5-2024

Optimizing Laying Hen Behavior, Bone Health, and Welfare Through Environmental and Nutritional Enrichments

Mallory Anderson
mga5@clemson.edu

OPTIMIZING LAYING HEN BEHAVIOR, BONE HEALTH, AND WELFARE THROUGH ENVIRONMENTAL AND NUTRITIONAL ENRICHMENTS

A Dissertation
Presented to
the Graduate School of
Clemson University

In Partial Fulfillment
of the Requirements for the Degree
Doctor of Philosophy
Animal and Veterinary Sciences

---

by
Mallory Grace Anderson
May 2024

Accepted by:
Dr. Ahmed Ali, Committee Chair
Dr. Jeryl Jones
Dr. Mireille Arguelles-Ramos
Dr. William Bridges

ABSTRACT

The objective of this dissertation was to evaluate the impact of environmental and nutritional enrichments on the behavior, bone health, and welfare of Hy-line brown and Hy-line W-36 laying hens. Laying hens are prone to experiencing a progressive increase in bone fragility due to the ongoing mobilization of calcium from the bones for eggshell formation. Over time, this increases their susceptibility for bone fracture, which is a welfare concern. Prior research suggests that exercise, especially during the developmental stage, can aid in minimizing bone fractures by strengthening muscles and increasing bone mass. Furthermore, nutrition plays an integral role in laying hen skeletal health. We investigated two interventions to prevent or reduce the negative effects observed on laying hen skeletal health: 1) environmental enrichment, which included the provision of multi-tier perches at various time points within the lifespan of laying hens and 2) nutritional enrichment, which involved supplementing the diet with boron. The first aim of this dissertation (described in Chapter 2) was to determine the effect of perch provision during the rearing phase on the activity and musculoskeletal health of pullets. Pullets (n=810) were either housed with or without multi-tier perches from 0 to 17 weeks of age (15 pens/treatment, 29 birds/pen). We expected pullets with perches to show improved musculoskeletal characteristics compared to pullets reared without perches. At 5, 11, and 17 weeks, birds were individually monitored for activity level over 3 consecutive days. At 11 and 17 weeks, 60 birds were euthanized for computed tomography (CT) scans to quantify tibiotarsal bone mineral density (BMD) and cross-sectional area (CSA). After CT scanning, birds were dissected for measurement of muscle size, tibiotarsal breaking strength, and tibiotarsal ash

percentage. Novel markers of bone mineralization (bone-specific alkaline phosphatase [BALP] and pro-collagen type 1 n-terminal propeptide [P1NP]) were measured from serum samples of 60 birds/week. Results indicated that pullets reared with perches from 0-17 weeks of age exhibited increased levels of vertical activity, with no significant effect on overall activity level. Pullets with perches had greater total and cortical BMD at week 11, with increased cortical bone CSA and higher total and cortical BMD at week 17 compared to pullets without perches. At week 11, pullets with perches had heavier leg muscles, with heavier triceps, biceps, pectoralis major and minor, and leg muscles at week 17 than pullets without perches. At both weeks, pullets with perches had greater tibiotarsal breaking strengths, higher ash percentages, and greater concentrations of BALP and P1NP than pullets without perches. These results indicate that activity resulting from perching elicits a beneficial impact on measures of pullet musculoskeletal health at both 11 and 17 weeks of age.

The second objective (described in Chapter 3) was to determine whether there were enduring impacts of perch provision timing on the musculoskeletal health of laying hens. Pullets (n=812) were housed under different conditions (7 pens/treatment, 29 birds/pen) with either continuous access to multi-tier perches from 0 to 40 weeks of age (CP), no access to perches (NP), early access to perches during the rearing phase from 0 to 17 weeks of age (EP), or solely during the laying phase from 17 to 40 weeks of age (LP). We expected hens from the CP group to exhibit improved musculoskeletal health as a result of perch-related activity compared to hens from the NP group, with hens from the EP group having improved musculoskeletal health compared to hens from the LP group due to increased

activity during the developmental stage. At weeks 24, 36, and 40 of age, birds were individually monitored for activity level over 3 consecutive days, and blood samples were collected from a separate set of 3 birds per pen to analyze serum concentrations of tartrate-resistant acid phosphatase 5b (TRACP-5b) and C-terminal telopeptide of type I collagen (CTX-I) as novel markers of bone demineralization. At 40 weeks of age, 3 birds per pen were euthanized for CT scans with further analysis including muscle weights, tibiotarsal breaking strength, and tibiotarsal ash percent. During week 24, hens from CP, EP, and LP pens had the highest overall activity compared to hens from NP pens, with no differences at week 36 or 40. During all weeks, hens from CP and LP pens had greater vertical and less horizontal activity compared to those from EP and NP pens. TRACP-5b and CTX-I concentrations did not differ at week 24 of age, with hens from CP pens having the lowest TRACP-5b and CTX-I concentrations compared to NP pens at 40 weeks of age. Total bone CSA did not differ between treatments, but CP had greater total BMD than NP with no differences between EP and LP pens. CP and LP hens had heavier biceps, pectoralis major, and leg muscle groups, as well as greater tibiotarsal breaking strengths than EP and NP pens. CP hens had higher tibiotarsal ash percentages compared to all other treatment groups. The results from this chapter indicate that the continuous provision of perches throughout the rearing and lay phase beneficially impacts activity level and measures of hen musculoskeletal health at 40 weeks of age, contributing to an overall improvement in laying hen welfare compared to no access to perches. Perch access during the early lay phase (17-40 weeks of age) had a positive impact on activity, muscle weight, and bone strength, but these benefits were not as great as those observed with continuous perch

access. Perch access during the rearing phase (0-17 weeks of age) was associated with a decline in measures of bone demineralization, but did not have an overarching beneficial impact on other measures of hen musculoskeletal health or activity at 40 weeks of age, suggesting there was not a long-term benefit of perch access during the developmental stage.

The third objective (within Chapter 4) was to determine the influence of perch provision timing on laying hen behavior, specifically anxiety and fearfulness. While providing perches may enhance biological functioning and animal welfare, their effectiveness could be age-dependent. This chapter investigated the effects of early and late perch access on anxiety and fear in hens through attention bias (AB) and tonic immobility tests (TI). Pullets (n=728) were raised with or without multi-tier perches either continuously (CP; 0-37 weeks), during only the rearing phase (EP; 0-17 weeks), during only the laying phase (LP; 17-37 weeks), or not at all (NP; no perch access). We expected hens from the CP group to show responses consistent with reduced anxiety and fear compared to hens from the NP group, with intermediate responses from hens in the EP and LP groups. AB was conducted in weeks 21 and 37 (n=84 birds/week) and TI was performed in weeks 20, 25, and 37 (n=112/week). CP hens fed quicker in the AB test than EP, LP, and NP hens at weeks 21 and 37. CP and NP feeding latencies were stable, while EP and LP fed faster at week 37 compared to 21. CP had the shortest TI duration at week 20, while both CP and LP had the shortest TI durations in weeks 25 and 37. Hens from LP pens showed increased anxiety levels at week 21 of age, which they adapted to by week 37, indicating that adaptation to a new adult environment requires at least 16 weeks. Also, LP

hens exhibited reduced fearfulness by 20 weeks of age compared to hens that lost their perch access (EP) or never had perch access (NP). At 25 and 37 weeks of age, LP hens showed similar fear levels as hens from CP pens, indicating that current perch access reduces fearfulness. Removing perches at 17 weeks of age (EP) increased fear at weeks 20 and 25 and anxiety at week 21, effects that disappeared by week 37 of age. Furthermore, birds from EP pens showed decreased anxiety at 37 weeks of age compared to NP birds, suggesting that perch access, even when removed at 17 weeks of age, is more beneficial to anxiousness at 37 weeks of age than not having access to perches at all. Our findings indicated that providing hens with multi-tier perches throughout their lifetime improved affective state by reducing anxiety and fearfulness, while no access to perches negatively impacted measures of emotion and affective state.

The last objective (described in Chapter 5) was to determine the effects of boron supplementation on pullet musculoskeletal health and performance parameters. Boron plays a role in the metabolism of calcium, which may help improve bone strength and prevent fracture. A total of 529 Hy-Line W-36 pullets were distributed across 24 pens and fed basal diets containing varying amounts of boron (C: (C: 0mg/kg; L: 50mg/kg; M: 100mg/kg; H: 150mg/kg) for 17 weeks. We expected pullets from the M group to show improved musculoskeletal health compared to the other treatment groups. Performance parameters (body weight, average daily weight gain/bird, and average daily feed intake/bird) were measured at weeks 4, 7, 10, 13, and 16, while all other measures were recorded at 11 and 17 weeks of age. Performance measures did not differ between treatment groups. Pectoralis major weights were higher in H pullets at 11 weeks of age, and we also

observed higher pectoralis major, minor, and leg muscle weights in H pullets at 17 weeks of age. Pullets fed the H diet had larger cortical CSA than the other treatment groups at 11 weeks of age. At 17 weeks of age, both H and M groups had larger cortical CSA than L and C groups, but the M group had slightly smaller cortical CSA. Pullets fed the H diet had higher BMD values than other treatment groups at 11 weeks of age. At 17 weeks of age, pullets fed the H diet had the highest total BMD values compared to the other treatment groups, and cortical BMD increased with increasing boron inclusion. Pullets fed the H diet had the highest tibia ash percentages and concentrations of BALP and P1NP. Pullets fed the M and H diets had greater failure load and maximum bending moment than pullets fed the L or C diet at 11 weeks of age, with H pullets having greater stiffness values than other groups. At 17 weeks of age, pullets fed the H diet had greater failure load and maximum bending moment compared to all other treatment groups. Our results imply that providing boron within the diet at 150mg/kg improves musculoskeletal characteristics of Hy-Line W-36 pullets up to 17 weeks of age, without impacting performance parameters.

DEDICATION

I dedicate this body of work to my parents, Carrol DeNure and Dr. Edwin Anderson. Without your constant support and fostering my creativity, I would not have grown into the animal lover and scientist I am today. I have endless gratitude and love for both of you. I also dedicate this dissertation to the flocks of birds with whom I spent countless hours, and whose sacrifice was made in the pursuit of science, animal welfare, and the greater good. We did our best not to become attached, yet one bird in particular made that challenging. G0080, affectionately known as Heidi, captivated us with such warmth and amiability that we could not resist giving her a name. Thank you.

ACKNOWLEDGMENTS

I would like to thank my advisor, Dr. Ali, for your guidance and mentorship throughout my time at Clemson. Your (sometimes) gentle nudges to push me out of my comfort zone helped me grow as a scientist and as a person, and for that I am endlessly appreciative. You were there for me through everything and somehow always knew when I needed a laugh. To my committee members, Drs Jones, Arguelles-Ramos, and Bridges, I thank you for the time you committed to my academic and personal growth. I truly could not have asked for a better committee to guide me through graduate school.

To my family and friends that have become family: Jon, Dave, Jesse, Zach, and Dezzi, you all supported me in ways that have not gone unnoticed. When I was silently having a mental breakdown or stressing about my research, at one time or another one of you calmed my nerves with a silly joke and reminded me that everything was going to be okay. Jon, you are the reason I made it this far and I am forever grateful that you are in my life. A heartfelt appreciation goes out to my crazy friends, Finley and Willie. Their excited welcomes, whether I'd been away for five minutes or five hours, never failed to bring a smile to my face and brighten every bad day. They are a constant reminder to find joy in everyday life.

Alexa, those infinite dusty hours spent at the feed mill or on the farm at CBH would have been far less joyous and full of laughter without you. Laughter that often left us in tears with our stomachs aching. I am deeply grateful for your friendship, support, and care over the past three years. I can't wait to watch the inevitable success in your career, because I know that's just what you will do.

Lastly, I would like to thank Clemson University, the Department of Animal and Veterinary Sciences, and all the faculty and staff I met along the way. I cannot thank the Clemson community enough for making South Carolina truly feel like home.

TABLE OF CONTENTS

Page

ABSTRACT i
DEDICATION viii
ACKNOWLEDGMENTS ix
TABLE OF CONTENTS xi
LIST OF TABLES xv
LIST OF FIGURES xix
Chapter 1 1
Animal Welfare 1
Laying Hen Behavior 5
Dynamics of Bone Biology in Laying Hens 7
Bone growth during the rearing phase 7
Bone growth during the laying phase 9
Factors Affecting Osteoporosis in Laying Hens 10
Modern housing for laying hens 10
Housing and exercise 12
Genetic selection 13
Compounding welfare concerns 15
Interventions to Improve Laying Hen Bone Health 16
Environmental enrichment 16
Nutritional enrichment 23
Laying Hen Welfare Assessment 27
Affective state 28
Bone health 32
References 40
Chapter 2 54
Abstract 55
Introduction 57
Materials and Methods 59
Ethics 59
Animals and housing 59
Treatments 60
Activity 61

Computed tomography (CT) image acquisition 61
Tibiotarsal CT image analysis 62
Muscle deposition 63
Breaking strength 64
Tibia ash percentage 65
Bone mineralization 66
Data processing and statistical analysis 66
Results 68
Activity 68
Musculoskeletal health 69
Discussion 76
Activity 76
Muscle deposition 77
Bone mineral density (BMD) and bone cross-sectional area (BCSA) 78
Breaking strength 79
Tibia ash percentage 80
Bone mineralization 81
Conclusions 82
References 84
Chapter 3 87
Simple Summary 88
Abstract 88
Introduction 91
Materials and Methods 93
Ethics 93
Animals and housing 93
Treatments 94
Activity 95
Musculoskeletal health 96
Data processing and statistical analysis 100
Results 102
Activity 102
Tibial bone mineral density (BMD) and cross-sectional area (CSA) 104
Muscle deposition 106
Tibia breaking strength 107
Tibia ash percentage 107
Bone demineralization 108
Discussion 109
Activity 109

Tibial bone mineral density (BMD) and cross-sectional area (CSA) 110
Muscle deposition 111
Tibia breaking strength 112
Tibia ash percentage 113
Bone resorption 113
Conclusions 114
References 118
Chapter 4 122
Simple Summary 123
Abstract 123
Introduction 125
Materials and Methods 128
Ethics 128
Animal and housing 128
Treatments 129
Attention bias test 129
Tonic immobility test 131
Statistical analysis 132
Results 133
Attention bias test 133
Tonic immobility test 138
General summary of results 141
Discussion 142
Attention bias 143
Tonic immobility 148
Conclusions 152
References 156
Chapter 5 161
Simple Summary 162
Abstract 162
Introduction 164
Materials and Methods 166
Ethics 166
Animals and housing 166
Treatments 167
Performance 168
Computed tomography (CT) image acquisition 169

Bone cross-sectional area (CSA) and bone mineral density (BMD) 169
Muscle deposition 169
Ash percentage 170
Breaking strength 170
Bone mineralization 171
Statistical analysis 171
Results 172
Performance 172
Bone cross-sectional area (CSA) and bone mineral density (BMD) 173
Muscle deposition 175
Ash percentage 177
Breaking strength 178
Bone mineralization 179
Discussion 180
Conclusions 184
References 189
Chapter 6 193
APPENDICES 197

LIST OF TABLES

Page

Table 1.1. The Five Freedoms and Provisions. Adapted from Brambell (1965) and Farm Animal Welfare Council (1993) [11,12]. 3

Table 1.2. Summary of studies evaluating the effect of perch provision on laying hen musculoskeletal health. WOA = weeks of age, BS = breaking strength, BMD = bone mineral density, BMC = bone mineral content, CSA = cross-sectional area, CT = computed tomography. 19

Table 1.3. Summary of studies evaluating boron as a feed additive in pullets or laying hens. WOA = weeks of age, B = boron, CSA = cross-sectional area, FCR = feed conversion ratio. 26

Table 1.4. Summary of studies evaluating laying hen affective state through an attention bias test. 31

Table 2.1. Activity of pullets housed with perches (P) or no perches (NP) for 3 consecutive days at weeks 5, 11, and 17 of age (n = 90 birds/week; g = gravitational force; f = frequency). 69

Table 2.2. Bone mineral density (BMD; mg/cm3) and bone cross-sectional area (BCSA; mm2) ± SEM for the total and cortical regions of the right tibiotarsus of pullets housed with perches (P) or no perches (NP) at 11 (n = 60 birds) and 17 (n = 60 birds) weeks of age. 72

Table 2.3. Breaking strength (N), stiffness (N/mm), and maximum bending moment (N/m) of pullets housed with perches (P) or no perches (NP) at weeks 11 (n = 60 birds) and 17 (n = 60 birds) of age. 73
Table 2.4. Tibia ash percent (%) of pullets housed with perches (P) or no perches (NP) at weeks 11 (n = 30 birds) and 17 (n = 60 birds) of age. 74
Table 3.1. Overall, vertical, and horizontal activity levels, and average daily vertical displacement per bird (F) of laying hens housed in continuous perch (CP), early perch (EP), late perch (LP), and no perch (NP) pens at weeks 24, 36, and 40 of age (n = 84/week). 103
Table 3.2. Tibial total, medullary, and cortical bone mineral density (BMD; mg/cm3) and cross-sectional area (CSA; mm2) ±SEM for the proximal, middle, and distal regions of the right tibiotarsus of laying hens. 105
Table 3.3. Mean weight (g) ±SEM of biceps brachii, triceps brachii, pectoralis major, pectoralis minor, and leg muscle group of laying hens. 106
Table 4.1. Summary of the attention bias (AB) testing methodology adapted from Campbell et al. [7]. Birds were tested in groups of three at 21 and 37 weeks of age. 131
Table 4.2. Simple summary of attention bias (AB) and tonic immobility (TI) results. Hens were kept in continuous perch (CP), early perch (EP), late perch (LP), or no perch (NP) housing environments. 141
Table 5.1. Ingredient percentage and calculated nutrient analysis of 4 basal diets used in the current experiment. 168

Table 5.2. Body weight, average daily weight gain, and average daily feed intake per bird (g) fed (mean ± SEM) a control diet (C), or a basal diet supplemented with low (L; 50mg/kg), medium (M; 100mg/kg), and high (H; 150mg/kg) boron at weeks 4, 7, 10, 13, and 16 weeks of age. 173

Table 5.3. Tibia cross-sectional area (CSA; mm^2) and bone mineral density (BMD; mg/cm^3) of pullets (mean ± SEM) fed a control diet (C), or a basal diet supplemented with low (L; 50mg/kg), medium (M; 100mg/kg), and high (H; 150mg/kg) boron at week 11 of age (n = 48). [a-d] Means with different superscripts within columns differ at $p < 0.05$. 174

Table 5.4. Tibia cross-sectional area (CSA; mm^2) and bone mineral density (BMD; mg/cm^3) of pullets (mean ± SEM) fed a control diet (C), or a basal diet supplemented with low (L; 50mg/kg), medium (M; 100mg/kg), and high (H; 150mg/kg) boron at week 17 of age (n = 48). [a-d] Means with different superscripts within columns differ at $p < 0.05$. 175

Table 5.5. Tibia breaking strength (N), stiffness (N/mm), and maximum bending moment (N/m) of pullets fed a control diet (C), or a basal diet supplemented with low (L; 50mg/kg), medium (M; 100mg/kg), and high (H; 150mg/kg) boron at weeks 11 and 17 of age (n = 48 birds/week). [a-c] Means with different superscripts within columns differ at $p < 0.05$..... 179

Table 6.1. Performance of laying hens housed in continuous perch (CP; perch access from 0-40 weeks of age), early perch (EP; perch access from 0-17 weeks of age), late perch (LP; perch access from 17-40 weeks of age), and no perch (NP; no perch access)

groups during the experiment described in Chapter 3. FCR = feed conversion ratio; ADFI = average daily feed intake; HDEP = hen-day egg production across weeks (week 24, 30, 36, 40). Values within the same week with unlike letters indicate significant differences ($p < 0.05$). 197

LIST OF FIGURES

Page

Figure 1.2. Four types of cells within the bone and their function. Adapted from Office of the Surgeon General (2004) and Wittkowske et al. (2016) [41,42]. 8

Figure 1.3. Schematic of the three bone types found in laying hens. Adapted from Soriano (2021) [43]. 9

Figure 1.4. Factors affecting laying hen bone health. 15

Figure 1.5. Schematic of mechanical test frame setup for a three-point bending test. Adapted from Awoyera et al. (2021) [172]. 36

Figure 1.6. Load-displacement plot produced by biomechanical strength testing. Adapted from Silva (2016) [173]. 38

Figure 2.1. Perch and adjustable rung heights in the perch (P) treatment groups during days A) 0-11, B) 11-19, and C) 19+ days of age. 60

Figure 2.2. Dorsal recumbent positioning of the birds on the hydroxyapatite phantom inside the V-shaped foam cradle for computed tomography image acquisition. 62

Figure 2.3. Steps of tibiotarsal image analysis. A) Division of the tibiotarsus into 4 segments to set proximal, middle, and distal locations, B) region of interest tracings for the tibiotarsus of a 17-week-old bird in the proximal location, and C) region of interest. 63

Figure 2.4. Instron configuration with rounded supports and breaking blade machined according to ANSI standards. 65

Figure 2.5. Weight (grams) of biceps brachii, triceps brachii, pectoralis major, pectoralis minor, and leg muscle group of 11-week-old pullets (n = 60 birds) housed with perches

(P) or no perches (NP). Results are presented as mean weight (grams) ± SEM. *Across bars indicates significant statistical differences at $p < 0.05$. 70
Figure 2.6. Weight (grams) of bicep brachii, triceps brachii, pectoralis major, pectoralis minor, and leg muscle group of 17-week-old pullets (n = 60 birds) housed with perches (P) or no perches (NP). Results are presented as mean weight (grams) ± SEM. *Across bars indicates significant statistical differences at $p < 0.05$. 70
Figure 2.7. Concentrations of bone-specific alkaline phosphatase (BALP) for pullets housed in perch (P) and no perch (NP) housing environments during weeks 11 and 17 (n = 90 birds/week). Results are presented as mean ± SEM. *Across bars indicates significant statistical differences at $p < 0.05$. 75
Figure 2.8. Concentrations of pro-collagen type 1 n-terminal propeptide (P1NP) for pullets housed in perch (P) and no perch (NP) housing environments during weeks 11 and 17 (n = 90 birds/week). Results are presented as mean ± SEM. *Across bars indicates significant statistical differences at $p < 0.05$. 75
Figure 3.1. Perch and adjustable rung heights in continuous perch (CP) treatment groups during days (a) 0–11, (b) 12–19, and (c) 20+ days of age. Perches were placed in late perch (LP) treatment groups beginning at 18 weeks of age with rungs at heights pictured in (c). 95
Figure 3.2. Steps of tibiotarsal image analysis. (a) Division of the tibiotarsus into 4 segments to set proximal, middle, and distal locations, (b) region of interest tracings for the tibiotarsus in the proximal location, and (c) region of interest placement in the 3 rods of hydroxyapatite phantom using the oval tool. 97

Figure 3.3. Mean tibia breaking strength (N) and stiffness (N/mm) of laying hens housed in continuous perch (CP), early perch (EP), late perch (LP), and no perch (NP) pens at 40 weeks of age (n = 84). a,b Means with differing superscripts indicate statistically significant differences between treatments within a parameter at $p < 0.05$..... 107
Figure 3.4. Mean tibia ash percent (%) of laying hens housed in continuous perch (CP), early perch (EP), late perch (LP), and no perch (NP) pens at 40 weeks of age (n = 84). a,b Means with differing superscripts indicate statistically significant differences between treatments at $p < 0.05$..... 108
Figure 3.5. Mean serum concentrations of (a) tartrate-resistant acid phosphatase 5b (TRACP-5b; U/L) and (b) C-terminal telopeptide of type I collagen (CTX-I; ng/L) of laying hens housed in continuous perch (CP), early perch (EP), late perch (LP), and no perch (NP) pens at 24, 36, and 40 weeks of age (n = 84/week). [a-d]Means with differing superscripts indicate statistically significant differences between treatments within week at $p < 0.05$. 109
Figure 4.1. Latency to begin feeding (0–300 s) for laying hens in CP (continuous perch), EP (early perch), LP (later perch), and NP (no perch) housing environments during the attention bias test at onset of lay at week 21 and peak-lay at 37 of age (n = 112 hens/week). [a-c]Different superscripts indicate statistically significant differences between treatments within the same week at $p < 0.05$. [x-z]Different superscripts indicate statistically significant differences between weeks within the same treatment at $p < 0.05$..... 134
Figure 4.2. Latency to resume feeding (0–120 s) expressed as (mean ± SEM) for laying hens in CP (continuous perch), EP (early perch), LP (late perch), and NP (no perch)

housing environments during the attention bias test at the onset of lay at week 21 and peak-lay at week 37 of age (n = 112 hens/week). [a–c] Different superscripts indicate statistically significant differences between treatments within the same week at $p <$ 0.05. [x–z] Different superscripts indicate statistically significant differences between weeks within the same treatment at $p < 0.05$ 135
Figure 4.3. Percentage (%) of laying hens (expressed as mean ± SEM) observed to begin feeding from CP (continuous perch), EP (early perch), LP (late perch), and NP (no perch) housing environments during the attention bias test at onset of lay at week 21 and peak-l lay at week 37 of age (n = 112 hens/week). [a–c] Different superscripts indicate statistically significant differences between treatments within the same week at $p < 0.05$. [x–z] Different superscripts indicate statistically significant differences between weeks within the same treatment at $p < 0.05$. 136
Figure 4.4. Percentage (%) of laying hens (expressed as mean ± SEM) observed to resume feeding from CP (continuous perch), EP (early perch), LP (late perch), and NP (no perch) housing environments during the attention bias test at onset of lay at week 21 and peak-lay at week 37 of age (n = 112 hens/week). The timer was reset to zero after the second alarm call was played to record latency to resume feeding. [a–c] Different superscripts indicate statistically significant differences between treatments within the same week at $p < 0.05$. [x–z] Different superscripts indicate statistically significant differences between weeks within the same treatment at $p < 0.05$. 137
Figure 4.5. Vigilance behavior scores (expressed as means ± SEM) for laying hens in CP (continuous perch), EP (early perch), LP (late perch), and NP (no perch) housing

environments during AB testing at onset of lay at week 21 and peak-lay at week 37 of age (n = 84 hens/week). [a–c] Different superscripts indicate statistically significant differences between treatments within the same week at $p < 0.05$. [x–z] Different superscripts indicate statistically significant differences between weeks within the same treatment at $p < 0.05$. 138

Figure 4.6. Tonic immobility duration (0–300 s) expressed as (mean ± SEM) for laying hens in CP (continuous perch), EP (early perch), LP (late perch), and NP (no perch) housing environments at the onset of lay at week 21, early-lay at week 25, and peak-lay at 37 w weeks of age (n = 112 hens/week). [a–c] Different superscripts indicate statistically significant differences between treatments within the same week at $p < 0.05$. [x–z] Different superscripts indicate statistically significant differences between weeks within the same treatment at $p < 0.05$. 139

Figure 4.7. Tonic immobility induction attempts (1–3) expressed as (mean ± SEM) for laying hens in CP (continuous perch), EP (early perch), LP (late perch), and NP (no perch) housing environments during the onset of lay at week 21, early-lay at week 25, and peak- lay at 37 weeks of age (n = 112 hens/week). [a–c] Different superscripts indicate statistically significant differences between treatments within the same week at $p < 0.05$. [x–z] Different superscripts indicate statistically significant differences between weeks within the same treatment at $p < 0.05$. 140

Figure 5.1. Muscle mean weight (g) ±SEM of pullets fed a control diet (C), or a basal diet supplemented with low (L; 50mg/kg), medium (M; 100mg/kg), and high (H; 150mg/kg)

boron at week 11 of age (n = 48 birds). [a-b] Means with different superscripts differ at p < 0.05..... 176

Figure 5.2. Muscle mean weight (g) ±SEM of pullets fed a control diet (C), or a basal diet supplemented with low (L; 50mg/kg), medium (M; 100mg/kg), and high (H; 150mg/kg) boron at week 17 of age (n = 48 birds). [a-b] Means with different superscripts differ at p < 0.05..... 177

Figure 5.3. Tibia ash percentage of pullets fed a control diet (C), or a basal diet supplemented with low (L; 50mg/kg), medium (M; 100mg/kg), and high (H; 150mg/kg) boron at weeks 11 and 17 of age (n = 48 birds/week). [a-b] Means with different superscripts differ at p < 0.05. 178

Figure 5.4. Serum concentrations of bone-specific alkaline phosphatase (BALP) and procollagen type 1 N-terminal propeptide (P1NP) in pullets fed a control diet (C), or a basal diet supplemented with low (L; 50mg/kg), medium (M; 100mg/kg), and high (H; 150mg/kg) boron at weeks 11 and 17 of age (n = 72 birds/week). [a-d] Means with different superscripts differ at p < 0.05. 180

Chapter 1

REVIEW OF LITERATURE

Animal Welfare

There is not a single definitive definition of animal welfare, however there is a consensus that it should encompass the animal's individual experiences and their perception of the environment [1]. Broom (1986) described welfare as "an individual's state as regards to its attempts to cope with its environment" [2], where coping refers to physiological and psychological stability through various coping mechanisms (behavioral, physiological, immunological, etc.) [3]. From this definition's standpoint, poor welfare occurs when an individual fails to cope with its environment and good welfare occurs when an individual succeeds to cope with its environment. Later on, animal welfare scientists felt as though Broom's definition was quite functional and some other scientists (i.e., Ian Duncan) argued that welfare should be about an individual's feelings [4]. Dawkins attempted to incorporate both aspects of Broom and Duncan's viewpoints, stating that "the feelings of the individual are the central issue in welfare, but other aspects such as the health of the individual are also important" [5,6].

