theory alter the bone characteristics and make it stronger. On the other hand, inactivity has been reported to promote osteoporosis in birds (Nightingale et al., 1972; Whitehead and Fleming, 2000). Computed tomographic analyses suggest cortical and trabecular regions of bone from adult laying hens housed in different systems had similar bone densities despite having significant differences in cortical areas (Jendral et al., 2008; Shipov et al., 2010), indicating that loading improves bone quality in laying hens by chiefly altering its structural property rather than mineral composition. Efforts to improve bone health by loading have often been studied when birds are already into the laying stage and although loading exercise during production has helped to reduce the amount of

structural bone loss, the incidences of osteoporosis and fracture in cage systems indicate that either the birds do not have enough bone mass at the time they enter into the laying cycle or the rate of resorption is too high to be compensated by loading exercises. Hence, one strategy to prevent osteoporosis can be targeted at achieving optimum bone mass before the birds enter into lay. Positive effects of exercise during growth in bone mass and mechanical properties have been reported in several human studies (Vuori, 1996; Bass et al., 2002). Pre-pubertal loading resulted in increased bone mineral content as well as wider cortical and periosteal area, suggesting periosteal bone formation in the humerus of female tennis players (Bass et al., 2002). Some studies have been conducted previously in experimental setting in male chicks (Biewener and Bertram, 1994; Judex and Zernicke, 2000) whereas there is gap of knowledge on response of loading conditions in pullet skeleton. Pullets housed in cages with perches had higher bone mineral content in tibia and humerus at 12 wk compared to the pullets of same age kept in cages without perches (Enneking et al., 2012), suggesting more bone formation in pullets using perches. The present study was aimed at comparing the differences in material, structural, and mechanical properties of tibia and humerus of pullets, housed in aviary system and conventional cages in a commercial setting. The hypothesis being tested was pullets raised in an aviary system would have increased peak bone mass at the start of lay compared to pullets reared in conventional cages.

MATERIALS AND METHODS

Birds, Management, and Sampling

The experimental protocol was approved by Michigan State University Institutional Animal Care and Use Committee. Chickens of Lohmann White strain were raised in a commercial setting. Pullets were housed in conventional pullet cages (Univent starter; Big Dutchman, Inc.) and aviary system (Natura rearing; Big Dutchman). Aviary birds were moved from conventional rearing to aviary rearing at 6 wk. Fifteen pullets were kept in each conventional cage with an area of 248 cm^2/ bird. For aviary pullets, 218 birds were housed per cage with space allocation of 160 cm^2/ bird from 0 to 9 wk, after which total cage space was increased to 249 cm^2/ bird. AV pullets had floor access from 6 wk onwards, providing additional space of 100 cm^2 per bird (Jones et al., 2014). Feeding, lighting, and health management were same for both groups and are explained in more detail by Jones et al. (2014) as the same flock was used for the current study. At 16 wk, 120 birds from each housing system were randomly sampled for bone analysis. Birds were euthanized by cervical dislocation and the right and left tibiae, humeri were excised, and samples from each bird were stored in separate plastic zip-lock bags at -20°C.

Brachial vein blood samples were collected from 30 randomly selected pullets of each housing system at 4, 8, and 12 wk, and 60 pullets were sampled in similar fashion at 16 wk from each housing system for quantification of systemic bone markers. Serum was separated and frozen at -20°C prior to analysis. Serum osteocalcin (marker of bone formation) and hydroxylysyl pyridinoline (marker of bone resorption) concentrations were quantified using commercially available ELISA tests (Quidel Corporation, San Diego, CA, USA). Samples with

values greater than the standard curve were diluted according to manufacturer's recommendations in the assay buffer for the analysis.

Computed Tomography and Bone Ash

Prior to analysis, the right tibia and humerus were thawed overnight, and a quantitative computed tomography scan of the bone along with surrounding soft tissues was taken using a BrightSpeed scanner (General Electric Healthcare, Princeton, NJ). Ends of the bone were located to obtain total bone length, which was then divided by 4 to set the location for the cross-sectional x-ray image at proximal (one-fourth), middle, and distal (three-fourths) regions. Mimics software (Materialise, Plymouth, MI) was used to measure total bone length and analyze the resulting 1 mm cross sections for cortical bone thickness, and cortical bone density at each of the 3 regions. The orientation of the cortical region in relation to the skeletal axis was identified. Cortical bone thickness and cortical bone density were measured for anterio-posterior and medio-lateral planes. Density of bone cortex was measured as an average density within a 10 mm × 20 mm region at each of anterior, posterior, medial, and lateral region, and a further average of whole cross-sectional slice was measured. Profile line feature of the software was used to calculate appropriate threshold density to mask only the cortical region for measurements. The Hounsfield unit values obtained from the quantitative computed tomography scans were converted to milligrams per cubic centimeter in reference to the standard phantoms that were scanned along with the bones. The phantoms had pre-defined densities ranging from low to high-density regions (0 $mg/cm^{3,}$, 75 mg/cm^3, and 150 mg/cm^3). The Hounsfield units of the phantoms after the scan were plotted against the standard density values to generate an equation, which then was used to convert the Hounsfield units of bone into milligrams per cubic centimeter.

Each sample, after quantitative computed tomography scans, was further analyzed for ash content. The bones were cleaned of surrounding muscles and soft tissues. Tibia was separated from fibula, and both humerus and tibia were cut into pieces to fit into a soxhlet for ether extraction. Ether extracted bone pieces were dried and weighed and placed in crucibles, and further dried at 105°C for 24 h in a DN-81 constant temperature oven (American Scientific, Portland, OR). Finally bones were placed in an ash oven (Thermolyne, 30400, Barnstead International, Dubuque, IA) at 600°C for 6 h and ash percentage was determined.

Mechanical Testing

Two days prior to mechanical testing, the left legs and wings were thawed at room temperature. The tibia and humerus were harvested and cleaned of all soft tissue. The bones were wrapped in saline soaked gauze and kept moist throughout all preparations and testing procedures. A uniform mid-diaphysis section, 20 mm long for the humerus and 30 mm for the tibia, was identified for testing and the remaining ends were potted in cups filled with polyester resin (Martin Senour Fibre Strand Plus 6371, Sherwin-Williams; Cleveland, OH). A custom rig secured the bones in alignment with the cups while the resin cured. After potting, anterior-posterior (AP) and medial-lateral (ML) outer dimensions of the bones were measured at the ends and center with digital calipers.

The potted specimens were installed in a pure bending fixture mounted on a servohydraulic testing machine (model 1331, Instron, Norwood, MA). Freely pivoting cups secured the potted ends and a crossbar resting on pins attached to the cups transferred the linear displacement of the testing machine actuator to rotation of the cups. This setup applied an equal bending moment to each end of the specimen, uniformly loading the test section in pure bending. An actuator preload of 2 N was applied to eliminate residual system compliance before bone

failure was induced with a 1 Hz, 10 mm haversine displacement. Load and displacement output of the actuator were recorded at 5000 Hz with a 100-lb load cell (model 1500ASK-100, Interface; Scottsdale, AZ) and a 6 in linear variable differential transformer mounted on the actuator (model HR 3000, Measurement Specialties; Hampton, VA). The tibiae were oriented with the lateral surface loaded in tension and humeri with the posterior surface loaded in tension. The orientation was selected based on the assumption that the tibiae were likely to break when landing with the distal end medial of the proximal, putting the lateral side in tension. For the humeri, the orientation was selected based on the supposition that wing flapping, namely, adduction was the action most likely to result in a fracture.

After fracture, the bone fragments were reassembled in order to measure anterior, posterior, medial, and lateral cortical thicknesses at the fracture site. Outer dimensions and diaphysis thicknesses were used to approximate the cortical cross-section as a hollow ellipse.

The material properties of the bones were determined based on classical beam theory with the exposed bone test section modeled as a uniform beam with a moment applied to each end. The computations required the bone's geometrical resistance to bending, called the second moment of area, to be computed using the expression

$$I = \frac{\pi a_0 {b_0}^3}{4} - \frac{\pi a_1 {b_1}^3}{4} \tag{1}$$

where a and b were the radii parallel and perpendicular to the neutral axis of the bone, and subscripts 0 and 1 denoted outer and inner dimensions of the bone, respectively. The applied bending moment to each end of the specimen was calculated from the actuator force using the expression

$$M = \frac{a_l F}{2} \tag{2}$$

where F was the actuator force applied to both cups, and a_l was the distance between the pivot and load application points on the cups (Figure 4). Bone-end deflection angle θ was calculated using the expression

$$\theta = \sin^{-1}\frac{d}{a_l} \tag{3}$$

where d was the actuator displacement. Whole-bone bending stiffness, K, was determined by the slope of a line fit to the initial, linear portion of the moment-bone rotation plot. This mechanical stiffness and bone geometry were substituted into classical beam equations to compute material stiffness, known as Young's modulus E, using the equation

$$E = (K)\frac{L}{2I} \tag{4}$$

where L was the length of the test section (Figure 1). The material strength of each bone was determined based on a computation of the maximum (failure) bending stress (σ_f) in the bone using the expression

$$\sigma_f = \frac{M_f b}{I} \tag{5}$$

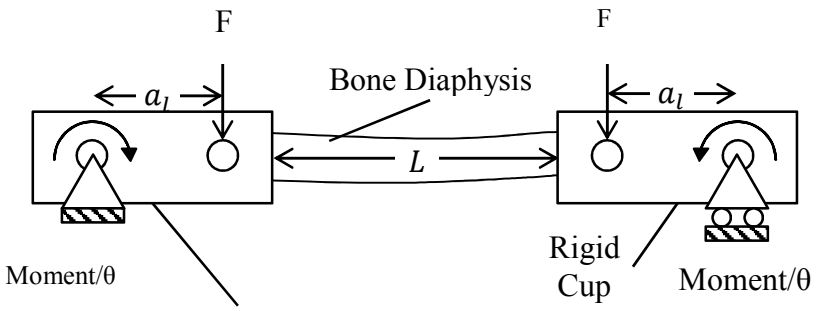
where M_f was the maximum bending moment exerted on each rigid cup at the ends of the specimen at failure (Figure 4).

Statistical Analysis

Data were analyzed by using the multivariate PROC MIXED analysis of SAS Version 9.3 (SAS Institute, Cary, NC). Repeated measures statement with the model including fixed effect of housing and section of bone, the interaction between housing and section, and the residual error was used to analyze all data other than length. Differences between means were tested using Fisher's least-square difference with significance accepted at $P < 0.05$. Data are

presented as least square means with their respective standard errors (LSM ± SEM). Mechanical data for tibia and humerus were analyzed using Student's *t*-test.

Figure 4: Pure bending mechanical test setup.



RESULTS

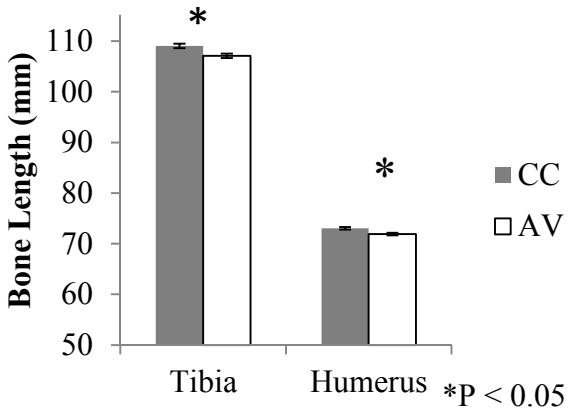
Bone Geometrical and Compositional Properties

Tibiae and humeri were longer in conventional-cage (CC) pullets compared to aviary (AV) pullets ($P < 0.05$; Figure 5A). However, cortical thickness of both tibiae and humeri were wider in AV pullets compared to CC pullets in proximal, middle, and distal sections along all anatomical planes except the antero-posterior plane in proximal tibia ($P < 0.05$; Table 2). Cortical thickness measured manually at the fracture site corroborated with QCT measurements for both bones with tibial and humeral cortex wider in AV birds compared to the CC birds ($P < 0.01$; Table 3 and 4). There was neither a difference in the medio-lateral nor antero-posterior outer dimensions of the tibia between housing systems (Table 3). Unlike the tibia, medio-lateral and antero-posterior outer dimensions of humerus were higher in AV birds compared to the CC birds ($P < 0.01$; Table 4). Cross-sectional areas of tibiae and humeri were greater in AV birds than those of CC birds, which eventually translated into higher values of second moments of area in bones of AV birds compared to CC birds ($P < 0.01$; Table 6). The difference in second moments of inertia between the housing conditions was more pronounced in humerus than in tibia.

The changes in geometrical properties of humerus of AV birds compared to humerus of CC birds were accompanied by changes in compositional parameters. AV birds had humeri with denser cortex compared to CC birds in all planes in proximal, middle and distal sections ($P < 0.01$; Table 5). Bone mineral content as measured by ash percentage of humerus was also higher in AV birds compared to the CC birds ($P < 0.05$; Figure 5B). Average cortical bone density of tibia was not different between the housing systems, except for distal tibia where AV birds had denser cortex compared to CC birds (Table 5).

Figure 5: (A) Mean total length; (B) percentage ash content of tibia and humerus, with respective standard error of the mean, of 16 wk Lohmann White pullets housed in cage-free aviary system (AV) and conventional cages (CC).

A.



B.

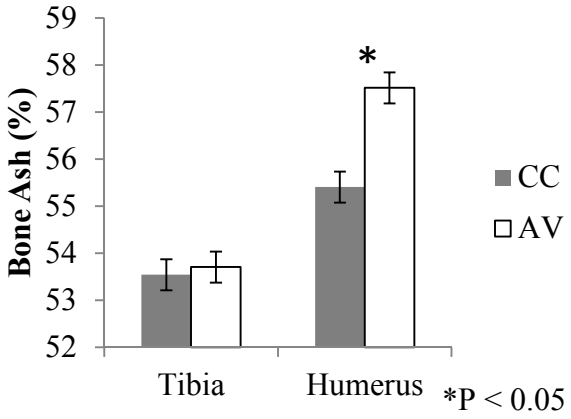


Table 2: Humerus and tibia cortical thickness (mm) of 16 wk Lohmann White pullets housed in cage-free aviary system and conventional pullet cages.

Bone type and housing	Planar orientation of bone			
	[2]Medial	Lateral	Anterior	Posterior
Humerus	LSM ± SEM	LSM ± SEM	LSM ± SEM	LSM ± SEM
Proximal				
AV	1.02 ± 0.02	1.02 ± 0.02	1.18 ± 0.02	1.29 ± 0.02
CC	0.86 ± 0.02	0.88 ± 0.02	0.91 ± 0.02	1 ± 0.02
P value	<.0001	<.0001	<.0001	<.0001
Mid				
AV	1.26 ± 0.02	1.21 ± 0.02	1.39 ± 0.02	1.45 ± 0.02
CC	1.03 ± 0.02	1.02 ± 0.02	1.07 ± 0.02	1.16 ± 0.02
P value	<.0001	<.0001	<.0001	<.0001
Distal				
AV	1.24 ± 0.02	1.40 ± 0.03	1.32 ± 0.02	1.37 ± 0.02
CC	1.03 ± 0.02	1.12 ± 0.03	1.06 ± 0.03	1.08 ± 0.02
P value	<.0001	<.0001	<.0001	<.0001
Tibia				
Proximal				
AV	1.52 ± 0.03	1.70 ± 0.03	1.52 ± 0.02	1.32 ± 0.02
CC	1.44 ± 0.03	1.57 ± 0.03	1.49 ± 0.02	1.36 ± 0.02
P value	0.0261	0.0007	0.4318	0.1334
Mid				
AV	1.59 ± 0.03	1.65 ± 0.03	1.54 ± 0.02	1.48 ± 0.02
CC	1.47 ± 0.03	1.55 ± 0.03	1.42 ± 0.02	1.39 ± 0.02
P value	0.0015	0.0124	<.0001	0.0032
Distal				
AV	1.53 ± 0.02	1.48 ± 0.02	1.23 ± 0.02	1.38 ± 0.02
CC	1.34 ± 0.02	1.36 ± 0.02	1.13 ± 0.02	1.25 ± 0.02
P value	<.0001	0.0002	<.0001	<.0001

[1]AV (Aviary system); CC (Conventional cages)

Table 3: Geometrical properties of humerus of 16 wk Lohmann White pullets housed in cage-free aviary system and conventional pullet cages.

Dependent variable	Housing		P value
	[1]AV	CC	
Geometrical properties	LSM ± SEM	LSM ± SEM	
Area (mm^2)	12.92 ± 0.12	9.42 ± 0.13	<.0001
Medial thickness (mm)	0.74 ± 0.01	0.54 ± 0.01	<.0001
Lateral thickness (mm)	0.75 ± 0.01	0.55 ± 0.01	<.0001
Anterior thickness (mm)	0.68 ± 0.01	0.52 ± 0.01	<.0001
Posterior thickness (mm)	0.70 ± 0.01	0.52 ± 0.01	<.0001
Average medio-lateral thickness (mm)	0.74 ± 0.01	0.55 ± 0.01	<.0001
Average antero-posterior thickness (mm)	0.69 ± 0.01	0.52 ± 0.01	<.0001
Average thickness (mm)	0.72 ± 0.01	0.53 ± 0.01	<.0001
Proximal medio-lateral diameter (mm)	7.52 ± 0.03	7.17 ± 0.03	<.0001
Mid medio-lateral diameter (mm)	6.82 ± 0.03	6.39 ± 0.03	<.0001
Distal medio-lateral diameter (mm)	6.88 ± 0.03	6.46 ± 0.04	<.0001
Prox antero-posterior diameter (mm)	5.98 ± 0.02	5.74 ± 0.02	<.0001
Mid antero-posterior diameter (mm)	5.79 ± 0.02	5.59 ± 0.02	<.0001
Distal antero-posterior diameter (mm)	5.92 ± 0.02	5.73 ± 0.02	<.0001
Average medio-lateral diameter (mm)	7.07 ± 0.03	6.68 ± 0.03	<.0001
Average antero-posterior diameter (mm)	5.90 ± 0.02	5.69 ± 0.02	<.0001
Average Diameter (mm)	6.49 ± 0.02	6.18 ± 0.02	<.0001

[1]AV (Aviary system); CC (Conventional cages)

Table 4: Geometrical properties of tibia of 16 wk Lohmann White pullets housed in cage-free aviary system and conventional pullet cages.

Dependent variable	Housing		P value
	[1]AV	CC	
Geometrical properties	LSM ± SEM	LSM ± SEM	
Area (mm^2)	14.16 ± 0.11	12.52 ± 0.12	<.0001
Medial thickness (mm)	0.90 ± 0.01	0.78 ± 0.01	<.0001
Lateral thickness (mm)	0.88 ± 0.01	0.78 ± 0.01	<.0001
Anterior thickness (mm)	0.80 ± 0.01	0.70 ± 0.01	<.0001
Posterior thickness (mm)	0.80 ± 0.01	0.70 ± 0.01	<.0001
Average medio-lateral thickness (mm)	0.89 ± 0.01	0.78 ± 0.01	<.0001
Average antero-posterior thickness (mm)	0.80 ± 0.01	0.70 ± 0.01	<.0001
Average thickness (mm)	0.84 ± 0.01	0.74 ± 0.01	<.0001
Proximal medio-lateral diameter (mm)	6.73 ± 0.03	6.69 ± 0.03	0.3825
Middle medio-lateral diameter (mm)	6.44 ± 0.03	6.37 ± 0.03	0.1121
Distal medio-lateral diameter (mm)	6.81 ± 0.03	6.73 ± 0.03	0.0912
Prox antero-posterior diameter (mm)	6.07 ± 0.03	5.94 ± 0.03	0.0029
Middle antero-posterior diameter (mm)	5.57 ± 0.02	5.51 ± 0.03	0.1252
Distal antero-posterior diameter (mm)	5.67 ± 0.03	5.67 ± 0.03	0.9947
Average medio-lateral diameter (mm)	6.66 ± 0.03	6.60 ± 0.03	0.1125
Average antero-posterior diameter (mm)	5.77 ± 0.02	5.71 ± 0.02	0.0831
Average Diameter (mm)	6.21 ± 0.02	6.15 ± 0.02	0.0585

[1]AV (Aviary system); CC (Conventional cages)

Table 5: Humerus and tibia cortical density (mg/cm^3) of 16 wk Lohmann White pullets housed in cage-free aviary system and conventional pullet cages.

Bone type and housing	Planar orientation of bone				
	Medial	Lateral	Anterior	Posterior	Average
Humerus Proximal	LSM ± SEM	LSM ± SEM	LSM ± SEM	LSM ± SEM	LSM ± SEM
AV	247.18 ± 7.09	259.25 ± 7.65	296.10 ± 8.47	280.36 ± 8.12	275.58 ± 7.08
CC	203.28 ± 7.37	219.36 ± 7.94	210.42 ± 8.80	217.62 ± 8.43	218.73 ± 7.28
P value	<.0001	0.0004	<.0001	<.0001	<.0001
Mid					
AV	376.93 ± 10.25	378.94 ± 10.17	422.61 ± 11.06	420.14 ± 10.79	391.72 ± 9.85
CC	300.48 ± 10.64	295.44 ± 10.55	318.91 ± 11.48	322.48 ± 11.20	304.60 ± 10.13
P value	<.0001	<.0001	<.0001	<.0001	<.0001
Distal					
AV	344.30 ± 9.20	422.85 ± 10.51	385.22 ± 10.10	395.52 ± 10.57	385.93 ± 9.47
CC	284.31 ± 9.46	331.42 ± 10.81	282.11 ± 10.38	289.55 ± 10.87	298.90 ± 9.74
P value	<.0001	<.0001	<.0001	<.0001	<.0001
Tibia Proximal					
AV	567.69 ± 31.23	589.16 ± 10.05	601.41 ± 10.85	557.46 ± 11.35	575.90 ± 9.99
CC	594.41 ± 31.23	571.07 ± 10.05	591.11 ± 10.85	569.89 ± 11.35	571.22 ± 10.03
P value	0.5449	0.2037	0.5021	0.4385	0.7409

Table 5 (cont'd).

