Evaluating perching biomechanics in commercial and random-bred strains of enriched-housed laying hens

by

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Abstract

Housing, including rearing environment and provided enrichments, impact the welfare of laying hens throughout their life. As the Canadian egg layer industry shifted away from conventional cages, an increase in keel bone damage (**KBD**) has raised welfare and economic concerns. Keel bone damage leads to reduced egg production, reduced egg quality, and negative affective states due to pain in laying hens. Our understanding of the genetic and environmental components that impact KBD is limited. The objective of our research was to identify genetic and environmental factors that contribute to KBD in enriched housed laying hens to help reduce the incidence below levels traditionally seen in conventional housing.

In our first study, commercial strains (Lohmann White, Shaver White, Lohmann Brown, ISA Brown) and random-bred strains (Shaver Rhode Island Red and Antique White Leghorn) were studied (n=20/treatment) to determine the impact of strain and perch shape (mushroom or round) on innate perching biomechanics at pre, peak, and end of lay, and secondly how strain affects KBD, body weight, footpad temperature, and bone composition. Perching kinematics, footpad temperature, bone parameters (femur and keel bone mineral content and density), and KBD severity were assessed. In addition, genotyping was conducted on tissue samples collected postmortem to identify causative mutations that negatively impact bone parameters. Single-SNP associations between bone parameters and SNPs located on candidate genes (*RANKL*, *RB1*, *SOST*, *POSTN*, *SOX9*, *PTHLH*, *OVALX*) were assessed. Rhode Island Red had greater femur bone mineral density and content than all other strains. Higher producing commercial strains had higher incidences of severe KBD. Round perches led to faster forward and backward and up and down movements compared to mushroom perches. Commercial strains moved up and down more and forward and backward less than random-bred strains. Up and down movements were correlated with higher incidences of severe KBD. Strain, but not perch shape, significantly impacted footpad temperature. Overall, round perches contributed to perching instability and random-bred and commercial strains had fundamentally different movement patterns while perching. Seven SNPs segregated and significant SNP effects on bone parameters from *RANKL*, *RB1*, *POSTN*, and *OVALX* genes were located, however, SNP effects were confounded by significant strain effects.

In our second study, we aimed to determine which strains experience certain perching biomechanics, higher bone strength, and keel health within enriched housed layers depending on perch design and pullet rearing system used. Lohmann White and Lohmann Brown pullets were placed in either pullet cages or floor pens at hatch with access to mushroom or round perches (n=9/treatment) and were transferred to an enriched battery at lay. Perching kinematics, body weights, bone parameters (femur and keel bone mineral content and density), bone breaking strength, and keel scores were evaluated. Behaviour observations were conducted every two weeks from seven until 69 weeks of age. Lohmann Brown spent more time perching from two to six months of age, when the keel is more susceptible to damage. There was a positive correlation between severity of deviation present and forward and backward keel motion. Keel and femur bone strength, content, and density were higher in Lohmann Brown in comparison to Lohmann White. Measured bone parameters were not protective against severe KBD as Lohmann Brown had more severe deviations and more fractures than Lohmann White. Round perches led to more severe KBD in comparison to mushroom perches. Increased forward and backward movements exhibited by Lohmann Brown were correlated with more severe KBD.

In conclusion, up and down movements and forward and backward movements correlated with a higher incidence of severe KBD. Mushroom perches are preferable to round perches for laying hens housed in furnished cages due to reduced KBD and increased stability while perching. Lower incidences of KBD despite lower bone mineralization indicate LW birds are more readily suited to furnished cages in comparison to LB birds. Higher producing, commercial strains had higher incidences of severe KBD. *POSTN*, *RANKL*, *RB1*, and *OVALX* are promising candidate genes for locating SNPs associated with bone parameters in laying hens.

Preface

Chapter 2 of this thesis was submitted to the journal of Poultry Science using the title “The effect of laying hen strain on perching biomechanics and keel bone damage”. E. DePaoli was responsible for data collection, data analysis, manuscript creation and editing. C. Bench was the supervisory author and was responsible for data collection, concept formation and manuscript formation. D. Korver contributed to manuscript editing.

Chapter 4 of this thesis was submitted to the journal of Applied Animal Behaviour Science using the title “Effect of rearing environment, strain and perch shape on perching behaviour, perching biomechanics, and keel bone damage in enriched-housed laying hens”. E. DePaoli was responsible for data collection, data analysis, manuscript creation and editing. C. Bench was the supervisory author and was responsible for data collection, concept formation and manuscript formation. D. Korver contributed to manuscript editing.

The research project, of which this thesis is a part, received research ethics approval from the University of Alberta Animal Care and Use Committee, AUP 3299.

Dedication

I dedicate this work to my Papa who throughout his life embodied an adventurous spirit and passion for agriculture. Thank you for always quoting the Serenity Prayer, having a Caramilk bar to share, and believing in everything I set my mind to. I think you would be proud.

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Table of Contents

List of Tables

List of Figures

List of Abbreviations

AHN	Adult hippocampal neurogenesis
AMPM	Morning Afternoon
BA	Back angle
BLAST	Basic local alignment search tool
BLUP	Best linear unbiased prediction
BMx	Back motion in the x-axis
BSy	Back speed in the y-axis
BSz	Back speed in the z-axis
CADD	Combined annotation-dependent depletion
COL2AI	*Collagen type II*
DXA	Dual x-ray absorptiometry
EFA	Egg Farmers of Alberta
FemurMC	Femur bone mineral content
FemurMD	Femur bone mineral density
GWAS	Genome wide association study
IRT	Infrared thermography
KA	Keel angle
KBD	Keel bone damage
KeelMC	Keel bone mineral content
KeelMD	Keel bone mineral density
KMx	Keel motion in the x-axis

KMy	Keel motion in the y-axis
KMz	Keel motion in the z-axis
KSy	Keel speed in the y-axis
KSz	Keel speed in the z-axis
LSMeans	Least Squares Means
MAS	Marker assisted selection
NFACC	National Farm Animal Care Council
OPG	Osteoprotegerin
OVALX	*Ovalbumin-related protein X*
POSTN	*Periostin*
PTH	*Parathyroid hormone*
PTHLH	*PTH-like hormone*
RANKL	*Receptor activator of nuclear factor kappa-B ligand*
RB1	*Retinoblastoma 1*
SIFT	Sorting intolerant from tolerant
SNPs	Single nucleotide polymorphisms
SOST	Sclerostin
SOX9	*SRY-Box 9*
VEP	Variant effect predictor

Chapter 1. Literature Review

1.1 Consumer and industry perspectives

Around 70 years ago there was a major change in how laying hens were bred and raised (Carter, 1964). As the demand for table eggs increased with rising global populations, traditional dual-purpose strains were replaced by lines bred from brown (Rhode Island Red) and white (White Leghorn) strains (Carter, 1964). Laying hens were bred for higher egg production levels and housed in battery cages (Leenstra et al., 2016) in contrast to the free-range systems used traditionally. Now, decades later, the industry is shifting once again with alternative housing systems becoming increasingly prevalent within the Alberta laying hen industry. By the end of 2021, 52% of laying hens were housed in alternative systems which include furnished cages, aviaries, free-run, and free-range systems (Egg Farmers of Alberta, 2021). Furnished cages are larger than battery cages and include features including perches, scratchpads, and nest boxes. Aviaries are multi-tier systems that provide areas for perching, dustbathing, and nesting. Free run simply indicates a hen can move freely around a barn, while free-range indicates a hen can access an outdoor environment. Alternative systems are all considered enriched environments as they provide opportunities for laying hens to perform natural behaviors through the inclusion of enrichments such as perches. As the understanding of welfare concerns associated with conventional battery cage housing became more prevalent and suitable alternatives were developed, regulatory bodies implemented changes (Tanaka and Hurnik, 1992; Baxter, 1994; Appleby, 2003). In Alberta, Egg Farmers of Alberta (**EFA**) adopted a mandatory housing policy that bans new conventional housing systems from being built (Egg Farmers of Alberta, 2021). By 2036, the Canadian National Farm Animal Care Council (**NFACC**) Layers and Pullets Code of Practice requires all hens to be in non-battery cage systems that allows for the expression of natural behaviors including scratching, nesting, and perching (NFACC, 2017).

The new EFA and NFACC requirements pose a challenge to Albertan producers, with roughly 48% of Albertan producers still using conventional cages. Meeting EFA and NFACC requirements will require producers to alter current barns or construct new barns. Switching from conventional (battery cages) to alternative (non-battery cages) housing is a large-scale undertaking that requires producers to invest in remodeling their barns. For many producers, enriched cages are seen as a safer financial option for producers taking into consideration renovation and production costs (Matthews and Sumner, 2015), and consumer demands (Bejaei et al., 2011). Average total costs of production are 36% higher in aviary systems in comparison to conventional housing, while enriched cages are only 13% higher (Matthews and Sumner, 2015). Contributing to the successful transition of the laying hen industry from conventional to alternative housing systems will help producers economically and support the egg laying industry. In 2020, Canada's egg industry contributed $1.3 billion to Canada's GDP (Egg Farmers of Canada, 2021). In Alberta, 78,739,611 dozen eggs were produced in 2022, a 4.5% increase from 2021 (Egg Farmers of Alberta, 2022).

1.2. Laying Hen Housing and Keel Bone Damage

Enriched housing provides welfare benefits in comparison to both conventional and aviary housing systems (Lay et al., 2011) and, within Alberta, scored best or high on most welfare parameters studied (C.J. Bench, unpublished data). When comparing furnished cages to conventional cages, laying hens have higher plumage cover (Abrahamsson and Tauson, 1997; Appleby et al., 2002) and healthier feet including shorter claws and less toe pad hyperkeratosis (Abrahamsson and Tauson, 1997; Appleby et al., 2002). In addition, furnished cages have lower mortality rates (Rodenburg et al., 2008), lower airborne dust concentrations (Rodenburg et al., 2008) and lower incidences of cannibalism and feather pecking compared to non-cage systems (Lay et al., 2011). Furnished cages allow laying hens to express behaviours such as perching while also limiting unwanted behaviours including cannibalism.

In addition to welfare parameters previously mentioned, housing type greatly impacts bone health. Furnished cages lead to stronger bones, including increased bone mineral density (Tactacan et al., 2009), and stronger humerus (Abrahamsson and Tauson, 1997) in comparison to conventional cages. Bone parameter differences may arise from the reduced opportunities for movement due to the barren design of conventional cages (Campbell, 2021) compared to the wider range of behavioral opportunities that are provided by furnished cages (Appleby et al., 2002). Despite the benefits to bone parameters in alternative systems, the incidence of keel bone damage (**KBD**) is higher compared to conventional cages (Petrik et al., 2015; Regmi et al., 2016). Conventional cages result in rates of KBD typically around 23% (Petrik et al., 2015;

Regmi et al., 2016) which is significantly lower than incidences seen in alternative housing. Wilkins et al. (2011) reported the incidence of fractures after dissection ranged from 36% in hens housed in furnished cages in contrast to 86% in free range systems. Saraiva et al. (2019) reported an incidence of KBD of 54.2% in hens housed in furnished cages compared to 60.4% in free-range systems. Despite the lower incidences of KBD in furnished cages compared to other alternative housing systems, conventional cages still have the lowest incidence of KBD. Ideally, through identifying both environmental and genetic factors that contribute to KBD, the incidence of KBD in furnished cage systems can be reduced below the 20% incidence seen in conventional cages.

Keel bone damage encompasses both keel bone deviations and keel bone fractures. Evaluating both is necessary to determine which factors contribute to KBD. Keel bone deviations are abnormally shaped keels, such as S and C-shaped curves (Harlander-Matauschek et al., 2015). Although not fully understood, deviations likely occur from pressure being exerted on the keel while perching (Stratmann et al., 2015). Predisposing factors may include late ossification of the keel (Buckner et al., 1948; Buckner et al., 1949) and weak bones (Habig et al., 2021). In addition, the presence of deviations may contribute to fractures via unequal bone loading on the keel when a bird flaps its wings (Harlander-Matauschek et al., 2015). Fractures are sharp breaks in the bone (Kittelsen et al., 2020). A variety of factors can contribute to the development of fractures including disease, inactivity, high and persistent egg production, and poor ossification due to the development of weaker bones that are more prone to break from environmental collisions (Toscano et al., 2020). It is important to note that although keel bone deviations and fractures are interlinked, to reduce KBD, the etiology of both needs to be fully understood.

1.3. Negative Impacts of Keel Bone Damage

Keel bone damage is of particular concern as it contributes to negative affective states due to pain (Nasr et al., 2012b; Armstrong et al., 2020; Wei et al., 2020) and reduced mobility (Nasr et al., 2012a; Wei et al., 2020), overall reducing laying hen welfare. The rate at which new neurons are integrated into the temporal lobe through adult hippocampal neurogenesis (**AHN**) can provide information on negative affective states (Armstrong et al., 2020). Adult hippocampal neurogenesis is sensitive to experiences, i.e., positive experiences increase neurogenesis

(Armstrong et al., 2020). Severe keel bone fractures notably lead to the downregulation of AHN potentially due to pain associated with keel fractures (Armstrong et al., 2020). Administering an analgesic drug to laying hens alters mobility in laying hens with fractures and not laying hens with healthy keels indicating fractures lead to pain impacting mobility (Nasr et al., 2012b). Hens with fractures move more slowly when walking and flying to and from perches compared to hens with no fractures (Nasr et al., 2012a). In addition, laying hens with fractures also spend more time resting in comparison to hens with no fractures (Nasr et al., 2012a; Wei et al., 2020) indicating that due to pain laying hens have restricted mobility. Pain can also contribute to stress and fear responses with physiological (e.g., blood tests) and behavioral (e.g., tonic immobility and novel object) tests being impacted by keel fractures (Wei et al., 2022). Laying hens with fractures had longer periods of tonic immobility and were less likely to approach a novel object (Wei et al., 2022). Higher concentrations of corticosterone, interleukin-1β, and interleukin-6 and lower levels of serotonin were indicative of higher stress levels (Wei et al., 2022). Finally, after a keel bone fracture occurs, hens can enter a negative affective state for three to four weeks due to pain, which can lead to immunosuppression as well as reduced egg production (Armstrong et al., 2020). Fractures and deviations are often grouped together when discussing KBD, which has led to a limited understanding of the specific consequences of deviations in comparison to fractures (Riber et al., 2018).

In addition to welfare concerns, keel fractures impact egg quality and production levels. At 61 weeks of age, severe keel bone fractures led to a significant drop in production (19.6%) compared to no change in production when hens were 37 weeks of age (Rufener et al., 2018). Hens with keel bone fractures lay fewer and lower quality eggs based on lower shell density, shell weight, egg weight, shell percentage, and egg surface area (Nasr et al., 2012a). Rufener et al. (2018) reported that laying hens with actively healing keel bone fractures biologically prioritize creating a lower number of quality eggs, reducing egg production. In addition, Wei et al. (2020) noted that keel fractures impact external egg qualities such as eggshell thickness and strength but not internal egg quality. Feed consumption also plays a role, with highest feed consumption occurring before the onset of lay, indicating that during structural bone formation (Hudson et al., 1993) feed intake is necessary to support bone formation (Candelotto et al., 2017). Higher feed consumption ensures more calcium is available in the body and has been linked to a lower susceptibility to fractures (Candelotto et al., 2017). Feed intake impacts egg production as a source of both energy and calcium. Reduced feed intake negatively impacts both the hen's ability to heal by reducing available calcium and thus, overall, the amount of available calcium for egg production. Fresher fractures have more of a negative impact on production as calcium is redirected away from production towards healing the fracture (Rufener et al., 2018). Overall, fresh fractures lead to decreased feed intake which means less calcium is available to both heal the keel and produce eggs.

1.4. Genetic Factors

Red jungle fowl are the living ancestor of the modern commercial laying hen and continue to inhabit areas of Southeast Asia (Kittelsen, 2020). Red jungle fowl have low incidences of KBD with only 8% of hens presenting with a fracture when raised in an aviary system (Kittelsen, 2020). As such, Red Jungle fowl have a considerably lower incidence of KBD in comparison to levels seen in modern laying hens housed in similar environments (86%; Wilkins et al., 2011). The consolidation of genetic companies has resulted in only a few key companies providing laying hens to the world's producers (Fernyhough et al., 2020). Intensive selection for egg production has also led to reduced genetic diversity in commercial laying hens (Muir et al., 2018). Considering the rapid intensification of the industry and strict laying hen breeding programs that have been used in the past century (Leenstra et al., 2016) it is not surprising that more than 50% of genetic diversity is no longer present in commercial strains (broilers and white and brown egg layer pure lines) in comparison to historical populations (Red Jungle Fowl and experimental breeds; Muir et al., 2008).

One of the main differences between the current commercial laying hen and heritage counterparts is a much higher level of egg production. Moderate producing lines (random-bred White Leghorn and New Hampshire) have lower incidences of keel bone fractures (41.1%) compared to higher producing counterparts (White Leghorn and Rhode Island Red; 66.4%; Habig et al., 2021). Candelotto et al. (2017) used experimental white and brown strains (specifics not provided), which had not been selected for egg production, that had a lower incidence of KBD (20%) in comparison to higher producing commercial counterparts, Bovans Brown, Dekalb White and ISA Dual Brown (90%; Candelotto et al., 2017). When egg production was halted using a synthetic agonist, laying hens had no fractures in contrast to a control group (40%;

Eusemann et al., 2018b). Fractures are likely impacted more than deviations by egg production as Eusemann et al. (2018a) noted no correlation between deviations and egg production, but higher egg production increased the incidence of keel bone fractures. As such, selection for higher rates of egg laying in modern laying hens has contributed significantly to the incidence of KBD, particularly fractures. By identifying other variations between modern and heritage populations, the factors contributing to KBD can be determined and more precise genetic selection can be conducted in order to reduce KBD.

Different strains of birds develop differently which leads to variation in body weight as well as variation in bone characteristics such as density (Regmi et al., 2016). Bone health and mineral density have been reported to be higher in brown layers compared to white layers (Habig et al., 2021). Heerkens et al. (2016) reported that ISA Brown laying hens in contrast to Dekalb White had higher incidence of keel bone fractures but a lower incidence of deviations. Behavioral differences between white and brown strains are also present. White layers engage in perching behaviors more than brown layers do and have better 3D movement skills and flight abilities (Scholz et al., 2014; Heerkens et al., 2016). However, white strains are typically flightier and more fearful which could lead to more panic responses leading to collisions with the perch (Habig et al., 2021). Locomotion, in particular, wing-associated locomotor behaviours (LeBlanc et al., 2018a) are more common in white strains compared to brown strains. LeBlanc et al. (2018b) reported that in order to navigate a challenge within their environment, such as a ramp, laying hens will modulate their locomotion. By determining what adaptations certain strains of laying hens have in order to handle the challenges presented by alternative housing, breeding programs can contribute to reducing the incidence of KBD in laying hen populations.

Bone strength characteristics have been shaped by genetic selection and impact the incidence of KBD (Fleming et al., 2006; Stratmann et al., 2016). Bone strength characteristics such as humeral strength, tibial strength, and keel radiographic density can be selected for by creating a selection program for birds with either higher or lower bone strength and density (Bone Index; Bishop et al., 2000). Bone characteristics have a high heritability of 0.4 (Bishop et al., 2000) indicating it is possible for genetics companies to select for laying hens with stronger bones. Following studies have used the Bone Index to create high and low bone strength lines to assess how the Bone Index impacts KBD (Fleming et al., 2006; Stratmann et al., 2016). Laying hens from the higher bone strength lines had fewer fractures compared to the lower bone strength lines (Fleming et al., 2006; Stratmann et al., 2016). Notably, Barred Plymouth Rock, a heritage strain of laying hen, has higher bone mineral content, cortical density, cortical thickness, and overall bone density (Regmi et al., 2016). Regmi et al. (2016) reported that bone characteristics had a significant impact on keel bone health, with heritage strains such as Barred Plymouth Rock presenting with lower keel deformity scores in comparison to modern strains (e.g., Hy-Line Brown and Silver Brown). With the knowledge that bone strength is highly heritable in laying hens, identifying causative mutations that impact bone strength and health is a logical next step for genetic companies to breed laying hens with stronger and healthier bones that are less susceptible to KBD.

In recent years, dual x-ray absorptiometry (**DXA**) has been used to assess body composition (Salas et al., 2012) and bone parameters (Shim et al., 2012) in poultry. Dual energy x-ray absorptiometry can reliably measure body composition in both deceased and living poultry (Schallier et al., 2019). DXA uses two x-ray beams with different energy levels and can quantify total tissue by attenuating those differences to distinguish between soft (muscle, fat) and hard (bone) tissue (Korine et al., 2004; Salas et al., 2012). Stratmann et al. (2016) utilized DXA to effectively assess different keel bone mineral densities between layer lines. Bone mineral density along with other microstructural parameters, such as bone volume, potentially impact KBD (Wei et al., 2021). Laying hens with lower bone mineral density had increased incidences of KBD (Habig et al., 2021). By effectively measuring bone density and strength, the impacts of bone parameters on KBD can be assessed to assist in selecting preferable phenotypes in laying hens to reduce KBD.

There is a need to investigate the genetic capacity to reduce KBD (Harlander-Matauschek et al., 2015). As KBD is a multi-faceted problem, genetic selection could focus on selecting for stronger bones. By identifying causative mutations on candidate genes that influence bone health, breeding programs can select for laying hens that are less likely to develop KBD. Specific genes are of particular interest regarding laying hen keel bone health. Single nucleotide polymorphisms (**SNP**s) are single nucleotide substitutions (i.e adenine instead of guanine). Substitutions can lead to deleterious effects leading to health issues (Liu et al., 2018b) and reduced production (Ye et al., 2022). Candidate genes can be selected for specific traits, such as bone mineral content, and then SNPs from the gene can be chosen for analysis. To understand the impacts of SNPs, an association analysis between an animal's genotype for certain SNPs and an associated phenotype is used (Ye et al., 2022).

Retinoblastoma 1 (***RB1***) impacts keel length by controlling body growth and osteogenic differentiation (Li et al., 2021; Zhang et al., 2011). *Sclerostin* (***SOST***) is involved in keel length and bone mineral content as the suppression of *SOST* facilitates bone formation and helps prevent fractures (Guo et al., 2017; Li et al., 2021). *Receptor activator of nuclear factor kappa-B ligand* (***RANKL***) also plays a role in bone mineral content due to its involvement in osteoclast differentiation and activation (Guo et al., 2017; Dale et al., 2015). *Periostin* (***POSTN***) impacts bone weight due to its role in the regulation of osteoblast proliferation and bone formation (Guo et al., 2017; Li et al., 2021). *SRY-Box 9* (***SOX9***) aids in chondrocyte development and maturation by promoting the expression of other genes, including *PTH-like hormone* (***PTHLH***) and *collagen type II* (***COL2AI***) which are important for endochondrogenesis, the formation of cartilage in early fetal development (Dale et al., 2015). PTH-like hormone regulates the differentiation of prehypertrophic chondrocytes into hypertrophic chondrocytes and is strongly influenced by artificial selection (Dale et al., 2015). Collagen type II, *SOX9*, *PTHLH*, and *RANKL* work together in developing and remodeling bones (Dale et al., 2015) which is crucial for effectively healing fractures (Wei et al., 2021). By identifying causative mutations on candidate genes associated with bone parameters genetic factors contributing to KBD can be incorporated in laying hen breeding programs.

1.5. Environmental Factors

Environmental factors including perch shape and rearing environment influence keel bone health in addition to genetic factors. By providing opportunities during rearing for pullets to engage in load-bearing exercise, the keel may become more resistant to KBD later in life. Non-cage rearing systems lead to higher bone mineral content and greater cross-sectional area of the radius and tibia from load-bearing exercise (Casey-Trott et al., 2017c) indicative of stronger bones. In addition, rearing pullets in non-cage environments contributes to the development of periosteal and endocortical bone layers, as well as increased skeleton size allowing for higher medullary bone deposition and bone mineralization (Casey-Trott et al., 2017c). Humeri in pullets housed in non-cage systems have increased bone density improving overall bone load-bearing capability (Regmi et al., 2015). In addition, the improvement in bone composition pre-lay provided by non-cage systems (Regmi et al., 2015; Casey-Trott et al., 2017b) has been reported to reduce KBD (Vits et al., 2005; Casey-Trott et al., 2017a). By looking at how different rearing environments for pullets impact their adaptation to their lay environment, additional information can be provided to producers on the ideal rearing environment to ensure their flocks have higher bone parameters and reduced incidences of KBD.

Perching may be an innate behaviour in poultry as pullets will use perches within the first week of life (Skanberg et al., 2021). Perching is a behavioral need in poultry (Weeks and Nicol, 2006) and is made possible by the tendon-lock mechanism (Quinn and Baumel, 1990). The tendon lock allows birds to firmly grasp onto the perch and remain balanced while doing so. Perches are important in allowing hens to roost at night (Olsson and Keeling, 2002) and escape conspecifics (Cordiner and Savory, 2001). Perching is an important behaviour during dark hours when hens spend 75% of their time perching compared to light hours when hens only spend 10% of their time perching (Liu et al., 2018a). Olsson and Keeling (2002) reported that laying hens are motivated to access perches at night, even if it requires them to open a heavy door to gain access to the perch. Early perch use is infrequent, increasing at around 2 weeks of age (Habinski et al., 2017). In cage-free systems, birds that have been housed with enrichments, such as ramps, show decreased hesitancy behaviors including crouching and wing-flapping when utilizing ramps compared to birds that had been housed without ramps (Norman et al., 2021). Early learning during the pullet phase allows pullets to navigate more complex locomotor challenges provided by enrichments, which reduces collisions decreasing the incidence of KBD (Norman et al., 2021).

While the behavioral benefits of perching are explained above, the inclusion of perches has been reported to lead to a significant increase in KBD. In addition, there is a need to understand how providing pullets with opportunities to perch impacts their bone development (Staaveren et al., 2019). When perches are provided during the lay phase, there is increased bone mineral deposition, however it is not enough to offset the increased incidence of KBD when perches are available (Hester et al., 2013). The inclusion of perches and the complexity of the perch (static vs swinging) increased the prevalence of KBD from 67% to 86% (Wilkins et al., 2011). Ascending onto the perch is responsible for most collisions in a furnished cage environment (Baker et al., 2020). In addition, the presence of deviations in the keel can be a risk factor for the development of fractures as unequal bone loading occurs (Harlander-Matauschek et al., 2015). Selection for larger bodies and smaller wings has led to increased pressure being exerted on the keel when laying hens are perching (Campbell, 2021). When perching, regardless of perch shape, hens exert twice the force (N/cm^2) on the keel bone compared to the feet (Pickel et al., 2011). Increased pressure on the keel results in increased keel deviations (Campbell, 2021).

Certain perch design elements such as shape may contribute to KBD (Pickel et al., 2011). Factors to consider include the maximum contact area between the bird and perch as well as the minimum amount of peak force (N/m^2) that is being placed on the keel and feet (Pickel et al., 2011). Keel bone damage was less prevalent when laying hens were provided with softer perches (plastic) that reduced force exerted on the keel (3.9 N/cm$^{2)}$ and increased contact areas between the keel and perch (2.1 cm^2) in contrast to perches made from metal or wood (6.02 N/cm^2 and 1.5 cm^2; Pickel et al., 2011). Polyurethane-coated perches led to reduced deviations and fractures (15.4%) compared to “hard”, non-coated perches (21.5%; Stratmann et al., 2015). Chen et al. (2014) have reported that hens prefer 3 cm wide, rectangular perches as they are easier to grip and reduce slipping or falling in contrast to larger round perches which were less desirable. Softer perches may work similarly to an automobile’s shock absorption system, allowing energy from collisions to be absorbed by the cushion instead of the keel (Stratmann et al., 2015). Overall, reducing peak pressure on the keel contributes to reducing the incidence of deviations.

Perches significantly increase the incidence of KBD in enriched housing, but there is a lack of information on how design features such as shape impact KBD with research focusing on other areas including perch hardness. As the industry navigates towards the widespread usage of perches in enriched housing it will be essential to understand how farmers should implement perches in their barns to promote perching behaviour and to reduce KBD. Current perch designs that are available to producers include round, square, oval and mushroom and are typically made in materials such as plastic or metal (Pickel et al, 2011). Materials such as plastic and metal, are chosen due to their ease to maintain and disinfect in between flocks. Producers need to consider a variety of factors when deciding on perches including cost, cleanliness and impacts on laying hens. By identifying how perching biomechanics are impacted by perch shape and how perch shape contributes to perch use and KBD, producers can select the perch that will work best for their flock.

1.6. Assessing Keel Bone Damage

A variety of techniques are used to assess KBD in laying hens including palpations (Stratmann et al., 2016), x-rays (Rufener et al., 2018), keel computed tomography (Regmi et al., 2016), and image analysis software (Eusemann et al., 2018a). Keel palpation is a common method of detecting KBD as it is minimally invasive and can be performed while a hen is still alive. Palpations can be used to assess the progression of KBD throughout a hen's lifetime while not requiring specialized equipment such as x-ray. Palpations are conducted by an experienced examiner who utilizes a point scoring system. Stratmann et al. (2016), used a 3-point scoring system that included non-damaged, intact keel bone, deviated keel bone and fractured keel bone. Upon assessment, fractures can either be fresh or healed. Fresh fractures are identified by a clear break in the bone while healing or healed fractures are identified by a callus (Kittelsen et al., 2020). Understanding how to properly palpate a keel bone is important to correctly assess KBD in living hens. Notably, there are issues with the identification and classification of KBD. There are high error rates (30%) with methods such as palpation and the validity of the results often depends on the level of experience and training the examiner has (Casey-Trott et al., 2015). Consensus among multiple assessors provides the highest level of reliability to palpation scores (Casey-Trott et al., 2015). Palpation as a means of assessing KBD can be more reliable through ensuring consistent and simplified scoring categories such as a 3-point scoring system (Stratmann et al., 2016) and by properly training assessors.

In addition to *in vivo* keel assessment, post-mortem keel assessments can be conducted to identify aspects of KBD that may not be palpable on examination such as fractures on the caudal end of the keel. Keel assessments can be conducted utilizing x-ray which uses a scoring system based on the severity of fractures present. Rufener et al. (2018) used x-ray while birds were still alive to determine keel bone fracture presence and severity on a 5-point scale going from no keel bone fracture to extremely severe on a tagged visual analog scale. Eusemann et al. (2018a) utilized x-ray to score for deviations and fractures separately. Deviations were scored on presence and severity by utilizing an image analysis software (AxioVision 4.8) to calculate the area of the deviation (Eusemann et al. 2018a). Image analysis software can also measure the angle of the deviation to determine severity (Regmi et al., 2016). Overall, it is preferable to pair palpations of the keel with both postmortem visual assessments and imaging such as x-rays.

1.7. Limitations in Keel Bone Damage Research and Filling the Gap

Keel fractures often still occur in cage systems where there are not many opportunities for collision events to occur indicating collisions are not the sole mechanism of damage (Wilkins et al., 2011; Petrik et al., 2015; Regmi et al., 2016; Saraiva et al., 2019). Factors such as pressure exerted on the keel while perching have been identified as a potential causative factor contributing to keel bone deviations (Pickel et al., 2011; Chen et al., 2014; Harlander-Matauschek et al., 2015). Some research has investigated movements while perching including balancing behaviour (LeBlanc et al., 2016), and stability while accessing a perch from aerial descent (Scholz et al., 2014). Rotational movements and wing movements are factors that assist a bird while balancing on a perch (LeBlanc et al., 2016). Scholz et al. (2014) considered side-to-side movements as indicators of instability when hens land on a perch. Notably, physical impairments in laying hens led to higher rotational movement to remain balanced while perching (LeBlanc et al., 2016). Although perching biomechanics have been preliminarily assessed, strong linkages to KBD incidence have not been identified as either KBD was not measured in a study (LeBlanc et al., 2016) or only visual assessment of video recordings was performed (Scholz et al., 2014). Thus, an opportunity for the use of more precise methods of assessing perching biomechanics could be used in order to identify ideal perching behaviour that minimizes impact on the keel bone.

3D kinematics is a tool that has been utilized in animal research to assess biomechanics including locomotor movements and body postures in livestock (Guesgen and Bench, 2018; Perez Marquez et al., 2020). Kinematics utilizes a three-dimensional co-ordinate system (xyz) to track specific points on a body (Beggs, 1983). By placing reflective markers onto key skeletal landmarks, cameras can track displacement (Caplen et al., 2012; Guesgen and Bench, 2018), angle (Navik et al., 2012) and speed (Provini and Abourachid, 2018). Kinematics has been used in wild bird studies for assessing terrestrial locomotion (Abourachid et al., 2019) and take-off for flight (Provini and Abourachid, 2018). In domestic poultry, kinematics has been used to assess space use (Mench and Blatchford, 2014), gait in broilers (Caplen et al., 2012; Bench, C.J., unpublished data), and stifle joint movement (Navik et al., 2012). Invasive measures were used to attach markers to the birds, including suturing markers onto the skin (Abourachid et al., 2019) or surgically implanting markers into the skeleton (Provini and Abourachid, 2018). Invasive measures are uncomfortable and likely cause pain for the animals, which could have impacted the movement exhibited. In addition, utilizing post-mortem joints to assess movement (Navik et al., 2012) limits recordings to one sampling per bird and may not be an accurate reflection of movements that would occur when a bird is alive. An alternative is taping markers onto key skeletal landmarks (Perez-Marquez et al., 2021; Perez-Marquez et al., 2020; Perez-Marquez et al., 2019; Guesgen and Bench, 2018) which is minimally invasive and does not cause pain.