Although consideration of feelings is important, welfare is comprised of more than just what an individual feels and is often quite difficult to measure alone. For example, distinguishing between an animal resting because it is satiated and content and an animal that is resting due to boredom. The three concepts of adaptation, stress, and biological needs may help further our understanding of welfare from an agricultural animal standpoint. Adaptation refers to how well an individual adapts to its environmental conditions through the use of regulatory systems [4]. An animal will better adapt to its environmental conditions when its needs are met. However,

adaptation does not necessarily equate to good welfare. An animal that adapts to its environment, but has difficulty doing so, would experience poor welfare. For example, a herd animal that requires social interaction with conspecifics that is housed alone for extended periods of time may adapt to this condition, but will likely experience depression and reduced biological function. Stress refers to "an environmental effect on an individual which overtaxes control systems and results in adverse consequences, eventually reduced fitness" [2,3]. When an individual is stressed, their welfare may be poor or temporarily poor without any long-lasting impacts on welfare [4]. Biological needs refer to an animal's need "to obtain a particular resource or respond to a particular environmental or bodily stimulus" [3]. Needs may be for resources (i.e., food, water, shelter) or for carrying out behaviors with an ultimate objective (i.e., perching at night to avoid predation) [4]. Meeting biological needs of an animal allows for effective functioning and good welfare. Biological needs of animals has led to the incorporation of the Five Freedoms, which provides a general guideline for review of animal welfare. The Five Freedoms are widely used in legislation, policy, and standards for humanely raising farm animals (Table 1.1) [7–9]. However, this concept is criticized for emphasizing preventing negative states rather than ensuring positive ones. For example, four of the five freedoms are freedoms from negative states. Furthermore, the Five Freedoms serve as a framework for caretakers to implement management practices aimed at sustaining an animal's life, rather than fostering an environment where animals flourish and experience a quality life [9,10].

Table 1.1. The Five Freedoms and Provisions. Adapted from Brambell (1965) and Farm Animal Welfare Council (1993) [11,12].

Freedom	**Ensured by providing**
From hunger, thirst, and malnutrition	Ready access to fresh water and a diet to maintain full health and vigor
From discomfort and exposure	An appropriate environment, including shelter and a comfortable resting area
From pain, injury, and disease	Prevention or rapid diagnosis and treatment
From fear and distress	Ensuring conditions and treatment which avoid mental suffering
To express normal behavior	Sufficient space, proper facilities, and company of the animal's own kind

Animal welfare research continues to progress, focusing on ensuring positive welfare rather than preventing negative welfare. A more comprehensive conceptualization of animal welfare is comprised of an interconnecting framework of three major components: affective states, biological functioning, and natural living (Figure 1.1) [11]. Each component overlaps with the others to provide a comprehensive approach to evaluating animal welfare. The affective state viewpoint refers to the animal's feelings and emotions, ranging from positive to negative. Within this viewpoint, animals that experience positive emotions, such as play, and are free from negative emotions, like pain, are thought to have good welfare. The concept of affective state relates to Duncan's viewpoint, where subjective positive feelings (pleasure) accompanied with the absence of negative feelings (or suffering) indicates good welfare [12]. Biological functioning pertains to aspects contributing to optimal health, which might include reproductive ability, nutritional status, and growth, alongside the animal's capacity to adapt to its surroundings. Focusing on this view, good welfare ensures that there are adequate nutrients within the animal's diet or that the animal

is developing normally. Poor biological functioning would still necessitate a concern for welfare even if there is no impact on affective state [12]. Lastly, natural living underscores an animal's ability to thrive in an environment conducive to expressing innate behavior. Some welfare scientists argue that providing natural living conditions that allows for expression of natural behaviors promotes biological functioning and elicits pleasurable feelings, resulting in positive affective states [13]. From this view, laying hens with access to structures within their environment that provide opportunities for perching, foraging, and dustbathing would have good welfare. This framework proposes that all three factors should be considered and animals should be kept in environmental conditions that allow them to "feel well, function well, and express species-specific behaviors" [12]. Furthermore, applying this framework in the real world requires guidance from experts to ensure animal welfare solutions are biologically relevant and optimized for that species needs. Ultimately, incorporating all three perspectives is essential for a comprehensive assessment of animal welfare.

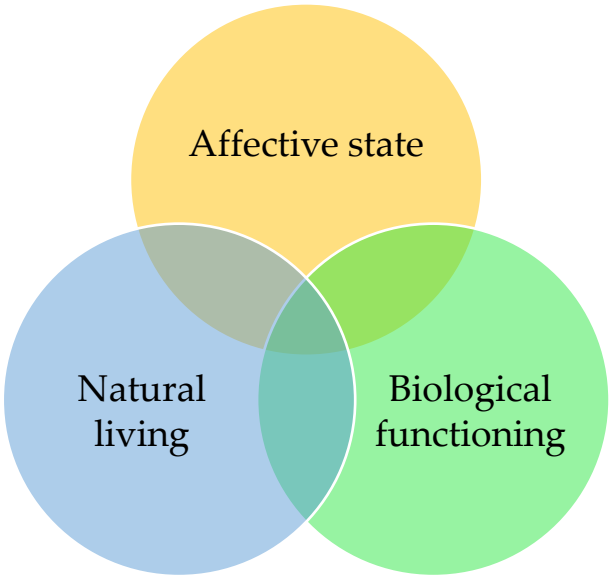

Figure 1.1. The three conceptions of animal welfare. Adapted from Fraser (2008) [13].

Laying Hen Behavior

Behavior itself evolves through natural selection and is strongly impacted by genes. Individuals whose behavior best equips them to survive will leave the most offspring who then inherit their parents' behavior. There are a few behavioral differences between domesticated poultry and their ancestors, the red jungle fowl, which are likely due to deliberate selection (*for* behaviors such as rapid growth, increased egg production, etc… and *against* others, such as broodiness), however others remain unchanged [14]. Those behaviors that withstand genetic selection likely did so because they are widespread and stable in the genotype and there has been no selection against them [14,15]. Modern domestic hens have retained many natural behaviors from their ancestors and are highly motivated to perform a variety of them. Some important behaviors to laying hens include: perching, nesting, dustbathing, and foraging.

Perching on elevated surfaces is a natural behavior observed in the red jungle fowl, serving as an anti-predatory measure and offering protection while resting (i.e., communal roosting)

[16,17]. Communal roosting likely evolved due to the reduced cost for thermoregulation and decreased risk for predation (dilution effect), where the presence of nearby birds reduces energy demands and increases predator detection and dilutes individual predation risk [18]. Hens have retained the motivation to perch even throughout persistent genetic selection for productivity and are highly motivated to do so, especially at night [19,20]. Pullets have been observed perching within the first week of life [21] and preventing hens from access to preferred perches can lead to frustration and reduced welfare [20]. Nesting behavior likely provided a selective advantage in the wild, as female birds typically search for safe areas to nest and lay eggs while protected [22]. Hens place great importance on enclosed nesting areas and their behavioral priority to have access to one increases as they get closer to laying an egg [23]. Dustbathing is a sequence of behaviors typically performed in areas with litter substrate, where birds first peck and scratch at a potential dustbathing area. Then, the bird will sit and pull substrate closer to their body, performing vertical wing shakes which causes particles of the substrate to land on the feathers. The bird will lay on their side and use their wings to push substrate along their body, usually accompanied with a rubbing motion of the legs. Lastly, the bird will stand and conclude the dustbathing sequence with a ruffle-shake that shakes off the substrate particles [24]. This behavior is thought to maintain feather condition by distributing lipids across the feathers and removing parasites [23]. Hens show a strong motivation for dustbathing, and will perform sham dustbathing (the sequence of dustbathing activity that is similar to dustbathing) on wire cage floors [25–27] and will work to gain access to litter in order to dustbathe [28]. After preventing access to suitable dustbathing substrates, hens will dustbathe for very long periods of time when finally provided with a substrate, indicating a build-up of motivation [26,27]. Furthermore, hens show preferences for dustbathing materials, such as peat and sand over sawdust or straw [24]. Foraging behavior is innate and

involves scratching and pecking at the ground [29]. Foraging behavior has been observed in 60% of active daylight in semi-wild red junglefowl [30]. This behavior persists even when an appropriate substrate is unavailable, suggesting a high motivation to exhibit foraging behavior [31,32]. Hens exhibit "contra free-loading", which means that they will work for food rather than receive "free" food from a feeder (i.e., peck and scratch within substrate to obtain feed rather than obtain feed from a trough) [23,33]. Perching, nesting, dustbathing, and foraging behaviors are important to consider when attempting to optimize laying hen welfare, as they are retained in the behavioral repertoire even throughout years of genetic selection and hens show high motivation to perform them.

Dynamics of Bone Biology in Laying Hens

Bone growth during the rearing phase

Immature laying hens (pullets) experience general growth and development during the rearing phase, which typically occurs from hatch until 15 to 18 weeks of age [34]. During this period, the skeleton is developed through a process called endochondral ossification, which refers to the longitudinal growth of the long bones [35]. Bones are comprised of a collagen matrix that surrounds a cellular component containing osteoblasts, osteoclasts, and osteocytes (Figure 1.2). Osteoblasts cells form the hydroxyapatite crystals within the bone matrix (the basis of bone itself), while osteoclasts are bone-resorbing cells involved in bone demineralization [35]. Osteoblasts produce bone spicules that combine to form a network of bone cavities that become filled with layers of cortical (structural) bone, resulting in a pneumatic bone (i.e., bone filled with air; Figure 1.3) [35]. This adaptation results in a lightweight skeleton that is well-adapted for flight. Some

osteoblasts remain within the bone matrix and differentiate into osteocytes, which are cells that maintain the bone matrix itself [36]. The activity of both osteoblasts and osteoclasts results in the production of trabecular bone (also known as cancellous or spongy bone) at the end of long bones [35,37]. During this period of growth, there is minimal bone remodeling, which refers to removing old bone and replacing it with new bone material [38].

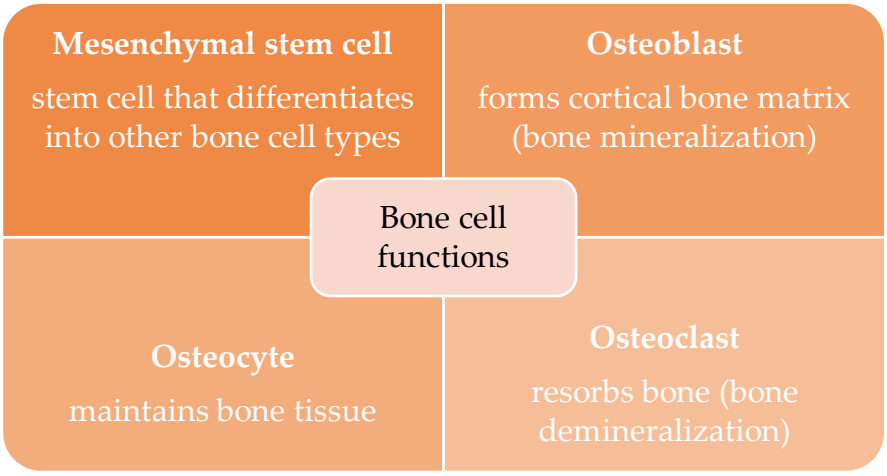

Figure 1.1. Four types of cells within the bone and their function. Adapted from Office of the Surgeon General (2004) and Wittkowske et al. (2016) [41,42].

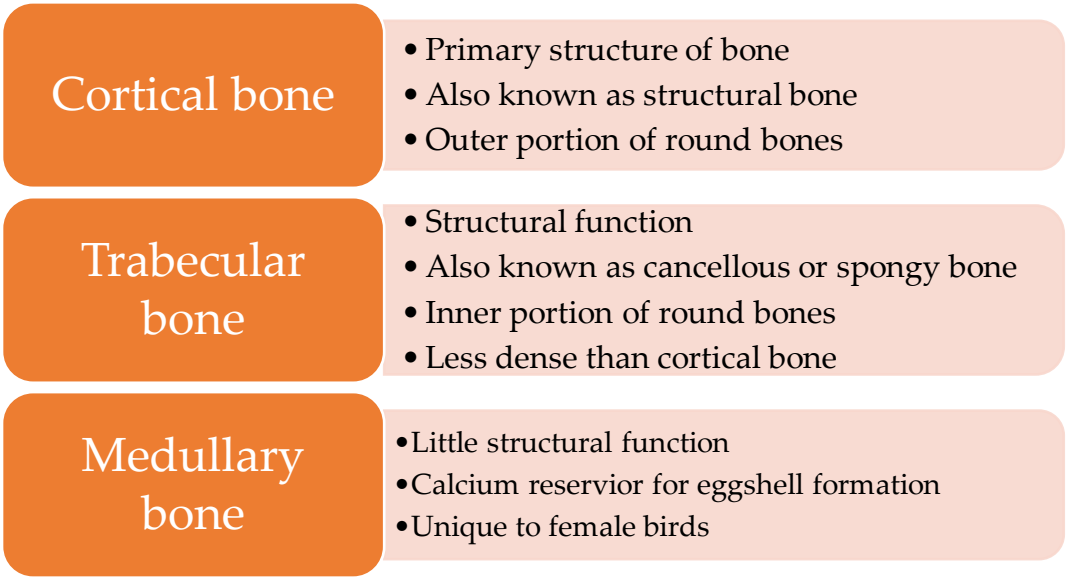

Figure 1.2. Schematic of the three bone types found in laying hens. Adapted from Soriano (2021) [43].

Bone growth during the laying phase

When pullets reach a sexually mature age (around 18 weeks of age), osteoblasts cease making cortical bone due to a surge in estrogen and begin producing medullary bone that is laid down within the cortical bone, specifically in the leg bones (Figure 1.3) [35]. Medullary bone is only found in female birds and crocodilians. Its purpose is to serve as a dependable calcium source for eggshell formation, rather than offering a structural support to the skeleton [35]. Medullary bone accumulates quickly during the initial stages of the laying phase and will amass continually throughout the laying phase [35]. Hens can only remodel cortical bone when estrogen concentrations are low, such as during breaks from the production cycle [35]. Genetic selection for high egg production means laying hens remain in a reproductively active state for an extended period (i.e., little to no interruptions in the production cycle), with some strains producing about 300 eggs per year [39,40]. Egg production can be biologically demanding, requiring copious amounts of calcium within the diet to sustain the high number of eggs laid by a single hen. Laying

hens require approximately 2.2 grams of calcium to form one eggshell [41]. About two-thirds of the required calcium is absorbed intestinally from the hen's diet, and the other one-third is resorbed by osteoclasts from the medullary and some cortical bone [41–43]. The calcium demand is highest when the egg is within the shell gland, which typically occurs at night when there is little calcium supplied from the digestive system [35,41]. The lack of calcium absorbed from the digestive system at night means that a much higher amount of calcium is resorbed from the medullary bone to form the eggshell [35,41]. Because osteoclasts are non-discriminative, some calcium is resorbed from the cortical bone alongside the medullary bone [35]. While medullary bone is metabolically active and has a high turnover rate, cortical bone has a much slower turnover rate [44]. Over time, the progressive decrease in cortical bone can increase hens' susceptibility to developing osteoporosis (i.e., a net resorption of cortical bone) [44]. Osteoporosis is a condition in which there is a progressive loss of mineralized cortical bone, which can cause bone fragility and susceptibility to fracture [44]. At the end of the production cycle, hens' have a very thin and fragile cortical bone, leading to several welfare concerns.

Factors Affecting Osteoporosis in Laying Hens

Modern housing for laying hens

Prior to streamlining housing, production, and genetics, laying hens were traditionally kept in small, free-range flocks. Over time, there was a movement towards intensive caged systems with birds in larger flocks in order to reduce disease and mortality. Mainly, this change occurred due to the demand for cheaper food after World War II because intensive housing was more economic and productive than the traditional free-range flocks. In recent years, with the rise in wealth and health among Europeans, there has been a heightened concern for the welfare of

agricultural animals, prompting a reassessment of our production systems to transition away from caged egg production. After 2012, producers in the United Kingdom were no longer able to house laying hens in conventional cages, but instead use alternative housing systems [45]. In the United States, conventional cages are still legal and many large companies still purchase their eggs from producers that utilize caged housing systems, but much of this is being phased out due to a consumer push for improved animal welfare standards (i.e., McDonalds moved to 100% cage-free eggs as of 2024 [46]). At the beginning of 2023, approximately 34.6% of U.S. table egg-laying flocks were cage free, with many states phasing out conventional cage systems (i.e., California, Oregon, Washington, Michigan, Massachusetts, Ohio, Rhode Island) [47].

There are five main types of housing systems for laying hens, which can be divided into two systems, cage and cage-free. Cage systems include conventional cages and furnished cages, while cage-free systems include barn management, aviaries, and conventional free-range or organic housing systems. Conventional cages (also known as battery cages) are comprised of a wire mesh sloped floor, automated feeders, drinkers, and egg collection, and houses around 6-7 hens per cage. Furnished cages (also referred to as enriched colony cages) share similarities with conventional cages, but with the addition of furniture to fulfill some motivations to perform natural behaviors, such as perches, nest boxes, and an area for various loose material to allow for foraging and dustbathing behavior [48]. Furnished cages typically allot more space than conventional cages [48]. Barns are usually one level, either with or without outdoor access, with litter or perforated floors and nest boxes. Aviaries are large buildings that house hundreds of thousands of hens that can move about freely within a tiered structure, increasing the amount of available vertical space [48]. Hens in this system also have access to perches, nest boxes, and wood shavings for bedding, which facilitates foraging and dustbathing behavior [48]. Conventional free-range and organic

housing systems differ in terms of stocking density in the European Union, where conventional allows 9 birds/m^2, but organic systems allow only 6 birds/m^2 [45]. Organic systems typically are awarded the label through a voluntary certification program, for example, eggs labelled with the USDA National Organic Program come from cage-free housing systems that have outdoor access, where hens are fed an organic diet of feed produced without pesticides or fertilizers [49].

Housing and exercise

The effects of exercise and type of housing system on the musculoskeletal system of laying hens are well established. As of December 2022, 65.9% of laying hens were housed in conventional cages in the U.S. [50]. Conventional housing systems have received criticism from consumers because they do not provide an environment that allows for the performance of natural behaviors, subsequently compromising hen welfare [51]. The low activity levels due to reduced freedom of movement results in weaker bones that are more susceptible to fracture than hens kept in larger housing systems with more freedom of movement [34,35,44,52,53]. For these reasons, the European Union banned the use of conventional cages for housing laying hens in 2012 [45]. Although conventional cages are not banned in the U.S., investigation into alternative systems is well underway as another option for housing laying hens. Alternative housing systems may provide a potential solution to improve bone health through increased freedom of movement.

Often, including opportunities to exercise is accomplished through the provision of perches or multiple tiers. Furnished cages and non-cage aviaries are two leading alternative housing system types that include perch access or are multi-tiered [48]. These housing systems aim to increase complexity, encourage activity, and provide areas to express natural behaviors [54]. Opportunities for exercise, such as jumping on and off perches, within complex alternative housing systems can

actually help strengthen the skeletal system through increased load-bearing exercise [54]. By providing space for exercise and subsequently stimulating the formation of structural bone, the integrity of the skeleton may be improved [55]. However, the freedom of movement offered in alternative systems can lead to increased collisions with other birds or structures within the housing environment [34,44,54]. As a byproduct of genetic selection, hens have a much smaller wing size to body ratio than their flight-adept ancestors, resulting in a more "clumsy" bird when navigating large and complex housing systems [54,56]. This uncovers a double-edged sword, where increased freedom of movement provides opportunities for exercise, performing natural behaviors, and strengthening bones, but also can increase the risk for trauma-related injuries. Therefore, the investigation of management strategies that improve bone development, but minimize the risk of physical damage is necessary.

Genetic selection

Modern laying hens (*Gallus gallus domesticus*) were domesticated from the red jungle fowl (*Gallus gallus*) that are native to south and southeast Asia around 9000 years ago [57,58]. Since then, there have been tremendous advancements in genetic improvement of the domestic chicken into a highly specialized laying hen. The junglefowl lays approximately 12 eggs per year and begins laying eggs around 42 weeks of age, while modern strains of laying hens produce more than 300 eggs per year and begin laying eggs around 16 weeks of age [59,60]. This results in a production output increase of 2400% over many years of genetic selection. But, this selection for productivity traits can lead to adverse side effects on behavior and physiology due to the reallocation of energy towards one or a few specific systems [61]. For example, hens are intensively selected for egg production, allocating most available resources towards reproduction.

However, we observe an undesirable impact on their adaptive immune response and genetic diversity [62,63].

Furthermore, modern laying hens have trouble adapting to new environments and coping with stressors, which could impact health and biological functioning [64]. Researchers believe that intense genetic selection for high egg production can contribute to skeletal issues, such as keel bone damage. However, specific strain, age, sex, nutrition, and physical exercise can all play a role in the development of bone fragility over time (Figure 1.4) [65,66]. Keel bone damage can manifest as either keel bone deviations (KBD) or keel bone fractures (KBF). KBD is characterized by a morphological change to the bone itself, such as an abnormally shaped keel not due to fracture, but due to pressure on the keel from perching on hard surfaces [67]. KBF are characterized as sharp, fragmented portions of the keel bone, affecting up to 97% of end-of-lay hens in alternative housing systems [68–70]. Strong genetic selection for high egg production may be linked to keel bone damage due to the early onset of lay and underdeveloped keel bone that is more susceptible to damage or fracture than a completely developed (ossified) keel bone [69,71,72]. Intense egg production may disrupt normal bone biology, leading to bone weakness in other areas of the skeleton, especially for birds at the end of the laying cycle.

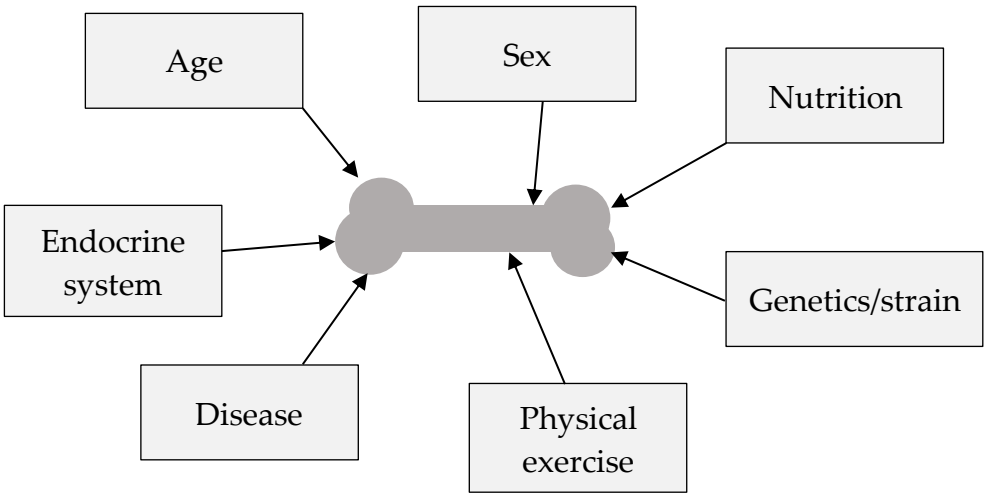

Figure 1.3. Factors affecting laying hen bone health.

Compounding welfare concerns

Osteoporosis is a significant animal welfare concern, as it can cause acute and chronic pain, as well as reduced mobility and production [35,44,73,74]. Old or healing bone fractures have been reported in 0 to 25% of birds, with nearly 100% having at least one fracture at the end of processing, reducing meat quality [75–78]. Therefore, osteoporosis is not just an animal welfare concern, but also an economic concern. The higher risk for bone fracture due to increased freedom of movement in alternative housing systems poses a major welfare concern. Furthermore, because hens have been so highly genetically selected for egg production, the resulting skeleton towards the end of lay is already weak, compounding the threat of bone fracture and other welfare issues. Given these health and welfare issues, it is imperative to delve deeper into management strategies to ease the physiological stressors placed on laying hens due to genetic selection for high egg production.

Interventions to Improve Laying Hen Bone Health

Environmental enrichment

Alongside the movement towards alternative housing systems for laying hens to increase behavioral expression and improve skeletal health comes some issues such as feather pecking, increased occurrence of keel bone damage, and mortality [79,80]. These undesirable behaviors and effects suggest that the biological needs of those laying hens are not met by current housing conditions or that the environment is not adequately designed for hens' locomotor or spatial navigation skills [81]. Providing environmental enrichments may ameliorate some of these behavioral issues and improve health as long as they are properly designed and biologically relevant. Newberry (1995) defined environmental enrichment as "an improvement in the biological functioning of captive animals resulting from modifications to their environment" [82]. Environmental enrichment aims to: "1) promote natural, species-specific behaviors, 2) minimize negative or abnormal behaviors, 3) improve the animal's ability to use their environment, and 4) improve the animal's ability to cope with stressors or challenges" [83]. van de Weerd and Day (2009) proposed that environmental enrichment should also encompass aspects of economics, stating that it should: "1) increase species-specific behavior, 2) maintain or improve health, 3) improve the economics of the production system, and 4) be practical to employ" [84]. There is ongoing investigation of practical enrichments for laying hens that increase complexity of the environment and improve their quality of life, health, and productivity. A few environmental enrichments evaluated for laying hens are ramps [85–87], swinging or flexible perches to improve balance and strength [88], brightly colored, stimulating novel objects to reduce fearfulness [89,90], and foraging substrates to promote species-specific ground pecking and foraging behavior [91,92]. Relevant to the present dissertation, perch provision has been suggested as an environmental

enrichment aimed to stimulate skeletal development and provide opportunity to express highly motivated perching behavior.

Provision of perches

The provision of perches within alternative housing systems is suggested as a solution to improve bone health of laying hens. Although perches are not required in housing systems within the United States, there are many recommendations for perch space per bird and height. For example, Certified Humane (a voluntary humane farm animal care certification program) requires 15cm of perch space per hen, and perches must be higher than 16cm and lower than 1m [93]. Perching involves load-bearing exercise, increasing the biomechanical load on the skeleton, thereby improving bone health [44,94]. In other words, the strength of a bone is dependent upon the load-bearing activity it experiences, and perching increases the amount of load-bearing activity performed by laying hens [95]. Aviary systems offer increased opportunity for exercise and can result in improved muscle and bone growth compared to pullets reared in conventional cages at 16 weeks of age [96,97]. Furthermore, pullets reared in cages with access to perches had higher mineral content of the tibia, humerus, and sternum and heavier leg muscle weights than caged pullets without perch access at 12 weeks of age, suggesting that providing access to perches positively affects pullet health by promoting the development of leg muscles and enhancing the mineral content of specific bones, all without compromising bone density [98]. Hens housed in an aviary system from 0-77 weeks of age had greater cortical thickness and density and stronger bones than hens reared in an aviary and moved to a conventional cage at 19 weeks of age, suggesting that movement limitation after the rearing phase causes a loss of bone mass and density [99].

The provision of perches during bone development (i.e., the rearing phase) may work as a proactive solution to slow or prevent the loss of structural bone later in life. By increasing bone mass before egg production, the skeletal system may better respond to calcium withdrawal during the lay phase and minimize the risk of future bone fractures. Metatarsal bones of 71-week-old hens with perch provision only during the rearing phase (0-17 weeks of age) were wider than hens without pullet perch access, possibly because of a higher activity level and load-bearing exercise during the crucial developmental stage when the skeleton undergoes rapid growth [100]. Aviary-reared hens had greater tibia and radius bone cross-sectional areas and mineral content compared to conventional hens at 73 weeks of age, implying that the aviary system provided enhanced exercise opportunities and led to better bone quality attributes by the end of the laying period [101].

Some researchers propose that the beneficial impact of perch provision to increase activity levels and improve bone health is not enough to prevent keel bone damage at the end of the lay period. For example, bone mineralization of 71-week-old hens improved when they had access to perches as adults (17-71 weeks of age). Nevertheless, the researchers observed a greater occurrence of keel bone deviations and fractures towards the end of the laying period. This suggests that while perch provision throughout the lay phase increased keel bone mineralization, this intervention was not sufficient to counteract the number of fractures in the keel at the end of the laying period [102]. Additionally, although cage-free environments improved tibia and keel bone integrity compared to conventional cages at 78 weeks of age, both housing types were associated with a high prevalence of keel deformities (>90%), suggesting that this positive impact is insufficient to prevent keel bone damage [103].

The beneficial impact of perches on the musculoskeletal health of birds can be strain-specific. For example, white-feathered strains exhibited heavier muscle weights and performed more

vertical transitions than brown-feathered strains in the same housing environment [104]. Additionally, white-feathered pullets exercised more in the rearing and laying phases and had higher odds of perching in the rearing phase than brown-feathered birds [105]. This indicates that strain can affect the types of locomotion that birds perform and could impact musculoskeletal development. In fact, tibia cortical thickness was greater in Barred Plymouth Rock hens compared to Hy-Line brown hens at 78 weeks of age [103]. A summary of previous studies investigating the impact of perch access on the musculoskeletal health of laying hens is provided in Table 1.2.

