Mechanoadaptation in chickens and mice

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Table of Contents

Abstract

The skeleton adapts in response to the mechanical demands placed on it via bone (re)modelling throughout an individual's lifespan. Osteocytes, the mechano-sensors in the bone, regulate bone (re)modeling by controlling osteoblast bone formation and osteoclast bone resorption during mechanotransduction. I used two animal models, egg laying chickens and mice to investigate bone adaptation to mechanical loading in the form of physical activity and controlled loading, respectively.

In my first study, I hypothesized that exercise - WAIR, from age of 2-21 weeks would increase bone mineral density and microstructure in two commercial genetic strains of chickens (Delkab white and Hyaline brown). I measured bone mineral density and microstructure of the humerus in 36-week-old chickens using Micro-CT. My results showed that the hypothesis was not supported in that there was no significant differences observed between WAIR treated chickens and the handled chickens nor between WAIR and control chickens. We did however observe a trend of increased Tb.vTMD in brown chickens that were WAIR trained compared to controls ($p=0.090$). In addition, within the brown chickens we observed a significantly greater Tb.vTMD ($p=0.007$) and Tb. Th ($p=0.018$) in the handled group (Tb. vTMD: 565.435 ± 37.25, Tb. Th: 7.864 ± 2.19) compared to the control group (Tb.vTMD: 507.377 ± 51.67 , Tb. Th: 5.758 ± 0.86). We did not measure any differences in bone outcomes in response to treatment in white chickens. Since the increase in Tb. vTMD is only a trend, and trabecular bone is more prevalent at metaphyseal regions, studies at this location are necessary to determine if WAIR enhances trabecular bone mass. In addition, these data suggests that perhaps increased duration or magnitude of WAIR training may be necessary to elicit a significant cortical bone response. The reason for the increase in Tb. vTMD due to handling is less clear. However, it may be due to the brown chickens exerting more muscle forces on the bones while being handled. Interestingly, there was a significant phenotypic difference between brown and white chickens within a treatment group for multiple outcome parameters. For example, within the WAIR treated group, we observed that cortical area, total area, cortical thickness, cortical bone volume and moments of inertia were significantly greater in the brown compared to the white chickens. Within the handled

group, we observed that cortical area, cortical thickness, cortical bone volume and moments of inertia were significantly greater in the brown compared to the white chickens. Within the control group, we observed that cortical area, cortical thickness and moments of inertia were significantly greater in the brown compared to the white chickens. These observations suggest that regardless of treatment, the brown chickens had significantly greater cortical bone mineral density and microstructure compared to the white chickens.

In my second study, I hypothesized that mice lacking the transcription factor, Brain and Muscle ARNT-like Protein 1 (*Bmal1*), would have an altered load-strain relationship compared to wild-type littermate mice. *Bmal1* is an essential element of the circadian clock and plays a crucial role in its functioning. I generated osteocyte-specific males and females *Bmal1*$^{-/-}$ mice and wild-type littermate controls (Dmp1Cre;Bmal$^{+/+}$ and Bmal$^{Fl/Fl}$ mice). I then assessed the load-strain relationship in these mice at 10 weeks of age. Results did not support the hypothesis; there were no difference in the *in vivo* tibial stiffness between Dmp1Cre;Bmal$^{Fl/Fl}$ mice compared to either WT mice, regardless of sex. These results suggest that the deletion of the *Bmal1* gene from the osteocyte does not have a significant impact on the whole bone stiffness properties. Further studies are warranted to determine if disruption of circadian rhythms alters bone geometry, material properties, and osteocyte networks in mice.

Résumé

Le squelette s'adapte à la force mécanique en se (re)modelant. Les ostéocytes, les mécano-capteurs de l'os, régulent le (re)modelage osseux en contrôlant la formation osseuse des ostéoblastes et la résorption osseuse des ostéoclastes. J'ai utilisé deux modèles animaux, des poules pondeuses et des souris, pour étudier l'adaptation des avec d'activité physique et de charge contrôlée, respectivement.

Dans ma première étude, j'ai émis l'hypothèse que l'exercice - Course Inclinée Assistée par l'Aile (CIAA), entre l'âge de 2 et 21 semaines, augmenterait la densité minérale osseuse et la microstructure de deux souches génétiques commerciales de poulets (Delkab white et Hyaline brown). J'ai mesuré la densité minérale osseuse et la microstructure de l'humérus chez des poulets de 36 semaines à l'aide du Micro-CT. Mes résultats ont montré que l'hypothèse n'était pas étayée car aucune différence significative n'a été observée entre les poulets manipulés ou traités au CIAA, ni entre les poulets témoins ou traités au CIAA. Nous avons cependant observé une tendance à l'augmentation du Tb.vTMD chez les poulets bruns qui ont été entraînés à la CIAA par rapport aux témoins (p=0,090). Nous avons observé une augmentation significative de la Tb.vTMD (p=0,007) et de la Tb.Th (p=0,018) dans le groupe manipulé (Tb.vTMD: 565,435±37,25, Tb.Th: 7,864±2,19) par rapport au groupe témoin (Tb.vTMD: 507,377±51,67, Tb.Th: 5,758±0,86). Nous n'avons pas mesuré de différences dans les résultats osseux en réponse au traitement chez les poulets blancs. Étant donné que l'augmentation de la Tb.vTMD n'est qu'une tendance, et que l'os trabéculaire est plus répandu dans les régions métaphysaires, des études à cet endroit sont nécessaires pour déterminer si la CIAA améliore l’os trabéculaire. En outre, ces données suggèrent qu'une augmentation de la durée ou de l'ampleur de l'entraînement CIAA pourrait être nécessaire pour obtenir une réponse significative de l'os cortical. La raison de l'augmentation de la Tb.vTMD due à la manipulation est moins claire, mais elle peut être due au fait que les poulets bruns exercent plus de forces musculaires sur les os lorsqu'ils sont manipulés.
Il existe des différences phénotypiques entre les poulets bruns et blancs pour plusieurs paramètres de résultats. Dans le groupe traité au CIAA, j’ai observé que la surface corticale, la surface totale, l'épaisseur corticale, le volume de l'os cortical et les moments

d'inertie étaient significativement plus élevés chez les poulets bruns que chez les poulets blancs. Les mêmes observations ont été faites avec des poulets bruns et blancs dans le groupe manipulé. Dans le groupe témoin, j'ai observé que la surface corticale, l'épaisseur corticale et les moments d'inertie étaient plus élevés chez les poulets bruns que les poulets blancs. Ces observations suggèrent qu'indépendamment du traitement, les poulets bruns avaient une densité minérale osseuse corticale élevés et une microstructure plus développée que les poulets blancs.

Dans ma deuxième étude, j'ai émis l'hypothèse que les souris dépourvues du facteur de transcription de l'horloge circadienne Brain-and-Muscle-ARNT-like-1 (*Bmal1*) présenteraient une relation charge-déformation altérée par rapport aux souris de type sauvage. J'ai généré des souris mâles et femelles avec une réduction de Bmal1 dans les ostéocytes et des témoins de type sauvage (Dmp1Cre;Bmal+/+ et BmalFl/Fl). J'ai ensuite évalué la relation charge-déformation chez ces souris à l'âge de 10 semaines. Les résultats ne confirment pas l'hypothèse; il n'y a pas de différence dans la rigidité tibiale *in vivo* entre les souris Dmp1Cre;BmalFl/Fl et les souris sauvage. Ces résultats suggèrent que la suppression du gène *Bmal1* dans l'ostéocyte n'a pas d'impact significatif sur les propriétés de rigidité de l'os entier. D'autres études sont nécessaires pour déterminer si les rythmes circadiens modifie la géométrie des os, les propriétés des matériaux et les réseaux d'ostéocytes chez la souris.

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Contributions of authors

Chapter 1 – Background and Rationale –
was completed by Meet Shah and revised by Prof. Dr. Bettina Willie.

Chapter 2 – The effect of WAIR on the humerus density and microstructure in egg laying hens.
Meet Shah – performed all microCT imaging, microCT analysis, and statistical analysis
Isabela Vitienes – assisted the microCT and analysis experiments
Bettina Willie – study design, supervisory support, funding procurements
Alexandra Harlander, Department of Animal Biosciences, University of Guelph, Ontario **–** conceptualized and supervised *in vivo* chicken study.
Tina Widowski, Department of Animal Biosciences, University of Guelph, Ontario **–** conceptualized and supervised *in vivo* chicken study.

Chapter 3 – Circadian rhythms in mechanoadaptation in mice.
Meet Shah – performed all the strain gauging and loading surgeries.
Catherine Julien – managing the mice colonies
Michael Bruccoleri – assisted all surgical procedures
Bettina Willie – study design, supervisory support, funding procurements
Dr. Nicolas Cermakian, Department of Psychiatry, Douglas Research Centre, Montreal – study design and knowledge of circadian rhythm.

In addition to the manuscripts that will result from the research presented in chapters 2 and 3 of this thesis, I also contributed on several other projects:

Meghan Morrell, Michael Bruccoleri, TBD… Catherine Julien, **Meet Shah**, Bettina Willie
Systemic administration of bone-targeting anabolic drug MES-1022 to accelerate early bone healing, *TBD*, In preparation.
My Contributions - Assisted with osteotomy surgeries.

Leanne van der Merwe, Michael Bruccoleri, **Meet Shah**, TBD…, Catherine Julien, Bettina Willie

Effect of loss of circadian rhythms in osteocytes on bone adaptation, *TBD*, In preparation.

My Contributions - Performed strain-gauging surgery and background strain gauging experiments.

List of figures:

List of Tables:

Chapter 1
Background and Rationale

1.1. Bone:

Bone is a dynamic, mineralized connective tissue demonstrating adaptive responses to external and internal stimuli [1, 2]. The skeletal system provides mechanical functions such as a structural framework to the body, locomotion through muscular kinetics, and protection of vital inner organs. Apart from being sites of hematopoiesis, bones also serve as integral reservoirs of growth factors and minerals that aid in the maintenance of homeostasis [1, 2].

1.1.1. Bone in humans:

The human skeletal system typically encompasses 270 bones at infancy, that upon fusion form the adult skeleton of 206 to 213 bones [2]. These bones form the appendicular skeleton (126 bones) and the axial skeleton (74 bones) [1]. Macroscopically, bones are categorized into long (including the clavicle, humerus, ulna, radius, metacarpal, femur, tibia, fibula, metatarsal and phalange), short (comprising bones of carpal, tarsal, patella, sesamoid), flat (bones of skull, mandible, scapula, sternum, rib), and irregular bones (including the vertebra, sacrum, coccyx, hyoid) [3].

Structurally, most bones demonstrate an outer superficial layer of organized dense, and solid compact or cortical bone, with an inner honeycomb-like meshwork of spongy marrow or cancellous trabecular bone [1, 3]. The healthy adult human skeleton comprises 80% cortical bone and 20% trabecular bone, however, the composition of each varies in all bones [1, 3].

Cortical bone is predominant in the long bones of the appendicular skeleton, comprised of osteons arranged as concentric lamellae in a cylindrical manner forming the Haversian system; organized in the direction of routine mechanical stresses [1, 3]. The diaphyseal shaft of the long bones includes the outer dense fibrous periosteum and the inner thin endosteum. The bone cells lining the periosteum and endosteum, are vital for osteogenesis, bone health, adaptation, remodeling, and repair. Healthy aging bones

demonstrate an increased remodeling of cortical bone, causing increased porosity and decreased bone mass [3]. Though cortical bone is less metabolically active, it is stronger and withstands higher impact forces than trabecular bone [1, 3].

The weight-bearing epiphyseal and metaphyseal ends of the long bones consist of trabecular bone within the cortical bones [1]. Unlike cortical bone, trabecular osteons are called packets that are arranged in plates and rods in a meshwork interspersed with marrow tissue. This porous structure of trabecular bone permits extensive yielding to stresses and low-grade forces [1].

Osteogenesis or ossification is a dynamic process that is initiated between the sixth and seventh weeks of intrauterine life and continues in adulthood till approximately twenty-five years of age [4]. Endochondral ossification is observed in the long and short bones and involves the transformation of precursor mesenchymal tissue to cartilaginous tissue, ultimately forming bone. Whereas intramembranous ossification is characterized by direct bone formation from mesenchymal tissue, such as in flat bones [1, 4].

The osteoblasts, osteocytes, osteoclasts, and bone lining cells of the mesoderm, constantly interact to form, maintain, remodel, resorb, and repair bone [1, 4]. Osteoblasts (4-6% of the total resident bone cells) or bone-forming anabolic cells are mesenchymal progenitors that secrete unmineralized matrix protein, osteoid, that calcifies to form bone [5]. Transforming growth factor beta (TGF-β) / bone morphogenic protein (BMP) signaling and Wnt signaling, are essential pathways that regulate osteoblastic activity and maintain balanced bone deposition and resorption [1, 4, 6]. The most abundant bone cells are the osteocytes (90-95% of total bone cells), which form from osteoblasts trapped in the unmineralized osteoid. It is worth mentioning that these cells have a lengthy lifespan and play a role in facilitating adaptive reactions to mechanical stress and hormonal signals in order to preserve the balance of bone in the body [7, 8]. Osteocytes crucially regulate the remodeling of bone by controlling osteoblastic and osteoclastic activity through sclerostin and RANKL [4, 5]. Primarily functioning as mechanosensors, osteocytes prove a vital role in micro-environmental stress detection and bone mechanotransduction [1, 9-11]. Osteoclasts, or bone-resorbing catabolic cells, arise from macrophages and function in response to damaged or disused bone. Osteoblasts and osteoclasts interact via the

RANK/RANKL/OPG pathway to maintain bone homeostasis and remodeling [1, 5]. Osteoporosis of bone occurs due to overactivity of the osteoclasts [4].

Bone growth and development is a dynamic physiological process involving longitudinal and radial growth, bone modeling, and remodeling. Longitudinal and radial growth occur during the childhood and adolescence stages, primarily in the long bones that function as the load-bearing bones of the skeleton. The epiphyseal and metaphyseal plates of the long bones routinely withstand physical loads during daily activity [1, 3]. Bone modeling involves constructive changes in bone owing to mechanical forces or physiological stresses. Modeling is chiefly orchestrated by osteoblastic activity, leading to the apposition of new bone [1, 3]. Bone remodeling, on the other hand, is a complex, restorative, and homeostatic process, involving simultaneous osteoclastic-osteoblastic activity to resorb old bone and form new bone [1, 3].

1.1.2. Measuring bone strength, structure and density:

A cascade of collaborative events underlying bone deposition, adaptation, resorption, and regulation, in response to mechanical stresses, at macroscopic and microscopic levels, influence bone strength [1]. Bone strength is the bone's resistance to fracture for a given load. Recent scientific advancements prove promising to examine, measure, and monitor bone strength, to enable studying skeletal health and preserve bone strength. Imaging methods are advantageous for longitudinal investigations because they enable many measurements to be taken from the same subject at different points in time.

Micro-CT is a technique used to examine the microarchitecture of trabecular and cortical bone at a very tiny scale. It offers detailed information on the structure of the bone with resolutions as fine as 1 to 6 μm. Micro-CT scanners involve rotating the specimen at specific angles between the x-ray source and detector [12]. The attenuation data obtained at each position are then reconstructed into a 3D array of x-ray attenuation. These attenuation values can be converted into mineral density values by incorporating suitable calibration phantoms. The outcomes measured in this study are bone volume fraction (BV/TV), trabecular thickness (Tb.Th), trabecular spacing (Tb.Sp), trabecular number (Tb.N), trabecular connectedness, and true tissue mineral density (TMD, which is the

mass of mineral divided by the volume of bone tissue) [12]. The advent of desktop *in vivo* micro-CT scanners has facilitated the analysis of the overall shape and microscopic structure of the bones in living animals. These scanners have made it possible to conduct longitudinal studies that investigate skeletal growth, adaptation, and response to therapy in the same animals. The scanners can achieve an isotropic resolution of up to roughly 10 μm [12]. However, achieving such resolutions requires longer scan periods and higher radiation doses. These studies have limitations such as their focus on tiny rodents and the requirement to control the amount of ionising radiation that the animals are exposed to.