Another limitation of 3D kinematics research includes creating 3-D recordings from 2-D recordings (Mench and Blatchford, 2014). With specialized 3D kinematics cameras and software 3-D recordings can be taken and assessed without the need for additional software (Perez-Marquez et al., 2021; Perez-Marquez et al., 2020; Perez-Marquez et al., 2019; Guesgen and Bench, 2018). Using 3-D kinematics, laying hen perching biomechanics can be evaluated; in particular, keel movements in relation to the perch. Forward and backward movements are akin to a rocking motion on the perch, side-to-side movements can reflect side-to-side stepping and balancing behaviours (Scholz et al., 2014) and up and down movements could be reflective of small collisions occurring between the keel and perch. In addition, assessing the speed of movement while a laying hen is perching could be indicative of instability while perching and increase risk for KBD if higher speed small-scale collisions between the keel and perch are occurring. Finally, perching angle indicates if a laying hen is leaning forward or backward while on the perch or if she can keep herself mostly upright. Overall, through 3-D kinematics technology, we can evaluate whether specific perching biomechanics are linked to KBD and create environments where unwanted movements are less likely to occur.

Based on previous literature assessing how pressure on the keel impacts keel deviations, particular additional methods for assessing pressure while perching could provide clarity on how pressure contributes to KBD. Infrared thermography utilizes specialized thermal cameras that are non-invasive and portable to detect infrared radiation that is emitted from an object (McCafferty, 2013). Infrared thermography can be used for assessing stress in poultry (Edgar et al., 2013; Moe et al., 2017; Weimer et al., 2020), bumblefoot in laying hens (Wilcox et al., 2009), and potential breast myopathies in broilers (Castilho et al., 2021). Pressure exerted on the keel is a factor contributing to keel deviations (Scholz et al., 2008; Stratmann et al., 2015). As such assessing how factors including strain variation, body weight and perch shape impact pressure exerted on the keel and in turn contribute to KBD. Pressure exerted on the keel could be assessed through differences in blood flow which can be captured by IRT, specifically through convection heat transfer (Perez Marquez et al., 2019). However, the presence of feathers can impact recordings, as they are not a solid surface and instead emit radiation from different layers within the feather matrix (McCafferty, 2011). Feathers provide insulation from heat loss which leads to lower temperature recordings on well feathered areas compared to bare skin (Cook et al., 2006). When conducting infrared recordings, selecting an area of the bird that has no feathers such as the feet (Wilcox et al., 2009) or comb (Edgar et al., 2011; Moe et al., 2017) is more reflective of physiological changes than a feathered area (McCafferty, 2011). Utilizing infrared thermography to assess pressure placed on the keel or the presence of injuries such as fractures would likely be ineffective due to the feather cover along the keel. Alternatively, as other studies have compared the pressure exerted on the footpad and keel while perching (Pickel et al., 2011), infrared thermography will likely be more accurate in assessing footpad temperature changes due to pressure exerted on the feet while perching instead of the keel.

1.8. Project Objectives

The objective of our research was to identify genetic and environmental factors that contribute to KBD in enriched housed laying hens to reduce the incidence of KBD below levels traditionally seen in conventional housing. The goals for this thesis were to:

1. Determine how strain differences between random-bred and commercial strains of laying hens impact perching biomechanics, bone parameters and pressure exerted on the footpad while perching.
2. Determine what ideal perching biomechanics lead to stability and reduced KBD while perching and what perch shape, strain and rearing environment contribute to an ideal perching behaviour using 3D kinematics software.

3. Identify SNPs of interest on candidate genes pertaining to bone health parameters and determine if specific causative mutations increase bone health parameters and reduce KBD.
4. Determine how rearing environment impacts bone quality and KBD in enriched housed laying hens and how perch shape and usage impacts perching biomechanics and KBD throughout the life cycle of a laying hen.

Overall, our research looks to assist producers in transitioning from conventional to enriched housing by identifying what strain, perch shape, and rearing environment is best for their flock to reduce KBD incidence.

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Chapter 2. The effect of laying hen strain on perching biomechanics and keel bone damage

2.1 Introduction

Historically, artificial selection has led to a lower level of genomic sequence diversity in domestic chickens (Rubin et al., 2010). Intensification of agricultural practices has led to the selection of specific strains for either egg or meat production in contrast to original dual-purpose breeds (Siegel et al., 2006). Egg production has been prioritized by genetics companies for many years creating highly productive laying hens that are well suited to conventional housing systems (battery cages; Fernyhough et al., 2019). However, conventional battery cages restrict opportunities for laying hens to exhibit important natural behaviours. In contrast, alternative housing (furnished cages, aviaries, free-run, and free-range) presents opportunities for laying hens to express natural behaviours such as perching via the provision of perches. Since 2014, Alberta's egg industry has adopted a mandatory housing policy that bans new conventional housing systems from being built (Egg Farmers of Alberta, 2021). As a result, by the end of 2021, 52% of laying hens were housed in alternative systems (Egg Farmers of Alberta, 2021). In addition, the National Farm Animal Care Council Layers and Pullets Code of Practice requires all laying hen housing systems to provide perches by 2036 (NFACC, 2017).

Environments that encourage weight-bearing exercise such as walking, and wing flapping aid in bone development leading to increased bone mineral content of the tibia and radius (Casey-Trott et al., 2017a). Despite the many welfare benefits of alternative housing that promote greater behavioral diversity compared to conventional housing, there are some welfare concerns. One main concern is the higher prevalence of keel bone damage (**KBD**; 36% - 90% of the flock; Regmi et al., 2016; Saraiva et al., 2019; Wilkins et al., 2011) in alternative systems compared with conventional battery cages (22-24% of the flock; Petrik et al., 2015; Regmi et al., 2016). As such, keel bone damage is one welfare concern that has been exacerbated by a shift to alternative housing. Due to selective breeding and artificial selection, modern laying hens may not be as well-equipped to handle the challenges presented by more complex housing environments (reviewed by Leenstra et al., 2016). In contrast, Leenstra et al. (2016) suggested that broadening phenotype selection in laying hen breeding programs could provide genotypes that are suitable for a range of laying hen housing systems. As such, the current study focuses on the innate perching biomechanics associated with KBD in various commercial and random-bred laying hen strains.

When sitting on the perch, laying hens exert twice the force (N/cm^2) onto the keel compared to their feet, despite the contact area between the keel and perch being much smaller than between the feet and perch (Pickel et al., 2011). Areas of the keel that are exposed to high levels of pressure while perching could be susceptible to chronic damage to the keel such as deviations. Wider perches distribute the force exerted on the keel by increasing the contact area between the perch and keel, in contrast to round-shaped perches which increase the peak force being exerted on the keel (Pickel et al., 2011). In addition, Chen et al., (2014) reported that hens preferred 3 cm wide, rectangular perches while round perches were less desirable. Thus, wider perches are thought to contribute to laying hen stability while perching and help to decrease the incidence of KBD. However, the use of new tools like 3D kinematics to study perching biomechanics on rounded versus wider perches has not been previously studied.

Certain laying hen strains may exhibit different innate biomechanical tendencies while perching, contributing to KBD. White strains exhibit more resting and perching behaviors compared to brown strains, overall exerting more pressure on their keels (Kozak et al., 2016). Thus, increased perching behaviour could contribute to increased KBD simply due to increased contact between the perch and keel. In contrast, white strains may be more stable while perching due to increased perch use which would reduce the severity of instability events while perching. Brown strains performed more balancing movements including side-to-side movement and wing flapping on perches (Scholz et al., 2014). By balancing more efficiently on perches, the risk of KBD may be reduced. As such, previous literature suggests strain variation impacts perching behaviors which could contribute to perching behaviors and in turn be protective against KBD.

The keel bone is a projection of the sternum in birds and serves as an attachment point for important muscles such as the supracoracoideus and pectoralis (Lowi-Merri et al., 2021). Keel bone damage is a concern in commercial laying hens and can be divided into two types: keel bone deviations and keel bone fractures. Deviations are when the keel has an abnormal structure not caused by fractures, such as S-shaped curves, and have been linked to pressure being exerted on the keel (Harlander-Matauschek et al., 2015). Keel bone deviations are a chronic issue, in contrast to fractures which are typically viewed as an acute issue linked to disease and environmental collisions (Toscano et al., 2020). In many studies, fractures and deviations are grouped together when discussing KBD, which has led to a limited understanding of the specific consequences of deviations in comparison to fractures (Riber et al., 2018). Harlander-Matauschek et al. (2015) hypothesizes that deviations may increase a hen's susceptibility to fractures due to unequal bone loading (amount of weight applied to the bone) when birds slip on the perch and vigorous wing-flapping occurs to regain their balance.

Developing a more complete understanding of risk factors and potential causes of keel deviations allows us to implement strategies to reduce fractures. After a keel bone fracture occurs, hens enter a negative affective state for three to four weeks due to pain, which can lead to immunosuppression and reduced egg production (Armstrong et al., 2020). Hens with fractures often move more slowly than hens without fractures, which is a potential indicator of restricted mobility or pain (Nasr et al., 2012). Hens with fractures also spend more time standing, sitting, and resting in comparison to hens without fractures (Nasr et al., 2012; Wei et al., 2020) indicating that due to pain laying hens have restricted mobility.

As a laying hen heals a keel bone fracture, producing a lower number of quality eggs is prioritized leading to reduced egg production (Rufener et al., 2018). In addition, Wei et al. (2020) noted that keel fractures impact external egg qualities such as eggshell thickness and strength. Fresher fractures have increased negative impacts on production at end of lay, as the hens are unable to meet both the physiological demands of healing the fracture and maintaining egg production (Rufener et al., 2018). Fractures are often concentrated on the caudal third or tip of the keel bone which is the last part of the bone to ossify between 28 to 40 weeks of age (Buckner et al., 1948; Buckner et al., 1949), which may contribute to an increased susceptibility to fractures (Habig et al., 2021). The incidence of KBD increases with egg production as birds at pre-lay have a lower incidence of KBD in comparison to birds at peak lay (Habig et al., 2021).

As laying hen housing becomes more complex through the addition of enrichments, breeding programs must consider a wider range of traits beyond egg production, such as bone health, for laying hens to thrive. Bone mineral density potentially impacts KBD (Wei et al., 2021). Habig et al. (2021) reported that laying hens with lower bone mineral density had increased incidences of

KBD. Dual x-ray absorptiometry (**DXA**) has been used to assess bone parameters (Shim et al., 2012; Stratmann et al., 2016) in poultry. DXA uses two x-ray beams with different energy levels and can quantify total tissue by attenuating those differences to distinguish between soft (muscle, fat) and hard (bone) tissue (Korine et al., 2004; Salas et al., 2012). By effectively measuring bone density and strength, the impacts of bone parameters on KBD can be assessed to assist in selecting preferable phenotypes in laying hens to reduce KBD.

Three-dimensional (3D) kinematics is a novel tool that is used to assess biomechanics including locomotor movements and body postures in livestock (Guesgen and Bench, 2018; Perez Marquez et al., 2020). By placing reflective markers onto key skeletal landmarks, cameras can track displacement (Caplen et al., 2012; Guesgen and Bench, 2018), angle (Navik et al., 2012) and speed (Provini and Abourachid, 2018). Forward and backward movements are akin to a rocking motion on the perch, rocking movements could contribute to the development of deviations. Side-to-side movements reflect side-to-side stepping and balancing behaviours, Scholz et al. (2014) used increased side-to-side movement as a perching instability indicator. Finally, up and down movements could be reflective of small collisions occurring between the keel and perch which could contribute to KBD. Angle indicates if a laying hen is leaning forward or backward while on the perch or if she can keep herself mostly upright. In addition, movement speed can be assessed as a potential indicator of instability. Higher speed, small-scale collisions between the keel and perch could increase risk for KBD. Using 3D kinematics, we can evaluate perching biomechanics of laying hens and linkages to KBD.

Pressure exerted on the keel is a factor contributing to keel deviations (Scholz et al., 2008; Stratmann et al., 2015), assessing pressure while perching could provide clarity on how pressure contributes to KBD. Infrared thermography cameras provide a non-invasive and high-resolution means of assessing temperature variations in living tissues and is an effective tool for detecting health concerns in poultry (McCafferty, 2012; Wilcox et al., 2009). Differences in blood flow can be captured by IRT, specifically through convection heat transfer (Perez Marquez et al., 2019), which could be used to assess pressure exerted on the keel. Areas such as the feet that are not feathered (Wilcox et al., 2009) provide accurate infrared recording on physiological changes in laying hens. As previous research compared pressure exerted on the keel and feet while perching (Pickel et al., 2011), pressure exerted on the footpad while perching could provide information on the pressure exerted on the keel.

The primary objective of this study was to determine how strain and perch shape impact perching biomechanics at different phases of lay. Secondly, to determine how strain affects KBD, bone composition, body weight and foot temperature. We hypothesize that strain and perch shape will influence perching biomechanics at different stages of lay.

2.2. Materials and methods

2.2.1. Animals and Housing

This study occurred at the University of Alberta Poultry Unit in Edmonton, Alberta, Canada in accordance with the University of Alberta Animal Use Protocol AUP 3299 and Canadian Council on Animal Care guidelines (CCAC, 2009). This study used a 6 x 2 factorial arrangement of treatments with measurements taken at 18 weeks (pre-lay), 29 weeks (peak-lay) and 70 weeks (end of lay). Measurement days such as 18 weeks (pre-lay) were confirmed across strains via egg production data collected by the University of Alberta Poultry Unit staff. Four commercial egg-laying strains (Lohmann White, Lohmann Brown, ISA Brown and Shaver White) and two University of Alberta Poultry Unit random-bred heritage strains (Shaver Rhode Island Red and Antique University of Saskatchewan White Leghorn) were evaluated. Commercial strains were housed from December 2019 until May 2021 and random-bred strains were housed from June 2020 until November 2021. Twenty birds from each strain were studied (n=20/strain). All birds were wing banded with an identification number at 18 weeks of age and housed to 70 weeks of age. Birds were split by strain into floor pen enclosures (3.9 m by 4.4 m) post-hatch. Enclosures consisted of feeders, waterers, and an elevated nest box with roosts, but did not include perches other than those associated with nest boxes. To limit familiarity bias, perches were not included in the home pen in order that kinematic recordings would reflect innate perching ability on each perch shape. During recordings, round and mushroom shaped perches were used. Each recording perch was 30 cm in length, 4.8 cm in diameter and 0.6 m tall, the recording round perch was constructed using 3.8 cm PVC pipe and the recording mushroom perch was a section of a Comfort Perch System (Lubing Systems, Cleveland, TN). All birds were housed with a day length of 14L:8D during lay and all birds were fed according to NRC requirements and provided water and feed *ad libitum* (NRC, 1994).

2.2.2. Data collection

Weight. Individual body weights were collected at pre-lay (18 weeks of age), peak of lay (29 weeks of age) and end of lay (70 weeks of age) just prior to 3-D kinematic imaging using a BW-2050 Weighing System (Weltech International Ltd., Cambridgeshire, UK). Weights were collected to assess how body weight impacted perching biomechanics and bone parameters as a covariate.

3-D Kinematics. Kinematic recordings were captured using six Vicon Nexus Bonita cameras (Bonita, Vicon Motion Systems Ltd., Denver, CO). Cameras were connected to a desktop computer running Vicon Nexus 2.12 software (Vicon Motion Systems Ltd., Denver, CO) and arranged in a semi-circle facing a recording perch. Recordings were captured at 18, 29 and 70 weeks of age representing phases of the lay cycle on both round and mushroom perches. Seven 14 mm diameter (Life Science Basic Kit, Vicon Motion Systems Ltd., Denver, CO) markers were placed on each bird after being removed from the home pen. Four back markers were placed on the pygostyle (B1), free thoracic vertebrae (B2), left (B3), and right iliac crest (B4) to form a back segment (Figure 1). Three keel markers were utilized along the keel at the caudal (K1) and cranial (K2) ends and one halfway between them (K3) to form a keel segment (Figure 1). Markers were placed at either end of each kinematic recording perch (P1 and P2) to form a perch segment. Before each recording, each bird was placed gently on the recording perch and checked to ensure it had performed a tendon lock and was securely on a perch. A recording was considered acceptable when a bird was on the perch for a minimum of 30 s following a tendon lock. The order of birds recorded was randomized and the recording process was repeated so that each bird had a recording on both a round and mushroom recording perch at each phase of lay.

After recording, kinematic clips were reconstructed using the Reconstruct pipeline in Nexus software. Recordings were labeled based upon a skeleton template that included all markers. The y-axis represented the forward and backward movement of a laying hen, while the x-axis represented side-to-side movement, and the z-axis represented up and down movement. Recordings were shortened to the middle 1000 frames, standardizing the length of recordings.

Labeled recordings were exported to ProCalc (Vicon Motion Systems Ltd., Denver, Colorado). The following metrics were assessed; back angle (**BA**), keel angle (**KA**), side-to-side back motion (**BMx;** mm), side-to-side keel motion (**KMx;** mm), forward and backward keel motion (**KMy;** mm), forward and backward back speed (**BSy;** mm/s), up and down back speed (**BSz;** mm/s), forward and backward keel speed (**KSy;** mm/s), up and down keel speed (**KSz;** mm/s), standing, and resting duration on the perch (Table 1).

Infrared Thermography. To assess footpad temperature as an indicator of foot pressure on the perch, infrared thermography (**IRT**) images were recorded after each 3-D kinematic recording. Images (160 x 120 pixels) were captured using a FLIR E40bx camera (Webmaster Teledyne FLIR LLC, Wilsonville, OR USA) at pre-lay (18 weeks of age), peak of lay (29 weeks of age) and end of lay (70 weeks of age). The camera was set to an emissivity of 0.98 which is used for live tissues and a resolution of 160 x 120 pixels. FLIR Tools software (Webmaster Teledyne FLIR LLC, Wilsonville, OR USA) was used to identify the hottest pixel (maximum temperature) from a 50-pixel circle digitally drawn on each footpad image (Perez-Marquez et al., 2019). Use of the maximum temperature was chosen to avoid any effect of feces or other debris on the feet which could have affected an average temperature measure. Following weighing, kinematics and IRT recording, kinematic markers were gently removed, and each bird was returned to its home pen.

Dual X-Ray Absorptiometry. Bone mineral content and density measurements were analyzed post-mortem at 70 weeks of age utilizing LUNAR Prodigy Dual X-Ray Absorptiometry (**DXA**; GE Healthcare Canada, Mississauga, ON, Canada). Scans were conducted on the keel and the left femur which was dissected and cleaned. Before scanning, the machine was calibrated to 2% ± the mean densities of a phantom spine with a known set of densities (GE Healthcare Canada, Mississauga, ON, Canada). The keel and femur of each bird were then placed horizontally into a plastic container filled with rice. Rice allowed for all bones to be imaged at the same angle. Keel bone mineral content (**KeelMC**), keel bone mineral density (**KeelMD**), femur bone mineral content (**FemurMC**) and femur bone mineral density (**FemurMD**) were measured and recorded.

Keel Bone Assessment. Digital keel bone imaging was used to determine the severity of keel bone deviations as a measure of KBD. Excised keels were cleaned of muscle tissue and were placed on a black background alongside a ruler to ensure a standard scale for image analysis. Right sagittal and ventral view images were recorded using a Canon PowerShot SX420 IS (20 Megapixel, Canon Canada Inc, Brampton, ON, Canada). Images of the keel were analyzed using ImageJ software (ImageJ, National Institutes of Health, 1997). Keel length was measured from the caudal tip to the cranial end. The number of deviations was noted for both the right sagittal and ventral view. The severity of each deviation was measured using the angle tool in ImageJ. Modified from Regmi et al. (2016), keels were scored based on the severity and number of deviations present on a 5 pt scale from 1 to 5 (5 being the most severe, indicated 5+ deviations present). Keel deviation severity was classified based on least to most severe deviation (178°-172° was a score of "1", 171°-156° was "2", 155°-142° was "3", 141°-126° was "4" and 125°-110° was "5"). Keel score was based on the most severe of the two scores. For example, a keel with a score of 3 for the number of deviations present and a 5 for the severity of the worst deviation received a final keel score of 5.

2.2.3. Statistical Analysis

Data were analyzed using SAS software (SAS ver 9.4, Cary, NC, USA). To test normality assumptions, a ProcUnivariate with a Kolmogorov-Smirnov test was run on all metrics. 3D-kinematic metrics with outliers that fell ± 3SD from the mean were removed from the data set. All metrics, except for keel scores, were then analyzed using a generalized linear mixed model (Proc Glimmix; SAS Institute, 2013), Type 3 test with the inverse (ilink) function specified. Distributions used were selected based on the lowest Akaike information criterion (AIC). Back angle, keel angle, keel length, and temperature were analyzed using a normal distribution. Keel bone mineral content, KeelMD, FemurMC, FemurMD, BMx, KMx, KMy, BSy, BSz, KSy, and KSz were analyzed using a gamma distribution. Standing and resting duration were analyzed using a lognormal distribution. Fixed variables in the glimmix model were strain and perch shape; weight was included as a covariate. All models were run to determine any interactions between strain x perch separately for each phase of lay. If an interaction was not significant, a subsequent model was run without the specific interaction. Least Squares Means (**LSMeans**) differences were adjusted for multiple comparisons using a Bonferroni test. Categorical data modeling using the catmod procedure were used for keel bone damage scores. Strain was the fixed variable. Pearson correlation coefficients were calculated using the corr procedure to compare different kinematic markers to identify which marker could be used as a proxy marker when the primary choice was not visible in a recording (e.g., due to dust). As a result, for keel metrics, K1 was identified as the primary marker and K3 was identified as the proxy marker (0.95; $P<0.001$), for back metrics B1 was identified as the primary marker and B2 was identified as the proxy marker (0.97; $P<0.001$). Pearson correlation coefficients were also calculated using the corr procedure between kinematic metrics, bone parameters, IRT metrics, weight, and keel scores. For this study, P-values < 0.05 were considered statistically significant, $0.05 > P > 0.10$ was considered a trend, and $P> 0.10$ was considered not statistically significant. Correlation coefficients were considered strong if > 0.80, moderate if between 0.50-0.80, and weak if < 0.50. A strong negative correlation was considered if < -0.80, moderate if between -0.50 and -0.80 and weak if < -0.50.

2.3. Results

2.3.1. Body Weight

At all stages of lay brown birds (Lohmann Brown, ISA Brown, and Rhode Island Red) weighed significantly more than white birds (Lohmann White, Shaver White, White Leghorn; Figure 2.2; $P<0.001$).

2.3.2. Bone Traits

All bone parameters were affected by strain (Table 2.2). Keel bone mineral density in each of the brown strains and White Leghorn was significantly denser than in Shaver White ($P<0.001$) and Rhode Island Red was denser than Lohmann White. There was a significant strain effect on KeelMC with Rhode Island Red having the highest mineral content, followed by ISA Brown, Lohmann Brown and White Leghorn. In contrast, both commercial white strains had the lowest mineral content ($P<0.001$). Femur bone mineral density ($P<0.001$) and FemurMC ($P<0.001$) were significantly denser in Rhode Island Red compared to all other strains. Rhode Island Red had significantly longer keels than all other strains, and ISA Brown had significantly longer keels than all white strains ($P<0.001$). Severity of deviations present was significantly impacted by strain with severe damage (i.e., score of 4-5) being most prevalent in Lohmann White (69%

of keels) followed by Lohmann Brown (60% of keels), Shaver White (45% of keels), White Leghorn (32% of keels), and ISA Brown (21% of keels). Notably, Rhode Island Red had no severe keel bone damage ($P<0.001$; Table 2.3). The number of deviations present was also significantly impacted by strain, with the highest number of deviations (4+) present in Shaver White (55% of keels) followed by Lohmann Brown (33% of keels), White Leghorn (26% of keels), Lohmann White (10% of keels). ISA Brown and Rhode Island Red did not present with any keels with four or more deviations. The number of deviations was weakly, negatively correlated with KeelMD (r -0.23; $P<0.01$), FemurMD (r -0.20; $P<0.05$) and FemurMC (r -0.19; $P<0.05$). Severity of deviations was weakly, negatively correlated with KeelMD (r -0.24; $P<0.01$), KeelMC (r -0.22; $P<0.05$), FemurMD (r -0.28; $P<0.01$) and FemurMC (r -0.30; $P<0.05$).

2.3.3. Infrared Thermography

Strain had a significant impact on footpad temperature ($P<0.001$; Figure 2.3). At pre-lay, Lohmann White and Shaver White had significantly colder feet than all other strains (Figure 2.3 (A)). At peak lay, Shaver White had significantly colder feet than other strains (Figure 2.3 (B)). At end of lay, Shaver White and White Leghorn had colder feet than ISA Brown, Lohmann Brown, and Lohmann White (Figure 2.3 (C)), and ISA Brown had hotter feet than Rhode Island Red, Shaver White, and White Leghorn ($P<0.001$). There was no correlation between weight and maximum footpad temperature on mushroom ($P>0.10$) or round perches ($P>0.10$). There was also no correlation between maximum footpad temperature and keel deviation severity score following perching on either the mushroom ($P>0.10$) or round ($P>0.10$) perches.

2.3.4. 3-D Kinematics

Speed. Pre-lay birds had faster forward and backward back speed on round perches (47.9 mm/s ± 3.38) than on mushroom perches (37.2 mm/s ± 2.90: $P<0.05$). As well as faster up and down back speed on round perches (48.9 mm/s ± 3.99) compared to mushroom perches (35.99 mm/s ± 3.09; $P<0.01$). White Leghorns had faster forward and backward back movement than Lohmann White ($P<0.05$; Table 2.4). Up and down keel speed was faster in White Leghorn than Rhode Island Red ($P<0.01$; Table 2.4). Forward and backward keel speed was faster in White Leghorn compared to Lohmann Brown and Rhode Island Red while Shaver White and ISA

Brown moved faster than Rhode Island Red (P<0.001; Table 2.4). Notably, there was a trend of White Leghorn having faster up and down back movement compared to other strains (P=0.06; Table 2.4).

In contrast to laying hens at pre and end of lay, peak of lay birds' movement speed was not significantly impacted by perch shape (P>0.10). At peak lay, only keel speed was impacted by strain. Up and down keel speed was significantly faster in Shaver White compared to Lohmann Brown (P<0.05; Table 2.5). Forward and backward keel movement was significantly faster in all other strains compared to Lohmann Brown (P<0.001; Table 2.5).

At end of lay, forward and backward back speed was faster on round perches (37.2 mm/s ± 2.31) compared to mushroom perches (29.4 mm/s ± 1.84; P<0.01). Up and down back speed was faster for birds perching on round perches (39.2 mm/s ± 3.19) compared to mushroom perches (29.1 mm/s ± 2.34: P<0.01). Forward and backward keel speed was faster on round perches (98.4 mm/s ± 7.86) compared to mushroom perches (69.7 mm/s ± 5.60; P<0.01). Up and down keel speed was faster on round perches (61.6 mm/s ± 4.73) in contrast to mushroom perches (46.6 mm/s ± 3.57; P<0.05).

End of lay birds' forward and backward back speed was impacted by strain; Shaver White and White Leghorn moved faster than Lohmann Brown (P<0.001; Table 2.6). Up and down keel speed was significantly impacted by strain at end of lay with Shaver White and White Leghorn moving faster than Lohmann Brown, Lohmann White, and Rhode Island Red. End of lay Shaver White hens moved faster than ISA Brown (P<0.001; Table 2.6). Forward and backward keel speed was significantly impacted by strain with Shaver White and White Leghorn moving faster than Lohmann Brown and Rhode Island Red, while only White Leghorn moved faster than ISA Brown (P<0.001; Table 2.6).

Angle. Notably, keel angle was not significantly affected by strain or perch shape (P>0.10). Pre lay birds did not have a significant difference in back angle (P<0.10). Birds at peak lay had a greater back angle while perching on round perches (93.2° ± 0.85) in comparison to mushroom perches (90.5° ± 0.85; P<0.05). Birds at end of lay had a greater back angle while perching on round perches (94.9° ± 1.00) in comparison to mushroom perches (90.4° ± 1.00; $P<0.01$). Strain did not have a significant effect on back angle at any stage of lay ($P<0.10$).

Motion. At pre lay (Figure 2.4 (A)) commercial strains had more up and down keel movement than random-bred strains ($P<0.01$). Pre lay birds had more up and down keel movement on mushroom perches (157.53 mm ± 3.85) than round perches (143.9 mm ± 3.50; $P<0.01$). Strain had a significant effect on side-to-side back motion at pre-lay, White Leghorn moved more than ISA Brown and Lohmann White ($P<0.001$; Figure 2.5 (A)). Side-to-side keel motion was not impacted by strain or perch shape at pre lay ($P>0.10$; Figure 2.6). At pre-lay there was a significant strain x perch shape interaction, with each of the Lohmann strains moving forward and backward less on round perches compared to mushroom perches ($P<0.001$) while other strains were not impacted by perch shape (Figure 2.7 (A)). Strain significantly impacted the amount of time spent perching with White Leghorn (4.37 cs ± 0.44) perching less than Rhode Island Red (6.2 cs ± 0.27), Lohmann White (6.9 cs ± 0.26), and Lohmann Brown (6.6 cs ± 0.20). Shaver White (6.1 cs ± 0.28) and ISA Brown (5.77 cs ± 0.40) did not perch a significantly different amount of time compared to other strains.

At peak lay, all other strains had more up and down keel movement than Rhode Island Red ($P<0.01$; Figure 2.4 (B)). Peak lay hens had more up and down keel movement on mushroom perches (192.5 mm ± 2.64) than round perches (180.97 mm ± 2.43; $P<0.001$). At peak lay there was a significant strain x perch shape interaction impacting side-to-side motion, ISA Brown and Lohmann White moved more on mushroom perches compared to round perches while other strains were not impacted by perch shape ($P<0.001$; Figure 2.5 (B)). Notably, at peak lay Lohmann White was the only strain that moved differently based on perch shape, with more side-to-side keel movement occurring on mushroom perches in comparison to round perches (Figure 2.6. (B); $P<0.01$). Regardless of perch shape White Leghorn moved forward and backward more than Shaver White and Lohmann White ($P<0.001$; Figure 2.7(B)). Rhode Island Red only moved forward and backward more than Lohmann Brown on mushroom perches but moved significantly more than Lohmann Brown and ISA Brown on round perches ($P<0.001$; Figure 2.7 (B)). At peak lay, amongst all strains Lohmann Brown moved forward and backward less than other commercial strains on both perch shapes ($P<0.001$). At peak lay perch shape and strain did not significantly impact perching duration ($P<0.10$).

At end of lay (Figure 2.4 (C)), commercial strains had more up and down keel movement than random-bred strains ($P<0.01$). End of lay birds had more up and down keel movement on mushroom perches (374.0 mm ± 3.08) than round perches (360.3 mm ± 2.97; $P<0.001$). Side to side back motion was not impacted by strain or perch shape at end of lay ($P>0.10$). Up and down keel motion was the only kinematic metric correlated with the number of deviations (r 0.2; $P<0.001$) and the severity of deviations present (r 0.3; $P<0.001$) at end of lay. Side-to-side keel motion was not impacted by strain or perch shape at end of lay ($P>0.10$; Figure 2.6). At end of lay, only strain had a significant effect, with Rhode Island Red, White Leghorn, and Lohmann Brown moving forward and backward more while perching than all other strains ($P<0.001$; Figure 2.7 (C)). At end of lay there was no significant strain or perch shape effects on side-to-side back motion ($P>0.10$). At end of lay perch shape significantly impacted perching duration with mushroom perches (6.9 cs ± 0.07) leading to more perching than round perches (6.7 cs ± 0.07).

2.4. Discussion

Bone Parameters. White Leghorn had the lowest prevalence of severe KBD compared to commercial white birds, and Rhode Island Red had the lowest prevalence of severe KBD compared to commercial brown birds. A potential explanation for the strain difference is egg production levels. Based on historical data collected between 2017 and 2020, random-bred strains have a lower rate of egg production at peak lay; Rhode Island Red (84%; K. Nadeau, unpublished data) and White Leghorn (80%; K. Nadeau, unpublished data). In contrast, commercial strains have higher egg production; Lohmann White (94%; Lohmann Tierzucht. 2019), Lohmann Brown (94%; Lohmann Tierzucht. 2019), ISA Brown (96%; Hendrix Genetics, 2023a), and Shaver White (95%; Hendrix Genetics 2023b). The previously reported relationship between lower egg production, bone health, and lower incidences of KBD support our results that the lower producing, random-bred strains have higher bone health parameters and lower incidences of KBD (Candelotto et al., 2017; Eusemann et al., 2022; Habig et al., 2021).

In this study, the heavier strains have denser bones, aligning with previous research (Habig et al., 2021). Brown strains (Lohmann Brown, ISA Brown, and Rhode Island Red) weigh more than white strains (Lohmann White, Shaver White, White Leghorn), which is reported by previous studies (Chew et al., 2021; Riczu et al., 2004). Both femur and keel bone mineral content and density were weakly, negatively correlated with final keel bone damage scores. Femur bone strength was indicative of stronger keels, and in turn, lower levels of severe KBD. Rhode Island Red and ISA Brown had the least severe KBD and the densest bones in contrast to Shaver White and Lohmann White who had significantly lower bone density and more severe KBD. Notably, ISA Brown had the lowest incidence of severe KBD in contrast to other commercial strains, which may be explained by ISA Brown higher bone parameters. Our results contrast with Fawcett et al. (2020) who report that the keels of brown birds ossify at a slower rate in comparison to white birds and have a higher incidence of KBD. In this study, Rhode Island Red and ISA Brown had the least severe KBD across strains and higher bone mineral content and density.