Mid					
AV	639.40 ± 12.80	655.86 ± 12.83	663.15 ± 33.76	632.14 ± 12.36	643.22 ± 12.19
CC	605.09 ± 12.80	617.84 ± 2.83	657.69 ± 33.76	602.55 ± 12.36	609.45 ± 12.30
P value	0.0594	0.0373	0.909	0.0919	0.0524
Distal					
AV	668.92 ± 13.29	605.15 ± 12.54	559.69 ± 12.02	590.98 ± 11.77	612.06 ± 11.90
CC	615.33 ± 13.29	574.85 ± 12.54	519.21 ± 12.02	545.93 ± 11.77	571.64 ± 11.96
P value	0.0048	0.0889	0.0181	0.0073	0.0174

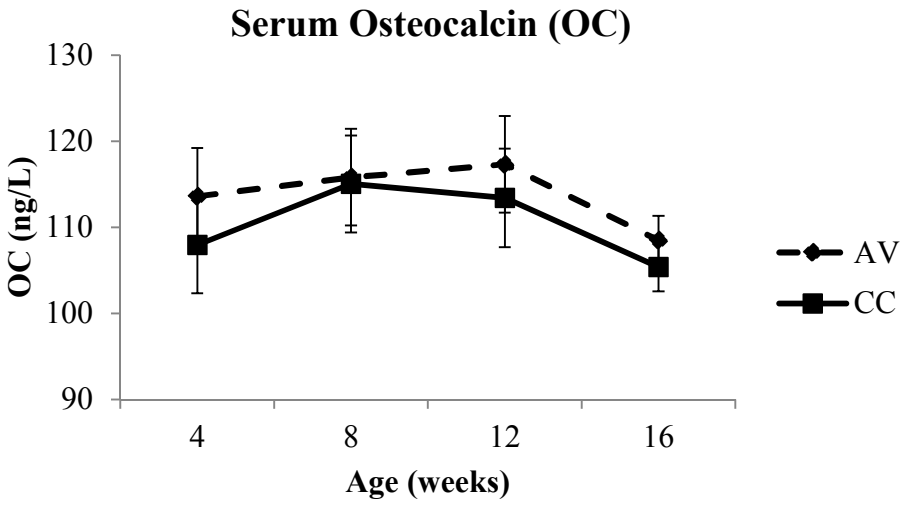
[1]AV (Aviary system); CC (Conventional cages)

Serum Bone Markers

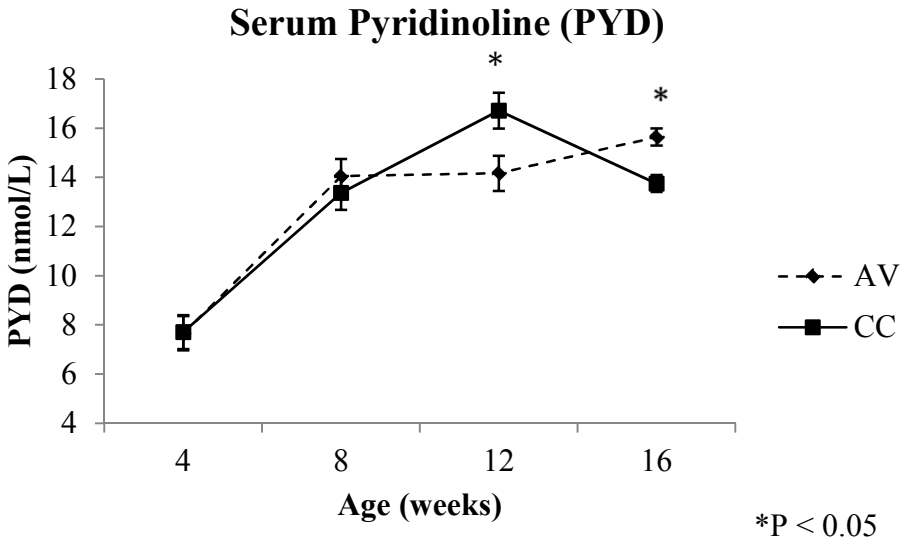
No effect of age or housing condition was observed for mean serum osteocalcin levels in the pullets (Figure 6A). Serum hydroxylysyl pyridinoline level increased from 4 to 8 weeks of age with no effect of housing system observed until 12 wk (Figure 6B). The hydroxylysyl pyridinoline concentration was 15.2% higher in caged pullets at 12 wk and the opposite was observed by 16 wk when the hydroxylysyl pyridinoline level was 12.2% higher in aviary pullets than caged pullets ($P < 0.05$; Figure 6B).

Figure 6: Effect of age and housing systems in systemic markers of bone formation and resorption of Lohmann White pullets housed in cage-free aviary system (AV) and conventional cages (CC). (A) Serum osteocalcin; (B) Pyridinoline.

A.



B.



*P < 0.05

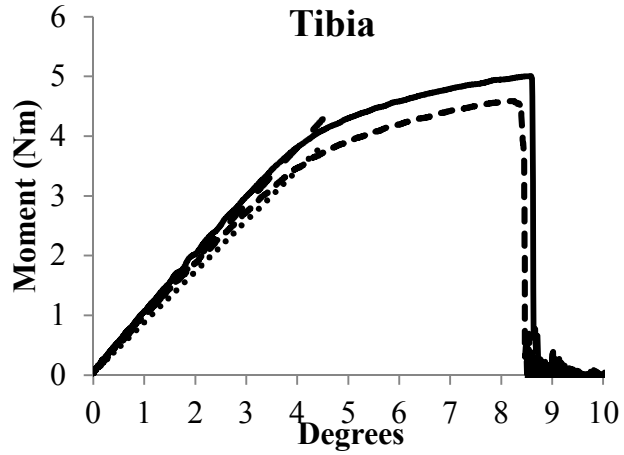
Bone Mechanical Properties

An overlay of representative moment-rotation data of a bone from each housing condition illustrates the general differences in bone mechanics up to failure (Figure 7A and B). The failure moment, M_f, was greater for AV tibia and humerus than that of the CC birds ($P < 0.001$; Figure 8A). As a result, aviary birds had stiffer tibiae and humeri compared to the caged birds, as represented by the slope of the curve (Figure 7A and B).

Aviary birds also had stronger bones with tibia material strength, σ_f, 3.7% greater than that of the CC tibia (P = 0.012) and humerus strength 6.3% greater than that of the CC humerus (P = 0.002; Figure 8B). There was no difference in Young's modulus, *E*, of the tibia between housing conditions (P = 0.4889; Table 6). In contrast, *E* of CC humeri was greater than that of AV humeri (P < 0.05; Table 6).

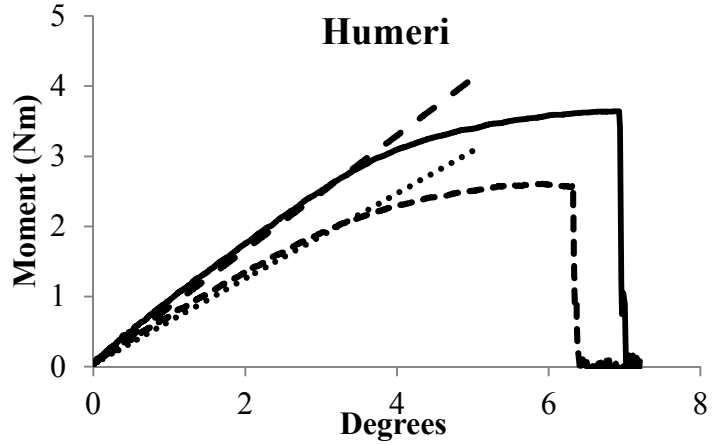
Figure 7: Representative moment-rotation curves showing tibia mechanical behavior in bending up to failure of 16 wk Lohmann White pullets housed in cage-free aviary system (AV) and conventional cages (CC). (A) behavior of the tibia; (B) behavior of the humerus. Stiffness was determined from the slope of the initial, linear portion of the curves.

A.



AV; AV stiffness; CC; CC stiffness

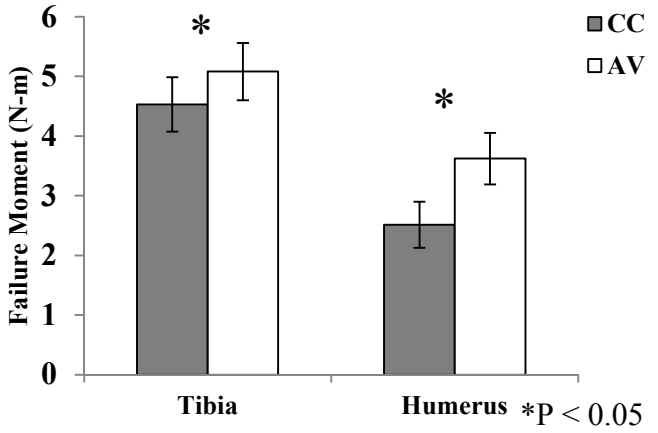
B.



AV; AV stiffness; CC; CC stiffness

Figure 8: (A) Ability of tibia and humerus to withstand bending moments; (B) breaking strength (least-squares mean ± standard error of the mean) of tibia and humerus of 16 wk Lohmann White pullets housed in cage-free aviary system (AV) and conventional cages (CC).

A.



B.

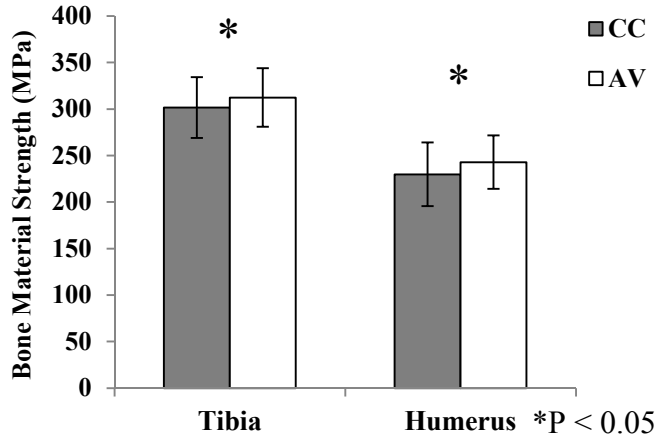
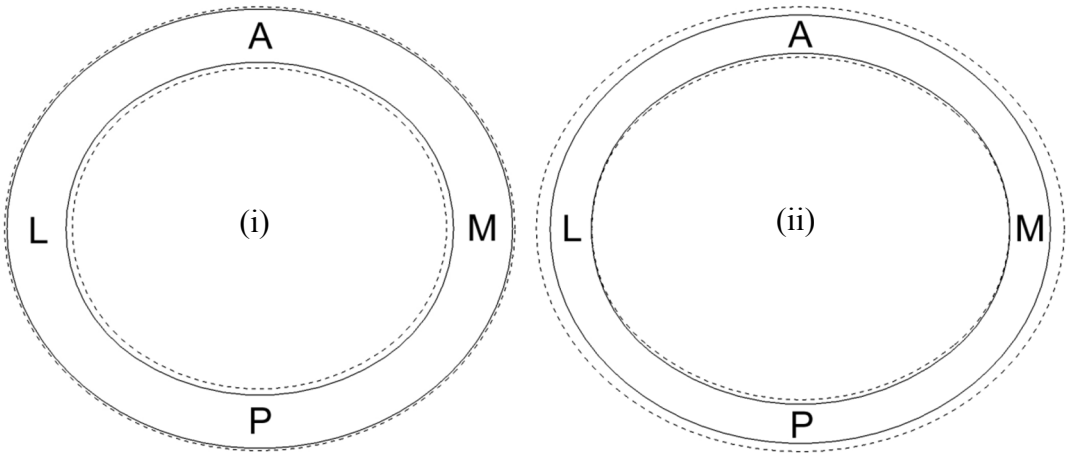


Table 6: Mechanical properties of tibia and humerus of 16 wk Lohmann White pullets housed in cage-free aviary system and conventional pullet cages.

Dependent variable	Housing		P value
	[1]AV	CC	
Tibia Mechanical properties	LSM ± SEM	LSM ± SEM	
Failure Moment (Nm)	5.08 ± 0.48	4.53 ± 0.46	<0.001
Failure Rotation (degree)	8.90 ± 1.15	8.57 ± 1.27	0.042
Stiffness (Nm/degree)	0.94 ± 0.10	0.85 ± 0.11	<0.001
Failure Stress (MPa)	312.40 ± 31.50	301.80 ± 32.70	0.012
Young's Modulus (GPa)	13.65 ± 1.20	13.74 ± 1.42	0.627
Second moment of inertia (mm^4)	59.91 ± 8.53	53.55 ± 8.02	<0.001
Humerus Mechanical properties			
Failure Moment (Nm)	3.62 ± 0.43	2.51 ± 0.39	<0.001
Failure Rotation (degree)	6.97 ± 1.05	6.49 ± 1.01	0.001
Stiffness (Nm/degree)	0.82 ± 0.11	0.61 ± 0.09	<0.001
Failure Stress (MPa)	242.90 ± 28.80	229.80 ± 34.30	0.002
Young's Modulus (GPa)	10.25 ± 1.22	10.77 ± 1.30	0.002
Second moment of inertia (mm^4)	46.25 ± 7.13	32.91 ± 5.64	<0.001

[1]AV (Aviary system); CC (Conventional cages)

Figure 9: Diagrammatic representation of bone cross-sections reconstructed using average measurements of 16 wk Lohmann White pullets housed in AV system (dashed line) and CCs (solid line): (i) = measurements for tibiae; (ii) = measurements for humeri.



A (Anterior); P (Posterior); M (Medial); L (Lateral)

DISCUSSION

Previous research exploring bone qualities in laying hens has often done so when the birds are in the laying stage. This work examines the effect of loading provided by difference in housing system in the tibia and humerus of growing pullets. The aviary (AV) system provides birds with more opportunity of dynamic loading exercises like running and flying which are not possible in cages. Early mechanical loading has been reported to result in narrower growth plates and shorter bones in broiler chicks (Reich et al., 2005). The common response of bones undergoing loading in compression is shortening in length and widening in cross-section (Seeman and Delmas, 2006), which was also the case in this study, even though the percentage change in length was very small. Conventionally housed pullets had longer tibiae and humeri at the end of the pullet phase compared to those kept in an aviary system, whereas aviary birds had greater bone width and cortical thickness. Measurements of bone thickness from quantitative computed tomography scans as well as the measurements taken after fracture found that bones from birds reared in aviary systems developed a thicker cortex than birds from conventional rearing systems.

In the tibiae, there was no difference in the outer (periosteal) dimensions between housing conditions. Thus, the increased cross-sectional area of AV tibiae was due to a narrowed medullary canal. Contrary to the results of this study, increase in cortical area in 8 wk old male white Leghorn chicks under controlled exercise regimen has been reported to be a result of periosteal deposition rather than endosteal apposition (Biewener and Bertram, 1994). In another study, the effect of high-impact exercise like jumping was limited to periosteal surface until 16 weeks in tarso-metatarsus of male leghorn chicks, however after that age, growth was more pronounced at endocortical surface (Judex and Zernicke, 2000). The varied response of growing

leg bone in White Leghorns to mechanical stimuli is likely to be the result of differences in age, sex, and the mechanical environment in which the birds are reared. Whereas such inward growth (as observed in present study) increases the second moment of area, the addition of bone more proximal to the neutral axis means that second moment of area differences were not as pronounced as cross-sectional area differences. The primary function of endosteal growth is to increase a bone's axial rigidity as has been observed in response to *in vivo* dynamic longitudinal compressive loading (Robling, et al., 2002). Thus, the cross-sectional geometry differences observed suggest that the additional loading on AV pullet tibiae may have been primarily along the axis of the bone.

The increased cross-sectional area in the humerus of AV birds was due to increased periosteal diameters with the endosteal dimensions remaining largely unchanged [Figure 9 (ii)]. In addition to increasing axial rigidity, outward expansion of the cortex greatly increases the second moment of area. The humeri second moment of area increased more dramatically than the cross-sectional area in AV housing conditions. An increased second moment of area is a characteristic response in bone that has been subjected to additional bending or torsional loads (Bass, et al., 2002; Ducher, et al., 2009) such as wing flapping which was possible in the AV systems. These findings suggest that humerus loading might be different to tibia, resulting in distinct growth patterns in each bone. The study results corroborate the findings that torsional resistance is the principal component to drive humerus structural design, while axial bending drives the structure of tibiotarsus in birds (de Margerie et al., 2004).

Structural improvement in AV pullets' humeri was accompanied by an increase in volumetric bone density and bone ash. The effect in cortical density of tibia was limited to the distal section and no difference was observed in the ash content. Concentration of hydroxylysyl

pyridinoline decreased in the pullets switched to AV until 12 wk but then increased to even higher levels compared to CC pullets at wk 16. Although net bone resorption is decreased in birds undergoing exercise (Fleming et al., 2006), why the level went up after 12 wk was unclear. Unlike deoxypyridinoline which is often used as bone-specific marker of collagen turnover, hydroxylysyl pyridinoline used in this study is not bone-specific and the ambiguous result might be due to collagen turnover in muscles and other organs of the growing pullets. Bone strength and modulus were calculated from the geometry and moment-bone rotation data to assess the material properties of the bones. Although the differences between groups for some of these quantities were statistically significant, the percentage changes were small. The statistically significant material differences were likely a product of the large sample sizes that boosted the sensitivity or due to the differences in structure and properties of organic matrix of the bone, which was not studied in this experiment. The changes in bone structure and density were not highly reflected by the mechanical testing when young bull calves were subjected to exercise (Hiney et al., 2004). The researchers suggested that physical measurements may provide more reliable assessment of bone mineral content than quantitative computed tomography, especially with smaller bones. Similar results were observed in mature White Leghorn roosters, where cortical areas and load bearing capacity were improved with exercise but the Young's modulus was not (Loitz and Zernicke, 1992). Laying hens housed in aviary houses have been reported to have significantly wider tibio-tarsal cortical area along with heavier bone mass, and denser tibia and humeri, compared to conventionally caged birds (Fleming et al., 2006). Whereas more recent quantitative computed tomography studies of bones in laying hens have demonstrated no changes in volumetric density of cortical and trabecular tissues between the housing systems despite having significant differences in structure (Jendral et al., 2008; Shipov et al., 2010),

which suggests that increased bone mineral content was a response to increased bone quantity and not a result of improved bone mineral density. Enneking et al. (2012) reported no difference in pooled data of various bones and ages for areal density, bone length, and width in pullets housed in cages with perches compared to pullets in cages without perches. However, the bone mineral content of tibia and humerus were significantly different at 12 wk between the groups.

This study indicated that skeletal loading provided by activities within pullet AV housing resulted in structural and material changes that improved the load-bearing capability and stiffness to the tibia and humerus. Providing greater access to activities including flying, perching, and running during pullet phase can be crucial to the increased bone quantity that might help prevent fractures due to osteoporosis in cage birds, and impact injuries during the production phase in the extensive systems.

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CHAPTER 3.

Housing conditions alter properties of the tibia and humerus during laying phase in Lohmann white leghorn hens

INTRODUCTION

Consumer awareness concerning food of animal origin has increased the importance of animal welfare in production systems. Among many issues concerning animal behavior and welfare, the conventional cage housing system for laying hens has come under particular scrutiny. Laying hens housed in conventional cages are prone to osteoporosis mainly due to restricted movement, lack of exercise, and the calcium demand for eggshell production (Fleming et al., 1994; Tauson and Abrahamsson, 1994; Whitehead and Fleming, 2000). Ethological and skeletal concerns for laying hens in conventional cages have prompted legislative changes in housing throughout the E. U. (Appleby, 2003). Egg producers in the U. S. are facing similar pressures leading to increased regulation of the egg industry at the state level (Mench et al., 2011). Several alternative housing systems are commercially available and thorough investigation is required to determine their impacts on bone quality.

The provision of greater space and perches in the alternative housing systems allows birds to perform activities like running, jumping and wing flapping, presumably providing greater loading to their bones and strengthening them (Leyendecker et al., 2005; Jendral et al., 2008; Sandilands et al., 2009; Norgaard-Nielsen, 1990). However, because of the wide variation in the age and strain of the birds used, rearing environment during the pullet phase and forms of alternative housing systems (enriched colony cages, floor pens, cage-free aviary or free range), clear inferences on the impact of loading on bone structure and mechanics cannot be drawn. Recently Silversides et al., (2012) reported the radius and tibia of hens housed in floor pens were denser and had greater cortical area but the probable changes in the mechanical properties of these bones were not studied. Besides, most of the prior studies have assessed the bone quality at the end of the hen's production period and no study has addressed how bones remodel during

production upon changing housing environment at the end of the pullet phase. With reports of high occurrence of keel fractures in non-cage aviary systems (Freire et al., 2003; Wilkins et al., 2004; Nicol et al., 2006; Moinard et al., 2004), hens housed in these systems might have to mobilize minerals to repair the fractures in addition to egg production. A middle ground with improved bone quality and reduced keel fractures may be found in the enriched colony cages and producers in the U. S. may find the information valuable in the decision-making when they look for alternative to conventional cages.

Previously we have reported the difference in bone properties between pullets housed in cage-free aviary systems and conventional cages at 18 weeks (Regmi et al., 2015). The current study was aimed at investigating the influence of laying period on mechanical, geometrical, and mineral compositions of humeri and tibiae from hens housed in conventional cages, enriched colony cages and cage-free aviary system. Specifically, we wanted to see if any changes occurred in the two groups of pullets when the ability to perform physical activity was altered during the laying period.

MATERIALS AND METHODS

The experimental procedures were approved by Institutional Animal Care and Use Committee of Michigan State University.

Birds, Management, and Sampling

At 19 wk of age, Lohmann White pullets reared in conventional cages were transferred to either conventional layer cages (CC) or enriched colony cages (EN) whereas those reared in cage-free aviary (AV) were transferred to an aviary system or to conventional cages (AC). The housing design and space allocation were the same as described by Jones et al., (2014). A population of 193,424 hens were housed in conventional cages with 6 birds per cage and provided with 568 cm^2 per bird. Enriched colony cages were stocked with a total of 46,795 hens at 60 hens per cage with 753 cm^2 cage space per bird. Each hen was provided with 18 cm of perch length space, 63 cm^2 of nest space and 26.5 cm^2 of scratch pad space. For the aviary system, 49,842 hens were housed with a minimum of 1,166.5 cm^2 of total area per bird. The hens were provided 15 cm of perch length and 86 cm^2 of nest space area on per bird basis. Nutritional and health management were carried out according to the breeder's guidelines with hens having ad libitum access to water and commercially available feed. The hens were maintained on 16:8 light and dark cycle during the lay cycle (Jones et al., 2014). At 77 weeks, 120 birds at random were sampled for bone analysis from each housing system. Hens were euthanized by cervical dislocation followed by excision of the right and left tibiae and humeri for further analysis.

Computed Tomography and Bone Ash

Quantitative computed tomography (QCT) was performed on 120 AV, EN, and CC hens and 100 AC hens. Right tibiae and humeri of the hens were scanned for measurement of total bone length, cortical thickness and cortical density at proximal, middle and distal sections of

each bone as described by Regmi et al., (2015). A threshold of 180 HU and 450 HU was used to measure cortical density of humeri and tibiae respectively. Samples were QCT scanned followed with analysis of ash content. Dry bone weight and ash percentage on dry bone basis of tibiae and humeri were analyzed as previously described (Regmi et al., 2015).

Mechanical Testing

Mechanical properties were evaluated in 120 hens from AV, EN and CC groups while 60 hens were studied from the AC group. The testing was carried out in a fixture, described previously in Regmi et al., (2015), that generated pure or uniform bending of the mid-diaphyseal section of the humerus and tibia. Briefly, anterior-posterior (AP) and medial-lateral (ML) outer dimensions of the bones were measured manually at the ends and center with digital calipers. Equal moments were applied at each end of the specimen and the bones were loaded up to failure for determination of the mechanical properties. The tibiae were loaded such that the lateral surfaces were in tension and humeri were loaded such that the posterior surface were in tension. The measured and calculated mechanical parameters were second moment of area (I), maximum or failure bending moment (M_f), stiffness (K), failure stress (σ_f), and Young's modulus (E). The bone fragments at the fracture site were used to measure anterior, posterior, medial, and lateral cortical thicknesses. Outer dimensions and diaphyseal thicknesses were used to approximate the cortical cross-section as a hollow ellipse.

The material properties of the bones were determined based on classical beam theory and second moment of area was computed using the expression

$$I = \frac{\pi a_0 {b_0}^3}{4} - \frac{\pi a_1 {b_1}^3}{4} \quad (6)$$

where a and b were the radii parallel and perpendicular to the neutral axis of the bone and subscripts 0 and 1 denoted outer and inner dimensions of the bone, respectively. The applied bending moment was determined from the actuator force using the expression

$$M = \frac{\sqrt{a_l{}^2 - d^2}F}{2} \tag{7}$$

where F was the actuator force applied to both cups and a_l was the distance between the pivot and load application points on the cups and where d was the actuator displacement. The bone-end deflection angle was calculated using the expression

$$\theta = \sin^{-1}\frac{d}{a_l} \tag{8}$$

Whole-bone (structural) bending stiffness, K, was the slope of linear portion of the moment-bone rotation plot. Material stiffness, known as Young's modulus, was computed using the equation

$$E = (K)\frac{L}{2I} \tag{9}$$

where L was the length of the test section. The material strength of each bone was determined based on a computation of the maximum (failure) bending stress (σ_f) in the bone using the expression

$$\sigma_f = \frac{M_f b}{I} \tag{10}$$

where M_f was the maximum bending moment exerted on each rigid cup at the ends of the specimen at failure.