1.1.3. Bone in chickens:

Poultry farming, a key component of the global agricultural sector, is broadly divided into layer poultry farming and broiler poultry farming and deals with the breeding, production, and processing of table eggs, chicken (Gallus gallus), and broiler meat [13, 14]. Chicken or the common laying hens are genetically modified descendants of the wild red jungle fowl of India [13, 14]. Egg laying is affected by various factors such as quality and quantity of feed, water consumption, intensity and duration of daylight and artificial light, management, and housing characteristics [15, 16].

A strong skeleton is integral to a productive laying hen. Increased incidence of skeletal weakness and bone fractures due to osteoporosis, in modern laying hens is a pressing animal welfare implication reported as cage layer fatigue [17]. This has been attributed to the lack of opportunity for physical activity of the birds due to battery cages since 1955 [18]. Subsequently developed alternative housing systems provided sufficient room for hens to run, perch, and flap wings, thereby stimulating bone health globally [19]. However, it may be deemed mandatory to further delve into understanding the complexities of the avian skeletal system and bone structure.

The skeletal system and bones of the hen are homologous to humans, bearing minor differences such as pneumatic bones and fused vertebrae to facilitate flight [13, 14]. The sternum or keel is modified to attach flight muscles, while ribs are modified to include an uncinate process to reinforce the rib cage [13, 14]. The arms and legs of hen share skeletal similarities with humans. Bone microstructure in chickens mimics

mammalian bones, involving osteoblasts, osteocytes, and osteoclasts, forming cortical and cancellous osseous structures [13, 14]. Hormone levels such as growth hormone, parathyroid hormone, calcitonin, and Vitamin D highly influence bone formation, bone health, and eggshell formation [13, 14]. The growth and development of the chicken skeleton can be broadly divided into two phases of their lifespan: the rearing period and the laying period [20]. The rearing period involves the transformation of pre-existing tissues to form bone, through prechondral (membranous) and chondral (cartilagenous) stages [13, 14, 20]. Though the majority of the chicken bones develop via the cartilaginous stage, the membranous stage is observed in the egg during embryonic development [13, 14, 20]. Chondroblasts secrete cartilaginous matrix at the head of the growth plate, that subsequently ossifies to form bone. In the avian skeleton, bone is primarily laid down in successive layers to form dense, compact bone surrounded by cellular periosteum [13, 14, 20]. Long bones demonstrate a hollow internal structure filled with bone marrow and air sacs. The formation of special cavities is observed in the compact bones, that form concentric layers of new bone, similar to the haversian system [13, 14, 20]. The rearing period culminates in osteoclastic activity for bone remodeling in response to mechanical stimuli [13, 14, 20].

The laying period is characterized by sexually mature hens with structural changes in the skeletal system to adapt for egg production [13, 14, 20]. The reproductive period of a hen is encountered by highly unstable secondary bone within marrow spaces called medullary bone, which also acts as a reservoir of calcium for strong and thick eggshells [13, 14, 20]. The surge of estrogen and androgen in serum stimulates medullary woven bone formation approximately two weeks before laying an egg and continues forming throughout her reproductive phase, emphasizing the importance of a high calcium diet [13, 14, 20]. Calcium depletion leads to weak skeletons and a decline in egg production, with thinner eggshells. Progressively, paralysis or cage layer fatigue is observed, which is associated with muscular paralysis, amplified fracture risk, and osteoporosis [13, 14, 20].

1.1.4. Bone in mice:

Among animal model studies, the common laboratory mouse (Mus musculus) is a key validated model for global biological research [21, 22]. Despite the small comparative size, the mouse has proved impactful for exploring human physiology and pathology, due to various reasons [21, 22]. Apart from being cost-effective models, with quickened lifespan (30 human years account for one mouse year), and easy handling, mice are biologically comparable to humans and share approximately 30,000 genes with human orthologues [21-23]. Among the various mice strains, the C57BL/6 mice are widely applied in academic research, especially for knockout or targeted mutant models [22].

Mice and humans demonstrate similar structural and functional roles of bone anatomy, histology, and physiology, bearing a few dissimilarities, such as skeletal size, tail morphology, and orientation of shoulder and pelvic girdle [24]. The mouse skeleton, however, has thinner articular cartilage with persistent growth plate remnants in long bones [24]. Another vital observation is the presence of circumferential lamella and the lack of osteons and Haversian remodeling in the mouse cortical bones [24]. The majority of mice bones develop by endochondral ossification and are classified similarly as long, flat, short tubular, and sesamoid bones.

Murine long bones, such as the femur and tibia, also have the epiphysis, diaphysis, and metaphysis. Bending forces predominate at the diaphysis, accounting for the thickest cortical bone [24]. Likewise, the metaphysis and epiphysis have thicker cancellous bones to sustain various compressive forces [24]. These similarities in the mouse and human bones aid scientific animal research in further understanding human bone physiology, modeling, and remodeling.

1.1.5. Table 1: Differences in bone of humans, chickens and mice: [13, 14, 24-27]

Factor	Human	Chicken	Mouse
Number of bones	206 to 213	120	200
Gross features of different types of bones			
Axial skeleton – Skull	22 cranial bones –	28 cranial bones – frontal, parietal,	8 cranial bones –

	Ethmoid, frontal, occipital, parietal, sphenoid, temporal, 14 facial bones	temporal, occipital, ethmoid, sphenoid, 14 facial bones	Occipital, interparietal, parietal, frontal, squamosal, 9 facial bones
Vertebral column	33 vertebrae – 7 cervical, 12 thoracic, 5 lumbar, 5 sacral, 4 coccygeal	Vertebrae – 14 cervical, 7 thoracic, 14 lumbo-sacral, 6 caudal	61 Vertebrae – 7 cervical, 13 thoracic, 6 lumbar, 4 sacral, 27-31 coccygeal
Ribs	12 pairs	7 pairs	13 or 14 pair
Appendicular skeleton – Girdles	Shoulder/ pectoral girdle - (scapula and clavicle) hip/ pelvic girdle - (ilium, ischium, and pubis).	Pectoral girdle – 3 bones (coracoid, scapula, furcula), sternum, pelvic girdle – 3 bones (ilium, ischium, pubis)	pectoral girdle – (dorsal scapula and ventral clavicle) pelvic girdle - innominate bones - fused ilium, ischium, and pubis
Limbs	Upper limb – humerus, radius, ulna, 8 carpals, 5 metacarpals, 14 phalanges/ each side. Lower limb – femur, tibia, fibula, 7 tarsals, 5 metatarsals, 14 phalanges/each side	Wings – humerus, radium, ulna, 3 digits/ each side Legs – femur, patella, tibiotarsus, fibula, tarsometatarsus, metatarsal, 4 digits/ each side.	Forelimb – humerus, radius and ulna, eight carpals, five metacarpals, five first phalanges, four second phalanges, and five third phalanges with their tips / side Hindlimb – femur, tibia and fibula, seven tarsals, five metatarsals, and the same number of phalanges as in the forepaw / side

Microscopic features			
Cortical bone	Osteons present	Osteons present	Osteons absent; porosities develop with age
Cancellous bone	Secondary spongiosa absent after skeletal maturity		Secondary spongiosa present during first several months of life and decreases thereafter
Growth plate	Present only during	Persists throughout life	Persists throughout life
Articular cartilage	Thickness depends on joint; uniform subchondral plate; tidemark present	Similar to human, depends on joint	Thinner than human; variability in thickness of subchondral plate; no clear tidemark (in skeletally immature animals)
Bone marrow	Hematopoietic marrow during development and in adult axial spine; fatty in adult appendicular skeleton	Hematopoietic marrow in axial and appendicular skeleton throughout life	Hematopoietic marrow in axial and appendicular skeleton throughout life
Woven bone	Present	Present	Present
Lamellar bone	Present	Present	Present
Medullary bone	Absent	Present – woven bone that acts as a labile source of calcium for eggshell formation. It lines structural bone and	absent

		also occurs as spicules within the marrow cavity.	
Brown fat	Mostly involutes but can persist in adulthood in neck region	Present throughout life	Present in adulthood largely in dorsal intrascapular region
Growth and development			
	Endochondral and intra-memberanous	Rearing period – prechondral (membranous) and chondral (cartilagenous) stages. Laying period – medullary bone formation	Endochondral

1.2. Mechanical loading on bone (mechanotransduction):

The remarkable capacity of bones to regenerate fractured or injured bone and to conform to mechanical loads to fulfill functional requirements has been well documented [28]. Through adaptive remodeling, the human skeleton continuously adjusts to its mechanical loading environment, demonstrating new bone formation in response to increased stress and losing bone in reaction to disuse and unloading [9]. Literature reveals that mechanical loading and stresses on bones are necessary for the ideal growth and development of strong, weight-bearing bones. The link between mechanical stress and the adaptation of bone microarchitecture was explained by Julius Wolff in 1892 [29]. It states that this adaptation is a dynamic, self-regulating process and that trabeculae formed near joints are dictated by the direction of stresses, which can be explained and predicted by mathematics [29]. Wolff's law can be used to explain why regions with high mechanical stress exhibit higher bone mass, whereas regions with low mechanical stress exhibit lower bone mass. Lack of load or exercise has been shown to decrease bone

mass to only 30-50% [30]. The macro- and microstructures of bones, such as bone length, cortical thickness, cross-section geometry, and bone curvature, have also been investigated to better understand this relationship.

These paradigms of bone modeling and remodeling that were first developed by Julius Wolff, refined by Wilhelm Roux (Wolff's Law), and extended by Harold Frost (Mechanostat Theory) continue to be at the core of new and current research [1]. The scientific and mathematical concepts of bone adaptation, maintenance, and repair caused by the load are acknowledged and understood. However, the cellular mechanisms of this phenomenon yet remain to be completely studied.

Bone cells play a crucial role in bone adaption and are thus influenced by mechanical stresses. Mechanotransduction can be categorised into the subsequent stages: The three main processes involved are: 1) detection of mechanical stresses, 2) conversion and transmission of mechano-biochemical signals, and 3) response of effector cells. The phases and mechanotransductive signals that occur within them happen at significantly diverse time intervals (ranging from seconds to minutes for [Ca2+]i transients and ATP release, hours for gene expression, days for cellular responses and weeks for tissue adaptation) [29].The initial stage of mechanosensation entails the transformation of a mechanical force exerted on the bone into a localised mechanical signal that is detected by bone cells. While marrow stromal cells, osteoclasts, and osteoblasts have all been observed to react to mechanical stimulation, osteocytes are particularly well-suited to sense mechanical strain and convert it into biochemical signals for resorption and formation due to their widespread distribution and interconnectedness within the bone matrix [29].

The cell bodies of osteocytes are situated within lacunae that are filled with fluid, and they are interconnected. Osteocytes are linked to osteoblasts and bone-lining cells on the surface of the bone by dendritic processes, which are surrounded by a canalicular network of channels in the bone matrix [29]. The prevailing belief is that the primary mechanical signal at the local level is strain, which can arise from either direct tissue deformation of the bone matrix or deformation of osteocytes through interstitial fluid flow [29]. Osteocytes have a greater level of sensitivity to fluid flow shear stress compared to other types of mechanical strain, such as substrate stretching. Research has

demonstrated that regulating the movement of fluid outside of the bone structure, without causing strain to the surrounding tissue, may produce a bone-forming response that is comparable to the effects of mechanical stress [29].

The last stage of mechanotransduction entails the tissue-level reaction carried out by the effector cells, namely the osteoblasts and osteoclasts, which are responsible for the formation and resorption of bone, respectively [29]. During the process of modelling, osteoclasts and osteoblasts are activated separately. However, during the process of remodelling, bone resorption and creation are closely linked in both space and time, occurring inside a tiny bone unit known as a basic multicellular unit (BMU) [29]. Remodelling takes place in three primary phases known as A-R-F: osteoclast activation, osteoclast resorption, and osteoblast formation [29]. The formation phase encompasses the synthesis of osteoid by osteoblasts, which is subsequently accompanied by the deposition of minerals inside and amidst the collagen fibres. Osteoclasts efficiently resorb human bone at a rate of around 40 μm per day, while osteoblasts construct human bone at a slower pace of roughly 1 μm per day [29]. Modelling has a substantial impact on the total number and form of bones, but remodelling often leads to little alterations in bone mass and structure [29]. In general, there is a consensus that bone adjusts to its mechanical surroundings by modifying its structure, material qualities, and arrangement [29]. In addition, bone cells, namely osteocytes, osteoblasts, and osteoclasts, function as detectors and implementers of these alterations.

1.2.1.Mechanoadaptation in chickens:

1.2.1.1. Types of chickens and ancestors:

The global classification of chicken breeds is extensive and includes country specific databases such as the Australian Poultry Standard (60 breeds), the British Poultry Standards (93 breeds), and the American Poultry Association (6 broad classes) [31]. The American Poultry Association classifies the commonly bred chicken as American, Asiatic, Continental, English, Mediterranean, and all other standard breeds (AOSB) [31]. The Lohmann white and Lohmann brown birds are the predominant strains utilised in Canada.

Broadly, the purpose of breeding chicken defines its housing and raising conditions. Within the poultry industry, chicken may be bred for the purpose of their meat (commonly

termed as broilers), egg-laying (referred to as layer hens), dual-purpose, and exhibits (also known as show poultry) [31]. Chicken that are raised to produce meat and eggs are generally factory-farmed and spend their short-lived lives indoors with lack of sunshine or fresh air. Layer hens usually are housed in groups of 4-10 in crowded battery cages, restricting movement and spread of wings [31]. Cage-free chicken on the other hand, enables the chicken to perch on multi-level aviaries that are housed indoors, proving a slight improvement over the battery cages, though not ideal [31].

1.2.1.2. Problem with egg layers:

Laying chickens possess a distinctive process for the formation of eggshells. Medullary bone is a kind of bone characterised by its weaving structure. It serves a crucial function as a storage site for calcium, which is later used in the formation of eggshells [20, 32, 33]. Nevertheless, it is important to note that it has less mechanical strength compared to structural bone (cortical and cancellous) and does not contribute to overall bone strength [20, 34]. This bone undergoes fast remodelling during the egg-laying cycle. It is actively produced in the bone marrow before the eggshell calcification begins in the shell gland [33].

Estrogen has a vital role in the formation of medullary bone. Endosteal bone-lining cells possess oestrogen receptors, and oestrogen influences their transformation into osteoblasts. [35, 36]. Parathyroid hormone (PTH) is increased in response to low levels of calcium in the blood due to the increased need for calcium in the formation of eggshells [37]. PTH promotes the breakdown of bone tissue by osteoclasts in the inner part of the bone, leading to an increase in the amount of calcium in the blood [38].

Osteoclasts facilitate bone resorption by removing the calcium from the inorganic matrix and breaking down the organic matrix of the medullary bone [39, 40]. During the late laying stage, laying hens have a loss of structural bone, which can result in severe skeletal issues like osteoporosis. This not only affects the health and well-being of the animals but also has economic implications [41-43].

1.2.1.3. Reasons for fracture and disuse:

The main flight muscles of birds are the pectoralis and supracoracoideus, specifically adapted to endure significant tension and strain during flying [44]. The pectoralis muscle is the greatest wing muscle, and it is hypothesised that it applies a force to the keel bone during wing-flapping. The pectoralis major and minor muscles are attached to the keel bone and connect to the humerus bone. These muscles are responsible for generating the greatest amount of mechanical force during flight [45]. Keel bone fractures are one of the most common fractures associated to the commercial egg lying hens. Significant heterogeneity exists in the susceptibility to keel bone fractures across various strains of laying hens, suggesting that genetics may play a role in determining susceptibility to such fractures [46]. While most studies investigate bone biology and mechanical strain of chicken bones like the tibiotarsus and the keel, little is known about the humerus bone which is a major entity in the flight response of the birds.