Infrared Thermography. Infrared thermography imaging was conducted to identify pressure exerted on the feet while perching. No effect of perch shape on footpad temperature was found, which may be due to the short period of time that birds were placed on the recording perch. If birds were placed onto the perch for five to ten minutes instead of 30 seconds, there may be a change in the thermal profile of the foot. However, there was a significant effect of strain at each stage of lay. Based on Campbell (2021), heavier bodies should exert more pressure on the keel and feet while perching, leading to higher footpad temperatures after perching. Based on our results indicating that brown strains were heavier than white strains, one might assume that brown strains would have consistently higher footpad temperatures than white strains. Although Shaver White had colder feet at all stages of lay, there was no distinct pattern of white strains having colder feet than all brown strains. In addition, body weight was not correlated with footpad temperature, indicating that differences in footpad temperature was not the result of pressure exerted on the keel while perching. Another physiological process that infrared thermography may be inadvertently measuring is day to day egg laying. Laying hens in lay have significantly higher blood flow than birds not in-lay (Hu et al., 2019). Differences in blood flow can be captured by IRT, specifically through convection heat transfer (Perez Marquez et al., 2019). In this study, before imaging, birds were not palpated to determine if they were forming an egg, which inhibited our ability to determine if temperature differences between strains were due to egg laying.

Perching Biomechanics. Birds were placed on round or mushroom perches, which were selected based on their current availability to producers and usage on commercial egg farms. Mushroom perches are wider than round perches and more evenly distribute the force exerted on the keel (Pickel et al., 2011). Deviations, in contrast to fractures, are caused by force exerted on the keel while perching over extended periods of time (Harlander-Matauschek et al., 2015). Of particular interest in this study was the directionality of movement that was occurring, the speed of movement, and the angles between the perch and the back and keel while perching.

When comparing the movement patterns of commercial and random-bred strains, commercial strains moved up and down more than random-bred strains at pre-lay and end of lay. In contrast, random-bred strains moved forward and backward more than commercial strains. Up and down keel motion was the only kinematic metric correlated with the number of deviations and the severity of deviations present at end of lay. Up and down movements are thought to reflect small collisions between the perch and keel, causing KBD (Stratmann et al., 2016). Fundamentally different patterns of movement occurred in random-bred vs commercial strains. Differences in movement patterns may be linked to genetic differences between random-bred and commercial populations of laying hens. Random-bred strains have not undergone the same extensive selection process as commercial strains. Commercial strains have been selected for living in conventional housing systems that do not provide opportunities for perching (Fernyhough et al., 2019). Forward and backward movements while perching may be preferable to up and down movements to remain balanced on the perch.

In addition to forward and backward and up and down movements, side-to-side movement has been previously used as an instability indicator (Scholz et al., 2014). Pre-lay was the only stage where strain had a significant impact on side-to-side back movement, where White Leghorn moved more than Lohmann Brown. Although weight was not significantly different between strains at pre-lay, White Leghorns were lighter than Lohmann Browns, which could potentially explain the variation in movement. Only at peak lay. Lohmann White on round perches moved less than all other strains on mushroom perches. Overall, very little variation was seen in side-to-side movement across stages of lay and strain, which could indicate side-to-side movements were not an effective measure of instability while perching or simply that there were no strain differences. Although a great deal of strain variation was reported in the perching biomechanics measured in this study, selecting for an ideal set of perching behaviors would be extremely difficult due to the polygenic nature of behaviour phenotypes (Chabris et al., 2015). Alternatively, assessing which environmental factors can be manipulated to encourage preferred perching biomechanics may be possible.

Laying hens on mushroom perches exhibited more movement while laying hens on round perches exhibited faster movements. At all stages of lay, birds had more up and down movement on mushroom perches compared to round perches. Up and down keel motion was the only kinematic metric correlated with the number of deviations and the severity of deviations present at end of lay. At peak lay, mushroom perches led to more side-to-side motion only for ISA Brown and Lohmann White. At pre-lay, Lohmann strains moved forward and backward more on mushroom perches while other strains were not impacted by perch shape. At end of lay, perch shape significantly impacted perching duration where mushroom perches led to more perching than round perches. In contrast, laying hens moved faster on round perches compared to mushroom perches. Wide perches (mushroom) are preferable for laying hens in comparison to round perches as they are easier to grip, providing increased stability (Chen et al., 2014; Pickel et al., 2011). Quicker movements may be indicators of instability while perching, which in turn increases the risk of KBD. Instability could contribute to birds spending more time resting on the perch to balance, which was reflected in back speed being negatively correlated with standing duration. Increased time spent resting on the perch could equate to increased pressure on the keel and in turn more severe deviations.

Keel and back angles were used as posture indicators, specifically, smaller angles were indicative of birds leaning forward on the perch while larger angles reflected birds leaning backwards on the perch. Round perches led to laying hens leaning backwards on the perch to a greater extent than mushroom perches at peak and end of lay. Leaning further back on round perches could be necessary for laying hens to remain balanced, which may put additional pressure onto the caudal end of the keel, which is particularly susceptible to fractures (Habig et al., 2021). However, the variation in back angle was small, with all values falling between 80 and 100 degrees. Back angle while perching was not significantly impacted by the presence of KBD. Strain and perch did not significantly impact keel angle, potentially due to the smaller range of motion of the keel [-126 to 141 mm] when compared to the back [-150 to 198 mm].

2.5. Conclusion

Overall, strain and perch shape significantly impacted perching biomechanics. Perching instability reflected through speed metrics showed that round perches contributed to faster movements and white strains moved faster than brown strains. Additionally, strain impacted movement patterns with commercial strains moving up and down more and forward and backward less than random-bred strains. Up and down movements correlated with a higher incidence of severe KBD, in comparison to forward and backward rocking and side-to-side movement which did not. Femur bone strength was reported to be indicative of stronger keels and in turn lower levels of severe KBD. In addition, higher producing, commercial strains had higher incidences of severe KBD. Perch shape did not impact footpad temperature, but variation amongst strains at different stages of lay indicate future research utilizing infrared thermography to assess biological functions such as egg production in laying hens could be successful. Future research should assess if certain perching biomechanics contribute to KBD. Scoring keels at the same time point that kinematic recordings are taking place will lead to a more complete understanding of the relationships between movement and movement speed and the development of KBD.

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Table 2.1. 3-D kinematic metrics and corresponding descriptions used to analyze perching biomechanics.

Metric	**Description**
Maximum back angle (BA; degrees)	Maximum angle between B1 and the center of the perch. If B1 was not present, B2 was used.
Maximum keel angle (KA; degrees)	Maximum angle between K1 and the center of the perch. If K1 was not present, K3 was used.
Maximum back speed in the y-axis (Bsy; mm/s)	Maximum speed of the midway point between B1 and B2 in the y-axis. If B1 and B2 were not present, the point between B3 and B4 was used.
Maximum back speed in the z-axis (BSz; mm/s)	Maximum speed of the midway point between B1 and B2. If B1 and B2 were not present, the point between B3 and B4 was used.
Maximum keel speed in the y-axis (KSy; mm/s)	Maximum speed of K1 in the y-axis. If K1 was not present, K3 was used.
Maximum keel speed in the z-axis (KSz; mm/s)	Maximum speed of K1 in the z-axis. If K1 was not present, K3 was used.
Maximum back motion in the x-axis (BMx; mm)	Greatest change in position of the midway point between B1 and B2 in the x-axis. If B1 and B2 were not present, the point between B3 and B4 was used.
Maximum keel motion in the x-axis (KMx; mm)	Greatest change in position of K1 in the x-axis. If K1 was not present, K3 was used.
Maximum keel motion in the z-axis (KMz; mm)	Greatest change in position of K1 in the z-axis. If K1 was not present, K3 was used.
Maximum keel motion in the y-axis (Kmy; mm)	Greatest change in position of K1 in the y-axis. If K1 was not present, K3 was used.
Duration of standing (cs)	Length of time hen was upright with keel not in contact with the perch.
Duration of perching (cs)	Length of time hen was resting with keel in contact with the perch.

Abbreviation: **B1**, pygostyle. **B2**, free thoracic vertebrae. **B3**, left iliac crest. **B4**, right iliac crest. **K1**, caudal keel. **K2**, cranial keel. **K3**, middle keel.

Table 2.2. LSMeans (± SEM) for bone parameters measured using dual x-ray absorptiometry post-mortem at 70 weeks of age in six laying hen strains**.**

Strain	**KeelMD (g/cm^2)**	**FemurMD (g/cm^2)**	**KeelMC (g)**	**FemurMC (g)**	**Keel Length (cm)**
ISA Brown[2]	0.20^{ab} ±0.007	0.31^{b} ± 0.014	2.7^{b} ± 0.19	3.3^{b} ± 0.18	9.2^{b} ± 0.15
Lohmann Brown[2]	0.20^{ab} ±0.006	0.32^{b} ± 0.013	2.6^{b} ± 0.19	3.4^{b} ± 0.18	8.9^{bc} ± 0.15
Rhode Island Red[1]	0.22^{a} ± 0.008	0.50^{a} ± 0.025	4.6^{a} ± 0.37	5.8^{a} ± 0.27	10.3^{a} ± 0.16
Lohmann White[2]	0.18^{bc} ±0.006	0.31^{b} ± 0.012	1.9^{c} ± 0.07	2.8^{b} ±0.17	8.3^{c} ± 0.15
Shaver White[2]	0.15^{c} ± 0.006	0.29^{b} ± 0.015	1.6^{c} ± 0.8	2.5^{b} ± 0.19	8.3^{c} ± 0.16
White Leghorn[1]	0.20^{ab} ± 0.008	0.34^{b} ± 0.019	2.6^{b} ± 0.18	3.4^{b} ± 0.28	8.4^{c} ± 0.15
P value	<0.001	<0.001	<0.001	<0.001	<0.001

Abbreviation: **KeelMD**, keel bone mineral density. **FemurMD**, femur bone mineral density. **KeelMC**, keel bone mineral content. **FemurMC**, femur bone mineral content.

[a-c] Values within columns with different letters are significantly different ($P<0.05$).

[1] Random-bred strains from University of Alberta Heritage Poultry Program.

[2] Commercial laying hen strains commonly housed in Alberta.

Table 2.3. Percentage (%) of birds by strain according to number of deviations (0 to 5) and severity of deviation (A to E) scored post-mortem at 70 weeks of age via visual assessment of excised keels. Keels were scored based on the severity of deviations (A to E) with E being the most severe. Keels were scored based on the number of deviations present (0 to 5) with 5 indicating 5 + deviations present. Strain had a significant effect on the severity of deviations (P<0.001) and the number of deviations present (P<0.001).

Strain	**Number of deviations**						**Deviation Severity**				
	0	1	2	3	4	5+	A	B	C	D	E
ISA Brown[2]	11%	26%	52%	11%	0%	0%	21%	37%	21%	21%	0%
Lohmann Brown[2]	5%	11%	31%	21%	11%	21%	6%	17%	17%	43%	17%
Rhode Island Red[1]	5%	37%	58%	0%	0%	0%	32%	58%	10%	0%	0%
Shaver White[2]	15%	0%	25%	5%	30%	25%	20%	20%	15%	45%	0%
Lohmann White[2]	10%	33%	26%	21%	10%	0%	5%	10%	16%	53%	16%
White Leghorn[1]	5%	32%	26%	11%	26%	0%	5%	53%	10%	32%	0%
P-value						<0.001					<0.001

[1] Random-bred strains from University of Alberta Heritage Poultry Program.

[2] Commercial laying hen strains commonly housed in Alberta.

Table 2.4. LSMeans (± SEM) of keel and back movement speed of six strains of laying hens recorded using kinematic camera while perching, along the z-axis (up and down movement) and y-axis (forward and backward movement) at pre-lay. Results were averaged across both round and mushroom perch shapes.

Strain	**BSz (mm/s)**	**BSy (mm/s)**	**KSz (mm/s)**	**KSy (mm/s)**
ISA Brown[2]	35.7 ± 6.51	36.2^{ab} ± 5.85	59.0^{ab} ± 8.18	123.8^{ab} ± 17.03
Lohmann Brown[2]	36.5 ± 6.85	39.6^{ab} ± 6.24	56.1^{ab} ± 8.05	77.1^{bc} ± 11.07
Rhode Island Red[1]	41.0 ± 5.01	40.4^{ab} ± 4.37	51.0^{b} ± 6.73	66.14^{c} ± 5.30
Lohmann White[2]	36.3 ± 4.38	35.9^{b} ± 3.91	63.2^{ab} ± 8.94	102.4^{abc} ±13.88
Shaver White[2]	42.1 ± 6.10	46.6^{ab} ± 5.96	75.4^{ab} ± 10.53	138.0^{ab} ± 18.73
White Leghorn[1]	67.2 ± 8.19	58.6^{a} ± 6.48	98.8^{a} ± 12.91	172.1^{a} ± 22.83
P-value	=0.06	<0.05	<0.01	<0.001

Abbreviation: **BSz**, back speed in the z-axis. **BSy**, back speed in the y-axis. **KSz**, keel speed in the z-axis. **KSy**, keel speed in the y-axis.

$^{a-d}$ Values within columns with different letters are significantly different ($P<0.05$).

[1] Random-bred strains from University of Alberta Heritage Poultry Program.

[2] Commercial laying hen strains commonly housed in Alberta.

Table 2.5. LSMeans (± SEM) of keel and back movement speed of six strains of laying hens recorded using kinematic camera while perching, along the z-axis (up and down movement) and y-axis (forward and backward movement) at peak-lay. Results were averaged across both round and mushroom perch shapes.

Strain	**BSz (mm/s)**	**BSy (mm/s)**	**KSz (mm/s)**	**KSy (mm/s)**
ISA Brown[2]	22.6 ± 6.51	30.2 ± 3.46	37.3^{ab} ± 8.18	75.5^{a} ± 10.54
Lohmann Brown[2]	23.2 ± 3.69	25.4 ± 3.07	29.2^{b} ± 8.05	38.1^{b} ± 5.30
Rhode Island Red[1]	35.1 ± 5.42	32.5 ± 3.77	51.3^{ab} ± 7.98	72.3^{a} ± 5.42
Lohmann White[2]	31.3 ± 4.60	35.7 ± 4.00	35.6^{ab} ± 5.49	65.3^{a} ± 9.17
Shaver White[2]	42.4 ± 6.63	42.2 ± 5.10	58.0^{a} ± 9.13	100.3^{a} ± 14.24
White Leghorn[1]	36.2 ± 5.83	33.9 ± 4.16	53.8^{ab} ± 8.62	93.8^{a} ± 13.52
P-value	=0.06	=0.08	<0.05	<0.001

Abbreviation: **BSz**, back speed in the z-axis. **BSy**, back speed in the y-axis. **KSz**, keel speed in the z-axis. **KSy**, keel speed in the y-axis.

$^{a\text{-}d}$ Values within columns with different letters are significantly different ($P<0.05$).

[1] Random-bred strains from University of Alberta Heritage Poultry Program.

[2] Commercial laying hen strains commonly housed in Alberta.

Table 2.6. LSMeans (± SEM) of keel and back movement speed of six strains of laying hens recorded using kinematic camera while perching, along the z-axis (up and down movement) and y-axis (forward and backward movement) at end of lay. Results were averaged across both round and mushroom perch shapes.

Strain	**BSy (mm/s)**	**KSz (mm/s)**	**KSy (mm/s)**
ISA Brown[2]	31.8^{ab} ± 3.29	47.2^{bc} ± 6.18	69.5^{bc} ± 9.53
Lohmann Brown[2]	22.3^{b} ± 2.34	42.2^{c} ± 5.59	67.2^{c} ± 9.18
Rhode Island Red[1]	33.9^{ab} ± 3.67	41.7^{c} ± 5.68	59.4^{c} ± 8.44
Lohmann White[2]	29.8^{ab} ± 3.08	39.6^{c} ± 4.99	70.4^{bc} ± 9.27
Shaver White[2]	45.8^{a} ± 4.67	86.8^{a} ± 11.65	121.7^{ab} ± 17.06
White Leghorn[1]	39.7^{a} ± 5.03	82.59^{ab} ± 11.25	135.1^{a} ± 19.48
P-value	<0.001	<0.001	<0.001

Abbreviation: **BSy**, back speed in the y-axis. **KSz**, keel speed in the z-axis. **KSy**, keel speed in the y-axis.

[a-d] Values within columns with different letters are significantly different ($P<0.05$).

[1] Random-bred strains from University of Alberta Heritage Poultry Program.

[2] Commercial laying hen strains commonly housed in Alberta.

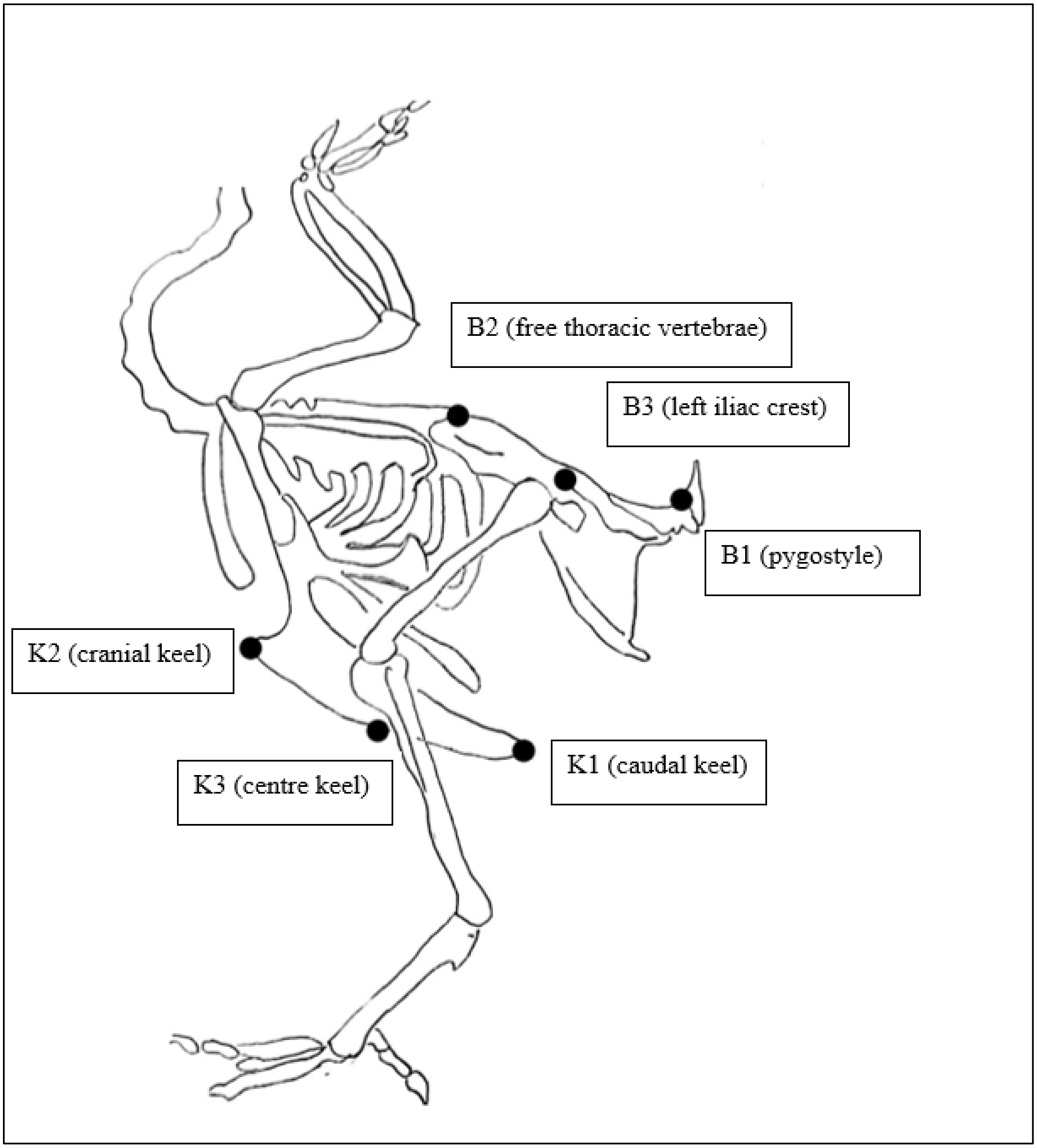
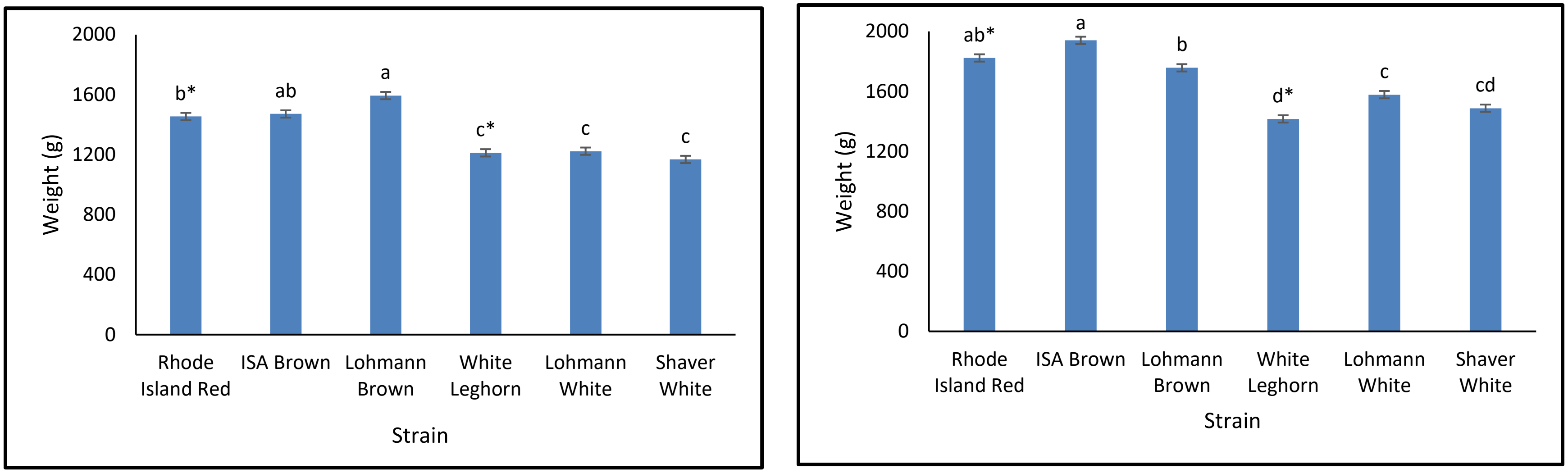


Figure 2.1. Image depicting the placement of kinematic markers on key skeletal landmarks, for the purpose of evaluating perching biomechanics using 3-D kinematic recordings. Marker B4 is located on the right iliac crest which is not visible in this figure.



A: Pre-lay (18 weeks of age)

B: Peak lay (29 weeks of age)

C: End of lay (70 weeks of age)

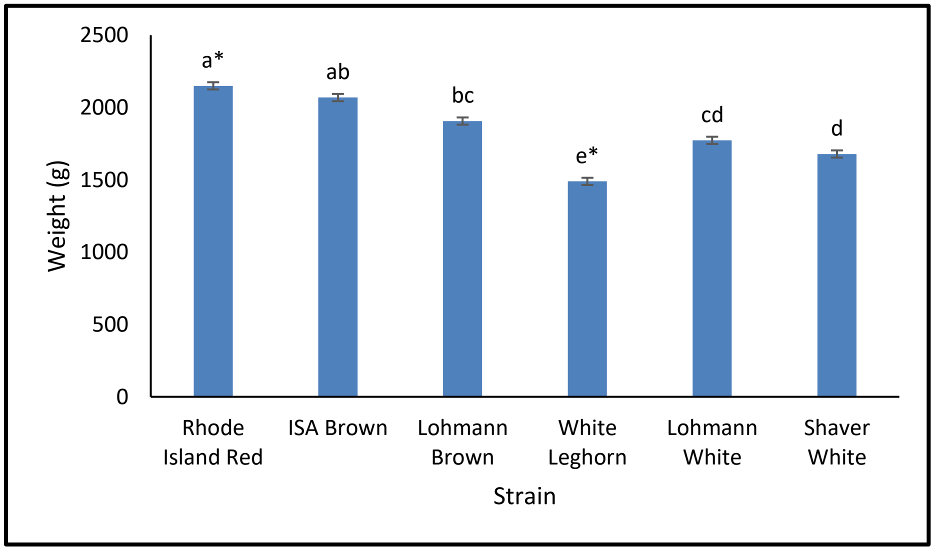
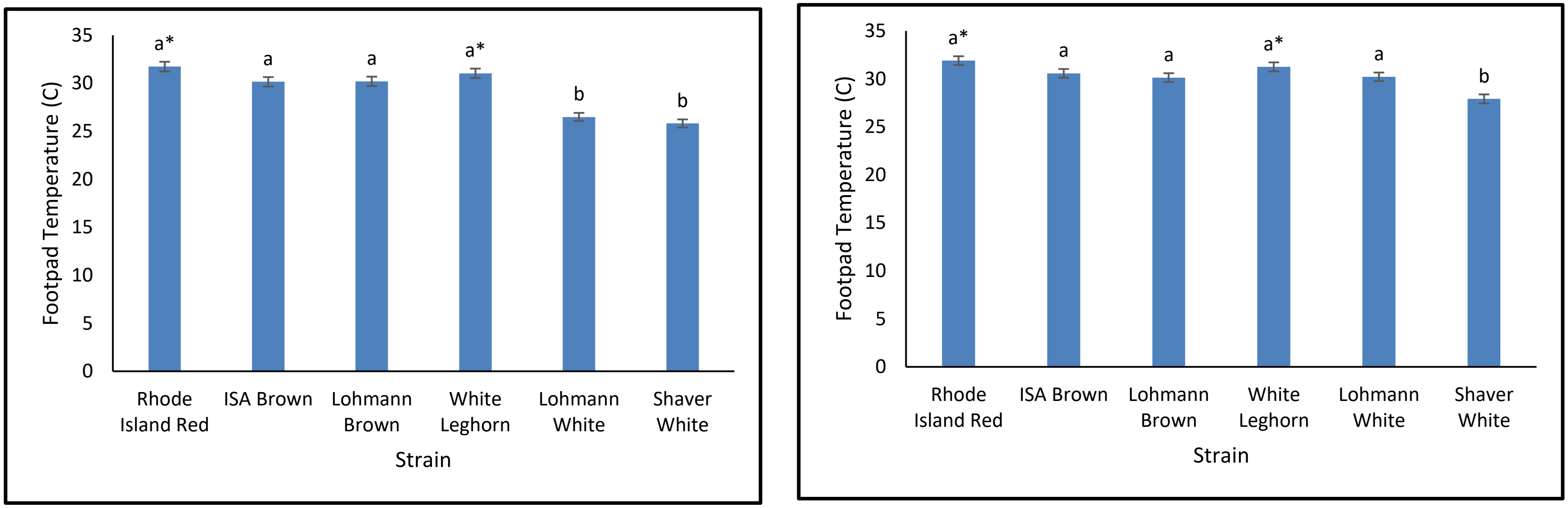


Figure 2.2: LSMeans (± SEM) of weight of six laying hen strains at pre-lay (A), peak lay (B), and end of lay (C). Effect of strain was significant at pre-lay ($P<0.001$), peak lay ($P<0.001$), and end of lay ($P<0.001$).

* Random-bred strains from University of Alberta Heritage Poultry Program.

A: Pre-lay (18 weeks of age)

B: Peak lay (29 weeks of age)



C: End of lay (70 weeks of age)

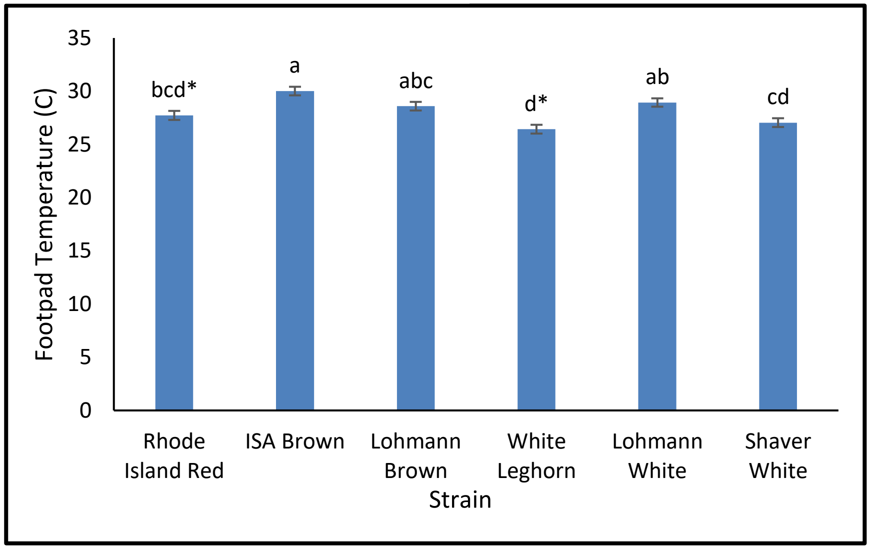
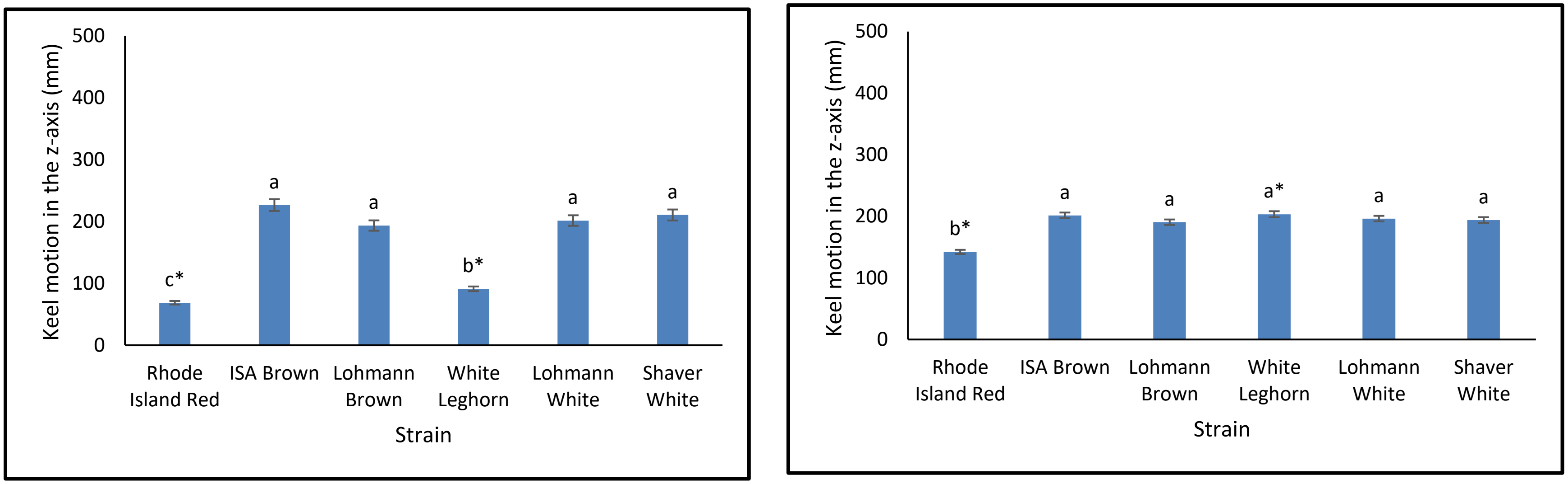


Figure 2.3. LSMeans (± SEM) of footpad temperature of six laying hen strains at pre-lay (A), peak lay (B), and end of lay (C). Effect of strain was significant at pre-lay ($P<0.001$), peak lay ($P<0.001$), and end of lay ($P<0.001$).

* Random-bred strains from University of Alberta Heritage Poultry Program.



A: Pre-lay (18 weeks of age)

B: Peak lay (29 weeks of age)

C: End of lay (70 weeks of age)

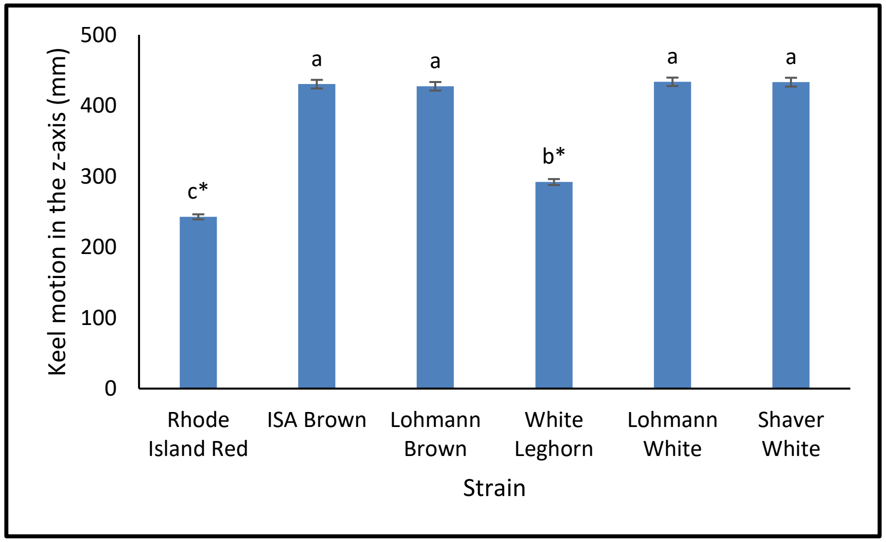
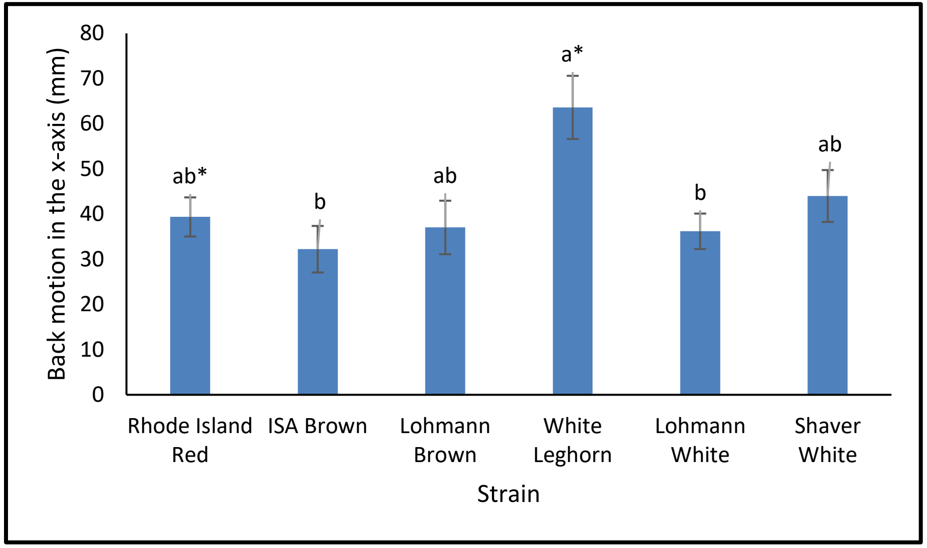


Figure 2.4: LSMeans (± SEM) of keel motion in the z-axis (up and down) while perching of six laying hen strains at pre-lay (A), peak lay (B), and end of lay (C). Effect of strain was significant at peak lay ($P<0.01$), pre-lay ($P>0.10$) and end of lay ($P>0.10$).