Statistical Analysis

Data were analyzed using the multivariate PROC MIXED analysis of SAS 9.3 (SAS Institute 2002, Cary, NC). Repeated measures statement with the model including fixed effect of

housing and section of bone, the interaction between housing and section, and the residual error was used to analyze the data. The body weights of hens soon after euthanasia and before collection of samples were measured and differences were only observed between EN and CC hens with EN hens being heavier. Since the influence of fixed effects was apparent on the body weight, it was not included as a covariate during the statistical analysis of the data. Differences between means were tested using Fisher's LSD with significance accepted at $P<0.05$. Values were represented as least square means with their respective standard error for the mean (LSM ± SEM).

RESULTS

Geometrical and Compositional Properties

The results for structural measurements of tibiae and humeri are presented in Tables 7 and 8. Effect of restriction in activities by placing aviary-reared pullets in conventional cages was observed in the cross-sectional geometry of tibiae and humeri between the AV and AC groups.

Humeri of AV birds had greater cortical cross-sectional area than AC birds along with greater diaphyseal cortical thickness ($P < 0.05$, Table 7). The changes, however, were not observed for the outer dimensions of humeri between the groups, as average antero-posterior and medio-lateral diameter were not different. The difference between humeri of AV and AC hens were also observed when cortical thickness was measured using images obtained from QCT scans (Table 8). Humeri of AV hens had a consistently thicker cortex across all the planes at mid diaphysis and along posterior and medial planes of the distal section when compared to AC hens ($P < 0.05$, Table 8). In addition to the structural differences, AV hens had greater volumetric density of cortical bone than AC hens ($P < 0.05$, Table 9). Geometrical measurements of tibiae between AV and AC hens were different for area and thicknesses. Tibiae of AV hens had greater cortical cross-sectional area and cortical thickness, however antero-posterior outer diameter was not different compared to AC hens ($P < 0.05$; Table 7). Unlike the diaphyseal cortical thickness measured manually, QCT measured cortical thickness at mid section was greater in AC hens than AV hens ($P < 0.05$, Table 8). The results for distal cortical thickness of tibiae between AV and AC hens were more variable. AC hens had thicker cortex along the anterior plane than AV hens whereas the result was opposite along the posterior plane ($P < 0.05$). Medio-lateral cortical thicknesses were not different between AV and AC hens. On the other hand, average density of

cortical bone was greater in AV hens compared to AC hens at mid and distal section of tibia ($P < 0.05$, Table 9).

Tibiae and humeri also responded to the provision of moderate level of movement during the laying period as elucidated by differences in bone properties of EN and CC hens. The humeri of EN hens had greater total cortical area than that of CC hens ($P < 0.05$), while no differences were observed for cortical thicknesses at mid diaphysis. Medio-lateral outer diameter of humeri was wider in EN hens than CC hens ($P < 0.05$). Cortical thickness of humeri between EN and CC hens were only different at the distal section along the antero-posterior plane with EN hens having thicker cortex ($P < 0.05$). The cortical bone density of humeri was not different between EN and CC hens. Unlike humeri, tibiae of EN and CC hens were not different for any of the geometrical parameters, whereas cortical tibiae were denser in EN hens than CC hens ($P < 0.05$). Ash content and dry bone weight of humeri were not different between EN and CC hens, whereas AV hens had heavier bones with greater ash content than AC hens ($P < 0.05$, Figure 10A and B). Ash percentage was lowest in tibia of EN hens compared to the rest of the group, whereas the humeri ash content was lowest in AC hens ($P < 0.05$, Figure 10A).

Overall comparison indicated cortical bone to be denser and thicker in the AV hens at the middle section of the bone among the 4 groups of hens ($P < 0.05$, Table 7, 8, and 9). The outer dimensions of the humeri and tibiae were greater in AV and AC hens than EN and CC hens ($P < 0.05$). Interestingly, humeri of the AC hens had cortical thickness value similar or even smaller than CC hens ($P < 0.05$, Table 7 and 8) whereas no differences in density of either tibiae or humeri was observed between those groups (Table 9).

Table 7: Geometrical properties of humeri and tibia of 77 wk Lohmann White hens housed in cage-free aviary, enriched colony cages, and conventional cages.

Bone type and geometrical properties	Housing			
	[1]AV	AC	CC	EN
Humeri	LSM ± SEM	LSM ± SEM	LSM ± SEM	LSM ± SEM
Cortical area (mm^2)	12.07 ± 0.11[a]	9.00 ± 0.16[d]	9.74 ± 0.12[c]	10.07 ± 0.12[b]
Medial thickness (mm)	0.64 ± 0.01[a]	0.44 ± 0.01[c]	0.55 ± 0.01[b]	0.55 ± 0.01[b]
Lateral thickness (mm)	0.66 ± 0.01[a]	0.49 ± 0.01[c]	0.57 ± 0.01[b]	0.57 ± 0.01[b]
Anterior thickness (mm)	0.61 ± 0.01[a]	0.45 ± 0.01[c]	0.52 ± 0.01[b]	0.54 ± 0.01[b]
Posterior thickness (mm)	0.62 ± 0.01[a]	0.44 ± 0.01[c]	0.52 ± 0.01[b]	0.54 ± 0.01[b]
Average M-L diameter (mm)	7.42 ± 0.03[a]	7.48 ± 0.04[a]	6.85 ± 0.03[c]	6.98 ± 0.03[b]
Average A-P diameter (mm)	6.14 ± 0.03[a]	6.07 ± 0.04[a]	5.75 ± 0.03[b]	5.80 ± 0.03[b]
Average Diameter (mm)	6.78 ± 0.02[a]	6.78 ± 0.03[a]	6.30 ± 0.02[c]	6.39 ± 0.02[b]
Tibia				
Cortical area (mm^2)	13.90 ± 0.13[a]	12.56 ± 0.21[b]	12.27 ± 0.13[b]	12.27 ± 0.14[b]
Medial thickness (mm)	0.83 ± 0.01[a]	0.76 ± 0.02[b]	0.75 ± 0.01[b]	0.75 ± 0.01[b]
Lateral thickness (mm)	0.81 ± 0.01[a]	0.74 ± 0.02[b]	0.72 ± 0.01[b]	0.71 ± 0.01[b]
Anterior thickness (mm)	0.77 ± 0.01[a]	0.67 ± 0.02[b]	0.68 ± 0.01[b]	0.67 ± 0.01[b]
Posterior thickness (mm)	0.78 ± 0.01[a]	0.64 ± 0.02[c]	0.69 ± 0.01[b]	0.69 ± 0.01[b]
Average M-L diameter (mm)	6.81 ± 0.03[b]	6.95 ± 0.04[a]	6.67 ± 0.03[c]	6.72 ± 0.03[c]
Average A-P diameter (mm)	5.92 ± 0.03[a]	5.90 ± 0.04[a]	5.80 ± 0.03[b]	5.81 ± 0.03[b]
Average Diameter (mm)	6.37 ± 0.02[a]	6.43 ± 0.04[a]	6.23 ± 0.02[b]	6.26 ± 0.02[b]

[abcd] Means within the same row lacking a common superscript differ significantly ($P<0.05$).

[1] AV (Cage-free aviary system); EN (Enriched colony cage); CC (Conventional cage); AC (Pullet aviary reared-Conventional cage laying)

Table 8: QCT measured cortical thickness (mm) of tibiae and humeri of 77 wk Lohmann White hens housed in cage-free aviary, enriched colony cages, and conventional cages.

Bone type and housing	Planar orientation of bone			
	Anterior	Posterior	Medial	Lateral
Humeri	LSM ± SEM	LSM ± SEM	LSM ± SEM	LSM ± SEM
Middle				
[1]AV	1.28 ± 0.02[a]	1.28 ± 0.02[a]	1.16 ± 0.02[a]	1.08 ± 0.02[a]
AC	1.06 ± 0.03[c]	1.00 ± 0.03[c]	0.99 ± 0.03[c]	0.97 ± 0.03[b]
CC	1.12 ± 0.02[bc]	1.15 ± 0.03[b]	1.02 ± 0.02[bc]	1.01 ± 0.02[b]
EN	1.17 ± 0.02[b]	1.21 ± 0.03[ab]	1.09 ± 0.02[b]	0.99 ± 0.02[b]
Distal				
AV	1.35 ± 0.03[a]	1.34 ± 0.03 [a]	1.16 ± 0.02[a]	1.15 ± 0.03
AC	1.27 ± 0.04[ab]	1.17 ± 0.03[b]	1.03 ± 0.03[c]	1.08 ± 0.04
CC	1.14 ± 0.03[c]	1.17 ± 0.03[b]	1.07 ± 0.02[bc]	1.16 ± 0.03
EN	1.23 ± 0.03[b]	1.31 ± 0.03 [a]	1.12 ± 0.02[ab]	1.12 ± 0.03
Tibiae				
Middle				
AC	1.51 ± 0.04	1.58 ± 0.04[a]	1.69 ± 0.05[a]	1.65 ± 0.04[a]
AV	1.43 ± 0.03	1.36 ± 0.04[b]	1.45 ± 0.04[c]	1.46 ± 0.04[c]
CC	1.53 ± 0.05	1.51 ± 0.05[ab]	1.66 ± 0.06[ab]	1.60 ± 0.05[ab]
EN	1.46 ± 0.03	1.4 ± 0.04[b]	1.54 ± 0.04[bc]	1.51 ± 0.04[bc]
Distal				
AC	1.36 ± 0.03[a]	1.09 ± 0.03[b]	1.51 ± 0.04	1.37 ± 0.03
AV	1.18 ± 0.03[b]	1.34 ± 0.03[a]	1.51 ± 0.04	1.34 ± 0.03
CC	1.23 ± 0.04[b]	1.43 ± 0.04[a]	1.48 ± 0.05	1.46 ± 0.04
EN	1.21 ± 0.03[b]	1.38 ± 0.03[a]	1.48 ± 0.04	1.41 ± 0.03

[abc] Means within the same column lacking a common superscript differ significantly ($P<0.05$).

[1]AV (Cage-free aviary system); EN (Enriched colony cage); CC (Conventional cage); AC (Pullet aviary reared-Conventional cage laying)

Table 9: Humeri and tibiae cortical density (mg/cm^3) of 77 wk Lohmann White hens housed in cage-free aviary, enriched colony cages, and conventional cages.

Bone type and housing	Planar orientation of bone				
	Anterior	Posterior	Medial	Lateral	Average
Humeri	LSM ± SEM	LSM ± SEM	LSM ± SEM	LSM ± SEM	LSM ± SEM
Middle					
[1]AV	792.98 ± 21.70^{a}	730.96 ± 20.33^{a}	696.57 ± 20.91^{a}	701.86 ± 20.62^{a}	734.65 ± 19.43^{a}
AC	655.66 ± 27.82^{b}	603.68 ± 26.43^{b}	595.25 ± 27.00^{b}	601.90 ± 27.28^{b}	591.48 ± 24.57^{b}
CC	670.66 ± 22.46^{b}	637.38 ± 21.05^{b}	624.15 ± 21.65^{b}	602.41 ± 21.26^{b}	634.89 ± 20.11^{b}
EN	670.49 ± 22.07^{b}	651.06 ± 20.68^{b}	620.85 ± 21.27^{b}	592.72 ± 21.07^{b}	633.16 ± 19.76^{b}
Distal					
AV	800.47 ± 21.53^{a}	773.07 ± 21.21^{a}	720.72 ± 21.31^{a}	770.72 ± 22.78	767.74 ± 19.73^{a}
AC	701.90 ± 26.71^{b}	645.58 ± 26.65^{b}	627.12 ± 27.32^{b}	733.61 ± 29.82	631.89 ± 24.47^{b}
CC	633.60 ± 22.29^{c}	629.71 ± 21.95^{b}	616.43 ± 22.06^{b}	698.77 ± 23.68	646.58 ± 20.52^{b}
EN	654.68 ± 21.90^{bc}	676.53 ± 21.57^{b}	608.85 ± 21.67^{b}	688.89 ± 23.27	648.06 ± 20.07^{b}
Tibiae					
Middle					
AV	1393.20 ± 25.31^{a}	1335.19 ± 25.56^{a}	1349.94 ± 26.95^{a}	1408.6 ± 26.65^{a}	1384.77 ± 25.53^{a}
AC	1237.23 ± 26.62^{b}	1273.25 ± 26.71^{a}	1289.09 ± 28.17^{ab}	1292.43 ± 27.85^{bc}	1218.61 ± 26.68^{c}
CC	1201.35 ± 34.76^{b}	1175.98 ± 35.10^{b}	1211.42 ± 37.02^{b}	1218.93 ± 36.60^{c}	1208.84 ± 35.06^{c}
EN	1344.30 ± 25.94^{a}	1289.92 ± 26.20^{a}	1311.84 ± 27.63^{a}	1322.61 ± 27.31^{b}	1312.62 ± 26.17^{b}
Distal					
AV	1273.59 ± 26.38^{a}	1322.85 ± 26.62^{a}	1422.47 ± 30.24^{a}	1335.86 ± 27.98^{a}	1361.67 ± 25.82^{a}
AC	1133.59 ± 27.40^{bc}	1045.52 ± 27.84^{c}	1246.98 ± 31.60^{c}	1151.3 ± 29.43^{c}	1139.12 ± 26.82^{c}
CC	1056.48 ± 36.01^{c}	1113.94 ± 36.35^{c}	1224.43 ± 41.53^{c}	1152.72 ± 38.42^{bc}	1164.2 ± 35.25^{c}
EN	1188.47 ± 26.87^{b}	1232.38 ± 27.12^{b}	1334.49 ± 30.99^{b}	1241.71 ± 28.68^{b}	1287.53 ± 26.30^{b}

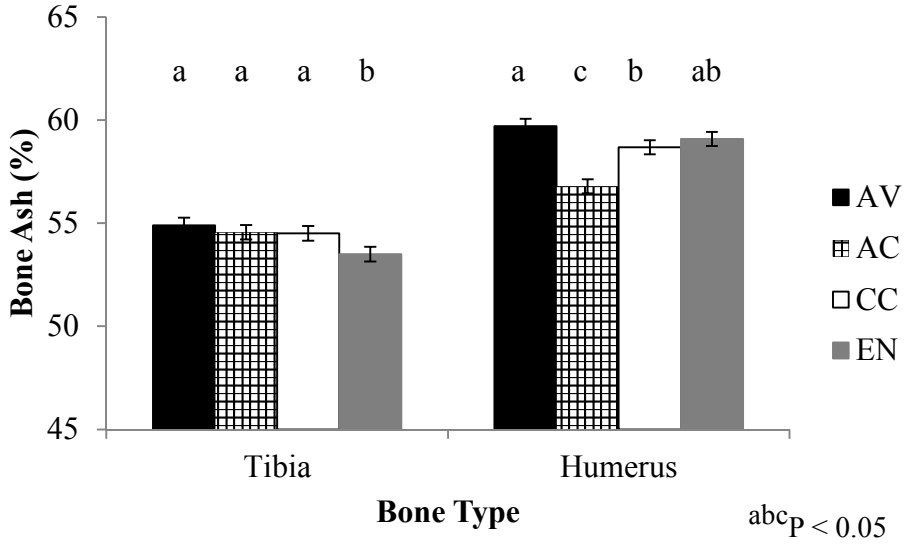
Table 9 (cont'd).

[abc] Means within the same column lacking a common superscript differ significantly ($P<0.05$).

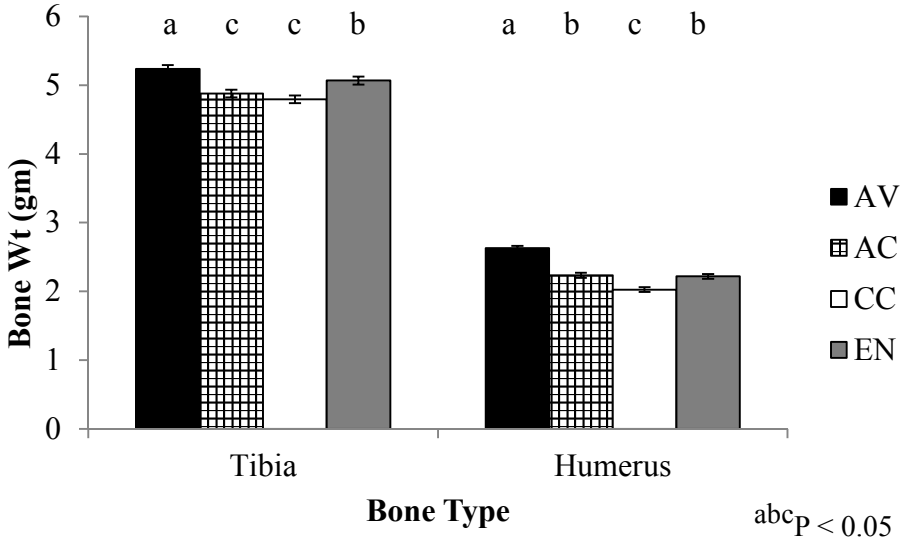
[1] AV (Cage-free aviary system); EN (Enriched colony cage); CC (Conventional cage); AC (Pullet aviary reared-Conventional cage laying)

Figure 10: (A) Percentage ash content and (B) dry bone weight of tibia and humerus of 77 Wk Lohmann White hens in AV (Cage-free aviary system), EN (Enriched colony cages), CC (Conventional cages) and AC (Pullet aviary reared-Conventional cage laying) groups.

A.



B.



Mechanical Properties

The results of mechanical testing of tibiae and humeri are presented in Table 10. The higher values of outer dimensions and thicker cortex of humeri in AV hens translated into higher values of second moments of area compared to AC hens ($P < 0.05$). The mechanical properties of whole humeri were greater in AV hens compared to the AC hens. The material strength of humeri indicated by failure stress was not different between AV and AC hens, while the material stiffness indicated by Young's modulus was greater in AV humeri than AC humeri ($P < 0.05$). The results for tibiae were varied and while properties such as failure moment, stiffness, and modulus of elasticity were greater in AV hens than AC hens ($P < 0.05$), material strength (failure stress) and second moment of area were not different between the groups.

Greater average outer diameter and cortical area meant that humeri second moments of area were higher in EN hens than in CC hens ($P < 0.05$). The humeri material strength was greater in EN hens ($P < 0.05$), whereas the humeri modulus of elasticity was not different between EN and CC hens. Tibiae of EN hens were stiffer (both the structural and material values) compared to CC hens, whereas other mechanical properties were not different between the groups ($P < 0.05$). The mechanical properties of tibiae and humeri indicate that between AC and CC hens, second moments of area were greater in AC hens while CC hens had a greater modulus of elasticity ($P < 0.05$).

Table 10: Mechanical properties of humeri and tibiae of 77 wk Lohmann White hens housed in cage-free aviary, enriched colony cages, and conventional cages.

Dependent variable	Housing			
	[1]AV	AC	CC	EN
Humeri Mechanical properties	LSM ± SEM	LSM ± SEM	LSM ± SEM	LSM ± SEM
Failure Moment (Nm)	3.90 ± 0.04[a]	2.83 ± 0.05[c]	2.81 ± 0.04[c]	3.17 ± 0.04[b]
Failure Rotation (degree)	5.45 ± 0.10[bc]	5.69 ± 0.14[ab]	5.34 ± 0.10[c]	5.77 ± 0.10[a]
Stiffness (Nm/degree)	0.85 ± 0.01[a]	0.58 ± 0.01[d]	0.67 ± 0.01[c]	0.70 ± 0.01[b]
Failure Stress (MPa)	264.05 ± 3.76[ab]	246.91 ± 5.22[bc]	254.30 ± 3.81[b]	272.30 ± 3.82[a]
Young's Modulus (GPa)	10.21 ± 0.15[b]	9.00 ± 0.21[c]	11.12 ± 0.15[a]	11.05 ± 0.15[a]
Second moment of area (mm^4)	48.32 ± 0.57[a]	37.33 ± 0.79[b]	34.73 ± 0.58[c]	36.82 ± 0.58[b]
Tibia Mechanical properties				
Failure Moment (Nm)	5.35 ± 0.08[a]	4.80 ± 0.12[b]	4.70 ± 0.08[b]	4.80 ± 0.08[b]
Failure Rotation (degree)	6.41 ± 6.41[b]	6.55 ± 6.55[ab]	7.03 ± 7.03[a]	6.51 ± 6.51[b]
Stiffness (Nm/degree)	1.06 ± 0.01[a]	0.88 ± 0.02[c]	0.92 ± 0.01[c]	0.96 ± 0.01[b]
Failure Stress (MPa)	331.49 ± 4.69	326.18 ± 7.49	329.32 ± 4.67	329.37 ± 4.82
Young's Modulus (GPa)	14.70 ± 0.15[b]	12.53 ± 0.23[c]	14.72 ± 0.14[b]	15.13 ± 0.15[a]
Second moment of area (mm^4)	62.19 ± 0.86[a]	60.76 ± 1.38[a]	54.01 ± 0.86[b]	54.90 ± 0.89[b]

[abcd] Means within the same row lacking a common superscript differ significantly ($P<0.05$).

[1] AV (Cage-free aviary system); EN (Enriched colony cage); CC (Conventional cage); AC (Pullet aviary reared-Conventional cage laying)

DISCUSSION

The compositional and structural properties of bones can be modified by various factors, particularly the loading environment, in addition to the genetic make-up of an animal. The effect of physical activity on bone properties is fairly well established from several human and animal studies. Bones are more responsive to loading exercises during growth and the results are often characterized by increased bone mass via periosteal and endosteal apposition. The changes in bone mineral density may not be apparent during growth whereas mature skeleton responds mainly by preventing bone loss and acquiring bone density (Bergmann et al., 2011). The same principles could be applied in modern laying hens, except they are different because of the egg-laying activity that begins with adulthood. The formation of medullary bones with the start of lay might change the response of structural bone to the physical activity provided during the laying period. The first part of the current study conducted in pullets clearly suggested that at the start of the laying phase, pullets reared in AV have increases in bone mass and density in both humeri and tibia (Regmi et al, 2015). The changes were then reflected in the mechanical properties with AV pullets having greater resistance to bending with the differences more pronounced in humeri than in tibiae. In the current study we examined the effect of different commercial housing conditions on the tibia and humerus of adult laying hens. The greater interest was to identify whether the bone mass gained by AV pullets is maintained at the end of the laying cycle when the extent of physical activity is either continued or discontinued. Furthermore, the enriched colony cages during the laying phase allowed us to observe the effects of moderate physical activities in the properties of tibia and humerus of hens previously housed in CC during pullet phase.