One crucial aspect to take into account regarding the well-being of laying hens is their housing and the activities related with it. While there is a lack of extensive study on the impact of physical activity on bone formation and resorption in laying hens, existing studies have indicated that reduced physical activity or confinement results in bone loss and decreased bone mineral density in both the femur and tibia of young female chickens [47].

1.2.1.4. Physical activity and bone physiology in chickens:

During the raising phase, which is a critical stage for bone growth, it is important to provide the chickens with opportunities to engage in natural behaviours including running, wing flapping, pecking and perching [48]. One effective approach to promote movement is incorporating perches in the rearing habitat. This practice has been found to enhance the bone mineral content of the tibia, as well as boost leg muscle strength and overall body weight [49].

1.2.1.5. Studies reporting the effect of housing on bone parameters:

Modern laying hens have been bred for a remarkable level of egg production, but their skeletal health is significantly affected by the high calcium requirements. Bones can

be evaluated using a mix of many structural and mechanical examination techniques, both in living organisms (*in vivo*) and outside of living organisms (*ex vivo*). Usually, the characteristics of leg, wing, and keel bones are assessed. Conventional caged layers experience limited mobility, leading to an imbalance between the breakdown of structural bone and the production of new bone, ultimately causing osteoporosis. Hens housed in alternative systems have the chance to engage in physical activity, which promotes bone formation. However, they are also more prone to experiencing keel fractures and deviations, which are likely caused by collisions or pressure. There has been a lack of extensive study undertaken in commercial housing systems to evaluate the skeletal health of hens, specifically the occurrence of keel injury in different types of systems [50]. Recent worldwide research undertaken within the past decade on both brown and white hen strains has consistently demonstrated that bone health is compromised in all housing settings [50]. Keel-bone injury is a significant problem because to its high occurrence rates, especially in multi-tiered systems. It causes discomfort, can change hen behaviour, and negatively impact both productivity and egg quality. Implementing management methods, such as the installation of ramps to facilitate access to perches and tiers, can help mitigate the occurrence of keel-bone injury to some extent. Enhancing bone strength can be achieved by providing activity opportunities, especially during the pullet raising phase. Genetic selection for enhanced bone strength may be essential for hens to effectively adjust to bigger housing systems, but the most effective approach for enhancing skeletal health is likely to involve many factors [50].

Comparative research was conducted to assess the egg quality, carcass, meat qualities, and bone properties of Taihang chickens in two distinct housing systems (traditional cages - CC or on the floor - FF housing) across different age groups [51].Their results demonstrated that the FF chickens superseded the CC birds among numerous parameters such as weight of hens' eggs, the height of albumen, and the Haugh unit, egg quantity, weight of the chickens, percentage of breast meat, weight and breaking strength of the humerus and tibia [51]. Overall, the age of hens and their interaction within the housing systems have the potential to impact the slaughter performance, egg quality, meat quality, and bone quality of Taihang chickens [51].

Staaveren et al. demonstrated that the development of novel technologies to allow pullets to perch and forage in these systems would become more crucial as Canada's laying hen housing system transitions from traditional cages to supplied cage and non-cage housing systems [52]. They also stated that for non-cage housing solutions, farmers should also follow explicit litter management rules to maintain good litter quality [52].

Rentsch et al. conducted a study to evaluate the effects of early environmental complexities and genetic strains on the development of laying hen locomotion [53]. The authors observed that the white chicks outperformed browns in both spatial tests, but aviary-reared birds outperformed those raised in traditional cages [53]. White pullets grown with high complexity performed better in 3D space compared to those raised with moderate or low complexity (High > Mid, Low), whereas browns did not. Brown chicks had lesser incentive for test participation than white chicks, which might explain why they performed differently as pullets [53]. Their findings suggest a gene-environment interaction in the development of spatial abilities in laying hens with white feathered pullets but not browns, which improve vertical navigation skills as spatial complexity increases [53].

1.2.1.6. Studies reporting the effect of exercise on bone parameters:

Domestic laying hens mostly use their hindlimbs for walking on land. Despite engaging in flapping flight, these creatures seem to use their maximum force during descent, which may result in a lack of control for manoeuvring and avoiding injury upon landing. Consequently, this might lead to harm in open rearing systems. WAIR is a technique where a bird uses its wings to help its hindlimbs while climbing an incline [54]. Training in WAIR can be a beneficial way to enhance a hen's power reserve and flying control. In this study, the researchers exposed chickens to a workout routine that included inclines in order to produce weight-induced aerobic resistance over a period of 16 weeks during their early development stage [54]. The researchers recorded the movement of the wings and body while the subject descended from a platform that was 155 cm high [54]. The hypothesis was that birds raised with exercise would have improved ability to adjust their wing and body movements in order to achieve slower and more controlled fall and landing. Birds with brown feathers displayed higher wing beat frequencies than birds with

white feathers, which aligns with the increased wing loading of brown-feathered birds. Additionally, birds trained with WAIR demonstrated larger beginning flight velocities compared to control birds [54]. This suggests that the WAIR training may have enhanced the ability to control flight speed and strengthen the muscles in the legs. Introducing inclination workouts throughout the raising process might enhance the well-being of adult laying hens. This is because a higher initial flight velocity would decrease the energy needed to sustain their body weight in the air, enabling hens to utilise their extra force for manoeuvring [54].

According to another study, farmed hens may inhabit conditions that limit the use of their wing muscles [55]. It is hypothesised that decreased wing movement and the resulting muscular weakening are risk factors for keel bone fractures and deviations. The researchers employed radio-frequency identification to quantify the amount of time spent at elevated resources such as feeders and nest-boxes [55]. They used ultrasonography to measure changes in muscle thickness in the breast and lower leg, radiography and palpation to identify fractures and deviations. These measurements were taken after subjecting the birds (both white-feathered and brown-feathered birds) to three different levels of immobilisation: no immobilisation, partial immobilisation using a one-sided wing sling, and full immobilisation in a cage. The researchers hypothesised that partially immobilised hens would lower their excessive resource utilisation, and that both groups of immobilised chickens would exhibit a drop in pectoralis thickness due to disuse, as well as an increase in the occurrence of fractures and deviations [55]. The use of nest-boxes significantly decreased by 42% after five weeks of partial immobilisation for brown-feathered hens. Completely unable to move, chickens with white feathers saw a 17% decrease in the thickness of their pectoralis muscle [55]. This study demonstrates a significant contrast between hens with white feathers and hens with brown feathers in their reaction to wing immobilisation and the corresponding muscle physiology [55].

There is a significant gap in the research done on the effects of WAIR on ramps greater than 40 degrees, to evaluate the bone microstructure of chickens' humerus. Hence, we proposed this study to elaborate on the effects of prolonged exercise improves the microstructure of the humerus bone, in egg laying hens.

1.2.2. Mouse as a model for studying mechanoadaptation:

Laboratory mice are a highly suitable animal model for biomedical research and comparative medicine studies due to their significant resemblances to humans in terms of anatomy and physiology. Similarly, mice and humans possess around 30,000 genes, with approximately 95% of these genes being common to all two species [23]. The utilisation of mice for research purposes offers economic benefits. Mice, due to their small size, require minimal space and resources for maintenance. Additionally, they have short gestation periods but produce a significant number of offspring. Furthermore, they exhibit rapid development to adulthood and have relatively short life spans. As an illustration, mice have a gestation period of around 19-21 days [23]. They may be weaned at three to four weeks old and become sexually mature by five to six weeks old. This enables the rapid generation of a large number of mice for research purposes. The availability of the whole nucleotide sequences for all three species has for comprehensive comparisons across species, which have been crucial for the discovery and characterisation of genes [23]. The utilisation of advanced molecular genetic techniques enables the manipulation of genes in mice. This allows for the suppression of gene expression (known as "knocking out") or the controlled expression of genes during specific stages of development or in specific tissues [23]. These techniques are employed to gain a deeper understanding of the normal function of genes and their involvement in disease [23].

1.2.2.1. Mechanoadaptation in mice:

In vivo compression tibial loading in mice has been utilised to evaluate bone adaptability and mechanotransduction in munerous loading investigations. To induce bone formation, it is necessary to achieve a strain magnitude that is larger than the minimum effective strain during loading [56]. The advantages of this model include: 1) the ability to apply and isolate a specific load to a targeted limb, allowing for precise description and quantification of the load unlike other exercise models, 2) the use of the opposite limb as a control within the same animal, and 3) the ability to study both cancellous and cortical bone adaptation. Additionally, this model may be utilised to examine individual, uninterrupted, stationary, and changing loading circumstances by modifying the load cycles and waveform [56].

Mineralized bone growth necessitates the application of dynamic stresses on a daily basis for a period of 1 to 2 weeks [57]. The commonly employed techniques for evaluating the results of these loading experiments are microcomputed tomography and histomorphometry. Multiple studies have documented the bone formation reaction to mechanical stress in these mouse models, encompassing both males and females, as well as young and fully matured C57Bl/6 mice, which are the most often utilised animal models. Female C57Bl/6 mice and female BALB/c mice, aged six weeks, had higher cortical bone volume compared to their counterparts of the same age. Additionally, both genetic strains shown a diminished bone production response to loading as they aged [58-62]. Mechano response of bones of conditional knock-in/knock-out mouse models can be studied using same loading model. The use of these mouse models plays a crucial role in the advancement of therapeutic approaches for the treatment of bone disorders, such as osteoporosis.

1.2.2.2. Advantages of the tibial loading model:

The mice are used for various loading experiments for tail vertebrae, tibias and ulnae using multiple loading protocols [63-65]. The ability of individual bones to endure regular pressures without breaking is created and maintained, at least partially, by functional adaptation to the stresses caused by these loads. Bones adapt to variations in mechanical stresses caused by changes in load by adjusting their remodelling processes to maintain the right structure and density of cortical and trabecular bone. While this notion is widely acknowledged, the specific processes that support it have yet to be determined. In order to tackle this issue, researchers have created animal models that allow for targeted loading. These are valuable for determining how loading affects the process of remodelling and enable the study of the processes that trigger and coordinate these events.

The initial endeavour to investigate the reaction of bone to specific and regulated artificial stresses was the insertion of Kirschner wires into the tibiae of rabbits. These investigations demonstrated that dynamic stress, as opposed to static loading, triggered adaptive responses in the bone. These reactions were independent of central nerve input and were endogenous to the bone itself.

Nevertheless, the process of incorporating these models had a significant drawback as it necessitated surgical intervention, resulting in trauma, heightened susceptibility to infection, and direct impact on bone cell metabolism. Therefore, it is desirable to have a calibrated model that enables regulated non-invasive loading.

These advancements, however, have facilitated the examination of trabecular bone and also emphasise the practicality of utilising laboratory animals. Therefore, it is beneficial to have non-invasive models that allow for the application of controlled mechanical stresses to a bone, enabling the examination of both cortical and trabecular responses in a suitable laboratory animal. While it offers convenience and control, four-point bending of the tibia depends on applying direct pressure to the periosteum of the diaphysis. Although cantilever-like bending of the mouse tibia and axial loading of rat ulna can address this drawback, neither method permits the study of loading responses in trabecular bone. The deficit may be remedied by applying non-invasive controlled axial stress to the mouse tibia, which enables the measurement of both trabecular and cortical responses. Furthermore, the tibia is the most often examined bone for studying the impact of disuse, ovariectomy, and corticosteroids on bone remodelling.

1.2.2.3. Studies using the tibial loading model:

Due to the various aforementioned advantages of this tibial loading mouse model, bone researchers have recently applied it in their practice.

Yang et al. conducted a study to analyse the gene expression, bone formation, and structural changes in the cancellous and cortical bone of female C57Bl/6 mice in response to mechanical loading [66]. The loading experiment demonstrated a considerable increase in cortical bone mass at the tibial midshaft across all force levels tested. However, only the high load had a notable impact on bone mass and bone production indices in the proximal metaphyseal cancellous bone. The finite element analysis revealed that the maximum tensile or compressive stresses that promoted bone formation in the proximal cancellous bone under high load were considerably higher than the strains that promoted bone formation in the midshaft cortical tissues under low load. These findings indicate that the level of strain stimulus that controls the structural, cellular, and

molecular responses of bone to loading may be higher for cancellous tissues compared to cortical tissues [66].

In ageing animals, the capacity of bone to adjust its mass and structure to meet the demands of bearing weight is reduced, leading to the typical bone loss seen in osteoporosis. A recent study by Galea et al. examined the alterations in gene expression patterns that are linked to the compromised adaptive response [67].

1.3. Circadian rhythms in bone:

The circadian clock is an intrinsic system found in the majority of animals that coordinates their physiology and behaviour with the rotation of the Earth [68].The suprachiasmatic nucleus (SCN) in the brain serves as the central clock in mammals, whereas most other tissues house the peripheral clock [69, 70]. The central clock is well recognized as the mechanism that coordinates and regulates the peripheral clock via neuronal and hormonal pathways [71-73]. Nevertheless, this notion was questioned when the specialised involvement of aryl hydrocarbon receptor nuclear translocator-like (*Bmal1*) in different tissues was uncovered, indicating that the peripheral clock, particularly peripheral *BMAL1*, may have its own distinct function in peripheral tissues that is not reliant on the central clock [74]. The central clock and the peripheral clock have a same molecular mechanism and consist of a self-regulating transcriptional-translational feedback loop [75].

The system consists of two positive components, *BMAL1* and circadian locomotor output cycles kaput (*CLOCK*), as well as two negative components, period circadian clock (*PER1, PER2, and PER3*) and cryptochrome (photolyase-like) (*CRY1 and CRY2*), as depicted in Figure 1. In general, the *BMAL1: CLOCK* transcription factor, which consists of two different proteins, promotes the transcription of genes associated to the circadian rhythm, such as *Per* and *Cry*. This activation occurs by binding to specific regions called E-box elements in the promoters of these genes. These E-box elements have a sequence similar to *CACGTG* or *CACGTT* [76-79]. *PER* and *CRY* combine to create a complex and subsequently hinder the transcriptional activity of *BMAL1: CLOCK* [80, 81]. Osteoblasts and osteoclasts exhibit the expression of clock genes [82, 83]. The deposition of minerals

in the bones of mice in calvarial organ cultures exhibited a 24-hour cycle and might be linked to the expression of clock genes [84].