Table 1.2. Summary of studies evaluating the effect of perch provision on laying hen musculoskeletal health. WOA = weeks of age, BS = breaking strength, BMD = bone mineral density, BMC = bone mineral content, CSA = cross-sectional area, CT = computed tomography.

Aim/hypothesis	Treatment	Variables	Results	Reference
Find out if supplying perches for caged brown hens would enhance bone strength and/or volume	16 hens housed in cages with or without perches from 18-72 weeks of age	At 72 WOA: Tibiotarsi BS Tarsometatarsi bone volume	No effect of perches on tibiotarsal BS Both groups of hens exhibited signs of osteoporosis, though it was more pronounced in birds housed in conventional cages lacking perches → tarsometatarsal trabecular bone volume was larger in hens with perches	Hughes et al. (1993) [106]
Investigate the impact of perch availability on the health, bone mineralization, muscle development, and stress levels of caged White Leghorn pullets	From 0-17 WOA P: cages with 2 round metal perches C: no perches in cage	3, 6, and 12 WOA: Bone mineralization and size of the tibia, femur, sternum, humerus, ulna, radius, and phalange using DEXA Breast and left leg muscle weights Foot health Body weight Right adrenal weight Packed cell volume	Perch access did not impact breast muscle weight, % breast or leg muscle, bone length or width, BMD, packed cell volume, adrenal weight, or hyperkeratosis of footpad/toes No difference in body weight, BMC, and leg muscle weight at 3 and 6 WOA, but at 12 WOA: body weight, BMC of tibia, sternum, and humerus, and left leg muscle weight increased in P pullets *Access to perches positively affected health of pullets by promoting deposition of leg muscle and enhancing mineral content of specific bones, without reducing bone density*	Enneking et al. (2012) [98]
Investigate whether the use of metal perches throughout or during certain stages of the White Leghorn's lifespan influences	T1: no perch access T2: perch access during lay phase (17-71 WOA)	71 WOA: Muscle weight Bone mineralization	T3: Heavier muscle dep. Of 71 WOA hens T2: increased bone mineralization of 71 WOA hens; higher incidence of keel deviation and keel fractures at end of lay	Hester et al. (2013) [102]

musculoskeletal health at the end of their laying period	T3: perch access during pullet phase (0-17 WOA) T4: continuous perch access (0-71 WOA)	Bone fracture incidence Keel bone deviation	*Increase in keel bone mineralization as a result of pullet and lay phase perch access was not beneficial enough to stop a greater incidence of keel bone fractures at end of lay*	
Investigate the impact of perch availability throughout or during specific life stages on physiological balance in caged White Leghorn hens	T1: no perch access T2: access to perches during lay phase (17-71 WOA) T3: access to perches during pullet phase (0-17 WOA) T4: continuous perch access	71 WOA: Plasma catecholamines and corticosterone Blood serotonin and Trp Fluctuating symmetry of shank length and width Adrenal weight	T3 hens had wider shanks than T1 *Early perch access improved skeletal development* *No stress response observed in 71 WOA hens in T4 compared to hens in other groups*	Yan et al. (2014) [100]
Investigate how different housing systems affect the bone quality of pullets	White leghorns from 0-16 WOA: Cage free aviary (AV) Conventional cages (CC)	At 16 WOA: Cortical bone density and thickness Periosteal and endosteal dimensions Serum osteocalcin and hydroxylysyl pyridinoline measured as markers of bone formation (at 4, 8, 12, 16 WOA)	Cortical bone density was higher in AV humeri and tibiae were denser in AV in the distal section compared to CC Greater humeri ash content in AV, no difference in tibae ash Denser cortex of tibiae and humeri in AV than CC Greater second moment area of tibiae and humeri in AV than CC Osteocalcin concentrations not different, but hydroxylsyl pyridinoline was higher in CC at 12 WOA than AV → effect switched for 16 WOA *The tibiae and humeri exhibit varied responses to weight-bearing activities during growth* *Enhanced weight bearing capability and stiffness in AV pullets are linked to augmented cross-sectional geometry*	Regmi et al. (2015) [97]
Determine the housing and strain effects on bone properties	Hy-line brown Hy-line silver brown Barred Plymouth rock hens in: Conventional cages (CC) Cage-free (CF) Cage-free with range access (R)	At 78 WOA: Dry weight % ash content Cortical density Cortical thickness Keel deformities	Tibiae cortical thickness was greater in barred Plymouth rock compared to Hy-line brown and silver No effect of housing on femur cortical density, but it was greater for middle and distal tibia of birds in R than CC Keel cortical density greater in CF and R than CC Housing system is linked with a high prevalence (over 90%) of keel deformities, and both housing conditions and genetics play a role in the specific type of deformity observed *Range and cage-free systems might have a positive effect on integrity of the tibia and keel bone compared to conventional cages, but the improvement might not be adequate to entirely prevent fractures or deformities of the keel bone*	Regmi et al. (2016) [103]
Investigate how housing systems affect the tibiae and humeri of Lohmann white hens	Pullets reared in aviary or conventional cages and transferred at 19 weeks to: Aviary (AV)	At 77 WOA: Cortical thickness Cortical density geometric properties	Greater cortical thickness and density in AV, but not different outer dimensions to AC EN had similar humeri cortical thickness and density, but wider outer dimensions than CC	Regmi et al. (2016) [99]

	Aviary reared-conventional cage adult (AC) Conventional (CC) Conventional cage reared-enriched colony cage adult (EN)		Tibial cortical geometry was same for EN and CC, but EN had denser tibial cortex Increased second moment of area in humeri of AV and EN than AC and CC AV hens had greater failure moment and stiffness than AC, same difference between EN and CC *Restricting movement leads to a decline in bone mass and density, whereas allowing moderate movement enhances certain bone quality parameters during adults hood*	
Investigate if various exercise opportunities during pullet rearing affect long-term bone quality characteristics in hens at the end of their laying period	Lohmann selected leghorn-lite pullets reared in: conventional or aviary system then transferred at 16 WOA to: Conventional (CC) Aviary (AV) FC-L (large furnished cage) FC-S (small furnished cage) Conventional cages (CC)	At 73 WOA: Wing and leg bones collected for CT and BS measures	AV hens exhibited larger total and cortical CSA for radius and tibia, higher total BMC of the radius, and greater cortical BMC of the tibia than CC hens Total and cortical BMD of the radius and tibia were greater in CC hens FC-L hens had greater total BMD for radius and tibia, and greater trabecular BMD for the radius than FC-S and CC Total BMC of tibia and cortical BMC of radius and tibia were greater in FC-L than CC Humerus of CC hens had greater BS than AV hens Tibia of FC-L and FC-S hens had greater BS than CC hens *The increased opportunities for exercise provided by the aviary rearing system resulted in improved bone quality characteristics throughout the laying period*	Casey-Trott et al. (2017) [101]
Determine whether differing chances for exercise during rearing influences pullet musculoskeletal characteristics	Lohmann Selected Leghorn-lite pullets were reared in either conventional cages or aviary system from 0-16 WOA	At 16 WOA: Keel bone and its muscles, radius, humerus, and tibia were dissected for CT and BS measures	Aviary pullets had greater keel metasternum and caudal tip cartilage lengths, and higher % of cartilage present than control Wing and breast muscle weights were greater in aviary vs control, but leg muscle weights were greater in the control Aviary had greater total bone density, total CSA, cortical CSA, total BMC, and cortical BMC than control pullets for the radius, humerus, and tibia Aviary pullets had greater BS compared to control for all bones *Enhanced chances for physical activity provided by the aviary rearing system led to increased muscle and bone development in pullets at 16 WOA*	Casey-Trott et l. (2017) [96]
White-feathered strains and pullets raised in the most intricate system were expected to demonstrate enhanced locomotion and musculoskeletal traits in comparison to brown-feathered strains and those reared in simpler systems	S1 (simplest) 3-tiered wire-floored brooding compartment with litter floors, terraces, ramps, and perches available at 37 days of age S2: wire-floored brooding compartment with three round metal	25, 68, and 112 days of age: Locomotion Muscle weights Breaking strength of tibia, femurs, radius, humerus	S3: most time spent locomoting during rearing, white strains in S3 performed highest rate of vertical transitions No difference in muscle weight between style White strains had heavier pectoralis major, minor, and lighter leg muscles than brown strains White strains and pullets in S3 had stronger tibiae and femurs than brown strains and pullets in S1	Pufall et al. (2021) [104]

	perches and a raised platform S3 (most complex): open-concept system with 6 perches and vertical panels that could become additional ramps or platforms		No differences in radius or humerus BS *Breed and variations in design of rearing aviaries influences the types of movements that pullets engage in, which could potentially affect their skeletal development*	
Investigate how environmental complexity during early life and genetic strain influence space utilization and exercise patterns: 1) chicks raised in highly complex brooding compartments expected to engage in more exercise, particularly activities involving wing-bearing loads, and utilize perches more frequently in the brooding phase, 2) pullets reared in aviaries with high complexity are anticipated to engage in more exercise and perching during the laying phase, 3) level of exercise and perching was expected to be higher in white strains compared to brown	Four brown- and white-strained flocks raised in 3 styles of aviaries with low, intermediate, or high complexity	Behavioral observations (weeks 1, 3, 5, 7, 11, 17): aerial locomotion, perching, dynamic load-bearing behavior, and wing-involved load-bearing behavior	During rearing, chicks in high complexity exercised most frequently → effect remained for white strains, but not brown, during the lay phase White pullets exercised more than brown pullets in rearing and laying phases White pullets had higher odds of perching than brown throughout rearing *Design of rearing aviaries can influence behavior during the rearing phase, but housing distinctions primarily impacted white pullets during the laying phase*	Rentsch et al. (2023) [105]
A review detailing the effects of cage and cage-free housing systems on critical welfare aspects for laying hens, including musculoskeletal health, disease susceptibility, feather pecking, and behavioral expression				Hartcher et al. (2017) [114]
A review detailing prevalent and successful strategies for enhancing farm environments to alleviate stress, enhance welfare, and boost productivity in laying hens				Xu et al. (2022) [108]
A review summarizing the skeletal health of laying hens across various housing systems				Campbell (2022) [54]
A review detailing studies on poultry perching and exploring the connection between perch design,				Bist et al. (2023) [112]

Nutritional enrichment

Although bone health is influenced by genetics and environment, nutrition can play a role in alleviating the welfare concerns observed in laying hens. If a nutritional intervention is not implemented until the laying phase, the effects will only be observed on medullary bone formation (13). This may not be ideal since the progressive loss of cortical bone ultimately leads to bone fragility and osteoporosis. Therefore, it is essential to provide nutritional interventions during the rearing phase when the skeleton is developing to observe a beneficial impact [110]. Chicks require at least 38 nutrients alongside appropriate metabolizable energy and water [111]. Macrominerals, such as calcium, phosphorus, sodium, potassium, manganese, chlorine, and sulfur, are required in the diet in large amounts [111]. Microminerals are required in small amounts in the diet and consist of copper, iodine, iron, manganese, selenium, and zinc [111]. Vitamins are required in small amounts within the diet because poultry cannot synthesize them [111]. Fat-soluble vitamins, such as vitamins A, D, E, and K, are kept within the body for extended durations, meaning they are needed intermittently within the diet, but raise concerns for toxicity due to their long storage time [111]. Water-soluble vitamins (thiamin, riboflavin, niacin, pantothenic acid, pyridoxine, biotin, folic acid, cobalamin, and choline) are not stored in the body for very long, thus need to be supplied within the diet frequently for normal energy and nutrient metabolism, health, and productivity [111].

During the rearing phase, appropriate inclusion rates of calcium, vitamin D, and phosphorus is essential for ensuring high bone quality [112]. Calcium is required at approximately 9g/kg of feed during the early rearing period and should be increased in the diet as the pullets reach

sexual maturity [112]. Calcium inclusion may help minimize cortical bone loss during the developmental stage. Vitamin D's active metabolite, 25-hydroxyvitamin D_3, is commercially available and has been studied to improve skeletal characteristics during the rearing phase due to its involvement in calcium and phosphorus absorption. One study discovered that 25-hydroxyvitamin D_3 supplementation during the rearing phase improved bone growth, increased bone size, and allowed for more mineral deposition within the cortical bone during the laying phase [113]. The ratio between calcium and phosphorus in poultry diets is quintessential, ensuring a 2:1 ratio of calcium to phosphorus [112]. Aside from these macrominerals and vitamins, some microminerals have been investigated to improve bone health. Zinc plays a vital role in the growth and development, bone health, egg quality, and immune function of laying hens. For example, zinc-methionine supplementation has shown to improve tibia cortical thickness in laying hens [114]. Copper plays a role in a multitude of physiological functions, including bone metabolism. In fact, a copper-dependent enzyme is responsible for initiating the process of covalent cross-linkage formation in elastin and collagen, crucial components of bones and other connective tissues [115]. A copper deficiency can cause bone loss, demineralization, and failure of bone to ossify due to reduced osteoblast function [116,117]. Manganese is essential and contributes significantly to growth, bone development, prevention of perosis, eggshell quality, and the maintenance of good performance [118]. Zinc, copper, and manganese are all constituents of proteins involved in intermediary metabolism, hormone secretions, and the immune system, which would have a beneficial effect on poultry health and biological functioning [119].

Aside from the required nutrients, some non-nutrient feed additives can be added to the diet for specific purposes or functions. For example, antioxidants can protect vitamin integrity and unsaturated fatty acid oxidation [111]. Other exogenous enzymes can increase nutrient availability

(e.g., phytase) or decrease the antinutritional effects of certain ingredients [111]. Probiotics have been shown to improve bone quality in broilers [120] and laying hens [121]. Essential fatty acids might have a significant role in sustaining a robust skeletal system and could enhance the ash content of laying hen tibiae [122]. Ultimately, there is ongoing investigation of many nutritional solutions to improve poultry bone health aside from the required nutrients found within a typical poultry diet.

Boron

Boron is a trace element and has been studied as a feed additive in broilers, but not recently in laying hens to determine its effects on bone health. Functions of boron may include increased growth rate, retention of calcium and phosphorus, and decreased vitamin D deficiency in broiler chickens [123]. The most recent study conducted in laying hens (in 2012) discovered that boron supplementation had a beneficial impact on bone resistance and copper supplementation improved eggshell quality [124]. In that study, boron supplementation at 60, 120, or 240mg/kg promoted some trace element (B, Cu, and Zn) deposition in the bones without impacting the amounts of Ca, P, and Mg, which may have led to the increased cortex thickness, shear force, stress, and fracture energy of the bones [124]. Furthermore, other older studies suggest that boron plays a role in the metabolism of calcium, which improves bone strength and subsequently reduce the incidence of fractures [125,126]. In laying hens, adding boron to the basal diet has been shown to improve bone characteristics, such as tibia calcium levels [127,128], calcium retention [129], shear stress of the tibia, shear fracture energy of the femur, tibia ash content [128,130], and even some egg quality parameters [128,129]. Given results from previous studies, it is imperative to gain a better understanding of how boron supplementation may play a role in skeletal health of the modern

pullet and laying hen. Previous studies investigating the effects of boron supplementation on various parameters, including bone health, of pullets or laying hens are summarized in Table 1.3.

Table 1.3. Summary of studies evaluating boron as a feed additive in pullets or laying hens. WOA = weeks of age, B = boron, CSA = cross-sectional area, FCR = feed conversion ratio.

Aim/hypothesis	Treatments	Variables measured	Results	Citation
Effect of boron on White Leghorn tibia, humerus, and radius bone strength characteristics	White Leghorns (last 28 days of production): B supplemented at 3.5, 7,14, 28, and 56mg/kg	Shear force, stress, fracture energy, CSA of tibia, radius, and humerus B levels in tissue samples Egg production	No significant effects on shear force, stress, fracture energy, CSAand body weight B found in breast, liver, and thigh	Wilson & Ruszler (1995) [138]
Effect of boron supplementation on laying hen egg production and tiba, femur, humerus, and radius qualities	White Leghorns (last 84 days of production): B supplemented at 0, 100, 200, and 400mg/kg	Shear force, stress, and fracture energy of the tibia, femur, radius, and humerus Body weight and feed consumption Egg characteristics Bone ash content B, calcium, and phosphorus concentrations in tissue samples	No effects on shear force, stress, and fracture energy Egg production, feed consumption, and body weight decreased at 400mg/kg B B increased in tissue samples in birds fed 400mg/kg B	Wilson & Ruszler (1996) [139]
Effect of boron on growing pullets	White Leghorn pullets: 50mg/kg B 100mg/kg B 200mg/kg B	Ash content Ultimate shear force, stress, and fracture energy of the tibia, femur, humerus, and radius	Tibia shear stress increased at 50 and 100mg/kg B Femur fracture energy increased at 50 and 100mg/kg B Tibia bone ash content increased at 50, 100, and 200mg/kg B, with the greatest at 50mg/kg B	Wilson et al. (1997) [130]
Long-term impact of boron on egg production and tibia and radius characteristics	White Leghorn (16 or 32-72 WOA): B supplemented at 50, 100, 200, and 400mg/kg	72 WOA: Body weight Feed consumption Ca, P, and B content of tissues Egg characteristics Tibia ash percent Tibia and radius qualities	Tibia shear force and stress increased with B supplementation at 32 WOA Tibia and radius shear fracture increased for birds fed 200mg/kg B starting at 32 WOA Decrease in body weight at 400mg B B concentration in the breast, liver, and thigh increased with increasing B Egg production and weight negatively impacted by 400mg/kg B	Wilson & Ruszler (1998) [137]
Effect of boron on performance of laying hens	Hysex-Brown layer hybrids (40 WOA): 0, 50, 100, 150, 200, 250 ppm B	Feed consumption FCR Egg production Body weight Egg weight Specific gravity Damaged egg ratio Biochemical characters	B supplementation did not impact egg production, egg weight, specific gravity, feed consumption, body weight Serum Ca increased at 250mg/kg B No difference between control and B in serum Mg and P values	Kurtoglu et al. (2006) [136]

Effect of boron on laying hen egg production, egg quality, performance, and bone characteristics	Barred Rock (4-64 WOA): B supplemented at 0, 25, 50, 100, and 200mg/kg feed	Egg production Egg weight Cracked eggs Body weight Egg quality parameters Tibia and femur strength Ash and calcium content of tibia and femur	50, 100, and 200mg/kg B showed decreased body weight than 0mg/kg at 64 WOA Albumen height and Haugh unit benefitted at 25 or 50mg/kg B Femur strength, tibia and femur ash and calcium content increased at 25 and 50mg/kg B	Mizrak et al. (2010) [128]
Determine effects of boron and copper on laying hen bone biomechanical properties, eggshell, qualities, and mineral concentrations in bone and plasma	Lohmann laying hens (26 WOA): B (0, 60, 120, 240mg/kg) Copper (0, 75, 150, 300mg/kg) For 16 weeks	Eggshell quality parameters Bone biomechanical properties Tibia and plasma mineral concentrations	B reduced eggshell thickness and improved trace element (B, Cu, Zn) distribution without negatively affecting bone Ca, P, and Mg B increased bone resistance (increased cortex thickness, shear force, shear stress, and fracture energy)	Olgun et al. (2012) [124]
Impact of dietary boric acid and boric and ascorbic acid together on laying hen egg traits, performance, blood serum, and egg yolk cholesterol concentrations and bone characteristics	Hy-Line white hens (59-65 WOA): Basal control diet Ascorbic acid (AA) supplement at 200mg/kg Boric acid (BA) supplement 120mg/kg AABA- supplement 200mg/kg AA + 120mg/kg BA	Body weight Feed efficiency Egg weight Eggshell index Egg breaking strength Eggshell thickness Egg albumen index Egg yolk index Egg Haugh unit Egg yolk weight Tibiae crude ash and phosphorus	Serum cholesterol concentration was reduced with ascorbic and boric acid supplementation either alone or combined Ascorbic and boric acid increased tibia calcium levels	Sizmaz et al. (2016) [127]
Determine the effect of boron as a feed additive to a diet deficient in calcium under standard management guidelines	White Leghorns (25 WOA): NC: Normal calcium LC: Low calcium NCB: Normal calcium with 40ppm B LCB: Low calcium with 40 ppm B	FCR Eggshell thickness Cracked egg production	FCR positively influenced by LCB Eggshell thickness was higher in B supplemented groups regardless of Ca Cracked egg production decreased with B than Ca inadequate groups B benefitted Ca retention regardless of Ca in diet LC had decreased retention of magnesium and B	Adarsh et al. (2021) [129]

Laying Hen Welfare Assessment

In order to accurately evaluate laying hen welfare, the three conceptions of animal welfare should be applied. The present dissertation aimed to measure all three: affective state, natural living, and biological functioning. Affective state, referring to an individual animal's long-term mood state, was evaluated through an attention bias test, which will be discussed in depth within

the following section. Natural living discusses the ability of a captive animal to express natural behaviors and live in an environment similar to which it would in the wild. This conception was achieved through the provision of perches to elicit opportunities to express highly motivated, species-specific perching behavior. Biological functioning focuses on measures of health and physiological function, which was investigated in pullets and laying hens through various bone health and development parameters.

Affective state

Affective states are characterized as enduring emotional states that persist without being triggered by a specific stimulus, event, or object [135]. More specifically, affective states are "the outcome of the accumulation of short-term emotional experiences, resulting in a 'running mean' of positions occupied across scales of valence and arousal over time" [136–138]. The overall positivity or negativity of temporary emotions experienced by an animal over its lifetime shapes its affective state. However, an animal in a positive affective state may still temporarily experience negative states due to punishment (e.g., loss of prey, hunger) [136]. Cognition is defined as the "mechanism by which animals acquire, process, store, and act on information from their environment" [139]. Affective states and cognitive processing often influence one another through overlapping brain regions [140–143]. Negative affective states will cause individuals to perceive ambiguous stimuli negatively [144], exhibit a greater tendency to concentrate on threatening stimuli, and recall negative memories more rapidly compared to those in positive affective states [145,146]. For example, an animal experiencing a negative affective state due to living in a threatening environment will interpret an ambiguous stimulus, such as a rustle in the grass, as a potential danger (like a predator). It will recall past experiences and react to seek safety [136,147].

In a similar scenario, an animal experiencing a positive affective state due to living in a comfortable environment may perceive the same ambiguous stimulus as a positive opportunity, such as finding food or encountering a potential mate. It will recall past experiences in similar situations and take action to obtain the food reward or attract a potential mate. When an animal's affective state influences cognitive functions, like judgement or attention, it is termed as a "cognitive bias" [143,148–150]. These biases serve as indicators of an animal's affective state and welfare, offering insights into how the animal perceives its environment [136,139,143,151–154].

Attention biases are described as "the differential allocation of attentional resources towards one stimulus compared to another" [149]. Anxiety is classified as an affective state disorder in humans, leading to heightened focus on adverse information [149]. For example, subjects with anxiety tend to direct their attention more towards threatening stimuli compared to subjects without anxiety [146,155]. Increased anxiety levels can be assessed by observing vigilance behaviors such as scanning, alertness, and posture, with vigilance being more evident in threatening situations than in non-threatening ones. For instance, cattle exhibit increased vigilance, indicated by spending more time with their heads upright, when subjected to threatening situations (aversive handling) compared to neutral situations (gentle handling) [156]. Thus, attentional biases can serve as indicators of how animals perceive and are able to cope with their housing environment and, consequently, their welfare [149].

A common method of attention bias testing is known as the "attentional probe or "dot probe" task [157]. In this test, two stimuli are simultaneously given to the animal: one positive (like high-value feed) and one negative (like a conspecific alarm call signaling a potential threat) [157]. By observing the animal's behavior afterwards, we can determine whether its attention is skewed towards either stimulus. For instance, if the animal takes longer to start feeding and exhibits

heightened vigilance behavior, this may indicate an attention bias towards the negative stimulus (potential threat) rather than the positive stimulus (feed), suggesting a negative affective state. In laying hens, attention bias testing was pharmaceutically validated, where hens receiving an anxiogenic drug exhibited responses consistent with increased anxiety compared to control hens receiving saline injections [158]. Similarly, hens that preferred to stay indoors showed higher attentional biases towards a conspecific alarm call than hens that preferred to range outdoors [159]. A table summarizing studies that evaluate laying hen affective state through an attention bias test are included in Table 1.4.

The provision of perches may improve laying hen affective state, as they are highly motivated to perform this behavior. Hens have exhibited a strong desire to access perches, as they have been observed pushing through weighted doors to access perches [20]. Providing housing systems that meet hens' motivational needs and induce positive experiences could prevent negative affective states. Hence, the capacity to engage in highly motivated, species-specific behaviors could promote positive affective states and consequently, good animal welfare. Furthermore, the inability to perform highly motivated behaviors early on in life may have long-term negative effects on the psychological well-being of laying hens. The early rearing environment is essential for a pullet's adaptation to their adult environment, which can impact how they cope with stressors and the ability to navigate a potentially complex environment [160]. Pullets are often reared with little to no complexity or enrichments within their environment and are transferred from these simple rearing environments to a complex adult aviary. This transition may impact their physiological well-being differently compared to pullets who are housed in the same environment for their entire life.

Table 1.4. Summary of studies evaluating laying hen affective state through an attention bias test.

Stimuli	Treatment	Measures of attention bias	Result	Reference
Conspecific alarm call (negative) and feed (positive)	N: anxiogenic drug C: saline	Latency to first step, vocalize, and feed following first and second playback of an alarm call # of steps and vocalizations Time spent eating	N had longer latencies to feed after both alarm calls than C No impact on first step and vocalizations after first call or latency to step after second call N had longer latencies to vocalize after second call than C Greater number of steps and vocalizations in N group than C C spent a longer time feeding than N	Campbell et al. (2019) [158]
Conspecific alarm call (negative) and mixed grain (positive)	N: hens that prefer to stay indoors P: hens that prefer to range outdoors	Latency to first step, vocalize, and feed following playback of an alarm call (hens must eat prior to playing the alarm call)	N had longer latency to step than P P had longer latency to vocalize than N Fewer P hens did not eat at all compared to N hens Of the N group that did eat, only 7% resumed eating after the alarm call playback compared to 36% of P hens	Campbell et al. (2019) [159]
Conspecific alarm call (negative) and feed, mealworms, and oats (positive)	N: conventional cages P: enriched floor pens	Number and latency to begin feeding following first alarm call, vigilant behaviors, Number and latency to resume feeding following second alarm call	Latency to begin feeding P > N Number of birds resume feeding N > P	Campbell et al. (2022) [161]

Bone health

There are a multitude of ways to quantitatively measure bone health in poultry. Traditionally, poultry bone health is measured *ex vivo*, as these techniques are invasive. For example, biomechanical strength testing to determine bone breaking strength and bone ashing to elucidate percent ash content require dissection of the bone of interest for further postmortem testing. Computed tomography (CT) is a technology used more widely within the poultry research field to measure bone health, as the technique is non-invasive. It is possible to perform CT scanning *in vivo* as long as the subject remains motionless for a short period, typically by physical restraint or an anesthetic. A summary of previous studies evaluating the impact of perch provision on laying hen bone health measured by CT, breaking strength, and ash percent is provided in Table 2. Ultimately, these measures of bone health are important to further our understanding of avian bone biology, especially for laying hens to help determine nutritional or management interventions to prevent bone loss.

Computed tomography

Traditional radiography is based on attenuation, which refers to the difference between x-ray energy emitted from its source and the energy received at the detector [37]. A radiographic image is 2-dimensional, based on the amount of X-ray energy that passes through an area of interest, and dependent upon tissue physical density and atomic composition. The X-ray attenuation is responsible for determining the coloration observed in radiographic and CT images. Low density tissues appear black (radiolucent, i.e., air or gas), high density tissues or materials with a high atomic mass appear white (radiopaque, i.e., bone, contrast agents such as barium or iodine), and intermediate density tissues appear grey. Because traditional radiography uses 2-

dimensional film, the resulting image per pixel is a weighted attenuation of all tissues the x-ray energy passes through [37]. This problem, where structures of interest overlap with other structures, is called superimposition and can be solved by determining X-ray attenuation from multiple angles through the use of computed tomography [37].