The differences in mechanical properties of bones from the AV hens compared to those of the AC hens could be attributed to both geometrical and compositional differences observed between the groups. Humeri and tibiae of hens kept in AV had similar outer dimensions but thicker cortex than AC hens, indicating that bone loss was mainly due to increased endosteal resorption in the latter. In addition to loss of bone mass, AC hens also lost a significant amount of bone density in both the tibiae and humeri. The aviary system used in this study was a multi-tier structure equipped with perches of different heights which provided opportunities for hens to perform high impact loading exercises like jumping and flying. The actual loading environment in commercial hen houses has not been studied. However, varying levels of locomotive and flight behaviors likely resulted in different structural, compositional and mechanical changes in the bones of end-of-lay hens. A 24 hr long video recording at peak, mid and end-lay from the same flock reported a total number of 1,588 flights in the AV system (Campbell et al., 2015). The stress and strains developed in pectoralis muscles during different stages of flight and wing-assisted incline running (Jackson et al., 2011) are the primary loading activities of humeri in AV hens. Fewer cycles of unusual loading conditions, like jumping, have been reported previously to dominate the adaptive response in bone rather than numerous cycles of normal loading conditions (Lanyon, 1992). On the other hand, wing movement is greatly restricted in conventional cages and hens are not able to maintain the same strength in humeri compared to the birds in perchery (Knowles and Broom, 1990). Experimental immobilization of wings in young pullets has been reported to result in cortical bone loss of humeri as early as 14 days after immobilization (Foutz et al., 1997). Subsequent loss of mechanical stability with decreased stiffness and modulus of elasticity were observed after 35 days of immobilization (Foutz et al., 1997). The results of the current study are also consistent with the loss of bone density and

decreased shear load observed after immobilization of broiler tibia (Foutz et al., 2007). In another study, floor-reared pullets were able to better protect bone thickness and density when moved to colony cages with perches than in conventional cages (Jendral et al., 2008; Silversides et al., 2012). Shipov et al. (2010) compared the humerus and tibia of 2 yr old laying hens kept in free range and conventional cages. Hens in a free-range system had bones with greater stiffness and were able to bear greater load to failure than hens in cages, which is consistent with the results of our study. Mechanical properties of bone can be attributed to its structure, composition, or to a combination of both (Sharir et al., 2008). Compositional parameters often studied as markers of bone health are bone mineral density and characteristics of collagen fibers, whereas thickness, diameter and cross-sectional area of cortices, trabeculae and medullary bones are indicators of structural integrity of the bone. Some mechanical properties, failure stress in particular, were not different for tibia between the groups in this study. In contrast to the results of the current study, Loitz and Zernicke (1992) reported no change in elastic modulus of mature White Leghorn roosters under an experimental exercise plan compared to that of a control group. Additional analysis of structure and nature of the organic components of tibiae and humeri may help explain the documented changes, but such work was beyond the scope of the current study. Furthermore, trabecular bone loss of over 10% has been reported to alter deformation characteristics of femoral cortical bone in adult laying hens (Reich and Gefen, 2006). Analysis of trabecular bone properties by high-resolution micro-computed tomography or histology may have helped us understand the mechanism in greater detail, but again was beyond the scope of the current work.

The increased second moment of area of the humeri of AV and EN hens, compared to AC and CC hens in the current study was typical of bones undergoing loading in compression

and torsion (Wainright et al., 1976). On the other hand, there was no effect of housing on the second moment of area for the tibia, most likely because of the similar effective radius between AV-AC and between EN-CC hens. While wing movement is greatly limited, hens in CC spend more time standing (Silversides et al., 2012) and because of the weight bearing nature of the tibia, a loading environment similar to that in alternative housing systems might have resulted in similar structural properties. Similar results were reported by Shipov et al., 2010 with humeri of free-range hens having greater second moment of area than caged hens, while the difference was not apparent in tibia. Interestingly, cortical thickness measured from QCT scans in mid tibia of AC hens was similar to CC hens but greater compared to AV hens. The reason for this observation is not understood. The accuracy of peripheral macro-QCT measurements particularly for bones containing high amount of medullary bone tissue like tibia and femur should be validated by other high-resolution tomography or microscopy techniques.

The bone properties of AC hens when compared to AV hens indicated that discontinuation of physical activity during the laying phase was detrimental. Hens from the AC group were not able to preserve the cortical area they had at the end of the pullet phase. The changes were drastic in humeri cortices at the end of lay with CC hens having greater cortical thickness and area than AC hens. The modulus of elasticity, in particular, for both the tibiae and the humeri was greater in CC hens than in AC hens whereas second moment area was greater in AC hens. This means the quality of the bone was better for CC hens despite AC hens having more bone quantity than CC hens. Studies involving human, rodents and hibernating mammals have shown contradicting results on persistence of bone benefits gained as a result of greater physical activity upon cessation of such activity (Kontulainen et al., 2001; Englund et al., 2009; Wojda et al., 2012). On the other hand, switching CC pullets to EN at the start of lay brought

about positive changes in the bones. The difference between humeri of EN and CC hens was observed for geometrical parameters but not for cortical density. Humeri of EN hens had greater outer dimensions and cortical area than CC hens but the cortical thickness was similar indicating the change in structure might be primarily due to medullary expansion by resorption from endosteal surface or periosteal apposition. Effect of loading on periosteal apposition in adult hens has not been reported and the general concept is that the structural bone formation ceases with the onset of lay (Whitehead, 2004). However recent observations, for example of keel bone tip (Regmi, unpublished data), suggest some structural bone formation might occur even after the onset of lay. Comparable to the adult hens in this study, expansion of the medullary cavity and periosteal apposition has been reported as a common adaptation to increased bone resorption in postmenopausal women (Ahlborg et al., 2003). In the case of the tibia, the difference was limited to increased volumetric density in EN hens, and that might have imparted greater whole bone (structural) and material stiffness compared to the tibia of CC hens. Increased total density, elastic modulus, and compressive strength index of proximal tibia metaphysis has been reported with exercise of moderate intensity after a period of disuse under rat hindlimb suspension model (Shirazi-Fard et al., 2014). The results of cortical density between EN and CC hens in our study are different from other studies conducted with laying hens in furnished cages and floor pens. Humeri cortical density was increased in furnished cages and floor pens compared to CC, but no difference was observed for tibiae (Leyendecker et al., 2005; Vits et al., 2005; Jendral et al., 2008; Shipov et al., 2010; Silversides et al., 2012). In a similar trial, Hester et al., (2013) could not detect the effect of installing metal perches in cages during pullet phase and/or laying phase in bone mineral content and density of tibiae and humeri measured by DEXA. However, an increase in volumetric bone mineral density of the tibia has been observed in adult female human

subjects with higher levels of physical activity (Uusi-Rasi et al., 2002). Measures of bone mineral density become more variable with age and confounding factors that affect calcium metabolism like diet, circulating estrogen concentrations (Hansen, 2002), and possibly the exercise regimen during growth could have resulted in non-uniformity between studies.

In conclusion, the results of the current study suggest that bone mass and density acquired during the pullet phase were only maintained during the laying cycle if the opportunities for movement was continued (AV). In contrast, limiting the movement during the laying phase (AC) induced bone loss and decreased mechanical stability of the bones. Providing moderate opportunities of movement during lay (EN) to pullets reared in CC may bring some improvement in mechanical properties of tibiae and humeri of laying hens. These results also indicate that the effect of load bearing activities on bone structure, density, and therefore the resistance to bending was different in tibiae and humeri. Future studies involving complete crossover housing designs will be required to explain cause and effect of changes in bone properties occurring at different points during the production cycle. A detailed study of the organic matrix of the bone is also warranted to get a more complete understanding for the changes in material and compositional properties of these bones.

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CHAPTER 4.

Influence of age and housing systems on properties of tibia and humerus of Lohmann white leghorns

INTRODUCTION

Bone properties of laying hens in different housing conditions have often been studied at a single time point, mostly at the end of the first production period (Leyendecker et al., 2005; Clark et al., 2008; Jendral et al., 2008). Such studies are insufficient to draw inferences on skeletal dynamics throughout the entire productive life of the bird because of the possible interaction between age and physical activity in a particular housing system. Previous work in our lab provided evidence that properties of tibia and humerus changes in response to housing environment and the difference was reported at 16 wk (Regmi et al., 2015a). In another study, we reported that bone mass and associated mechanical changes in tibia and humerus are only maintained if the level of physical activity is continued throughout production. We also observed in the same study that housing hens in enriched colony cages (moderate physical activity) after rearing them in conventional cages during pullet stage prevents bone loss to a certain extent (Regmi et al., 2015b). These results suggest a possible interaction between age and the housing conditions. Dual energy x-ray absorptiometry scans have been used to follow bone mineral density in White Leghorns throughout the production period (Schreiweis et al., 2004). Bone mineral density is an indicator of the condition of the mineral portion of the bone but does not provide a complete picture of mechanical integrity of the bone.

In the wake of consumer concerns regarding egg production systems and legislative changes around the country, egg producers in the U. S. are exploring newer housing systems for laying hens. However, there is a dearth of information regarding how body systems cope to the demands of production at different points throughout the production in these newer houses. Aviary systems with multi-level perches have been associated with incidence of old fractures ranging from 49 to 74% in a variety of extensive housing systems in farms within E. U. and

Canada (Freire et al., 2003; Nicol et al., 2006; Wilkins et al., 2004; Petrik et al., 2015). Almost 90% of the breaks sustained by laying hens are to the furculum and the keel (Gregory and Wilkins, 1996). The prevalence of keel injuries were slightly lower in enriched colony cages than in aviary systems, but still ranged from 33 to 62% (Rodenberg et al., 2008; Vits et al., 2005). Accumulation of keel bone fractures increases until around 50 wk (Petrik et al., 2015), which means that fracture repair is occurring in these hens at the same time they are in active production. The impact of this event on the overall integrity of the skeletal system has not been studied yet.

The aim of this study was to evaluate changes in tibia and humeri properties at different time points throughout the production in commercial housing systems – conventional cage, enriched colony cage, and aviary system. Biochemical markers were monitored throughout the productive life of the birds as an overall indicator of bone formation and bone resorption.

MATERIALS AND METHODS

The experimental procedures were approved by Institutional Animal Care and Use Committee of Michigan State University.

Birds, Management, and Sampling

Lohmann White hens were raised in a commercial setting. Pullets were housed in conventional pullet cages and an aviary system. The stocking density and space allowance were same as used by Regmi et al. (2015a) for the first flock. Briefly, 15 pullets/cage were kept in conventional cages with an area of 248 cm^2/bird. Aviary pullets were stocked at 218 birds per colony unit with space allocation of 160 cm^2/ bird from 0 to 9 wk, after which total cage space was increased to 249 cm^2/bird. AV pullets had floor access from 6 wk onwards, providing additional space of 100.13 cm^2 /bird. Feeding, lighting, and health management were same for both groups of pullets.

At 19 wk of age, pullets reared in conventional cages were continued in conventional layer cages or transferred to enriched colony cages. Aviary pullets were moved to multi-tier aviary housing system. The housing design and space allocation were the same as described by Zhao et al., (2015). In conventional cages, hens were housed at 6 hens/cage with a space allocation of 516 cm^2/bird. Hens in enriched colony cages were housed at 60 birds/colony unit and were provided a total space of 752 cm^2/bird whereas, 142 hens/colony unit were housed in the aviary system with a minimum space allocation of 1,253 cm^2/bird. Nutritional and health management were carried out according to the breeder's guidelines with hens having ad libitum access to water and commercially available feed. The hens were maintained on 16:8 light and dark cycle during the lay cycle.

Hens were randomly sampled from each housing system and were euthanized by cervical dislocation. Body weight was measured immediately after euthanasia and tibia and humerus from each bird were collected. Sixty hens/housing system at 18 wk and 72 wk, and 30 hens/housing system at 26 wk and 56 wk were collected for bone property analysis as well as serum concentrations of osteocalcin and hydroxylysyl pyridinoline. Serum samples to analyze bone marker were also collected at 4, 8, and 12 wk. Prior to analysis, bones with surrounding soft tissues were kept frozen. Osteocalcin and hydroxylysyl pyridinoline were quantified using ELISA and the procedure is mentioned in details by Regmi et al. (2015a).

Computed Tomography and Bone Ash

Quantitative computed tomography (QCT) was performed on the right tibia and humerus of the hens. Scan parameters were set at a tube voltage of 120 kV and current of 220 mAs and the final images had the voxel resolution of 195 μm. The images were then imported to MIMICS software and a threshold mask of 450 and 180 Hounsfield Units were selected for separating cortical tissues of tibia and humerus respectively. The threshold Hounsfield Units were chosen using the 'Profile line' feature of the software. The profile line, when drawn across a bone cross-section, generates a graph with a range of Hounsfield Units at different areas of the bone. The thresholds were then chosen based on the average Hounsfield Units across the cortical area. For the purpose of consistency, same threshold was used for all tibiae (and humeri) regardless of some individual variations. Average cortical density was measured at a 0.625 mm thick cross-sectional slice at each of proximal (one-fourth), middle, and distal (three-fourths) section along the length of the bone. Mineral content of the whole bone was approximated with analysis of ash content. Dry bone weight and ash percentage on dry bone basis of tibiae and humeri were analyzed as previously described (Regmi et al., 2015a).

Mechanical Testing

Mechanical testing was carried out in a pure bending fixture with uniform loading, described previously in Regmi et al. (2015). A thirty-millimeter section of tibia diaphysis and a 20 millimeter section of humerus diaphysis was used to analyze the failure properties. Briefly, anterior-posterior (AP) and medial-lateral (ML) outer dimensions of the bones were measured at the ends and center with digital calipers. Tibiae were loaded with the lateral surface in tension whereas humeri were loaded with the posterior surface in tension until failure to calculate mechanical properties. The measured and calculated mechanical parameters were second moment of area (I), maximum or failure bending moment (M_f), stiffness (K), failure stress (σ_f), and Young's modulus (E). Mathematical equations used to calculate mechanical and material properties were described in detail by Regmi et al. (2015a). In addition to the properties analyzed in Chapter 2, yield bending moment and energy require to failure were calculated in this study. Yield bending moment was determined as the point at which the response of bone to applied moment deviated from a linear and elastic response by 4% in moment. Cortical thickness along the anterior, posterior, lateral, and medial planes was measured at the fracture site. Outer dimensions and diaphyseal thicknesses were used to approximate the cortical cross-section as a hollow ellipse.

Statistical Analysis

Data were analyzed using the multivariate PROC MIXED analysis of SAS 9.3 (SAS Institute 2002, Cary, NC). Repeated measures statement with the model including fixed effect of housing system and age, the interaction between housing and age, and the residual error was used to analyze the data. Differences between means were tested using Fisher's least-square

difference with null hypothesis rejected at $P < 0.05$. Values were represented as least square means with their respective standard error for the mean.

RESULTS

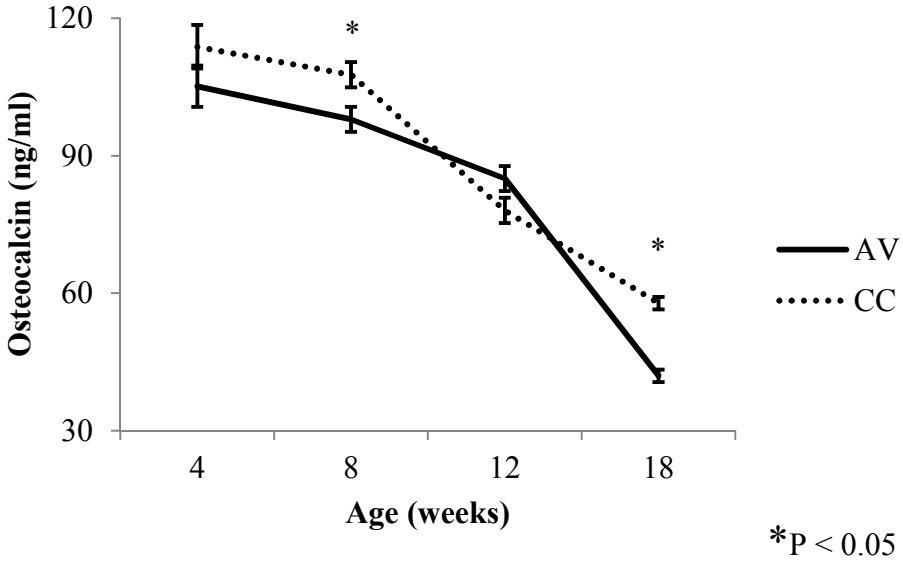
Body weight of hens from different housing systems was not different (AV – 1.58 ± 0.02 kg; CC – 1.58 ± 0.02 kg; EN – 1.55 ± 0.02 kg). The effect of age was observed with hens at 56 wk being heavier than 26 and 72 wk (26 wk – 1.54 ± 0.02 kg; 56 wk – 1.60 ± 0.02kg; 72 wk – 1.56 ± 0.01 kg; $P < 0.05$).

Serum Osteocalcin and Pyridinoline Concentrations

The results of serum bone marker analysis are presented in Figure 11 and 12. Mean serum osteocalcin (OC) concentration decreased progressively with age in all groups of hens. Osteocalcin concentration was greater in CC hens than AV hens at 8, 18, and 26 wks age ($P < 0.05$, Figure 11A and B). At other times throughout the production cycle, OC concentration was not different between the housing types. Serum pyridinoline (PYD) concentration, on the other hand, was greater in AV birds than CC birds during the pullet phase ($P < 0.05$, Figure 11B). During the laying phase, PYD concentration was greater in CC hens than AV hens at 26 wks but serum PYD increased progressively in AV hens thereafter ($P < 0.05$, Figure 12B). The concentration of PYD decreased in CC hens after 26 wks age until 56 wks and then plateaued.

Figure 11: (A) Serum osteocalcin and (B) pyridinoline concentration during pullet stage of Lohmann White hens housed in cage-free aviary rearing system (AV) and conventional pullet cages (CC).

A.



B.

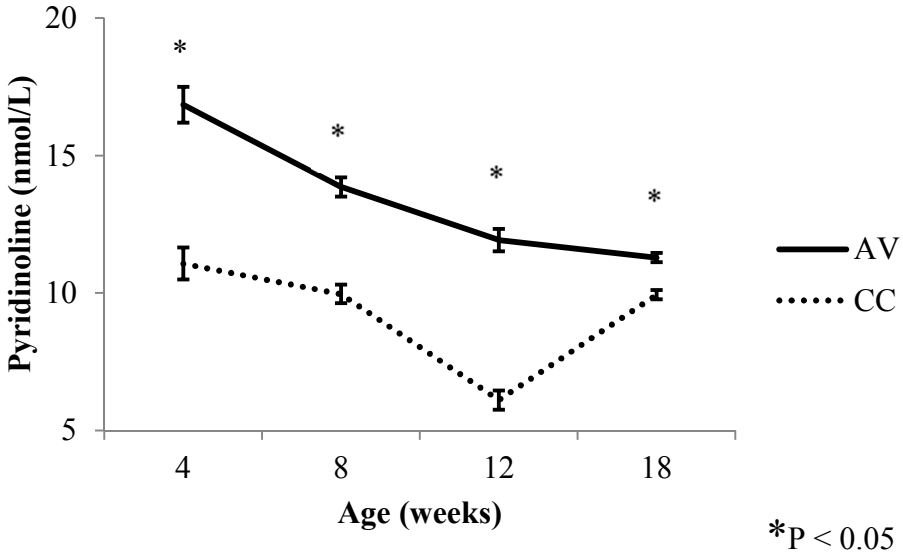
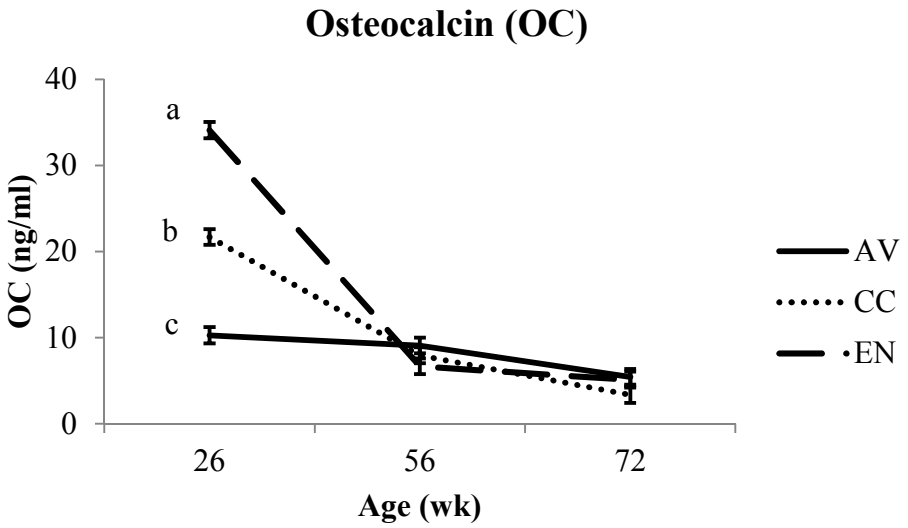
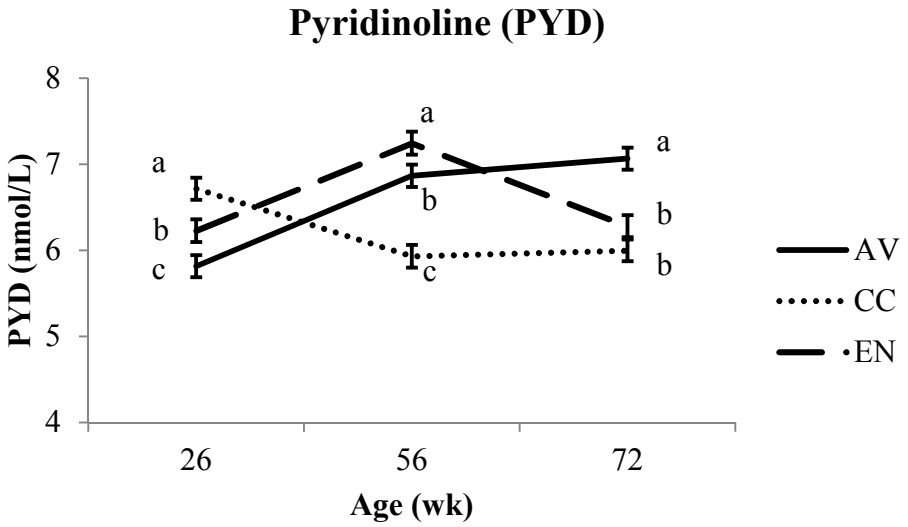


Figure 12: (A) Serum osteocalcin and (B) pyridinoline concentration during laying stage of Lohmann White hens housed in cage-free aviary (AV), enriched colony cages (EN), and conventional cages (CC).

A.



B.



Geometrical and Compositional Properties

Housing system and age influenced the geometrical measurements of tibiae and humeri of laying hens (Table 11 to 15). Hens housed in the aviary system (AV) had greater cortical cross-sectional area and cortical thickness of tibiae and humeri than hens housed in conventional cages (CC) at 18 wks age and the same effect was maintained until the birds were 72 wks old ($P < 0.05$, Table 11, 12, and 13). At 18 wks, tibiae outer dimensions were not different between the groups except that the antero-posterior diameter of distal diaphysis was 2% greater in CC hens than AV hens ($P < 0.05$, Table 11). During the laying phase, an age by housing interaction was observed for some of the outer dimension parameters. Average diameter of AV tibiae was 2.9% greater than CC tibiae ($P < 0.05$) at 56 wk whereas no differences were observed at 26 and 72 wks ($P < 0.05$, Table 14). Unlike tibiae, humeri outer dimensions were greater in AV hens compared to the CC hens at the end of the pullet phase and the difference was maintained until 72 wks age ($P < 0.05$, Table 11 and 12). Tibiae and humeri geometry responded differently to the overall effect of age. Cortical area and thickness of tibia decreased with age (Table 12 and 13). During the laying phase, 26 wk old hens had thickest cortices and 72 wk old hens had the thinnest whereas the 56 wk old hens were intermediate ($P < 0.05$). Humeri cortical area, on the other hand, did not change during the laying phase. Geometrical parameters for which housing by age interaction was significant, linear and quadratic effects of age within each housing system were estimated (Table 15). Negative linear effect of age was observed for humeri diameter of AV hens whereas negative quadratic effect of age was observed for average outer diameter of tibiae and humeri of CC hens. A negative linear effect of age was also observed for tibia cortical area of CC hens. Within housing systems, tibia cortical area of AV hens was not different at 26 and 56 wks but decreased at 72 wks ($P < 0.05$) whereas in CC hens, tibia cortical area decreased

from 26 to 56 wks but did not change therafter ($P < 0.05$, Table 14). A similar response was observed for average diameter within the housing system for both tibiae and humeri.