* Random-bred strains from University of Alberta Heritage Poultry Program.

A: Pre-lay (18 weeks of age)



B: Peak lay (29 weeks of age)

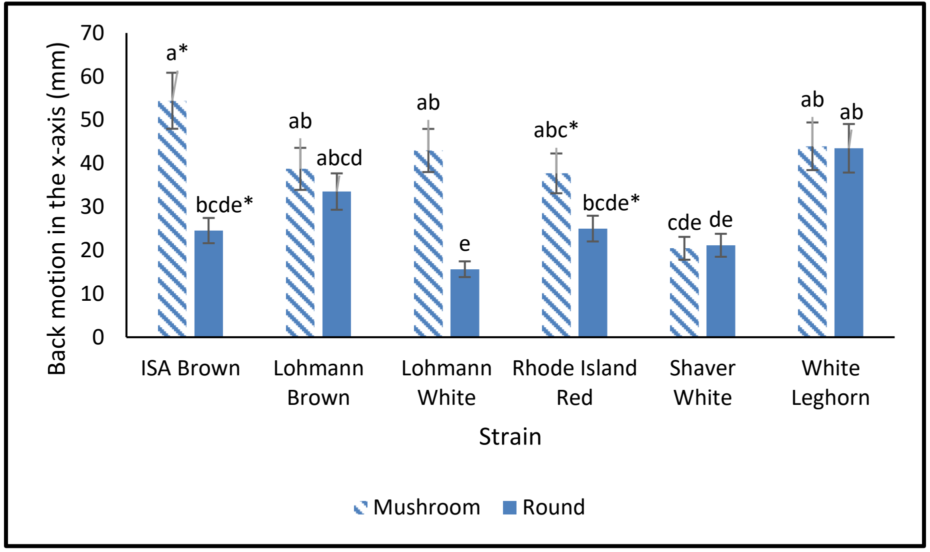
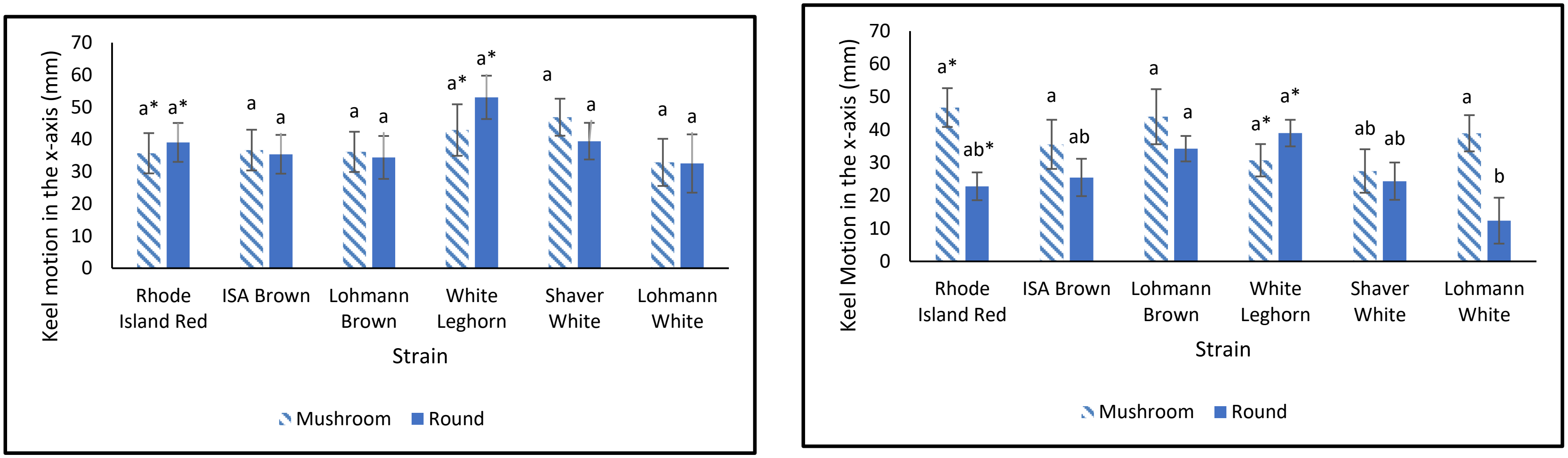


Figure 2.5: LSMeans (± SEM) of back motion in the x-axis (side-to-side) while perching of six laying hen strains at pre-lay (A) and peak lay (B). Effect of strain was significant at pre lay (P<0.01). There was a significant peak of lay strain x perch shape interaction (P<0.001). There was no significant effects of strain or perch shape at end of lay (P>0.10).

* Random-bred strains from University of Alberta Heritage Poultry Program.


A: Pre-lay (18 weeks of age)

B: Peak lay (29 weeks of age)

C: End of lay (70 weeks of age)

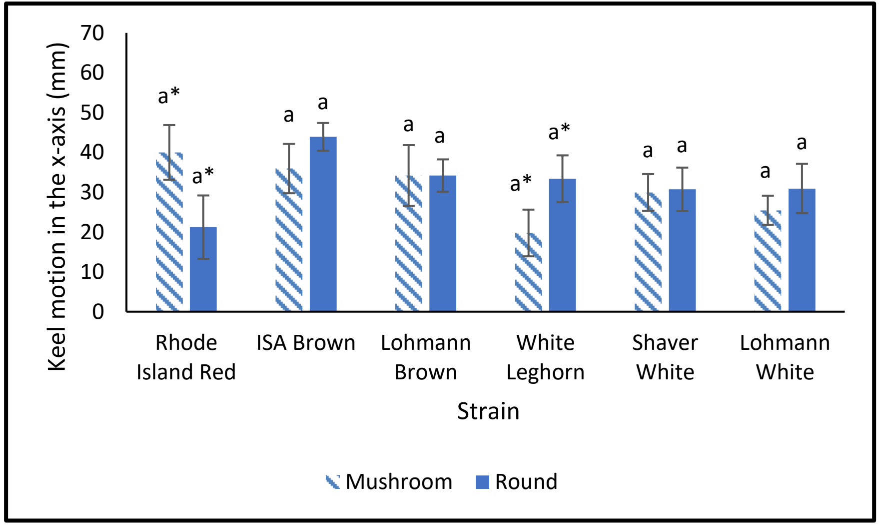
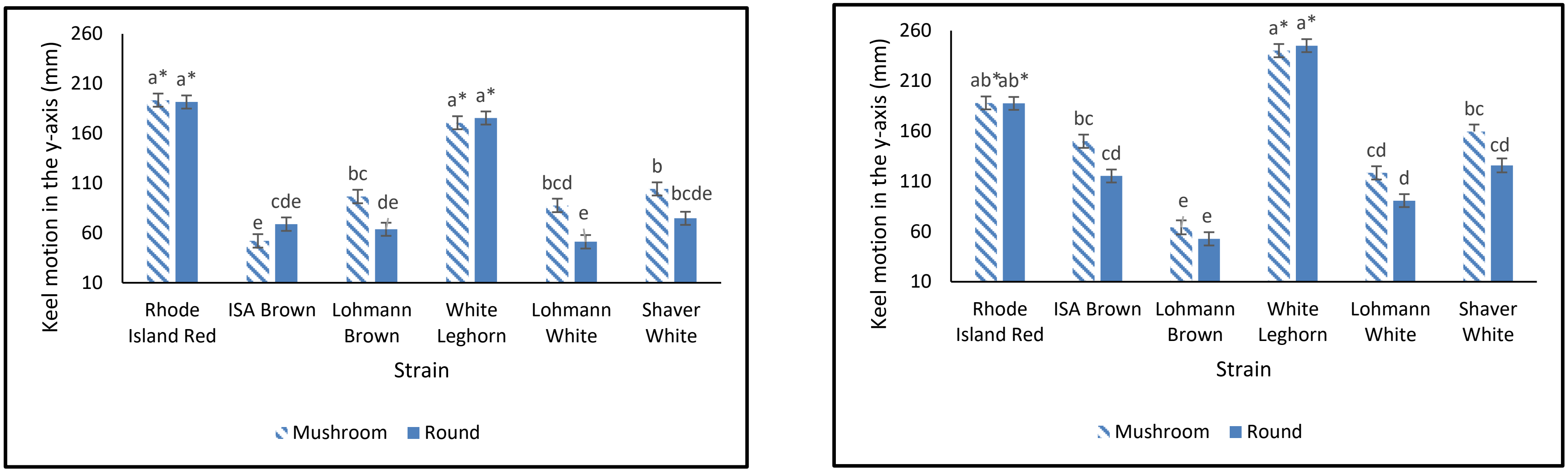


Figure 2.6: LSMeans (± SEM) of keel motion in the x-axis (side-to-side) while perching on round or mushroom perches of six laying hen strains at pre-lay (A), peak lay (B), and end of lay (C). Effect of strain and perch shape interaction was significant at peak lay ($P<0.01$). There was no significant perch shape or strain effects at pre-lay ($P>0.10$) and end of lay ($P>0.10$).

* Random-bred strains from University of Alberta Heritage Poultry Program.


A: Pre-lay (18 weeks of age)

B: Peak lay (29 weeks of age)

C: End of lay (70 weeks of age)

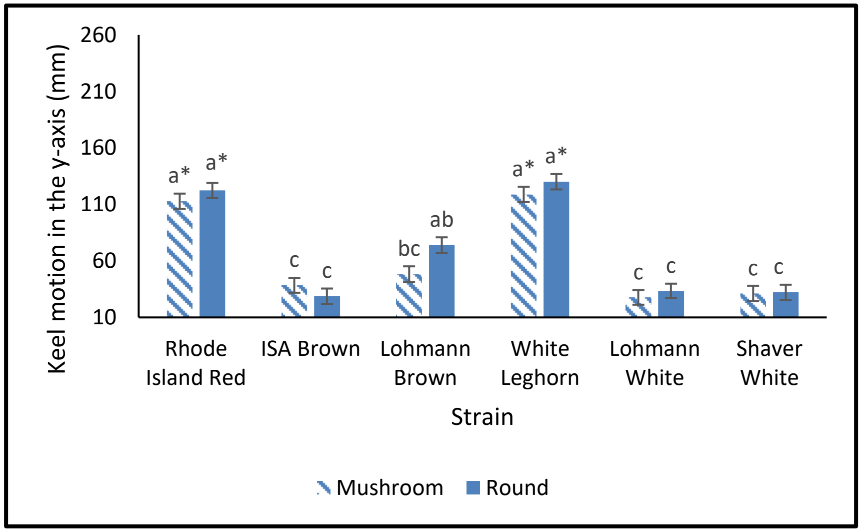


Figure 2.7: LSMeans (± SEM) of keel motion in the y-axis (forward and backward) while perching on round or mushroom perches of six laying hen strains at pre-lay (A) peak lay (B) and end of lay (C). Effect of strain and perch shape interaction was significant at pre lay ($P<0.001$) and peak lay ($P<0.001$), but only strain had a significant effect at end of lay ($P<0.001$).

Chapter 3. Single-SNP association analysis of potential causative mutations in candidate genes for keel bone damage in six strains of laying hen

3.1. Introduction

Commercial laying hen genetics are controlled by two main companies in the Western market: Hendrix Genetics and Erich Wesjohann Group GmbH (Fernyhough et al., 2019). Due to the proprietary nature of laying hen genetics, it can be difficult to assess which traits are prioritized in laying hen breeding programs. In addition, the proprietary nature of genetic differences between commercial strains limits our knowledge of strain variation. Laying hens have been selected for high egg production levels. Between 2010 and 2017, the number of eggs produced per hen housed increased by 87 eggs (Preisinger, 2017). High demand for egg production has led to weaker bones and high incidences of osteoporosis (Toscano et al., 2020) potentially contributing to the development of deviations and fractures (Candelotto et al., 2017; Habig et al., 2021).

Poultry breeding originated with selection for breed standards such as feather colour and comb shape (Carter, 1964). Phenotypic selection was less effective for sex-limited characteristics such as egg quality, that were essential for the development of laying hens (Wolc, 2014). Due to chickens' short generation interval and large number of offspring, paired with the development of best linear unbiased prediction (**BLUP**) and selection indexes, rapid changes in poultry breeding programs occurred. Best linear unbiased predictions were developed from Henderson's Mixed Model type III (Henderson, 1953) and inverse pedigree relationship matrixes (Henderson, 1976). By considering pedigree information and observed phenotypic information of an individual, poultry breeders were able to estimate the breeding value of an animal based on phenotypic traits like egg production or hatchability (Henderson, 1975). In addition, to account for a variety of traits that contribute to a breeding goal, selection indexes were created. Selection indexes consider the breeding value of multiple traits on an animal and take into consideration the importance of each trait to rank an animal on breeding potential (Wolc, 2014). Overall, traditional breeding methods led to high egg production in laying hens but was limited by a lack of information on certain traits' performance or progeny records and did not rely on information on genes themselves.

Initially, following the discovery that specific DNA markers influenced desired traits, marker assisted selection (**MAS**) became popular (Wolc, 2014). Although effective for certain traits that are influenced by only a small number of genes, MAS was not effective for most traits. The vast majority of traits are influenced by a large number of genes that each have small, additive effects (Wolc et al., 2014). Meuwissen et al. (2001) theorized that utilizing a large number of markers, known as single nucleotide polymorphisms (**SNPs**), for predicting breeding value would be highly accurate and more effective in selecting for sex-limited traits which is called genomic selection. Single nucleotide polymorphisms are variations at specific points in a genome, for example guanine instead of adenine (Borstring and Morling, 2013). Diseases and traits of interest can be investigated through analyzing the association between SNPs and certain phenotypes (e.g., KBD). Consequently, genomic selection was proven to be effective in enhancing genetic gain in poultry breeding programs across a variety of traits (Wolc et al., 2011) and the first round of genomic selection was conducted commercially in 2013 (Hy-Line Int., 2013). A common methodology for detecting trait-SNP associations is genome-wide association studies (**GWAS**), however power calculations have shown a minimum of 100 individuals is needed for detecting trait-SNP associations with some traits requiring sample sizes higher than 100 million individuals (Visscher et al., 2017). Alternatively, single SNP association studies have been conducted in a variety of species including humans (Yuferov et al., 2022), cattle (Ye et al., 2022) and geese (Melak et al., 2021). Bone strength and density have been linked to reduced incidences of KBD (Regmi et al., 2016). Identifying specific SNPs that contribute to keel bone damage can assist genetic companies in selecting for laying hens who are less likely to experience KBD and when laying hens do experience KBD, healing will occur more quickly.

The heritability of bone strength characteristics has been reported to be moderate to high in chickens (0.4; Bishop et al., 2000) and high in humans (0.85; Ralston, 2002). Bishop et al. (2000) used bone parameters including humeral strength, tibial strength, and keel radiographic density to create a Bone Index in laying hens. The Bone Index has been used in various studies to highlight how high bone strength lines and low bone strength lines differ (Bishop et al., 2000, Fleming et al., 2006; Stratmann et a., 2016). Notably, high bone strength lines selected using the Bone Index have resulted in laying hens with fewer fractures and more intact keel bones compared to the low strength line (Fleming et al., 2006; Stratmann et al., 2016). Bone characteristics are heritable and contribute to reduced KBD. Research conducted in humans and mice has identified thousands of SNPs associated with bone mineral density and osteoporosis, indicating these phenotypes are highly polygenic (Kemp et al., 2017; Morris et al., 2019). Genomic selection is ideal for assessing polygenic traits in contrast to other methods such as MAS (Meuwissen et al., 2001). Of particular interest are causative mutations, mutations that contribute to a specific disease or phenotype (Boycott et al., 2013). By identifying causative mutations associated with lower bone mineral content and density, healthier laying hens with a reduced capacity of developing KBD can be bred.

Bone formation and remodeling are complex and crucial for laying hens to maintain egg production levels while supporting overall bone health. Most skeletal bone is comprised of two types of structural bone; cortical (outer region) and trabecular (interior bone matrix; Whitehead, 2004). However, in sexually mature laying hens, estrogen stimulates medullary bone production providing a source of calcium that can be utilized for eggshell formation (Whitehead, 2004). Medullary bone is unique to birds and crocodilians, specifically providing a source of calcium for shell formation (Whitehead, 2004). Structural bone formation ceases, while gradually throughout lay, the structural bone content of the hen declines as osteoclastic resorption of the bone continues, leading to weakened bones (Whitehead, 2004). The parathyroid hormone receptor is stimulated by parathyroid hormone (**PTH**) to activate osteoclasts for structural and medullary bone to be resorbed. Mechanical loading on bones leads to bone remodelling, which requires parathyroid hormone to bind to receptors on both osteoblasts and osteocytes resulting in the secretion of receptor activator of nuclear factor kappa-B ligand (**RANKL**) which leads to preosteoclasts differentiating into osteoclasts leading to bone resorption (Khosla, 2001). Bone remodeling is inhibited by osteoprotegerin (**OPG**) which binds to RANKL and recruits osteoblasts. In addition, PTH will inhibit sclerostin production through osteocytes preventing bone formation (Raggatt and Partridge, 2010). Protection from excessive bone resorption, proper bone formation and remodeling could help reduce the risk of KBD and speed up healing when keel fractures occur.

Through previous genome-wide association studies; *RANKL* (Guo et al., 2017; Dale et al., 2015), *SRY-Box 9* (***SOX9*; **Dale et al., 2015), *PTH-like hormone* (***PTHLH*; **Dale et al., 2015) *retinoblastoma 1* (***RB1*;** Li et al., 2021; Zhang et al., 2011), *sclerostin* (***SOST***; Guo et al., 2017; Li et al., 2021), and *periostin* (***POSTN*; **Guo et al., 2017; Li et al., 2021) have been selected for their impact on bone parameters. The objectives of our study were to determine if causative mutations in SNPs from candidate genes could be identified in relation to KBD in laying hens. We hypothesized that keel bone mineral content (**KeelMC**), keel bone mineral density (**KeelMD**), femur bone mineral content (**FemurMC**), femur bone mineral density (**FemurMD**) and keel length would be associated with specific SNP genotypes and contribute to an increased incidence of KBD.

3.2. Materials and Methods

This study occurred at the University of Alberta Poultry Unit in Edmonton, Alberta, Canada in accordance with the University of Alberta Animal Use Protocol AUP 3299 and Canadian Council on Animal Care guidelines (CCAC, 2009). Four commercial egg-laying strains (Lohmann White, Lohmann Brown, ISA Brown and Shaver White) and two University of Alberta Poultry Unit random-bred heritage strains (Shaver Rhode Island Red and Antique University of Saskatchewan White Leghorn) were evaluated. Commercial strains were housed from December 2019 until May 2021 and random-bred strains were housed from June 2020 until November 2021. Twenty birds from each strain were studied (n=20/treatment) and housed to 70 weeks of age. Birds were split by strain into floor pen enclosures post-hatch. Enclosures consisted of feeders, waterers, and an elevated nest box with roosts. All birds were housed with a day length of 14L:8D during lay and all birds were fed according to NRC requirements and provided water and feed *ad libitum* (NRC, 1994).

3.2.1. Data collection

Bone mineral content and density measurements were analyzed post-mortem at 70 weeks of age utilizing LUNAR Prodigy Dual X-Ray Absorptiometry (**DXA**; GE Healthcare Canada, Mississauga, ON, Canada). Scans were conducted on the keel and the left femur which was dissected and cleaned. Before scanning, the machine was calibrated to 2% ± the mean densities of a phantom spine with a known set of densities (GE Healthcare Canada, Mississauga, ON, Canada). The keel and femur of each bird were then placed horizontally into a plastic container filled with rice, to ensure all bones were imaged at the same angle. Keel bone mineral content, KeelMD, FemurMC, and FemurMD were measured and recorded. Digital keel bone imaging was used to determine the length of each keel using a Canon PowerShot SX420 IS (20 Megapixel, Canon Canada Inc, Brampton, ON, Canada). Excised keels were cleaned of muscle tissue and were placed on a black background alongside a ruler to ensure a standard scale for image analysis. Keel length was measured from the caudal tip to the dorsal end using ImageJ software (ImageJ, National Institutes of Health, 1997).

3.2.2. Bioinformatics

As previously described, all candidate genes were selected based on previous research findings that highlighted the association between each gene and important bone traits that could impact a laying hens' susceptibility to keel bone damage. In addition, *ovalbumin-related protein X* (***OVALX***) was selected as a control gene. A control gene was utilized to ensure genotyping was optimized for other candidate genes, if the control SNP does not segregate in a genotyping assay this indicates a genotyping error has occured. Also known as *SERPINB14*, and located on chromosome two, this gene is associated with egg weight egg breaking strength and shell thickness (Dunn et al., 2009) and eggshell crystal size (Dunn et al., 2012).

The Ensembl genome database was used to identify potential SNPs of interest on the chosen candidate genes (*SOX9, RB1, PTHLH, POSTN, SOST, RANKL*; Cunningham et al., 2022). All SNPs were evaluated based on a variety of scores (Table 3.1). The variant effect predictor (**VEP**) tool in Ensembl was used to find SNPs that were predicted to result in missense or frameshift, which are considered high impact due to a change in the amino acid sequence (Cunningham et al., 2022). In addition, we used the sorting intolerant from tolerant (**SIFT**) program within Ensembl to select deleterious SNPs with a score of 0; indicating an increased likelihood for disease such as osteoporosis. All potential SNPs combined annotation-dependent depletion (**CADD**) scores were determined, only SNPs with a score of >10 was chosen. A high CADD score is indicative of variants that are more likely to cause disease than expected by random chance and are not stabilized by selection (Kircher et al., 2014). Potential SNPs were screened based on a database containing the genomes of all University of Alberta Heritage

Poultry breeds, to determine if segregation occurred. Basic Local Alignment Search Tool (**BLAST**) scores were also used to determine if any regions of similarity existed between chosen SNPs and the rest of the genome. Lower BLAST scores were preferred, indicating a reduced likelihood of issues arising during genotyping such as the genotyping assay focusing on the wrong SNP.

In total, there were seven SNPs run from six different candidate genes and the control gene; rs740376108 (*PTHLH*), rs317151697 (*RB1*), rs314956063 (*SOST*), rs15490317 (*RANKL*), rs733873794 (*POSTN*), rs13975174 (*POSTN*), and rs733219382 (*SOX9*). In addition, a control SNP was used rs315395834 (*OVALX*).

3.2.3. Tissue Sampling

Whole birds were stored post-mortem in a -20°C freezer until tissue sampling occurred. Skin samples were removed from the leg of each bird for a total of 116 samples (Rhode Island Red (n=18), White Leghorns (n=19), ISA Brown (n=20), Shaver White (n=19), Lohmann White (n=20), and Lohmann Brown (n=20)). Skin samples were placed in a -80°C freezer.

3.2.4. DNA Extraction

DNA was extracted using QIAGEN DNeasy Blood & Tissue Kit (QIAGEN, Valencia, CA). A 25 mg sample of skin was mixed with 180 µL of Buffer ATL and 20 µL of proteinase K. Samples were then incubated overnight at 56°C. Following incubation, 200 µL of Buffer ATL was vortexed into the sample. Then 200 µL of 100% ethanol was added and the sample was centrifuged at 12,000 rpm. 500 µL of Buffer AW1 was added and the sample was centrifuged again at 12,000 rpm. 500 µL of Buffer AW2 was added and the mixture was centrifuged at 14,000 rpm twice, and finally 70 µL of Buffer AE was added and the sample was centrifuged at 14,000 rpm. A DNA concentration of 100 ng/µL was required for analysis. Sample DNA concentrations were measured using NanoDrop® ND-1000 UV-Vis Spectrophotometer (Thermo Fischer, Boston, MA, USA). Overall, 75.0% of samples were within the 100 – 299 ng/µL range, 82.76% of samples were within the 100 – 349 ng/µL range, and 6.04% of samples had a concentration >400 ng/ µL.

3.2.5. Genotyping Assay

For genotyping, TaqMan™ Fast Advanced Master Mix (Thermo Fischer, Boston, MA, USA) and a MicroAmp Fast Optical 96-well reaction plate were used (Thermo Fischer, Boston, MA, USA). The online database Ensembl was used to determine each SNPs flanking sequence which was then used as the primer. Each well contained 5 μL of TaqMan, 0.5 μL of assay probe and 4.5 μL of DNA. All samples were genotyped using a StepOnePlus™ Real-Time PCR System (Thermo Fischer, Boston, MA, USA). TaqMan utilizes a nucleic-acid probe complementary to the chosen SNP, that is labeled with two dyes that release a fluorescent signal which are measured and are indicative of the genotype present in a sample. Thermocycle conditions were as follows; 60°C for 30 s, 95°C for 10 min, 95°C for 15 s, 60°C for 1 m, and 60°C for 30 s for 40 cycles.

3.2.6. Statistical Analysis

Phenotype data (bone parameters) were analyzed using SAS software (SAS ver 9.4, Cary, NC, USA). To test normality assumptions, a ProcUnivariate with a Kolmogorov-Smirnov test was run on all metrics. All metrics were then analyzed using a generalized linear mixed model (Proc Glimmix; SAS Institute, 2013), Type 3 test with the inverse (ilink) function specified. Distributions used were selected based on the lowest Akaike Information Criterion (AIC). Keel length was analyzed using a normal distribution. Keel bone mineral content, KeelMD, FemurMC, and FemurMD, were analyzed using a gamma distribution. Fixed variables in the glimmix model were strain with weight included as a covariate. Least Squares Means (**LSMeans**) differences were adjusted for multiple comparisons using a Bonferroni test.

This study used ASReml-W (VSN International, Hemel Hempstead, UK) to conduct single-SNP association analyses. The following model was used (Gilmour et al., 2015):

$$y = 1\mu + Xb + S\alpha + Za + e$$

Where y is a vector of phenotypic values; 1 is a vector of ones; μ is the overall mean for bone parameter phenotypes; X is a design matrix that relates fixed effects to the observations; b is the vector of fixed effects including strain; Z is a design matrix associating a with the phenotypic records (response variables); a is the vector of random additive genetic effects that is assumed to be normally distributed; S is a vector of SNP genotypes coded 0, 1, 2; α is the additive SNP effect; and e is a vector of residual effects. The additive genetic effects and the residual effects are both assumed to be normally distributed.

Initially, the data was run pooled across all strains with strain as a fixed effect. A second analysis was completed pooling results by bird feather colour (brown or white) with colour treated as a fixed effect. For this study, P-values < 0.05 were considered statistically significant, $0.05 > P > 0.10$ were considered a trend, and $P < 0.10$ was considered not statistically significant.

3.3. Results and Discussion

3.3.1. Phenotypic statistics

All bone parameters were affected by strain as reported in Chapter 2 (see Table 2.2). Keel bone mineral density in each of the brown strains and White Leghorn was significantly denser than in Shaver White (P<0.001) and Rhode Island Red was denser than Lohmann White. There was a significant strain effect on KeelMC with Rhode Island Red having the highest mineral content, followed by ISA Brown, Lohmann Brown and White Leghorn. In contrast, both commercial white strains had the lowest mineral content (P<0.001). Femur bone mineral density (P<0.001) and FemurMC (P<0.001) were significantly denser in Rhode Island Red compared to all other strains. Rhode Island Red had significantly longer keels than all other strains, and ISA Brown had significantly longer keels than all white strains (P<0.001).

3.3.2. Selection of candidate genes

Selected candidate genes had been previously identified in the literature for their relationship to bone health. *Receptor activator of nuclear factor kappa-B ligand* is located on chromosome one and plays a role in bone mineral content due to its involvement in osteoclast differentiation and activation (Guo et al., 2017; Dale et al., 2015). Receptor activator of nuclear factor kappa-B ligand is secreted to recruit osteoclasts to develop the marrow region of the bone. However, when RANKL is inhibited by OPG it inhibits the activity and differentiation of osteoclasts and preosteoclasts (Dale et al., 2015). *SRY-Box 9* is located on chromosome 18 and aids in chondrocyte development and maturation by promoting the expression of other genes including

PTH-like hormone and *collagen type II* (***COL2AI***; Dale et al., 2015). *Collagen type II* and *SOX9* are important for the formation of cartilage in early fetal development (Dale et al., 2015). *PTH-like hormone* regulates the differentiation of prehypertrophic chondrocytes into hypertrophic chondrocytes (Dale et al., 2015). *Collagen type II*, *SOX9*, *PTHLH* and *RANKL* work closely together in developing and remodeling bones (Dale et al., 2015).

In addition to the genes previously identified, three other genes have also been notably linked to bone parameters. *Retinoblastoma 1* located on chromosome one controls body growth and osteogenic differentiation in chickens (Li et al., 2021; Zhang et al., 2011) and inhibits bone formation and remodeling in mice (Li et al., 2022). Thus, *RB1* may impact keel length in laying hens. *Sclerostin* located on chromosome 27 primarily regulates bone homeostasis through the Wnt/β-catenin signaling pathway (Jiao et al., 2023), and the suppression of *SOST* facilitates bone formation and helps prevent fractures (Guo et al., 2017; Li et al., 2021) highlighting *SOST*s' involvement in keel length and bone mineral content. Finally, *periostin* located on chromosome one may impact bone weight due to its role in the regulation of osteoblast proliferation and bone formation (Guo et al., 2017; Li et al., 2021).

In total, five SNPs segregated; rs740376108 (*PTHLH*), rs317151697 (*RB1*), rs314956063 (*SOST*), rs15490317 (*RANKL*), and rs13975174 (*POSTN*). In addition, the control SNP rs315395834 (*OVALX*) segregated. However, rs733873794 (*POSTN*), and rs733219382 (*SOX9*) did not segregate. Segregation occurs when there is variation in a population's genotypes for a SNP. Frequency of each SNP genotype was determined by pooling all data (Table 3.2), by strain colour (eg. Brown vs White; Table 3.3), by commercial vs random bred (Table 3.4) and by strain (Table 3.5).

There were notable differences between genotype and allele frequency when comparing pooled white vs brown and commercial vs random-bred results. *OVALX*, *POSTN*, *RANKL* strongly segregated when comparing white and brown strains, demonstrated by a lower number of heterozygotes. Brown strains have been reported to have significantly stronger and denser bones than white strains (Fawcett et al., 2020, Habig et al., 2021) which supports our results demonstrating segregation between brown and white strains. Only *RB1,* when pooling commercial vs random-bred strains, had similar results (Table 3.6). Notably, strains with higher frequencies of allele C on *RB1,* also had higher bone parameter measures. Within white strains, random-bred White Leghorns had the highest bone parameters and in turn had the highest C allele frequency in comparison to commercial white strains. In addition, within brown strains, random-bred Rhode Island Reds had the highest bone parameters and in turn had the highest C allele frequency in comparison to commercial white strains. The small sample size in this study could explain the high number of homozygotes for certain pooled groups. Higher levels of homozygosity can in part be explained by reduced genetic diversity in laying hen populations as artificial selection has progressed (Muir et al., 2018) and laying hens short generation intervals (Baes et al., 2019). *OVALX*, *POSTN*, *RANKL*, and *RB1* may be genes of interest of future studies exploring variation between strains in relation to bone parameters and KBD.

3.3.3. Single-SNP association analysis

Phenotypic variance, SNP variance, SNP effect and p-values for pooled data is detailed in Table 3.6. Phenotypic variance, SNP variance, SNP effect and p-values for data pooled by feather colour is detailed in Table 3.7. Notably, rs315395834 (*OVALX*) accounted for 11.7% of phenotypic variation in KeelMC (P<0.05; Figure 3.1), and 20.2% of phenotypic variation in KeelMD (P<0.01; Figure 3.2) when comparing pooled data. When comparing data pooled by feather color, rs13975174 (*POSTN*) accounted for 14.2% of phenotypic variation in FemurMC (P<0.05; Figure 3.3). rs15490317 (*RANKL*) accounted for 10.6% of phenotypic variation in FemurMC (P<0.05; Figure 3.4), 14.4% of phenotypic variation in FemurMD (P<0.01; Figure3.5), 8.7% of phenotypic variation in KeelMC (P<0.05; Figure 3.6), and 8.9% of phenotypic variation in Keel Length (P<0.05; Figure 3.7). Finally, rs317151697 (*RB1*) accounted for 8.6% of phenotypic variation in KeelMC (P<0.05; Figure 3.8). The heritability for all SNPs was 0.09 ± 0.12 which is considered very low (Bishop et al., 2000) indicating the SNPs do not account for variation seen in the bone parameters measured. Notably, *OVALX* not only segregated in our populations but was also found to have an impact on bone parameters despite previous research highlighting associations with egg weight, egg breaking strength and shell thickness (Dunn et al., 2009) and eggshell crystal size (Dunn et al., 2012). Based on the segregation and single-SNP association analyses of SNPs located on *POSTN*, *RANKL*, *RB1,* and

OVALX, future research should investigate more SNPs located on these genes as they could potentially impact bone parameters and KBD in laying hens.