Computed tomography is also based on the attenuation of x-ray energy based on tissue density or atomic mass and provides information on the distribution of radiographic densities within an object of interest. The CT machine includes an X-ray tube, patient table, gantry with a ring of X-ray sensitive detectors, and a computer. The x-ray tube rotates around an opening through which the area of interest moves using a motorized table. The X-ray tube emits a beam of X-ray energy that passes through the area of interest at each table position using multiple rotations. The degree of X-ray absorption is recorded for each rotation by an array of X-ray energy detectors positioned opposite the X-ray tube. The CT operator creates a scout image and selects the beginning and end locations for the scan, as well as technical parameters such as slice thickness, field of view, and filter/algorithm [37]. A CT slice is created at each angle based on X-ray energy absorbed in each detector. Each slice is divided into voxels, and each voxel is converted into a 2-dimensional pixel for display. Modern CT software can create 3-dimensional models of the skeleton or area of interest [162]. In order to do this, the detectors convert the recorded X-ray energy into CT numbers for each voxel. CT uses Hounsfield units, a measure relative to the density of water, and provides a quantitative scale for describing the radiodensity of a certain object. A phantom with a known hydroxyapatite density value is included with the subject of interest to convert densities to CT values [162]. Low density tissues appear black (hypoattenuating), high density tissues appear white (hyperattenuating), and intermediate density tissues appear grey. Software processing allows scientists to view structures of interest within different anatomical

planes. Because CT involves acquiring scans in a single axial section from multiple angles, the pixel is not impacted by the density of neighboring tissues. The X-ray beam used to measure an area of interest has a beam width, which allows software processing to calculate volumetric bone density [37]. Furthermore, CT scanning allows for cortical and medullary bone width or cross-sectional area measurements [162]. A summary of studies evaluating laying hen bone health by computed tomography as a result of perch provision is included in Table 1.2.

When performing diagnostic imaging, a variety of artifacts may manifest causing issues with the interpretation of the image due to an abnormality (77, 78). A few of the common artifacts encountered are: motion, ring, metal, beam hardening, and partial volume averaging. Motion artifacts occur when the subject moves during image acquisition (i.e., breathing, swallowing, moving body part, heart pulsing) and can cause blurring and double contour [163]. A faster gantry rotation or more x-ray sources may reduce the effect of motion artifacts. Ring artifacts occur due to a miscalibration of one of the detectors within the CT machine, causing a bright ring to appear within the center of the image [164]. Ring artifacts can be minimized by recalibrating or replacing the detector [165]. Metal artifacts typically occur due to the presence of metals with a high atomic number (i.e., iron or platinum) and are caused by beam hardening, scatter effects, and poisson noise [165]. Beam hardening and scatter effects result in dark streaks appearing between two high attenuation objects (i.e., metal and bone), surrounded by bright streaks [165]. Beam hardening typically happens as a polychromatic x-ray beam passes through an object, where low-energy photons are selectively absorbed more than high-energy photons, resulting in predominately high-energy photons remaining. This results in "hardening" of the beam and results in dark streaks between high attenuating tissues or objects within the CT image [165]. Scatter effects occur because x-ray photons change direction and are absorbed by a detector other than the one originally

intended to receive them [165]. To minimize the effects observed by beam hardening and scatter, a filter can be applied so that the beam is hardened before it reaches the area of interest. Furthermore, increasing the energy of the x-ray beam or using iterative reconstruction algorithms may also help minimize the effects of these artifacts [165,166]. Poisson noise is the result of a statistical error resulting in low photon counts, and causes bright and dark streaks to appear towards the direction of greatest attenuation [165]. Effects from poisson noise can be reduced by using iterative reconstruction techniques or combining data from multiple scans [165]. Partial volume averaging artifacts occur when tissues of different absorption energy are on the same CT voxel, producing an average attenuation of the CT volumes [167]. This can cause blurring of the edges and reduced contrast within the image. To minimize this artifact, use a smaller CT slice thickness, as this improves the ability to distinguish between structures within the CT image [167].

Bone biomechanical testing by three-point bending test

Bones are anisotropic (having different properties in different directions) and viscoelastic (exhibiting both viscous and elastic characteristics). Its properties vary with direction for both cortical and trabecular bone and are time (rate) dependent. Therefore, the type of load applied during biomechanical testing influences the mechanical behavior of bone. The mechanical test frame is composed of a moving crosshead, load cell, machine base, and motor (Figure 1.5). The specimen is placed upon fulcra attached to the machine base. Once the test has begun, the load cell attached to the crosshead moves downwards at a preset speed toward the test specimen. The motor controls the rate at which the crosshead moves up or down. During the test, load and displacement values are recorded and stored in a data file. Long bones are tested under three-point bending or four-point bending scenarios. Three-point bending tests are typically used for homogenous

materials and will apply load at the midpoint of the specimen, which is supported by two fulcra (Figure 1.5). Because the load is placed in the middle of the specimen, the length to sample diameter ratio is important (≥10 is recommended) [168]. Four-point bending tests apply equal load at two points on the bone [169].

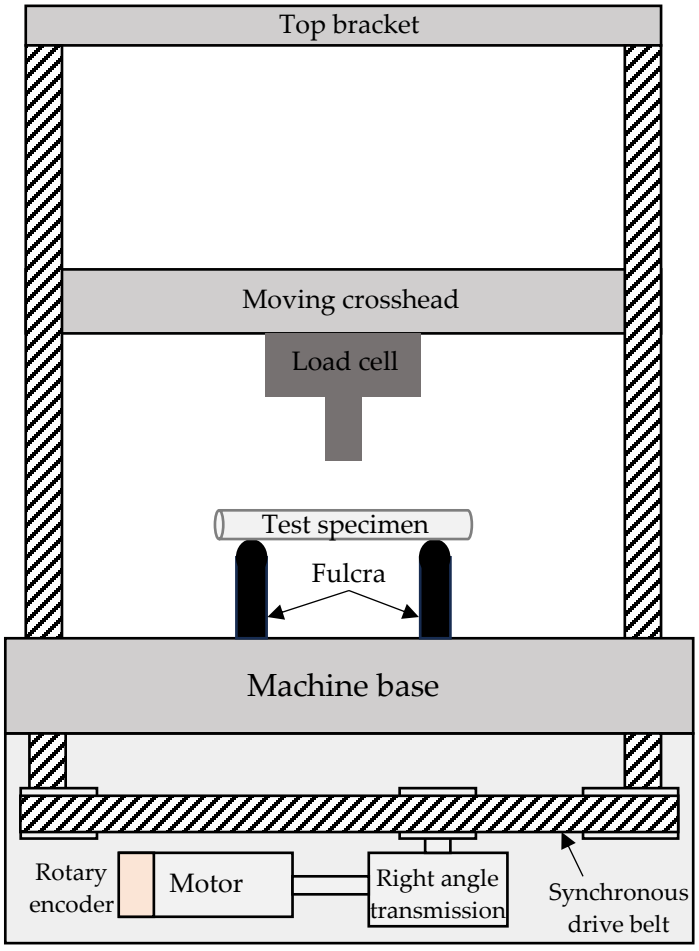

Figure 1.4. Schematic of mechanical test frame setup for a three-point bending test. Adapted from Awoyera et al. (2021) [172].

The resulting load vs. displacement plot provides the basic structural parameters of stiffness, yield load, ultimate load, post-yield displacement, and work to fracture (Figure 1.6). Stiffness refers to the amount a specimen will deform for a given applied force and corresponds to the slope of the linear portion of the load-displacement curve. Stiffness indicates the bone's resistance to displacement during the elastic region [170]. Elastic deformation disappears upon the removal of external force (i.e., the curve area to the left of the yield point in Figure 1.6), whereas plastic deformation remains even after the removal of external forces (i.e., the curve area to the right of the yield point in Figure 1.6). Yield load refers to the stress level at the yield point, marking the onset of plastic deformation of the material [171]. In other words, yield load measures how much force the specimen can withstand before it breaks [170]. A common characteristic of breaking strength is failure (ultimate) load, which refers to the amount of force required to break the bone [172]. Post-yield displacement refers to the displacement occurring from the yield point to the fracture point and serves as a measure of ductility [170]. Ductility is the ability of a material to deform plastically before fracture (i.e., the material can absorb great amounts of energy before fracturing). Work to fracture is the total area beneath the load-displacement curve and signifies the amount of work required to induce fracture [170]. Bone ash has been correlated with CT measures of bone mineral content in laying hens, therefore could be a useful tool in assessing mineral content in individual birds over a long period of time [162].

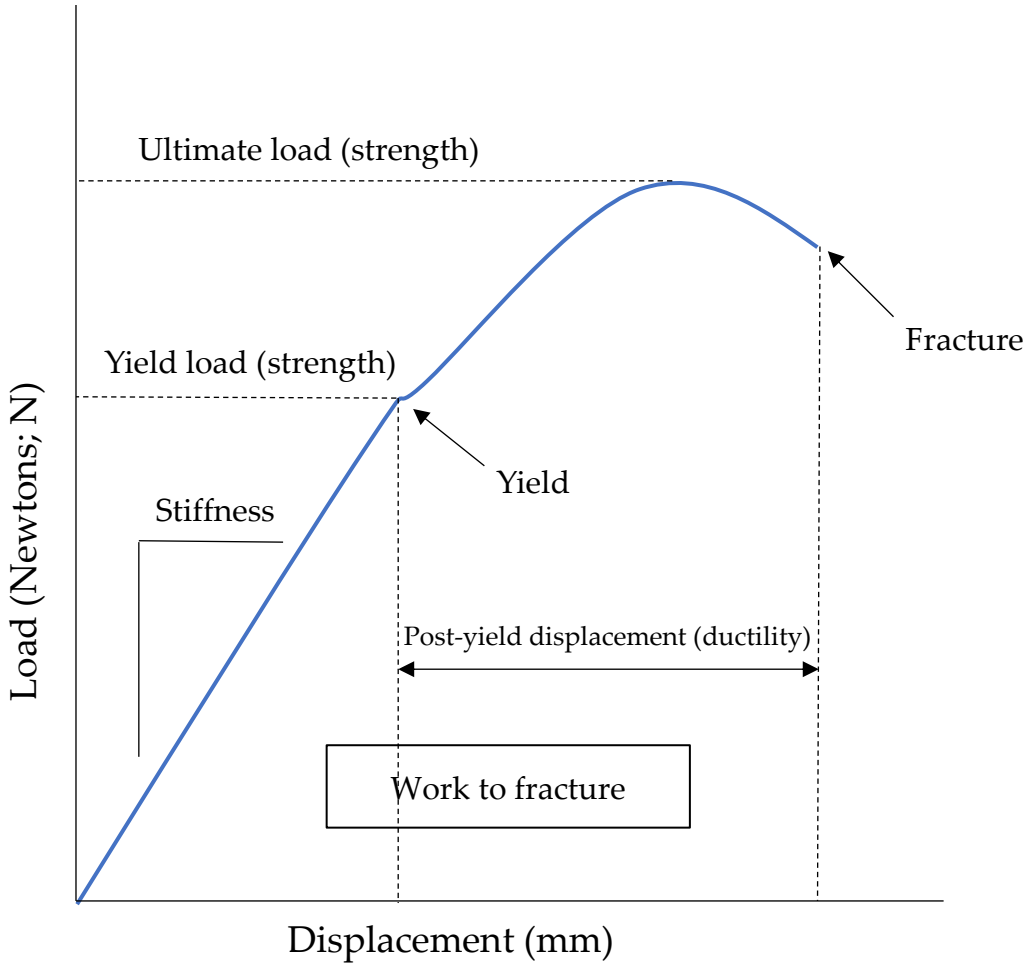

Figure 1.5. Load-displacement plot produced by biomechanical strength testing. Adapted from Silva (2016) [173].

Ash percentage

Bone is composed of the matrix and cells, such as osteoblasts, osteoclasts, and osteocytes. Organic and inorganic phases make up the bone matrix, with the organic bone matrix being 90% collagen and 10% amorphous ground substances (i.e., extracellular fluid). The inorganic phase makes up approximately 65% of the matrix's mass and is composed of hydroxyapatite ($Ca_{10}[PO4]_6[OH]_2$) [173]. Hydroxyapatite is mainly composed of calcium and phosphate [173]. The process of ashing bones leaves behind inorganic compounds (calcium and phosphate) and can be used as an indicator of bone mineralization [174]. Bones with a higher ash percent or content

contain more inorganic materials and thus have a higher rate of mineralization compared to bones with a low ash percent [175]. Bones with higher mineralization are stronger and healthier, and poor bone mineralization is associated with an increased fracture risk [175]. Therefore, ash percentage can be used to indicate overall bone health.

Aside from being a postmortem evaluation, there are some disadvantages to using ash percent as a measure of bone health. The type of bone used for ashing can impact results, as different bones are comprised of varying mineral amounts and may be more or less sensitive to changes in diet [176]. Furthermore, the method of sample preparation can influence ash determination data. The flesh and connective tissue must be removed prior to ashing. This can be performed in a variety of ways (i.e., enzymatic maceration [177], autoclaving [178], or boiling [179]) and is subject to variability between researchers performing the tissue removal. There are also an array of methods to extract the fat from the bone prior to ashing, such as soaking in diethyl alcohol for 48 hours [180], a two phase extraction process with ethanol for 48 hours followed by ethel for 48 hours [181], or a Soxhlet method for 16 hours [182], among other methodologies. These variations in methods for bone processing can impact results obtained from ashing and make it difficult to compare results between studies [183].

References

1. Carenzi, C.; Verga, M. Animal Welfare: Review of the Scientific Concept and Definition. *Ital J Anim Sci* **2009**, *8*, 21–30, doi:10.4081/ijas.2009.s1.21.
2. Broom, D.M. Indicators of Poor Welfare. *British Veterinary Journal* **1986**, *142*, 524–526, doi:10.1016/0007-1935(86)90109-0.
3. Broom, D.M.; Johnson, K.G. *Stress and Animal Welfare*; Springer Netherlands: Dordrecht, 1993; ISBN 978-0-412-39580-2.
4. Broom, D.M. A History of Animal Welfare Science. *Acta Biotheor* **2011**, *59*, 121–137, doi:10.1007/s10441-011-9123-3.
5. Dawkins, M.S. The Science of Animal Suffering. *Ethology* **2008**, *114*, 937–945, doi:10.1111/j.1439-0310.2008.01557.x.
6. Dawkins, M.S. From an Animal's Point of View: Motivation, Fitness, and Animal Welfare. *Behavioral and Brain Sciences* **1990**, *13*, 1–9, doi:10.1017/S0140525X00077104.
7. Bayvel, A.C.D. The OIE Animal Welfare Strategic Initiative–Progress, Priorities and Prognosis. In Proceedings of the Proceedings of the Global Conference on Animal Welfare: An OIE Initiative; Office International des Epizooties (OIE): Paris, France, 2004; pp. 12–16.
8. Animal Welfare Act 2006 Available online: https://www.legislation.gov.uk/ukpga/2006/45/contents.
9. Mellor, D.J. Updating Animalwelfare Thinking: Moving beyond the "Five Freedoms" towards "A Lifeworth Living." *Animals* **2016**, *6*, 21, doi:10.3390/ani6030021.
10. Lawrence, A.B.; Vigors, B.; Sandøe, P. What Is so Positive about Positive Animal Welfare?—A Critical Review of the Literature. *Animals* 2019, *9*, 783.
11. Fraser, D. Understanding Animal Welfare. *Acta Vet Scand* 2008, *50*, 1–7.
12. Weary, D.; Robbins, J. Understanding the Multiple Conceptions of Animal Welfare. *Animal Welfare* **2019**, *28*, 33–40, doi:10.7120/09627286.28.1.033.
13. Bracke, M.B.M.; Hopster, H. Assessing the Importance of Natural Behavior for Animal Welfare. *J Agric Environ Ethics* **2006**, *19*, 77–89, doi:10.1007/s10806-005-4493-7.
14. Appleby, M.C.; Mench, J.A.; Hughes, B.O. *Poultry Behaviour and Welfare*; CABI Pub., 2004; ISBN 9780851996677, 0851996671.
15. Appleby, M.C.; Duncan, I.J.H. Development of Perching in Hens. *Biology of Behaviour1* **1989**, *14*, 157–168.
16. Blokhuis, H.J. Rest in Poultry. *Appl Anim Behav Sci* **1984**, *12*, 289–303, doi:10.1016/0168-1591(84)90121-7.
17. Eklund, B.; Jensen, P. Domestication Effects on Behavioural Synchronization and Individual Distances in Chickens (Gallus Gallus). *Behavioural Processes* **2011**, *86*, 250–256, doi:10.1016/j.beproc.2010.12.010.
18. Beauchamp, G. The Evolution of Communal Roosting in Birds: Origin and Secondary Losses. *Behavioral Ecology* **1999**, *10*, 675–687, doi:10.1093/beheco/10.6.675.
19. Campbell, D.L.M.; Makagon, M.M.; Swanson, J.C.; Siegford, J.M. Perch Use by Laying Hens in a Commercial Aviary. *Poult Sci* **2016**, *95*, 1736–1742, doi:10.3382/PS/PEW111.
20. Olsson, I.A.S.; Keeling, L.J. Night-Time Roosting in Laying Hens and the Effect of Thwarting Access to Perches. *Appl Anim Behav Sci* **2000**, *68*, 243–256.

21. Skånberg, L.; Kjærsgaard Nielsen, C.B.; Keeling, L.J. Litter and Perch Type Matter Already from the Start: Exploring Preferences and Perch Balance in Laying Hen Chicks. *Poult Sci* **2021**, *100*, 431–440, doi:10.1016/J.PSJ.2020.11.041.
22. Wood-Gush, D.G.M. *The Behaviour of the Domestic Fowl.*; Heinemann Educational Books Ltd., 1971; ISBN 9780435.
23. Weeks, C.A.; Nicol, C.J. Behavioural Needs, Priorities and Preferences of Laying Hens. *Worlds Poult Sci J* **2006**, *62*, 296–307, doi:10.1079/WPS200598.
24. Shields, S.J.; Garner, J.P.; Mench, J.A. Dustbathing by Broiler Chickens: A Comparison of Preference for Four Different Substrates. *Appl Anim Behav Sci* **2004**, *87*, 69–82, doi:10.1016/j.applanim.2004.01.003.
25. Hughes, B.O.; Duncan, I.J.H. The Notion of Ethological "Need", Models of Motivation and Animal Welfare. *Anim Behav* **1988**, *36*, 1696–1707, doi:10.1016/S0003-3472(88)80110-6.
26. Vestergaard, K. Dust-Bathing in the Domestic Fowl - Diurnal Rhythm and Dust Deprivation. *Applied Animal Ethology* **1982**, *8*, 487–495, doi:10.1016/0304-3762(82)90061-X.
27. Vestergaard, K.S.; Damm, B.I.; Abbott, U.K.; Bildsøe, M. Regulation of Dustbathing in Feathered and Featherless Domestic Chicks: The Lorenzian Model Revisited. *Anim Behav* **1999**, *58*, 1017–1025, doi:10.1006/anbe.1999.1233.
28. Widowski, T.M.; Duncan, I.J.H. Working for a Dustbath: Are Hens Increasing Pleasure Rather than Reducing Suffering? *Appl Anim Behav Sci* **2000**, *68*, 39–53, doi:10.1016/S0168-1591(00)00088-5.
29. Campbell, D.L.M.; Dyall, T.R.; Downing, J.A.; Cohen-Barnhouse, A.M.; Lee, C. Rearing Enrichments Affected Ranging Behavior in Free-Range Laying Hens. *Front Vet Sci* **2020**, *0*, 446, doi:10.3389/FVETS.2020.00446.
30. Dawkins, M.S. Time Budgets in Red Junglefowl as a Baseline for the Assessment of Welfare in Domestic Fowl. *Appl Anim Behav Sci* **1989**, *24*, 77–80.
31. Moroki, Y. Impact of Flooring Type on the Sham Dustbathing Behaviour of Caged Laying Hens. *Appl Anim Behav Sci* **2020**, *230*, 105066, doi:10.1016/j.applanim.2020.105066.
32. Merrill, R.; Nicol, C. The Effects of Novel Floorings on Dustbathing, Pecking and Scratching Behaviour of Caged Hens. *Animal Welfare* **2005**, *14*, 179–186, doi:10.1017/S096272860002933X.
33. Duncan, I.J.H.; Wood-Gush, D.G.M. Thwarting of Feeding Behaviour in the Domestic Fowl. *Anim Behav* **1972**, *20*, 444–451, doi:doi.org/10.1016/S0003-3472(72)80007-1.
34. Janczak, A.M.; Riber, A.B. Review of Rearing-Related Factors Affecting the Welfare of Laying Hens. *Poult Sci* **2015**, *94*, 1454–1469, doi:10.3382/PS/PEV123.
35. Whitehead, C.C. Overview of Bone Biology in the Egg-Laying Hen. *Poult Sci* **2004**, *83*, 193–199, doi:10.1093/PS/83.2.193.
36. Nahian, A.A.A.M. Histology, Osteocytes. In *StatPearls [Internet]*; StatPearls Publishing: Treasure Island (FL), 2024.
37. Korver, D.R.; Saunders-Blades, J.L.; Nadeau, K.L. Assessing Bone Mineral Density in Vivo: Quantitative Computed Tomography. *Poult Sci* **2004**, *83*, 222–229, doi:10.1093/ps/83.2.222.
38. Hadjidakis, D.J.; Androulakis, I.I. Bone Remodeling. *Ann N Y Acad Sci* **2006**, *1092*, 385–396, doi:10.1196/annals.1365.035.

39. Dudde, A.; Krause, E.T.; Matthews, L.R.; Schrader, L. More Than Eggs – Relationship Between Productivity and Learning in Laying Hens. *Front Psychol* **2018**, *9*, doi:10.3389/fpsyg.2018.02000.
40. Alders, R.G.; Dumas, S.E.; Rukambile, E.; Magoke, G.; Maulaga, W.; Jong, J.; Costa, R. Family Poultry: Multiple Roles, Systems, Challenges, and Options for Sustainable Contributions to Household Nutrition Security through a Planetary Health Lens. *Matern Child Nutr* **2018**, *14*, doi:10.1111/mcn.12668.
41. Liu, Y.; Uyanga, V.A.; Jiao, H.; Wang, X.; Zhao, J.; Zhou, Y.; Lin, H. Effects of Feeding Strategies on Eggshell Quality of Laying Hens during Late Laying Period. *Poult Sci* **2023**, *102*, 102406, doi:10.1016/j.psj.2022.102406.
42. Bain, M.M.; Nys, Y.; Dunn, I.C. Increasing Persistency in Lay and Stabilising Egg Quality in Longer Laying Cycles. What Are the Challenges? *Br Poult Sci* **2016**, *57*, 330–338, doi:10.1080/00071668.2016.1161727.
43. Gloux, A.; Le Roy, N.; Même, N.; Piketty, M.L.; Prié, D.; Benzoni, G.; Gautron, J.; Nys, Y.; Narcy, A.; Duclos, M.J. Increased Expression of Fibroblast Growth Factor 23 Is the Signature of a Deteriorated Ca/P Balance in Ageing Laying Hens. *Sci Rep* **2020**, *10*, 21124, doi:10.1038/s41598-020-78106-7.
44. Whitehead, C.C.; Fleming, R.H. Osteoporosis in Cage Layers. *Poult Sci* **2000**, *79*, 1033–1041, doi:10.1093/PS/79.7.1033.
45. Council Directive 1999/74/EC.
46. McDonald's Achieves Goal of Sourcing 100% Cage-Free Eggs in the U.S. Available online: https://corporate.mcdonalds.com/corpmcd/our-stories/article/mcdonalds-achieves-goal-sourcing-100cagefree-eggs-us.html (accessed on 26 February 2024).
47. U.S. Egg Production and Hen Population Available online: https://unitedegg.com/facts-stats/ (accessed on 26 February 2024).
48. National Research Council Comparing Hen Housing Practices and Their Effects on Various Domains. In *Committee on a Framework for Assessing the Health, Environmental, and Social Effects of the Food System*; Nesheim, M.C., Oria, M., Yih, P.T., Eds.; National Academies Press (US): Washington, D.C., 2015.
49. Barnes, R. Eggstra! Eggstra! Learn All About Them Available online: https://www.usda.gov/media/blog/2012/04/06/eggstra-eggstra-learn-all-about-them#:~:text=Organic%3A%20Eggs%20marked%20with%20the,without%20conventional%20pesticides%20or%20fertilizers. (accessed on 26 February 2024).
50. United Egg Producers Facts and Stats 2023.
51. Appleby, M.C. The EU Ban on Battery Cages: History and Prospects. In *The state of animals II: 2003*; Salem, D.J., Rowan, A.N., Eds.; Humane Society Press: Washington, DC, 2003; pp. 159–174.
52. Whitehead, C.C. *Bone Biology and Skeletal Disorders in Poultry*; Poultry science symposium series; Carfax Publishing Co, 1992;
53. Fleming, R.H.; McCormack, H.A.; McTeir, L.; Whitehead, C.C. Relationships between Genetic, Environmental and Nutritional Factors Influencing Osteoporosis in Laying Hens. *Br Poult Sci* **2006**, *47*, 742–755, doi:10.1080/00071660601077949.
54. Campbell, D.L.M. Skeletal Health of Layers across All Housing Systems and Future Research Directions for Australia. *Anim Prod Sci* **2020**, *61*, 883–892, doi:10.1071/AN19578.

55. Sharma, M.K.; White, D.; Chen, C.; Kim, W.K.; Adhikari, P. Effects of the Housing Environment and Laying Hen Strain on Tibia and Femur Bone Properties of Different Laying Phases of Hy-Line Hens. *Poult Sci* **2021**, *100*, 100933, doi:10.1016/j.psj.2020.12.030.
56. Baker, S.L.; Robison, C.I.; Karcher, D.M.; Toscano, M.J.; Makagon, M.M. Keel Impacts and Associated Behaviors in Laying Hens. *Appl Anim Behav Sci* **2020**, *222*, 104886, doi:10.1016/j.applanim.2019.104886.
57. Eda, M. The Osteological Microevolution of Red Junglefowl and Domestic Fowl under the Domestication Process. *Quaternary International* **2022**, *626–627*, 14–21, doi:10.1016/j.quaint.2020.10.001.
58. Wang, M.-S.; Thakur, M.; Peng, M.-S.; Jiang, Y.; Frantz, L.A.F.; Li, M.; Zhang, J.-J.; Wang, S.; Peters, J.; Otecko, N.O.; et al. 863 Genomes Reveal the Origin and Domestication of Chicken. *Cell Res* **2020**, *30*, 693–701, doi:10.1038/s41422-020-0349-y.
59. Höhne, A.; Petow, S.; Bessei, W.; Schrader, L. Contrafreeloading and Foraging-Related Behavior in Hens Differing in Laying Performance and Phylogenetic Origin. *Poult Sci* **2023**, *102*, 102489, doi:10.1016/j.psj.2023.102489.
60. Kittelsen, K.E.; Gretarsson, P.; Jensen, P.; Christensen, J.P.; Toftaker, I.; Moe, R.O.; Vasdal, G. Keel Bone Fractures Are More Prevalent in White Leghorn Hens than in Red Jungle Fowl Hens—A Pilot Study. *PLoS One* **2021**, *16*, e0255234, doi:10.1371/journal.pone.0255234.
61. Beilharz, R.G.; Luxford, B.G.; Wilkinson, J.L. Quantitative Genetics and Evolution: Is Our Understanding of Genetics Sufficient to Explain Evolution? *Journal of Animal Breeding and Genetics* **1993**, *110*, 161–170, doi:10.1111/j.1439-0388.1993.tb00728.x.
62. Cheema, M.; Qureshi, M.; Havenstein, G. A Comparison of the Immune Response of a 2001 Commercial Broiler with a 1957 Randombred Broiler Strain When Fed Representative 1957 and 2001 Broiler Diets. *Poult Sci* **2003**, *82*, 1519–1529, doi:10.1093/ps/82.10.1519.
63. Muir, W.M.; Wong, G.K.-S.; Zhang, Y.; Wang, J.; Groenen, M.A.M.; Crooijmans, R.P.M.A.; Megens, H.-J.; Zhang, H.; Okimoto, R.; Vereijken, A.; et al. Genome-Wide Assessment of Worldwide Chicken SNP Genetic Diversity Indicates Significant Absence of Rare Alleles in Commercial Breeds. *Proceedings of the National Academy of Sciences* **2008**, *105*, 17312–17317, doi:10.1073/pnas.0806569105.
64. Väisänen, J.; Jensen, P. Social versus Exploration and Foraging Motivation in Young Red Junglefowl (Gallus Gallus) and White Leghorn Layers. *Appl Anim Behav Sci* **2003**, *84*, 139–158, doi:10.1016/j.applanim.2003.07.001.
65. Eusemann, B.K.; Baulain, U.; Schrader, L.; Thöne-Reineke, C.; Patt, A.; Petow, S. Radiographic Examination of Keel Bone Damage in Living Laying Hens of Different Strains Kept in Two Housing Systems. *PLoS One* **2018**, *13*, e0194974, doi:10.1371/journal.pone.0194974.
66. Candelotto, L.; Stratmann, A.; Gebhardt-Henrich, S.G.; Rufener, C.; van de Braak, T.; Toscano, M.J. Susceptibility to Keel Bone Fractures in Laying Hens and the Role of Genetic Variation. *Poult Sci* **2017**, *96*, 3517–3528, doi:10.3382/ps/pex146.
67. Harlander-Matauschek, A.; Rodenburg, T.B.; Sandilands, V.; Tobalske, B.W.; Toscano, M.J. Causes of Keel Bone Damage and Their Solutions in Laying Hens. *Worlds Poult Sci J* **2015**, *71*, 461–472, doi:10.1017/S0043933915002135.