Housing system influenced the cortical measurements of tibiae and humeri between hens housed in enriched colony cages (EN) and CC. The difference in tibia cortical area betweeen EN and CC hens was only apparent at 72 weeks when cortical area was 7.4% greater in EN hens than CC hens ($P < 0.05$, Table 12). Overall housing effects were also observed for cortical thicknesses along the lateral and posterior surface of the tibiae with EN hens having greater thicknesses than CC hens ($P < 0.05$). Similarly, humeri cortical area and thickness were also greater in EN hens than CC hens ($P < 0.05$, Table 13) whereas the outer dimensions were not different.

In addition to the structural differences, AV hens had denser tibia cortices than CC hens ($P < 0.05$, Table 16). The difference in tibiae cortical density between AV and CC hens was limited to the distal section whereas no differences were observed between cortical density of EN and CC hens. Average volumetric density of humeri cortical bone was also greater in AV hens than CC hens ($P < 0.05$). Mid-diaphyseal humeri cortical density was greater in AV hens than CC hens at 26 and 56 wks but not at 72 wks of age ($P < 0.05$). Between EN and CC hens, humeri average cortical density was greater in EN hens compared to CC hens at proximal and mid diaphysis but not at the distal diaphysis ($P < 0.05$). Tibiae and humeri dry bone weight were greater in AV and EN hens than CC hens ($P < 0.05$, Figure 13A and B). Tibiae dry bone weight in AV hens peaked at 26 wks whereas humeri dry bone weight peaked at 56 wks ($P < 0.05$, Figure 13A). Dry bone weight of both tibiae and humeri kept increasing with age in CC hens ($P < 0.05$, Figure 13A and B). Tibia ash percentage was not different between housing systems (Fig 14A). Humeri ash percentage was greater in AV hens than CC hens ($P < 0.05$, Figure 14A) but

no difference was observed between EN and CC hens. Age related changes in ash percentage were of opposite nature in tibiae and humeri. Tibiae ash percentage was not different between 18, 26, and 56 wks age but increased at 72 wks ($P < 0.05$, Figure 14B). Humeri ash percentage, on the other hand, peaked at 18 wks and then declined with lowest value at 56 wks age ($P < 0.05$, Figure 14B).

Table 11: Geometrical properties of tibiae and humeri of 18 wk old Lohmann White pullets housed in cage-free aviary and conventional cages.

	Bone type					
Dependent Variable	Humeri			Tibia		
	[1]AV	CC	P value	AV	CC	P value
Geometrical properties						
Area (mm^2)	12.39 ± 0.13	8.78 ± 0.13	< 0.01	15.14 ± 0.17	12.76 ± 0.18	< 0.01
Medial thickness (mm)	0.71 ± 0.01	0.47 ± 0.01	< 0.01	1.01 ± 0.02	0.81 ± 0.02	< 0.01
Lateral thickness (mm)	0.69 ± 0.01	0.47 ± 0.01	< 0.01	0.96 ± 0.02	0.80 ± 0.02	< 0.01
Anterior thickness (mm)	0.60 ± 0.01	0.46 ± 0.01	< 0.01	0.86 ± 0.01	0.68 ± 0.02	< 0.01
Posterior thickness (mm)	0.65 ± 0.01	0.50 ± 0.01	< 0.01	0.87 ± 0.01	0.71 ± 0.01	< 0.01
Average M-L thickness (mm)	0.70 ± 0.01	0.47 ± 0.01	< 0.01	0.99 ± 0.01	0.81 ± 0.01	< 0.01
Average A-P thickness (mm)	0.62 ± 0.01	0.48 ± 0.01	< 0.01	0.86 ± 0.01	0.70 ± 0.01	< 0.01
Average thickness (mm)	0.66 ± 0.01	0.48 ± 0.01	< 0.01	0.92 ± 0.01	0.75 ± 0.01	< 0.01
Proximal M-L diameter (mm)	7.74 ± 0.04	7.33 ± 0.04	< 0.01	6.88 ± 0.04	6.94 ± 0.04	0.28
Mid M-L diameter (mm)	6.82 ± 0.03	6.35 ± 0.03	< 0.01	6.34 ± 0.03	6.34 ± 0.03	0.93
Distal M-L diameter (mm)	7.72 ± 0.04	7.33 ± 0.05	< 0.01	6.92 ± 0.04	6.82 ± 0.04	0.09
Prox A-P diameter (mm)	6.19 ± 0.03	5.82 ± 0.03	< 0.01	6.10 ± 0.03	6.08 ± 0.03	0.75
Mid A-P diameter (mm)	5.71 ± 0.03	5.48 ± 0.03	< 0.01	5.47 ± 0.03	5.48 ± 0.03	0.74
Distal A-P diameter (mm)	5.85 ± 0.03	5.74 ± 0.04	0.03	5.45 ± 0.03	5.56 ± 0.03	0.01
Average M-L diameter (mm)	7.43 ± 0.03	7.00 ± 0.03	< 0.01	6.71 ± 0.03	6.68 ± 0.04	0.56
Average A-P diameter (mm)	5.92 ± 0.03	5.68 ± 0.03	< 0.01	5.66 ± 0.03	5.71 ± 0.03	0.25
Average Diameter (mm)	6.67 ± 0.03	6.34 ± 0.03	< 0.01	6.19 ± 0.03	6.20 ± 0.03	0.83

[1]AV (Cage-free aviary system); CC (Conventional cage)

Table 12: Geometrical properties of humeri of Lohmann White hens housed in cage-free aviary, enriched colony cages, and conventional cages at different ages.

Dependent variable and bone type	Housing				Age (weeks)			
	[1]AV	CC	EN	P Value	26	56	72	P value
Humeri	Mean ± SD	Mean ± SD	Mean ± SD		Mean ± SD	Mean ± SD	Mean ± SD	
Area (mm^2)	12.07 ± 0.11[a]	9.53 ± 0.11[c]	10.25 ± 0.11[b]	<0.01	10.53 ± 0.12	10.66 ± 0.12	10.66 ± 0.09	0.67
Medial thickness (mm)	0.63 ± 0.01[a]	0.51 ± 0.01[c]	0.57 ± 0.01[b]	<0.01	0.57 ± 0.01	0.57 ± 0.01	0.57 ± 0.01	0.73
Lateral thickness (mm)	0.66 ± 0.01[a]	0.56 ± 0.01[c]	0.60 ± 0.01[b]	<0.01	0.60 ± 0.01	0.61 ± 0.01	0.61 ± 0.01	0.80
Anterior thickness (mm)	0.62 ± 0.01[a]	0.52 ± 0.01[c]	0.55 ± 0.01[b]	<0.01	0.55 ± 0.01[b]	0.57 ± 0.01[a]	0.57 ± 0.01[a]	0.04
Posterior thickness (mm)	0.60 ± 0.01[a]	0.52 ± 0.01[c]	0.55 ± 0.01[b]	<0.01	0.55 ± 0.01	0.56 ± 0.01	0.56 ± 0.01	0.53
[2]M-L thickness (mm)	0.64 ± 0.01[a]	0.54 ± 0.01[c]	0.59 ± 0.01[b]	<0.01	0.58 ± 0.01	0.59 ± 0.01	0.59 ± 0.01	0.75
A-P thickness (mm)	0.61 ± 0.01[a]	0.52 ± 0.01[c]	0.55 ± 0.01[b]	<0.01	0.55 ± 0.01	0.56 ± 0.01	0.57 ± 0.01	0.10
Average thickness (mm)	0.63 ± 0.01[a]	0.53 ± 0.01[c]	0.57 ± 0.01[b]	<0.01	0.57 ± 0.01	0.58 ± 0.01	0.58 ± 0.01	0.29
Prox M-L diameter (mm)	7.86 ± 0.03[a]	7.23 ± 0.03[b]	7.27 ± 0.03[b]	<0.01	7.55 ± 0.04[a]	7.41 ± 0.04[b]	7.40 ± 0.03[b]	<0.01

Table 12 (cont'd).

Mid M-L diameter (mm)	6.96 ± 0.03[a]	6.27 ± 0.03[b]	6.32 ± 0.03[b]	<0.01	6.51 ± 0.03	6.51 ± 0.03	6.53 ± 0.02	0.71
Distal M-L diameter (mm)	7.55 ± 0.04[a]	6.93 ± 0.04[b]	6.97 ± 0.04[b]	<0.01	7.29 ± 0.05[a]	7.14 ± 0.05[b]	7.02 ± 0.03[c]	<0.01
Prox A-P diameter (mm)	6.21 ± 0.02[a]	5.80 ± 0.02[b]	5.82 ± 0.02[b]	<0.01	5.96 ± 0.03	5.96 ± 0.03	5.91 ± 0.02	0.15
Mid A-P diameter (mm)	5.99 ± 0.03[a]	5.62 ± 0.03[b]	5.67 ± 0.03[b]	<0.01	5.73 ± 0.03	5.75 ± 0.03	5.80 ± 0.02	0.17
Distal A-P diameter (mm)	6.22 ± 0.04[a]	5.92 ± 0.04[b]	5.93 ± 0.04[b]	<0.01	6.01 ± 0.04	6.05 ± 0.04	6.00 ± 0.03	0.56
Average M-L diameter (mm)	7.46 ± 0.03[a]	6.80 ± 0.03[b]	6.85 ± 0.03[b]	<0.01	7.11 ± 0.03[a]	7.02 ± 0.03[b]	6.98 ± 0.02[b]	<0.01
Average A-P diameter (mm)	6.14 ± 0.03[a]	5.78 ± 0.03[b]	5.81 ± 0.03[b]	<0.01	5.90 ± 0.03	5.92 ± 0.03	5.90 ± 0.02	0.87
Average Diameter (mm)	6.80 ± 0.02[a]	6.29 ± 0.02[b]	6.33 ± 0.02[b]	<0.01	6.50 ± 0.02	6.47 ± 0.02	6.44 ± 0.02	0.13

[abc]Means within the same row lacking a common superscript differ significantly (P<0.05).

[1]AV (Cage-free aviary system); EN (Enriched colony cage); CC (Conventional cage)

[2]M-L (Medio-lateral); A-P (Antero-posterior)

Table 13: Geometrical properties of tibiae of Lohmann White hens housed in cage-free aviary, enriched colony cages, and conventional cages at different ages.

Dependent variable	Housing				Age (weeks)			
	[1]AV	CC	EN	P Value	26	56	72	P value
Humeri	Mean ± SD	Mean ± SD	Mean ± SD		Mean ± SD	Mean ± SD	Mean ± SD	
Area (mm^2)	12.07 ± 0.11[a]	9.53 ± 0.11[c]	10.25 ± 0.11[b]	<0.01	10.53 ± 0.12	10.66 ± 0.12	10.66 ± 0.09	0.67
Medial thickness (mm)	0.63 ± 0.01[a]	0.51 ± 0.01[c]	0.57 ± 0.01[b]	<0.01	0.57 ± 0.01	0.57 ± 0.01	0.57 ± 0.01	0.73
Lateral thickness (mm)	0.66 ± 0.01[a]	0.56 ± 0.01[c]	0.60 ± 0.01[b]	<0.01	0.60 ± 0.01	0.61 ± 0.01	0.61 ± 0.01	0.80
Anterior thickness (mm)	0.62 ± 0.01[a]	0.52 ± 0.01[c]	0.55 ± 0.01[b]	<0.01	0.55 ± 0.01[b]	0.57 ± 0.01[a]	0.57 ± 0.01[a]	0.04
Posterior thickness (mm)	0.60 ± 0.01[a]	0.52 ± 0.01[c]	0.55 ± 0.01[b]	<0.01	0.55 ± 0.01	0.56 ± 0.01	0.56 ± 0.01	0.53
[2]M-L thickness (mm)	0.64 ± 0.01[a]	0.54 ± 0.01[c]	0.59 ± 0.01[b]	<0.01	0.58 ± 0.01	0.59 ± 0.01	0.59 ± 0.01	0.75
A-P thickness (mm)	0.61 ± 0.01[a]	0.52 ± 0.01[c]	0.55 ± 0.01[b]	<0.01	0.55 ± 0.01	0.56 ± 0.01	0.57 ± 0.01	0.10
Average thickness (mm)	0.63 ± 0.01[a]	0.53 ± 0.01[c]	0.57 ± 0.01[b]	<0.01	0.57 ± 0.01	0.58 ± 0.01	0.58 ± 0.01	0.29
Prox M-L diameter (mm)	7.86 ± 0.03[a]	7.23 ± 0.03[b]	7.27 ± 0.03[b]	<0.01	7.55 ± 0.04[a]	7.41 ± 0.04[b]	7.40 ± 0.03[b]	<0.01

Table 13 (cont'd).

Mid M-L diameter (mm)	6.96 ± 0.03[a]	6.27 ± 0.03[b]	6.32 ± 0.03[b]	<0.01	6.51 ± 0.03	6.51 ± 0.03	6.53 ± 0.02	0.71
Distal M-L diameter (mm)	7.55 ± 0.04[a]	6.93 ± 0.04[b]	6.97 ± 0.04[b]	<0.01	7.29 ± 0.05[a]	7.14 ± 0.05[b]	7.02 ± 0.03[c]	<0.01
Prox A-P diameter (mm)	6.21 ± 0.02[a]	5.80 ± 0.02[b]	5.82 ± 0.02[b]	<0.01	5.96 ± 0.03	5.96 ± 0.03	5.91 ± 0.02	0.15
Mid A-P diameter (mm)	5.99 ± 0.03[a]	5.62 ± 0.03[b]	5.67 ± 0.03[b]	<0.01	5.73 ± 0.03	5.75 ± 0.03	5.80 ± 0.02	0.17
Distal A-P diameter (mm)	6.22 ± 0.04[a]	5.92 ± 0.04[b]	5.93 ± 0.04[b]	<0.01	6.01 ± 0.04	6.05 ± 0.04	6.00 ± 0.03	0.56
Average M-L diameter (mm)	7.46 ± 0.03[a]	6.80 ± 0.03[b]	6.85 ± 0.03[b]	<0.01	7.11 ± 0.03[a]	7.02 ± 0.03[b]	6.98 ± 0.02[b]	<0.01
Average A-P diameter (mm)	6.14 ± 0.03[a]	5.78 ± 0.03[b]	5.81 ± 0.03[b]	<0.01	5.90 ± 0.03	5.92 ± 0.03	5.90 ± 0.02	0.87
Average Diameter (mm)	6.80 ± 0.02[a]	6.29 ± 0.02[b]	6.33 ± 0.02[b]	<0.01	6.50 ± 0.02	6.47 ± 0.02	6.44 ± 0.02	0.13

[abc]Means within the same row lacking a common superscript differ significantly (P<0.05).

[1]AV (Cage-free aviary system); EN (Enriched colony cage); CC (Conventional cage)

[2]M-L (Medio-lateral); A-P (Antero-posterior)

Table 14: Age by housing type interaction for tibiae properties of Lohmann White hens housed in cage-free aviary, enriched colony cages, and conventional cages at different ages.

Dependent variable and housing type		Age (weeks)		
Tibiae		26	56	72
Area (mm^2)		Mean ± SD	Mean ± SD	Mean ± SD
	[1]AV	$14.00 \pm 0.26^{a\ xy}$	$14.29 \pm 0.26^{a\ x}$	$13.64 \pm 0.18^{a\ y}$
	CC	$12.44 \pm 0.26^{b\ x}$	$11.47 \pm 0.26^{b\ y}$	$11.30 \pm 0.18^{c\ y}$
	EN	12.25 ± 0.27^{b}	11.93 ± 0.29^{b}	12.14 ± 0.18^{b}
	P value	0.04		
Average antero-posterior diameter (mm)				
	AV	5.72 ± 0.04	5.80 ± 0.04^{a}	5.71 ± 0.03
	CC	5.82 ± 0.04^{x}	$5.66 \pm 0.04^{b\ y}$	5.75 ± 0.03^{xy}
	EN	5.71 ± 0.04	5.70 ± 0.05^{ab}	5.77 ± 0.03
	P value	0.04		
Average Diameter (mm)				
	AV	6.25 ± 0.04^{x}	$6.31 \pm 0.04^{a\ x}$	6.21 ± 0.03^{y}
	CC	6.26 ± 0.04^{x}	$6.13 \pm 0.04^{b\ y}$	6.22 ± 0.03^{xy}
	EN	6.16 ± 0.04	6.16 ± 0.05^{b}	6.22 ± 0.03
	P value	0.03		
Humeri				
Average antero-posterior diameter (mm)				
	AV	$6.18 \pm 0.05^{a\ x}$	$6.18 \pm 0.05^{a\ x}$	$6.05 \pm 0.03^{a\ y}$
	CC	5.79 ± 0.05^{b}	5.75 ± 0.05^{b}	5.81 ± 0.04^{b}
	EN	5.74 ± 0.05^{b}	5.83 ± 0.05^{b}	5.85 ± 0.04^{b}
	P value	0.04		

Table 14 (cont'd).

Average Diameter (mm)				
	AV	$6.84 \pm 0.04^{a\,x}$	$6.83 \pm 0.04^{a\,x}$	$6.72 \pm 0.03^{a\,y}$
	CC	$6.35 \pm 0.04^{b\,x}$	$6.22 \pm 0.04^{c\,y}$	$6.30 \pm 0.03^{b\,y}$
	EN	6.32 ± 0.04^{b}	6.36 ± 0.04^{b}	6.31 ± 0.03^{b}
	P value	0.05		

[ab]Means within the same column lacking a common superscript differ significantly ($P<0.05$).

[xy]Means within the same row lacking a common superscript differ significantly ($P<0.05$).

[1]AV (Cage-free aviary system); EN (Enriched colony cage); CC (Conventional cage)

Table 15: Linear and quadratic effect of age on housing system for tibiae and humeri properties of Lohmann White hens housed in cage-free aviary, enriched colony cages, and conventional cages.

Bone type and dependent variable		[1]Q_L	Q_Q	SEM (Q_L)	SEM (Q_Q)	P value (Q_L)	P value (Q_Q)
Tibia							
Young's Modulus (GPa)							
	[2]AV	2.27	-2.32	0.33	0.63	<0.01	<0.01
	CC	2.33	0.39	0.32	0.62	<0.01	0.53
	EN	1.91	0.34	0.33	0.69	<0.01	0.62
Second moment of area (mm^4)							
	AV	-2.71	8.38	1.79	3.44	0.13	0.02
	CC	-4.89	-6.64	1.78	3.39	0.01	0.05
	EN	0.92	-3.24	1.81	3.77	0.61	0.39
Area (mm^2)							
	AV	-0.36	0.95	0.32	0.61	0.26	0.12
	CC	-1.14	-0.81	0.32	0.60	<0.01	0.18
	EN	-0.11	-0.53	0.32	0.67	0.72	0.43
Average antero-posterior diameter (mm)							
	AV	0.005	0.16	0.05	0.10	0.93	0.13
	CC	-0.07	-0.25	0.05	0.10	0.22	0.01
	EN	0.05	-0.07	0.05	0.11	0.31	0.53
Average Diameter (mm)							
	AV	-0.04	0.17	0.05	0.10	0.45	0.09
	CC	-0.04	-0.22	0.05	0.10	0.38	0.03
	EN	0.07	-0.05	0.05	0.11	0.20	0.62
Humeri							
Young's Modulus (GPa)							
	AV	1.77	-1.59	0.32	0.6	<0.01	0.01
	CC	1.45	0.83	0.32	0.62	<0.01	0.18
	EN	1.38	-0.24	0.32	0.61	<0.01	0.70

Table 15 (cont'd).

Average antero-posterior diameter (mm)							
	AV	-0.13	0.13	0.06	0.12	0.04	0.26
	CC	0.02	-0.10	0.06	0.12	0.76	0.4
	EN	0.10	0.07	0.06	0.12	0.09	0.54
Average Diameter (mm)							
	AV	-0.13	0.11	0.05	0.10	0.01	0.28
	CC	-0.05	-0.21	0.05	0.10	0.34	0.04
	EN	0.004	0.08	0.05	0.10	0.94	0.42

[1]Q_L (Estimate of linear effect); Q_Q (Estimate of quadratic effect)

[2]AV (Cage-free aviary system); EN (Enriched colony cage); CC (Conventional cage)

Table 16: Age by housing type interaction for average volumetric density (mg/cm^3) of tibiae and humeri of Lohmann White hens housed in cage-free aviary, enriched colony cages, and conventional cages.

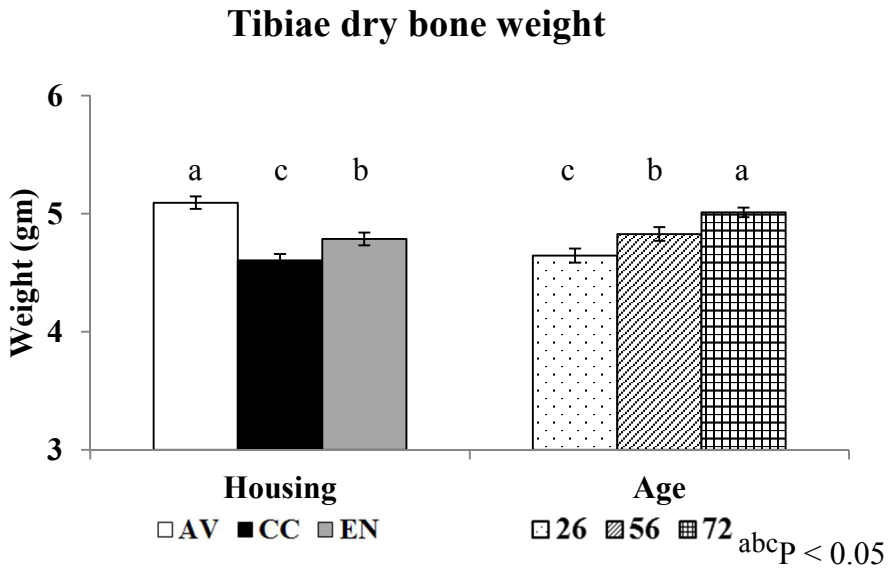
Bone type and section variable	Age and housing type									P value
	26 weeks			56 weeks			72 weeks			
Humeri	[1]AV	CC	EN	AV	CC	EN	AV	CC	EN	
Proximal	586.15 ± 16.61^a	462.61 ± 14.80^b	493.68 ± 15.76^b	599.14 ± 15.76^a	420.68 ± 16.31^b	446.98 ± 16.61^b	496.68 ± 12.59^a	448.06 ± 11.54^b	480.11 ± 12.33^{ab}	<0.01
Middle	795.02 ± 24.46^a	629.48 ± 21.87^b	666.31 ± 24.03^b	772.90 ± 24.03^a	534.65 ± 24.46^b	563.90 ± 24.46^b	624.24 ± 17.45	577.70 ± 16.99	624.28 ± 17.45	<0.01
Distal	843.79 ± 23.44^a	635.13 ± 22.70^b	682.05 ± 23.84^b	842.64 ± 23.84^a	588.53 ± 23.84^b	583.16 ± 24.71^b	674.47 ± 17.31^a	609.11 ± 17.16^b	620.94 ± 16.86^b	<0.01
Tibia										
Proximal	1323.26 ± 26.28	1287.56 ± 26.28	1255.77 ± 26.28	1330.6 ± 26.28	1201.74 ± 26.28	1178.06 ± 26.28	1292.71 ± 18.74	1215.38 ± 18.59	1252.06 ± 18.28	0.09
Middle	1511.80 ± 33.40	1429.97 ± 32.84	1383.47 ± 32.84	1507.42 ± 33.4	1299.42 ± 32.84	1270.76 ± 32.84	1463.31 ± 23.42	1301.24 ± 23.42	1338.50 ± 23.03	0.13
Distal	1453.65 ± 33.08^a	1360.21 ± 33.08^b	1319.36 ± 33.08^b	1463.84 ± 33.08^a	1238.61 ± 33.64^b	1219.12 ± 33.08^b	1401.54 ± 23.59^a	1252.5 ± 23.39^b	1309.06 ± 23.01^b	0.05

[ab]Means within the same row lacking a common superscript differ significantly for each age group (P<0.05).