Despite significant results from the single-SNP association analysis, results were confounded by significant strain effects. Due to the genetic differences that exist between the strains used in the study and different proportions of individual hens per strain having relatively high or low bone parameter measurements, our results can be equated to sampling differences. Previous studies have resolved confounding strain effects by identifying a particular breed that led to a significant strain effect and removing it from pooled results (Kim et al., 2000). In this study, no one strain was responsible for the significant variation in bone parameters, thus removing a strain from the analysis was not effective. Even when results were looked at by individually assessing white and brown strains or removing Rhode Island Red from analysis, strain differences were still significant. Due to strain, we cannot say with confidence that the SNP effects reported in this study were in fact significant or influenced the phenotypes. In future work, utilizing a less rigorous SNP selection criteria and investigating a wider range of SNPs may provide more information on what causative mutations are at work on the candidate genes selected.

Overall, a few different approaches can be used to mitigate the impact of a significant strain effect. In this study an identity matrix was used due to the small number of SNPs that were analyzed and lack of information pertaining to the sires and dams of each individual bird, which would not have been feasible in this study. Alternatively, creating a relationship matrix through the analysis of more SNPs on a candidate gene may be possible. In addition, this study had a relatively small sample size (n=116) in comparison to other poultry genetic research (n=1,534; Li et al., 2021). Large sample sizes are particularly important in population genomics in order to generate robust results (Subramanian, 2016). As such, when designing future studies assessing causative mutations projects should aim to have larger sample sizes to strengthen results.

3.4. Conclusions

Single-SNP association analysis was used to identify potential causative mutations associated with bone parameters in laying hens. *PTHLH*, *OVALX*, *POSTN*, *RANKL*, *RB1*, *SOST*, and *SOX9* were selected from the literature due to previous association with bone parameters of interest.

Genotyping assays revealed segregation in seven SNPs with variation between brown vs white and random-bred vs commercial strains. Supporting previous literature, brown and white strains were significantly different from one another, and brown strains had significantly stronger and denser bones in comparison to white strains. Notably, allele C on *RB1* may contribute to bone parameters and was more prevalent in random-bred strains in comparison to commercial strains. Although results showed some significant SNP effects, strain was a confounding factor. Our results highlight that causative mutations responsible for differences in bone parameters may be located elsewhere on *POSTN*, *RANKL*, *RB1*, and *OVALX*. Alternatively, by assessing a larger number of SNPs or increasing the sample size more complete candidate gene genotyping would reduce the impact of confounding factors.

3.5. References

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Table 3.1. Variant effect predictor (VEP), sorting intolerant from tolerant (SIFT), and combined annotation dependant depletion (CADD) scores of all selected SNPs.

SNP	**VEP**	**SIFT**	**CADD**
rs740376108 (*PTHLH*)	Missense	0.03-0.07	18
rs317151697 (*RB1*)	Missense	0	19
rs314956063 (*SOST*)	Missense	0	18
rs15490317 (*RANKL*)	Missense	0	16
rs733873794 (*POSTN*)	Missense	0	15
rs13975174 (*POSTN*)	Intron variant	0	1
rs733219382 (*SOX9*)	Missense	0	17
rs315395834 (*OVALX*)	Missense	0	17

Abbreviation. ***PTHLH***, *PTH-like hormone.* ***OVALX,*** *ovalbumin-related protein X.* ***POST***, *periostin.* ***RANKL***, *receptor activator of nuclear factor kappa-B ligand****.*** ***RB1,*** *Retinoblastoma 1.* ***SOST,*** *sclerostin.*

Table 3.2. Count of each genotype for *PTHLH*, *OVALX*, *POSTN*, *RANKL*, *RB1*, and *SOST* when all data is pooled across strains.

	Genotype			
	0	1	2	Total
PTHLH	43	44	29	116
OVALX	44	27	45	116
POSTN	58	9	48	115
RANKL	27	31	58	116
RB1	73	16	27	116
SOST	68	33	15	116

Abbreviation. ***PTHLH**, PTH-like hormone.* ***OVALX,** ovalbumin-related protein X.* ***POST**, periostin.* ***RANKL**, receptor activator of nuclear factor kappa-B ligand**.** **RB1,** Retinoblastoma 1.* ***SOST,** sclerostin.*

Table 3.3. Count of each genotype and allele frequency for *PTHLH* (A), *OVALX* (B), *POSTN* (C), *RANKL* (D), *RB1* (E), and *SOST* (F) when data is pooled by strain color, brown (ISA Brown, Lohmann Brown, and Rhode Island Red) and white (Lohmann White, Shaver White, and White Leghorn).

A.

PTHLH	**C/C**	**T/C**	**T/T**	**C**	**T**	**Total**
Brown	34	23	1	0.78	0.22	58
White	9	21	28	0.34	0.66	58
Total	43	44	29	/	/	116

B.

OVALX	**C/C**	**T/C**	**T/T**	**C**	**T**	**Total**
Brown	44	12	2	0.89	0.11	58
White	0	15	43	0.13	0.87	58
Total	44	27	45	/	/	116

C.

POSTN	**C/C**	**T/C**	**T/T**	**C**	**T**	**Total**
Brown	0	9	48	0.08	0.92	57
White	58	0	0	1.00	0.00	58
Total	58	9	48	/	/	115

D.

RANKL	**A/A**	**G/A**	**G/G**	**A**	**G**	**Total**
Brown	5	26	27	0.31	0.69	58
White	53	5	0	0.96	0.04	58
Total	58	31	27	/	/	116

E.

RB1	**C/C**	**C/G**	**G/G**	**C**	**G**	**Total**
Brown	54	4	0	0.97	0.03	58
White	19	12	27	0.43	0.57	58
Total	73	16	27	/	/	116

F.

SOST	**A/A**	**G/A**	**G/G**	**A**	**G**	**Total**
Brown	3	16	39	0.19	0.81	58
White	12	17	29	0.35	0.65	58
Total	15	33	68	/	/	116

Abbreviation. ***PTHLH**, PTH-like hormone.* ***OVALX,** ovalbumin-related protein X.* ***POST**, periostin.* ***RANKL**, receptor activator of nuclear factor kappa-B ligand**.*** ***RB1,*** Retinoblastoma 1. ***SOST,** sclerostin.* **A**, adenine. **C**, cytosine. **G**, guanine. **T**, thymine.

Table 3.4. Count of each genotype and allele frequency for *PTHLH* (A), *OVALX* (B), *POSTN* (C), *RANKL* (D), *RB1* (E), and *SOST* (F) when data is pooled by commercial (Lohmann Brown, Lohmann White, ISA Brown, and Shaver White) vs random-bred (Rhode Island Red and White Leghorn).

A.

PTHLH	**C/C**	**T/C**	**T/T**	**C**	**T**	**Total**
Commercial	29	26	24	0.53	0.47	79
Random-Bred	14	18	5	0.62	0.38	37
Total	43	44	29	/	/	116

B.

OVALX	**C/C**	**T/C**	**T/T**	**C**	**T**	**Total**
Commercial	36	19	24	0.58	0.42	79
Random-Bred	8	8	21	0.32	0.68	37
Total	44	27	45	/	/	116

C.

POSTN	**C/C**	**T/C**	**T/T**	**C**	**T**	**Total**
Commercial	39	5	34	0.52	0.48	78
Random-Bred	19	4	14	0.57	0.43	37
Total	58	9	48	/	/	115

D.

RANKL	**A/A**	**G/A**	**G/G**	**C**	**T**	**Total**
Commercial	38	16	25	0.58	0.42	79
Random-Bred	20	15	2	0.74	0.26	37
Total	58	31	27	/	/	116

E.

RB1	**C/C**	**C/G**	**G/G**	**C**	**T**	**Total**
Commercial	36	16	27	0.56	0.44	79
Random-Bred	37	0	0	1.00	0.00	37
Total	73	16	27	/	/	116

F.

SOST	**A/A**	**G/A**	**G/G**	**C**	**T**	**Total**
Commercial	1	16	62	0.11	0.89	79
Random-Bred	14	17	6	0.28	0.72	37
Total	15	33	68	/	/	116

Abbreviation. ***PTHLH**, PTH-like hormone.* ***OVALX,** ovalbumin-related protein X.* ***POST**, periostin.* ***RANKL**, receptor activator of nuclear factor kappa-B ligand.* ***RB1,*** Retinoblastoma 1. ***SOST,** sclerostin.* **A**, adenine. **C**, cytosine. **G**, guanine. **T**, thymine.

Table 3.5. Count of each genotype for PTHLH (A), OVALX (B), POSTN (C), RANKL (D), RB1 (E), and SOST (F) when data is pooled by strain.

A.

PTHLH	**Lohmann Brown**	**Lohmann White**	**Shaver White**	**ISA Brown**	**Rhode Island Red**	**White Leghorns**	**Total**
T/T	0	5	19	0	1	4	29
T/C	0	15	0	11	2	6	44
C/C	20	0	0	9	15	9	43
Total	20	20	19	20	18	19	116

B.

OVALX	**Lohmann Brown**	**Lohmann White**	**Shaver White**	**ISA Brown**	**Rhode Island Red**	**White Leghorns**	**Total**
C/C	19	0	0	17	8	0	44
C/T	1	1	14	3	8	0	27
T/T	0	19	5	0	2	19	45
Total	20	20	19	20	18	19	116

C.

POSTN	**Lohmann Brown**	**Lohmann White**	**Shaver White**	**ISA Brown**	**Rhode Island Red**	**White Leghorns**	**Total**
C/C	0	20	19	0	0	19	58
C/T	1	0	0	4	4	0	9
T/T	18	0	0	16	14	0	48
Total	19	20	19	20	18	19	115

D.

RANKL	**Lohmann Brown**	**Lohmann White**	**Shaver White**	**ISA Brown**	**Rhode Island Red**	**White Leghorns**	**Total**
G/G	11	0	0	14	2	0	27
G/A	8	0	2	6	12	3	31
A/A	1	20	17	0	4	16	58
Total	20	20	19	20	18	19	116

E.

RB1	**Lohmann Brown**	**Lohmann White**	**Shaver White**	**ISA Brown**	**Rhode Island Red**	**White Leghorns**	**Total**
C/C	20	0	0	16	18	19	73
C/G	0	6	6	4	0	0	16
G/G	0	14	13	0	0	0	27
Total	20	20	19	20	18	19	116

F.

SOST	**Lohmann Brown**	**Lohmann White**	**Shaver White**	**ISA Brown**	**Rhode Island Red**	**White Leghorns**	**Total**
G/G	14	10	19	19	6	0	68
G/A	5	10	0	1	10	7	33
A/A	1	0	0	0	2	12	15
Total	20	20	19	20	18	19	116

Abbreviation. ***PTHLH**, PTH-like hormone.* ***OVALX,** ovalbumin-related protein X.* ***POST**, periostin.* ***RANKL**, receptor activator of nuclear factor kappa-B ligand**.*** ***RB1,** Retinoblastoma 1.* ***SOST,** sclerostin.* **A**, adenine. **C**, cytosine. **G**, guanine. **T**, thymine.

Table 3.6. Frequency of allele C of *RB1* by strain in contrast to bone parameters measured post-mortem at 70 weeks of age as reported in Table 2.2.

RB1	**Lohmann Brown**	**Lohmann White**	**Shaver White**	**ISA Brown**	**Rhode Island Red**	**White Leghorns**
C allele	1.0	0.15	0.16	0.9	1.0	1.0
KBMD	ab	bc	c	ab	a	ab
FBMD	b	b	b	b	a	b
FBMC	b	b	b	b	a	b
KBMC	b	c	c	b	a	b
Keel Length	bc	c	c	b	a	c

Abbreviation: **KeelMD**, keel bone mineral density. **FemurMD**, femur bone mineral density. **KeelMC**, keel bone mineral content. **FemurMC**, femur bone mineral content. **C**, cystine. ***RB1***, *retinoblastoma 1*.

a-c Values within columns with different letters are significantly different ($P<0.05$).

Table 3.7. Single SNP association analysis results including P-value, SNP effect, phenotypic variance and SNP variance pooled by strain color.

	P-value	**SNP effect**	**Phenotypic variance**	**SNP variance**
PTHLH				
KBMD	0.10	0.00	0.00	0.53
KBMC	0.10	0.12	0.82	0.83
Length	0.10	0.06	0.11	1.77
FBMC	0.10	0.05	1.25	0.10
FBMD	0.10	0.01	0.01	1.04
RB1				
KBMD	0.10	-0.01	0.00	2.13
KBMC	**0.001**	-0.39	0.75	8.60
Length	0.10	-0.02	0.11	0.15
FBMC	0.10	-0.03	1.25	0.03
FBMD	0.10	0.01	0.01	0.38
OVALX				
KBMD	0.10	-0.01	0.00	2.05
KBMC	0.07	0.32	0.80	6.47
Length	0.09	0.11	0.11	5.48
FBMC	0.10	0.19	1.24	1.52
FBMD	0.10	0.01	0.01	0.74
SOST				
KBMD	0.10	0.00	0.00	0.24
KBMC	0.09	-0.29	0.80	4.03
Length	0.05	-0.12	0.11	5.68
FBMC	0.10	-0.34	1.22	3.74
FBMD	0.10	-0.03	0.01	3.58
POSTN				
KBMD	0.10	0.00	0.00	0.00
KBMC	0.10	-0.13	0.82	1.06
Length	0.10	-0.01	0.11	0.02
FBMC	**0.05**	-0.59	1.21	14.23
FBMD	0.10	-0.03	0.01	8.69
RANKL				
KBMD	0.10	0.00	0.01	0.14
KBMC	**0.05**	0.38	0.78	8.74
Length	0.10	0.07	0.11	2.34
FBMC	**0.01**	0.52	1.18	10.56
FBMD	**0.001**	0.04	0.01	14.39

Significant P-values are **bolded**

Abbreviation: **KeelMD**, keel bone mineral density. **FemurMD**, femur bone mineral density. **KeelMC**, keel bone mineral content. **FemurMC**, femur bone mineral content. ***PTHLH**, PTH-like hormone.* ***OVALX,** ovalbumin-related protein X.* ***POST**, periostin.* ***RANKL**, receptor activator of nuclear factor kappa-B ligand.* ***RB1,*** *Retinoblastoma 1.* ***SOST,*** *sclerostin.*

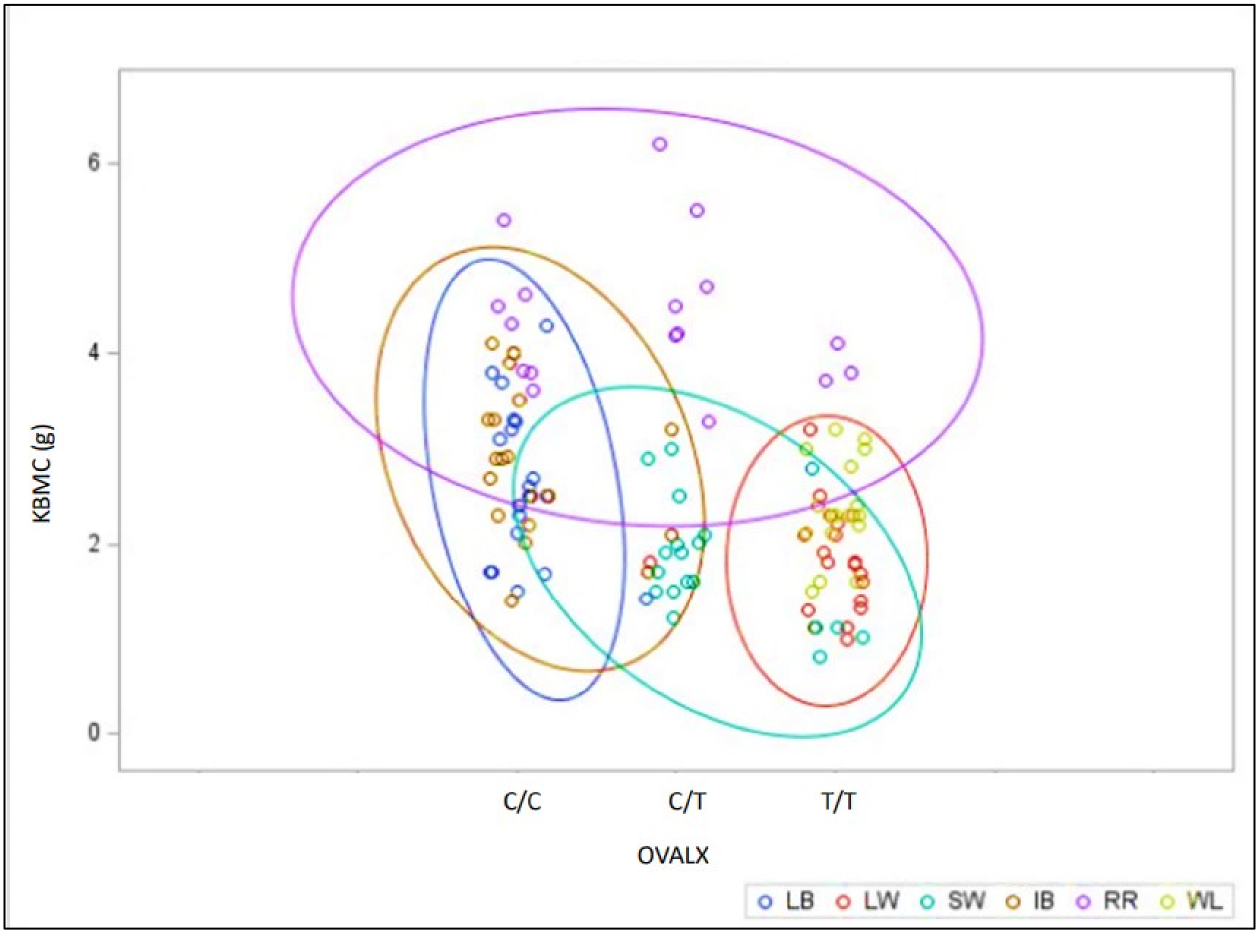
Table 3.8. Single SNP association analysis results including P-value, SNP effect, phenotypic variance and SNP variance pooled.

	P-value	SNP effect	Phenotypic variance	SNP variance
PTHLH				
KBMD	0.10	0.01	0.00	1.92
KBMC	0.10	0.13	0.48	1.69
Length	0.10	0.02	0.08	0.15
FBMC	0.10	-0.30	0.87	5.10
FBMD	0.10	-0.02	0.00	3.39
RB1				
KBMD	0.08	-0.01	0.00	11.64
KBMC	0.10	-0.30	0.47	7.98
Length	0.10	0.08	0.08	3.00
FBMC	0.10	0.33	0.88	5.28
FBMD	0.08	0.04	0.00	11.57
OVALX				
KBMD	**0.01**	-0.02	0.00	20.24
KBMC	**0.05**	-0.33	0.47	11.77
Length	**0.05**	-0.13	0.08	10.64
FBMC	0.10	-0.35	0.87	7.08
FBMD	0.10	-0.02	0.00	5.49
SOST				
KBMD	0.10	0.00	0.00	0.01
KBMC	0.10	0.08	0.48	0.58
Length	0.10	0.01	0.08	0.03
FBMC	0.10	-0.01	0.89	0.01
FBMD	0.10	0.00	0.00	0.07
POSTN				
KBMD	0.10	0.00	0.00	1.11
KBMC	0.10	0.13	0.48	1.72
Length	0.10	0.07	0.08	3.01
FBMC	0.10	-0.25	0.88	3.47
FBMD	0.10	-0.01	0.00	1.72
RANKL				
KBMD	0.10	0.00	0.00	0.65
KBMC	0.10	-0.04	0.48	0.17
Length	**0.05**	-0.12	0.08	8.94
FBMC	0.10	0.03	0.89	0.06
FBMD	0.10	0.01	0.00	0.70

Significant P-values are **bolded**

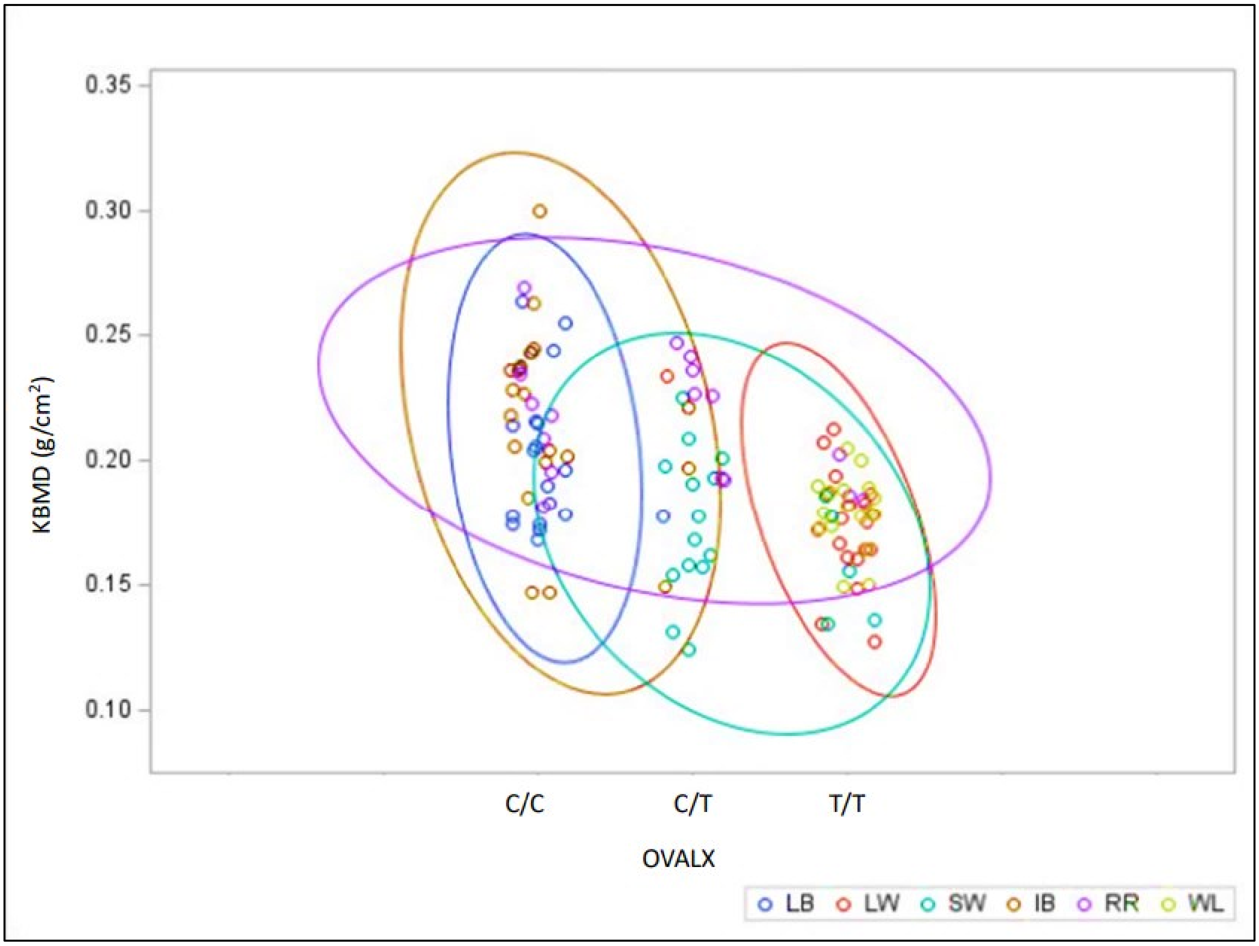
Abbreviation: **KeelMD**, keel bone mineral density. **FemurMD**, femur bone mineral density. **KeelMC**, keel bone mineral content. **FemurMC**, femur bone mineral content. ***PTHLH**, PTH-like hormone.* ***OVALX,** ovalbumin-related protein X.* ***POST**, periostin.* ***RANKL**, receptor activator of nuclear factor kappa-B ligand.* ***RB1,** Retinoblastoma 1.* ***SOST,** sclerostin.*

Figure 3.1. Relationship between rs315395834 on the gene *OVALX* and KBMC across six strains of laying hens. rs315395834 accounted for 11.7% of phenotypic variation in KBMC (P<0.05).



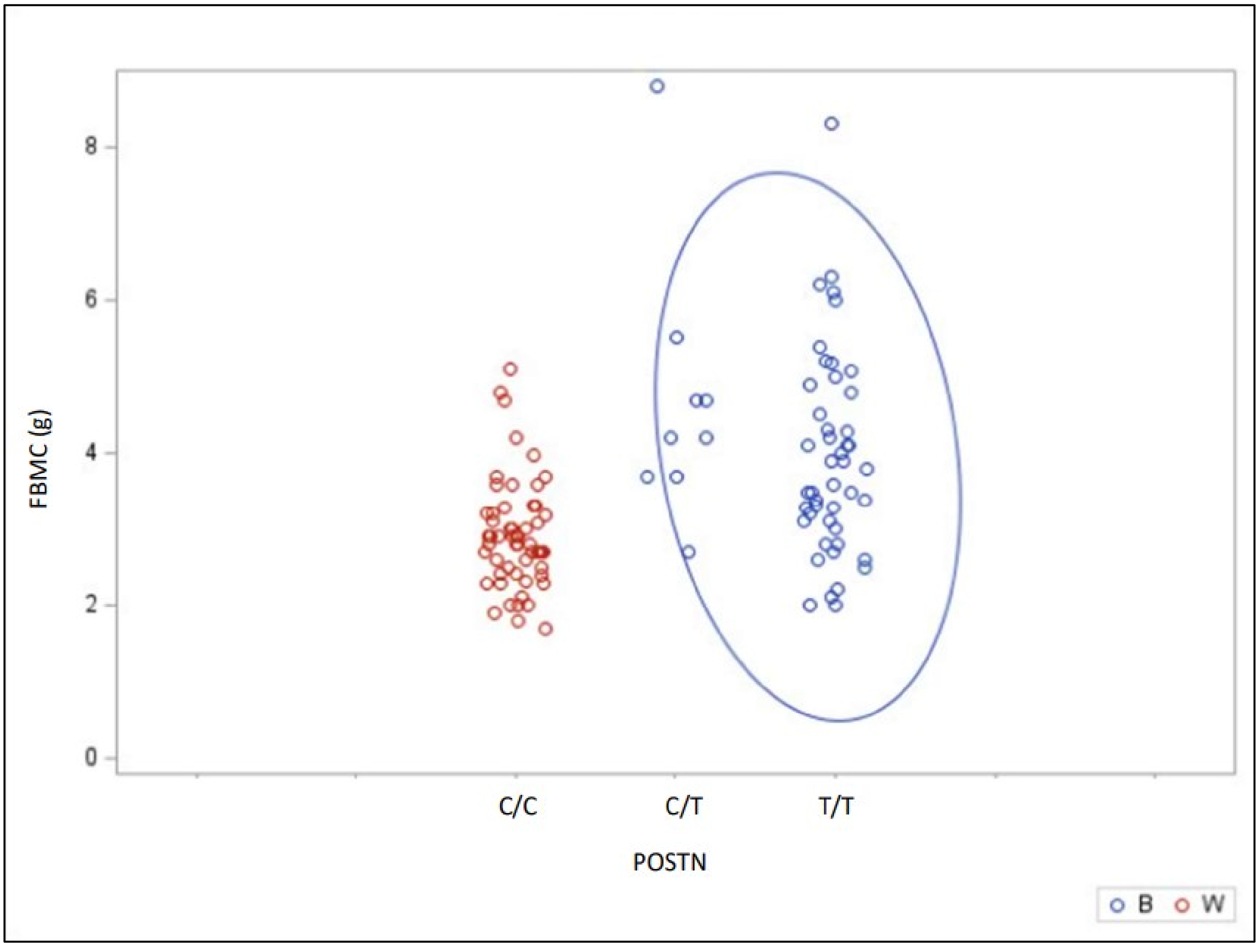
Abbreviations: **KBMC**, Keel Bone Mineral Content. **LB**, Lohmann Brown. **LW**, Lohmann White. **SW**, Shaver White. **IB**, ISA Brown. **RR**, Rhode Island Red. **WL**, White Leghorn. **C**, cytosine. **T**, thymine. ***OVALX,*** *ovalbumin-related protein X.*

Figure 3.2: Relationship between rs315395834 on the gene *OVALX* and KBMD across six strains of laying hens. rs315395834 accounted for 20.2% of phenotypic variation in KBMD (P<0.01).



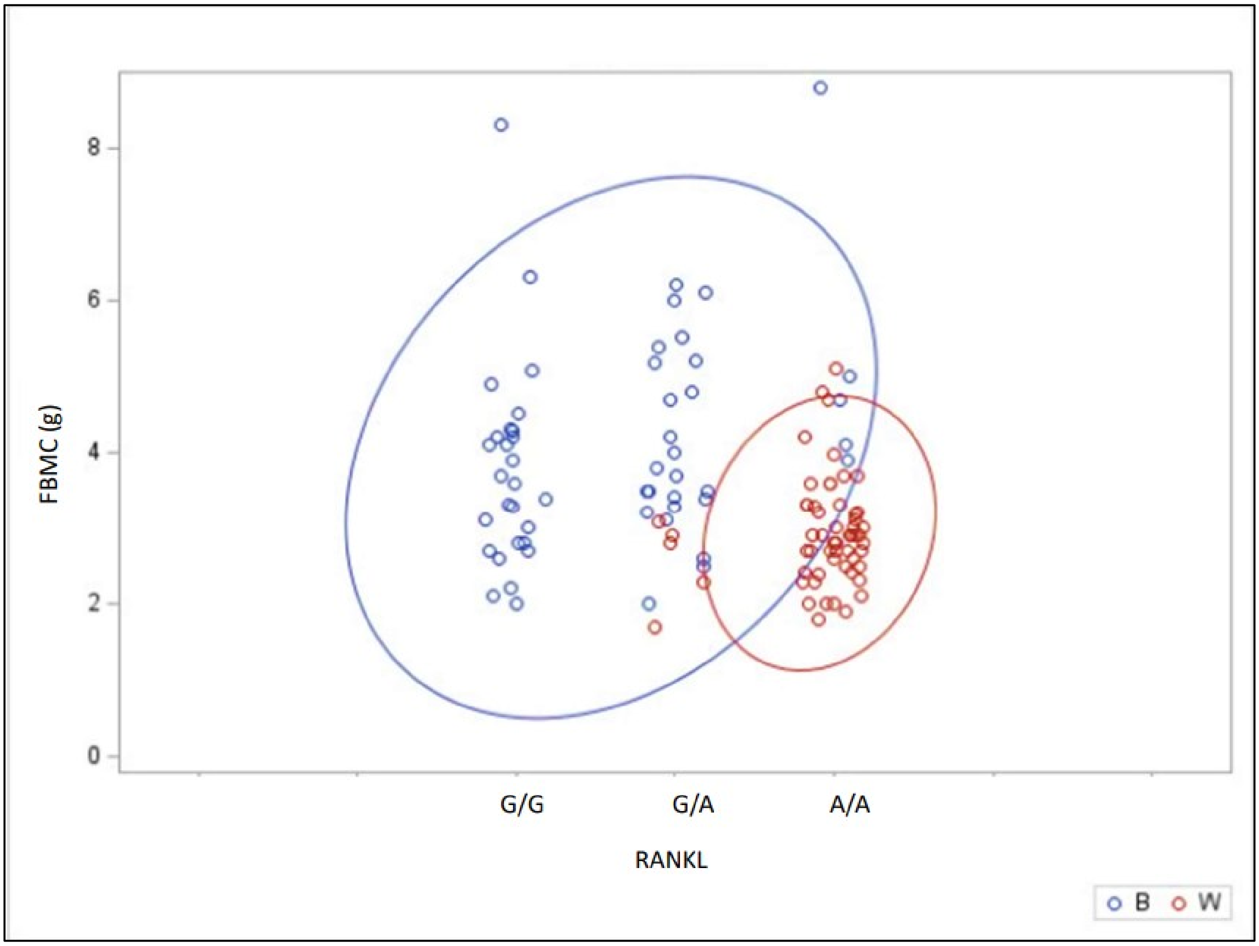
Abbreviations: **KBMD**, Keel Bone Mineral Density. **LB**, Lohmann Brown. **LW**, Lohmann White. **SW**, Shaver White. **IB**, ISA Brown. **RR**, Rhode Island Red. **WL**, White Leghorn. **C**, cytosine. **T**, thymine. ***OVALX,*** *ovalbumin-related protein X.*

Figure 3.3: Relationship between rs13975174 on the gene *POSTN* and FBMC across brown (ISA Brown, Lohmann Brown, and Rhode Island Red) and white strains (Lohmann White, Shaver White, and White Leghorn) of laying hens. rs13975174 accounted for 14.2% of phenotypic variation in FBMC ($P<0.05$).