68. Rodenburg, T.; Tuyttens, F.; de Reu, K.; Herman, L.; Zoons, J.; Sonck, B. Welfare Assessment of Laying Hens in Furnished Cages and Non-Cage Systems: An on-Farm Comparison. *Animal Welfare* **2008**, *17*, 363–373, doi:10.1017/S096272860002786X.
69. Baur, S.; Rufener, C.; Toscano, M.J.; Geissbühler, U. Radiographic Evaluation of Keel Bone Damage in Laying Hens—Morphologic and Temporal Observations in a Longitudinal Study. *Front Vet Sci* **2020**, *7*, doi:10.3389/fvets.2020.00129.
70. Casey-Trott, T.; Heerkens, J.L.T.; Petrik, M.; Regmi, P.; Schrader, L.; Toscano, M.J.; Widowski, T. Methods for Assessment of Keel Bone Damage in Poultry. *Poult Sci* **2015**, *94*, 2339–2350, doi:10.3382/ps/pev223.
71. Gebhardt-Henrich, S.; Fröhlich, E. Early Onset of Laying and Bumblefoot Favor Keel Bone Fractures. *Animals* **2015**, *5*, 1192–1206, doi:10.3390/ani5040406.
72. Thøfner, I.; Hougen, H.P.; Villa, C.; Lynnerup, N.; Christensen, J.P. Pathological Characterization of Keel Bone Fractures in Laying Hens Does Not Support External Trauma as the Underlying Cause. *PLoS One* **2020**, *15*, e0229735, doi:10.1371/journal.pone.0229735.
73. Petrik, M.T.; Guerin, M.T.; Widowski, T.M. On-Farm Comparison of Keel Fracture Prevalence and Other Welfare Indicators in Conventional Cage and Floor-Housed Laying Hens in Ontario, Canada. *Poult Sci* **2015**, *94*, 579–585, doi:10.3382/PS/PEV039.
74. Habig, C.; Henning, M.; Baulain, U.; Jansen, S.; Scholz, A.M.; Weigend, S. Keel Bone Damage in Laying Hens—Its Relation to Bone Mineral Density, Body Growth Rate and Laying Performance. *Animals* **2021**, *11*, 1546, doi:10.3390/ani11061546.
75. Gregory, N.G.; Wilkins, L.J.; Tucker, S.A.; Alvey, D.M.; Belyavin, C.G. Effect of Lighting Pattern during Lay on Bone Strength and the Prevalence of Broken Bones in End of Lay Hens. *Journal of Applied Poultry Research* **1993**, *2*, 103–106, doi:10.1093/japr/2.2.103.
76. Gregory, N.; Wilkins, L.; Alvey, D.; Tucker, S. Effect of Catching Method and Lighting Intensity on the Prevalence of Broken Bones and on the Ease of Handling of End-of-Lay Hens. *Veterinary Record* **1993**, *132*, 127–129, doi:10.1136/vr.132.6.127.
77. Gregory, N.G.; Wilkins, L.J.; Eleperuma, S.D.; Ballantyne, A.J.; Overfield, N.D. Broken Bones in Domestic Fowls: Effect of Husbandry System and Stunning Method in End-of-lay Hens. *Br Poult Sci* **1990**, *31*, 59–69, doi:10.1080/00071669008417231.
78. Budgell, K.L.; Silversides, F.G. Bone Breakage in Three Strains of End-of-Lay Hens. *Can J Anim Sci* **2004**, *84*, 745–747, doi:10.4141/A04-040.
79. Lay, D.C.; Fulton, R.M.; Hester, P.Y.; Karcher, D.M.; Kjaer, J.B.; Mench, J.A.; Mullens, B.A.; Newberry, R.C.; Nicol, C.J.; O'Sullivan, N.P.; et al. Hen Welfare in Different Housing Systems. *Poult Sci* **2011**, *90*, 278–294, doi:10.3382/ps.2010-00962.
80. Weeks, C.A.; Lambton, S.L.; Williams, A.G. Implications for Welfare, Productivity and Sustainability of the Variation in Reported Levels of Mortality for Laying Hen Flocks Kept in Different Housing Systems: A Meta-Analysis of Ten Studies. *PLoS One* **2016**, *11*, e0146394, doi:10.1371/journal.pone.0146394.
81. Campbell, D.L.M.; Goodwin, S.L.; Makagon, M.M.; Swanson, J.C.; Siegford, J.M. Failed Landings after Laying Hen Flight in a Commercial Aviary over Two Flock Cycles. *Poult Sci* **2016**, *95*, 188–197, doi:10.3382/ps/pev270.
82. Newberry, R.C.R.C. Environmental Enrichment - Increasing the Biological Relevance of Captive Environments. *Appl Anim Behav Sci* **1995**, *44*, 229–243, doi:10.1016/0168-1591(95)00616-Z.

83. Riber, A.B.; Van De Weerd, H.A.; De Jong, I.C.; Steenfeldt, S. Review of Environmental Enrichment for Broiler Chickens. *Poult Sci* 2018, *97*, 378–396.
84. Van De Weerd, H.A.; Day, J.E.L. A Review of Environmental Enrichment for Pigs Housed in Intensive Housing Systems. *Appl Anim Behav Sci* **2009**, *116*, 1–20, doi:10.1016/j.applanim.2008.08.001.
85. Pettersson, I.C.; Weeks, C.A.; Norman, K.I.; Nicol, C.J. The Ability of Laying Pullets to Negotiate Two Ramp Designs as Measured by Bird Preference and Behaviour. *PeerJ* **2017**, *5*, e4069, doi:10.7717/PEERJ.4069.
86. LeBlanc, C.; Tobalske, B.; Bowley, S.; Harlander-Matauschek, A. Development of Locomotion over Inclined Surfaces in Laying Hens. *Animal* **2018**, *12*, 585–596, doi:10.1017/S1751731117001896.
87. Heerkens, J.L.T.; Delezie, E.; Ampe, B.; Rodenburg, T.B.; Tuyttens, F.A.M. Ramps and Hybrid Effects on Keel Bone and Foot Pad Disorders in Modified Aviaries for Laying Hens. *Poult Sci* **2016**, *95*, 2479–2488, doi:10.3382/ps/pew157.
88. LeBlanc, S.; Tobalske, B.; Quinton, M.; Springthorpe, D.; Szkotnicki, B.; Wuerbel, H.; Harlander-Matauschek, A. Physical Health Problems and Environmental Challenges Influence Balancing Behaviour in Laying Hens. *PLoS One* **2016**, *11*, e0153477, doi:10.1371/journal.pone.0153477.
89. Jones, R.B.; Waddington, D. Modification of Fear in Domestic Chicks, Gallus Gallus Domesticus, via Regular Handling and Early Environmental Enrichment. *Anim Behav* **1992**, *43*, 1021–1033, doi:10.1016/S0003-3472(06)80015-1.
90. Hartcher, K.M.; Tran, M.K.T.N.; Wilkinson, S.J.; Hemsworth, P.H.; Thomson, P.C.; Cronin, G.M. Plumage Damage in Free-Range Laying Hens: Behavioural Characteristics in the Rearing Period and the Effects of Environmental Enrichment and Beak-Trimming. *Appl Anim Behav Sci* **2015**, *164*, 64–72, doi:10.1016/j.applanim.2014.12.011.
91. Tahamtani, F.M.; Nordgreen, J.; Brantsæter, M.; Østby, G.C.; Nordquist, R.E.; Janczak, A.M. Does Early Environmental Complexity Influence Tyrosine Hydroxylase in the Chicken Hippocampus and "Prefrontal" Caudolateral Nidopallium? *Front Vet Sci* **2016**, *3*, doi:10.3389/fvets.2016.00008.
92. Dixon, L.; Duncan, I.; Mason, G. The Effects of Four Types of Enrichment on Feather-Pecking Behaviour in Laying Hens Housed in Barren Environments. *Animal Welfare* **2010**, *19*, 429–435, doi:10.1017/S0962728600001913.
93. Humane Farm Animal Care *Humane Farm Animal Care Standards for Production of Egg Laying Hens*; 2014;
94. Yuan, Y.; Chen, X.; Zhang, L.; Wu, J.; Guo, J.; Zou, D.; Chen, B.; Sun, Z.; Shen, C.; Zou, J. The Roles of Exercise in Bone Remodeling and in Prevention and Treatment of Osteoporosis. *Prog Biophys Mol Biol* **2016**, *122*, 122–130, doi:10.1016/j.pbiomolbio.2015.11.005.
95. Webster, A.B. Welfare Implications of Avian Osteoporosis. *Poult Sci* **2004**, *83*, 184–192, doi:10.1093/ps/83.2.184.
96. Casey-Trott, T.M.; Korver, D.R.; Guerin, M.T.; Sandilands, V.; Torrey, S.; Widowski, T.M. Opportunities for Exercise during Pullet Rearing, Part I: Effect on the Musculoskeletal Characteristics of Pullets. *Poult Sci* **2017**, *96*, 2509–2517, doi:10.3382/PS/PEX059.

97. Regmi, P.; Deland, T.S.; Steibel, J.P.; Robison, C.I.; Haut, R.C.; Orth, M.W.; Karcher, D.M. Effect of Rearing Environment on Bone Growth of Pullets. *Poult Sci* **2015**, *94*, 502–511, doi:10.3382/PS/PEU041.
98. Enneking, S.A.; Cheng, H.W.; Jefferson-Moore, K.Y.; Einstein, M.E.; Rubin, D.A.; Hester, P.Y. Early Access to Perches in Caged White Leghorn Pullets. *Poult Sci* **2012**, *91*, 2114–2120, doi:10.3382/ps.2012-02328.
99. Regmi, P.; Smith, N.; Nelson, N.; Haut, R.C.; Orth, M.W.; Karcher, D.M. Housing Conditions Alter Properties of the Tibia and Humerus during the Laying Phase in Lohmann White Leghorn Hens. *Poult Sci* **2016**, *95*, 198–206, doi:10.3382/ps/pev209.
100. Yan, F.F.; Hester, P.Y.; Cheng, H.W. The Effect of Perch Access during Pullet Rearing and Egg Laying on Physiological Measures of Stress in White Leghorns at 71 Weeks of Age. *Poult Sci* **2014**, *93*, 1318–1326, doi:10.3382/ps.2013-03572.
101. Casey-Trott, T.M.; Korver, D.R.; Guerin, M.T.; Sandilands, V.; Torrey, S.; Widowski, T.M. Opportunities for Exercise during Pullet Rearing, Part II: Long-Term Effects on Bone Characteristics of Adult Laying Hens at the End-of-Lay. *Poult Sci* **2017**, *96*, 2518–2527, doi:10.3382/ps/pex060.
102. Hester, P.Y.; Enneking, S.A.; Haley, B.K.; Cheng, H.W.; Einstein, M.E.; Rubin, D.A. The Effect of Perch Availability during Pullet Rearing and Egg Laying on Musculoskeletal Health of Caged White Leghorn Hens. *Poult Sci* **2013**, *92*, 1972–1980, doi:10.3382/PS.2013-03008.
103. Regmi, P.; Nelson, N.; Steibel, J.P.; Anderson, K.E.; Karcher, D.M. Comparisons of Bone Properties and Keel Deformities between Strains and Housing Systems in End-of-Lay Hens. *Poult Sci* **2016**, *95*, 2225–2234, doi:10.3382/ps/pew199.
104. Pufall, A.; Harlander-Matauschek, A.; Hunniford, M.; Widowski, T.M. Effects of Rearing Aviary Style and Genetic Strain on the Locomotion and Musculoskeletal Characteristics of Layer Pullets. *Animals* **2021**, *11*, 634, doi:10.3390/ani11030634.
105. Rentsch, A.K.; Harlander, A.; Siegford, J.M.; Vitienes, I.; Willie, B.M.; Widowski, T.M. Rearing Laying Hens: The Effect of Aviary Design and Genetic Strain on Pullet Exercise and Perching Behavior. *Frontiers in Animal Science* **2023**, *4*, doi:10.3389/fanim.2023.1176702.
106. Hughes, B.O.; Wilson, S.; Appleby, M.C.; Smith, S.F. Comparison of Bone Volume and Strength as Measures of Skeletal Integrity in Caged Laying Hens with Access to Perches. *Res Vet Sci* **1993**, *54*, 202–206, doi:10.1016/0034-5288(93)90057-M.
107. Hartcher, K.M.; Jones, B. The Welfare of Layer Hens in Cage and Cage-Free Housing Systems. *Worlds Poult Sci J* **2017**, *73*, 767–782, doi:10.1017/S0043933917000812.
108. Xu, D.; Shu, G.; Liu, Y.; Qin, P.; Zheng, Y.; Tian, Y.; Zhao, X.; Du, X. Farm Environmental Enrichments Improve the Welfare of Layer Chicks and Pullets: A Comprehensive Review. *Animals* **2022**, *12*, 2610, doi:10.3390/ani12192610.
109. Bist, R.B.; Subedi, S.; Chai, L.; Regmi, P.; Ritz, C.W.; Kim, W.K.; Yang, X. Effects of Perching on Poultry Welfare and Production: A Review. *Poultry* **2023**, *2*, 134–157, doi:10.3390/poultry2020013.
110. Adhikari, R.; White, D.; House, J.D.; Kim, W.K. Effects of Additional Dosage of Vitamin D3, Vitamin D2, and 25-Hydroxyvitamin D3 on Calcium and Phosphorus Utilization, Egg Quality and Bone Mineralization in Laying Hens. *Poult Sci* **2020**, *99*, 364–373, doi:10.3382/ps/pez502.

111. Korver, D. Nutritional Requirements of Poultry Available online: https://www.merckvetmanual.com/poultry/nutrition-and-management-poultry/nutritional-requirements-of-poultry (accessed on 7 February 2024).
112. Fleming, R.H. Nutritional Factors Affecting Poultry Bone Health. *Proceedings of the Nutrition Society* **2008**, *67*, 177–183, doi:10.1017/S0029665108007015.
113. Chen, C.; Kim, W.K. The Application of Micro-CT in Egg-Laying Hen Bone Analysis: Introducing an Automated Bone Separation Algorithm. *Poult Sci* **2020**, *99*, 5175–5183, doi:10.1016/j.psj.2020.08.047.
114. Niknia, A.D.; Vakili, R.; Tahmasbi, A.M. Role of Zinc-Methionine Chelate on Bone Health and Eggshell Quality in Late–Phase Laying Hens. *All Life* **2023**, *16*, doi:10.1080/26895293.2022.2162609.
115. Kothapalli, C.R.; Ramamurthi, A. Lysyl Oxidase Enhances Elastin Synthesis and Matrix Formation by Vascular Smooth Muscle Cells. *J Tissue Eng Regen Med* **2009**, *3*, 655–661, doi:10.1002/term.214.
116. Ciosek, Ż.; Kot, K.; Rotter, I. Iron, Zinc, Copper, Cadmium, Mercury, and Bone Tissue. *Int J Environ Res Public Health* **2023**, *20*, 2197, doi:10.3390/ijerph20032197.
117. Xu, X.; Pin, S.; Shedlock, J.; Harris, Z.L. Copper. In *Encyclopedia of Human Nutrition*; Elsevier, 2005; pp. 471–476.
118. Olgun, O. Manganese in Poultry Nutrition and Its Effect on Performance and Eggshell Quality. *Worlds Poult Sci J* **2017**, *73*, 45–56, doi:10.1017/S0043933916000891.
119. Pereira, C.G.; Rabello, C.B.-V.; Barros, M.R.; Manso, H.E.C.C.C.; Santos, M.J.B. dos; Faria, A.G.; Oliveira, H.B. de; Medeiros-Ventura, W.R.L.; Silva Júnior, R.V.; Carvalho, C.C.D.; et al. Zinc, Manganese and Copper Amino Acid Complexed in Laying Hens' Diets Affect Performance, Blood Parameters and Reproductive Organs Development. *PLoS One* **2020**, *15*, e0239229, doi:10.1371/journal.pone.0239229.
120. Selim, S.; Abdel-Megeid, N.S.; Khalifa, H.K.; Fakiha, K.G.; Majrashi, K.A.; Hussein, E. Efficacy of Various Feed Additives on Performance, Nutrient Digestibility, Bone Quality, Blood Constituents, and Phosphorus Absorption and Utilization of Broiler Chickens Fed Low Phosphorus Diet. *Animals* **2022**, *12*, 1742, doi:10.3390/ani12141742.
121. Yan, F.F.; Murugesan, G.R.; Cheng, H.W. Effects of Probiotic Supplementation on Performance Traits, Bone Mineralization, Cecal Microbial Composition, Cytokines and Corticosterone in Laying Hens. *Animal* **2019**, *13*, 33–41, doi:10.1017/S175173111800109X.
122. Akbari Moghaddam Kakhki, R.; Shouldice, V.L.; Price, K.R.; Moats, J.; Kiarie, E.G. N-3 Fatty Acids Fed to ISA Brown and Shaver White Breeders and Their Female Progeny during Rearing: Impact on Egg Production, Eggshell, and Select Bone Attributes from 18 to 42 Weeks of Age. *Poult Sci* **2020**, *99*, 3959–3970, doi:10.1016/j.psj.2020.03.061.
123. Hunt, C.D.; Nielsen, F.H. Interaction Between Boron and Cholecalciferol in the Chick. In *Trace Element Metabolism in Man and Animals*; Springer Berlin Heidelberg: Berlin, Heidelberg, 1981; pp. 597–600.
124. Olgun, O.; Yazgan, O.; Cufadar, Y. Effects of Boron and Copper Dietary Supplementation in Laying Hens on Egg Shell Quality, Plasma and Tibia Mineral Concentrations and Bone Biomechanical Properties. *Rev Med Vet (Toulouse)* **2012**, *163*, 335–342.
125. Elliot, M.A.; Edwards, H.M. Studies to Determine Whether an Interaction Exists Among Boron, Calcium, and Cholecalciferol on the Skeletal Development of Broiler Chickens. *Poult Sci* **1992**, *71*, 677–690, doi:10.3382/ps.0710677.

126. Rossi, A.F.; Miles, R.D.; Damron, B.L.; Flunker, L.K. Effects of Dietary Boron Supplementation on Broilers. *Poult Sci* **1993**, *72*, 2124–2130, doi:10.3382/ps.0722124.
127. Sizmaz, O.; Yildiz, G. Influence of Dietary Boric Acid and Ascorbic Acid on Performance, Egg Traits, Cholesterol and Bone Parameters of Laying Hens. *Ankara Üniv Vet Fak Derg* **2016**, *63*, 151–156.
128. Mizrak, C.; Yenice, E.; Can, M.; Yildririm, U.; Atik, Z. Effects of Dietary Boron on Performance, Egg Production, Egg Quality, and Some Bone Parameters in Layer Hens. *S Afr J Anim Sci* **2010**, *40*.
129. Adarsh, V.; Dintaran, P.; Shivakumar, G.N.K.; Vijayarangam, E.A.; Kumar, D.D.; Nagaraj, K.; Eknath, J.S. Effect of Boron Supplementation on Laying Performance of White Leghorn Hens Fed Diet with and without Adequate Level of Calcium. *Trop Anim Health Prod* **2021**, *53*, 444, doi:10.1007/s11250-021-02878-x.
130. Wilson, J.H.; Ruszler, P.L. Effects of Boron on Growing Pullets. *Biol Trace Elem Res* **1997**, *56*, 287–294.
131. Wilson, J.H.; Ruszler, P.L. Effects of Dietary Boron on Poultry Bone Strength. *Transactions of the ASAE* **1995**, *38*, 167–170.
132. Wilson, J.H.; Ruszler, P.L. Effects of Dietary Boron Supplementation on Laying Hens. *Br Poult Sci* **1996**, *37*, 723–729, doi:10.1080/00071669608417902.
133. Wilson, J.H.; Ruszler, P.L. Long Term Effects of Boron on Layer Bone Strength and Production Parameters. *Br Poult Sci* **1998**, *39*, 11–15, doi:10.1080/00071669889312.
134. Kurtoğlu, V.; Kurtoğlu, F.; Coşkun, B. Effects of Boron Supplementation on Performance and Some Serum Biochemical Parameters in Laying Hens. **2006**.
135. Russell, J.A. Core Affect and the Psychological Construction of Emotion. *Psychol Rev* **2003**, *110*, 145–172, doi:10.1037/0033-295X.110.1.145.
136. Mendl, M.; Burman, O.H.; Paul, E.S. An Integrative and Functional Framework for the Study of Animal Emotion and Mood. *Proceedings of the Royal Society B* **2010**, *277*, 2895–2904, doi:10.1098/rspb.2010.0303.
137. Nettle, D.; Bateson, M. The Evolutionary Origins of Mood and Its Disorders. *Current Biology* **2012**, *22*, doi:https://doi.org/10.1016/j.cub.2012.06.020.
138. Trimmer, P.C.; Paul, E.S.; Mendl, M.T.; McNamara, J.M.; Houston, A.I. On the Evolution and Optimality of Mood States. *Behavioral Sciences* **2013**, *3*, 501–521, doi:10.3390/bs3030501.
139. Roelofs, S.; Boleij, H.; Nordquist, R.E.; van der Staay, F.J. Making Decisions under Ambiguity: Judgement Bias Tasks for Assessing Emotional State in Animals. *Front Behav Neurosci* **2016**, *10*, 119, doi:10.3389/fnbeh.2016.00119.
140. Kleinginna, P.R.; Kleinginna, A.M. A Categorized List of Emotion Definitions, with Suggestions for a Consensual Definition. *Motiv Emot* **1981**, *5*, 345–379, doi:10.1007/BF00992553.
141. Lazarus, R.S. Thoughts on the Relations Between Emotion and Cognition. *American Psychologist* **1982**, *37*, 1019–1024, doi:10.1037/0003-066X.37.9.1019.
142. Dolcos, F.; Denkova, E. Dissociating Enhancing and Impairing Effects of Emotion on Cognition. In *Emerging Trends in the Social and Behavioral Sciences: An Interdisciplinary, Searchable, and Linkable Resource*; Scott, R., Kosslyn, S., Eds.; John Wiley & Sons, Inc., 2015; pp. 1–18.
143. Mendl, M.; Burman, O.H.P.O.H.; Parker, R.M.A.R.M.A.; Paul, E.S.E.S. Cognitive Bias as an Indicator of Animal Emotion and Welfare: Emerging Evidence and Underlying

Mechanisms. *Appl Anim Behav Sci* **2009**, *118*, 161–181, doi:10.1016/j.applanim.2009.02.023.
144. MacLeod, A.K.; Byrne, A. Anxiety, Depression, and the Anticipation of Future Positive and Negative Experiences. *J Abnorm Psychol* **1996**, *105*, 286–289, doi:10.1037/0021-843X.105.2.286.
145. Mineka, S.; Watson, D.; Clark, L.A. Comorbidity of Anxiety and Unipolar Mood Disorders. *Annu Rev Clin Psychol* **1998**, *49*, 377–412, doi:10.1146/annurev.psych.49.1.377.
146. Mogg, K.; Bradley, B.P. A Cognitive-Motivational Analysis of Anxiety. *Behaviour Research and Therapy* **1998**, *36*, 809–848, doi:10.1016/S0005-7967(98)00063-1.
147. Mendl, M.; Paul, E.S. Animal Affect and Decision-Making. *Neurosci Biobehav Rev* **2020**, *112*, 144–163, doi:10.1016/j.neubiorev.2020.01.025.
148. Paul, E.S.; Harding, E.J.; Mendl, M. Measuring Emotional Processes in Animals: The Utility of a Cognitive Approach. *Neurosci Biobehav Rev* 2005, *29*, 469–491.
149. Crump, A.; Arnott, F.; Bethell, E.J. Affect-Driven Biases as Animal Welfare Indicators: Review and Methods. *Animals* **2018**, *8*, 136, doi:10.3390/ani8080136.
150. Košťál, Ľ.; Skalná, Z.; Pichová, K. Use of Cognitive Bias as a Welfare Tool in Poultry. *J Anim Sci* **2020**, *98*, S63–S79, doi:10.1093/jas/skaa039.
151. Carver, C.S. Affect and the Functional Bases of Behavior: On the Dimensional Structure of Affective Experience. *Society for Personality and Social Psychology* **2001**, *5*, 345–356, doi:10.1207/S15327957PSPR0504_4.
152. Carver, C.S. Pleasure as a Sign You Can Attend to Something Else: Placing Positive Feelings within a General Model of Affect. *Cogn Emot* **2003**, *17*, 241–261, doi:10.1080/02699930302294.
153. Bethell, E.J. A "How-To" Guide for Designing Judgment Bias Studies to Assess Captive Animal Welfare. *Journal of Applied Animal Welfare Science* **2015**, *18*, S18–S42, doi:10.1080/10888705.2015.1075833.
154. Baciadonna, L.; McElligott, A.G. The Use of Judgement Bias to Assess Welfare in Farm Livestock. *Animal Welfare* **2015**, *24*, 81–91, doi:10.7120/09627286.24.1.081.
155. Eysenck, M.W.; Mogg, K.; May, J.; Richards, A.; Mathews, A. Bias in Interpretation of Ambiguous Sentences Related to Threat in Anxiety. *J Abnorm Psychol* **1991**, *100*, 144–150, doi:10.1037/0021-843X.100.2.144.
156. Welp, T.; Rushen, J.; Kramer, D.L.; Festa-Bianchet, M.; de Passile, A.M.B. Vigilance as a Measure of Fear in Dairy Cattle. *Appl Anim Behav Sci* **2004**, *87*, 1–13, doi:10.1016/j.applanim.2003.12.013.
157. Yiend, J. The Effects of Emotion on Attention: A Review of Attentional Processing of Emotional Information. *Cogn Emot* **2010**, *24*, 3–47, doi:10.1080/02699930903205698.
158. Campbell, D.L.M.; Taylor, P.S.; Hernandez, C.E.; Stewart, M.; Belson, S.; Lee, C. An Attention Bias Test to Assess Anxiety States in Laying Hens. *PeerJ* **2019**, *2019*, doi:10.7717/peerj.7303.
159. Campbell, D.L.M.; Dickson, E.J.; Lee, C. Application of Open Field, Tonic Immobility, and Attention Bias Tests to Hens with Different Ranging Patterns. *PeerJ* **2019**, *7*, e8122, doi:10.7717/peerj.8122.
160. Gunnarsson, S.; Yngvesson, J.; Keeling, L.J.; Forkman, B. Rearing without Early Access to Perches Impairs the Spatial Skills of Laying Hens. *Appl Anim Behav Sci* **2000**, *67*, 217–228, doi:10.1016/S0168-1591(99)00125-2.

161. Campbell, A.M.A.M.; Johnson, A.M.A.M.; Persia, M.E.M.E.; Jacobs, L. Effects of Housing System on Anxiety, Chronic Stress, Fear, and Immune Function in Bovan Brown Laying Hens. *Animals* **2022**, *12*, 1803, doi:10.3390/ani12141803.
162. Robison, C.I.; Karcher, D.M. Analytical Bone Calcium and Bone Ash from Mature Laying Hens Correlates to Bone Mineral Content Calculated from Quantitative Computed Tomography Scans. *Poult Sci* **2019**, *98*, 3611–3616, doi:10.3382/ps/pez165.
163. Patel, S.H.; Moore, T.A. Motion Artifact on Computed Tomography Scan Suggesting an Unstable 3-Column Spine Injury: Case Report of a "near Miss" Root Cause of Unneeded Surgery. *Patient Saf Surg* **2013**, *7*, 35, doi:10.1186/1754-9493-7-35.
164. Botz, B.; Dawes, L. Ring Artifact. In *Radiopaedia.org*; Radiopaedia.org, 2008.
165. Boas, F.E.; Fleischmann, D. CT Artifacts: Causes and Reduction Techniques. *Imaging Med* **2012**, *4*, 229–240.
166. Hsieh, J.; Molthen, R.C.; Dawson, C.A.; Johnson, R.H. An Iterative Approach to the Beam Hardening Correction in Cone Beam CT. *Med Phys* **2000**, *27*, 23–29, doi:10.1118/1.598853.
167. Murphy, A.; Garg, V. Partial Volume Averaging (CT Artifact). In *Radiopaedia.org*; Radiopaedia.org, 2014.
168. ANSI/ASAE *ANSI/ASAE S459 MAR1992 (R2017) Shear and Three-Point Bending Test of Animal Bone*; St. Joseph, 2017;
169. Vahle, J.L.; Ma, Y.L.; Burr, D.B. Skeletal Assessments in the Nonhuman Primate. In *The Nonhuman Primate in Nonclinical Drug Development and Safety Assessment*; Bluemel, J., Korte, S., Schenck, E., Weinbauer, G.F., Eds.; Elsevier Inc., 2015; pp. 605–625.
170. Silva, M.J. *Bone Mechanical Testing by Three-Point Bending*; 2016;
171. Abdewi, E.F. Mechanical Properties of Reinforcing Steel Rods Produced by Zliten Steel Factory. In *Reference Module in Materials Science and Materials Engineering*; Elsevier, 2017.
172. Kim, W.K.; Donalson, L.M.; Herrera, P.; Woodward, C.L.; Kubena, L.F.; Nisbet, D.J.; Ricke, S.C. Research Note: Effects of Different Bone Preparation Methods (Fresh, Dry, and Fat-Free Dry) on Bone Parameters and the Correlations Between Bone Breaking Strength and the Other Bone Parameters. *Poult Sci* **2004**, *83*, 1663–1666, doi:10.1093/ps/83.10.1663.
173. El-Ghannam, A.; Ducheyne, P. Bioactive Ceramics. In *Comprehensive Biomaterials*; Elsevier, 2011; pp. 157–179.
174. Muszyński, S.; Kasperek, K.; Świątkiewicz, S.; Arczewska-Włosek, A.; Wiącek, D.; Donaldson, J.; Dobrowolski, P.; Arciszewski, M.B.; Valverde Piedra, J.L.; Krakowiak, D.; et al. Assessing Bone Health Status and Eggshell Quality of Laying Hens at the End of a Production Cycle in Response to Inclusion of a Hybrid Rye to a Wheat–Corn Diet. *Vet Sci* **2022**, *9*, 683, doi:10.3390/vetsci9120683.
175. Bauman, W.A.; Cardozo, C.P. Immobilization Osteoporosis. In *Osteoporosis*; Elsevier, 2013; pp. 1139–1171.
176. Scholey, D.V.; Burton, E.J. The Effect of Bone Choice on Quantification of Mineralization in Broiler Chickens up to 6 Weeks of Age. *Journal of Applied Poultry Research* **2017**, *26*, 485–490, doi:10.3382/japr/pfx020.
177. Shastak, Y.; Witzig, M.; Hartung, K.; Bessei, W.; Rodehutscord, M. Comparison and Evaluation of Bone Measurements for the Assessment of Mineral Phosphorus Sources in Broilers ,. *Poult Sci* **2012**, *91*, 2210–2220, doi:10.3382/ps.2012-02179.