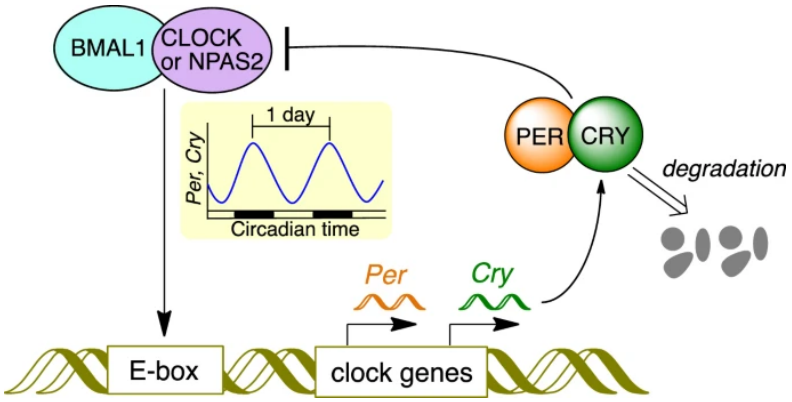

Figure 1: Schematic illustration depicting the feedback loop of circadian cycle. [85]

1.3.1. Studies evaluating circadian rhythm in mice:

The proper functioning of several organs, including bone, relies heavily on physical pressures. Remarkably, the timing of physical activity throughout the day influences the functioning and genetic activity in several organs as a result of circadian rhythms. Tissues, like bone, have circadian clocks that contain circadian clock genes [86]. These clock genes regulate particular genes in the tissue, leading to the production of tissue-specific genes in a rhythmic manner (known as clock-controlled genes). Bouchard et al. postulated that the adaptive response of bone to mechanical pressure is governed by circadian cycles. Initially, mice were subjected to a simulated load and then euthanized after 8 hours [86]. This resulted in the collection of tissues at certain time points known as zeitgeber Zeit (ZT)2, 6, 10, 14, 18, and 22. The cortical bone of the tibiae obtained from these mice exhibited daily fluctuations in the expression of core clock genes and important genes associated to osteocytes and osteoblasts, such as *Sost* and *Dkk1*, which are known to be regulated by the body's internal clock. The serum bone turnover indicators did not exhibit any rhythmic patterns. Furthermore, the mice were subjected to a solitary instance of *in vivo* loading either at ZT2 or ZT14, and were then euthanized after 1, 8, or 24 hours [86]. When loaded at ZT2, there was an increase in *Sost*

expression. However, loading at ZT14 resulted in downregulation of both *Sost* and *Dkk1*. Furthermore, the mice were subjected to daily tibial loading *in vivo* for a duration of 2 weeks. This loading was provided either in the morning (ZT2, during the resting phase) or in the evening (ZT14, during the active phase). MicroCT imaging was conducted in living organisms on days 0, 5, 10, and 15, whereas conventional histomorphometry was conducted specifically on day 15. All the measurements taken demonstrated a strong reaction to the applied load [86]. However, only the timelapse morphometry based on microCT scans revealed that loading at ZT14 led to a more significant increase in endocortical bone production compared to animals loaded at ZT2. The reduced expression of *Sost* and *Dkk1*, together with the small yet substantial time-of-day specific increase in adaptive bone production, indicates that circadian clocks play a role in influencing bone mechanoresponse [86].

Another study by Samsa at al. revealed that mice lacking *BMAL1* exhibit a phenotype characterised by reduced bone density, which is not present at birth but deteriorates gradually as they age [87]. The accelerated ageing of these mice is linked to the development of osseous bridges that form between the metaphysis and the epiphysis, leading to a reduction in the length of their long bones. The authors demonstrate by micro-computed tomography that *Bmal1*−/− mice have decreased cortical and trabecular bone volume, as well as other micro-structural parameters, and a reduced bone mineral density [87]. The histological analysis reveals that a lack of *BMAL1* leads to a decreased population of functional osteoblasts and osteocytes in living organisms. The isolation of mesenchymal stem cells produced from the bone marrow of *Bmal1*−/− mice reveals a diminished capacity to transform into osteoblasts in a laboratory setting. This is likely the reason for the observed decrease in both osteoblasts and osteocytes and may be a factor contributing to the reported osteopenia [87]. This work provides evidence for the involvement of the circadian clock in the control of bone balance and demonstrates that a lack of *BMAL1* leads to a phenotype characterised by reduced bone mass [87].

Additional mice study by Chen et al. indicates that while clinical investigations have demonstrated the influence of circadian rhythm on bone resorption through daily changes, the specific molecular mechanism responsible for circadian clock-dependent bone resorption remains unidentified [88]. In order to elucidate the function of circadian rhythm

in the process of bone resorption, the aryl hydrocarbon receptor nuclear translocator-like (*Bmal1),* which is a representative gene involved in circadian regulation, was selectively deactivated in osteoclasts [88]. Osteoclast-specific *Bmal1*-knockout animals exhibited a phenotype characterised by increased bone density as a result of decreased osteoclast differentiation. An in vitro test demonstrated that *BMAL1* increased the transcription of nuclear factor of activated T cells, cytoplasmic, calcineurin-dependent 1 (*Nfatc1*) by binding to an E-box element on the *Nfatc1* promoter, in collaboration with circadian locomotor output cycles kaput (*CLOCK*), a heterodimeric partner of *BMAL1*. Additionally, it has been demonstrated that members of the steroid receptor coactivator (SRC) family may interact with and enhance the transcriptional activity of *BMAL1:CLOCK* [88]. The findings indicate that bone resorption is regulated by osteoclastic *BMAL1* through its interactions with the SRC family and its binding to the *Nfatc1* promoter [88].

1.4. Hypothesis and aims:

Rationale of the chicken study:

Therefore, the goal of this study was to assess whether physical exercise in form of WAIR influences humerus bone microstructure and density. This work is focused on chickens undergoing WAIR from 2 to 21weeks of age.

Aim of chicken study:

Aim: To determine if 19 weeks of WAIR leads to improved bone health in the humerus of egg laying hens.

Hypothesis: Hens that performed WAIR will have enhanced bone mineral density and microstructure in the humerus compared to non trained controls.

Rationale of the mice study:

Before tibial loading study can be performed on mice lacking *Bmal1* in their osteocytes, we must understand the load strain relationship in these mice by performing strain gauging studies. This knowledge will allow us to then identify the load level required

to engender anabolic strains in the osteocyte-specific *Bmal1*$^{-/-}$ mice and their littermate controls.

Aim of mice study:

Aim: Generate osteocyte-specific *Bmal1*$^{-/-}$ mice and assess the load-strain relationship.

Hypothesis: Loss of Bmal in osteocytes will lead to altered load-strain relationship in osteocyte-specific *Bmal1*$^{-/-}$ mice compared to littermate controls.

Chapter 2

The effect of Wing Assisted Incline Running (WAIR) on the humerus density and microstructure in egg laying hens.

Preface: Bone can adapt to mechanical stimuli through a process of (re)modeling that occurs throughout an organism's life. Although this adaptive response has been documented numerously in mammmals, less is known about mechanoadaptation in birds. In our first manuscript, I employed egg-laying chickens to investigate bone mechanoadaptation in response to physical activity in the form of WAIR. I studied the bone formation response by examining the bone mineral density and microstructure in the mid-diaphysis of the humerus bone.

Title: The effect of Wing Assisted Incline Running on the humerus density and microstructure in egg laying hens.

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Abstract

Purpose: To evaluate and compare the effects of physical exercise in the form of WAIR, on the bone mineral density and microstructure of the humerus bones, in two commercial genetic strains of chickens, from 2 to 21 weeks of age.

Materials and Methods: Female chicks of each genetic strain (Lohmann lite=Dekalb white-feathered birds (n=27); Lohmann brown=Hyline brown-feathered birds (n-27), were sorted separately into 8 identical floor pens. At one week of age, each pen was allocated to one of two treatment groups: WAIR or control. From week 2 till 21 weeks of age, n=9 of each genetic strain was either 1) trained on inclined ramps greater than 40° to perform wing-assisted incline running (Treatment A), 2) handled for an equivalent period of time (e.g., wings and legs palpated, mock measures of weight, photography of wings) but not trained on inclined ramps (Treatment B), or 3) were neither trained nor handled (Treatment C). Upon euthanasia, at week 36, the humerus bones were dissected and analysed using micro-computed tomography to evaluate intact bone microstructure of mid-diaphyseal humeri bones. The scans were further reconstructed using Xamflow software to study cortical bone parameters such as: cortical thickness (Ct.Th), cortical area (Ct.Ar), total cross-sectional area inside the periosteal envelope (Tt.Ar), cortical volumetric tissue mineral density (Ct.vTMD), and principal moments of inertia (Imax, Imin), cortical total volume (Ct.TV), cortical bone mineral density (Ct.BMD), and the trabecular bone parameters included: trabecular thickness (Tb.Th), trabecular number (Tb.N), trabecular separation (Tb.Sp), trabecular bone volume (Tb.BV), and trabecular volumetric tissue mineral density (Tb.vTMD). Medullary bone parameters included were: medullary area (Md.Ar) and medullary total volume (Md.TV).

Results: There was no significant differences observed between WAIR treated chickens and the handled chickens nor between WAIR and control chickens. We did however observe a trend of increased Tb.vTMD in brown chickens that were WAIR trained compared to controls (p=0.090). In addition, within the brown chickens we observed a significantly greater Tb.vTMD (p=0.007) and Tb. Th (p=0.018) in the handled group (Tb. vTMD: 565.435 ± 37.25, Tb. Th: 7.864 ± 2.19) compared to the control group (Tb.vTMD: 507.377 ± 51.67 , Tb. Th: 5.758 ± 0.86).

Conclusions: WAIR did not significantly affect cortical, trabecular, or medullary bone properties in the diaphyseal region of the humerus. Interestingly, however, handling alone led to an increase in trabecular bone mineral density and improved microstructure in brown chickens. Furthermore, genetic variation between chicken strains influenced bone characteristics, with brown chickens exhibiting superior cortical bone parameters compared to their white counterparts.

Keywords. WAIR, bone mineral density, microstructure, micro-computer tomography, cortical bone, trabecular bone, chicken.

2.1. Introduction:

The Chicken Farmers of Canada oversees the production and marketing of the Canadian poultry and egg industry (https://agriculture.canada.ca/en/sector/animal-industry/poultry-and-egg-market-information/chicken). This national organization marked 2,826 regulated Canadian chicken producers, accounting for 1.34 billion kilograms of chicken in 2022, with a contributing revenue of CAD 3.8 billion [1]. Poultry farmers also witnessed 866.5 million dozen eggs produced in 2022 [2]. Canada has a widespread network of over 1200 egg farms, with an annual estimate of 25 million laying hens [3].

Numerous factors such as the quality and quantity of feed, water consumption, intensity and duration of daylight and artificial light, management, and housing characteristics, impact the skeletal system of the hens and ultimately reflect upon their productive egg laying ability. [4, 5]

Commercial hens and pullets, which are young chickens, before reaching sexual maturity, have traditionally been confined to cramped conventional cages. These cages provide little space for the hens to stand or sit. They do not permit them to move, run, perch, or flap their wings. The limited access to physical exercise given to the pullets raised in conventional cages is likely a contributing factor to their susceptibility to bone fragility. [6, 7].

There is a particular interest in the egg-farming sector to study the mechanical and adaptive behaviour of the bones of commercial egg-laying hens. Similar to mammals, the skeletal structure of chickens (and other avian species) grows by elongation via

endochondral ossification and cross-sectional expansion through intramembranous ossification [8]. Chicken bones are composed of osteocytes and undergo intracortical remodeling (Hudson HA, 1993). The formation of secondary osteons in female chickens starts with the commencement of sexual maturity, typically around 16-18 weeks of age [8].

Research has demonstrated that the bone biology of chickens undergoes significant changes when they begin laying eggs to adapt to the demands of egg production [8-10]. It is thought that this involves a transition in osteoblast activity from producing cortical bone to producing medullary bone, which serves as the primary supply of calcium for eggshells, while the activity of osteoclasts stays constant [11]. Hens have a decrease in bone density, resulting in bones that are more fragile and prone to fractures [8]. This decline in bone density persists until the conclusion of the laying period, which might perhaps account for the higher occurrence of fractures in aging layers.

It has been demonstrated that providing more chances for physical activity during the early development stage enhances muscle growth and increases the strength of long bones, which remains consistent until the conclusion of the laying period [12, 13]. Developing pullets with increased bone mass is a potential strategy to decrease fracture occurrences in laying hens since it leads to the formation of more robust bones. Pullets that are provided with more chances for physical activity throughout their early development may experience more mechanical stress on their bones, which can stimulate higher bone apposition. This, in turn, might help prevent the occurrence of bone fractures later in life [12, 13]. By implementing this method, these young female chickens can experience a consistent decrease in bone density throughout the period of egg production, without their bones reaching a dangerously fragile state that is prone to breaking.

Osteoporosis in laying hens has been a significant welfare problem since the 1980s due to the correlation between osteoporosis and a high occurrence of fractures during the laying phase [14-17]. In chickens, osteoporosis is believed to start between 16 and 31 weeks of age when histological evidence shows a decrease of around 50% in the volume of cancellous (trabecular) bone [18]. This reduction occurs when the creation of medullary bone becomes dominant. The continuous generation of eggs is thought to contribute to a

higher occurrence of osteoporosis from 31 to 42 weeks of age due to significant depletion of calcium stores in the skeleton [19]. This aligns with findings of an increase in fractures around 40 weeks of age [20].

The manifestations of osteoporosis are often apparent only in situations of extreme severity, initially referred to as "cage layer fatigue" (Couch, 1955), leading to spontaneous immobility, deaths, and substandard eggshell quality. During the laying phase, the decrease in the number of structural mineralized bone tissues leads to an increase in bone fragility and susceptibility to fractures [8].

Fractures in laying hens can have several negative effects. They can limit the hens' ability to move, as well as their intake of food and water. Fractures can also put pressure on the spinal cord and restrict normal respiratory function if the ribs and keel bone are fractured. Additionally, fractures are likely to cause both acute and chronic pain in the hens. The mortalities associated with osteoporosis are thought to occur owing to two main factors: paralysis caused by spine degeneration and the impairment of muscular function resulting from insufficient metabolic calcium stores [8].

Enhancing the physical activity of adult laying hens through the inclusion of perches and elevated dust baths in cages, as well as providing them with aviary systems that allow for increased movement and flight, has been shown to enhance bone strength and composition [21-27].

While the implementation of a more intricate housing system for adults has shown some positive results in enhancing bone health, an alternative strategy for avoiding osteoporosis is to focus on the pullet stage, which is the era of musculoskeletal development.

Engaging pullets in physical activity during their early development enhances their skeletal strength and potentially allowing for higher levels of medullary bone calcium stores [12]. This, in turn, helps to prevent skeletal depletion in later stages of life. Initial findings indicate that raising pullets in non-cage systems enhances the overall bone composition and strength of their bones at 16 weeks [18, 28]. Additionally, Regmi et al. (2016) demonstrated that the favourable effect on cortical bone thickness and density observed at 16 weeks was maintained during the laying period [13].

In humans too, osteoporosis is now becoming recognized as a paediatric illness in the field of human medicine. The early stages of the disease develop during childhood and adolescence, and the symptoms become apparent in maturity [29]. Understanding bone biology, associated problems with disuse osteoporosis and high fracture rates in young chickens, may help solve questions related to human osteoporosis as well.

Aim and Rationale:

Aim:

To determine if 19 weeks of WAIR leads to improved bone health in the humerus of egg laying hens.

Hypothesis: Hens that performed WAIR will have enhanced bone mineral density and microstructure in the humerus compared to non-trained controls.

Rationale:

Therefore, the goal of this study was to assess whether physical exercise in form of WAIR influences humerus bone microstructure and density. This work is focused on chickens undergoing WAIR from 2 to 21weeks of age.

2.2. Methods:

All animal trials were carried out by the Guelph Poultry Research Station Standard Operating Procedures (Guelph, ON, Canada). The *in vivo* experimental procedures (eg. housing, WAIR program) and non-bone health related results (eg. body kinematic results) have been previously published by colleagues at the University of Guelph, who performed the study [30]. Details related to the animal model, WAIR exercise program and sample collection are briefly described below as well as the novel bone analyses performed as part of this thesis.

2.2.1. Animal model:

Female chicks of each genetic strain (Lohmann [LSL] lite=Dekalb white-feathered birds (n=27); Lohmann brown [LB]=Hyline brown-feathered birds (n-27), Figure 2) were sorted separately into 8 identical floor pens (183 cms L x 244 cms W x 290 cms H), within

a room at the Poultry Research Center, Guelph, ON, Canada. All birds had equal access to food and received a standard age-appropriate crumble diet ad lib. Each bird was assigned and labelled with a unique identification number.