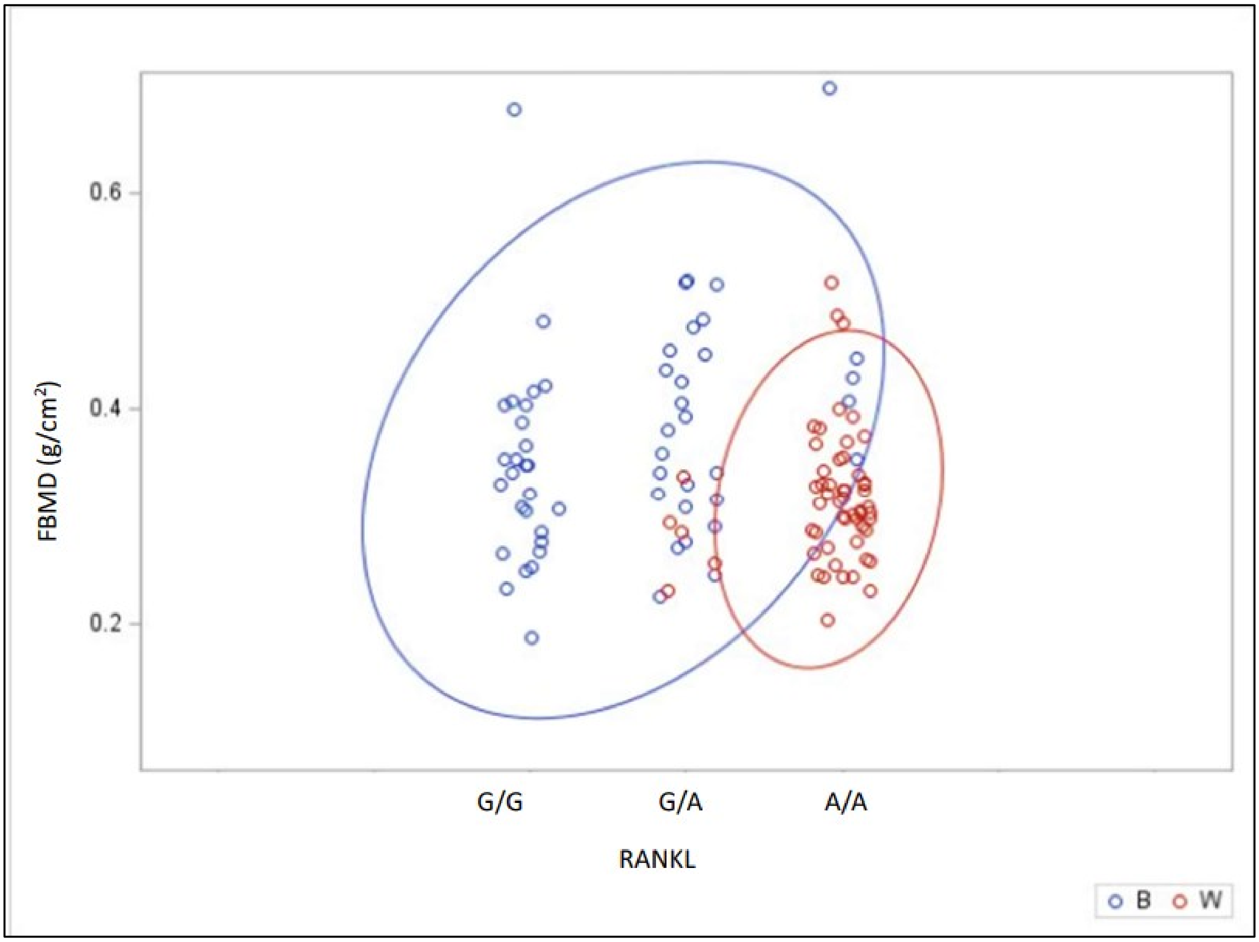
Abbreviations: **FBMC**, Femur Bone Mineral Content. **B**, Brown. **W**, White. **C**, cytosine. **T**, thymine. ***POST**, periostin*

Figure 3.4.: Relationship between rs15490317 on the gene *RANKL* and FBMC across brown (ISA Brown, Lohmann Brown, and Rhode Island Red) and white strains (Lohmann White, Shaver White, and White Leghorn) of laying hens. rs15490317 accounted for 10.6% of phenotypic variation in FBMC (P<0.01).



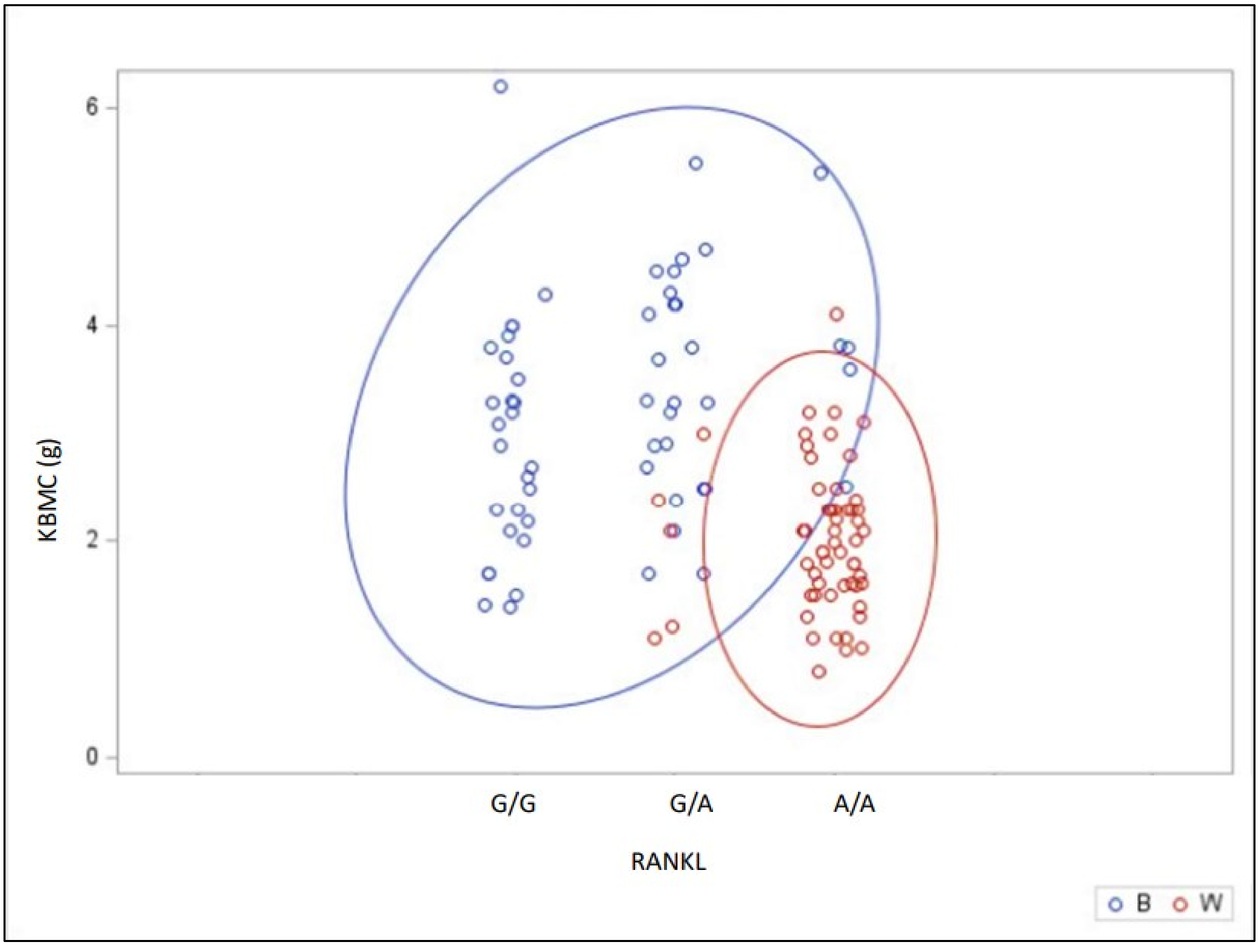
Abbreviations: **FBMC**, Femur Bone Mineral Content. **B**, Brown. **W**, White. **A**, adenine. **G**, guanine. ***RANKL**, receptor activator of nuclear factor kappa-B ligand.*

Figure 3.5. Relationship between rs15490317 on the gene *RANKL* and FBMD across brown (ISA Brown, Lohmann Brown, and Rhode Island Red) and white strains (Lohmann White, Shaver White, and White Leghorn) of laying hens. rs15490317 accounted for 14.4% of phenotypic variation in FBMD (P<0.01).



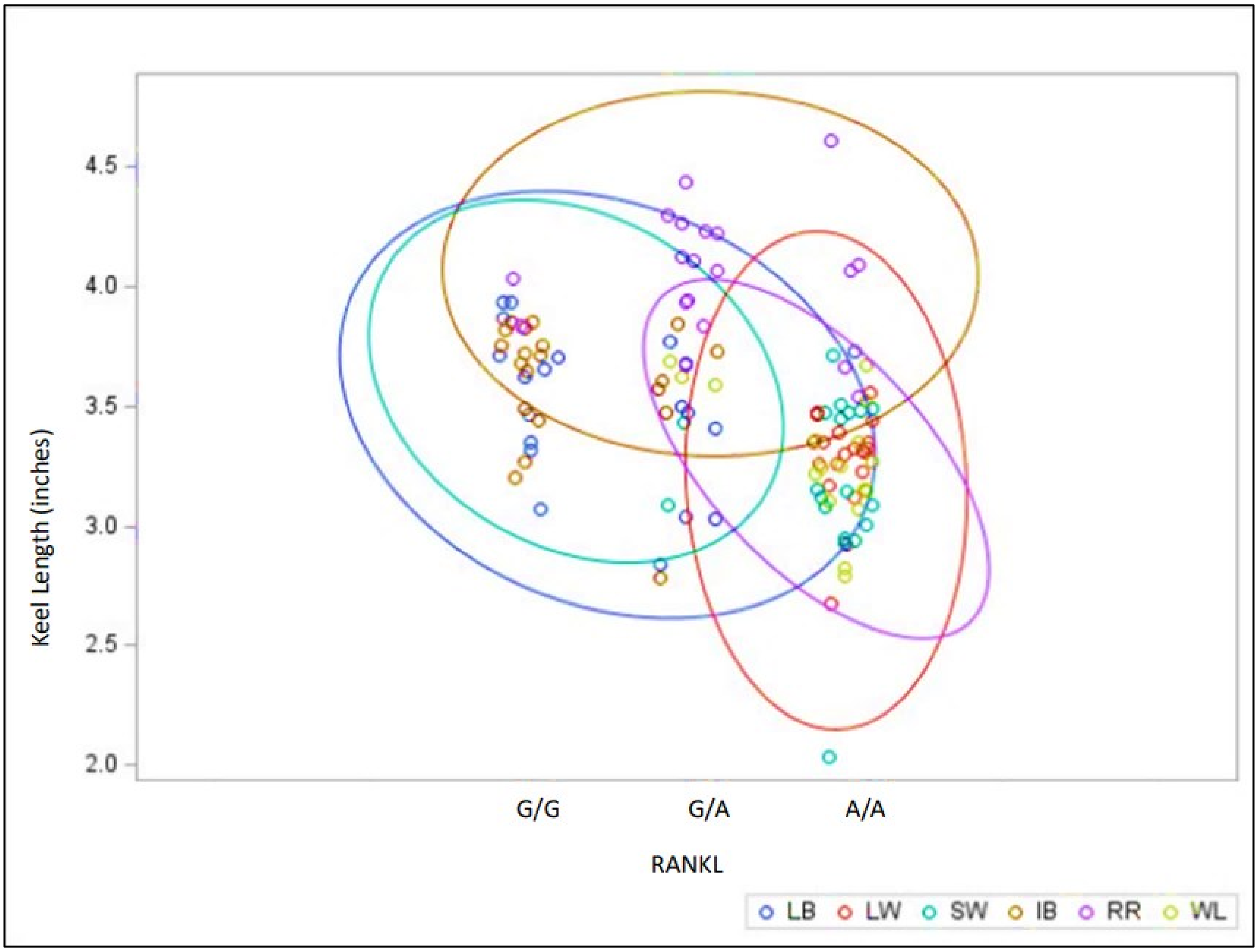
Abbreviations: **FBMD**, Femur Bone Mineral Density. **B**, Brown. **W**, White. **A**, adenine. **G**, guanine. ***RANKL**, receptor activator of nuclear factor kappa-B ligand.*

Figure 3.6. Relationship between rs15490317 on the gene *RANKL* and KBMC across brown (ISA Brown, Lohmann Brown, and Rhode Island Red) and white strains (Lohmann White, Shaver White, and White Leghorn) of laying hens. rs15490317 accounted for 8.7% of phenotypic variation in KBMC (P<0.05).



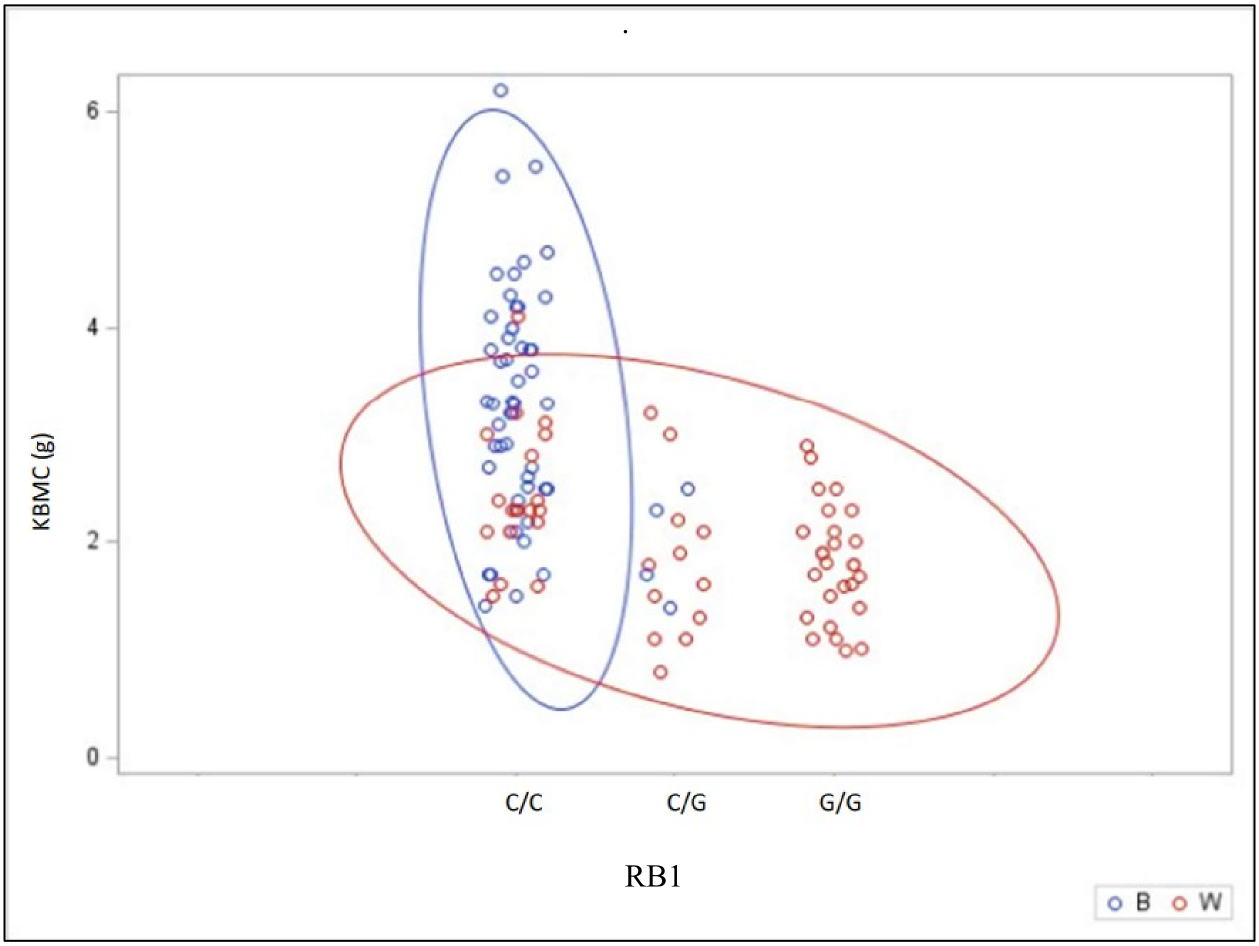
Abbreviations: **KBMC**, Keel Bone Mineral Content. **B**, Brown. **W**, White. **A**, adenine. **G**, guanine. ***RANKL****, receptor activator of nuclear factor kappa-B ligand*.

Figure 3.7. Relationship between rs15490317 on the gene *RANKL* and Length across six strains of laying hens. rs15490317 accounted for 8.9% of phenotypic variation in Keel Length (P<0.05).



Abbreviations: **LB**, Lohmann Brown. **LW**, Lohmann White. **SW**, Shaver White. **IB**, ISA Brown. **RR**, Rhode Island Red. **WL**, White Leghorn. **A**, adenine. **G**, guanine. ***RANKL**, receptor activator of nuclear factor kappa-B ligand.*

Figure 3.8. Relationship between the SNP rs317151697 on the gene *RB1* and KBMC across brown (ISA Brown, Lohmann Brown, and Rhode Island Red) and white strains (Lohmann White, Shaver White, and White Leghorn) of laying hens. rs317151697 accounted for 8.6% of phenotypic variation in KBMC (P<0.01).



Abbreviations: **KBMC**, Keel Bone Mineral Content. **B**, Brown. **W**, White. **C,** cystine. **G**, guanine. ***RB1**, Retinoblastoma 1.*

Chapter 4. Effect of rearing environment, strain and perch shape on perching behaviour, perching biomechanics, and keel bone damage in enriched-housed laying hens

4.1. Introduction

As the Canadian laying hen industry shifts from conventional battery cages towards alternative systems (furnished cages, aviary, free-run, and free range) there has been a notable increase in the incidence of keel bone damage (**KBD**; 36% - 90% of the flock; Regmi et al., 2016; Saraiva et al., 2019; Wilkins et al., 2011) in contrast to conventional housing (22-24% of the flock; Petrik et al., 2015; Regmi et al., 2016). Keel bone damage presents in laying hens as fractures and/or deviations. Fractures have been linked to acute events including collisions (Wilkins et al., 2011) whereas deviations are thought to be caused, in part, by chronic pressure exerted on the keel from behaviours such as perching (Pickel et al., 2011). Fractures lead to reduced egg production (Nasr et al., 2012a; Rufener et al., 2018), reduced egg quality (Rufener et al., 2018; Wei et al., 2020) and negative affective states due to pain (Nasr et al., 2012b; Armstrong et al., 2020). Deviations can contribute to an increased incidence of fractures (Casey-Trott et al., 2017a), potentially from the unequal amount of weight applied to the bone when slipping and vigorous wing-flapping that occur while perching (Harlander-Matauschek et al., 2015).

Furnished cages provide similar welfare benefits as free-range or aviary housing by providing enrichments (Tauson, 2005; Lay et al., 2011). Although furnished cages result in a lower incidence of KBD (36%-54% of the flock; Wilkins et al., 2011; Saraiva et al., 2019) in comparison to aviary or free-range housing (60-90%; Wilkins et al., 2011; Saraiva et al., 2019) the incidence of KBD is still high. In particular, the inclusion of perches in alternative systems increases the incidence of KBD by 24 to 54% in comparison to conventional cages (Wilkins et al., 2011). Perch shape can also impact the incidence of KBD; wider perches (e.g., mushroom or rectangular) distribute the force exerted on the keel by increasing the contact area between the perch and keel which reduces KBD severity (Pickel et al., 2011). In addition, Chen et al. (2014) reported that laying hens prefer rectangular and mushroom perches over round perches. To reduce the negative welfare and economic impacts of KBD, exploring the effects of perch shape in furnished cage systems could help reduce the incidence of KBD below the 20% average in conventionally housed flocks (Petrik et al., 2015; Regmi et al., 2016).

Other factors such as rearing environment and laying hen strain can impact the incidence and severity of KBD. Non-cage rearing systems provide opportunities for load-bearing exercise leading to higher bone mineral content and greater cross-sectional area of the radius and tibia may be protective against KBD (Casey-Trott et al., 2017c). Opportunities for exercise, including wing-flapping and jumping, provided by non-cage systems during rearing contribute to the development of pullet bones, including the periosteal and endocortical bone layers, as well as increased bone surface area allowing for increased medullary bone deposition and bone mineralization (Casey-Trott et al., 2017c). In addition, higher bone strength at pre-lay provided by non-cage systems (Regmi et al., 2015; Casey-Trott et al., 2017b) has been reported to be associated with reduced KBD (Vits et al., 2005; Casey-Trott et al., 2017a). Ensuring pullet rearing and lay environments have enrichments such as perches can provide welfare benefits and lead to increased perch use during the lay phase (Hester et al., 2013; Liu et al., 2018). In addition, variation among laying hen strains contributes to differences in KBD and bone composition. Brown strains weigh more than white strains (Riczu et al., 2004; Chew et al., 2021). Bones in brown strains also ossify more slowly than white strains, which could lead to the development of more severe deviations contributing to more severe KBD (Fawcett et al., 2021).

Perching is considered a behavioral need for laying hens (Weeks and Nicol, 2006), but long-term contact between the keel and perch likely leads to keel deviations (Harlander-Matauschek et al., 2015). Rocking movements of the keel, paired with high levels of pressure exerted on the keel while perching (twice the amount of force (N/cm^2) compared to the feet (Pickel et al., 2011)), could contribute to KBD. While pressure exerted onto the keel or feet when perching (Pickel et al., 2011; Chen et al., 2014) has been researched, more research on balance or biomechanics while perching should be conducted. Other perching biomechanics including movement and movement speed can be assessed utilizing three-dimensional (**3D**) kinematic technology (e.g., Guesgen and Bench, 2018; Perez Marquez et al., 2020). By assessing how specific movements such as forward and backward keel rocking or up and down small scale "collisions" between the perch and keel contribute to KBD, factors including perch shape and strain can be assessed for how they might contribute to unwanted perching biomechanics.

The objectives of the current study were to determine, within enriched-housed layers, which strain (Lohmann Brown vs Lohmann White) exhibited perching biomechanics that did not contribute to KBD, bone strength, and keel health depending on perch shape (mushroom vs round) and pullet rearing system used (cage vs floor pen). We hypothesized that strain, perch shape and pullet rearing environment would contribute to perching instability reflected by changes in movement speed and directionality leading to KBD.

4.2. Materials and Methods

4.2.1. Ethical note

This study occurred at the University of Alberta Poultry Unit in Edmonton, Alberta, Canada in accordance with the Canadian Council on Animal Care guidelines under AUP 3299 (CCAC, 2009). General health and wellbeing of animals were monitored daily by trained barn staff and the research team.

4.2.2. Animals and Housing

This study used a 2 x 2 x 2 randomized complete block design with repeated measures. Two commercial egg-laying strains, Lohmann White (**LW**; n=360) and Lohmann Brown (**LB**; n=360) were evaluated. Laying hens were provided with either a round or mushroom shaped perch and housed in either a pullet cage or floor pen. Throughout lay, all hens were housed in furnished cages. There were nine replications of each treatment combination.

Laying hens were kept from January 2022 to May 2023. Within each strain, birds were split at hatch randomly and equally between the cage and floor pen rearing environments. There were eight floor pens (2.3 m by 5.5 m) divided into two perch shape treatments (mushroom and round) with 45 birds per pen (10.9 m^2/pullet). Floor pens consisted of feeders, waterers, and four perches (84.5 cm x 3.5 cm x 7 cm; 15 cm of perch/pullet). There were 36 cages (53.3 cm x 25 cm x 119.4 cm) divided into two perch shape treatments (mushroom and round) with 10 birds per cage (128 cm^2/pullet). Rearing cages included a water line, feed trough and two perches (25 cm x 3.5 cm x 7 cm; 10 cm of perch/pullet).

At 17 weeks of age, all birds were moved into a Tecno Plus battery (AGCO, Duluth, GA, USA) containing nest curtains, a scratchpad, two perches (64 cm x 3.5 cm x 6 cm), a water line, and a feed trough. The battery consisted of 72 cages (10 birds per cage) and three tiers, treatments were balanced for side, tier level, and end vs middle positions along the battery. Treatment groups from the rearing phase were maintained through the lay phase. Birds were housed to 70 weeks of age, with a day length of 14L:8D during lay. All birds were fed according to NRC requirements and were provided with water and feed *ad libitum* (Lohmann Tierzucht, 2019). At 51 weeks of age due to reduced eggshell quality, supplemental oyster shell was top-dressed into the feed troughs in line with standard practice at the University of Alberta Poultry Unit.

4.2.3. Data collection

Weight. Weights were collected at eight separate time points (2, 4, 6, 8, 10, 12, 14, and 16 months of age) using a BW-2050 Weighing System (Weltech International Ltd., Cambridgeshire, UK). Weights were collected to assess how body weight impacted perching biomechanics and bone parameters as a covariate. A focal group consisting of two birds from each cage and nine birds from each floor pen (20%; n=144) which were wing-banded with an identification number at 8 weeks of age. Focal birds were used for kinematic recordings, weights, keel palpations, and bone measurements.

Behaviour. From seven weeks of age to 69 weeks of age, behaviour observations occurred biweekly. Enclosure (floor pen or cage) was treated as the experimental unit. Behaviours recorded included standing on perch, resting on perch, standing on other, resting on other, squabble, and displacement (Table 4.2). Only standing and resting on perch were observed frequently enough to be included in the subsequent statistical analysis. Instantaneous scan sampling was used for standing on perch, resting on perch, standing on other, resting on other, and one-zero sampling for squabble and displacement (Martin and Bateson, 2007). Before in barn observation periods, observers were trained on the definition of the behaviours and tested following training to ensure at least 90% compliance with the lead study observer.

During the rearing phase, pullets housed in cages were observed by three observers, eight times a day for six days. There were 36 pullet cages with nine replicates of each treatment combination. Four observation sessions were held in the morning (8:00 h to 11:00 h) and four in the afternoon (13:00 h to 16:00 h). In total each treatment was observed 1,296 times. Concurrently, pullets housed in floor pens were recorded via SWPRO-640 video cameras situated in each floor pen (Swann Communications U.S.A Inc., Santa Fe Springs, CA, USA). There were eight floor pens with two replicates per treatment combination. Video recordings were analyzed by one trained observer, with each floor pen observed 24 times a day. In total each treatment was observed 288 times.

During the lay phase, laying hens housed in a Tecno enriched battery (n=72 cages) were observed by three observers cycling through eight times a day on 26 observations days. Four observation sessions were held in the morning (8:00 h to 11:00 h) and four in the afternoon (13:00 h to 16:00 h). In total, each treatment (n=9 floor pens) was observed 5,616 times.

Bone Parameters. Dual X-Ray Absorptiometry (**DXA**) was used to determine bone mineral content and density (Schreiweis et al., 2005). Radiographic bone mineral density and content measurements of the dissected and cleaned keel and left femur were analyzed post-mortem at 70 weeks of age utilizing LUNAR Prodigy DXA (GE Healthcare Canada, Mississauga, ON, Canada). Before scanning, the machine was calibrated to 2% ± the mean densities of a phantom spine with a known set of densities (GE Healthcare Canada, Mississauga, ON, Canada). The keel and femur of each focal bird were then placed horizontally into a plastic container filled with rice. By placing bones in rice, each scan was taken at the same angle for each sample. Keel bone mineral content (**KeelMC**), keel bone mineral density (**KeelMD**), femur bone mineral content (**FemurMC**) and femur bone mineral density (**FemurMD**) were measured and recorded. Bone breaking strength was measured post-mortem at 70 weeks of age utilizing an Instron Materials Tester (Model 4411, Instron Corp., Norwood, MA, USA) fitted with a 50 kg load cell and loading rate of 30 mm/min. Bones were placed dorsal side up on supports placed 5 cm apart. Tests were performed on the left femur which was dissected and cleaned. Load at maximum compressive load (**Load**; kgf) and compressive extension at break (**Extension**; mm) were measured and recorded.

Keel Bone Assessment. Between 20 to 70 weeks of age, all focal birds (n=144 with 18 birds per treatment and two focal birds per Tecno cage) were assessed via manual palpation for presence of fractures on the ventral surface of the keel, severity of deviations and body condition score. Keel bone assessment protocol was modified from Casey-Trott et al. (2015) and Butterworth et al. (2009). Hens were restrained in an inverted position so that their ventral surface was facing towards the assessor. The feathers were pushed back so that the keel could be visually inspected and assessed by touch. Keels were palpated and scored for the presence of fractures; 0 (absence of fracture) or 1 (presence of fracture). Fractures were indicated by sharp bends, swelling, or detached/semi-detached bone fragments. Finally, keels were scored for the presence and severity of deviations; 0 (straight, 180°, line), 1 (mild deviation of less than 1 cm from the normal line), or 2 (severe deviation of more than 1cm from the normal line). For body condition scores, the keel prominence was visually assessed and ranked as 2 (normal/fat), 1 (lean), or 0 (emaciated, tented).

Post-mortem digital keel bone imaging and palpation were also used to assess the presence of deviations and fractures of excised keels. Digital keel bone imaging was used to determine the severity and number of keel bone deviations present. Right sagittal and ventral view images of excised keels were recorded using a Canon PowerShot SX420 IS (20 Megapixel, Canon Canada Inc, Brampton, ON, Canada). Images of the keel were analyzed using ImageJ software (ImageJ, National Institutes of Health, 1997). The number of deviations were recorded, taking into consideration both the right sagittal and ventral keel views. The severity of each deviation was measured using the angle tool in ImageJ. Modified from Regmi et al. (2016), keels were scored based on deviation severity on a 5-point scale from 1 to 5 (5 being the most severe), and number of deviations present (1 to 5). Keel deviation severity was classified based on least to most severe deviation (178°-172° was a score of "1", 171°-156° was "2", 155°-142° was "3", 141°-126° was "4" and 125°-110° was "5"). All keels were also assessed for the number of fractures present which was based on the total number of both healed and fresh fractures.

3-D Kinematics. Each recording perch, round or mushroom, was 30 cm in length, 4.8 cm diameter and 60 cm tall. The round recording perch was constructed using 3.8 cm PVC pipe and the mushroom recording perch was a section of a Comfort Perch System (Lubing Systems, Cleveland, TN). At 2, 4, 6, and 8 months of age, kinematic recordings were captured using six Vicon Nexus Bonita cameras (Bonita, Vicon Motion Systems Ltd., Denver, CO). Cameras were arranged in a semi-circle facing a recording perch and connected to a laptop computer running Vicon Nexus 2.12 software (Vicon Motion Systems Ltd., Denver, CO) and. Starting at 10 months of age, two Vicon Nexus Vero cameras (Vero, Vicon Motion Systems Ltd., Denver, CO) were added so at least two cameras could see each marker despite the extra feather coverage at 10 months of age. Kinematic recordings were conducted a day after weights were collected at each sampling time point on the respective perch that was present in the home environment.

Seven 14 mm diameter (Life Science Basic Kit, Vicon Motion Systems Ltd., Denver, CO) markers were placed on each focal bird (Figure 4.2). Four back markers were placed on the pygostyle (B1), free thoracic vertebrae (B2), left (B3), and right iliac crest (B4) to form a back segment. Three keel markers were utilized along the keel at the caudal (K1) and cranial (K2) ends and one halfway between the two points (K3) to form a keel segment. Markers were also placed at either end of each kinematic recording perch (P1 and P2) and used to form a perch segment. Before each recording, each bird was placed gently on the recording perch and checked to ensure it had performed a tendon lock and was securely on the perch. A recording was considered acceptable when a bird was on the perch for a minimum of 30s following a tendon lock.

After recording, kinematic clips were reconstructed using the Reconstruct pipeline in Nexus software and labeled using a skeleton template that included all seven anatomical markers. The y-axis represented the forward and backward movement of a laying hen, the x-axis represented side-to-side movement, and the z-axis represented up and down movement. Recordings were shortened to the middle 1000 frames, standardizing the length of recordings. Labeled recordings were exported to ProCalc (Vicon Motion Systems Ltd., Denver, Colorado). In total, ten metrics were assessed (Table 4.1). Back angle (**BA**), keel angle (**KA**), side-to-side back motion (**BMx;** mm), side-to-side keel motion (**KMx;** mm), forward and backward keel motion (**KMy;** mm), forward and backward back speed (**BSy;** mm/s), up and down back speed (**BSz;** mm/s), forward and backward keel speed (**KSy;** mm/s), and up and down keel speed (**KSz;** mm/s).

4.2.4. Statistical Analysis

Data were analyzed using SAS software (SAS ver 9.4, Cary, NC, USA). To test normality assumptions, a ProcUnivariate with a Kolmogorov-Smirnov test was run on all metrics. 3D-kinematic metrics with outliers that fell ± 3SD from the mean were removed from the data set. Kinematic metrics, DXA metrics and body weight were analyzed using a generalized linear mixed model (Proc Glimmix; SAS Institute, 2023), Type 3 test with the inverse (ilink) function specified. Distributions used were selected based on the lowest Akaike information criterion (AIC). Back angle, KA, KMy, KMz, FemurMD, FemurMC, KeelMD, and KeelMC were analyzed using a gamma distribution. Back speed in the y-axis, KSy, BSz, KSz, BMx, and KMx were analyzed using a lognormal distribution. Fixed variables in the glimmix model were strain, rearing housing, and perch shape; weight was included as a covariate. Age was treated as a repeated measure. All models were run to determine any interactions present between fixed variables. If an interaction was not significant, a subsequent model was run without the specific interaction. Least Squares Means (**LSMeans**) differences were adjusted for multiple comparisons using a Bonferroni test. Keel score metrics were analyzed using a logistic regression model using a Logistic procedure in SAS (Proc Logistic; SAS Institute, 2023). Fixed variables in the model were strain, rearing housing, perch shape, and their interaction(s).

Pearson correlation coefficients calculated using the corr procedure to compare and identify kinematic markers that were used as proxy markers when the primary marker was not visible in a recording (e.g., due to feather cover). For keel metrics, K1 was identified as the primary marker and K3 was identified as the proxy marker (see Chapter 2). For back metrics, B1 was identified as the primary marker and B2 was identified as the proxy marker (see Chapter 2). Proxy markers had significant correlations that were higher than 0.9. Pearson correlation coefficients were also calculated using the corr procedure between kinematic metrics, bone parameters, and keel scores.