178. Kim, E.J.; Amezcua, C.M.; Utterback, P.L.; Parsons, C.M. Phosphorus Bioavailability, True Metabolizable Energy, and Amino Acid Digestibilities of High Protein Corn Distillers Dried Grains and Dehydrated Corn Germ. *Poult Sci* **2008**, *87*, 700–705, doi:10.3382/ps.2007-00302.
179. Ruiz-Feria, C.A.; Arroyo-Villegas, J.J.; Pro-Martinez, A.; Bautista-Ortega, J.; Cortes-Cuevas, A.; Narciso-Gaytan, C.; Hernandez-Cazares, A.; Gallegos-Sanchez, J. Effects of Distance and Barriers between Resources on Bone and Tendon Strength and Productive Performance of Broiler Chickens. *Poult Sci* **2014**, *93*, 1608–1617, doi:10.3382/ps.2013-03421.
180. Perney, K.M.; Cantor, A.H.; Straw, M.L.; Herkelman, K.L. The Effect of Dietary Phytase on Growth Performance and Phosphorus Utilization of Broiler Chicks. *Poult Sci* **1993**, *72*, 2106–2114, doi:10.3382/ps.0722106.
181. Payne, R.L.; Lavergne, T.K.; Southern, L.L. A Comparison of Two Sources of Phytase in Liquid and Dry Forms in Broilers. *Poult Sci* **2005**, *84*, 265–272, doi:10.1093/ps/84.2.265.
182. Li, J.; Yuan, J.; Miao, Z.; Song, Z.; Yang, Y.; Tian, W.; Guo, Y. Effect of Dietary Nutrient Density on Small Intestinal Phosphate Transport and Bone Mineralization of Broilers during the Growing Period. *PLoS One* **2016**, *11*, e0153859, doi:10.1371/journal.pone.0153859.
183. Alkhtib, A.; Sanni, C.O.; Burton, E.; Scholey, D. Evaluating Different Bone Processing Methods Used in Assessing Mineralization in Broilers. *Journal of Applied Poultry Research* **2023**, *32*, 100363, doi:10.1016/j.japr.2023.100363.
184. Chen, C.; Turner, B.; Applegate, T.J.; Litta, G.; Kim, W.K. Role of Long-Term Supplementation of 25-Hydroxyvitamin D3 on Egg Production and Egg Quality of Laying Hen. *Poult Sci* **2020**, *99*, 6899–6906, doi:10.1016/j.psj.2020.09.020.
185. Liu, D.; Veit, H.; Wilson, J.; Denbow, D. Long-Term Supplementation of Various Dietary Lipids Alters Bone Mineral Content, Mechanical Properties and Histological Characteristics of Japanese Quail. *Poult Sci* **2003**, *82*, 831–839, doi:10.1093/ps/82.5.831.
186. Bhattarai, H.K.; Shrestha, S.; Rokka, K.; Shakya, R. Vitamin D, Calcium, Parathyroid Hormone, and Sex Steroids in Bone Health and Effects of Aging. *J Osteoporos* **2020**, *2020*, 1–10, doi:10.1155/2020/9324505.
187. Hunt, C.D. Dietary Boron Modified the Effects of Magnesium and Molybdenum on Mineral Metabolism in the Cholecalciferol-Deficient Chick. *Biol Trace Elem Res* **1989**, *22*, 201–220, doi:10.1007/BF02916650.
188. Fassani, E.; Bertechini, A.; Brito, J.; Kato, R.; Fialho, E.; Geraldo, A. Boron Supplementation in Broiler Diets. *Rev Bras Cienc Avic* **2004**, *6*, 213–217, doi:10.1590/S1516-635X2004000400004.
189. Kurtoğlu, F.; Kurtoğlu, V.; Çelik, İ.; Keçeci, T.; Nizamlioğlu, M. Effects of Dietary Boron Supplementation on Some Biochemical Parameters, Peripheral Blood Lymphocytes, Splenic Plasma Cells and Bone Characteristics of Broiler Chicks given Diets with Adequate or Inadequate Cholecalciferol (Vitamin D). *Br Poult Sci* **2005**, *46*, 87–96, doi:10.1080/00071660400024001.
190. Kurtoğlu, V.; Kurtoğlu, F.; Coşkun, B. Effects of Boron Supplementation of Adequate and Inadequate Vitamin D3-Containing Diet on Performance and Serum Biochemical Characters of Broiler Chickens. *Res Vet Sci* **2001**, *71*, 183–187, doi:10.1053/rvsc.2001.0517.

191. Nielsen, F.H.; Shuler, T.R. Studies of the Interaction between Boron and Calcium, and Its Modification by Magnesium and Potassium, in Rats. *Biol Trace Elem Res* **1992**, *35*, 225–237, doi:10.1007/BF02783768.
192. Nielson, F. *Nutrition Today*. 1992, pp. 6–12.
193. *Hy-Line W-36 Management Guide*; 2020;
194. Harrison, C.; Jones, J.; Bridges, W.; Anderson, G.; Ali, A.; Mercuri, J. Associations among Computed Tomographic Measures of Bone and Muscle Quality and Biomechanical Measures of Tibiotarsal Bone Quality in Laying Hens. *Am J Vet Res* **2023**, 1–9, doi:10.2460/ajvr.23.05.0109.
195. Anderson, M.G.; Johnson, A.M.; Harrison, C.; Arguelles-Ramos, M.; Ali, A. Impact of Perch Provision Timing on Activity and Musculoskeletal Health of Laying Hens. *Animals* **2024**, *14*, 265, doi:10.3390/ani14020265.
196. Hothorn, T.; Bretz, F.; Westfall, P. Simultaneous Inference in General Parametric Models. *Biometrical Journal* **2008**, *50*, 346–363, doi:10.1002/BIMJ.200810425.
197. Cheng, J.; Peng, K.; Jin, E.; Zhang, Y.; Liu, Y.; Zhang, N.; Song, H.; Liu, H.; Tang, Z. Effect of Additional Boron on Tibias of African Ostrich Chicks. *Biol Trace Elem Res* **2011**, *144*, 538–549, doi:10.1007/s12011-011-9024-y.
198. Devirian, T.A.; Volpe, S.L. The Physiological Effects of Dietary Boron. *Crit Rev Food Sci Nutr* **2003**, *43*, 219–231, doi:10.1080/10408690390826491.
199. Bai, Y.; Hunt, C.D. Dietary Boron Enhances Efficacy of Cholecalciferol in Broiler Chicks. *The Journal of Trace Elements in Experimental Medicine* **1996**, *9*, 117–132, doi:10.1002/(SICI)1520-670X(1996)9:3<117::AID-JTRA4>3.0.CO;2-P.
200. Nielsen, F.H. Dietary Fat Composition Modifies the Effect of Boron on Bone Characteristics and Plasma Lipids in Rats. *BioFactors* **2004**, *20*, 161–171, doi:10.1002/biof.5520200305.
201. Whitehead, C.C.; Fleming, R.H.; Julian, R.J.; Sørensen, P. Skeletal Problems Associated with Selection for Increased Production. *Poultry Genetics, Breeding, and Biotechnology* **2003**, *3*, 29–52.
202. Bozkurt, M.; Küçükyilmaz, K.; Çath, A.U.; Çinar, M.; Çabuk, M.; Bintaş, E. Effects of Boron Supplementation to Diets Deficient in Calcium and Phosphorus on Performance with Some Serum, Bone and Fecal Characteristics of Broiler Chickens. *Asian-Australas J Anim Sci* **2012**, *25*, 248, doi:10.5713/AJAS.2011.11211.
203. Magnusson, P.; Arlestig, L.; Paus, E.; Di Mauro, S.; Testa, M.P.; Stigbrand, T.; Farley, J.R.; Nustad, K.; Millan, J.L. Monoclonal Antibodies against Tissue-Nonspecific Alkaline Phosphatase. *Tumor Biology* **2002**, *23*, 228–248, doi:10.1159/000067254.
204. Chavassieux, P.; Portero-Muzy, N.; Roux, J.-P.; Garnero, P.; Chapurlat, R. Are Biochemical Markers of Bone Turnover Representative of Bone Histomorphometry in 370 Postmenopausal Women? *J Clin Endocrinol Metab* **2015**, *100*, 4662–4668, doi:10.1210/jc.2015-2957.
205. Shipov, A.; Sharir, A.; Zelzer, E.; Milgram, J.; Monsonego-Ornan, E.; Shahar, R. The Influence of Severe Prolonged Exercise Restriction on the Mechanical and Structural Properties of Bone in an Avian Model. *The Veterinary Journal* **2010**, *183*, 153–160, doi:10.1016/j.tvjl.2008.11.015.
206. Kolakshyapati, M.; Flavel, R.J.; Sibanda, T.Z.; Schneider, D.; Welch, M.C.; Ruhnke, I. Various Bone Parameters Are Positively Correlated with Hen Body Weight While Range

Access Has No Beneficial Effect on Tibia Health of Free-Range Layers. *Poult Sci* **2019**, *98*, 6241–6250, doi:10.3382/ps/pez487.
207. Manet, M.W.E.; Kliphuis, S.; Nordquist, R.E.; Goerlich, V.C.; Tuyttens, F.A.M.; Rodenburg, T.B. Brown and White Layer Pullet Hybrids Show Different Fear Responses towards Humans, but What Role Does Light during Incubation Play in That? *Appl Anim Behav Sci* **2023**, *267*, 106056, doi:10.1016/j.applanim.2023.106056.
208. De Haas, E.N.; Bolhuis, J.E.; de Jong, I.C.; Kemp, B.; Janczak, A.M.; Rodenburg, T.B. Predicting Feather Damage in Laying Hens during the Laying Period. Is It the Past or Is It the Present? *Appl Anim Behav Sci* **2014**, *160*, 75–85, doi:10.1016/j.applanim.2014.08.009.
209. Moinard, C.; Statham, P.; Green, P.R. Control of Landing Flight by Laying Hens: Implications for the Design of Extensive Housing Systems. *Br Poult Sci* **2004**, *45*, 578–584, doi:10.1080/00071660400006321.
210. Jackson, B.E.; Tobalske, B.W.; Dial, K.P. The Broad Range of Contractile Behaviour of the Avian Pectoralis: Functional and Evolutionary Implications. *Journal of Experimental Biology* **2011**, *214*, 2354–2361, doi:10.1242/jeb.052829.
211. McCoy, H.; Kenney, M.A.; Montgomery, C.; Irwin, A.; Williams, L.; Orrell, R. Relation of Boron to the Composition and Mechanical Properties of Bone. *Environ Health Perspect* **1994**, *102*, 49–53, doi:10.1289/ehp.94102s749.

Chapter 2

INFLUENCE OF PERCH PROVISION DURING REARING ON ACTIVITY AND MUSCULOSKELETAL HEALTH OF PULLETS[1]

[1] Anderson, MG, Johnson AM, Harrison C, Jones J, Ali A. Influence of perch provsion during rearing on activity and musculoskeletal health of pullets. Accepted to *PLOS One*.

Abstract

Prior research suggests exercise during pullet rearing can mitigate lay-phase bone fractures by strengthening muscles, enhancing balance, and increasing bone mass. This study aimed to confirm that Hy-Line brown pullets with multi-tier perches show increased activity and improved musculoskeletal health. Pullets (n=810) were randomly allocated to housing systems, either with multi-tier perches (P; n=15 pens) or without (NP; n=15 pens), spanning from 0-17 weeks of age. At 5, 11, and 17 weeks, individual birds were meticulously monitored for activity using accelerometers over three consecutive days (n=90 randomly selected birds/week). At 11 and 17 weeks, 60 birds underwent euthanasia and computed tomography (CT) scans to ascertain tibiotarsal bone mineral density and cross-sectional area measurements. Post-CT scanning, birds were dissected for muscle size, tibiotarsal breaking strength, and tibiotarsal ash percentage measurements. Additionally, serum concentrations of bone-specific alkaline phosphatase and procollagen type 1 N-terminal propeptide were assessed as novel markers of bone mineralization (n=90 birds/week). Pullet group P exhibited heightened vertical activity ($P<0.05$), with no discernible differences in overall activity ($P>0.05$) during weeks 5, 11, and 17 compared to group NP. Tibiotarsal bones of P pullets demonstrated superior total and cortical bone mineral density at week 11, alongside increased cortical bone cross-sectional areas and heightened total and cortical bone mineral densities at week 17 ($P<0.05$) compared to NP pullets. At week 11, P pullets displayed larger leg muscles, including triceps, pectoralis major and minor, and leg muscles at week 17 ($P<0.05$) compared to NP pullets. Notably, at both weeks, P pullets' tibiae exhibited greater breaking strengths, higher ash percentages, and elevated concentrations of bone-specific alkaline phosphatase and procollagen type 1 N-terminal propeptide compared to NP pullets ($P<0.05$). The study findings underscore the benefits of providing multi-tier perches for pullets,

serving as a valuable tool for enhancing bird activity and musculoskeletal health preceding the lay phase.

Keywords: pullet, musculoskeletal health, perch, activity, bone health

Introduction

Laying hens experience pronounced biological stress to meet the calcium requirements for eggshell formation. To prepare for this impending calcium demand, the function of osteoblasts changes from forming cortical (structural) bone to depositing medullary bone within the cortical bone (mainly in the long bones, such as the femur and tibia) in pullets nearing sexual maturity, due to a surge in estrogen hormone (1,2). Medullary bone is intended as a reliable source of calcium for eggshell formation, and the amount of medullary bone builds up rapidly during the early stages of lay (1). The supply of medullary bone can be replenished by dietary calcium whereas cortical bone cannot, except if the amount of estrogen decreases and egg production ceases (3–5). Osteoclasts mobilize calcium for eggshell formation mainly from medullary bone, but will take calcium from cortical bone where medullary bone is thin (6,7). Over time, mobilization of the medullary and unreplenishable cortical bone can cause osteoporosis, a major welfare problem in the laying hen industry (8–10).

Housing systems with perches and greater freedom of movement may offer a potential solution to the negative effects of osteoporosis by providing opportunities for exercise during rearing. Pullets that frequently perform exercise-related activities may be better prepared for the strenuous requirements of the lay phase through improved musculoskeletal health at an earlier age. Bone is typically strengthened when weight-bearing load is applied or when the bone is strained by muscle contractions to induce bone remodeling (11,12). Perches can increase activity and load-bearing exercise, as pullets are highly motivated to perch on elevated surfaces (13,14). A few previous studies document the beneficial effect of perches on pullet musculoskeletal health. For example, bone mineral content and leg muscle weights were greater in 12-week-old White Leghorn pullets reared with perches compared to those without, indicating exercise via perch use

had a beneficial impact on bone mineralization and muscle deposition (15). Aviary-reared LSL-Lite pullets with access to perches had improved muscle weights and better bone quality than conventional cage-reared pullets at 16 weeks of age (16). Furthermore, structural bone density of the humeri and tibiae of aviary-reared pullets were greater than for conventional cage-reared White Leghorn pullets, with the former having stronger humeri (17). Conversely, inactivity has shown to increase the incidence of osteoporosis (8). However, the literature lacks information on how multi-tier perches may affect the musculoskeletal health of brown-feathered pullet strains. By providing a structure for perching that contained three levels, birds would be able to perform behaviors such as wing-flapping, walking, running, and jumping which would strengthen their musculoskeletal system. The incorporation of a multi-tier perch particularly in earlier age may encourage birds to perch, practice their balance, and jump up from one rung to reach the next, undergoing more strenuous exercise compared to a single-level perch. In addition, by increasing activity from loading and unloading exercises associated with perching, we hypothesized that pullets would experience improved bone density, mineralization, and muscle deposition at the start of the lay period, possibly reducing the incidence of osteoporosis or bone fracture later in life. The primary objectives encompassed the comparative analysis of musculoskeletal health metrics in brown-feathered pullets accommodated with or without multi-tier perches. Additionally, we endeavored to assess pullet activity levels utilizing a body-worn accelerometer and explore novel biomarkers of bone mineralization. These biomarkers, not previously examined, afford a distinctive perspective, contributing to a comprehensive understanding of the musculoskeletal health and activity profile of Hy-Line brown pullets.

Materials and Methods

Ethics

This experiment was approved by and conducted in accordance with requirements of the Clemson University Institutional Animal Care and Use Committee (protocol #: AUP2021-0068).

Animals and housing

This experiment was conducted in a ventilation- and temperature-controlled poultry house at the Morgan Poultry Center, Clemson, South Carolina, USA, from December 2021 to March 2022. Day-old Hy-Line brown chicks (n = 810) were randomly allocated across 30 pens (27 birds/pen) until 17 weeks of age. Pens (5.2m^2) contained 7.6cm of clean pine wood shavings as bedding. From 0 to 3 weeks of age, feed was provided in tube feeders and water in gallon drinkers, and for the first week of life, supplementary feed trays were provided. After 3 weeks, feed was provided in circular adjustable hanging feeders, and water was available in automatic cup drinkers. Feed and water were provided ad libitum. For the first 3 weeks of age, heat was provided by one focal electric brooder per pen and a gas-fired brooder for the entire house. The temperature was initially set at 35-36°C at day 0, then reduced by 2-3°C every week until 3 weeks of age when brooders were removed. Temperature was reduced weekly until 6 weeks of age to 21°C, then maintained until the end of the study, following the standard breed guidelines (Hy-Line, 2022). The light was provided by one 60-watt incandescent overhead lightbulb per pen and each pen was kept on a decreasing light schedule starting at 20L:4D during the first week and was decreased by increments of either 1.5 or 3 hours until 10L:14D from 7 weeks of age until the end of the study when birds were 17 weeks old (18).

Treatments

From 0 to 17 weeks of age, 15 pens were provided with perches while the remaining 15 pens were without perches. This resulted in two treatment groups: perch (P) and no perch (NP). The perch structure was constructed to be adjustable with perch rungs made of 5×5cm pressure-treated wooden lumber. Each perch structure contained 3 rungs of varying height, each 165.1cm in length, resulting in 495.3cm of total perch space and approximately 19cm of perch space per bird. In the P group, rung heights and distance between rungs were gradually increased concurrently with the growth of the birds to ensure they were easily accessible. For the first 11 days of age, the 3 rungs were 15.2cm, 22.8cm, and 30.4cm high off the ground (Fig 2.1A). For the next 8 days, the 3 rungs were 22.8cm, 38.1cm, and 54.6cm high off the ground (Fig 2.1B). The perch rungs were altered once more on day 19 of age to 38.1cm, 62.2cm, and 88.4cm high, with a 12.7cm distance between each perch rung (Fig 2.1C).

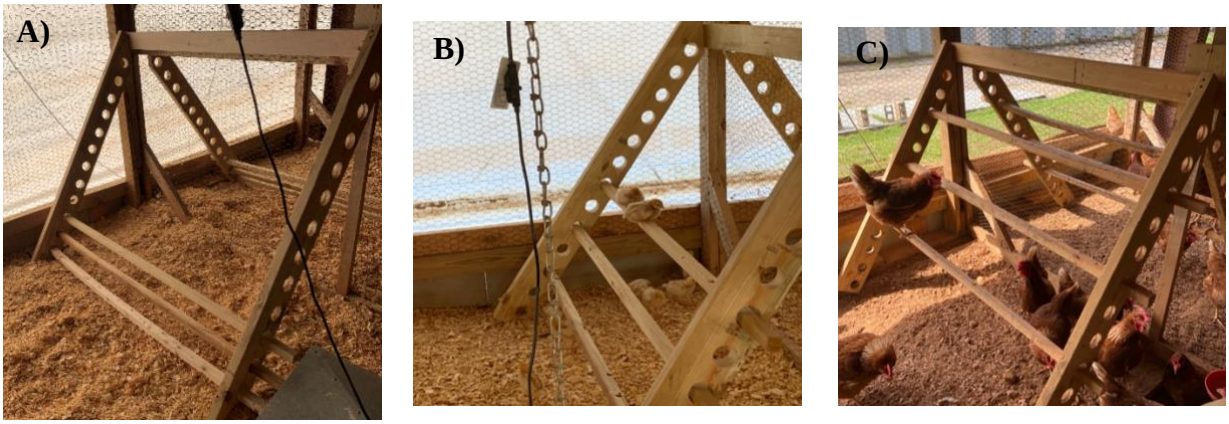

Figure 2.1. Perch and adjustable rung heights in the perch (P) treatment groups during days A) 0-11, B) 11-19, and C) 19+ days of age.

Activity

Bird activity was monitored over 3 consecutive days during weeks 5, 11, and 17 of age (n = 90 birds/week). At each time point, 3 birds per pen were caught after the lights went off. Birds were selected from among different resources and perch levels in an attempt to sample hens that were representative of the flock in that pen. Birds were fitted with a harness that was used to secure the accelerometer. An acceleration data logger (Onset HOBO PendantG acceleration data loggers, Onset Computer Corporation, Bourne, MA, USA) was inserted inside each harness. The loggers used in the current study were 58 ×33×23mm in size and 16g in weight, with a ±3g; 29.4m/s² measuring range, and ± 0.105g; 1.03m/s² accuracy level when operating between –20°C and 70°C. Loggers were oriented on the hens, so the X-axis captured forward and backward movement (craniocaudal movement), the Y-axis captured sideways movement (mediolateral movement), and the Z-axis captured vertical movement (dorsoventral movement) of the hens. Loggers were firmly secured inside the harness to reduce noise in the data due to the movement of the loggers themselves and to prevent changes in logger orientation. After fitting focal birds with harnesses and accelerometers, hens were given 1 day to habituate to wearing the equipment. During this period, hens were monitored to ensure that vests were not impacting behavior and locomotion abilities. After acclimation, loggers recorded hens' movement across 3 consecutive days (72 hours) at each time point, with a scanning frequency of 20 Hz (−3g to +3g) in 3 axes.

Computed tomography (CT) image acquisition

At 11 and 17 weeks of age, 2 birds per pen per week (n = 60) were euthanized on-farm by CO_2 inhalation, placed in a cooler of ice, and immediately transported to Godley-Snell Research Center on Clemson University's campus. Upon arrival, birds were individually placed inside a V-

shaped foam cradle in a dorsal recumbent position atop a hydroxyapatite calibration phantom (QRM Quality Assurance in Radiology and Medicine, Möhrendorf Germany). The head and the legs of the bird were extended in opposite directions and were taped to maintain this positioning in the foam cradle during image acquisition (Fig 2.2). CT images were acquired using a helical mode, head 0-10kg protocol, 0.5mm slice thickness, and bone and soft tissue reconstruction algorithms. CT images were acquired using a Toshiba Aquilion TSX-101A, 16-slice scanner (GE Healthcare, Chicago IL, USA). Birds were dissected immediately after CT scanning and frozen at -29°C for further testing.

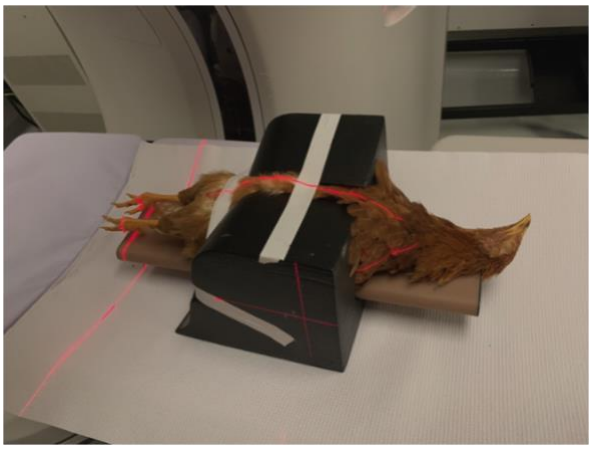

Figure 2.2. Dorsal recumbent positioning of the birds on the hydroxyapatite phantom inside the V-shaped foam cradle for computed tomography image acquisition.

Tibiotarsal CT image analysis

For each CT study, measurements of the right tibiotarsal bone and muscle were made using a standardized CT image analysis protocol previously published by (19). Cross-sectional density (HU) and area (mm) of the total and medullary components of the tibiotarsal bone were recorded at predefined proximal, middle, and distal transverse slice locations using hand-traced

regions of interest (Figs 2.3A and B). The cross-sectional area (CSA) of the muscle group surrounding the tibiotarsus at each of the predefined proximal, middle, and distal locations was also measured. The CT densities for each of the rods in the bone calibration phantom were recorded using the oval ROI tool (Fig 2.3C). The CT densities in HU were then converted to hydroxyapatite values using graphical analysis techniques described in Harrison et al. (19).

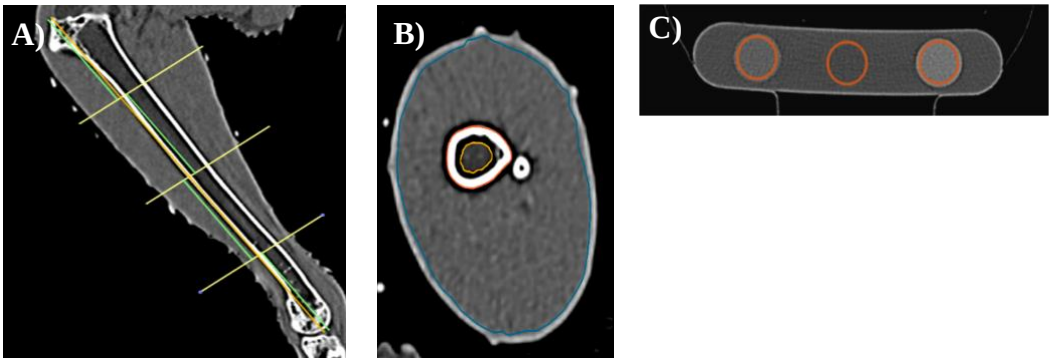

Figure 2.3. Steps of tibiotarsal image analysis. A) Division of the tibiotarsus into 4 segments to set proximal, middle, and distal locations, B) region of interest tracings for the tibiotarsus of a 17-week-old bird in the proximal location, and C) region of interest.

Muscle deposition

Birds were removed from a –29°C freezer and allowed to thaw at refrigerated temperature for approximately 48 hours before dissection. The separation of muscles followed procedures described by Casey-Trott et al. (16) and with the assistance of a veterinarian (A.A.) to ensure consistent muscle specimen collection. Birds were opened by cutting the skin below the bottom of the keel bone and peeling it back to expose the interior of the bird. To remove the right bicep and triceps brachii, the skin of the wing was peeled back, and a blunt dissection was made along the line of demarcation between the biceps and triceps. The bicep and triceps were gently

freed from the bone, and the proximal and distal tendons were cut at the bone level. To separate the pectoralis muscles, fascia was cut along the line of demarcation, separating the fats from the pectoralis muscles and severing all the attachment at the origin (Crania sternum, furcula, and sternal ribs), and at the insertion of the major (proximal ventral surface of the humerus) and of the minor (proximal dorsal surface of the humerus). The left leg muscles, tendons, and ligaments were detached from the bone, the Achilles tendon was severed, and the fascia along the synsacrum was detached. All muscles were immediately weighed upon removal. The left tibiae were frozen at -29°C for ash percentage, and the right tibiae were frozen at -20°C for breaking strength measures.

Breaking strength

Mechanical properties of the right tibiotarsi were assessed using a three-point bending test as specified by the American National Standards Institute (ANSI) standards for the application of 3 point bending on animal bones (20). Testing was performed using an Instron Dynamic and Static Material Test system (Model 5944, Instron Corp., Canton, MA, USA) equipped with a 500N load cell and Automated Material Test System software. Prior to testing, previously frozen legs were thawed at refrigerator temperature. Muscles surrounding the tibiotarsus were carefully dissected, tibiotarsal length and diameter at the midpoint were recorded, and the bones were wrapped in saline-soaked paper towels until testing.