WAIR Exercise program:

At one week of age (WOA), each pen was allocated to one of two treatment groups: WAIR or control. The WAIR group was familiarised and acclimated to the ramp apparatus (150 cm in length x 10 cm in width x 115 cm in height; (Figure 3) for a period of 2 to 4 weeks of age. The workout regimen started when the chicks reached an age of 5 weeks old. A ramp with an elevation of 60 degrees was employed from 5 to 9 WOA, and then the gradient was raised to 65 degrees from 10 WOA to 21 WOA. The chicks were mandated to engage in physical activity twice a week, with a minimum of one day of recuperation in between. During the exercise, every chick started its run from a start box and proceeded via a walkway to reach the base of the ramp. The duration of the ascent on the ramp was measured from the moment the chicks lifted both feet off the ground (i.e., both feet on the ramp) until both feet reached the top of the ramp. The duration of ascending the ramp was utilised to compute velocity and instantaneous deceleration. After completing 10 runs up the ramp and meeting the exercise requirement, the chicks were returned to their pen for the day, marking the conclusion of their daily activity. The exercise requirement for the chicks was considered fulfilled if, after completing a minimum of 10 runs, there were two consecutive runs with a velocity that was 40% lower than the quickest run. The control group undertook a handling technique as part of a wider experiment in order to standardise the way all birds were treated, therefore reducing the impact of human contact on the results. The control group was familiarised with the handling technique at 3 and 4 weeks of age, whereas the WAIR group was familiarised with the ramp apparatus and experienced comparable handling. The handling procedure commenced at 5 WOA, during which the chicks were transferred across 3 transport boxes. Initially, a complete group of birds was captured and confined within the initial container. One chick from the control group was transferred to the second crate when exercise commenced for one of the chicks in the WAIR group, and then relocated to the third crate once exercise was finished. After the chicks in the pen finished the handling treatment for the day, they were

let free in their designated enclosure and given a food incentive, much like the WAIR birds.

2.2.2. Sample collection:

Starting on week 2 (until 21 weeks of age), one third of the birds (9 birds) of each genetic strain was either 1) trained on inclined ramps greater than 40° to perform wing-assisted incline running (Treatment A), 2) handled for an equivalent period of time (e.g., wings and legs palpated, mock measures of weight, photography of wings) but not trained on inclined ramps (Treatment B), or 3) were neither trained nor handled (Treatment C).

The identification numbers of the birds were documented. Birds were euthanized at 36 weeks of age. The dissection was carried out by the University of Guelph and the dissected humerus (Figure 4) of these birds were bagged and transported to McGill University for analysis. Upon receiving the bone samples, they were stored in – 20 ℃.

2.2.3. Micro-computed tomography:

Micro-computed tomography was performed by the Bruker SkyScan 1276 at The Shriners Hospital for Children (Montreal, QC, Canada).

Prior to micro-CT, each humerus bone was removed from its bag, cleaned to remove any soft tissue and placed in a custom-made falcon tube. The bone was positioned in the centre of the tube using paper rolls.

The bone was then securely fixed in the centre of the scanner cassette with the proximal head of humerus bone at the distal end of the cassette (Figure 5). All bones were positioned and scanned in the same manner, with scanning parameters set as: 15 µm voxel size, 80 kV, 200 µA, 360° _scanning, frame averaging of 2 and rotational step of 0.6°.

Each day, after the final scan of the day, phantom of 0.25 and 0.75 mg HA/cm^3 density were scanned at the same scanning parameters. This was used to calibrate all the scans to units of mg HA/cm^3.

2.2.4. Selection of volume of interest:

After micro-CT scanning, a representative volume of interest (VOI) was defined. The aim of the study was to evaluate intact bone microstructure of Humerus bone at 1 region. Considering the size of the humerus bone, a VOI of 5 % of the humerus length at the mid-diaphysis was chosen as an appropriate representative of the cortical and trabecular bone microstructure.

2.2.5. Micro-computed tomography analysis of the cortical and trabecular bone:

Bruker XRM solutions include all software needed to reconstruct scans from 2D projection images into 3D volumes (NRECON) and to conduct 2D and 3D analyze (Xamflow) of the bone microstructure.

Since the scan resolution was 15 µm, the total humerus length was calculated by multiplying the number of slices in the scan by 0.015. To determine a global threshold, the histograms of a subset of 12 humerus bone scans were analyzed. For each scan, we computed the OTSU threshold as well as variations of this value (80% OTSU, 90% OTSU, ….) Mean thresholds for each variation were computed from the subset, applied and results visually inspected to select the optimal version. A global threshold value of 0.3683 mgHA/cm3 was chosen to segment cortical bone from soft tissue. A global threshold value of 0.2982 mgHA/cm3 was chosen to segment trabecular bone from soft tissue. Xamflow software was used to analyze all the reconstructed scans.

Table 2: The bone parameters recorded by micro-CT included the following:

Cortical bone parameters	**Trabecular bone parameters**	**Medullary bone parameters**
Cortical thickness (Ct.Th)	Trabecular thickness (Tb.Th)	Medullary area (Md.Ar)
Cortical area (Ct.Ar)	Trabecular number (Tb.N),	Medullary total volume (Md.TV)

Total cross-sectional area inside the periosteal envelope (Tt.Ar)	Trabecular separation (Tb.Sp),	
Cortical volumetric tissue mineral density (Ct.vTMD)	Trabecular bone volume (Tb.BV),	
Principal moments of inertia (Imax, Imin)	Trabecular volumetric tissue mineral density (Tb.vTMD)	
Cortical total volume (Ct.TV)		
Cortical bone mineral density (Ct.BMD)		

2.3. Statistical analysis:

Two-way ANOVA was performed on micro-CT data (prior to normalization for step frequency) to determine effects of treatment group and genotype (and interaction term) post-hoc comparisons were performed using Tukey-Kramer correction and significance was set at 0.05.

2.4. Results:

2.4.1. WAIR or handling had no effect on cortical bone mineral density and microstructure:

ANOVA showed that treatment had a significant effect on Ct.vTMD between all three groups ($p=0.029$). However, post-hoc Tukey-Kramer paired comparisons did not show any significant differences between the groups. The means of Ct.vTMD among all the groups are: WAIR group (brown: 777.86±13.08, white: 772.48±18.79), handled group (brown: 768.16±13.15, white: 767.67±9.84), and the control group (brown: 762.90±8.25, white: 764.03±10.85). However, besides this, there were no significant effects on any other measured cortical bone parameters, among all groups in brown and white chickens (Figure 6).

2.4.2. Handling increased trabecular bone mineral density and microstructure in brown chickens:

ANOVA and post-hoc Tukey-Kramer comparison demonstrated a trend of increased Tb.vTMD in brown chickens that were WAIR trained compared to controls ($p=0.09$). The means of Tb.vTMD in brown chickens were: WAIR group (551.43±25.49) and control group (507.37±51.67). In addition, within the brown chickens we observed a significantly greater Tb.vTMD ($p=0.007$) and Tb. Th ($p=0.018$) in the handled group (Tb. vTMD: 565.435 ± 37.25, Tb. Th: 7.864 ± 2.19) compared to the control group (Tb.vTMD: 507.377 ± 51.67 , Tb. Th: 5.758 ± 0.86). We did not measure any differences in bone outcomes in response to treatment in white chickens (Figure 7).

2.4.3. WAIR or handling had no effect on medullary bone mineral density and microstructure:

ANOVA and post-hoc Tukey-Kramer comparison showed no significant effects on any measured medullary bone parameters, among all treatment groups in brown and white chickens.

2.4.4. Genetic strain influences the cortical bone microstructure:

Our analysis demonstrated a significant phenotypic difference between brown and white chickens within a treatment group for multiple outcome parameters. Within the WAIR treated group, we observed that Ct.Ar ($p<0.0001$), Tt.Ar ($p=0.064$), Ct.Th ($p=0.007$), Ct.BV ($p=0.053$), Imax ($p<0.0001$), and Imin ($p=0.002$) were significantly greater in the brown compared to the white chickens. The mean and standard deviation of these measured parameters were: Ct.Ar (brown: 15.19±1.45; white: 12.38±0.69), Tt.Ar (brown: 44.33±3; white: 38.46±3.58), Ct.Th (brown: 0.72±0.06; white: 0.61±0.04), Ct.BV (brown: 310.89±28.35; white: 256.75±12.16), Imax (brown: 117.13±13.91; white: 83.13±7.33), and Imin (brown: 72.16±8.27; white: 55.71±3.81).

Within the handled group, we observed that Ct.Ar ($p<0.0001$), Ct.Th ($p=0.006$), Ct.BV ($p=0.028$), Imax ($p<0.0001$), and Imin ($p<0.0001$) were significantly greater in the brown compared to the white chickens. The means of these measured parameters were: Ct.Ar

(brown: 14.99±1.05; white: 11.99±1.16), Ct.Th (brown: 0.69±0.04; white: 0.59±0.05), Ct.BV (brown: 305.44±22.73; white: 248.46±23.28), Imax (brown: 121.70±13.13; white: 84.10±8.94), and Imin (brown: 77.48±9.12; white: 55.02±6.71).

Within the control group, we observed that Ct.Ar (p=0.000), Ct.Th (p=0.001), Ct.vBMD (p=0.035), Imax (p=0.001), and Imin (p=0.076) were significantly greater in the brown compared to the white chickens. The means of these measured parameters were: Ct.Ar (brown: 14.99±1.70; white: 12.17±1.11), Ct.Th (brown: 0.72±0.07; white: 0.61±0.03), Ct.vBMD (brown: 266.05±36.69; white: 221.15±18.76), Imax (brown: 111.13±16.03; white: 85.32±12.31), and Imin (brown: 69.04±10.87; white: 58.64±8.05).

2.5 Discussion:

The ability of bone to function under loads is dependent on the structure, properties of bone material and the amount of forces exerted on bone by the adjoining muscles and tendons. It has been observed that the muscle load on bone is directly proportional to the amount of bone mechaoadaptation or (re)modeling [31]. Regular exercise is known to substantially increase muscle mass that in turn increases bone density [31].

The keel bone in chickens is highly susceptible to fractures [8, 32]. Sufficient literature exists on the examination of bone microstructure and density of the keel bone. The humerus of the chicken is closely associated with its flight and wing-flapping. Hens that performed WAIR were observed to have higher initial flight velocity while descent, while brown feathered birds demonstrated 1.05 times faster wing beating frequency than the white chickens [30]. However, limited data is available on the microstructure of the humerus bone.

This study aimed to evaluate if physical exercise in the form of WAIR for 19 weeks, would improve bone health in the humerus of egg laying hens. These chickens were subjected to WAIR from 2 to 21 weeks of age. Their mid-diaphyseal humeri region was studied to further understand the bone microstructure and density.

2.5.1. Treatment had no effects on bone microstructural:

We observed no significant differences between WAIR treated chickens and the handled chickens nor between WAIR and control chickens. Since the increase in Tb.

vTMD is only a trend, and trabecular bone is more prevalent at metaphyseal regions, studies at the metaphyseal region are necessary to determine if WAIR enhances trabecular bone mass. In addition, these data suggests that perhaps increased duration or magnitude of WAIR training may be necessary to elicit a significant cortical bone response. The reason for the increase in Tb. vTMD due to handling is less clear. However, it may be due to the brown chickens exerting more muscle forces on the bones while being handled. Additional studies examining the effects of WAIR on bone mass and microstructure at other anatomical locations (eg. keel, tibiotarsus) are warranted.

2.5.2. Genetic strain influences the bone microstructure:

Our study suggests that the humeri of brown chickens had significantly greater cortical bone mineral density and microstructure compared to the white chickens.
Although identical in many ways, several studies have indicated that white and brown chickens respond differently to the availability of equipment like as perches, more complicated housing alternatives, and extra room for movement. White-feathered chickens often have lower wing loading (body weight per unit wing area) than brown-feathered birds [30]. Thus, the brown birds exert higher magnitude of force while flight and wing-flapping, that could possibly suggest a higher cortical bone parameters when compared to white birds. The possibility of brown chickens resisting being handled as a factor for increased cortical bone density, needs to be further evaluated.

2.6 Limitations:

This study lacks the baseline data for micro-CT parameters of all sample bones. Therefore, any comparative evaluations could not be conducted. Perhaps the increased duration or magnitude of WAIR training may be mandatory to elicit a significant osteogenic bone response.

The metaphyseal humerus regions should be scanned and studied for a better understanding of trabecular bone microstructure.

2.7 Conclusion:

In summary, this study showed that exercise in form of WAIR did not influence the cortical, trabecular or medullary bone parameters of the diaphyseal region of the humerus bone. Surprisingly, handling led to increased trabecular bone mineral density and microstructure in brown chickens. Also, the genetic strain of chickens influenced the bone microstructure, where the browns had a higher cortical bone parameters when compared to white chickens. This data will be helpful for poultry farmers to further understand the WAIR exercise regimen for increased bone welfare of the chicken.

2.8 Figures and tables:

Figure 2: Genetic strains of Lohmann brown [LB]: Hyline brown-feathered birds and Lohmann [LSL] lite: Dekalb white-feathered birds chickens (Images used with permission from I. Vitienes).

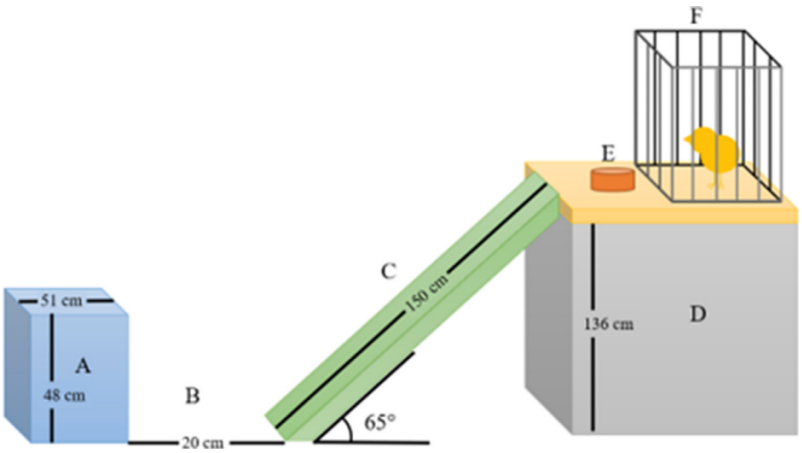


Figure 3: Schematic representation of WAIR exercise, where chickens initiate the exercise program on the box (A), move down the walkway (B) and to the ramp (that is set at 65 degrees) (C). The chicken then walks up the ramp apparatus at a height of 136 cms (D) where it receives food reward (E) and social reward (F) [30].

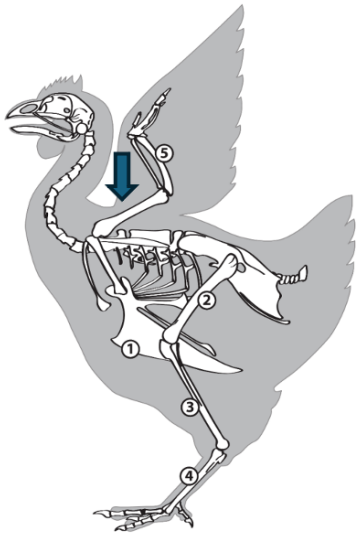
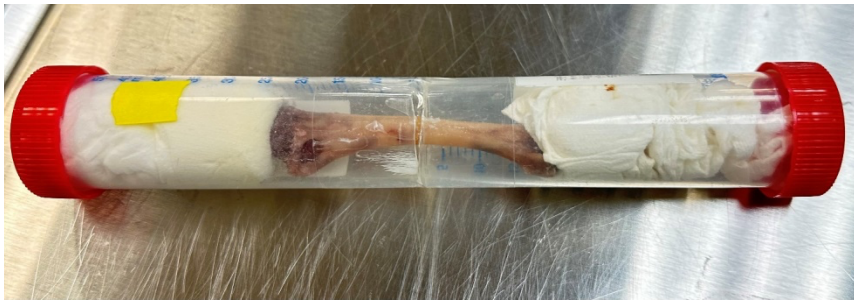


Figure 4: Schematic representation of chicken skeleton with the blue arrow depicting the volume of interest in the intact humerus bone at mid diaphyseal region.