[1]AV (Cage-free aviary system); EN (Enriched colony cage); CC (Conventional cage)

Figure 13: (A) Tibia and (B) humeri dry bone weight of Lohmann White hens housed in cage-free aviary, enriched colony cages, and conventional cages at different ages (weeks).

A.



B.

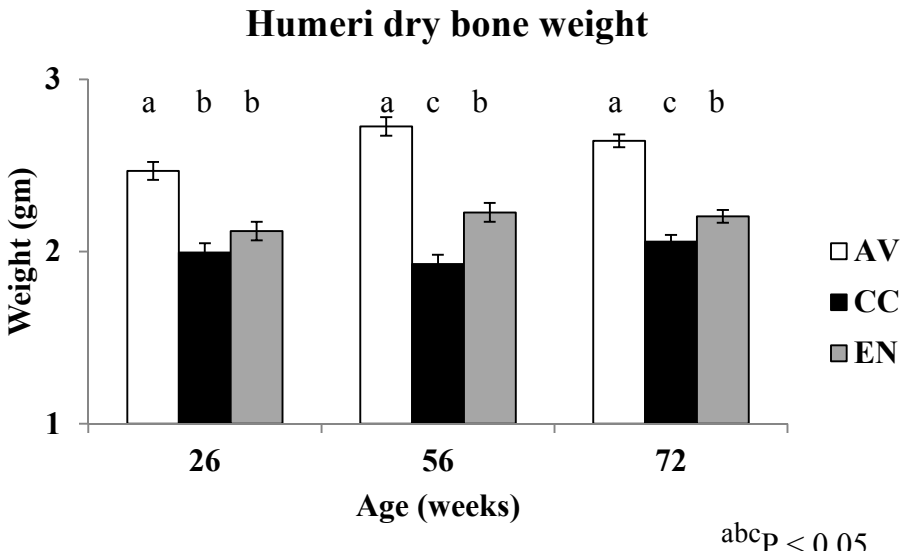
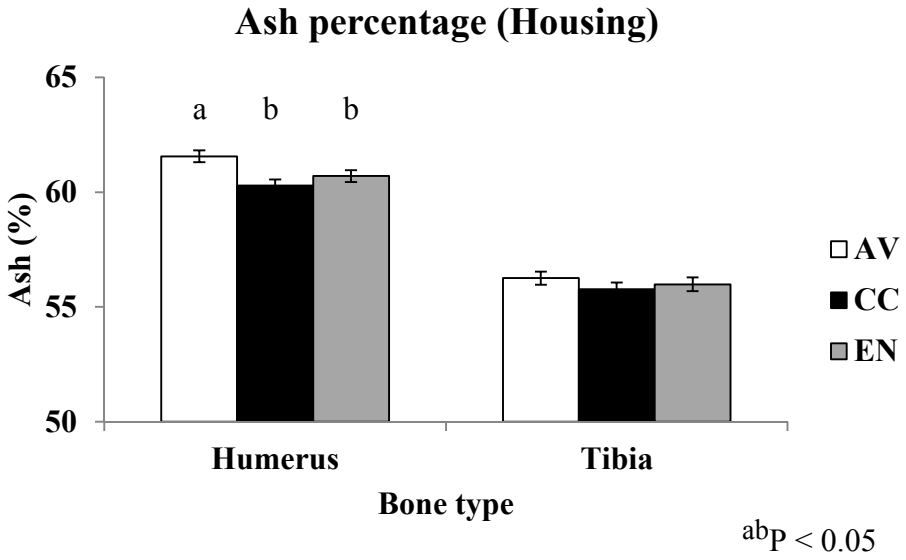


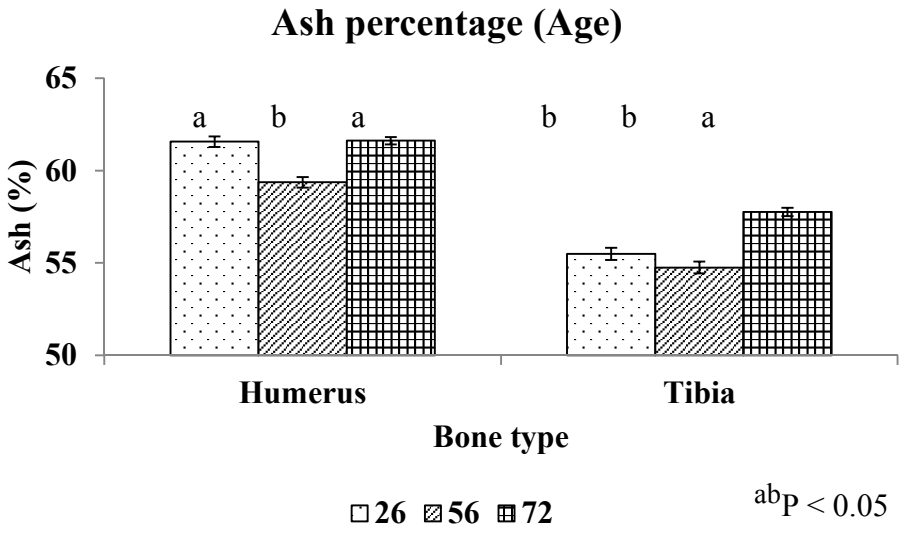
Figure 14: (A) Humerus and tibia ash percentage of White Leghorn hens housed in cage-free aviary, enriched colony cages, and conventional cages; (B) Humerus and tibia ash percentage of White Leghorn hens at different ages during the laying period.

A.



$^{ab}P < 0.05$

B.



$^{ab}P < 0.05$

Mechanical Properties

Tibiae and humeri mechanical properties of pullets are presented in Table 17 and that of layers are presented in Tables 18 to 20. Whole bone mechanical properties like failure moment, stiffness, yield bending moment, and energy to failure was greater for tibiae and humeri of AV hens than CC hens ($P < 0.05$, Tables 17, 18, and 19). Tibiae failure rotation was greater in AV hens than CC hens at 18 wks age ($P < 0.05$) but was not different at 26, 56, and 72 wks age. Humeri failure rotation was not different between AV and CC hens. Tibiae material strength indicated by failure stress was not different between the housing systems whereas humeri material strength was higher in AV hens compared to CC hens only at 18 wks ($P < 0.05$). Tibiae and humeri of AV hens were more resistant to bending than CC hens as indicated by greater second moment area ($P < 0.05$). Housing and age interaction was observed for tibiae second moment of area (Table 20). Tibiae of AV hens had greater second moment of area at 26 and 56 wks compared to 72 wks whereas tibiae of CC hens had greater second moment of area at 26 wks compared to 56 and 72 wks ($P < 0.05$). Interaction was also observed for the Young's modulus of elasticity indicative of material stiffness. Elastic modulus of both tibiae and humeri was greater in CC hens than AV hens at 18 and 56 wks but not different at 26 and 72 wks ($P < 0.05$). Within housing system, quadratic effect of age was observed for tibiae and humeri elastic modulus of AV hens whereas linear response was observed in CC hens (Table 15). Quadratic response of age was also observed for tibiae second moment of area. Tibiae and humeri elastic modulus for AV hens at 26 and 56 wks were smaller than at 72 wks age whereas for CC hens it kept increasing with age ($P < 0.05$, Table 20).

Differences in mechanical properties of tibia between EN and CC hens were limited to stiffness and second moment of area. Tibia stiffness was greater (3.5%) in EN hens than CC hens

while the decreased (6%) second moment of area in tibia of CC hens compared to EN hens was only observed at 72 wks ($P < 0.05$, Table 18). Humeri of EN hens had greater failure moment (12%) and rotation (5%), stiffness (6.7%), yield bending moment (11%), energy to failure (19%) and second moment of area (6.7%) than CC hens ($P < 0.05$, Table 19).

Stiffness, yield bending moment, and elastic modulus of tibiae and humeri increased with age whereas energy to failure and failure rotation decreased with age ($P < 0.05$, Table 18 and 19). Humeri elastic modulus and stiffness increased progressively at 26, 56 and 72 wks age whereas tibiae elastic modulus and stiffness were only greater at 72 wks compared to 26 and 56 wks age ($P < 0.05$). Yield bending moment for both bones was greater at 72 wks compared to 26 and 56 wks age ($P < 0.05$). Humeri failure rotation and energy to failure decreased progressively with age whereas for tibiae those properties were similar at 56 and 72 wks, which were smaller than at 26 wks age ($P < 0.05$).

Table 17: Mechanical properties of tibiae and humeri of 18 wk Lohmann White pullets housed in cage-free aviary and conventional cages.

	Bone type					
Dependent Variable	Humeri			Tibia		
	[1]AV	CC	P value	AV	CC	P value
Mechanical properties	Mean ± SD	Mean ± SD		Mean ± SD	Mean ± SD	
Failure Moment (Nm)	3.69 ± 0.04	2.60 ± 0.04	< 0.01	5.45 ± 0.06	4.87 ± 0.06	< 0.01
Failure Rotation (degree)	6.91 ± 0.12	6.69 ± 0.12	0.20	10.15 ± 0.15	9.44 ± 0.15	0.01
Energy	555.30 ± 13.47	385.19 ± 14.07	< 0.01	1286.60 ± 24.69	1061.62 ± 25.37	< 0.01
Yield Torque	3.18 ± 0.04	2.22 ± 0.04	< 0.01	4.33 ± 0.04	3.86 ± 0.04	< 0.01
Stiffness (Nm/degree)	0.71 ± 0.01	0.54 ± 0.01	< 0.01	0.89 ± 0.01	0.83 ± 0.01	< 0.01
Failure Stress (MPa)	252.94 ± 3.79	240.42 ± 3.96	0.02	332.34 ± 4.91	326.75 ± 5.04	0.43
Young's Modulus (GPa)	9.08 ± 0.11	9.75 ± 0.11	< 0.01	12.07 ± 0.12	12.79 ± 0.13	< 0.01
Second moment of area (mm^4)	45.4 ± 0.63	31.72 ± 0.66	< 0.01	63.45 ± 1.07	56.12 ± 1.10	< 0.01

[1]AV (Cage-free aviary system); CC (Conventional cage)

Table 18: Mechanical properties of tibiae of Lohmann White hens housed in cage-free aviary, enriched colony cages, and conventional cages at different ages.

Dependent variable	Housing				Age (weeks)			
	[1]AV	CC	EN	P Value	26	56	72	P Value
Mechanical properties	Mean ± SD	Mean ± SD	Mean ± SD		Mean ± SD	Mean ± SD	Mean ± SD	
Failure Moment (Nm)	5.55 ± 0.07[a]	4.84 ± 0.07[b]	4.96 ± 0.07[b]	<0.01	5.18 ± 0.07	5.00 ± 0.08	5.17 ± 0.05	0.13
Failure Rotation (degree)	7.84 ± 0.16	7.94 ± 0.16	7.82 ± 0.17	0.85	8.84 ± 0.17[a]	7.58 ± 0.18[b]	7.18 ± 0.12[b]	<0.01
Energy	971.36 ± 29.68[a]	865.2 ± 29.37[b]	864.12 ± 31.43[b]	0.02	1038.1 ± 32.64[a]	840.37 ± 33.89[b]	822.21 ± 22.74[b]	<0.01
Yield Torque	4.75 ± 0.05[a]	4.08 ± 0.05[b]	4.18 ± 0.05[b]	<0.01	4.27 ± 0.05[b]	4.26 ± 0.05[b]	4.48 ± 0.04[a]	<0.01
Stiffness (Nm/degree)	0.98 ± 0.01[a]	0.86 ± 0.01[c]	0.89 ± 0.01[b]	<0.01	0.87 ± 0.01[b]	0.90 ± 0.01[b]	0.97 ± 0.01[a]	<0.01
Failure Stress (MPa)	345.02 ± 3.98	348.57 ± 3.94	358.12 ± 4.22	0.07	352.00 ± 4.38[ab]	341.73 ± 4.55[b]	357.99 ± 3.05[a]	0.01
Young's Modulus (GPa)	13.89 ± 0.14[b]	14.34 ± 0.14[a]	14.63 ± 0.15[a]	<0.01	13.29 ± 0.15[c]	14.11 ± 0.16[b]	15.46 ± 0.11 [a]	<0.01
Second moment of area (mm^4)	61.39 ± 0.77[a]	52.24 ± 0.76[b]	52.98 ± 0.82[b]	<0.01	56.73 ± 0.85[a]	55.37 ± 0.88[ab]	54.51 ± 0.59[b]	0.10

[abc]Means within the same row lacking a common superscript differ significantly (P<0.05).

[1]AV (Cage-free aviary system); EN (Enriched colony cage); CC (Conventional cage)

Table 19: Mechanical properties of humeri of Lohmann White hens housed in cage-free aviary, enriched colony cages, and conventional cages at different ages.

Dependent variable	Housing				Age (weeks)			
	[1]AV	CC	EN	P Value	26	56	72	P value
Mechanical properties	Mean ± SD	Mean ± SD	Mean ± SD		Mean ± SD	Mean ± SD	Mean ± SD	
Failure Moment (Nm)	4.09 ± 0.04[a]	2.94 ± 0.04[c]	3.29 ± 0.04[b]	<0.01	3.42 ± 0.04	3.40 ± 0.04	3.50 ± 0.03	0.12
Failure Rotation (degree)	6.26 ± 0.10[b]	6.31 ± 0.10[b]	6.65 ± 0.10[a]	<0.01	7.49 ± 0.11[a]	6.19 ± 0.11[b]	5.55 ± 0.08[c]	<0.01
Energy	531.73 ± 11.83[a]	396.51 ± 12.10[c]	471.18 ± 11.89[b]	<0.01	563.67 ± 13.14[a]	438.93 ± 13.00[b]	396.82 ± 9.27[c]	<0.01
Yield Torque	3.70 ± 0.04[a]	2.57 ± 0.04[c]	2.86 ± 0.04[b]	<0.01	2.95 ± 0.04[b]	2.99 ± 0.04[b]	3.19 ± 0.03[a]	<0.01
Stiffness (Nm/degree)	0.80 ± 0.01[a]	0.60 ± 0.01[c]	0.64 ± 0.01[b]	<0.01	0.63 ± 0.01[c]	0.68 ± 0.01[b]	0.74 ± 0.01[a]	<0.01
Failure Stress (MPa)	283.28 ± 3.34	274.55 ± 3.41	284.18 ± 3.38	0.09	278.50 ± 3.71	280.73 ± 3.69	282.78 ± 2.62	0.64
Young's Modulus (GPa)	9.58 ± 0.14[b]	10.04 ± 0.14[a]	10.12 ± 0.14[a]	0.01	9.20 ± 0.15[c]	9.80 ± 0.15[b]	10.74 ± 0.11[a]	<0.01
Second moment of area (mm^4)	48.57 ± 0.58[a]	34.57 ± 0.59[c]	36.88 ± 0.58[b]	<0.01	39.77 ± 0.64	40.40 ± 0.64	39.84 ± 0.45	0.73

[abc]Means within the same row lacking a common superscript differ significantly (P<0.05).

[1]AV (Cage-free aviary system); EN (Enriched colony cage); CC (Conventional cage)

Table 20: Age by housing type interaction for humeri properties of Lohmann White hens housed in cage-free aviary, enriched colony cages, and conventional cages at different ages.

Dependent variable and housing type		Age (weeks)		
		26	56	72
Tibiae		Mean ± SD	Mean ± SD	Mean ± SD
Young's Modulus (GPa)				
	[1]AV	13.15 ± 0.27^{y}	$13.12 \pm 0.27^{b\ y}$	15.41 ± 0.19^{x}
	CC	13.11 ± 0.26^{z}	$14.47 \pm 0.26^{a\ y}$	15.44 ± 0.19^{x}
	EN	13.62 ± 0.27^{z}	$14.74 \pm 0.30^{a\ y}$	15.52 ± 0.18^{x}
	P value	0.01		
Second moment of area (mm^4)				
	AV	$61.35 \pm 1.47^{a\ xy}$	$64.19 \pm 1.47^{a\ x}$	$58.64 \pm 1.02^{a\ y}$
	CC	$55.79 \pm 1.44^{b\ x}$	$50.03 \pm 1.44^{b\ y}$	$50.90 \pm 1.04^{c\ y}$
	EN	$53.06 \pm 1.50^{b\ xy}$	$51.90 \pm 1.66^{b\ y}$	$53.98 \pm 1.01^{b\ x}$
	P value	0.01		
Humeri				
Young's Modulus (GPa)				
	[1]AV	8.96 ± 0.26^{y}	$9.05 \pm 0.26^{b\ y}$	10.73 ± 0.18^{x}
	CC	9.18 ± 0.26^{y}	$10.32 \pm 0.26^{a\ x}$	10.62 ± 0.18^{x}
	EN	9.47 ± 0.26^{y}	$10.04 \pm 0.26^{a\ y}$	10.85 ± 0.19^{x}
	P value	0.04		

[ab]Means within the same column lacking a common superscript differ significantly ($P<0.05$).

[xy]Means within the same row lacking a common superscript differ significantly ($P<0.05$).

[1]AV (Cage-free aviary system); EN (Enriched colony cage); CC (Conventional cage)

DISCUSSION

The results of this study demonstrates that bone mass and mechanical gains in tibia and humerus of hens in the aviary system (AV) over the conventional cages (CC) at the end of the pullet phase were maintained until the end of production. The differences in tibia and humerus properties between AV and CC birds at the end of pullet phase corroborates the results observed in the first flock housed in the same conditions (Regmi et al., 2015a). Tibiae and humeri responded differently to the housing conditions. Periosteal apposition was more apparent in humeri of AV hens as evident by increased cortical area as well as outer dimension. Tibiae structural changes as a result of more activity (in AV system) were most probably endocortical gain as the difference was limited to cortical area and thickness but not in outer diameter. The result of cortical gains increased the resistance to bending or the second moment of area in AV hens compared to CC hens. Tibiae and humeri of AV hens also had increased resistance to deformation (increased stiffness) and greater energy required to failure than CC hens. Failure stress, a measure of material strength of the bone, was not different between the housing systems whereas Young's modulus or material stiffness was greater in CC hens compared to AV hens at 18 wks and 56 wks age. The results of material properties suggest that the increased whole bone mechanical strength in AV hens is probably due to bone quantity or greater bone mass rather than increased intrinsic bone quality. Tibia cortical density was not different between the housing systems at the mid-diaphysis whereas humeri cortical density of AV hens was greater than CC hens. Tibiae and humeri of 2 yrs old hens in free-range and CC have previously been reported to have mechanical and structural results similar to our study (Shipov et al., 2010). Failure load, stiffness, and yield stress of tibiae and humeri of free-range hens were greater than CC hens. Cortical area and thickness were also greater in free-range hens than CC hens. Tibia

density was greater in CC hens than free-range hens while humeri density was not different between housing system (Shipov et al., 2010). The difference in density results compared to the current study was probably due to the age of the bird (104 wks vs 72 wks).

Hens housed in enriched colony cages (EN) at the start of laying phase (18 wks) had increased tibiae and humeri cortical area and thickness compared to the CC hens. Cortical density difference, however, was only observed for humeri. Hens in commercial systems can load their tibia in three possible ways – standing or body weight (present in all systems), low impact loading activities like walking and running (possible in EN and AV but limited in CC), and high impact loading activities like jumping (limited in EN but very unlikely in CC). Loading environment in EN and CC for tibiae was probably not different enough to elicit density changes (Silversides et al., 2012). Wing movement and flapping is greatly limited in CC compared to EN and could have resulted in density and structural difference in the humeri. The difference in cortical measurements in EN and CC hens indicate that providing moderate exercise during laying phase reduce the extent of endosteal resorption. The results agree with the findings of other studies comparing furnished cages and floor pens to CC (Leyendecker et al., 2005; Vits et al., 2005; Jendral et al., 2008; Silversides et al., 2012).

Failure load or the whole bone breaking strength did not change with age, however, properties like energy to failure and failure rotation decreased. Other properties like stiffness, Young's modulus and yield bending moment increased with age. These results indicate that the bone become less brittle with age and require less energy to fracture. Increase in stiffness accompanied by decrease in toughness was observed in laying hens housed in barn, free-range, free-range with suspended perches (DEFRA, 2008). Increased stiffness was related to increase in bone mineral density measured by DEXA and decrease in bone collagen content with age. In our

study, cortical density did not increase linearly with age however dry bone weight increased implying that medullary bone content and its calcification over time might have contributed to increased stiffness, particularly in case of tibiae. Cortical thinning of tibiae was observed with increasing age in the current study whereas age had no effect on the cortical area of humeri. This difference in tibiae and humeri was probably a result of higher medullary content in tibiae compared to humeri. Medullary bone has high concentration of osteoclasts because of its role in daily calcium turnover during production (van de Velde et al., 1984) making the exposed endocortical surface of tibiae more vulnerable to resorption (Whitehead, 2004).

Serum and urinary concentration of hydroxylysylpyridinoline (PYD) and DPD (deoxypyridinoline) have been used in monitoring bone resorption or collagen turnover in osteoporosis and other metabolic bone disease (Garnero and Delmas, 1998). Unlike DPD, PYD is not bone-specific but PYD is absent in skin and the ratio of PYD to DPD has been found to be similar in normal and known patients of metabolic bone diseases (Uebelhart et al., 1990; Robbins, 1995). In this study we used PYD to monitor systemic collagen turnover along with osteocalcin (OC) as a marker of bone formation. Mean serum OC concentration showed an overall trend of declining with age in both housing systems and was not different between the housing systems at 56 and 72 wks. Interesting housing by age interaction was observed for PYD concentration. Pyridinoline concentration increased in CC hens between 12 and 18 wks and declined thereafter before saturating at 56 wks whereas in AV hens PYD concentration was greater at 56 and 72 wks compared to 26 wks age. These changes coincided with some mechanical parameters and structural parameters. Tibiae cortical area and second moment of area in AV hens decreased after 56 wks. Similar changes in CC hens occurred between 26 and 56 wks but no difference was observed between 56 and 72 wks. Hens in AV seem to better cope

with rigors of early and peak production compared to CC hens. The decline in tibiae and humeri properties in AV hens after 56 wks might be a response to egg production. The incidences of keel bone fractures in hens housed in systems with perches and floor pens similar to the AV system of this study have been reported to be highest at around 50 weeks (Scholz et al., 2008, Petrik et al., 2015) whereas keel fractures have been fairly low in conventional cages (Petrik et al., 2015). The combination of keel bone fractures and active egg production might have resulted in increased resorption from tibiae and humeri to repair keel fractures ultimately causing decline of bone properties after 56 wks in AV hens.

In conclusion, the influence of housing system and age was observed for structural and mechanical properties of humeri. Humeri of aviary hens (AV) had thicker and denser cortical bone as early as 18 wks age than humeri of hens kept in conventional cages (CC) and the changes were maintained until the end of the cycle. These changes also translated into superior mechanical properties in AV hens; stiffness, resistance to bending (second moment of inertia), and energy to failure in particular. On the other hand, moving CC reared pullets to enriched colony cages (EN) at the start of laying cycle improved most of mechanical properties of humeri while the effect was limited to increased stiffness in case of tibia. The structural changes observed in the tibia were also less prominent than it was observed for humeri. Age-related changes in bone properties indicated that bones become more stiff with age but at cost of toughness and ultimately require less energy to failure/fracture. Also, the difference between EN and CC bone properties becomes more obvious towards the end of the cycle marked by difference in cortical thickness and related cross-sectional second moment of area.

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CHAPTER 5.