Rounded support pins and breaking blades were manufactured based on ANSI/ASAE S459 MAR1992 (R2017) standards for the application of 3-point bending on animal bones (20). A furculum width of 4cm was used (Fig 2.4). This width did not adhere to the ANSI standards, but was decided upon based on a consensus among coauthors. Due to the anatomy of the laying hen tibiotarsus, a 4cm width ensured that the tibiotarsus was able to rest on the furculum in a manner

in which the load would be applied to the midpoint of the bone evenly in the craniocaudal plane. The crosshead speed used was 3mm/min, and the test was carried out to failure. Load and displacement data were collected and were used to obtain the breaking strength (N), stiffness (N/mm), and maximum bending moment (N/m).

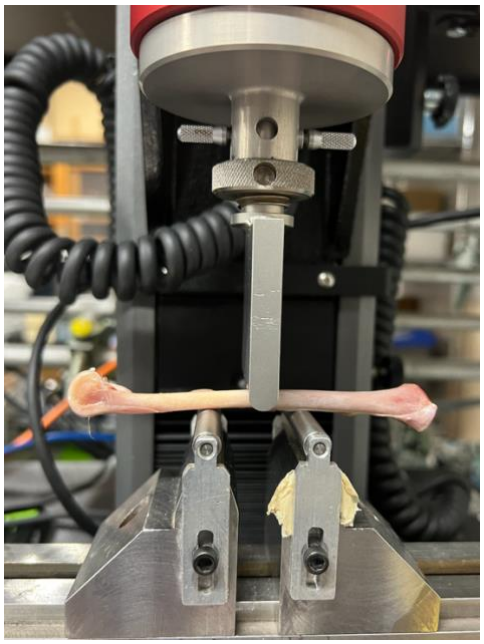

Figure 2.4. Instron configuration with rounded supports and breaking blade machined according to ANSI standards.

Tibia ash percentage

Left tibiotarsi of euthanized birds were thawed approximately 24 hours prior to data collection. The bones were cleaned from any surrounding muscles and soft tissues, and tibiae were separated from the fibula and were cut into 3 pieces to fit into a Soxhlet chamber for ether extraction. Ceramic crucibles were air-dried for one hour and then placed in a desiccator for another hour. The weight of the dried crucibles was recorded. Left tibiae were dried at 100°C for one hour, placed in a desiccator for another hour, and their weight was recorded. Tibiae were

then placed inside the dried ceramic crucibles and ashed (ashing oven: Thermolyne 30400, Barnstead International, Dubuque, IA, USA) for 6 hours at 600°C. The ash was placed in a desiccator for one hour, and then the ash weight was recorded. The percentage of tibia ash was calculated by dividing the tibia ash weight by the tibia dry weight and multiplying by 100.

Bone mineralization

During weeks 11 and 17 of age, blood samples were collected from the brachial wing vein of 3 birds per pen per week (n = 90). Whole blood samples were transferred to 1.5mL Eppendorf tubes, and serum was separated at 6000 rpm for 10 minutes at 4˚C. Serum samples were analyzed for levels of bone-specific alkaline phosphatase (BALP) and procollagen type 1 N-terminal propeptide (P1NP) using commercial ELISA kits Nanjing Jiancheng Institute of Bioengineering (Nanjing, China) and MyBioSource (San Diego, CA, USA), respectively.

Data processing and statistical analysis

The raw accelerometer data, consisting of the date, time, and the related impulse in the X, Y, and Z dimensions, were downloaded from the devices (HOBOware Graphing & Analysis Software, Bourne, MA, USA) at the end of each 3 day observation period. Data on hens' vertical (a_z: dorsoventral movement across vertical levels), horizontal (a_x: craniocaudal movement within the same vertical level), and lateral movement (a_y: mediolateral movement within the same vertical level) during light hours were obtained directly from loggers. Hens' triaxial movement (A_s) was calculated by summing and averaging raw movement data as follows.

$$A_s = \sqrt{a_x^2 + a_y^2 + a_z^2}$$

Acceleration data were post-processed using MATLAB (MATLAB and Statistics Toolbox Release 2012, The MathWorks, Inc., Natick, MA, USA). In order to accurately calculate the incidence of massive acceleration shifts on the vertical (z) axis that represents perching, data were smoothed from noisy components by removing all minor acceleration fluctuations using a loop function.

$$A_i = \frac{1}{3}\sum_{j=i-1}^{i+1} A_j A_i' = \begin{cases} \mu, if\ |A_i - \mu| < t \\ \mu, if\ |A_i - \mu| \geq t \end{cases}$$

Data smoothing included the passing of the raw acceleration values (*Aj*) through an asymmetrical 3-point-moving average low-pass filter (*I* = the middle point in the 3-point-moving average low-pass filter) and through a step function to define thresholds used to remove minor fluctuations (t = threshold values of minor fluctuations, i.e., between 0.001 and 0.043g). After processing data, perching events were recognized by detecting massive shifts in acceleration in the z-axis of activity. In order to precisely detect acceleration shifts due to perching and define thresholds for minor fluctuations in the z-axis, timestamped videos of birds while perching were obtained and compared with the corresponding activity data. Using the approach enabled us to locate shifts in z-axis acceleration mainly caused by perching and define the threshold cutoff points to remove minor fluctuation.

Data were analyzed using the R software (version 3.3.1) with the package "stats" (R Core Team, 2013). To test for the main effects of treatment (P and NP) and the age of the birds (activity; weeks 5, 11, and 17, bone demineralization, and tibiotarsal BMD and CSA: weeks 11, and 17) on each variable, generalized linear mixed-effects models (GLMMs) were conducted using the "lme4" package (21). In each GLMM, the interaction term between main effects was also tested

as a fixed effect, and bird ID, pen, and day for activity were tested as random effects, with the family set to "Quasibinomial" for proportion data (ash %) and "Poisson" for the other data. Tukey's HSD multiple comparison procedure was used for post hoc comparisons using the "multcomp" package (22) The "DHARMa" package was used for proportion data (ash %) to test residual distribution and assumptions for GLMM, while the Shapiro–Wilk test was utilized (i.e., activity (g), breaking strength (N), stiffness (N/mm)) for the normality analysis of the model residuals. Statistical significance was set at $p < 0.05$. Descriptive statistics were calculated using the "psych package", and data are presented as mean ± standard error of the mean (SEM).

Results

Activity

At weeks 5, 11, and 17 of age, pullets housed with perches showed increased vertical activity and average daily vertical displacement per bird compared to pullets housed without perches (Table 2.1). Furthermore, pullets housed with perches had decreased horizontal activity compared to pullets housed without perches at weeks 5, 11, and 17 of age (Table 2.1). Overall activity levels did not differ between treatment groups at any week of age ($p > 0.05$; Table 2.1).

Table 2.1. Activity of pullets housed with perches (P) or no perches (NP) for 3 consecutive days at weeks 5, 11, and 17 of age (n = 90 birds/week; g = gravitational force; f = frequency).

Parameter Week/Treatment		Overall Activity (g)	Vertical Activity (g)	Horizontal Activity (g)	Daily Vertical Displacement (f)
Week 5	Perch (P)	1.72±0.36	0.73±0.15*	0.99±0.12	26.88±6.85*
	No Perch (NP)	1.65±0.33	0.15±0.11	1.50±0.22*	5.36±1.66
	P-value	0.423	0.024	0.032	0.001
Week 11	Perch (P)	1.43±0.32	0.61±0.16*	0.82±0.18	15.96±5.85*
	No Perch (NP)	1.38±0.41	0.16±0.12	1.22±0.32*	3.22±1.96
	P-value	0.355	0.019	0.035	0.001
Week 17	Perch (P)	1.36±0.35	0.52±0.13*	0.84±0.11	13.85±4.52*
	No Perch (NP)	1.29±0.38	0.11±0.09	1.18±0.19*	2.52±1.01
	P-value	0.256	0.011	0.021	0.001
P-value	Week	0.219	0.153	0.185	0.287
	Treatment	0.426	0.003	0.001	0.002
	Week × Treatment	0.328	0.001	0.001	0.001

*Means within the same column (parameter), week of age, and across rows (treatments) indicate statistically significant differences ($P < 0.05$).

Musculoskeletal health

Muscle deposition

At week 11 of age, pullets housed with perches had greater leg muscle group weights compared to pullets without perches ($p = 0.041$; Fig 2.5). There were no differences between treatments for biceps brachii, triceps brachii, pectoralis major, or pectoralis minor weights at week 11 of age ($p > 0.05$).

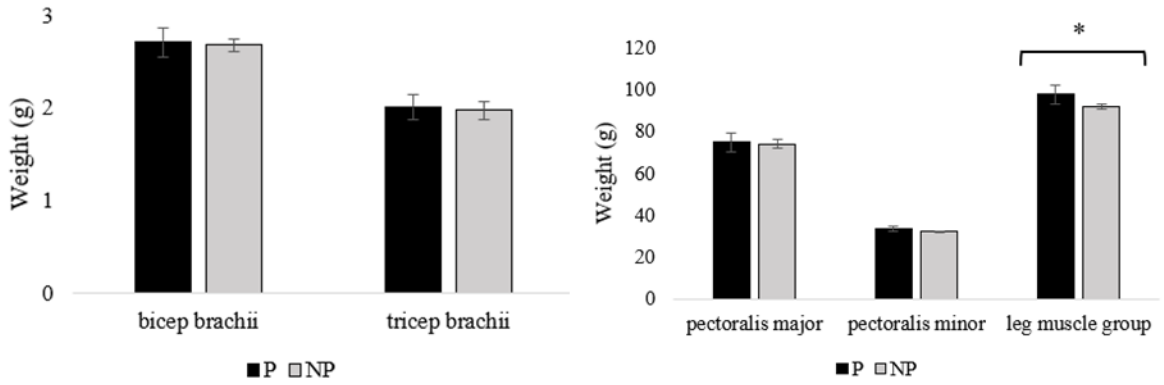

Figure 2.5. Weight (grams) of biceps brachii, triceps brachii, pectoralis major, pectoralis minor, and leg muscle group of 11-week-old pullets (n = 60 birds) housed with perches (P) or no perches (NP). Results are presented as mean weight (grams) ± SEM. *Across bars indicates significant statistical differences at $p < 0.05$.

At week 17 of age, pullets housed in P pens had greater triceps brachii ($p = 0.041$), pectoralis major ($p = 0.032$), pectoralis minor ($p = 0.039$), and leg muscle group ($p = 0.021$) weights compared to pullets from NP pens (Fig 2.6). There were no differences between treatments for bicep brachii weights at week 17 of age ($p > 0.05$).

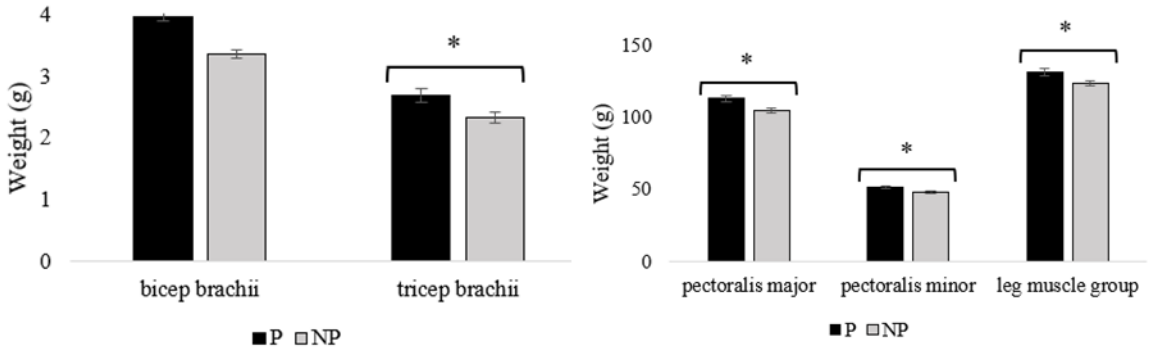

Figure 2.6. Weight (grams) of bicep brachii, triceps brachii, pectoralis major, pectoralis minor, and leg muscle group of 17-week-old pullets (n = 60 birds) housed with perches (P) or no perches (NP). Results are presented as mean weight (grams) ± SEM. *Across bars indicates significant statistical differences at $p < 0.05$.

Bone mineral density (BMD) and bone cross-sectional area (BCSA)

At week 11 of age, pullets housed with perches had greater cortical BCSA at the proximal section of the tibia and greater total and cortical BMD at all regions, with a tendency for a larger cortical BMD at the proximal section compared to pullets housed without perches (Table 2.2). At week 17 of age, pullets housed with perches had greater cortical BCSA, and total and cortical BMD at all sections of the tibia compared to pullets housed without perches (Table 2.2).

Table 2.2. Bone mineral density (BMD; mg/cm3) and bone cross-sectional area (BCSA; mm2) ± SEM for the total and cortical regions of the right tibiotarsus of pullets housed with perches (P) or no perches (NP) at 11 (n = 60 birds) and 17 (n = 60 birds) weeks of age.

Parameter		Bone Cross Sectional Area (mm^2)					
Week/Treatment		Total			Cortical		
		Proximal	Middle	Distal	Proximal	Middle	Distal
Week 11	Perch (P)	59.69±0.96	45.86±1.03	47.85±1.15	40.30±1.03	29.46±1.15	30.76±1.16
	No Perch (NP)	58.95±1.02	45.36±1.12	47.03±1.16	37.96±1.55	28.19±1.12	29.06±1.03
	P-value	0.351	0.152	0.216	0.043	0.253	0.152
Week 17	Perch (P)	62.69±1.06	47.99±0.79	49.58±0.88	44.64±1.58	35.98±1.23	36.07±1.03
	No Perch (NP)	61.52±0.75	46.13±0.88	48.63±1.01	40.99±1.16	33.69±1.03	34.26±1.81
	P-value	0.152	0.143	0.215	0.036	0.041	0.039
P-value	Week	0.096	0.263	0.199	0.423	0.039	0.096
	Treatment	0.185	0.258	0.452	0.023	0.046	0.036
	Week×Treatment	0.253	0.326	0.235	0.044	0.044	0.043
Parameter		Bone Mineral Density (mg/cm^3)					
Week/Treatment		Total			Cortical		
		Proximal	Middle	Distal	Proximal	Middle	Distal
Week 11	Perch (P)	340.84±19.17	420.63±27.76	304.92±23.10	372.10±20.93	449.70±29.68	352.85±26.73
	No Perch (NP)	276.25±19.89	321.82±24.86	243.13±20.97	324.73±15.83	383.31±21.92	308.65±20.26
	P-value	0.002	0.012	0.031	0.093	0.039	0.044
Week 17	Perch (P)	599.68±40.34	859.55±67.00	938.19±71.47	1196.28±134.58	2055.04±271.27	1651.45±250.19
	No Perch (NP)	537.88±21.07	761.35±20.04	707.66±24.17	953.69±107.29	1324.22±174.80	1103.99±167.25
	P-value	0.011	0.012	0.021	0.013	0.011	0.011
P-value	Week	0.013	0.014	0.036	0.026	0.023	0.034
	Treatment	0.011	0.021	0.021	0.018	0.012	0.021
	Week×Treatment	0.021	0.011	0.011	0.016	0.011	0.013

Breaking strength

At week 11 of age, pullets housed with perches had greater breaking strength and maximum bending moment compared to pullets housed without perches (Table 2.3). At week 17 of age, pullets housed with perches had greater breaking strength and stiffness compared to pullets housed without perches (Table 2.3). There were no differences between stiffness at week 11 of age ($p > 0.05$) or maximum bending moment at week 17 of age ($p > 0.05$; Table 2.3).

Table 2.3. Breaking strength (N), stiffness (N/mm), and maximum bending moment (N/m) of pullets housed with perches (P) or no perches (NP) at weeks 11 (n = 60 birds) and 17 (n = 60 birds) of age.

Parameter		Breaking strength (N)	Stiffness (N/mm)	Max. bending moment (N/m)
Week/Treatment				
Week 11	Perch (P)	171.71±9.89	184.98±8.67	502.37±22.63
	No Perch (NP)	153.05±3.78	178.16±13.26	437.08±16.59
	P-value	0.021	0.103	0.013
Week 17	Perch (P)	276.63±10.31	289.96±12.45	681.51±32.47
	No Perch (NP)	220.02±8.14	240.59±18.95	660.14±25.22
	P-value	0.031	0.029	0.135
P-value	Week	0.523	0.031	0.043
	Treatment	0.029	0.041	0.029
	Week × Treatment	0.017	0.038	0.046

*Means within the same column (parameter), week of age, and across rows (treatments) indicate statistically significant differences ($P < 0.05$).

Tibia ash percentage

At week 11 of age, pullets housed with perches had higher ash percent compared to pullets without perches (Table 2.4). Similarly, at week 17 of age, pullets housed with perches had higher ash percent compared to pullets without perches (Table 2.4).

Table 2.4. Tibia ash percent (%) of pullets housed with perches (P) or no perches (NP) at weeks 11 (n = 30 birds) and 17 (n = 60 birds) of age.

Parameter Week/Treatment		Bone Ash (%)
Week 11	Perch (P)	54.36±0.12
	No Perch (NP)	53.98±0.17
	P-value	0.039
Week 17	Perch (P)	54.96±0.21
	No Perch (NP)	54.19±0.19
	P-value	0.023
P-value	Week	0.089
	Treatment	0.031
	Week × Treatment	0.022

Bone mineralization

During week 11, birds housed in P pens had higher levels of BALP ($p = 0.032$) and P1NP ($p = 0.026$) compared to birds housed in NP pens (Figs 2.7 and 2.8). Similarly, during week 17, birds housed in P pens had higher levels of BALP ($p = 0.011$) and P1NP ($p = 0.016$) than birds housed in NP pens (Figs 2.7 and 2.8). There were no differences in treatments between weeks 11 and 17 ($p = 0.542$; Figs 2.7 and 2.8).

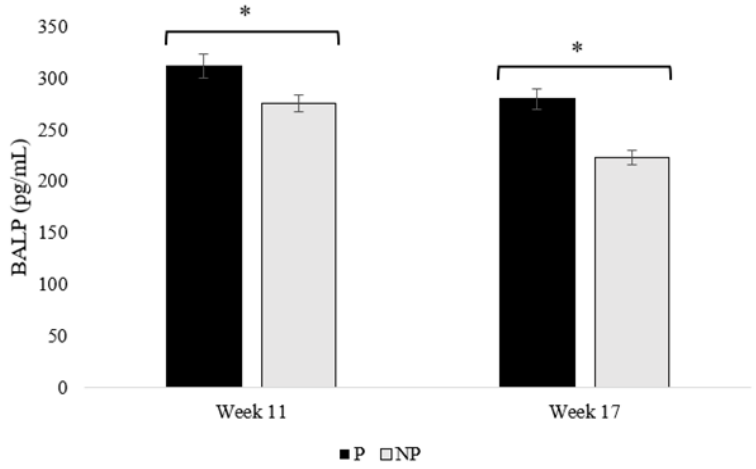

Figure 2.7. Concentrations of bone-specific alkaline phosphatase (BALP) for pullets housed in perch (P) and no perch (NP) housing environments during weeks 11 and 17 (n = 90 birds/week). Results are presented as mean ± SEM. *Across bars indicates significant statistical differences at $p < 0.05$.

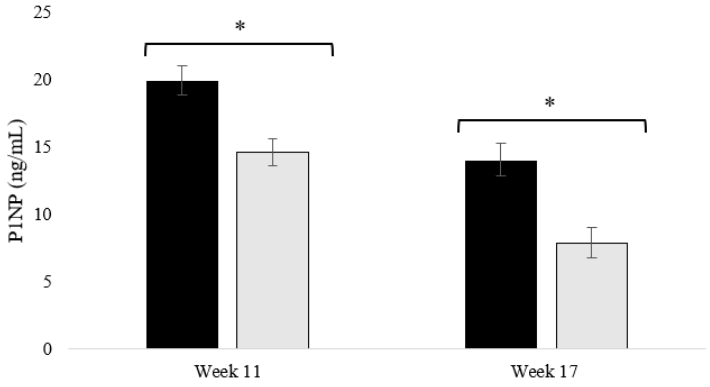

Figure 2.8. Concentrations of pro-collagen type 1 n-terminal propeptide (P1NP) for pullets housed in perch (P) and no perch (NP) housing environments during weeks 11 and 17 (n = 90 birds/week). Results are presented as mean ± SEM. *Across bars indicates significant statistical differences at $p < 0.05$.

Discussion

The objective of this study was to investigate the effects of access to a multi-tier perch during rearing on Hy-Line brown pullet activity and musculoskeletal health. Our results suggest that access to perches during rearing increases vertical activity levels and improves aspects of musculoskeletal health, which may benefit pullets as they enter the lay phase.

Activity

The addition of multi-tier perches to a floor pen environment increased the vertical activity of pullets, as well as the average daily vertical displacement per bird at weeks 5, 11, and 17 of age. The increase in vertical activity level stimulated by the addition of perches is in agreement with previous studies, especially considering that pullets are highly motivated to perch on elevated surfaces (15,23,24). The vertical movement of perching behavior is performed by wing-assisted jumping, which is a form of load-bearing exercise that can strengthen the musculoskeletal system (16,17). Although overall activity levels did not differ between treatment groups, the increase in vertical activity seen in pullets reared with perches suggests they were reaching higher areas of the pen and performing load-bearing exercises more often compared to pullets without perches, which could improve their musculoskeletal health.

Muscle deposition

We observed greater leg muscle group weights of pullets reared with perches at 11 weeks of age with no differences in the weights of other muscles, suggesting they were engaging the leg muscles more than the breast or wing muscles. In agreement, previous research found that when averaging muscle weights between 3, 6, and 12 week old pullets, the only observed difference between pullets reared in conventional cages with perches and those without was between thigh, not breast muscle weights (15). At 17 weeks of age, we observed greater triceps brachii, pectoralis major and minor, and leg muscle group weights in pullets housed with perches than those without. By 17 weeks of age, pullets were likely using their wings to assist in jumping on and off perches, whereas pullets without perches had no such opportunity to engage the wing and breast muscles. Previous research reports that wing, breast, and leg muscle weights differ between aviary-reared and conventional cage-reared pullets at 16 weeks of age (16). However, one previous study did not find a difference in pectoralis major or minor, bicep, or leg muscle group weights between pullets housed in an open-concept barn with platforms, ramps, and at least six perches and those housed in a single level wire-floor brooding compartment with only two perches at 10 and 16 weeks of age (25). Interestingly, this previous study found that brown-feathered strains had lower pectoralis major weights, but higher leg muscle group weights compared to white-feathered strains regardless of housing type (25). The brown-feathered birds used their leg muscles more, resulting in increased leg muscle weights, whereas the white-feathered birds performed more wing-associated behaviors, using their pectoral muscles more than brown-feathered strains, resulting in the increased pectoral weights (25). This is in line with our study, where brown-feathered pullets had heavier leg muscles than those without perches due to increased load-bearing exercise involving the legs. Ultimately, muscle development does seem to take time and depend on genetic

makeup, where first the leg muscles are mostly engaged theoretically to jump on perches at 11 weeks of age, and second, the wing and breast muscles are also engaged to assist in load bearing exercise associated with jumping on and off the elevated perch rungs.

Bone mineral density (BMD) and bone cross-sectional area (BCSA)

At week 11 of age, pullets housed with perches had greater cortical BCSA at the proximal section of the tibia, and a greater total and cortical BMD compared to pullets housed without perches. Furthermore, at week 17 of age, pullets housed with perches had greater cortical BCSA at all regions of the tibia, and greater total and cortical BMD compared to pullets housed without perches. These results indicate that at 11 and 17 weeks of age, pullets reared with perches showed improved bone mass compared to those without. In our study, pullets with access to perches also exhibited more vertical activity and vertical displacement (i.e., jumping) behavior at 5, 11, and 17 weeks of age compared to pullets without access to perches, likely resulting in the beneficial effect observed on BMD and BCSA. This is because load bearing exercise associated with perching can positively impact bone development (26,27). Our findings in brown-feathered pullets support previous literature, where white-feathered pullets housed in furnished cages with platforms and terraces had higher bone mineral densities than pullets in conventional cages at 4, 12, and 16 weeks of age (28). Furthermore, pullets provided opportunities for load bearing exercise in the form of wing-assisted jumping and increased vertical activity showed higher bone mineral density compared to pullets without opportunity to perform such exercise (16,17,29–31). Furthermore, 16-week-old pullets reared in an aviary system showed thicker cortices in the tibia and humerus compared to those reared in conventional cages (17). Our findings indicate a higher amount of structural bone in pullets housed with perches. This greater amount of total and cortical bone

density and area resulting from the perch treatment will likely benefit pullets as they reach the lay phase compared to pullets reared without perches. As pullets enter the lay phase, osteoclasts mobilize calcium from the cortical and medullary bone to be used for eggshell formation. Over time, this prolonged loss of nutrients from the bones results in weakness and susceptibility to fracture. By having a large cortical bone density and area (indicating strong bones) before the start of the lay phase, pullets may be less prone to fracture later on in their adult life. Indeed, it has been indicated that providing perches to pullets can have a long-term beneficial impact on musculoskeletal health of adult laying hens (32–34).

Breaking strength

Pullets housed with perches showed greater breaking strength at 11 and 17 weeks of age, greater maximum bending moment at 11 weeks of age, and greater stiffness at 17 weeks of age compared to pullets housed without perches. The greater breaking strength observed at both testing weeks indicates that pullets reared with perches had stronger bones as early as 11 weeks of age compared to those without perches, which is in line with our activity, muscle deposition, BMD, and BCSA findings. Also in agreement, previous studies demonstrate that the force required to fracture the humerus and tibia of aviary-reared pullets is higher than for conventional cage-reared pullets (16,35). Additionally, pullets reared in an open-concept barn with platforms, ramps, and at least six perches had stronger tibiae and femurs compared to pullets in in a single level wire-floor brooding compartment with only two perches at 10 and 16 weeks of age (25). Considering that pullets housed with multi-tier perches were performing more vertical activity and vertical displacement per day, it follows that their tibiae would be stronger than pullets without access to multi-tier perches. In pullets reared with perches, we observed a greater maximum bending

moment (elasticity) at 11 weeks of age and a greater stiffness (rigidity) at 17 weeks of age compared to pullets reared without perches. Previous research discovered similar results, where 16-week-old pullets reared in aviaries had higher stiffness values compared to pullets reared in conventional cages (17). Bone is a complex material, and its strength and health stems from the delicate balance between rigidity and elasticity (36). The bone must be stiff (rigid) enough to withstand applied force and allow for load-bearing exercise, but also elastic and flexible enough to absorb energy (36–38). Therefore, these interplaying variables indicate a tibia that is more resistant to fracture, strong enough to facilitate complex locomotion (i.e., jumping to and from perch rungs of varying heigh and distance), and flexible enough to absorb shock without failing. Ultimately, the bone breaking strength, maximum bending moment and stiffness measures indicate that rearing pullets with perches beneficially alters bone composition so that the overall bone is stronger, more flexible, and the force required to fracture increases.

Tibia ash percentage

At weeks 11 and 17 of age, pullets housed with perches had a higher ash percent compared to pullets without perches, suggesting improved bone quality which is also in agreement with and reflected by our previous measures of musculoskeletal health. Ash percent is used to measure the amount of minerals in the bone, translating to overall bone health, and has been highly correlated to quantitative computed tomography calculated tibial bone mineral content in laying hens (39). One previous study found no difference in tibia ash percent between 16-week-old White Leghorn pullets housed in aviary or conventional cage systems, but they did find differences in humerus ash percent, indicating the tibia and humerus respond differently to load-bearing exercise during development (17). As previously discussed, our brown-feathered pullets reared with perches had

heavier leg muscle weights than those without access to perches, so it does track that the tibia ash percent would be greater in the group of pullets with access to perches than those without due to increased activity levels. Although we did not analyze humerus ash percent, the significant difference in tibia ash percent suggests that providing multi-tier perches during development does improve tibiotarsal bone mineral content through increased vertical activity.

Bone mineralization

We observed higher levels of BALP and P1NP in birds reared with perches compared to those without perches at weeks 11 and 17 of age. Although a novel measure of bone mineralization in poultry, higher concentrations of BALP and P1NP can indicate greater rates of bone mineralization, as they are both markers of bone formation (40,41). BALP is produced by osteoblasts and is a specific marker of bone formation and osteoblast activity (40,42,43). During the secretion of collagen, which forms the basis of the bone matrix, the N-terminal propeptide (P1NP) is cleaved off and indicates bone formation activity (41,44,45). However, abnormally high levels of BALP and P1NP may suggest underlying problems such as bone disease (46). But, most other measures of bone health and quality were improved in pullets reared with perches, supporting this was not the case. Based on our review of the literature, this is the first study to evaluate the effect of perches on biomarkers of bone formation in pullets and indicates a positive effect of activity associated with perch use on bone mineralization.