Behaviour observation data was analyzed using a generalized linear mixed model (Proc Glimmix; SAS, 2023), Type 3 test with the inverse (ilink) function specified. A multinomial distribution was used. Fixed variables in the model were time of day (**AMPM**), strain, rearing environment, and perch shape. Results were analyzed by age. To quantify the strength of association between behaviours of interest and fixed effects, odds ratios were used. Odds ratios greater than one indicate an events odds are higher for the treatment in the numerator while odds ratios less than one indicate an events odds are lower for the treatment in the numerator. Behavioral data was analyzed in three data sets: floor pen rearing phase, pullet cage rearing phase, and lay phase. Data for statistical analysis was calculated as follows:

$$\frac{\#\ of\ hens\ performing\ behaviour}{\#\ of\ hens\ in\ enclosure} = frequency\ of\ the\ behaviour\ (\%)$$

For this study, P-values of $P < 0.05$ were considered statistically significant, $0.05 > P > 0.10$ were considered a trend, and $P > 0.10$ was considered not statistically significant. Correlation coefficients were considered strong if $r > 0.80$, moderate if between 0.50 and 0.80, and weak if r

< 0.50. A strong negative correlation was considered if r < -0.80, moderate if between -0.50 and -0.80 and weak if r < -0.50.

4.3. Results

4.3.1. Body Weight

There was a significant strain effect with LB birds (1.65 kg ± 0.047) weighing more than LW birds (1.39 kg ± 0.040; P<0.001).

4.3.2. Bone Parameters

Keel bone deviation scores are reported in Table 4.3 and keel fracture scores are reported in Table 4.4. Lohmann Brown was more likely to have more severe deviations (P<0.01), more deviations (P<0.01), and more fractures than Lohmann White (P<0.01). Laying hens housed with round perches were more likely to have more severe deviations (P<0.05) and tended to have a higher number of deviations in contrast to laying hens housed with mushroom perches (P=0.09). Notably, rearing environment did not have any effect on keel bone damage scores (P>0.10).

Body condition score was significantly, positively correlated with severity of deviation score (r 0.35, P<0.001; Table 4.5). In addition, fracture presence was significantly positively correlated with severity of deviation present (r 0.14, P<0.001; Table 4.5). All bone parameters were affected by strain (Table 4.6). Lohmann Browns had significantly denser and stronger bones; FemurMD (P<0.001), FemurMC (P<0.001), KeelMD (P<0.001), KeelMD (P<0.001), and Load (P<0.001) than LW (Table 2). While LW had significantly greater Extension than LB (P<0.05; Table 4.6). Rearing environment, perch shape and body weight, in contrast, did not significantly impact the bone parameters measured (P>0.10).

4.3.3. 3-D Kinematics

Angle. Keel angle was greater in laying hens housed in floor pens (103.52° ± 1.402) than those housed in pullet cages (99.01° ± 1.341; P<0.05). Laying hens housed in floor pens had a greater back angle (94.63° ± 0.619) than those housed in pullet cages (92.59° ± 0.598°; P<0.05). Laying hens housed with round perches had a greater back angle (94.61° ± 0.613) than those housed with mushroom perches (92.61° ± 0.604; P<0.05).

Motion. There was a negative correlation between severity of deviation present and side-to-side keel motion (r -0.13; P<0.001). Side-to-side keel movement (x-axis) was not significantly impacted by any main effects. There was a negative correlation between severity of deviation present and side-to-side back motion (-0.19; P<0.001). Back motion in the x-axis was not significantly impacted by any main effects.

There was no correlation between up and down keel movement and keel deviation scores (P<0.10). Laying hens perching on mushroom perches (6.10 mm ± 0.004) had more up and down keel movement (z-axis) than birds perching on round perches (6.08 mm ± 0.004; P<0.001). In addition, brown birds moved up and down more (6.09 mm ± 0.004) than white birds (6.00 mm ± 0.004; P<0.05).

There was a positive correlation between severity of deviation present and forward and backward keel motion (r 0.24; P<0.001). White birds moved forward and backward more (283.78 mm ± 4.285) than brown birds (260.53 mm ± 3.923; P<0.001). There was a trend for laying hens housed in floor pens to move more (277.24 mm ± 4.178) than those housed in pullet cages (266.67 mm ± 4.023; P=0.07).

Speed. There was a negative correlation between severity of deviation present and forward and backward keel movement speed (r -0.12; P<0.001). Forward and backward keel movement speed (y-axis) was significantly impacted by strain with LW moving faster (4.27 mm/s ±0.047) than LB (3.99 mm/s ± 0.048; P<0.001).

There was a weak, negative correlation between severity of deviation present and up and down keel movement speed (r -0.11; P<0.001). Up and down keel movement speed (z-axis) was significantly impacted by strain with LW (3.85 mm/s ± 0.043) moving faster than LB (3.69 mm/s ± 0.042; P<0.001).

There was a negative correlation between severity of deviation present and forward and backward back speed (r -0.24; P<0.001). Forward and backward back speed (y-axis) was significantly impacted by strain with LW (3.54 mm/s ± 0.041) moving faster than LB (3.29 mm/s ± 0.039; P<0.001).

There was a negative correlation between severity of deviation present and up and down back movement speed (r -0.12; P<0.001). Up and down back speed (z-axis) was significantly impacted by perch shape with laying hens housed with round perches (3.42 mm/s ± 0.045) moving faster than those housed with mushroom perches (3.16 mm/s ± 0.045; P<0.001).

4.3.4. Behaviour

During the rearing phase (7 weeks to 17 weeks of age), perch shape, strain, and time of day had significant effects on perching behaviours when pullets were housed in floor pens (Table 4.7). In floor pens, LB pullets perched (odds ratios: 0.00 to 0.11; P<0.001) and stood less (odds ratios: 0.08 to 0.36; P<0.001) than LW pullets. Floor pen pullets perched on mushroom perches less than round perches at 9 weeks of age (odds ratios: 0.48; P,0.05), 13 weeks (odds ratios: 0.24; P<0.05). However, at 17 weeks of age floor pen pullets perched on mushroom perches more than round perches (odds ratios: 1.07; P<0.001). In addition, floor pen pullets stood on mushroom perches less than round perches (odds ratios: 0.21 to 0.35; P<0.001). Overall, more perching occurred in the morning compared to the afternoon (odds ratios: 3.56 to 12.04; P<0.001).

During the rearing phase (7 weeks to 17 weeks of age), perch shape, strain, and time of day had significant effects on perching behaviours when pullets were housed in cages (Table 4.8). In pullet cages, LB pullets perched more than LW pullets (odds ratios: 1.28 to 16.15; P<0.001). Lohmann Brown pullets stood more on perches, from 13 to 17 weeks of age, than LW pullets (odds ratios: 1.50 to 2.26; P<0.001 to P<0.01). However, at seven and nine weeks of age, LB pullets stood less on perches than LW pullets (odds ratios: 0.72; P<0.05). At seven, nine, 11, and 15 weeks of age, pullets perched less on mushroom perches compared to round perches (odds ratio: 0.5 to 0.77; P<0.01 to P<0.05). Overall, pullets stood less on mushroom perches compared to round perches (odds ratios: 0.28 to 0.62; P<0.001).

During peak lay (19 to 43 weeks of age) and end of lay (45 to 69 weeks of age) perch shape, rearing environment, strain, and time of day had significant effects on perching behaviours (Tables 4.9 and 4.10). Lohmann Browns perched more during peak lay (odds ratios: 1.63 to 6.90; P<0.001) and more during end of lay than LW (odds ratios: 3.28 to 5.36; P<0.001). At end of lay, laying hens perched more on mushroom perches compared to round perches (odds ratios: 1.23 to 1.45; P<0.001 to P<0.05). During peak lay, laying hens housed in cages perched more than those housed in floor pens except at 25 and 31 weeks of age (odds ratios: 1.33 to 1.84; $P<0.05$ to $P<0.001$). During peak and end of lay, laying hens were perching less in the morning compared to the afternoon (odds ratios: 0.02 to 0.56; $P<0.001$). During peak lay laying hens were less likely to be standing on the perch in the morning compared to the afternoon (odds ratios: 0.6 to 0.8) but during end of lay perch shape did not significantly impact standing at each week measured. During peak and end of lay, laying hens housed in cages were less likely to stand on the perch only 40% of the time (odds ratios: 0.8 to 0.83; $P<0.001$ to $P<0.05$). At 19 and 21 weeks of age LB stood more than LW (odds ratios: 1.22; $P<0.05$). In contrast, from 27 to 33 weeks of age, LB stood less than LW (odds ratios: 0.58 to 0.74; $P<0.001$). At 65 and 67 weeks of age LB stood more than LW (odds ratios: 1.25 to 1.32; $P<0.001$ to $P<0.05$). During peak of lay, laying hens stood less on mushroom perches compared to round perches (odds ratios: 0.2 to 0.46) and stood less on mushroom perches compared to round perches at end of lay (odds ratios: 0.31 to 0.47; $P<0.001$ to $P<0.05$).

4.4. Discussion

Lohmann White and LB strains were selected based on a previous study that compared four commercial and two random-bred strains of laying hen (Appendix 1). Based on visual assessments of kinematic recordings, Lohmann Brown had the least amount of instability events (e.g., moving from perching to standing, wing flapping). Both strains also displayed minimal rocking movements which were of particular interest in this study as forward and backward rocking movements could lead to deviations (Harlander-Matauschek et al., 2015). Round and mushroom perches were selected based on current availability to producers and use on commercial egg farms (Liu et al., 2018). Mushroom perches are wider than round perches and more evenly distribute the force exerted on the keel, a wider area leads to lower peak force exerted on the keel (Pickel et al., 2011). However, a wider contact area between the perch and keel while perching on mushroom perches means there is more contact area between the perch and the keel. Long-term contact between the keel and perch likely leads to deviations in the keel (Harlander-Matauschek et al., 2015).

Typically, one-quarter to one-half of laying hens will be perching at any time during the day (Appleby et al., 2002), which is in line with the amount of perch use that was recorded throughout this study. Overall, more perching occurred during the morning in contrast to the afternoon across rearing, peak and end of lay but the same results were not observed with standing. A potential explanation is that perching on the perch is a resting behaviour, while laying hens standing on the perch could have been drinking water or performing other behaviours while stood on the perch. Regardless of rearing environment, LB perched less than LW and pullets with mushroom perches perched less than pullets with round perches. Laying hens at the end of lay, when observations were pooled by lay phase, used mushroom perches more than round perches. Our results indicated that round perches contributed to more severe deviations and are used less frequently during peak and end of lay. Laying hens prefer wider perches over round perches due to the more comfortable gripping surface provided by rectangular or mushroom shaped perches (Chen et al., 2014). White strains have been reported to exhibit more resting and perching behaviors compared to brown strains (Kozak et al., 2016) which differs from our results at peak and end of lay. Overall, LB used the perch more than LW at peak and end of lay. Generally laying hens housed in cages exhibited more perching behaviour than laying hens housed in floor pens, potentially due to the similarities between their rearing and lay environments. Alternatively, floor pens have increased opportunities for behaviours, including dust bathing, in contrast to pullet cages. As such, perches in pullets' cages may have been the most engaging aspect of their environment and familiarity and usage of perches increased accordingly.

Although not analyzed, it was anecdotally noted that during the pullet phase, pullets were more likely to jump or fall from the perch. Perching may be an innate behaviour in poultry as pullets will use perches within the first week of life (Skanberg et al., 2021), but it may take anywhere from five to seven weeks for acclimation to occur (Liu et al., 2018). Our results highlight that although perching is a behavioral need in laying hens (Weeks and Nicol, 2006) and is exhibited from day one, pullets and laying hens become more stable while perching with age. While learning how to perch, pullets are more likely to exhibit faster and greater indicators of instability while perching. In addition, differences between LW and LB were present in speed metrics that were recorded. Lohmann Whites moved faster while perching than LB. White strains tend to be more fearful and flightier (Uitdehaag et al., 2008; Uitdehaag et al., 2011; Habig et al., 2021) which, when paired with the manual restraint required during marker placement for recordings, could have contributed to the faster movements exhibited by LW.

Lohmann Browns are significantly heavier than LW, in line with previous research which reports brown layers are heavier than white layers (Riczu et al., 2014; Chew et al., 2021). Brown strains are reported to have significantly stronger and denser bones than white strains (Fawcett et al., 2020, Habig et al., 2021). In this study, LB had significantly stronger and denser bones than LW. Rearing environment did not significantly impact the bone parameters measured. Our results contrast with previous studies that report rearing systems that provide opportunities for load-bearing exercise (e.g., non-cage systems) increases bone mineral content (Hester et al., 2013; Casey-Trott et al., 2017a). One possible explanation is that both pullets in the cage and in the floor pen treatments were provided with perches in this study, potentially leading to wing-flapping and jumping which may contribute to greater bone mineral content and breaking strength (Jendral et al., 2008; Tactacan et al., 2009; Casey-Trott et al., 2017b). Notably, the keel does not fully ossify until well into lay (28 to 40 weeks of age; Buckner et al., 1948; Buckner et al., 1949), making the caudal tip of the keel particularly susceptible to deviations prior to 28 weeks of age. The benefits of the floor pen may have only been present during the start of lay and as DXA scans were only conducted post-mortem, we were not able to assess bone mineralization throughout the lay period. Overall, if there were beneficial effects on bone mineralization and strength from the additional space provided by floor pens during rearing, those effects did not persist until end of lay.

Lohmann Brown had more severe KBD than Lohmann White. Our results indicate that despite having higher bone mineralization and bone breaking strength, brown birds had more fractures and severe deviations. Other studies report that increased bone mineralization is not protective enough against KBD when laying hens are housed with perches, potentially due to perch design and placement (Hester et al., 2013). Higher bone mineralization is not associated with fracture healing, as bone loss following fractures is recorded (Osipov et al., 2018). As such, the higher bone mineralization in LB hens cannot be explained by increased bone mineralization from fracture healing. One potential explanation is that brown strains' keels ossify more slowly than white strains, thereby increasing susceptibility to KBD (Fawcett et al., 2020).

Based on correlations between keel bone scoring throughout the trial and kinematic recordings, increased forward and backward keel motion was correlated with keel bone damage severity. Forward and backward keel motions were akin to a rocking motion of the keel. When perching, regardless of perch shape, hens exert twice the force (N/cm^2) on the keel bone in comparison to the feet (Pickel et al., 2011). As such, forward and backward movements while the keel is in contact with the perch contribute to KBD severity.

4.5. Conclusion

In conclusion, strain, perch shape, and rearing environment significantly impacted perching biomechanics. Lohmann Whites moved faster than LB, which was not correlated with KBD and LW had the lowest incidences of severe keel bone damage. Most notably, increased forward and backward movement led to more severe keel bone damage. In addition, mushroom perches led to less severe KBD and were preferred by laying hens at peak and end of lay. Lohmann Brown had significantly stronger and denser bones than LW, while rearing environment did not significantly impact the bone parameters measured. Higher bone density and strength were not protective against keel bone damage. Based on the incidence of KBD, Lohmann White may be more suited to furnished cage housing in comparison to LB. Future research utilizing 3D kinematics technology in poultry could investigate the provision of perches during the rearing phase on the development of perching behaviour.

4.6. References

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Table 4.1. 3-D kinematic metrics and corresponding descriptions used to analyze perching biomechanics.

Metric	Description
Maximum back angle (BA; degrees)	Maximum angle between B1 and the center of the perch. If B1 was not present, B2 was used.
Maximum keel angle (KA; degrees)	Maximum angle between K1 and the center of the perch. If K1 was not present, K3 was used.
Maximum back speed in the y-axis (BSy; mm/s)	Maximum speed of the midway point between B1 and B2 in the y-axis. If B1 and B2 were not present, the point between B3 and B4 was used.
Maximum back speed in the z-axis (BSz; mm/s)	Maximum speed of the midway point between B1 and B2. If B1 and B2 were not present, the point between B3 and B4 was used.
Maximum keel speed in the y-axis (KSy; mm/s)	Maximum speed of K1 in the y-axis. If K1 was not present, K3 was used.
Maximum keel speed in the z-axis (KSz; mm/s)	Maximum speed of K1 in the z-axis. If K1 was not present, K3 was used.
Maximum back motion in the x-axis (BMx; mm)	Greatest change in position of the midway point between B1 and B2 in the x-axis. If B1 and B2 were not present, the point between B3 and B4 was used.
Maximum keel motion in the x-axis (KMx; mm)	Greatest change in position of K1 in the x-axis. If K1 was not present, K3 was used.
Maximum keel motion in the z-axis (KMz; mm)	Greatest change in position of K1 in the z-axis. If K1 was not present, K3 was used.
Maximum keel motion in the y-axis (KMy; mm)	Greatest change in position of K1 in the y-axis. If K1 was not present, K3 was used.

Abbreviation: **B1**, pygostyle. **B2**, free thoracic vertebrae. **B3**, left iliac crest. **B4**, right iliac crest. **K1**, caudal keel. **K2**, cranial keel. **K3**, middle keel.

Table 4.2. Ethogram of laying hen behaviours used during behaviour observations.

Behaviour	**Description**
Mutually exclusive behaviours	*By actor*
Perching on perch	Bird is resting on top of perch and feet and legs are tucked underneath body. Keel touching the perch.
Standing on perch	Bird is either fully upright or crouching with legs and feet visible on top of perch. Keel is not touching the perch.
Perching on other*	Bird is resting on top of water line or feeder and feet and legs are tucked underneath body. Keel touching the water line or feeder.
Standing on other*	Bird is either fully upright or crouching with legs and feet visible on top of the water line or feeder. Keel is not touching the water line or feeder.
Not mutually exclusive behaviours	*By recipient*
Squabble*	A bird engaging in one of the other behaviours is approached by 1 or more birds and one or both parties rapidly pecks and/or wing flaps and/or increases vocalizations.
Displacement *	Due to a squabble the bird falls off or leaves the place it was standing or perching.

*Behaviours not used in analysis

Table 4.3. Percentage (%) of birds by treatment according to number of deviations (0 to 4) and severity of deviation (1 to 5) scored post-mortem at 70 weeks of age via visual assessment of excised keels.

	Number of Deviations					**Severity of Deviations**				
	0	1	2	3	4	1	2	3	4	5
Treatment										
CBM	6%	67%	21%	6%	0%	10%	39%	22%	29%	0%
CBR	0%	52%	35%	13%	0%	5%	32%	55%	9%	0%
CWM	50%	44%	6%	0%	0%	41%	37%	22%	0%	0%
CWR	11%	61%	22%	6%	0%	16%	38%	24%	22%	0%
FBM	17%	44%	22%	11%	6%	11%	67%	0%	22%	0%
FBR	18%	47%	29%	0%	6%	9%	65%	26%	0%	0%
FWM	39%	39%	22%	0%	0%	32%	57%	11%	0%	0%
FWR	17%	67%	16%	0%	0%	14%	52%	9%	11%	14%

Abbreviations: **C,** cage. **F,** floor pen. **B,** brown. **W,** white. **M,** mushroom. **R,** round.

Table 4.4. Percentage (%) of birds by treatment according to number of fractures (0 to 7) post-mortem at 70 weeks of age via visual assessment of excised keels.

	Number of Fractures							
	0	1	2	3	4	5	6	7
Treatment								
CBM	11%	33%	33%	11%	6%	6%	0%	0%
CBR	28%	28%	11%	16%	11%	0%	0%	6%
CWM	55%	28%	11%	6%	0%	0%	0%	0%
CWR	39%	28%	16%	11%	6%	0%	0%	0%
FBM	16%	39%	22%	6%	0%	11%	6%	0%
FBR	22%	17%	11%	28%	16%	6%	0%	0%
FWM	55%	27%	6%	6%	6%	0%	0%	0%
FWR	33%	33%	17%	11%	6%	0%	0%	0%

Abbreviations: **C,** cage. **F,** floor pen. **B,** brown. **W,** white. **M,** mushroom. **R,** round.

Table 4.5. Pearson correlation coefficients of bone parameters measured postmortem at 70 weeks of age.

	Load	**Extension**	**KBMD**	**KBMC**	**FBMD**	**FBMC**	**Number**	**Severity**	**Total**
Load	1.00	-0.40 <.001	0.36 <.001	0.45 <.001	0.75 <.001	0.75 <.001	-0.12 0.10	-0.03 0.10	-0.22 0.01
Extension	-0.40 <.001	1.00	-0.12 0.10	-0.21 0.01	-0.45 <.001	-0.49 <.001	0.03 0.10	0.04 0.10	0.27 0.01
KBMD	0.36 <.001	-0.12 0.10	1.00	0.82 <.001	0.45 <.001	0.44 <.001	0.21 0.01	0.22 0.01	0.15 0.07
KBMC	0.45 <.001	-0.21 0.01	0.82 <.001	1.00	0.57 <.001	0.58 <.001	0.14 0.10	0.19 0.05	0.12 0.10
FBMD	0.75 <.001	-0.45 <.001	0.45 <.001	0.57 <.001	1.00	0.90 <.001	-0.01 0.10	0.04 0.10	-0.09 0.10
FBMC	0.75 <.001	-0.49 <.001	0.44 <.001	0.58 <.001	0.90 <.001	1.00	-0.02 0.10	0.06 0.10	-0.07 0.10
Number	-0.12 0.10	0.03 0.10	0.21 0.01	0.14 0.10	-0.01 0.10	-0.02 0.10	1.00	0.60 <.0001	0.41 <.0001
Severity	-0.03 0.10	0.04 0.10	0.22 0.01	0.19 0.05	0.04 0.10	0.06 0.51	0.60 0.001	1.00	0.32 0.001
Total	-0.22 0.01	0.28 0.01	0.15 0.07	0.116 0.10	-0.09 0.10	-0.07 0.10	0.41 0.001	0.32 0.001	1.00

Abbreviation: **Load**, total compressive load at break. **Extension**, maximum extension at break. **KBMD**, keel bone mineral density. **FBMD**, femur bone mineral density. **KBMC**, keel bone mineral content. **FBMC**, femur bone mineral content. **Number**, number of keel deviations. **Severity**, severity of keel deviations. **Total**, number of keel fractures.

Table 4.6. LSMeans (± SEM) of bone mineralization and strength of two laying hen strains measured postmortem at 70 weeks of age.

	Load (kgf)	**Extension (mm)**	**KBMD (g/cm^2)**	**KBMC (g)**	**FBMD (g/cm^2)**	**FBMC (g)**
Strain						
Lohmann Brown	20.03±0.625^a	11.60 ± 0.304^b	0.10±0.003^a	2.08±0.084^a	0.23±0.006^a	3.50±0.094^a
Lohmann White	16.32±0.506^b	12.50± 0.325^a	0.07±0.002^b	1.19±0.049^b	0.18±0.004^b	2.6±0.069^b
P value	<0.001	<0.05	<0.001	<0.001	<0.001	<0.001

Abbreviation: **KBMD**, keel bone mineral density. **FBMD**, femur bone mineral density. **KBMC**, keel bone mineral content. **FBMC**, femur bone mineral content.

$^{a\text{-}b}$ Values within columns with different letters are significantly different ($P<0.05$).

Table 4.7. Odds ratio of pullets perching and standing on perch in floor pens from seven to 17 weeks of age. The value in the odds ratio column indicates the association between a treatment and a behaviour occurring. For example, at 7 weeks of age birds in the morning were 3.56 times more likely to be perching on a perch than birds in the afternoon.

Perching on Perch					**Standing on Perch**				
Effect	**Odds ratio**	**95% Confidence Limits**		**Pr > F**	**Effect**	**Estimate**	**95% Confidence Limits**		**Pr > F**
Age=7					**Age=7**				
A vs P	3.56	1.72	7.37	0.001	A vs P	2.98	1.41	6.31	0.01
B vs W	0.01	0.00	0.02	0.001	B vs W	0.18	0.08	0.40	0.001
M vs R	0.69	0.34	1.41	0.10	M vs R	0.24	0.11	0.52	0.001
Age=9					**Age=9**				
A vs P	5.05	2.40	10.63	0.001	A vs P	0.42	0.19	0.93	0.05
B vs W	0.01	0.00	0.03	0.001	B vs W	0.08	0.03	0.22	0.001
M vs R	0.48	0.24	0.96	0.05	M vs R	0.25	0.11	0.57	0.001
Age=11					**Age=11**				
A vs P	12.04	5.35	27.10	0.001	A vs P	0.82	0.43	1.56	0.10
B vs W	0.00	0.00	0.01	0.001	B vs W	0.13	0.06	0.26	0.001
M vs R	1.07	0.55	2.07	0.10	M vs R	0.21	0.11	0.42	0.001
Age=13					**Age=13**				
A vs P	3.85	1.82	8.14	0.001	A vs P	0.47	0.25	0.86	0.05
B vs W	0.08	0.03	0.19	0.001	B vs W	0.36	0.19	0.66	0.001
M vs R	0.46	0.23	0.94	0.05	M vs R	0.24	0.13	0.46	0.001
Age=15					**Age=15**				
A vs P	1.97	0.99	3.94	0.06	A vs P	0.79	0.42	1.49	0.10
B vs W	0.11	0.05	0.25	0.001	B vs W	0.28	0.15	0.54	0.001
M vs R	0.55	0.27	1.10	0.09	M vs R	0.35	0.18	0.67	0.01
Age=17					**Age=17**				
A vs P	5.68	2.55	12.64	0.001	A vs P	1.71	0.91	3.23	0.10
B vs W	0.06	0.03	0.16	0.001	B vs W	0.09	0.04	0.18	0.001
M vs R	0.24	0.11	0.53	0.001	M vs R	0.27	0.14	0.52	0.001

Abbreviation: **A vs P**, AM vs PM. **B vs W**, Lohmann Brown vs Lohmann White strain. **M vs R**, mushroom perch vs round perch.

Age is measured in weeks.

Table 4.8. Odds ratio of pullets perching and standing on perch in pullet cages from seven to 17 weeks of age. The value in the odds ratio column indicates the association between a treatment and a behaviour occurring. For example, at age 7 birds in the morning were 1.02 times more likely to be perching on a perch than birds in the afternoon.

Perching on Perch					Standing on Perch				
Effect	**Odds ratio**	**95% Confidence Limits**		**Pr > F**	**Effect**	**Estimate**	**95% Confidence Limits**		**Pr > F**
Age=7					**Age=7**				
A vs P	1.02	0.79	1.32	0.10	A vs P	1.22	0.92	1.62	0.10
B vs W	0.36	0.28	0.46	0.001	B vs W	0.73	0.55	0.97	0.05
M vs R	0.63	0.49	0.82	0.01	M vs R	0.53	0.40	0.70	0.001
Age=9					**Age=9**				
A vs P	1.42	1.06	1.89	0.10	A vs P	0.76	0.56	1.02	0.07
B vs W	1.28	0.96	1.71	0.001	B vs W	0.72	0.53	0.97	0.05
M vs R	0.50	0.37	0.67	0.01	M vs R	0.47	0.35	0.63	0.001
Age=11					**Age=11**				
A vs P	2.33	1.80	3.03	0.001	A vs P	0.62	0.47	0.81	0.01
B vs W	3.93	3.00	5.14	0.001	B vs W	0.93	0.71	1.22	0.10
M vs R	0.77	0.60	1.00	0.05	M vs R	0.28	0.21	0.37	0.001
Age=13					**Age=13**				
A vs P	5.99	4.23	8.49	0.001	A vs P	0.88	0.67	1.15	0.10
B vs W	12.42	8.49	18.17	0.001	B vs W	1.50	1.15	1.96	0.01
M vs R	0.94	0.68	1.29	0.10	M vs R	0.30	0.23	0.40	0.001
Age=15					**Age=15**				
A vs P	2.46	1.75	3.46	0.001	A vs P	0.74	0.55	0.99	0.05
B vs W	16.15	10.25	25.46	0.001	B vs W	2.26	1.67	3.06	0.001
M vs R	0.70	0.50	0.97	0.05	M vs R	0.28	0.21	0.39	0.001
Age=17					**Age=17**				
A vs P	2.76	2.04	3.72	0.001	A vs P	1.37	1.07	1.76	0.05
B vs W	8.12	5.84	11.29	0.001	B vs W	1.54	1.20	1.98	0.001
M vs R	1.30	0.97	1.75	0.08	M vs R	0.62	0.48	0.80	0.001

Abbreviation: **A vs P**, AM vs PM. **B vs W**, brown strain vs white strain. **M vs R**, mushroom perch vs round perch.

Age is measured in weeks.

Table 4.9. Odds ratio of pullets perching on perch in the lay phase from 19 to 70 weeks of age. The value in the odds ratio column indicates the association between a treatment and a behaviour occurring. For example, at age 19 birds housed in cages were 1.47 times more likely to be perching on a perch than birds housed in the afternoon.

Effect	Estimate	95% Confidence Limits		Pr > F	Effect	Estimate	95% Confidence Limits		Pr > F
Age=19					**Age=45**				
A vs P	0.21	0.16	0.29	0.001	A vs P	0.82	0.66	1.02	0.08
C vs F	1.47	1.11	1.94	0.01	C vs F	1.19	0.96	1.48	0.001
B vs W	6.90	4.93	9.66	0.001	B vs W	4.23	3.37	5.32	0.001
M vs R	1.07	0.81	1.41	0.10	M vs R	1.45	1.17	1.80	0.01
Age=21					**Age=47**				
A vs P	0.20	0.14	0.27	0.001	A vs P	0.28	0.22	0.35	0.001
C vs F	1.32	1.00	1.74	0.05	C vs F	1.27	1.01	1.58	0.05
B vs W	2.17	1.63	2.88	0.001	B vs W	5.37	4.21	6.83	0.001
M vs R	1.01	0.77	1.33	0.10	M vs R	0.98	0.78	1.22	0.10
Age=23					**Age=49**				
A vs P	0.36	0.23	0.56	0.001	A vs P	0.36	0.29	0.44	0.001
C vs F	1.76	1.17	2.69	0.01	C vs F	1.02	0.83	1.25	0.10
B vs W	1.63	1.09	2.45	0.05	B vs W	3.30	2.67	4.09	0.001
M vs R	1.07	0.72	1.60	0.10	M vs R	1.29	1.05	1.59	0.05
Age=25					**Age=51**				
A vs P	0.04	0.02	0.08	0.001	A vs P	0.47	0.38	0.58	0.001
C vs F	1.22	0.87	1.71	0.10	C vs F	0.87	0.71	1.07	0.10
B vs W	2.37	1.67	3.35	0.001	B vs W	4.08	3.27	5.08	0.001
M vs R	0.86	0.62	1.20	0.10	M vs R	1.09	0.89	1.35	0.10
Age=27					**Age=53**				
A vs P	0.11	0.07	0.18	0.001	A vs P	0.26	0.21	0.33	0.001
C vs F	1.67	1.17	2.37	0.01	C vs F	1.24	0.99	1.55	0.06
B vs W	2.79	1.93	4.04	0.001	B vs W	5.52	4.32	7.05	0.001
M vs R	0.71	0.50	1.00	0.05	M vs R	1.33	1.06	1.67	0.05
Age=29					**Age=55**				
A vs P	0.02	0.01	0.03	0.001	A vs P	0.39	0.31	0.48	0.001
C vs F	1.56	1.19	2.05	0.01	C vs F	1.24	1.00	1.52	0.05
B vs W	3.20	2.42	4.23	0.001	B vs W	3.88	3.11	4.83	0.10
M vs R	0.87	0.67	1.14	0.10	M vs R	1.17	0.95	1.45	0.001
Age=31					**Age=57**				
A vs P	0.05	0.03	0.07	0.001	A vs P	0.46	0.38	0.57	0.001
C vs F	1.27	0.98	1.66	0.08	C vs F	0.88	0.72	1.07	0.10
B vs W	2.48	1.89	3.25	0.001	B vs W	4.42	3.58	5.45	0.001
M vs R	0.71	0.55	0.93	0.05	M vs R	1.30	1.06	1.58	0.05
Age=33					**Age=59**				

A vs P	0.23	0.18	0.30	0.001	A vs P	0.15	0.12	0.19	0.001
C vs F	1.79	1.43	2.25	0.001	C vs F	1.11	0.89	1.38	0.1
B vs W	2.77	2.20	3.48	0.001	B vs W	3.55	2.81	4.48	0.001
M vs R	0.88	0.70	1.10	0.10	M vs R	1.23	1.00	1.51	0.05
Age=35					**Age=61**				
A vs P	0.21	0.16	0.26	0.001	A vs P	0.35	0.28	0.43	0.001
C vs F	1.38	1.10	1.73	0.01	C vs F	1.08	0.88	1.32	0.1
B vs W	3.81	3.00	4.84	0.001	B vs W	3.87	3.13	4.79	0.001
M vs R	0.93	0.74	1.16	0.10	M vs R	1.23	1.00	1.51	0.05
Age=37					**Age=63**				
A vs P	0.16	0.11	0.22	0.001	A vs P	0.56	0.45	0.69	0.001
C vs F	1.84	1.40	2.43	0.001	C vs F	1.13	0.91	1.39	0.1
B vs W	3.93	2.92	5.28	0.001	B vs W	4.00	3.21	4.99	0.001
M vs R	0.93	0.71	1.22	0.10	M vs R	1.27	1.03	1.57	0.05
Age=39					**Age=65**				
A vs P	0.31	0.25	0.40	0.001	A vs P	0.49	0.40	0.60	0.001
C vs F	1.33	1.06	1.67	0.05	C vs F	1.13	0.92	1.39	0.1
B vs W	3.24	2.56	4.11	0.001	B vs W	4.95	3.96	6.19	0.001
M vs R	1.16	0.92	1.45	0.10	M vs R	1.41	1.14	1.74	0.01
Age=41					**Age=67**				
A vs P	0.50	0.40	0.63	0.001	A vs P	0.16	0.12	0.20	0.001
C vs F	1.44	1.15	1.80	0.01	C vs F	1.46	1.15	1.84	0.01
B vs W	3.21	2.54	4.07	0.001	B vs W	3.28	2.57	4.20	0.001
M vs R	1.07	0.86	1.34	0.10	M vs R	1.32	1.05	1.67	0.05
Age=43					**Age=69**				
A vs P	0.29	0.23	0.37	0.001	A vs P	0.43	0.34	0.53	0.001
C vs F	1.58	1.25	1.98	0.001	C vs F	1.03	0.83	1.29	0.10
B vs W	5.23	4.07	6.72	0.001	B vs W	4.76	3.74	6.06	0.001
M vs R	0.83	0.66	1.04	0.10	M vs R	1.26	1.01	1.58	0.05

Abbreviation: **A vs P**, AM vs PM. **C vs F**, pullet cage vs floor pen. **B vs W**, brown strain vs white strain. **M vs R**, mushroom perch vs round perch.