A



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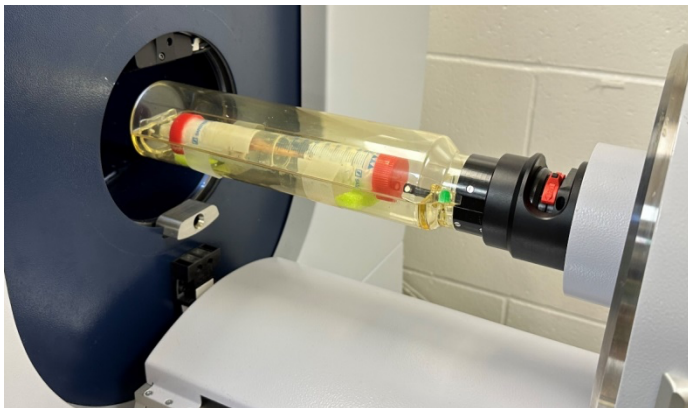

Figure 5: Scanning of humerus in micro-CT. (A) The clean humerus bone is positioned in the centre of a custom-made Falcon tube. (B) The Falcon tube is then placed into the cassette and fixed to position using tapes, with the proximal head of the bone at the distal end of the cassette. The humerus is then scanned using the *in vivo* micro CT scanner.

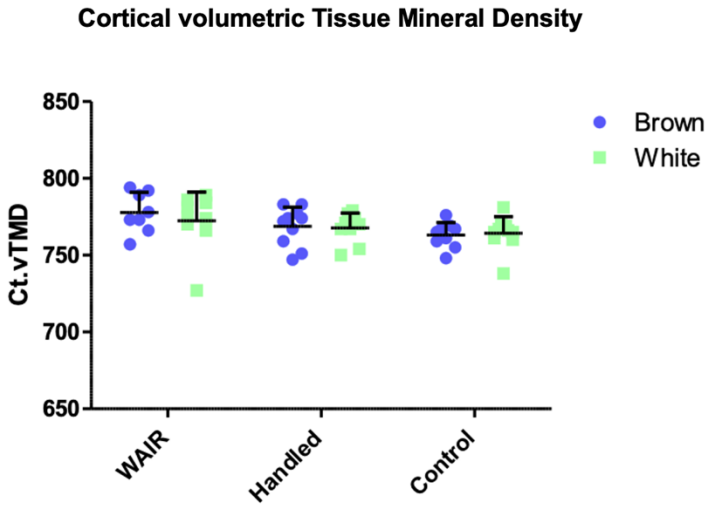

Figure A

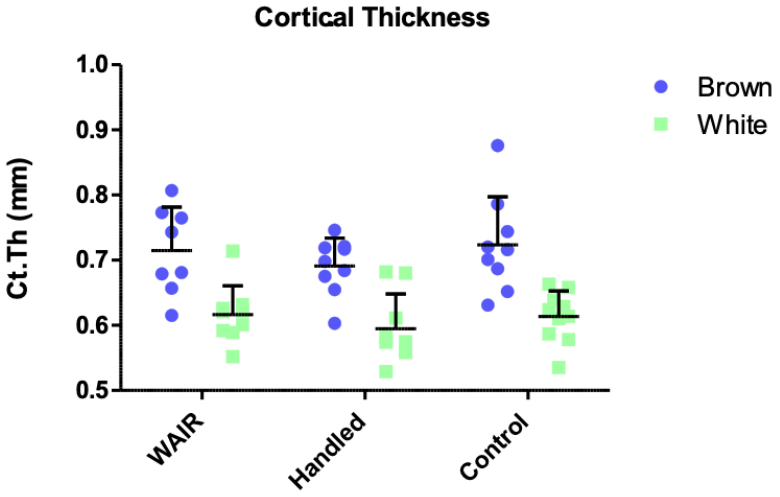

Figure B

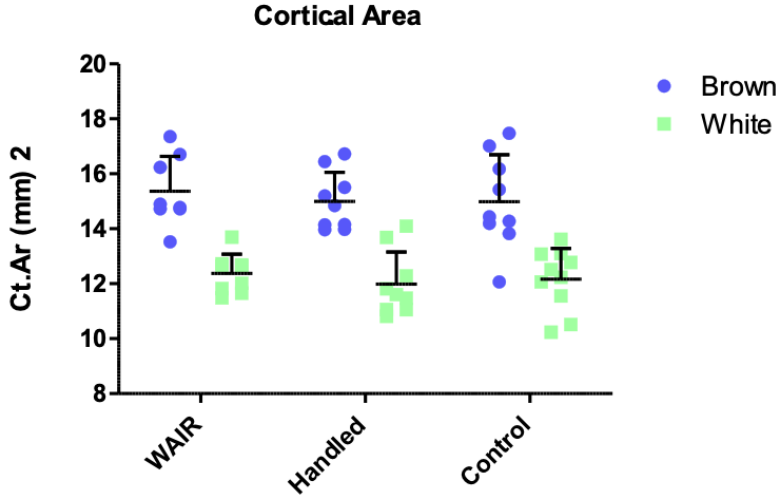

Figure C

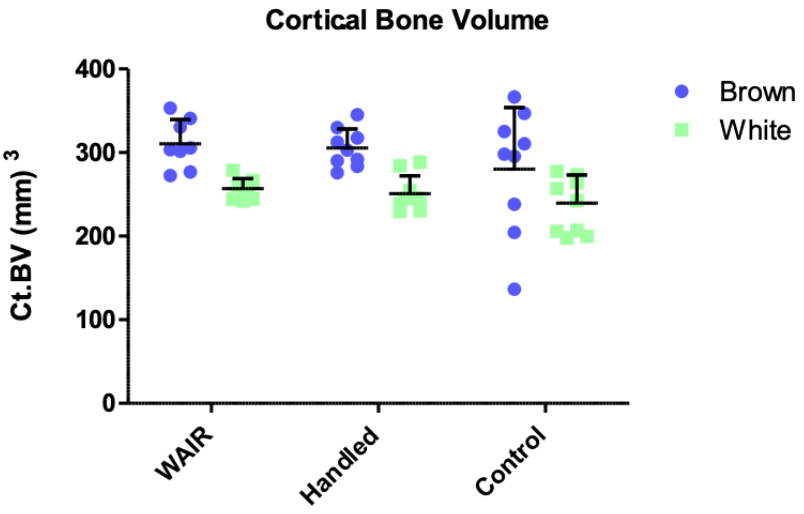

Figure D

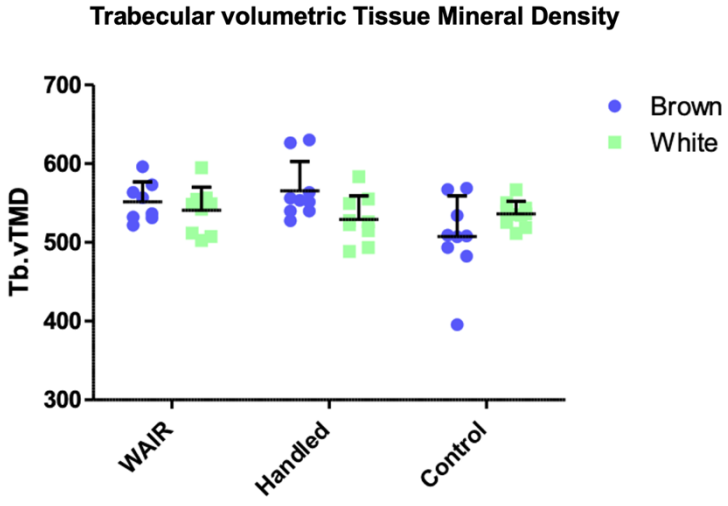

Figure E

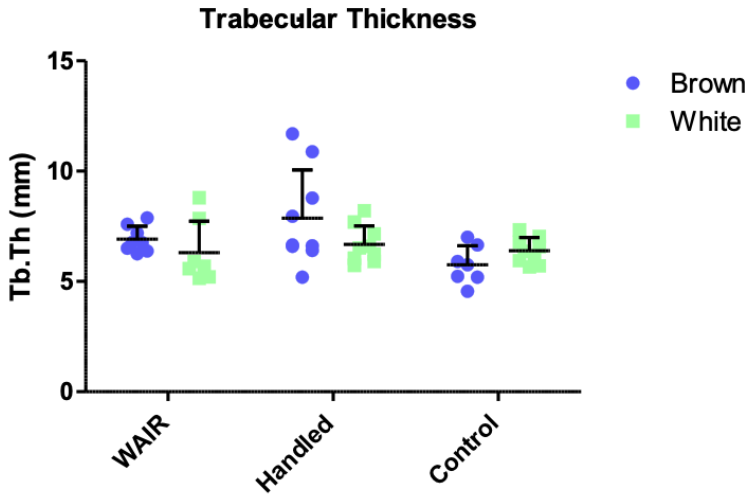

Figure F

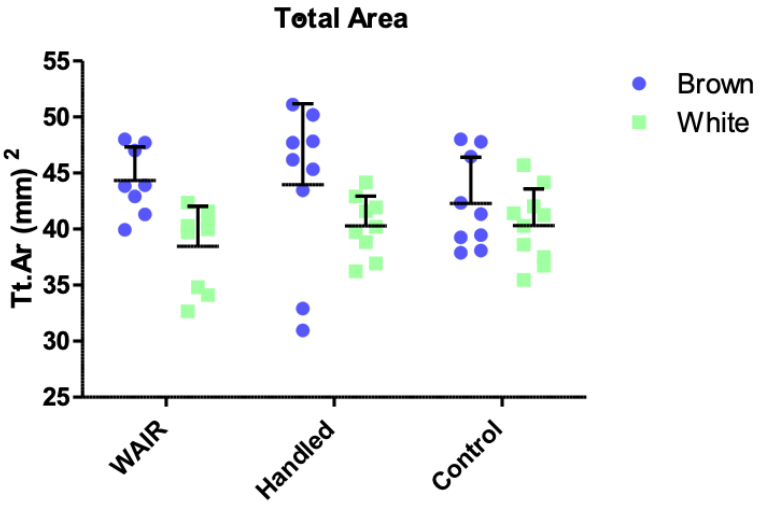

Figure G

Figure 6: Mean and standard deviation of bone micro-CT parameters. (A-D) showing cortical bone parameters; (A) Cortical volumetric tissue mineral density; (B) Cortical

thickness; (C) Cortical area; (D) Cortical bone volume. (E-F) showing trabecular bone parameters; (E) Trabecular volumetric tissue mineral density; (F) Trabecular thickness; (G) shows total area of humerus bone at mid diaphyseal region.

Table 3: Mean and Std. Dev. of brown chickens with 3 treatment groups

Brown Chickens									
	WAIR (treament-A)			Handled (treatment- B)			Control (treatment- C)		
Cortical									
Ct.vTMD	777.87		13.09	768.16		13.15	762.90		8.26
Ct.Ar (mm^2) [a]	15.19	±	1.45	15.00	±	1.06	14.99	±	1.70
Tt.Ar (mm^2) [a]	44.33	±	3.00	44.09	±	7.04	42.32	±	4.11
Ct.Th (mm) [a]	0.72	±	0.07	0.73	±	0.08	0.73	±	0.08
I_{max} (mm^4) [a]	117.13	±	13.91	121.71	±	13.13	111.14	±	16.04
I_{min} (mm^4) [a]	72.16	±	8.28	77.49	±	9.13	69.04	±	10.88
Trabecular									
Tb.vTMD	551.4	±	25.5	565.4	±	37.3	507.4	±	51.7
Tb.Th (µm)	6.9	±	0.6	7.9	±	2.2	5.8	±	0.9
Tb.N (1/mm) [a]	132.2	±	41.9	148.9	±	24.6	115.0	±	28.6
Tb.Sp (µm)	128.6	±	41.8	144.3	±	25.4	114.9	±	26.9
Tb.BV	5.3	±	1.6	10.4	±	5.7	10.9	±	18.5
Medullary									
Md.Ar	29.1	±	3.4	29.1	±	6.4	27.3	±	3.9
Md.TV	5.5	±	1.7	10.7	±	5.9	13.2	±	24.9

Table 4: Mean and Std. Dev. of white chickens with 3 treatment groups

White Chickens									
	WAIR (treament-A)			Handled (treatment- B)			Control (treatment- C)		
Cortical									
Ct.vTMD	772.48		18.80	767.68		9.85	764.04		10.86
Ct.Ar (mm^2) [a]	12.38	±	0.69	11.99	±	1.17	12.17	±	1.11
Tt.Ar (mm^2) [a]	38.46	±	3.59	40.28	±	2.65	40.32	±	3.26
Ct.Th (mm) [a]	0.62	±	0.04	0.59	±	0.05	0.61	±	0.04
I_{max} (mm^4) [a]	83.13	±	7.33	84.10	±	8.94	85.32	±	12.32
I_{min} (mm^4) [a]	55.71	±	3.82	55.03	±	6.72	58.65	±	8.06
Trabecular									
Tb.vTMD	540.8	±	29.4	529.1	±	30.0	536.3	±	15.8
Tb.Th (µm)	6.3	±	1.4	6.7	±	0.8	6.4	±	0.6
Tb.N (1/mm) [a]	133.2	±	35.4	124.7	±	21.5	137.8	±	34.8
Tb.Sp (µm)	131.2	±	35.4	120.7	±	21.4	131.8	±	30.7
Tb.BV	7.9	±	4.9	6.7	±	5.3	5.1	±	4.1
Medullary									
Md.Ar	26.1	±	3.7	28.3	±	2.6	28.2	±	2.6
Md.TV	8.1	±	5.0	6.9	±	5.4	5.3	±	4.3

2.9. References:

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Chapter 3

Circadian rhythms in mechanoadaptation in mice.

Preface: The skeleton continuously adapts to mechanical stimuli through a process of bone (re)modeling that persists throughout an individual's lifespan. This (re)modeling is regulated by osteocytes, the bone's mechanosensors, which modulate osteoblast and osteoclast activity during mechanotransduction. In this study, mice were used to investigate the mechanical strains engendered in the bone during controlled in vivo mouse tibial mechanical loading. The focus of the second manuscript was to test the hypothesis that mice deficient in the transcription factor Brain and Muscle ARNT-like Protein 1 (*Bmal1*) would exhibit an altered load-strain relationship compared to their wild-type counterparts.

Title: Circadian rhythms in mechanoadaptation in mice.

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Abstract

Purpose: To understand the load strain relationship in osteocyte-specific *Bmal1*$^{-/-}$ mice models, by performing strain gauging studies. This knowledge will allow us to then identify the load level required to engender anabolic strains in the osteocyte-specific *Bmal1*$^{-/-}$ mice and their littermate controls.

Materials and Methods: Male and Female 10 week old mice of 3 genotypes namely Dmp1Cre;Bmal$^{+/+}$, Bmal$^{Fl/Fl}$ and Dmp1Cre;Bmal$^{Fl/Fl}$ (n=6 mice/genotype/sex) were generated and analysed. Single element strain gauges were custom-made to fit the tibia of the mouse and were surgically glued to the tibia of the mouse. The relationship between applied compression and bone tissue deformation (mechanical strain) for the right and left tibia was established for young, postpubescent (10-week-old) male and female mice of the three different genotypes, for dynamic compressive loads of -3N, -6N, -9N & -12N. The slopes of load-strain regressions were analyzed to determine the *in vivo* tibial stiffness. Stiffness for each load level was calculated as a change in load over the change in strain during the loading portion of the waveform. *In vivo* stiffness at each of the load levels measured was averaged to get an average *in vivo* tibial stiffness per mouse.