Comparison of bone properties between strains and housing systems in 78 wk old laying hens

INTRODUCTION

The mechanical and functional failure of a bone can be attributed to genetic factors and environmental factors; particularly the failure to adapt to the nature of the loading environment the bone is subjected to (Pearson and Lieberman, 2004). In the present scenario, commercial laying hens have been dealing with a combination of selection programs favoring egg production and less than ideal housing designs causing skeletal instability and failure. Laying hens in the U.S. average almost 70 eggs more than they used to 50 years ago (USDA-NASS, 2012; Perez et al., 1991). The modern strains of laying hens are under constant negative calcium balance (Neijat et al., 2011) and if the hens preserve their medullary bone at the expense of structural bone when calcium deficient (Taylor and Moore, 1954), the result is thinning of cortices observed at the later stages of the laying cycle (Hudson et al., 1993; Whitehead, 2004). Structural loss of bone, commonly known as osteoporosis, is exacerbated when the high producing hens are kept in cages (Whitehead, 2004). In recent years, studies involving other housing systems have provided some evidences that providing opportunities for loading exercises can help increase bone mass in pullets (Regmi et al., 2015) and decrease bone resorption in adult hens (Shipov et al., 2010). Newer housing systems like aviaries and furnished cages, however, have come under intense scrutiny throughout the E.U. because of high prevalence of keel bone fractures and deformities (Freire et al., 2003; Wilkins et al., 2004; Nicol et al., 2006). The principal cause of keel fractures is hypothesized to be a result of collisions with perches and other structures within the alternative housing systems (Wilkins et al., 2011). On the other hand, keel breakage has been reported in hens housed in conventional cages (Sherwin et al., 2010; Hester et al., 2013) indicating the problem might be multifactorial. Since Bishop et al. (2000) reported that bone properties in laying hens can be moderately to strongly heritable, there has been a growing interest to separate

the contribution of genetics and housing systems on the skeletal parameters. One way to explore the aspects of selection and housing system is to compare the modern strain of hens with heritage breeds across different housing systems. A Rhode Island Red crossed with a Plymouth Rock was reported to have wider bone areas and greater ash content compared to modern white strains (Silversides et al., 2012) but the keel bone properties were not evaluated in that study. Therefore the aim of the present study was to evaluate the influence of strains and housing systems on the bone properties of end-of-lay hens including an assessment of keel bone deformities.

MATERIALS AND METHODS

The experimental procedure was approved by Institutional Animal Care and Use Committee of North Carolina State University (NCSU). The birds used in this study were a part of the North Carolina Layer Performance and Management Test.

Birds, Management, and Sampling

The details of housing layout, diets and management are provided in the single cycle report of the 38th North Carolina Layer Performance and Management Test (Anderson, 2011). Briefly, day old Hy-Line Brown (HB) and Hy-Line Silver Brown (SB) female chicks obtained from Hy-Line International (Mansfield, GA, USA and Dallas Center, IA, USA respectively) and Barred Plymouth Rock (BR) female chicks hatched at NCSU (Raleigh, NC, USA) were used in the study. The pullets for cage (CC) facilities were randomly assigned to growing cages whereas the pullets for the cage-free (CF) and range (R) facilities were reared on litter. The grower cage facility was an environmentally controlled house that provided a rearing space of 310 cm^2 and 4.7 cm feeder space/bird. The pullets raised on litter had a space allocation of 929 cm^2 including access to roosts in order to promote roosting behavior and the use of nest boxes. At 17 wks of age pullets were transferred to respective hen houses. Caged hens were stocked at a density of either 471 cm^2 or 497 cm^2 based on the dimension of the cage. The cage-free system was a slat-litter facility with floor space of 929 cm^2 and feeder space of 2.5 cm per bird. Pullets raised for range were randomly selected and moved to range huts of similar dimension as slat-litter facility at 12 wks of age. Range huts had a paddock 21.3 m x 21.3 m size that provided a total useable space of 8.1 m^2 (929 cm^2 + 8 m^2). Lighting, feeding, health and husbandry practices were consistent across all housing systems. At 78 weeks, 60 random birds from each housing and

strain combination were weighed and euthanized by cervical vertebra dislocation. Right leg (tibia and femur) and breast (keel bone) were collected and frozen at -20°C for further analysis.

Tibia and Femur Analysis

The right leg was thawed overnight at room temperature before quantitative computed tomography (QCT) scans were performed. Tibia and femur were scanned together along with the surrounding soft tissues using a GE BrightSpeed scanner (General Electric Healthcare, Princeton, NJ). Scan parameters were set at 120 kV tube voltage and 244 mAs and image data were calibrated to Hounsfield Units (HU) using reconstruction kernel specific to bone. The final CT images had a matrix size of 512 x 512 and a voxel size of ~ 0.27 mm^3 and were analyzed using MIMICS® software (Materialise, Plymouth, MI). Total bone length was measured and subsequently divided into 4 parts and cortical thickness (CBT) and density (CBD) were measured at a 0.625 mm thick slice in proximal, middle and distal section of the bone. A threshold mask was chosen based on several trials with different HU to separate cortical tissues from trabecular and medullary tissues with an idea to cover maximum cortical area without selecting the medullary cavity. A threshold mask of 450 HU was considered consistent to be used across the samples. Cortical thickness was measured along the antero-posterior and medio-lateral planes of each cross-sectional bone slice and CBD was measured at the same locations using a 10 x 20 mm rectangular box. Average CBD of the whole cross-section was also measured for each slice. A standard hydroxyapatite phantom was scanned along with the bones to convert the HU values of the bone images into density (mg/cm^3) values. After QCT was completed, tibia and femur were ashed in hot furnace and fat-free ash percentage was calculated (Regmi et al., 2015).

Keel Analysis

Keel computed tomography was conducted with similar image acquisition parameters to tibia and femur. The length of carina sterni (Figure 15) of the keel bone was measured from the proximal tip to the distal tip and was divided into four equal regions. Average density of keel was calculated at proximal (one-fourth), middle, and distal (three-fourths) sections. A threshold mask of 250 HU were used to segment keel bone from surrounding tissues using the 'Profile line' feature of the image analysis software as mentioned in Chapter 4. In addition to the density measurements, 3D models of keel (Figure 15) were developed to measure the greatest angle at twist in the carina sterni. The angle of deviation and the presence or absence of fractures was used to score the keel deformity on a scale of '0' to '4'. The description of the individual scores are as follows - Score '0': straight keel with angle of carina sterni between 175 and 180° and without any visible twists, indentations or fractures; Score '1': angle of carina sterni between 155 and 175° and/or presence of indentations but without any healed or unhealed fractures; Score '2': moderately twisted with or without fracture and angle of carina sterni between 140 and 155°; Score '3': Severely twisted keel with angle of carina sterni < 140° and mostly healed fractures; Score '4': Complete mid-keel fractures with disjointed bone fragements (Figure 16A to 16E). Percentage ash content and dry bone weight of keel were calculated similar to tibia and femur. The details of methods used to calculate ash percentage on dry bone basis are explained in Chapter 2. Briefly, breast muscles were removed and keel bone was separated from the ribs, coracoid, and clavicle. The keel bone was then placed in a soxhlet for ether extraction. Ether extracted bones were dried and finally ashed in hot furnace.

Figure 15: Anatomical representation of the keel bone on a 3D model (labeling based on Fleming et al., 1994).

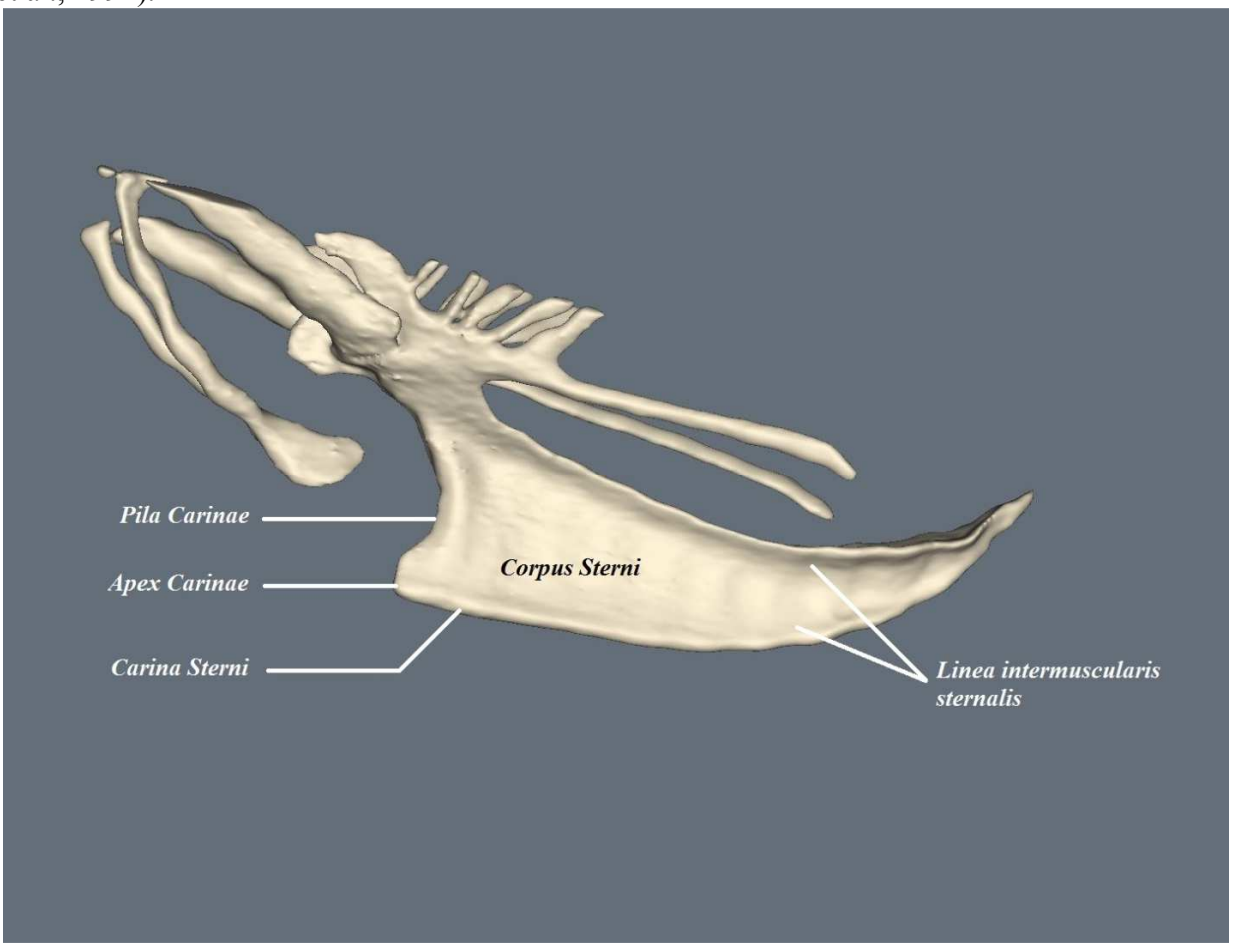


Figure 16: Keel bone deformity scoring system. (A) Score ‘0’: straight keel with angle of carina sterni between 175 and 180° and without any visible twists, indentations or fractures; (B) Score ‘1’: angle of carina sterni between 155 and 175° and/or presence of indentations but without any healed or unhealed fractures; (C) Score ‘2’: moderately twisted with or without fracture and angle of carina sterni between 140 and 155°; (D) Score ‘3’: Severely twisted keel with angle of carina sterni < 140° and mostly healed fractures; (E) Score ‘4’: Complete mid-keel fractures with disjointed bone fragements.

A. B. C.

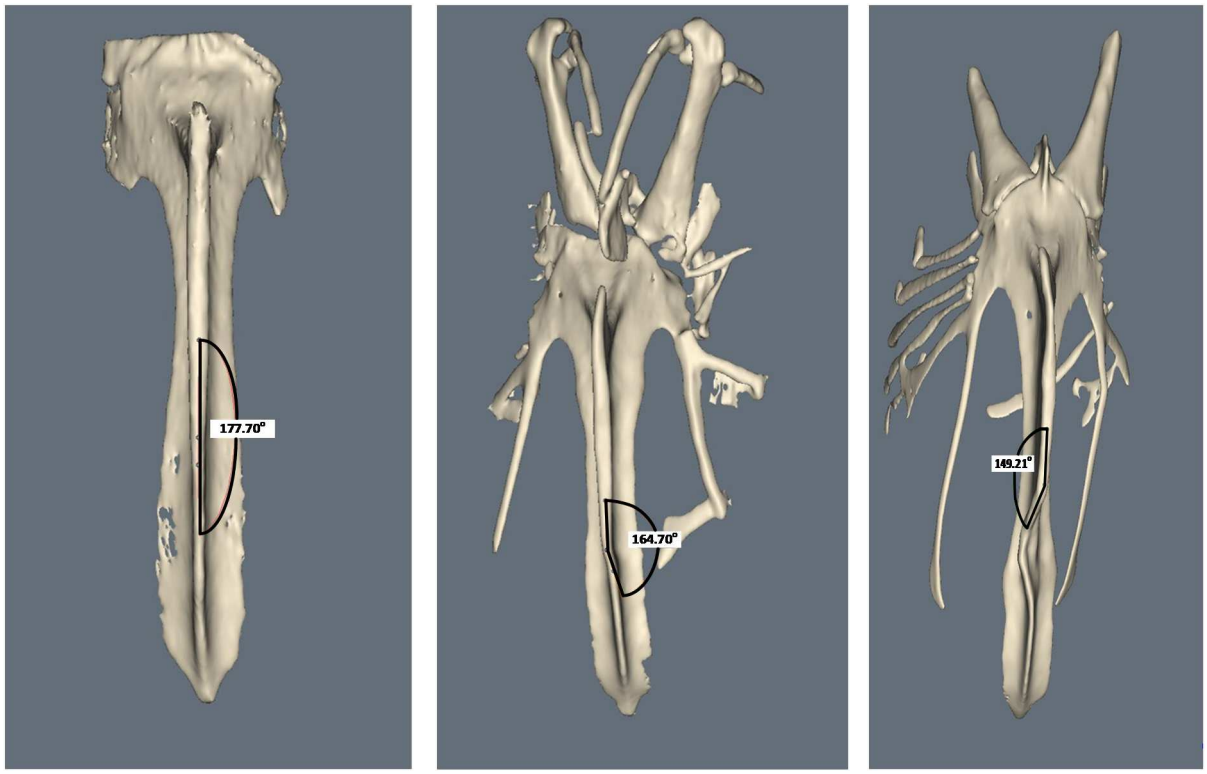
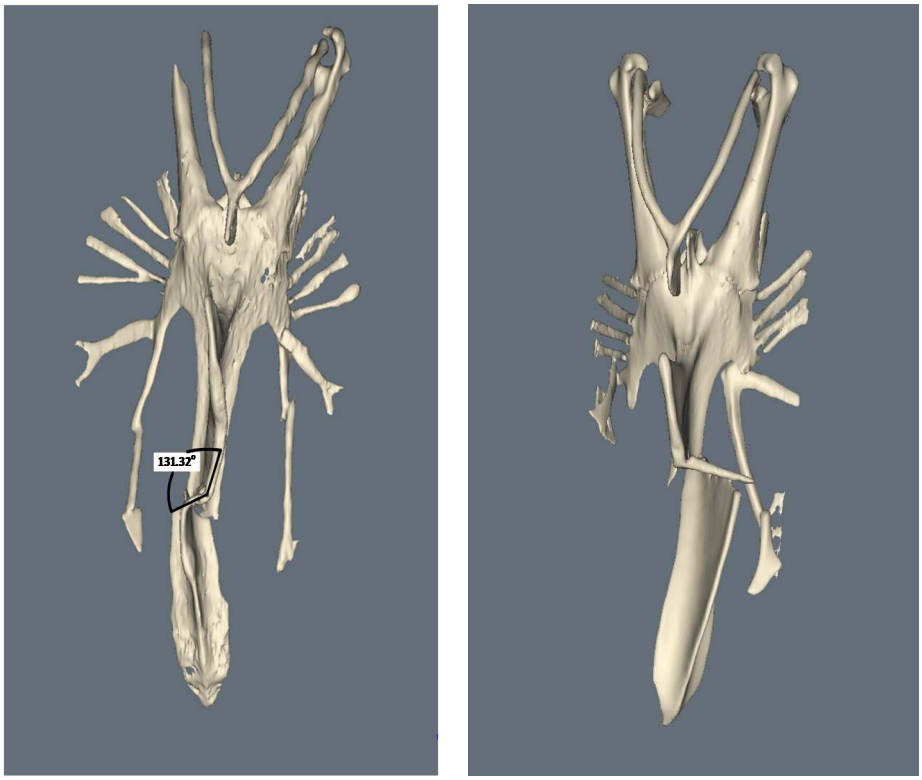


Figure 16 (cont'd).

D. E.



Statistical Analysis

A statistical model including the main effects of housing system and genetic strain, their interaction and the residual error was used to analyze the dependent variables using the multivariate PROC MIXED analysis of SAS 9.3 (SAS Institute 2002, Cary, NC). Means were separated using Tukey's post hoc adjustment with significance accepted at $P < 0.05$. The data for deformity score of the keel bone was analyzed as nominal categorical variable using PROC LOGISTIC and the results of maximum likelihood estimates ($P < 0.05$) were used to analyze the influence of the fixed effects. To analyze the effect of housing system, CC was used as the reference category and was compared against CF and R whereas BR was used as the reference for the strain effect. If the likelihood of an event was significant, the data were then presented as odd ratio estimates along with their respective range values.

RESULTS

Hens housed in conventional cages (CC) were heavier than cage-free (CF) (2.11 kg vs. 1.97 kg; $P < 0.05$) while the free-range (R) hens were intermediate (2.01 kg). Body weight of hens across genetic strains was not different ($P = 0.0538$).

Tibia and Femur Properties

Effects of housing system and genetic strain were observed for the bone properties of tibia and femur. Interactions between the main effects were not significant for any bone parameters. Tibia cortical bone thickness (CBT) was smaller along the posterior (11%) and lateral surface (13%) at the mid-diaphysis in the CC hens compared to CF and R hens (Table 21; $P < 0.05$). At the proximal section of the bone, CBT was greater in R hens along the posterior and medial plane compared to CC hens ($P < 0.05$) whereas CF hens had the intermediate values. Tibiae of CF hens had the greatest CBT along the lateral surface at the distal section whereas CC hens had the thinnest cortex ($P < 0.05$). The result was not different between the housing types along other anatomical surfaces at the distal tibiae. The result of strain-wise comparison for CBT was consistently greater in Barred Plymouth Rock (BR) hens and HB and SB hens (Table 21; $P < 0.05$). Cortical thickness was not different between the contemporary HB and SB strains.

Average volumetric density of tibiae cortex was greatest in R hens whereas CC hens had the least dense tibia at the middle and distal section (Table 22; $P < 0.05$). Cage-free hens had intermediate CBD, which was not different from CC and R hens. Similar differences were observed along the posterior and lateral surfaces at the middle section and along all surfaces at the distal section except the lateral surface. Cortical density at proximal tibia was similar in all the housing systems. There was no difference in the CBD of femur between the housing systems except along the anterior surface of proximal section where the response was similar to that observed for tibiae. When comparisons were made across genetic strains, tibiae and femur of BR

hens had greater cortical density than Hy-Line Brown (HB) and Hy-Line Silver Brown (SB) hens (Table 23; $P < 0.05$) whereas CBD of HB and HS hens were similar.

Tibia and femur dry bone weight and ash percentage, as an indicator of mineral content of the whole bone, was not different for the housing systems. Among the strains, tibiae and femur of BR hens were heavier than SB and HB hens (Table 24; $P < 0.05$). Similarly, tibia ash percentage was greater for BR hens compared to HB and SB hens ($P < 0.05$). Femur ash percentage was greatest in BR hens and smallest in SB hens while HB hens had the intermediate values ($P < 0.05$).

Table 21: Tibia cortical thickness (mm) of 78 wk hens housed in conventional cages, cage-free system, and free-range system.

Treatment	Planar orientation of bone			
	Anterior	Posterior	Medial	Lateral
Housing[1]	LSM ± SEM	LSM ± SEM	LSM ± SEM	LSM ± SEM
Proximal				
CF	1.99 ± 0.06	1.93 ± 0.05^{ab}	2.12 ± 0.05^{ab}	2.50 ± 0.09
CC	1.86 ± 0.06	1.77 ± 0.06^{b}	1.97 ± 0.05^{b}	2.29 ± 0.09
R	2.01 ± 0.06	1.96 ± 0.05^{a}	2.16 ± 0.05^{a}	2.47 ± 0.09
P value	0.12	0.03	0.02	0.20
Middle				
CF	1.99 ± 0.05	1.99 ± 0.05^{a}	2.13 ± 0.06	2.21 ± 0.06^{a}
CC	1.89 ± 0.05	1.79 ± 0.05^{b}	1.96 ± 0.06	1.95 ± 0.06^{b}
R	2.03 ± 0.05	1.99 ± 0.05^{a}	2.13 ± 0.06	2.20 ± 0.06^{a}
P value	0.12	0.01	0.06	0.01
Distal				
CF	1.76 ± 0.04	1.65 ± 0.04	1.81 ± 0.05	1.86 ± 0.04^{a}
CC	1.67 ± 0.04	1.56 ± 0.04	1.65 ± 0.05	1.68 ± 0.04^{b}
R	1.76 ± 0.04	1.57 ± 0.04	1.73 ± 0.05	1.81 ± 0.04^{ab}
P value	0.16	0.22	0.06	0.01
Strain[2]				
Proximal				
HB	1.72 ± 0.06^{b}	1.71 ± 0.05^{b}	1.90 ± 0.05^{b}	2.30 ± 0.09^{b}
SB	1.80 ± 0.06^{b}	1.74 ± 0.06^{b}	1.98 ± 0.05^{b}	2.35 ± 0.09^{ab}
BR	2.33 ± 0.06^{a}	2.20 ± 0.05^{a}	2.37 ± 0.05^{a}	2.61 ± 0.09^{a}
P value	<.0001	<.0001	<.0001	<.0001
Middle				
HB	1.76 ± 0.05^{b}	1.74 ± 0.05^{b}	1.90 ± 0.06^{b}	1.93 ± 0.06^{b}
SB	1.85 ± 0.05^{b}	1.75 ± 0.05^{b}	1.95 ± 0.06^{b}	1.97 ± 0.06^{b}
BR	2.30 ± 0.05^{a}	2.29 ± 0.05^{a}	2.38 ± 0.06^{a}	2.45 ± 0.06^{a}
P value	<.0001	<.0001	<.0001	<.0001
Distal				
HB	1.60 ± 0.04^{b}	1.46 ± 0.04^{b}	1.54 ± 0.05^{b}	1.63 ± 0.04^{b}
SB	1.60 ± 0.04^{b}	1.46 ± 0.04^{b}	1.58 ± 0.05^{b}	1.62 ± 0.04^{b}
BR	1.99 ± 0.04^{a}	1.85 ± 0.04^{a}	2.07 ± 0.05^{a}	2.10 ± 0.04^{a}
P value	<.0001	<.0001	<.0001	<.0001

^{ab} Means within the same column lacking a common superscript differ significantly (P<0.05).

[1]CF (Cage-free system); CC (Conventional cage); R (Free-range)

[2]HB (Hyline Brown); SB (Hyline Silver Brown); BR (Barred Plymouth Rock)

Table 22: Volumetric bone density (mg/cm^3) of cortical bone of tibia and femur of 78 wk hens housed in conventional cages, cage-free system, and free-range system.