Age is measured in weeks.

Table 4.10. Odds ratio of pullets standing on perch in the lay phase from 19 to 70 weeks of age. The value in the odds ratio column indicates the association between a treatment and a behaviour occurring. For example, at age 19 brown were 1.22 times more likely to be perching on a perch than white birds.

Effect	Estimate	95% Confidence Limits		Pr > F	Effect	Estimate	95% Confidence Limits		Pr > F
Age=19					**Age=45**				
A vs P	0.91	0.77	1.09	0.10	A vs P	0.93	0.78	1.10	0.10
C vs F	0.77	0.64	0.92	0.01	C vs F	0.86	0.72	1.02	0.09
B vs W	1.22	1.02	1.46	0.05	B vs W	1.11	0.93	1.32	0.10
M vs R	0.22	0.19	0.27	0.001	M vs R	0.40	0.34	0.48	0.001
Age=21					**Age=47**				
A vs P	0.72	0.60	0.86	0.001	A vs P	0.69	0.58	0.82	0.001
C vs F	0.80	0.67	0.96	0.05	C vs F	0.83	0.70	0.99	0.05
B vs W	1.23	1.02	1.47	0.05	B vs W	0.85	0.72	1.02	0.08
M vs R	0.22	0.18	0.27	0.001	M vs R	0.48	0.40	0.57	0.001
Age=23					**Age=49**				
A vs P	0.71	0.59	0.84	0.001	A vs P	1.19	1.00	1.42	0.05
C vs F	0.91	0.76	1.09	0.10	C vs F	0.91	0.77	1.08	0.10
B vs W	1.41	1.18	1.69	0.001	B vs W	0.92	0.77	1.09	0.10
M vs R	0.27	0.23	0.33	0.001	M vs R	0.41	0.34	0.49	0.001
Age=25					**Age=51**				
A vs P	0.63	0.53	0.75	0.001	A vs P	1.18	0.99	1.41	0.06
C vs F	0.83	0.70	0.99	0.05	C vs F	0.81	0.68	0.96	0.05
B vs W	1.03	0.87	1.23	0.10	B vs W	1.08	0.91	1.28	0.10
M vs R	0.20	0.16	0.24	0.001	M vs R	0.38	0.32	0.46	0.001
Age=27					**Age=53**				
A vs P	0.74	0.62	0.88	0.001	A vs P	0.88	0.74	1.04	0.10
C vs F	1.12	0.94	1.34	0.10	C vs F	0.83	0.69	0.98	0.05
B vs W	0.70	0.59	0.83	0.001	B vs W	0.81	0.68	0.97	0.05
M vs R	0.22	0.19	0.27	0.001	M vs R	0.41	0.35	0.50	0.001
Age=29					**Age=55**				
A vs P	0.67	0.56	0.80	0.001	A vs P	0.99	0.83	1.17	0.10
C vs F	0.97	0.81	1.16	0.10	C vs F	0.82	0.69	0.97	0.05
B vs W	0.59	0.50	0.71	0.001	B vs W	1.01	0.85	1.19	0.10
M vs R	0.28	0.23	0.34	0.001	M vs R	0.34	0.29	0.41	0.001
Age=31					**Age=57**				
A vs P	0.87	0.73	1.04	0.10	A vs P	0.79	0.66	0.94	0.01
C vs F	1.00	0.84	1.19	0.10	C vs F	0.79	0.67	0.94	0.01
B vs W	0.58	0.49	0.69	0.001	B vs W	1.10	0.92	1.30	0.10
M vs R	0.25	0.21	0.30	0.001	M vs R	0.42	0.35	0.50	0.001

Age=33					**Age=59**				
A vs P	0.68	0.57	0.81	0.001	A vs P	0.90	0.76	1.07	0.10
C vs F	0.98	0.82	1.17	0.10	C vs F	0.86	0.73	1.03	0.10
B vs W	0.74	0.62	0.89	0.001	B vs W	1.00	0.84	1.19	0.10
M vs R	0.46	0.39	0.56	0.001	M vs R	0.42	0.35	0.50	0.001
Age=35					**Age=61**				
A vs P	0.59	0.50	0.70	0.001	A vs P	1.12	0.94	1.34	0.10
C vs F	1.07	0.90	1.28	0.10	C vs F	0.83	0.70	0.99	0.05
B vs W	0.86	0.72	1.02	0.08	B vs W	0.99	0.83	1.18	0.10
M vs R	0.39	0.32	0.46	0.001	M vs R	0.31	0.26	0.37	0.001
Age=37					**Age=63**				
A vs P	0.67	0.56	0.79	0.001	A vs P	0.86	0.72	1.02	0.08
C vs F	0.90	0.76	1.07	0.10	C vs F	0.96	0.80	1.14	0.10
B vs W	1.04	0.87	1.24	0.10	B vs W	1.15	0.96	1.37	0.10
M vs R	0.28	0.23	0.34	0.001	M vs R	0.44	0.37	0.52	0.001
Age=39					**Age=65**				
A vs P	0.81	0.68	0.96	0.05	A vs P	1.15	0.97	1.37	0.10
C vs F	0.82	0.69	0.98	0.05	C vs F	0.86	0.72	1.02	0.09
B vs W	0.95	0.80	1.13	0.10	B vs W	1.25	1.05	1.48	0.05
M vs R	0.36	0.30	0.43	0.001	M vs R	0.40	0.34	0.48	0.001
Age=41					**Age=67**				
A vs P	0.93	0.78	1.10	0.10	A vs P	0.88	0.74	1.05	0.10
C vs F	0.90	0.76	1.08	0.10	C vs F	0.96	0.81	1.15	0.10
B vs W	0.95	0.80	1.13	0.10	B vs W	1.32	1.11	1.57	0.01
M vs R	0.49	0.41	0.59	0.001	M vs R	0.32	0.27	0.38	0.001
Age=43					**Age=69**				
A vs P	1.04	0.88	1.24	0.10	A vs P	0.65	0.55	0.78	0.001
C vs F	0.83	0.70	0.99	0.05	C vs F	1.09	0.91	1.29	0.10
B vs W	1.07	0.90	1.27	0.10	B vs W	1.14	0.96	1.36	0.10
M vs R	0.41	0.34	0.49	0.001	M vs R	0.36	0.30	0.42	0.001

Abbreviation: **A vs P**, AM vs PM. **C vs F**, pullet cage vs floor pen. **B vs W**, brown strain vs white strain. **M vs R**, mushroom perch vs round perch.

Age is measured in weeks.

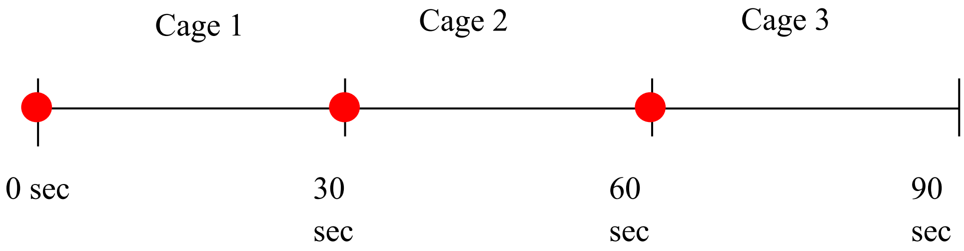

Figure 4.1. Pictorial depiction of sampling methodology used during behaviour observations. Perching and standing on perch or other behaviours were recorded using instantaneous scan sampling as indicated by the dots on the timeline. Each dot represents a sample point, between each dot during the 30 sec sample interval one-zero sampling occurred. One-zero sampling was observed as a discrete yes/no for displacement and squabble behaviours.

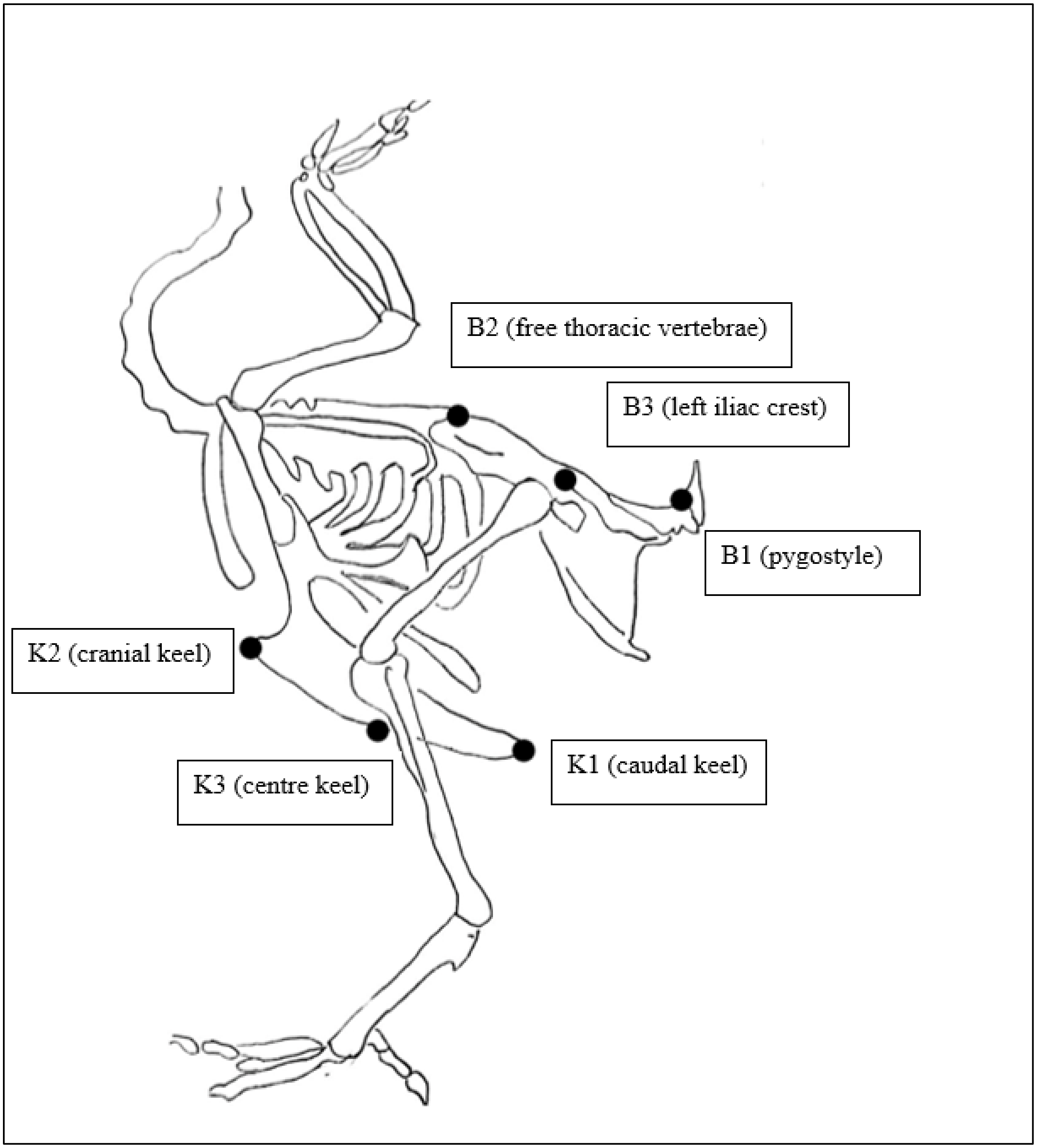

Figure 4.2. Image depicting the placement of kinematic markers on key skeletal landmarks, for the purpose of evaluating perching biomechanics using 3-D kinematic recordings. Marker B4 is located on the right iliac crest which is not visible in this figure.

5. General Discussion

5.1. Introduction

As the egg laying industry in Canada transitions away from conventional battery cages (Leenstra et al., 2016), and towards alternative systems (furnished cages, aviaries, free-run, and free-range) keel bone damage (**KBD**) has become an increasingly prevalent welfare and economic issue impacting the egg laying industry. Historically, laying hens have been bred for high production levels (Muir et al., 2018) and raised in conventional housing that limits the expression of natural behaviours (Lay et al., 2011). In alternative housing systems, the incidence of KBD is 13 to 63 % than in conventional housing systems (Wilkins et al., 2011; Petrik et al., 2015; Regmi et al., 2016; Saraiva et al., 2019). Keel bone damage encompasses both deviations and fractures. Deviations are thought to be caused by pressure exerted on the keel (Stratmann et al., 2015) while perching. Deviations may also contribute to fracture susceptibility in laying hens (Harlander-Matauschek et al., 2015). Fractures have notable negative impacts on laying hens, including negative affective states due to pain (Nasr et al., 2012b; Armstrong et al., 2020; Wei et al., 2022), reduced mobility (Nasr et al., 2012a; Wei et al., 2020), and decreased egg production (Nasr et al., 2012a). The shift towards alternative housing has highlighted the need for industry members, including producers and genetics companies, to consider whether some stains are suited to certain housing environments. When aiming to reduce the incidence of KBD in a flock, factors such as strain and housing conditions must be evaluated to identify which strains are best suited for furnished cages.

Many genetic and environmental factors can contribute to KBD, including collision interactions with furnishings (Habig et al., 2021), bone strength (Stratmann et al., 2016; Candelotto et al., 2017), pressure exerted on the keel while perching (Stratmann et al., 2015), rearing environment (Casey-Trott et al., 2017b; Casey-Trott et al., 2017c), and perch design elements such as hardness (Stratmann et al., 2015) and shape (Pickel et al., 2011). In addition, different strains of laying hen have different bone strengths (Habig et al., 2021), perching behaviours (LeBlanc et al., 2018a), body weights (Chew et al., 2018), and egg productions (Candelotto et al., 2017: Habig et al., 2021) which all may play a role in the incidence of KBD in a flock. Ideally, we want to see incidences of KBD below 20% in a flock, which is on par or lower than the incidence we see in conventional cages (Petrik et al., 2015; Regmi et al., 2016). To lower KBD, interactions between genetic factors, such as laying hen strain, and environmental factors, such as rearing environment, must be investigated to identify how KBD can be reduced in furnished cages.

The purpose of this thesis was: firstly, to determine how strain differences between random-bred and commercial strains of laying hen impact perching biomechanics, bone parameters, and pressure exerted on the footpad while perching. Secondly, to determine which ideal perching biomechanics lead to stability and reduced KBD while perching, and which perch shape, strain, and rearing environment contribute to an ideal perching behaviour using 3D kinematics software. Thirdly, to identify SNPs of interest on candidate genes pertaining to bone health parameters and determine if specific causative mutations impact bone health parameters and reduce KBD. Finally, to determine how rearing environment impacts bone quality and KBD in enriched housed laying hens and how perch shape and usage impacts perching biomechanics and KBD throughout the life cycle of a laying hen. The following sections will explore each objective and discuss future work and applications of the research conducted.

5.2. Random-bred and commercial strain differences

The previously reported relationship between lower egg production, bone health, and lower incidences of KBD, indicates that random-bred strains would likely have higher bone health parameters and lower incidences of KBD (Candelotto et al., 2017; Eusemann et al., 2020; Habig et al., 2021) due to lower levels of egg production. In Chapter 2, White Leghorn had the lowest prevalence of severe KBD when compared to commercial white birds and Rhode Island Red had the lowest prevalence of severe KBD when compared to commercial brown birds. From historical data collected at the University of Alberta Poultry unit, the random-bred strains have lower egg production in contrast to the commercial strains used by 10-16% (K. Nadeau, unpublished data; Lohmann Tierzucht. 2019; Hendrix Genetics, 2023a; Hendrix Genetics 2023b). In part, variation seen in KBD and bone parameters between random-bred and heritage strains may be explained by the substantial differences in egg production levels. In both Chapter 2 and 4, brown strains had heavier, denser, and stronger bones compared to white strains. Brown strains have been previously identified in the literature as having significantly stronger and denser bones than white strains (Fawcett et al., 2020, Habig et al., 2021) which was supported by our results. Brown strains weighed significantly more than white strains in both Chapter 2 and 4, which aligns with previous research (Chew et al., 2021; Riczu et al., 2004). In Chapter 2, Rhode Island Red and ISA Brown had the least severe KBD and the densest bones in contrast to commercial white strains (Shaver White and Lohmann White) which had significantly lower bone density and more severe KBD. Notably, ISA Brown had the lowest level of severe KBD and higher bone parameters in contrast to other commercial strains. Results in Chapter 2 indicated that higher bone parameters contributed to lower levels of KBD. In contrast, Chapter 4 results indicated that despite having higher bone mineralization and bone breaking strength, brown birds had more fractures and severe deviations. The inclusion of perches in Chapter 4 likely negate any protective effects of increased bone mineralization seen in Chapter 2 (Hester et al., 2013). In brown strains, the higher incidence of KBD could be explained by larger bodies which leads to increased pressure exerted on the keel when laying hens are perching (Campbell, 2021). Additionally, the keels of brown birds ossify at a slower rate in comparison to white birds, making the keel more susceptible to deviations and fractures (Fawcett et al., 2020). Overall, brown strains were heavier and had higher bone parameter scores than white strains, but the impacts on KBD varied between both trials indicating that although bone strength may contribute to KBD, other environmental factors including perch shape likely play a major role.

Overall, commercial and random-bred strains had fundamentally different patterns of movement while perching. Commercial strains moved up and down more than random-bred strains. In contrast, random-bred strains moved forward and backward more than commercial strains. Forward and backward movements while perching may be reflective of more natural perching behaviors as random bred strains have not undergone the same extensive selection process as commercial strains that did not prioritize perching behaviors (Fernyhough et al., 2019). Due to selective breeding and artificial selection, commercial laying hens may not be as well-equipped to exhibit ideal perching behaviours in contrast to their random-bred counterparts (Leenstra et al., 2016). White strains across speed metrics had faster movements in both trials. A potential explanation is when paired with the manual restraint required during marker placement for recordings, temperament could have contributed to the faster movements exhibited by LW. Although temperament was not assessed in this thesis, white strains have been reported to be flightier and more fearful (Uitdehaag et al., 2008; Uitdehaag et al., 2011; Habig et al., 2021). In addition, white strains were lighter than brown strains, making it easier for white birds to move more quickly while on the perch. Quicker movements may be indicators of instability while perching and in turn increase the risk of KBD. Instability could contribute to birds spending more time resting on the perch to balance, which was reflected in Chapter 2, where back speed was negatively correlated with standing duration; the faster a hen moves the less time is spent standing. Increased time spent resting on the perch could equate to more pressure on the keel and in turn more severe deviations (Stratmann et al., 2016). Behaviour is a highly polygenic trait (Chabris et al., 2015), as such utilizing genetic selection to control unwanted perching behaviour is unrealistic. Focusing on how environmental factors such as perch shape, rearing, and lay environment impact perching behaviour will be more effective in encouraging perching behaviours that do not contribute to KBD.

In Chapter 2 infrared thermography imaging was conducted to identify pressure exerted on the feet while perching. No effect of perch shape on footpad temperature was found. However, there was a significant effect of strain at each stage of lay. Based on Campbell (2021), larger bodies exert more pressure on the keel while perching, indicating that brown strains are expected to have consistently warmer footpad temperatures than white strains. There was no distinct pattern of all white strains having colder feet than all brown strains. Potentially, the time that birds were placed onto the perch in Chapter 2 (30 seconds) was not enough to lead to a change in the thermal profile of the foot. In addition, body weight was not correlated with footpad temperature, indicating that differences in footpad temperature may not be the result of pressure exerted on the keel while perching.

Based on the results of Chapter 2, infrared thermography was not used in Chapter 4. Despite results indicating that infrared thermography might not be an effective tool in assessing footpad pressure, it should not be discounted for future poultry research. Another physiological process that infrared thermography may be inadvertently measuring is egg laying. Laying hens in lay have significantly higher blood flow than birds not in-lay (Hu et al., 2019). Differences in blood flow can be captured by IRT, specifically through convection heat transfer (Perez Marquez et al., 2019). In this study, before imaging, birds were not palpated to determine if they were going to lay an egg, which inhibits our ability to determine if temperature differences between strains were due to egg laying.

5.3. Ideal perching biomechanics

A novel aspect of this thesis was the investigation of perching biomechanics and identifying an ideal set of perching behaviours that lead to reduced KBD. In Chapter 2, differences in perching biomechanics when comparing six strains at three stages of lay were assessed. Three movement patterns were measured: up and down, side-to-side, and forward and backward. Up and down keel movements reflect repetitive small scale “collisions” between the perch and keel which may contribute to the development of deviations (Stratmann et al., 2016). Side-to-side movement is used as an instability indicator when laying hens are accessing perches (Scholz et al., 2014). Forward and backward “rocking” movements potentially exacerbate force exerted on the keel while perching over extended periods of time contributing to deviations (Harlander-Matauschek et al., 2015). Slower and fewer movements while perching was considered ideal as a reflection of increased stability and a reduced likelihood of KBD in this thesis. Contradictorily, fewer, and slower movements could also be indicative of KBD as a reduction in locomotion and an increase in resting behaviours are both symptoms of KBD (Nasr et al., 2012; Wei et al., 2020).

In Chapter 2, KBD scores were conducted post-mortem, which limited our ability to assess relationships between perching biomechanics and KBD. Additionally, laying hens were not housed with perches in Chapter 2, which means KBD was not caused by perching on a perch. Potentially, the presence of deviations may have been from other features in the environment including nest boxes and hanging feeders, on which it was noted laying hens would rest. In Chapter 2 the only perching metric that was correlated with KBD was up and down movement. Up and down movement was correlated with both the number of deviations present and the severity of those deviations. Although not directly measured, KBD may lead to increased up and down movements due to pain when the keel contacts with the perch. In Chapter 4, KBD was assessed alongside each kinematic recording, allowing for correlations between perching biomechanics and incidence of KBD to be made. Increased forward and backward keel motion was correlated with keel bone damage severity. When perching, regardless of perch shape, hens exert twice the force (N/cm^2) on the keel bone compared to the feet (Pickel et al., 2011). As such, our results indicated forward and backward movements while the keel is in contact with the perch contributed to KBD severity. From our results, fewer up and down and forward and backward movements were considered a part of the ideal perching phenotype to reduce KBD.

Contrary to our initial assumptions, factors such as side-to-side movement and speed did not play a role in contributing to KBD. Speed may be more accurate in assessing the incidence of fractures and not deviations. However, due to limitations with physical palpation, it is likely not all fractures were accurately captured in contrast to deviations as the dorsal side of the keel could not be palpated. In Chapter 4, speed metrics were not correlated with the incidence of deviations which aligns with the theory that chronic continuous pressure exerted on the keel leads to deviations (Stratmann et al., 2016).

5.4. Causative mutations

A literature search provided information on potential candidate genes that are reported to impact bone parameters. This thesis hypothesized that candidate genes associated with bone health could contribute to the incidence of KBD in a flock. As such, based on a selection protocol that utilized VEP, SIFT, CADDand BLAST scores, seven SNPs were selected for analysis from six candidate genes selected. *SOX9* was the only candidate gene that did not have a SNP segregate. rs740376108 located on *PTHLH*, a gene that regulates the differentiation of prehypertrophic chondrocytes into hypertrophic chondrocytes and is strongly influenced by artificial selection (Dale et al., 2015), segregated. rs317151697 located in *RB1*, a gene that controls body growth and osteogenic differentiation in chickens (Li et al., 2021; Zhang et al., 2011) and inhibits bone formation and remodeling in mice (Li et al., 2022), segregated. rs15490317 located on *RANKL,* a gene that plays a role in bone mineral content due to its involvement in osteoclast differentiation and activation (Guo et al., 2017; Dale et al., 2015), segregated. rs314956063 located on *SOST*, a gene that regulates bone homeostasis through the Wnt/β-catenin signaling pathway (Jiao et al., 2023), and when suppressed facilitates bone formation and helps prevent fractures (Guo et al., 2017; Li et al., 2021), segregated. Finally, rs13975174, located on *POSTN*, a gene that impacts bone weight due to its role in the regulation of osteoblast proliferation and bone formation (Guo et al., 2017; Li et al., 2021), segregated.

There were notable differences between genotype frequency when comparing pooled white vs brown strains genotyping assay results. *POSTN*, *RANKL*, and *RB1* were notably different when comparing white and brown strains, demonstrated by significant differences in allele frequency. Strains with higher frequencies of allele C on *RB1* had higher bone parameter measures. Notably, White Leghorn and Rhode Island Red had the highest bone parameters and in turn had the highest C allele frequency in comparison to white and brown commercial strains respectively. The differences between the groupings may be due to reduced genetic diversity in laying hen populations (Muir et al., 2018) and shorter generation intervals (Baes et al., 2019). Results in Chapter 2 and 4 highlighted that brown strains had stronger and denser bones compared to white strains, which pointed to differences in genetics between the two groups. In addition, SNPs in *POSTN, RANKL, RB1*, and *OVALX* were reported to be associated with phenotypic variance in bone parameters measured. However, results were confounded by strain accounting for a significant amount of variation reported in bone parameters measured. In this study, no one strain was responsible for the significant variation in bone parameters, and due to the small sample size used (n=20/treatment) and low number of SNPs assessed per gene (1-3), we cannot say with confidence that the SNP effects reported in this study were significant and influenced the phenotypes. Future research may aim to further investigate potential causative mutations located on *POSTN*, *RANKL*, *RB1*, and *OVALX* based on our preliminary results.

5.5. Rearing environment and perch shape

As previous research has explored the beneficial effects of load-bearing exercise during rearing on the incidence of KBD in laying hens (Hester et al., 2013; Casey-Trott et al., 2017a) it was surprising to note that rearing environment did not have a significant impact on the bone parameters measured or KBD scores. If there were beneficial effects on bone mineralization and strength from the additional space provided by floor pens during rearing, those effects had not persisted until end of lay. As noted in the discussion of Chapter 4, there are a few possible explanations for our results contrasting with previous research. Both rearing environment treatment groups were provided with perches, which has been shown to encourage the expression of wing-flapping and jumping, contributing to greater bone mineral content and breaking strength (Jendral et al., 2008; Tactacan et al., 2009; Casey-Trott et al., 2017b). Additionally, the benefits of floor pens may have only been present during the start of lay whereas DXA scans were only conducted post-mortem. The caudal tip of the keel does not fully ossify until well into lay (28 to 40 weeks of age; Buckner et al., 1948; Buckner et al., 1949), making it susceptible to deviations prior to 28 weeks of age. Generally, laying hens housed in cages exhibited more perching behaviour than laying hens housed in floor pens, potentially due to the similarities between their rearing and lay environments which provided increased familiarity with their environment and supported the expression of perching behaviours. Alternatively, floor pens likely provided opportunities for laying hens to perform a variety of additional behaviors such as dust bathing that cages did not. As such, laying hens had fewer activities to engage in when in cages leading to increased time spent perching and had to cross over perches to move around the cage.

Round and mushroom perches were selected based on their current availability to producers and use on commercial egg farms. Mushroom perches are wider than round perches and more evenly distribute the force exerted on the keel. Based on our initial ideal perching phenotype, reduced pressure exerted on the keel would reduce the incidence of deviations (Pickel et al., 2011). Thus, it was expected that the wider mushroom perch would lead to less KBD. Overall, mushroom perches led to less severe deviations and were used more by laying hens than round perches. Chen et al. (2014) notes that laying hens prefer wider perches over round perches due to the wider gripping surface provided by rectangular or mushroom shaped perches, which may explain the increased mushroom perch use seen in Chapter 4. Perch shape affected movement and movement speed, with laying hens on mushroom perches exhibiting more movement in contrast to laying hens on round perches exhibiting faster movements. Laying hens moved faster on round perches compared to mushroom perches. However, movement speed was not correlated with KBD severity in Chapter 2 or 4, indicating the directionality and degree of movement is a more likely contributor to KBD than movement speed. Based on our results, mushroom perches are preferable for use in alternative housing due to their association with reduced KBD, increased stability while perching, and increased use by laying hens.

5.6. Future Research

The research discussed in this thesis lays important groundwork for future work investigating how various environmental and genetic factors contribute to KBD in laying hens. By measuring additional metrics in association with 3-D kinematic recordings, the associations between perching biomechanics and KBD will be clearer. A limitation of the current study was that pressure exerted on the keel while perching was not measured. Pressure exerted on the keel has been widely discussed in this thesis, however pressure exerted on the keel while perching was never directly measured. Assessing pressure utilizing pressure sensors placed on the perch

(Pickle et al., 2011) in addition to kinematic recordings will provide clarity on if certain biomechanics lead to increased pressure exerted on the keel contributing to KBD. In addition, identifying the laying status of each hen prior to infrared and kinematic recordings could potentially account for some strain variation reported in this thesis. The presence of an egg in the oviduct while perching may alter a hen's center of gravity. Increased weight towards the back of a laying hen could contribute to tipping backwards on a perch and requiring more efforts to remain balanced while perching which may be reflected by a larger back angle between the back and perch. 3-D kinematics would be effective in determining if egg laying impacts perching biomechanics in laying hens. In addition to the impacts of egg laying on perching biomechanics, egg laying may impact body temperature. Laying hens in lay have significantly higher blood flow than birds not in-lay (Hu et al., 2019) resulting in hotter body temperatures. Conducting behaviour observations in Chapter 4 provided a general assessment of perching behaviours exhibited by laying hens in each treatment group. However, utilizing low-frequency tracking systems (Montalcini et al., 2023) on focal birds would provide precise data on perch usage and interactions with the perch such as collisions or falls. Movement tracking throughout a trial with routine kinematic recordings would highlight how an individual laying hens perching behaviour and usage contributes to KBD.

Future research utilizing 3D kinematics technology in poultry could investigate the provision of perches during the rearing phase on the development of perching behaviour. Younger birds moved faster and more frequently than older birds. Although not analyzed, it was anecdotally noted, in Chapter 4, that during the pullet phase, pullets were more likely to jump or fall from the perch. Perching may be an innate behaviour in poultry as pullets will use perches within the first week of age (Skanberg et al., 2021) but it may take anywhere from five to seven weeks for acclimation to enrichments to occur (Liu et al., 2018). As such, our results highlight that perching behaviours stabilize with age. While learning how to properly perch, pullets are more likely to exhibit faster and greater movements. A period of instability may be indicative of a period of trial-and-error learning where a pullet can begin to associate certain perching behaviors with increased stability. By assessing how learning impacts perching behaviour, studies could determine how to best equip pullets for the challenges presented by rearing and lay environments.

To locate causative mutations contributing to KBD, future researchers could conduct a more thorough analysis of genes such as *RANKL*, *POSTN*, *OVALX*, and *RB1* due to their promising results in Chapter 3. Our results were confounded by strain effects and due to a limited number of samples, a proper relationship matrix could not be developed for statistical analysis. As such, in future research, a few different approaches can be used to mitigate a confounding strain effect. Future projects should aim to create a relationship matrix through the analysis of more SNPs on a candidate gene or by determining the sires and dams of all animals used. Determining sires and dams would likely be difficult in a standard university research setting so future work should focus on broadening the number of SNPs that are used. In addition, future projects should aim to have larger sample sizes, as appropriate sample sizes are important in population genomics to generate robust results (Subramanian, 2016).

5.7. General Conclusions

Overall, strain, perch shape, and rearing environment significantly impacted perching biomechanics. Up and down movements and forward and backward movements correlated with a higher incidence of severe KBD. Additionally, strain impacted movement patterns with commercial strains moving up and down more and forward and backward less than random-bred strains. Round perches led to more severe KBD, indicating wider mushroom perches are preferable for laying hen housing. Perching instability was also reflected through speed metrics, although not associated with KBD. White strains moved faster than brown strains which was not correlated with KBD. Round perches contributed to faster movements. Brown and random-bred strains had significantly stronger and denser bones compared to white and commercial strains. Differing results in our studies show that although bone strength may reduce KBD severity other factors such as perching biomechanics and perch usage likely have an effect. Leenstra et al. (2016) suggested that certain laying hen phenotypes may be more well suited to specific laying hen environments. Lower incidences of KBD despite lower bone mineralization and a smaller body size indicate LW birds are more readily suited to furnished cages in comparison to LB birds. In addition, higher producing, commercial strains had higher incidences of severe KBD in contrast to lower producing random-bred strains. Perch shape did not impact footpad temperature, but variation amongst strains at different stages of lay indicate future research utilizing infrared thermography to assess biological functions such as egg production in laying hens could be successful. *POSTN*, *RANKL*, *RB1*, and *OVALX* are promising candidate genes for locating SNPs associated with bone parameters in laying hens. Research looking to identify causative mutations associated with KBD should ensure suitable sample sizes as well as more complete candidate gene genotyping to reduce the impact of confounding factors such as strain that were identified in this thesis.

5.8. References

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Appendix 1: Percentage of instability behaviours exhibited by each strain of laying hen during 3-D kinematic recordings on both round and mushroom perches. All behaviours are mutually exclusive.

	Falling	**Sidestepping**	**Slipping**	**Restless**	**Rocking**
ISA Brown	3%	39%	39%	68%	8%
Lohmann Brown	3%	24%	24%	56%	6%
Shaver White	0%	46%	38%	84%	16%
Lohmann White	2%	67%	29%	76%	14%
Rhode Island Red	0%	33%	0%	58%	3%
White Leghorn	10%	34%	48%	95%	9%

Falling, focal bird falls from the perch during the recording. **Sidestepping**, focal bird moves along perch from side to side. **Slipping**, focal bird exhibits repetitive, rapid movements in any direction. **Restless**, focal bird moves between a perching and standing position more than once throughout a recording. **Rocking**, continual forward and backward movement of the focal bird throughout the recording.