Results: There were no difference in the *in vivo* tibial stiffness between Dmp1Cre;Bmal$^{Fl/Fl}$ mice compared to either WT mice, regardless of sex. These results suggest that the deletion of the *Bmal1* gene from the osteocyte does not have a significant impact on the whole bone stiffness properties.

Conclusions: In summary, this study showed that the deletion of *Bmal1* from the osteocytes had no significant role *in vivo* tibial stiffness. There was a lack of significant difference observed in the *in vivo* tibial stiffness between the different genotypes in the male and female mice. Further studies are warranted to determine if disruption of circadian rhythms alters bone geometry, material properties, and osteocyte networks in mice.

Keywords. BMAL1, osteocyte, strain gauge, Circadian rhythm, strain-load relationship, tibial stiffness.

3.1. Introduction:

In 1892, Julius Wolff, an anatomist and orthopaedic surgeon, proposed the idea that bones had the ability to adjust and conform to their mechanical surroundings. He stated a law- "*Every change in formor function of bone or of their function alone is followed by certain definite changes in their internal architecture, and equally definite alteration in their external conformation, in accordance with mathematical laws.*"

Architectural changes in bones, similar to those reported by Wolff, were theorized to happen as a result of a dynamic adaptation process in response to alterations in the mechanical stimuli caused by bearing weight. Harold Frost expanded upon this notion in 1960, and his ideas serve as the foundation for the current "mechanostat" theory of bone adaptation [1]. The Mechanostat theory is a term describing the way in which mechanical loading influences bone structure by changing the mass (amount of bone) and architecture (its arrangement) to provide a structure that resists habitual loads with an economical amount of material [1].

Bone remodelling necessitates precise regulation of two processes, namely bone creation by osteoblasts and bone resorption by osteoclasts, in order to uphold a robust skeletal structure [2]. The osteocyte is a crucial regulatory cell that influences and participates in bone remodelling by coordinating the activity of both osteoblasts and osteoclasts [3]. The mechanical environment has a significant impact on bone mass, as evidenced by the loss of bone in individuals who are paralysed or bedridden. Bone cells play a crucial role in bone adaptation, with osteocytes serving as the sensors for mechanical strain in the bone. The application of mechanical forces influences the quantity and function of cells, therefore adapting the material characteristics (such as strength, elasticity, and toughness) as well as the size and form of the bone, enabling it to withstand the exerted forces [4]. Osteocytes play a role in mechano-transduction. Specific deletion of osteocytes in mice leads to elevated cortical porosity and decreased trabecular bone, while also providing resistance against bone loss caused by unloading [5]. Exercise is perhaps the most effective and economical strategy for enhancing bone health [4]. Interestingly our research group showed that the time of the day that the mechanical stimuli is applied affects the amount of bone formation and resorption [4].

The circadian clock is an inherent mechanism present in most animals that synchronises their physiology and behaviour with the Earth's rotation [6]. The suprachiasmatic nucleus (SCN) functions as the primary circadian pacemaker in mammals, although other organs include secondary pacemakers [7, 8]. The central clock is widely acknowledged as the mechanism that synchronises and controls the peripheral clock through neuronal and hormonal pathways [9-11]. However, this idea was challenged when the specific role of aryl hydrocarbon receptor nuclear translocator-like (*Bmal1*) in various tissues was discovered. This suggests that the peripheral clock, specifically peripheral *BMAL1*, may have its own unique function in peripheral tissues that does not depend on the central clock [12]. The central clock and the peripheral clocks share the same molecular mechanism and are composed of a self-regulating transcriptional-translational feedback loop [13].

The system comprises two positive constituents, *BMAL1* and circadian locomotor output cycles kaput (*CLOCK*), together with two negative constituents, period circadian clock (*PER1, PER2, and PER3*) and cryptochrome (photolyase-like) (*CRY1* and *CRY2)*. Typically, the *BMAL1: CLOCK* transcription factor, composed of two distinct proteins, facilitates the transcription of genes related to circadian rhythms, such as *Per* and *Cry*. The activation process takes place through the binding of particular areas known as E-box elements in the gene promoters. These E-box elements contain a sequence that closely resembles *CACGTG* or *CACGTT* [14-17]. *PER* and *CRY* interact to form a compound that inhibits the transcriptional activity of *BMAL1: CLOCK*. Osteoblasts and osteoclasts demonstrate the manifestation of clock genes [18, 19]. The mineral deposition in the bones of mice in calvarial organ cultures displayed a circadian rhythm lasting 24 hours and might potentially be associated with the activation of clock genes [20].

Researchers have utilised experimental mouse models to investigate the effects of regulated and recurrent stress on bone remodelling [21-26]. The development of the *in vivo* mouse tibial loading model has the added benefit of allowing the investigation of mechanobiological processes in both the metaphyseal cortico-cancellous and diaphyseal cortical tissues inside the same bone [27-30]. The cortical strain engendered in the bone

by loading in these models have been evaluated using beam theory, surface strain gauge measurements, or surface digital image correlation [31, 32].

Osteoblast-specific *Bmal1* knockdown mice, which have impaired clock function only in osteoblasts, but not in other cells, exhibit a low bone mineral density (BMD) phenotype. This is caused by elevated levels of bone resorption. On the other hand, osteoclast-specific conditional *Bmal1* knockdown mice display a high BMD phenotype due to reduced bone resorption [33, 34]. With substantial work conducted in this field, certain research gaps yet exist. The characterization of the phenotype of mice lacking clock function specifically in osteocytes remains unclear. It is yet to be determined if loss of Bmal1 in osteocytes alters the bone (re)modeling response to loading.

Aim and Rationale:

Aims of mice study:

Aim: Generate osteocyte-specific *Bmal1*$^{-/-}$ mice and assess the load-strain relationship.
Hypothesis: Loss of *Bmal* in osteocytes will lead to altered load-strain relationship in osteocyte-specific *Bmal1*$^{-/-}$ mice compared to littermate controls.

Rationale of the mice study:

Before tibial loading study can be performed on mice lacking *Bmal1* in their osteocytes, we must understand the load strain relationship in these mice by performing strain gauging studies. This knowledge will allow us to then identify the load level required to engender anabolic strains in the osteocyte-specific *Bmal1*$^{-/-}$ mice and their littermate controls.

3.2. Methods:

3.2.1. Animal model:

Male and Female 10 week old mice of 3 genotypes namely Dmp1Cre;Bmal$^{+/+}$, Bmal$^{Fl/Fl}$ and Dmp1Cre;Bmal$^{Fl/Fl}$ were used. N=6 mice/genotype/sex were generated and analysed.

3.2.2. Strain gauge preparation:

Single element strain gauges (EA-06-015LA-120, Micro-measurements, USA) were prepared under a microscope to have a custom-made size that fits the tibia of the mouse (Figure 8). Two wires of equal lengths were soldered to the strain gauge and the other ends were soldered to the pin which connects to the computer software. The strain gauge was then calibrated in the Wintest software.

3.2.3. Strain gauge surgery:

During surgery, the mouse was put under isoflurane gas inhalation chamber for anesthesia (3 parts isoflurane, 2 parts oxygen). Once under deep sedation, the mouse was weighed, injected with saline and veterinary ointment was applied to the eyes. The mouse was transferred to the operating table and an isoflurane mask was placed on the mouse's head to cover the nose and mouth. The legs along with the back and neck of the mouse were shaved to have a clean surgical site.

A clean incision was made at the anterior mid diaphyseal region of the left tibia. That specific region of the bone was isolated from the muscles and the fascia. The bone was cleaned using a degreaser. The prepared strain gauge was then glued to the tibia using super glue. The strain gauge wire was then passed through the back of the mouse, exiting near the base of the neck (Figure 9).

3.2.4. Measuring load-strain relationship:

The relationship between applied compression and bone tissue deformation (mechanical strain) for the right and left tibia was established for young, postpubescent (10 week old) male and female mice of the three different genotypes.

Dynamic compressive loads of -3N, -6N, -9N & -12N between the flexed knee & ankle using an in-vivo loading device (TA Instruments Testbench ElectroForce, USA) were applied. No tibial fractures occurred within the loading range of -3N to -12 N. The load cycle consists of a triangular waveform of 50 cycles (initiating at -1N preload), with each cycle of 0.15 seconds. The load rate is dependent on the load value. Load-strain values are recorded using WinTest software (Figure 10). The slopes of load-strain regressions were analyzed to determine the *in vivo* tibial stiffness. We calculated stiffness for each

load level as the change in load over the change in strain during the loading portion of the waveform. These values were then averaged across four consecutive load cycles. *In vivo* stiffness (Figure 11) at each of the load levels measured was averaged to get an average *in vivo* tibial stiffness per mouse [35].

The strain level of +1200 microstrain was selected for future two-week tibial loading experiments based on previous evidence demonstrating its ability to stimulate bone formation in the mouse tibia at the medial midshaft, which is about two to three times the stresses experienced by the medial tibia during regular walking in mice [36].

3.3. Statistical analysis:

ANOVA was performed on the *in vivo* tibial stiffness values to determine effects of age and genotype (and interaction term) post-hoc comparisons were performed using Tukey-Kramer correction and significance was set at 0.05.

3.4. Results:

3.4.1. Deletion of *Bmal1* has no significant impact on bone stiffness:

Our results did not support the hypothesis; there were no difference in the *in vivo* tibial stiffness between Dmp1Cre;BmalFl/Fl mice compared to either WT mice, regardless of sex.

3.4.2. Gender of the mouse plays no role in the effects seen after deletion of *Bmal1*:

3.4.2.1. Females:

The mean and standard deviation of the *in vivo* tibial stiffness performed on all groups of mice are: Dmp1Cre;$Bmal^{+/+}$: - 0.0089± 0.0005 N/με, $Bmal^{Fl/Fl}$: - 0.0093 ± 0.0014 N/με and Dmp1Cre;$Bmal^{Fl/Fl}$ - 0.0076 ± 0.0139 N/με (as depicted in Figure 11).

3.4.2.2. Males:

The mean and standard deviation of the *in vivo* tibial stiffness performed on all groups of mice are: Dmp1Cre;$Bmal^{+/+}$: - 0.0086± 0.0011 N/με, $Bmal^{Fl/Fl}$: - 0.0090 ± 0.0018 N/με and Dmp1Cre;$Bmal^{Fl/Fl}$: - 0.0076 ± 0.0015 N/με (as depicted in Figure).

3.5. Discussion:

These results suggest that the deletion of the *Bmal1* from the osteocyte does not have a significant impact on the whole bone *in vivo* stiffness properties. It was surprising that we did not see differences in whole bone *in vivo* stiffness. However, differences may exist in the whole bone geometry and tissue material properties of bone, which should be analyzed in future. The other possibility is that loss of *Bmal1* in osteocytes does not affect bone geometry, mass, and material properties.

The curvature of bones plays a crucial role in determining the amount of stress and stiffness experienced by the bones when they are subjected to external forces. The axial components of the muscle and ground response forces exert multi-directional bending moments on the limb bones by acting around the longitudinal curvature of the bone. The moments generated by these forces generally result in bending stresses, which are the primary factor in skeletal loading for a variety of animals [35]. Future studies are needed to analyze these properties in the mice.

3.6. Limitations:

This study is a prerequisite for a future two-week loading analysis to determine if there is an altered mechanoresponse in the mice. The insignificant difference in the results could also be due to a smaller sample size. Further studies are warranted to determine if disruption of circadian rhythms alters bone geometry, material properties, and osteocyte networks in mice.

3.7. Conclusion:

In summary, this study showed that the deletion of *Bmal1* from the osteocytes had no significant role *in vivo* tibial stiffness. There was a lack of significant difference

observed in the *in vivo* tibial stiffness between the different genotypes in the male and female mice.

3.8. Figures and tables:

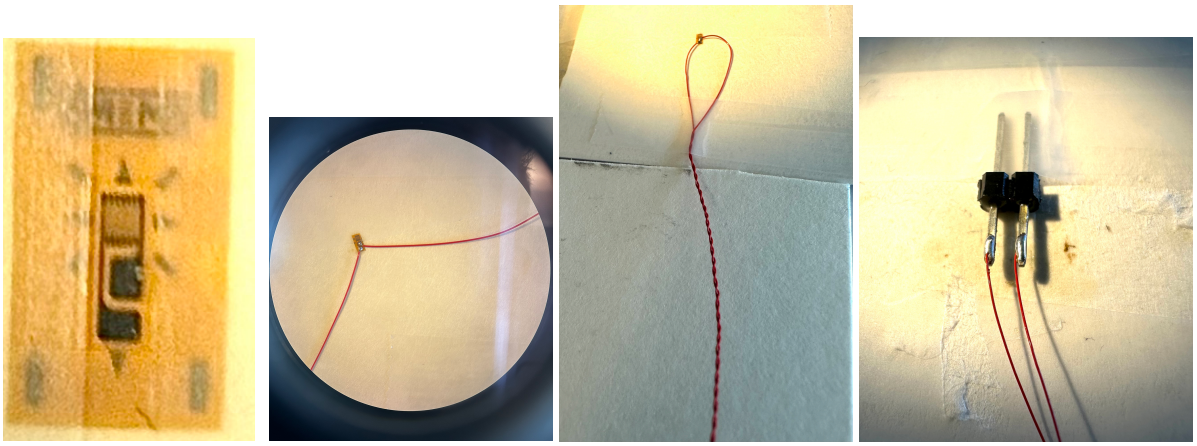

Figure 7: Left) Single element strain gauges (EA-06-015LA-120, Micro-measurements, USA) were prepared under a microscope to have a custom-made size that fits the tibia of the mouse. Left middle) Image shows soldering of the wires to the strain gauge. Right middle) Image shows the braiding of the wires. Right) Two wires of equal lengths were soldered to the strain gauge and the other ends were soldered to the pin which connects to the computer software.

A

B

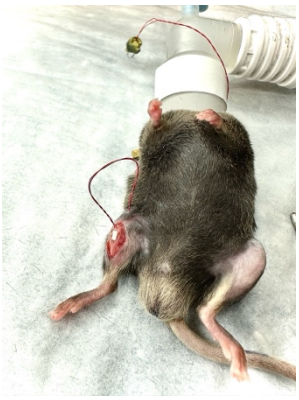

Figure 8: Strain gauge surgery. (A) Armamentarium and set up for strain gauging surgery. (B) Mouse under an anaesthesia mask with a strain gauge glued at the anterior mid diaphyseal region of tibia.

Figure 9: *In vivo* tibial loading to determine load-strain relationship. The tibia of the anaesthetised mouse is placed between the two arms of the *in vivo* loading machine.

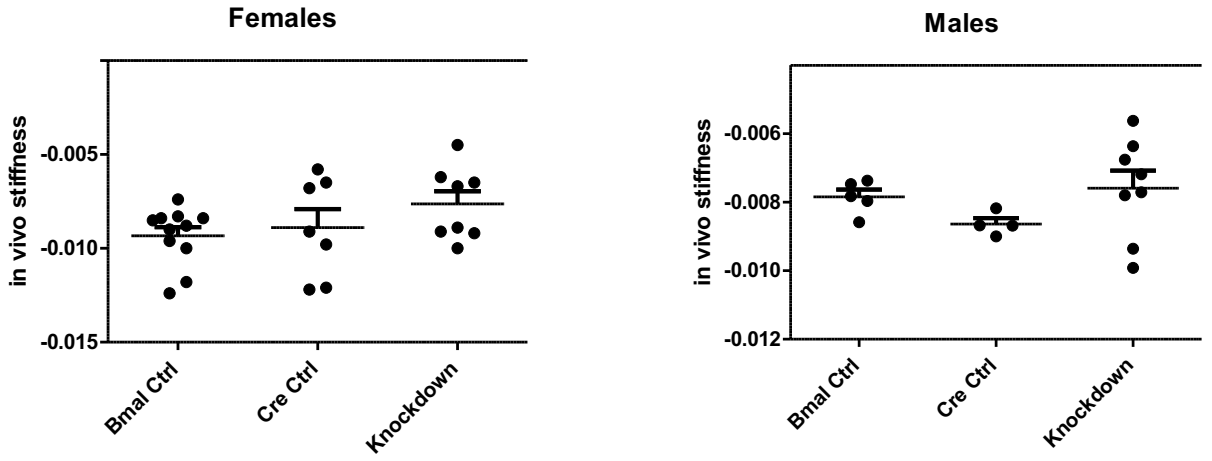

Figure 10: *In vivo* stiffness of 10 week old (female, male) *Bmal* control ($Bmal^{Fl/Fl}$), Cre Control ($Dmp1Cre;Bmal^{+/+}$ *),* and knockdown *(*$Dmp1Cre;Bmal^{Fl/Fl}$*)*mice.