Bone type and housing	Planar orientation of bone				
	Anterior	Posterior	Medial	Lateral	Average
Tibia	LSM ± SEM	LSM ± SEM	LSM ± SEM	LSM ± SEM	LSM ± SEM
Proximal					
[1]CF	679.24 ± 16.48	670.39 ± 14.84	676.05 ± 14.37	697.41 ± 15.15	678.83 ± 15.74
CC	686.39 ± 16.61	678.17 ± 14.96	680.93 ± 14.49	673.26 ± 15.27	682.96 ± 15.87
R	710.24 ± 16.46	707.03 ± 14.96	713.75 ± 14.36	721.18 ± 15.13	709.27 ± 15.73
P value	0.38	0.19	0.13	0.09	0.34
Middle					
CF	755.79 ± 17.19	738.31 ± 17.10^{ab}	759.09 ± 17.66	768.03 ± 16.74^{ab}	767.09 ± 15.51^{ab}
CC	735.67 ± 17.33	721.86 ± 17.24^{b}	730.82 ± 17.81	728.42 ± 16.88^{b}	735.36 ± 15.63^{b}
R	783.80 ± 17.17	786.54 ± 17.08^{a}	783.76 ± 17.64	790.53 ± 16.72^{a}	799.61 ± 15.49^{a}
P value	0.14	0.02	0.11	0.03	0.02
Distal					
CF	738.84 ± 15.24^{ab}	712.62 ± 16.46^{ab}	765.90 ± 17.11^{ab}	763.68 ± 16.89	762.95 ± 15.43^{ab}
CC	724.17 ± 15.37^{b}	673.37 ± 16.60^{b}	740.75 ± 17.25^{b}	729.21 ± 17.03	729.11 ± 15.55^{b}
R	787.69 ± 15.23^{a}	730.38 ± 16.44^{a}	806.73 ± 17.09^{a}	784.98 ± 16.87	793.29 ± 15.41^{a}
P value	0.01	0.05	0.03	0.07	0.02
Femur					
Proximal					
CF	727.89 ± 16.97^{ab}	695.25 ± 16.61	693.07 ± 15.40	670.84 ± 15.52	681.77 ± 13.88
CC	699.72 ± 17.86^{b}	660.39 ± 17.49	662.54 ± 16.21	647.99 ± 16.34	663.60 ± 14.61
R	765.17 ± 17.64^{a}	707.40 ± 17.08	708.64 ± 15.99	683.53 ± 15.96	696.80 ± 14.26

Table 22 (cont'd).

P value	0.04	0.14	0.12	0.29	0.27
Middle					
CF	710.38 ± 17.66	735.59 ± 15.25	682.34 ± 15.83	696.33 ± 16.18	708.83 ± 15.34
CC	709.45 ± 18.59	703.67 ± 16.05	669.51 ± 16.66	680.29 ± 17.03	690.16 ± 16.15
R	724.82 ± 18.15	744.47 ± 15.68	707.44 ± 16.27	704.76 ± 16.63	724.16 ± 15.93
P value	0.80	0.17	0.26	0.58	0.33
Distal					
CF	624.26 ± 16.47	682.69 ± 15.93	623.97 ± 15.75	650.03 ± 15.3	644.94 ± 14.44
CC	643.99 ± 17.34	681.76 ± 16.77	610.55 ± 16.82	648.01 ± 16.11	652.74 ± 15.20
R	626.29 ± 16.93	704.82 ± 16.38	629.31 ± 16.19	668.03 ± 15.73	655.97 ± 14.84
P value	0.67	0.53	0.71	0.61	0.86

[ab]Means within the same column lacking a common superscript differ significantly ($P<0.05$).

[1]CF (Cage-free system); CC (Conventional cage); R (Free-range)

Table 23: Volumetric bone density (mg/cm^3) of cortical bone of tibia and femur of 78 wk hens of three different genetic strains.

Bone type and strain	Planar orientation of bone				
	Anterior	Posterior	Medial	Lateral	Average
Tibia	LSM ± SEM	LSM ± SEM	LSM ± SEM	LSM ± SEM	LSM ± SEM
Proximal					
[1]HS	650.96 ± 16.46^b	633.60 ± 14.96^b	650.26 ± 14.36^b	635.05 ± 15.13^b	642.61 ± 15.73^b
SB	655.07 ± 16.61^b	661.54 ± 14.96^b	664.49 ± 14.49^b	656.92 ± 15.27^b	665.93 ± 15.87^b
BR	769.83 ± 16.48^a	760.45 ± 14.84^a	755.98 ± 14.37^a	799.88 ± 15.15^a	762.52 ± 15.74^a
P value	<0.01	<0.01	<0.01	<0.01	<0.01
Middle					
HS	708.48 ± 17.17^b	684.11 ± 17.08^b	707.66 ± 17.64^b	691.58 ± 16.72^b	708.50 ± 15.49^b
SB	731.68 ± 17.33^b	716.97 ± 17.24^b	727.82 ± 17.81^b	742.62 ± 16.88^b	741.85 ± 15.63^b
BR	835.09 ± 17.19^a	845.63 ± 17.10^a	838.19 ± 17.66^a	852.78 ± 16.74^a	851.71 ± 15.51^a
P value	<0.01	<0.01	<0.01	<0.01	<0.01
Distal					
HS	703.81 ± 15.23^b	640.25 ± 16.44^b	707.63 ± 17.09^b	687.39 ± 16.87^b	700.04 ± 15.41^b
SB	731.56 ± 15.37^b	681.31 ± 16.60^b	753.43 ± 17.25^b	730.05 ± 17.03^b	740.75 ± 15.55^b
BR	815.34 ± 15.24^a	794.81 ± 16.46^a	852.32 ± 17.11^a	860.42 ± 16.89^a	844.56 ± 15.43^a
P value	<0.01	<0.01	<0.01	<0.01	<0.01
Femur					
Proximal					
HS	650.07 ± 17.81^b	605.84 ± 17.25^b	622.06 ± 15.99^b	606.74 ± 16.12^b	611.96 ± 14.40^b
SB	696.54 ± 17.09^b	655.49 ± 16.73^b	656.73 ± 15.67^b	627.07 ± 15.64^b	648.56 ± 13.98^b
BR	846.17 ± 17.57^a	801.72 ± 17.20^a	785.46 ± 15.95^a	768.55 ± 16.08^a	781.66 ± 14.37^a
P value	<0.01	<0.01	<0.01	<0.01	<0.01
Middle					
HS	644.32 ± 18.33^b	654.06 ± 15.83^b	613.09 ± 16.43^b	628.39 ± 16.80^b	634.88 ± 15.93^b
SB	665.70 ± 17.78^b	691.90 ± 15.36^b	639.91 ± 15.94^b	650.34 ± 16.30^b	670.61 ± 15.61^b

Table 23 (cont'd).

BR	834.63 ± 18.29[a]	837.78 ± 15.79[a]	806.28 ± 16.39[a]	802.64 ± 16.76[a]	817.65 ± 15.89[a]
P value	<0.01	<0.01	<0.01	<0.01	<0.01
Distal					
HS	560.87 ± 17.10[b]	606.63 ± 16.54[b]	562.67 ± 16.36[b]	602.73 ± 15.89[b]	588.74 ± 14.99[b]
SB	567.06 ± 16.59[b]	637.59 ± 16.05[b]	571.61 ± 15.87[b]	618.98 ± 15.41[b]	609.34 ± 14.54[b]
BR	766.60 ± 17.06[a]	825.05 ± 16.50[a]	729.56 ± 16.55[a]	744.35 ± 15.85[a]	755.57 ± 14.95[a]
P value	<0.01	<0.01	<0.01	<0.01	<0.01

[ab]Means within the same column lacking a common superscript differ significantly (P<0.05).

[1]HB (Hyline Brown); SB (Hyline Silver Brown); BR (Barred Plymouth Rock)

Keel Bone Properties

Hens housed in CC had lower average density of keel bone compared to the CF and R hens (Table 25; $P < 0.05$). Dry bone weight of the keel was smaller for CC hens than CF and R hens (Table 24; $P < 0.05$). The effect of strain on density was only significant at the mid cross-section of the keel with BR hens having the most dense keel and SB having the least dense keel ($P < 0.05$). Barred Plymouth Rock hens showed a strong trend to have keel bone with greater density at proximal ($P = 0.07$) and distal ($P = 0.06$) sections as well. The angle of carina sterni of the keel was greater (closer to 180°) in BR hens than other two strains ($P < 0.05$). Keel bone ash percentage was greater in BR hens compared to SB hens (Table 24; $P < 0.05$) whereas housing system did not influence the ash content.

The effect of housing system and strain was also observed in the individual keel bone scores. The distribution of keel deformity scores is presented in Table 26. The odds of CF hens to have a score of '2' rather than '1' were greater compared to CC hens whereas the odds of CF hens to get a score of '4' rather than '3' were lesser compared to CC hens ($P < 0.05$, Table 27). The keel deformity scores of R hens were not different from either CF or CC hens. Between genetic lines, the odds of having a score of '2' and '3' rather than a score of '1' were greater in HB and SB hens compared to BR hens ($P < 0.05$). Hyline Brown and SB hens were at lesser odds to have a score of '4' rather than '3' compared to BR hens ($P < 0.05$).

Table 24: Dry bone weight (g) and ash percentage of tibia, femur, and keel bone of 78 wk hens housed in conventional cages, cage-free system, and free-range system.

Dependent variable and treatment	Bone type		
	Tibia	Femur	Keel
Dry bone Wt	LSM ± SEM	LSM ± SEM	LSM ± SEM
[1]Housing			
CF	7.51 ± 0.23	6.40 ± 0.18	7.94 ± 0.17[a]
CC	7.02 ± 0.21	6.13 ± 0.23	6.12 ± 0.17[b]
R	7.49 ± 0.20	6.71 ± 0.18	8.02 ± 0.17[a]
P value	0.19	0.13	<0.01
[2]Strain			
HB	6.73 ± 0.21[b]	5.99 ± 0.20[b]	7.18 ± 0.17
SB	7.01 ± 0.21[b]	6.05 ± 0.19[b]	7.42 ± 0.17
BR	8.27 ± 0.23[a]	7.21 ± 0.20[a]	7.48 ± 0.17
P value	<0.01	<0.01	0.40
Ash Percentage			
Housing			
CF	58.92 ± 0.59	56.52 ± 0.78	54.97 ± 0.59
CC	57.59 ± 0.54	55.35 ± 0.98	54.79 ± 0.59
R	57.87 ± 0.51	56.63 ± 0.75	55.82 ± 0.59
P value	0.23	0.55	0.42
Strain			
HB	56.54 ± 0.52[b]	55.59 ± 0.86[a]	54.42 ± 0.59[b]
SB	57.84 ± 0.53[b]	54.68 ± 0.81[b]	54.78 ± 0.59[ab]
BR	60.00 ± 0.59[a]	58.23 ± 0.86[a]	56.38 ± 0.59[a]
P value	<0.01	0.01	0.04

[ab]Means within the same column lacking a common superscript differ significantly (P<0.05).

[1]CF (Cage-free system); CC (Conventional cage); R (Free-range)

[2]HB (Hyline Brown); SB (Hyline Silver Brown); BR (Barred Plymouth Rock)

Table 25: Average volumetric density (mg/cm^3) of the keel bone of three different strains of 78 wk hens housed in conventional cages, cage-free system, and free-range system.

Treatment	Bone section		
	Proximal	Middle	Distal
[1]Housing	LSM ± SEM	LSM ± SEM	LSM ± SEM
CF	501.44 ± 14.26[a]	473.23 ± 13.94[a]	484.77 ± 15.67[a]
CC	453.95 ± 12.86[b]	400.67 ± 12.57[b]	409.88 ± 14.14[b]
R	506.13 ± 14.14[a]	467.47 ± 13.82[a]	471.77 ± 15.71[a]
P value	0.01	<0.01	<0.01
[2]Strain			
HS	479.39 ± 13.37	436.88 ± 13.07[ab]	443.34 ± 14.69
SB	469.47 ± 14.10	429.42 ± 13.78[b]	437.88 ± 15.50
BR	512.67 ± 13.82	475.06 ± 13.51[a]	485.20 ± 15.36
P value	0.07	0.04	0.06

[ab]Means within the same column lacking a common superscript differ significantly (P<0.05).

[1]CF (Cage-free system); CC (Conventional cage); R (Free range)

[2]HB (Hyline Brown); SB (Hyline Silver Brown); BR (Barred Plymouth Rock)

Table 26: Frequency of keel deformity score distribution across housing system and genetic strains of 78 wk hens.

	[1]Housing						[2]Strain					
Score	CF		CC		R		BR		HB		SB	
	[3]n_{CF}	%	n_{CC}	%	n_R	%	n_{BR}	%	n_{HB}	%	n_{SB}	%
0	4	2.60	5	3.25	2	1.30	8	5.19	2	1.30	1	0.65
1	15	9.74	27	17.53	18	11.69	24	15.58	18	11.69	18	11.69
2	18	11.69	7	4.55	10	6.49	1	0.65	19	12.34	15	9.74
3	8	5.19	6	3.90	10	6.49	4	2.60	10	6.49	10	6.49
4	3	1.95	13	8.44	8	5.19	14	9.09	5	3.25	5	3.25
Total	48	31.17	58	37.66	48	31.17	51	33.12	54	35.06	49	31.82

[1]CF (Cage-free system); CC (Conventional cage); R (Free-range)

[2]HB (Hyline Brown); SB (Hyline Silver Brown); BR (Barred Plymouth Rock)

[3]n (Categorical frequency of hens for each keel deformity score within housing and genetic strain)

Table 27: Odd ratio comparisons for keel deformity scores of 78 wk hens of three different genetic strains housed in conventional cages, cage-free system, and free-range system.

Treatment wise comparisons	Score	Odd ratio estimate	95% confidence limits		P value
Reference score 1					
[1]House CF vs CC	2	5.99	1.89	19.06	<0.01
[2]Strain HB vs BR	2	29.75	3.53	250.85	<0.01
Strain HB vs BR	3	3.697	0.98	14.00	0.05
Strain SB vs BR	2	25.46	2.97	218.77	<0.01
Strain SB vs BR	3	3.81	1.00	14.52	0.05
Reference score 3					
House CF vs CC	4	0.13	0.02	0.71	0.02
Strain HB vs BR	4	0.12	0.02	0.59	<0.01
Strain SB vs BR	4	0.11	0.02	0.56	<0.01

[1]CF (Cage-free system); CC (Conventional cage); R (Free-range)

[2]HB (Hyline Brown); SB (Hyline Silver Brown); BR (Barred Plymouth Rock)

DISCUSSION

Housing systems for laying hens play a significant factor in the development of or the prevention of osteoporosis. The contrasting feature of the housing systems used in the current study was availability of space to the birds thereby providing them opportunities to varying level of physical activity. Previous studies comparing bone properties of laying hens in different housing systems have fairly established that hens housed in conventional cages have less strong bone parameters compared to their counterparts housed in alternative systems (Jendral et al., 2008; Shipov et al., 2010; Silversides et al., 2012; Regmi et al., 2015). The nature of the effects on a particular bone, however, has been variable. Tibiae of hens in conventional cages from the present study had thinner cortex compared to the cage-free and range hens. Corroborating to the results of this study, tibia cortical area has been reported to be smaller in caged hens compared to the hens in free range and furnished colony cages (Jendral et al., 2008; Shipov et al., 2010). Unlike the result of this study, a comparison between 4 strains of hens in floor pens and conventional cages reported no effect of housing for tibia cortical area (Silversides et al., 2012). Despite a difference in tarsometatarsus bone volume, Hughes et al. (1993) reported no difference in the bone volume of tibiae when perches were added in the cages. Cortical density of tibia was not different between the housing systems at the proximal section of the bone whereas density at middle and distal sections were greater in R hens compared to the CC hens while CF had tibia with intermediate bone density. The possibility of different level of physical activity in the housing systems used in the study and the graded response of bone density changes indicate that the extent of movement is probably the contributing factor (Knowles and Broom, 1990). Silversides et al. (2012) observed no changes in cortical density at the proximal section of tibia in brown hens raised in floor pens compared to their caged counterparts but the white hens had

denser cortex in floor pens. Although the exact nature of loading pattern in our study was not studied, site-specific skeletal response to loading has been observed in human and other animal models (Heinonen et al., 2002; Kuruvilla et al., 2008). Iwamoto et al., (1999) reported exercise-induced bone formation of the distal cancellous bone to be greater as compared to the proximal site in tibiae of young rats. Insertion points of muscles and other soft tissues along with the natural curvature of the bone surface creates variation in the stress experienced by the bone at that particular region (Dodge et al., 2012) and might be the reason why we observed differences at some locations but not at others (proximal vs. middle or distal). Whole bone parameters like dry bone weight and ash percentages were not affected by the housing systems in this study despite density and structural changes. Tibia, femur and in some extent keel bone are known to have variable amount of medullary bone in laying hens. The difference in the content of medullary bone might have influenced the result of bone weight and ash content (Clark et al., 2007).

The effect of genetic strains was consistent and quite pronounced in all the bone parameters studied with BR hens having greater cortical density, cortical thickness and mineral content compared to the contemporary strains. Most of the previous comparisons between genetic lines have been made with brown and white strains where brown hens were reported to have greater bone density and bone strength (Riczu et al., 2004; Habig and Distl, 2013). Similar to the results of this study, non-commercial strain (cross between Rhode Island Red and Barred Plymouth Rock; RIR x BR) was observed to have greater ash percentage and dry bone weight than commercial brown and white hens (Silversides et al., 2012). Egg production of BR hens was only 50.7% whereas egg production was much higher in HB (81.3%) and SB (83.5%) hens (Anderson, 2011), which could have resulted in the difference in bone properties. Silversides et

al. (2012) did not observe any differences between RIR x BR and Lohmann Brown hens in terms of cortical area and density of tibia however the difference in egg production between the strains were very narrow (78.3% vs. 85.7%) in their study (Singh et al., 2009).

Housing systems and genetic strains influenced both the severity and the type of keel bone deformities in the current study. Hens housed in conventional cages were less likely to incur severe old fractures and deformities despite having lighter and less denser keel compared to the hens housed in cage-free and range houses. Similar to the results of this study, flocks housed in furnished cages had the lowest prevalence (36%) despite also having significantly weaker bones, while flocks housed in systems equipped with multilevel perches exhibited the highest levels of damage (over 80%) and the highest severity scores (Wilkins et al., 2011). Recently, Petrik et al. (2015) reported fracture prevalence of keel bone to be 28.4% in cages and 48.3% in floor pens. Caged hens in this study were more likely to have fresh/unhealed fractures compared to the CF hens. The incidences of keel damage in furnished cages and aviaries are associated with trauma caused by flights and landing accidents sustained during the production period (Wilkins et al., 2011). While there is less possibility of traumatic events in cages, fresh fractures in CC hens most likely occurred during the peri-mortem period while catching, euthanizing and removing the keel bones because of the osteoporotic nature of the bone (Keutgen et al., 1999). Between genetic strains, keel bone of HB and SB hens were less dense with lower ash content and were related to more severe deformity scores compared to BR hens. Keel bone damage has been associated with genetic component in experiments conducted in hens selected for bone quality (Fleming et al., 2004), between parent stock and commercial hens (Kappeli et al., 2011) or between brown and white strains (Vits et al., 2005). This is first study to our knowledge comparing keel deformities in contemporary strains (HB and SB) with a heritage breed with

significantly lower egg production (BR) and the result supports that genetics can be a factor in addition to housing design in occurrence and prevalence of keel deformities.

The results of this study provide further evidence that housing system and genotype of the hens both influence the bone properties while the response may vary with individual bone type. The results of keel bone analysis suggest that the mechanism underlying keel bone damage might be different in different housing systems. Every housing system and genotype was associated with some sort of keel deformities indicating the intrinsic biomechanical nature of the keel bone might be a contributing factor in causing deformities. Longitudinal studies with tomographic scanning of live hens for keel damage is possible and further studies are warranted to identify the cause, prevalence and timing of the keel fractures.

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CHAPTER 6.

Final summary

The chapters of this dissertation focus on the skeletal impacts of conventional cage housing type predominantly in use for laying hens in the US and compare the results with newer housing types. The four studies that make the backbone of this dissertation look at different architectural, compositional, and mechanical properties of bones in conventional cages, enriched colony cages, and cage-free aviary systems. Chapters 2, 3, and 4 stem from analyzing tibiae and humeri of two consecutive flocks of Lohmann White hens housed on a commercial setting. The final chapter was based on a study at the university facility and looked at keel bone deformities and fractures in different housing by strain combinations.

The objective of the first paper was to evaluate if opportunities to perform greater physical activity as in an aviary system will bring positive structural and mechanical changes during the pullet phase. The results indicated that pullets in AV housing had improved load-bearing capability and stiffness of the tibia and humerus than cage-reared pullets. The differences were of greater magnitude in humerus than in tibia. Cross-sectional second moment of area, in particular, was specifically greater in bones of pullets in AV system compared to that of CC pullets indicating cortical area and diameter can be a major determinant of mechanical stability of a bone during the pullet phase.

The next experiment was conducted to answer if differences observed at the end of the pullet phase were maintained at the end of the laying period. The experimental design consisted of crisscrossing the housing system at the beginning of egg production. The bone mass and density acquired during the pullet phase were only maintained during the laying cycle if the opportunities for movement was continued (AV). In contrast, limiting the movement during the laying phase (AC) induced bone loss and decreased mechanical stability of the bones. Bone density at humeri mid-diaphysis was 19% less in AC hens compared to the AV hens whereas

cortical area was 25% lower in AC hens. On the other hand, providing moderate opportunities of movement during lay (EN) to pullets reared in CC may bring some improvement in mechanical properties of tibiae and humeri of laying hens.

The third experiment was conducted on the second flock of the same genetic strain and using the same set of commercial housing set up with an objective to evaluate any housing by age interaction in humeri and tibia properties. The main effects of housing system were similar to what was observed in the first two studies. Humeri of aviary hens (AV) had thicker and denser cortical bone as early as 18 wks age than humeri of hens kept in conventional cages (CC) and the changes were maintained until the end of the cycle. These changes were also observed in bones' mechanical properties with AV hens having greater stiffness, resistance to bending (second moment of area), and energy to failure. Moving CC reared pullets to enriched colony cages (EN) at the start of the laying cycle improved most of the mechanical properties of humeri while the effect was limited to increased stiffness in the case of tibia. Both tibia and humerus became more stiff with age but at cost of toughness and ultimately required less energy to failure/fracture. Age of the laying hen was observed to have contrasting effect on bone properties in different housing conditions. Age had linear effect on tibia mechanical parameters like elastic modulus and second moment of area in CC hens whereas the effect was quadratic in AV hens.

The final study was conducted to study housing and strain differences in bone properties of contemporary and heritage laying hens. The results indicated that the response to both housing and genotype vary with individual bone type. One of the key findings was that the mechanism underlying keel bone damage might be different in cage and cage-free housing environments. Each housing system and genotype was associated with some sort of keel deformities indicating

the intrinsic biomechanical nature of the keel bone might be a contributing factor in causing deformities.

Egg industry in the United States is accommodating the newer housing system sooner rather than later with some of the biggest producers already announcing a move to the cage-free systems. A stable skeletal system is not only important for the egg production but also for efficient mobility of birds in the cage-free environment and hence, the know-how of the complex relationship between bone dynamics, nutritional requirement, and management practices is mandated. The results of studies presented in this dissertation are a first attempt to look at bone parameters of great detail in different housing type in a commercial setting and provides a base in with further research can be built upon. Future studies involving complete crossover housing designs will help to explain cause and effect of changes in bone properties occurring at different points during the production cycle. A detailed study of the organic matrix of the bone is also warranted to get a more complete understanding for the changes in material and compositional properties of these bones. Longitudinal studies with tomographic scanning of live hens for keel damage is possible and further studies are warranted to identify the cause, prevalence and timing of the keel fractures.