Table 5 Chapter 3: Mean and Std. Dev. of *in vivo* stiffness in female mice

Females								
	Bmal Control			Cre Control			Knockout	
	n=11			n=7			n=8	
In vivo stiffness (N/με)	-0.0093	±	0.0014	-0.0089	±	0.0005	-0.0076	±	0.0139

Table 6 Chapter 3: Mean and Std. Dev. of *in vivo* stiffness in male mice

Males								
	Bmal Control			Cre Control			Knockout	
	n=8			n=4			n=5	
In vivo stiffness (N/με)	-0.009	±	0.0018	-0.0086	±	0.0011	-0.0076	±	0.0015

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Chapter 4

Global discussion and Conclusion

This thesis describes the mechanoadaptation to loading in chickens (in the form of physical activity) and mice (in the form of controlled loading). The skeletal system undergoes bone (re)modeling during an individual's lifespan to adapt to the mechanical demands imposed on it. Osteocytes, which are the cells responsible for sensing mechanical forces in the bone, manage the process of bone remodelling by directing the creation of new bone by osteoblasts and the breakdown of bone by osteoclasts by mechanotransduction. We explored the effects of wing assisted inclined running in chickens aged 2-21 weeks (Chapter 2) to study the humerus bone mineral density and microstructure in 36-week old chickens. In Chapter 3, we aimed to study the load-strain relationship on mice tibia, specifically lacking *Bmal1* gene in their osteocytes.

Regarding the first aim of my thesis, I performed micro-CT analysis of dissected humerii bones of white and brown chickens that underwent WAIR. The results showed that WAIR had no effect on cortical and medullary bone mineral density or microstructure. This finding was surprising since other studies have shown that increased opportunity for physical activity in the form of more complex housing, can significantly enhance bone health in chickens. Chickens kept in traditional battery cages exhibit significantly reduced bone strength compared to chickens housed in alternative systems [20]. Hens housed in cages equipped with perches or in low-level aviary systems have enhanced leg bone strength, but only little increases in wing bone strength [20]. On the other hand, birds that have the ability to fly in high-level aviaries demonstrate significantly higher enhancements in wing bone strength [20]. Trott et al., aimed to investigate the impact of varying exercise opportunities throughout the raising phase on the musculoskeletal features of pullets [48]. Two groups of 588 Lohmann Selected Leghorn-Lite pullets were raised in either normal, conventional cages (Conv) or an aviary rearing system (Avi) from the time they were one day old until they reached 16 weeks of age. At 16 weeks, the keel bone, muscles, and long bones of the wings and legs were obtained to compare muscle growth variations across rearing regimens and examine bone quality features using quantitative computed

tomography (QCT) and bone breaking strength (BBS) testing. Aviary-raised pullets had significantly higher values for total bone density, total cross-sectional area, cortical cross-sectional area, total bone mineral content, and cortical bone mineral content compared to conventionally-raised pullets for the radius, humerus, and tibia ($P < 0.001$). The Avi pullets exhibited significantly higher BBS than the Conv pullets for the radius, humerus, and tibia ($P < 0.01$). My study was different from Trott et al. since I used WAIR and they used housing. Perhaps the chickens were more willing to move about or do physical activities inside their aviary instead of taking them out of their home pen.

In addition, Shipov et al., evaluated the effects of chronic exercise limitation and high calcium requirements on the micro-structural, compositional, and mechanical aspects of the avian skeleton [89]. A comparison was made between the tibiae and humeri of 2-year-old laying hens kept in conventional caging (CC) and free-range (FR) housing systems utilising mechanical testing and micro-computed tomography scanning. Examinations of cortical, cancellous, and medullary bone were conducted. Mechanical analysis demonstrated that the tibiae and humeri of birds in the FR group had superior mechanical characteristics compared to those in the CC group. Additionally, microCT imaging revealed that the bones of the FR group had bigger cortical areas and smaller medullary portions. Anderson et al., demonstrated that hyaline brown pullets on multi-tier perches exhibit heightened activity levels and enhanced musculoskeletal well-being [90]. The study period spanned from 0 to 17 weeks of age. The tibiotarsal bones of pullets on multi-tier perches (P) exhibited higher overall and cortical bone mineral density at week 11, coupled with increased cortical bone cross-sectional areas and elevated total and cortical bone mineral densities at week 17 ($P<0.05$). Significantly, throughout both weeks, the tibiae of P pullets had higher breaking strengths. Again, these studies differed from mine in that they used different types of housing methods to evaluate bone mineral density and microstructure in these chickens.

In addition to studies that have looked at housing to elicit physical activity and subsequent improved bone mass, a few studies have also examined the effect of exercise. Judex and Zernicke measured the mechanical conditions of the middle part of the rooster's tarsometatarsus bone during high-speed running [91]. They also investigated whether brief periods of this exercise-induced mechanical environment can

lead to beneficial alterations in the structure, strength, and mineral content of the cortical bone. At 9 weeks old, the roosters were divided into two groups: controls (n = 9) and runners (n = 8). Following an 8-week period of running, there were no discernible differences in mid diaphyseal areal and mechanical characteristics, as well as normalised ash weight, between the group of runners and the control group. The statistics indicate that decreasing the number of loading cycles can alleviate the negative reaction previously found in this model during prolonged operation. This study also confirms the principle that the mechanical environment created by exercise must be significantly different from the usual environment in order to cause meaningful changes. Thus, similar to my results, this study did not reveal any significant differences in the mid diaphyseal region of the tarsometatarsus bone mineral density and mechanical characteristics.

Several research studies have investigated the implementation and use of WAIR training ramps in both young and mature laying hen groups, examining the positive effects on cognitive function and bone health [92-94]. The majority of these research focused on examining the usage of ramps within the housing rather than taking the chickens out of the home pens, both in passive and voluntary contexts [95-97]. However no studies using WAIR training have previously examined the effect of the exercise on bone mass or microstructure in humerii or any other bone for that matter. Our colleagues at the University of Guelph recently showed using the same cohort of chickens as I have analyzed in this thesis that WAIR training led to greater initial flight velocity compared to control birds, suggesting WAIR may strengthen the leg muscles. Thus, additional investigation of leg bones are needed to determine if WAIR enhanced bone mass in the lower limbs.

The reason that I did not see alterations in humoral cortical bone mineral density or microstructure in response to WAIR might be because of the WAIR protocol used. The chickens underwent ten minutes a day for two days in the week, for 17 weeks WAIR training that involved a run in the start box, making their way to the base of the ramp. These chickens then ascended the ramp. They completed ten such runs in one exercise regime and returned to their home pens. Perhaps longer durations of daily training are needed to enhance bone mass. One limitation with our study was that we did not have baseline micro-CT data for the chickens prior to the WAIR training.

The WAIR exercise program ended at 21 weeks of age, and these chickens were euthanized at 36 weeks of age. During this period, the chickens continued normal daily activities. This may have led to loss of any beneficial bone adaptation that occurred due to WAIR training, since the mechanical signalling on the bone due to WAIR was not present anymore for 15 weeks

My results also showed that handling increased the trabecular bone mineral density and microstructure only in brown chickens, but not in white. The handling involved human interactions like palpating chicken wings and transferring to and from cages. This result may be due to the physical resistance offered by the muscles of the chicken to these aforementioned handling activities. No other studies that I am aware of have examining the effect of handling on the humerus bone mass or microstructure.

My results also stated that brown chickens had enhanced cortical area, total area, and cortical bone volume compared to white chickens. This finding is similar to that reported by Hong et al., in the same cohort of chickens. They showed that the brown chickens have higher wing loading than the white chickens. Hence, they brown chickens have increased power output for wing beating frequency than white chickens [54].

Regarding the second aim, I performed strain gauging surgeries on male and female 10-week old mice with deletion of *Bmal1* gene, specifically in their osteocytes. The results did not show a significant difference in the *in vivo* tibial stiffness between male and female mice. Although other studies have shown differences in bone mass and microstructure in other *Bmal* global or conditional KOs no one has examined mechanical behavior in these mice. Thus, we are currently examining the bone phenotype in these mice. Future studies are also underway to examine the response of these mice to unloading as well as loading.

Two research groups have reported the bone phenotype of mice with *Bmal1* knocked out in osteoblasts, however they did not study the mechanical behavior (ie. in vivo stiffness) of the bones, which is something that should be further investigated. Takarada et al. discovered a decrease in bone mass in mice that had *Bmal1* knocked down in their osteoblast cells. This reduction in bone mass was observed when the animals were 8 weeks old. Specifically, the researchers discovered reduced bone mass, cortical volume, trabecular number, and trabecular thickness in the tibia, as well as decreased bone volume in the vertebrae [98].

Qian et al., focused to examine the direct impact of *Bmal1*, the primary activator of the peripheral circadian clock system, on bone growth and remodelling phases. This was achieved by utilising similar mice with a particular gene deletion of *Bmal1* in osteoblasts. Surprisingly, when *Bmal1* was removed from osteoblasts, it resulted in several anomalies in bone metabolism. One of these abnormalities was a gradual increase in trabecular bone mass, which was observed as early as 8 weeks. This increase was characterized by an 82.3% rise in bone mineral density and a 2.8-fold increase in bone volume per tissue volume. As these mice got older, there was a continued growth in trabecular bone mass, whereas cortical bone mass declined by around 33.7%. This was accompanied by the development of kyphoscoliosis and deformed intervertebral discs. The elevated trabecular bone mass was ascribed to an augmentation in the quantity and function of osteoblasts, together with a reduction in the formation of osteoclasts [99]. We also focused on young growing mice (10-12 week old). Future studies should also examine the bone phenotype and mechanical behavior in these different conditional *Bmal* KO mouse models after peak bone mass is achieved, since the phenotype may change with age.

In addition to these mouse models, other mouse models could be investigated to examine the role of circadian rhythms on bone mechanical behavior. Fu et al. studied Leptin, a hormone, that regulates the process of bone remodelling, which is responsible for maintaining a consistent bone mass as part of the body's homeostatic function. Mice that did not have molecular-clock components in their osteoblasts had increased bone mass, indicating that circadian control may also affect bone remodelling. Clock genes could facilitate the leptin-dependent control of bone growth through sympathetic modulation. Their research demonstrated that the activity of clock genes in osteoblasts is controlled by both the sympathetic nervous system and leptin [83].

The phenotypic investigations of mice with a deletion of the *Bmal1* gene in osteoclasts have shown conflicting results, even though the identical animals with the *Ctsk* promoter-Cre driver were used. Xu et al., observed mice with a particular deletion of the *Bmal1* gene in osteoclasts, and they stated that they had a phenotype characterised by increased bone mass, which was caused by a decrease in the formation of osteoclasts. Through a cell-based assay, it was discovered that *BMAL1* increased the transcription of

nuclear factor of activated T cells, cytoplasmic, calcineurin-dependent 1 (*Nfatc1*) by binding to an E-box element on the *Nfatc1* promoter. This binding occurred in collaboration with circadian locomotor output cycles kaput (*CLOCK*), which is a heterodimer partner of *BMAL1*. Furthermore, it has been demonstrated that members of the steroid receptor coactivator (SRC) family may interact with and enhance the transcriptional activity of *BMAL1:CLOCK*. The findings indicated that osteoclastic *BMAL1* regulated bone resorption via interacting with the SRC family and binding to the *Nfatc1* promoter [88]. In contrast, Tsang et al. did not find any alterations in bone mass in the femur or osteoclast differentiation *in vitro* [100].

Ko et al., concluded that the functioning of the digestive system is greatly affected by disturbances in the circadian rhythm [101]. Given that the gut has an impact on bone remodelling, the objective of their study was to investigate if disturbing circadian signalling in colon epithelial cells had an effect on bone. Consequently, researchers evaluated the physical, operational, and biological characteristics of bone in 8-week-old Ts4-Cre and Ts4-Cre;Bmal1fl/fl (*cBmalKO*) mice, in which the clock gene *Bmal1* is absent in colon epithelial cells. The amount of trabecular bone in both the axial and appendicular regions was considerably reduced in *cBmalKO* mice compared to *Ts4*-Cre animals at 8 weeks of age. This effect was observed only in male mice and not in female mice. In the similar manner, the overall mechanical characteristics of the bones were impaired in male mice with *cBmalKO*. The processes at the tissue level that were involved in inhibiting bone growth while maintaining normal resorption were detected by serum markers and dynamic histomorphometry. This research shows that when the *Bmal1* gene is specifically deleted in colon epithelial cells, male mice are unable to develop trabecular and cortical bone. Again, none of these studies examined the mechanical behavior (ie. *in vivo* stiffness) of the bones, which is something that should be further investigated.

In conclusion, my first study examining WAIR in egg laying hens showed that the current training protocol did not lead to enhanced cortical bone mineral density or microstructure in the humori. This protocol requires further investigation to inform poultry farmers how exercise in form of WAIR can improve bone health of chickens. This will lead to reduced fractures and increased bone mineral density in egg laying chickens.

In conclusion, my second study examined the mechanical behavior of tibiae from mice with *Bmal1* KO in osteocytes. These data will help us to further examine mechanoadaptation in the mice and eventually develop molecular targets for *Bmal* as well as give us more knowledge to help patients who need physical activity-based treatments to enhance bone mass.

Chapter 5

Future Work

It may be necessary to increase the length or intensity of WAIR training in order to produce a noticeable bone response that promotes bone growth. An increased load magnitude, or running frequency, or duration of WAIR might elicit a bone response. Thus, future studies should examine how altering these different loading parameters may affect the bone formation response. Additionally, we looked at brown and white chickens WAIR trained from 2 to 21 weeks of age. Further studies are needed to determine if the response to loading changes with age in chickens as it has been shown to be reduced with age in mammals (ie. mice and humans). In addition, the training occurred from age 2-21 weeks of age, but euthanasia and analysis of the bones only occurred at 36 weeks of age.. Thus, the effects of the WAIR training may have been attenuated over this time period, as the bone readapted to normal loading conditions.

We only focused our analysis on the diaphysis of the humeri. However, the metaphyseal region of a long bone houses most of the trabecular bone. WAIR may have had an effect on the trabecular metaphyseal bone and not on the cortical diaphyseal bone. It is recommended to do scans and analysis of the metaphyseal areas of the humerus to have a more comprehensive understanding of the effect of WAIR of trabecular bone. Future experiments for WAIR should involve a protocol of blood collection at regular intervals of time, in order to assess changes in blood bone markers.

In addition, studies in mice have shown that different anatomical sites have a different response to loading partly due to the habitual loading conditions experienced by the bone [59, 102]. Thus, other bones such as the femur and tibia should be examined to determine if WAIR had an effect on them.

With regards to my second study, our lab is currently working on a two-week loading experiment that uses data from my *in vivo* strain gauging tests to examine mechanoresponse in the mice. The *Bmal1* knockdown mice may have reduced response to mechanical loading due to the deletion of this gene, specifically in osteocytes. This may further exhibit reduced bone formation compared to the wild type controls after the two-

week loading treatment. Additional research is necessary to ascertain if the disturbance of circadian rhythms affects the shape, physical characteristics, and connections between bone cells in mice.

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