Conclusions

In the current study, we observed significantly elevated levels of vertical locomotor activity, enhanced muscular tissue deposition, increased bone mineral density, improved bone biomechanical characteristics, elevated tibia ash content, and heightened bone mineralization in Hy-Line brown pullets provided with multi-tier perches compared to those deprived of access to perches. These discernible enhancements in pullets suggest that weight-bearing physical activity resulting from interaction with perches exerts a beneficial influence on the musculoskeletal properties of pullets at both 11 and 17 weeks of age. Providing pullets with multi-tier perches from 0 to 17 weeks of age promotes exercise, improves musculoskeletal health, and stimulates vertical activity, subsequently better preparing them for the lay phase and potentially reducing the risk of bone fractures in the future. These findings are in agreement with previous studies in white-feathered strains (16,17). Subsequent studies should aim to enhance our understanding of the long-term impacts of perching interventions on pullet welfare and bone health.

Declaration of competing interests

The authors declare no competing interests.

Acknowledgement

This study was supported by the United Sorghum Checkoff Program (project # RG002-21) and from the National Institute of Food and Agriculture, U.S. Department of Agriculture, Hatch project #NC1029, approved as Technical Contribution No. 7236 of the Clemson university Experiment Station. This project was partially funded by the Charles Lee Morgan Mem Student

Assistance Quasi Endowment. We would like to thank all the staff and student workers at the Morgan Poultry Center for their time and effort on this project.

References

1. Whitehead CC. Overview of bone biology in the egg-laying hen. Poult Sci. 2004 Feb 1;83(2):193–9.
2. Beck MM, Hansen KK. Role of estrogen in avian osteoporosis. Poult Sci. 2004;83(2):200–6.
3. Hudson HA, Britton WM, Rowland GN, Buhr RJ. Histomorphometric Bone Properties of Sexually Immature and Mature White Leghorn Hens with Evaluation of Fluorochrome Injection on Egg Production Traits. Poult Sci. 1993 Aug;72(8):1537–47.
4. Fleming RH, McCormack HA, McTeir L, Whitehead CC. Medullary bone and humeral breaking strength in laying hens. Res Vet Sci. 1998 Jan;64(1):63–7.
5. Beck MM, Hansen KK. Role of estrogen in avian osteoporosis. Poult Sci. 2004 Feb;83(2):200–6.
6. Habig C, Weigend A, Baulain U, Petow S, Weigend S. Influence of Age and Phylogenetic Background on Blood Parameters Associated With Bone Metabolism in Laying Hens. Front Physiol. 2021 Apr 29;12:592.
7. Fleming RH, McCormack HA, McTeir L, Whitehead CC. Relationships between genetic, environmental and nutritional factors influencing osteoporosis in laying hens. Br Poult Sci. 2006 Dec 15;47(6):742–55.
8. Whitehead CC, Fleming RH. Osteoporosis in Cage Layers. Poult Sci. 2000 Jul 1;79(7):1033–41.
9. Whitehead CC, Fleming RH, Julian RJ, Sørensen P. Skeletal problems associated with selection for increased production. Poultry Genetics, Breeding, and Biotechnology. 2003;3:29–52.
10. Petrik MT, Guerin MT, Widowski TM. On-farm comparison of keel fracture prevalence and other welfare indicators in conventional cage and floor-housed laying hens in Ontario, Canada. Poult Sci. 2015 Apr 1;94(4):579–85.
11. Weiss E. Reading the Bones. University Press of Florida; 2017.
12. Frost HM. Bone “mass” and the “mechanostat”: A proposal. Anat Rec. 1987 Sep;219(1):1–9.
13. Newberry RC, Estevez I, Keeling LJ. Group size and perching behaviour in young domestic fowl. Appl Anim Behav Sci. 2001 Jul 28;73(2):117–29.
14. Olsson IAS, Keeling LJ. Night-time roosting in laying hens and the effect of thwarting access to perches. Appl Anim Behav Sci [Internet]. 2000 [cited 2022 Nov 14];68:243–56. Available from: www.elsevier.comrlocaterapplanim
15. Enneking SA, Cheng HW, Jefferson-Moore KY, Einstein ME, Rubin DA, Hester PY. Early access to perches in caged White Leghorn pullets. Poult Sci. 2012 Sep;91(9):2114–20.
16. Casey-Trott TM, Korver DR, Guerin MT, Sandilands V, Torrey S, Widowski TM. Opportunities for exercise during pullet rearing, Part I: Effect on the musculoskeletal characteristics of pullets. Poult Sci. 2017 Aug 1;96(8):2509–17.
17. Regmi P, Deland TS, Steibel JP, Robison CI, Haut RC, Orth MW, et al. Effect of rearing environment on bone growth of pullets. Poult Sci. 2015 Mar 1;94(3):502–11.

18. Hy-Line Brown Management Guide [Internet]. 2016. Available from: www.hylinena.com
19. Harrison C, Jones J, Bridges W, Anderson G, Ali A, Mercuri J. Associations among computed tomographic measures of bone and muscle quality and biomechanical measures of tibiotarsal bone quality in laying hens. Am J Vet Res. 2023 Sep 26;1–9.
20. ANSI/ASAE. ANSI/ASAE S459 MAR1992 (R2017) Shear and Three-Point Bending Test of Animal Bone. St. Joseph; 2017.
21. Bates D, Mächler M, Bolker B, Walker S. Fitting Linear Mixed-Effects Models Using lme4. J Stat Softw. 2015;67(1).
22. Hothorn T, Bretz F, Westfall P. Simultaneous Inference in General Parametric Models. Biometrical Journal. 2008 Jun 1;50(3):346–63.
23. Gunnarsson S, Yngvesson J, Keeling LJ, Forkman B. Rearing without early access to perches impairs the spatial skills of laying hens. Appl Anim Behav Sci. 2000 Apr;67(3):217–28.
24. Brantsæter M, Nordgreen J, Rodenburg TB, Tahamtani FM, Popova A, Janczak AM. Exposure to Increased Environmental Complexity during Rearing Reduces Fearfulness and Increases Use of Three-Dimensional Space in Laying Hens (Gallus gallus domesticus). Front Vet Sci. 2016 Feb 29;3.
25. Pufall A, Harlander-Matauschek A, Hunniford M, Widowski TM. Effects of Rearing Aviary Style and Genetic Strain on the Locomotion and Musculoskeletal Characteristics of Layer Pullets. Animals. 2021 Feb 27;11(3):634.
26. Patel VS, Judex S, Rubin J, Rubin CT. Mechanisms of exercise effects on bone quantity and quality. In: Bilezikian JP, Martin TJ, Clemens TL, Rosen CJ, editors. Principles of Bone Biology. 4th ed. Academic Press; 2020. p. 1759–84.
27. Yuan Y, Chen X, Zhang L, Wu J, Guo J, Zou D, et al. The roles of exercise in bone remodeling and in prevention and treatment of osteoporosis. Prog Biophys Mol Biol. 2016 Nov;122(2):122–30.
28. Khanal T, Bédécarrats GY, Widowski T, Kiarie EG. Rearing cage type and dietary limestone particle size: I, effects on growth, apparent retention of calcium, and long bones attributes in Lohmann selected Leghorn-Lite pullets. Poult Sci. 2020 Sep;99(9):4454–65.
29. Campbell DLM, De Haas EN, Lee C. A review of environmental enrichment for laying hens during rearing in relation to their behavioral and physiological development. Poult Sci. 2019 Jan 1;98(1):9–28.
30. Khanal T, Bedecarrats GY, Kiarie EG. Cage type and mineral nutrition had independent impact on skeletal development in Lohmann LSL-Lite pullets from hatch to 16 weeks of age. Animal Nutrition. 2021 Sep;7(3):631–40.
31. Jendral MJ, Korver DR, Church JS, Feddes JJR. Bone Mineral Density and Breaking Strength of White Leghorns Housed in Conventional, Modified, and Commercially Available Colony Battery Cages. Poult Sci. 2008 May;87(5):828–37.
32. Hester PY, Enneking SA, Haley BK, Cheng HW, Einstein ME, Rubin DA. The effect of perch availability during pullet rearing and egg laying on musculoskeletal health of caged White Leghorn hens. Poult Sci. 2013 Aug 1;92(8):1972–80.
33. Casey-Trott TM, Korver DR, Guerin MT, Sandilands V, Torrey S, Widowski TM. Opportunities for exercise during pullet rearing, Part II: Long-term effects on bone

characteristics of adult laying hens at the end-of-lay. Poult Sci. 2017 Aug;96(8):2518–27.
34. Neijat M, Casey-Trott TM, Robinson S, Widowski TM, Kiarie E. Effects of rearing and adult laying housing systems on medullary, pneumatic and radius bone attributes in 73-wk old Lohmann LSL lite hens. Poult Sci. 2019 Jul;98(7):2840–5.
35. DeLand TS, Dudgeon RE, Orth MW, Karcher DM, Haut RC. Effect of Housing System on Properties of Pullet Bones. In: ASME 2012 Summer Bioengineering Conference, Parts A and B. American Society of Mechanical Engineers; 2012. p. 551–2.
36. Hart NH, Newton RU, Tan J, Rantalainen T, Chivers P, Siafarikas A, et al. Biological basis of bone strength: anatomy, physiology and measurement. J Musculoskelet Neuronal Interact. 2020 Sep 1;20(3):347–71.
37. Currey JD. How Well Are Bones Designed to Resist Fracture? Journal of Bone and Mineral Research. 2003 Apr 2;18(4):591–8.
38. Seeman E, Delmas PD. Bone Quality — The Material and Structural Basis of Bone Strength and Fragility. New England Journal of Medicine. 2006 May 25;354(21):2250–61.
39. Robison CI, Karcher DM. Analytical bone calcium and bone ash from mature laying hens correlates to bone mineral content calculated from quantitative computed tomography scans. Poult Sci. 2019 Sep;98(9):3611–6.
40. Magnusson P, Arlestig L, Paus E, Di Mauro S, Testa MP, Stigbrand T, et al. Monoclonal Antibodies against Tissue-Nonspecific Alkaline Phosphatase. Tumor Biology. 2002;23(4):228–48.
41. Chavassieux P, Portero-Muzy N, Roux JP, Garnero P, Chapurlat R. Are Biochemical Markers of Bone Turnover Representative of Bone Histomorphometry in 370 Postmenopausal Women? J Clin Endocrinol Metab. 2015 Dec 1;100(12):4662–8.
42. Brown JP, Don-Wauchope A, Douville P, Albert C, Vasikaran SD. Current use of bone turnover markers in the management of osteoporosis. Clin Biochem. 2022 Nov;109–110:1–10.
43. Sarvari BKD, Sankara Mahadev D, Rupa S, Mastan SA. Detection of Bone Metastases in Breast Cancer (BC) Patients by Serum Tartrate-Resistant Acid Phosphatase 5b (TRACP 5b), a Bone Resorption Marker and Serum Alkaline Phosphatase (ALP), a Bone Formation Marker, in Lieu of Whole Body Skeletal Scintigraphy with Technetium99m MDP. Indian Journal of Clinical Biochemistry. 2015 Jan 20;30(1):66–71.
44. Koivula MK, Risteli L, Risteli J. Measurement of aminoterminal propeptide of type I procollagen (PINP) in serum. Clin Biochem. 2012 Aug;45(12):920–7.
45. Parfitt AM, Simon LS, Villanueva AR, Krane SM. Procollagen type I carboxy-terminal extension peptide in serum as a marker of collagen biosynthesis in bone. Correlation with iliac bone formation rates and comparison with total alkaline phosphatase. Journal of Bone and Mineral Research. 2009 Dec 3;2(5):427–36.
46. Liu K, Wang K, Wang L, Zhou Z. Changes of lipid and bone metabolism in broilers with spontaneous femoral head necrosis. Poult Sci [Internet]. 2021 Mar 1 [cited 2022 Aug 18];100(3):100808. Available from: /pmc/articles/PMC7936160/

Chapter 3

INFLUENCE OF PERCH PROVISION DURING REARING ON ACTIVITY AND MUSCULOSKELETAL HEALTH OF PULLETS[2]

[2] Anderson MG, Johnson, AM, Harrison C, Arguelles-Ramos M, Ali A. Impact of Perch Provision Timing on Activity and Musculoskeletal Health of Laying Hens. *Animals.* 2024; 14(2):265. https://doi.org/10.3390/ani4020265

Simple Summary

In this study, we investigated the enduring impacts of perch provision timing on the musculoskeletal health of laying hens. A total of 810 pullets experienced different housing conditions: continuous access to multi-tier perches from 0 to 40 weeks (CP), no perch access (NP), early access during the rearing phase from 0 to 17 weeks (EP), or solely during the laying phase from 17 to 40 weeks (LP). Monitoring from week 24 to 40 included individual activity levels, blood sample collection for bone demineralization markers, and euthanasia for computed tomography scans at 40 weeks. Results showed that hens with continuous perch access demonstrated higher overall activity at 24 weeks and improved musculoskeletal health at 40 weeks compared to those with no access. Late perch access also positively affected activity, muscle deposition, and bone strength. Conversely, early access did not yield long-term impacts on activity or musculoskeletal health except for intermediate responses in bone demineralization. These findings highlight the importance of timing in perch provision, emphasizing that continuous or late access enhances the well-being and musculoskeletal health of laying hens in comparison to no access at all. Early access to perches did not have a long-term beneficial effect on the activity or musculoskeletal health of laying hens. The study suggests that optimizing perch exposure timing can contribute to sustained improvements in the physical condition of laying hens throughout their reproductive lifespan.

Abstract

Laying hens can experience a progressive increase in bone fragility due to the ongoing mobilization of calcium from bones for eggshell formation. Over time, this escalates their

susceptibility to bone fracture, which can reduce their mobility and cause pain. The provision of perches as an exercise opportunity could potentially enhance bone strength, but the timing of exposure to perches during the birds' development may modulate its impact. The objective of this study was to investigate the enduring impacts of perch provision timing on the musculoskeletal health of laying hens. A total of 812 pullets were kept in different housing conditions (seven pens/treatment, 29 birds/pen) with either continuous access to multi-tier perches from 0 to 40 weeks of age (CP), no access to perches (NP), early access to perches during the rearing phase from 0 to 17 weeks of age (EP), or solely during the laying phase from 17 to 40 weeks of age (LP). At weeks 24, 36, and 40 of age (n = 84 birds/week), three birds per pen were monitored for individual activity level, and blood samples were collected from a separate set of three birds per pen to analyze serum levels of tartrate-resistant acid phosphatase 5b (TRACP-5b) and C-terminal telopeptide of type I collagen (CTX-I) as markers of bone demineralization. At 40 weeks of age, three birds per pen (n = 84) were euthanized for computed tomography scans to obtain tibial bone mineral density (BMD) and cross-sectional area (CSA) with further analysis including muscle deposition, tibial breaking strength, and tibial ash percent. During week 24, hens from CP, EP, and LP pens had the highest overall activity compared to hens from NP pens ($p < 0.05$) with no differences between treatments for overall activity level during weeks 36 or 40 ($p > 0.05$). During weeks 24, 36, and 40, hens from CP and LP pens showed greater vertical and less horizontal activity compared to hens from EP and NP pens ($p < 0.05$). TRACP-5b and CTX-I concentrations did not differ between treatments at week 24 of age ($p > 0.05$). Hens from CP pens had the lowest TRACP-5b and CTX-I concentrations at 36 weeks of age with EP and LP hens showing intermediate responses and NP hens having the highest concentration ($p < 0.05$). At 40 weeks of age, CP hens had the lowest TRACP-5b and CTX-I concentrations compared to NP hens ($p <$

0.05). Total bone CSA did not differ between treatments ($p > 0.05$), but CP had greater total BMD than NP ($p < 0.05$) with no differences between EP and LP treatments. CP and LP hens had larger biceps brachii, pectoralis major, and leg muscle groups as well as greater tibial breaking strengths than EP and NP treatments ($p < 0.05$). CP hens had higher tibial ash percentages compared to EP, LP, and NP ($p < 0.05$). Our results indicate that providing continuous perch access improves the musculoskeletal health and activity of laying hens at 40 weeks of age compared to no access and that late access to perches has a beneficial impact on activity, muscle deposition, and bone strength.

Keywords: laying hen; perch provision; musculoskeletal health; activity level

Introduction

A major welfare concern in the laying hen industry is osteoporosis, which refers to the progressive decrease in structural bone, leading to increased susceptibility to bone fractures [1,2]. As pullets reach sexual maturity, osteoblasts begin forming medullary bone, which is intended as a reliable source of calcium for eggshell formation [1]. During calcium mobilization, osteoclasts resorb both medullary and structural bone so that over time, the hen remains reproductively active; there is a progressive decrease in structural bone, resulting in bone fragility [1,2]. The increase in bone fragility increases the susceptibility of the bones to fracture [2]. Fractures can reduce hen mobility and cause acute or chronic pain [3,4]. Solutions to reduce the occurrence of osteoporosis by improving bone strength in laying hens include dietary interventions [5,6,7,8,9], genetic selection for bone quality [10,11,12,13] and providing perches as an opportunity for exercise to increase activity levels [14,15,16,17,18,19].

The type of housing system can impact the activity level and musculoskeletal health of laying hens. For example, hens housed in conventional cages lack the opportunity for exercise and may be more susceptible to osteoporosis compared to hens housed in alternative systems [2,12,16]. In the United States, while the inclusion of perches within poultry housing is not compulsory, it is noteworthy that their incorporation is recommended according to guidelines provided by the United Egg Producers, contrasting with the regulatory framework in the European Union, where the provision of perches is obligatory. Providing more opportunities for load-bearing exercise, such as incorporating multi-tier perches into alternative housing systems, can increase hen activity and improve musculoskeletal health [12,15]. Laying hens are highly motivated to perch, mainly as a means of defense from predators or to avoid aggression from conspecifics [20]. Hens showed frustration-related behaviors when deprived of access to perches [19,20]. Moreover, the ability to

perch is important in laying hens reared in aviary systems, where resources (such as food, water, and nest boxes) can be provided in different vertical levels of the system. Perching is a highly motivated behavior that can be used to stimulate activity, improve musculoskeletal health, and reduce the occurrence of osteoporosis in laying hens [19,21,22].

With an increased freedom for movement and exercise comes an increased risk for keel bone injuries, which up to 80% of laying hens may experience [23,24]. Keel bone injuries are a complex welfare problem with a multitude of interplaying risk factors such as genetics, nutrition, and environment [25,26]. Keel bone fractures can frequently occur due to falls or collisions with furniture within complex housing environments or also due to other short-duration traumatic events [27,28]. In contrast, keel bone deformities typically occur from prolonged mechanical pressure load during perching [29]. The exact factors influencing keel bone injuries in laying hens are complex, and the extent to which physical activity at certain ages plays a role in susceptibility to keel bone damage is unknown [24,25].

A major contributing factor influencing a hen's ability to perch is the age during which they are exposed to perches. Without prior experience with perches, young chicks show a poor ability to use perches later in life [17,27]. Early access to perches may provide pullets with proper exposure to and practice perching, increasing muscle mass and bone strength. Perch access during rearing may improve the birds' ability to use perches better later in life and also result in stronger musculoskeletal systems at the start of lay, reducing the risk of osteoporosis as an adult. For example, some previous studies found the benefits of rearing pullets in alternative housing systems on bone composition and strength at 16 weeks of age [18,28], where the beneficial impact on pullet bone composition observed at 16 weeks of age continued during the laying phase [29]. Furthermore, rearing pullets in conventional cages with perches resulted in some benefits to bone

health in 71-week-old hens [15]. Rearing in alternative housing systems has also been shown to reduce keel bone damage during the laying phase [30,31]. By focusing on strengthening bones during development, we may observe hen bones that are better equipped to handle the mobilization of calcium in a proactive approach to prevent osteoporosis.

This study aimed to observe the long-term effects of perch provision timing, either during only the rearing phase, only the laying phase, both phases, or neither on the musculoskeletal health and activity of adult laying hens. Pullets were reared either with or without access to multi-tier perches until 17 weeks of age, at which point half of the pullets with perches transitioned to pens without perches, and half of the pullets without perches transitioned to pens with perches. We hypothesized that pullets with continuous access to perches would show improved musculoskeletal health and increased activity compared to pullets without access to perches, and pullets with perch access during only the rearing or only the laying phase showing intermediate responses.

Materials and Methods

Ethics

This experiment was approved by Clemson University's Institutional Animal Care and Use Committee (protocol #AUP2021-0068).

Animals and housing

This experiment was conducted at Clemson University's poultry facility in South Carolina, USA. Day-old Hy-Line brown chicks (n = 840) were randomly allocated across 30 pens (28 birds/pen) until 17 weeks of age. Pens (5.2 m^2) contained 7.6 cm of clean pine wood shavings as bedding. Trough feeders were provided for the first 3 weeks of age at which point hanging feeders

were used. Birds had ad libitum access to water and feed. From 0 to 3 weeks of age, feed was provided in tube feeders and water in gallon drinkers. For the first week of life, supplementary feed trays were provided. After 3 weeks, feed was provided in circular hanging feeders and water was available in automatic cup drinkers. For the first 3 weeks of age, heat was provided by one focal electric brooder per pen and a gas-fired brooder for the entire house. The temperature was initially set at 35–36 °C at day 0; then, it progressively reduced by 2–4 °C every week until 3 weeks of age when brooders were removed. Temperature was reduced weekly until 6 weeks of age to 21 °C, and then they were maintained until the end of the study, following the standard breed guidelines [32]. Light was provided by one 60-watt incandescent overhead lightbulb per pen, and each pen was kept on a decreasing light schedule starting at 20 L:4D during the first week and was decreased by increments of either 1.5 or 3 h until 10 L:14D from 7 weeks of age until the end of the study when birds were 40 weeks old [32].

Treatments

From 0 to 17 weeks of age, 15 pens (420 birds) were provided with perches while the remaining 15 pens (420 birds) were without perches. At 17 weeks of age, half of the birds with perches transitioned to pens without perches, and half of the birds without perches transitioned to pens with perches until 40 weeks of age. This resulted in four treatment groups (7 pens/treatment and 28 birds/pen after accounting for mortality during weeks 0–17): continuous perch (CP; perch access from 0–40 weeks of age), no perch (NP; no perch access from 0–40 weeks of age), early perch (EP; perch access from 0–17 weeks of age), and late perch (LP; perch access from 17–40 weeks of age). The perch structure was constructed to be adjustable with perch rungs made of 5 × 5 cm pressure-treated wooden lumber. Each perch structure contained 3 rungs of varying height, each 165.1 cm in length, resulting in 495.3 cm of total perch space and approximately 19 cm of

perch space per bird. In the CP group, rung heights and distance between rungs were gradually increased concurrently with the growth of the birds to ensure they were easily accessible. For the first 11 days of age, the 3 rungs were 15.2 cm, 22.8 cm, and 30.4 cm high off the ground (Figure 3.1a). For the next 8 days, the 3 rungs were 22.8 cm, 38.1 cm, and 54.6 cm high off the ground (Figure 3.1b). The perch rungs were altered once more on day 20 of age to 38.1 cm, 62.2 cm, and 88.4 cm high with a 12.7 cm distance between each perch rung (Figure 3.1c).

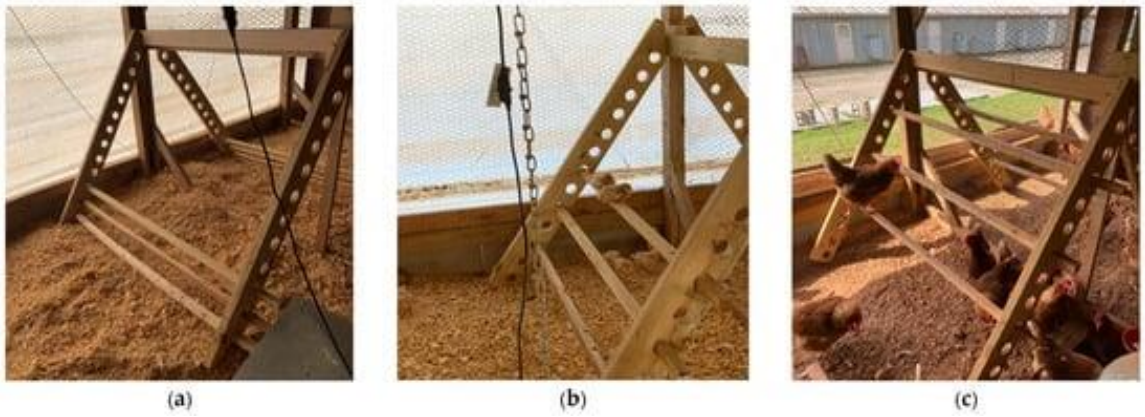

Figure 3.1. Perch and adjustable rung heights in continuous perch (CP) treatment groups during days (a) 0–11, (b) 12–19, and (c) 20+ days of age. Perches were placed in late perch (LP) treatment groups beginning at 18 weeks of age with rungs at heights pictured in (c).

Activity

Bird activity was monitored using an accelerometer over 3 consecutive days during weeks 24, 36, and 40 of age (n = 84 birds/week). At each time point, 3 birds/pen were caught after the lights went off. Birds were selected from among different resources and perch levels in an attempt to sample hens that were representative of the flock in that pen. Birds were fitted with a harness that was used to secure the accelerometer. An acceleration data logger (Onset HOBO PendantG acceleration data loggers, Onset Computer Corporation, Bourne, MA) was inserted inside each harness. The loggers used in the current study were 58 × 33 × 23 mm in size and 16 g in weight

with a ±3 g; 29.4 m/s^2 measuring range and ±0.105 g; 1.03 m/s^2 accuracy level when operating between −20 and 70 °C. Loggers were oriented on the hens, so the *X*-axis captured forward and backward movement (craniocaudal movement), the *Y*-axis captured sideways movement (mediolateral movement), and the *Z*-axis captured vertical movement (dorsoventral movement) of the hens. Loggers were firmly secured inside the harness to reduce noise in the data due to the movement of the loggers themselves and to prevent changes in logger orientation. After fitting focal birds with harnesses and accelerometers, hens were given 1 day to habituate to wearing the equipment. During this period, hens were monitored to ensure that vests were not impacting behavior and locomotion abilities. After acclimation, loggers recorded hens' movement across 3 consecutive days (72 h) at each time point with a scanning frequency of 20 Hz (±3 g to +3 g) in 3 axes.

Musculoskeletal health

Computed tomography (CT) image acquisition

At 40 weeks of age, 3 birds per pen (n = 84) were euthanized on-farm by CO_2 inhalation, placed in a cooler of ice, and immediately transported to the Godley-Snell Research Center on Clemson University's campus. Upon arrival, birds were individually placed inside a V-shaped foam cradle in a dorsal recumbent position atop a hydroxyapatite calibration phantom (QRM Quality Assurance in Radiology and Medicine, Möhrendorf Germany). The head and the legs of the bird were extended in opposite directions and were taped to maintain this positioning in the foam cradle during image acquisition. CT images were acquired using a helical mode, head 0–10 kg protocol, 0.5 mm slice thickness, and bone and soft tissue reconstruction algorithms. CT images were acquired using a Toshiba Aquilion TSX-101A, 16-slice scanner (https://www.gehealthcare.com, accessed on 16 November 2023, GE Healthcare, Chicago IL,

USA), a single bird scan and image construction required approximately 7 min. Birds were dissected immediately after CT scanning and frozen at −20 °F for further testing.

Tibiotarsal CT image analyses

For each CT study, measurements of the right tibiotarsal bone and muscle were made using a standardized CT image analysis protocol previously published by [33]. Cross-sectional density (HU) and area (mm) of the total and medullary components of the tibiotarsal bone were recorded at predefined proximal, middle, and distal transverse slice locations using hand-traced regions of interest (Figure 3.2a,b). The cross-sectional area (CSA) of the muscle group surrounding the tibiotarsus at each of the predefined proximal, middle, and distal locations was also measured. The CT densities for each of the rods in the bone calibration phantom were recorded using the oval ROI tool (Figure 3.2c). The CT densities in HU were then converted to hydroxyapatite values using graphical analysis techniques described in [33].

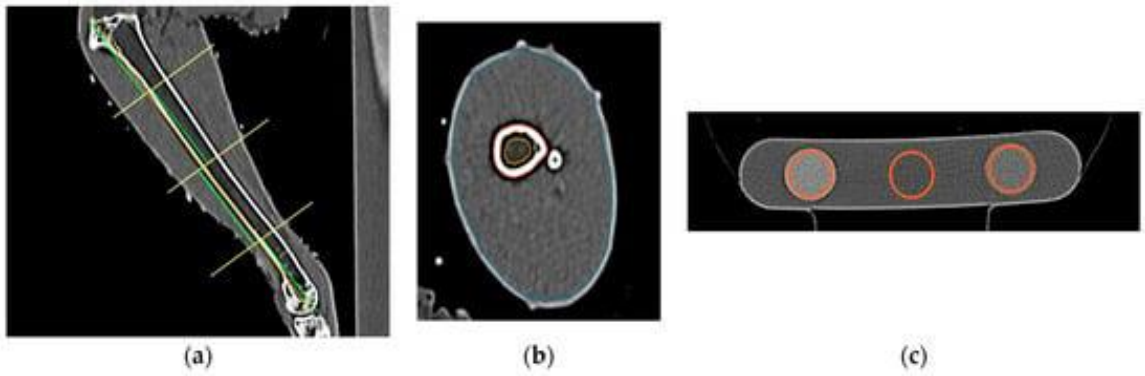

Figure 3.2. Steps of tibiotarsal image analysis. (a) Division of the tibiotarsus into 4 segments to set proximal, middle, and distal locations, (b) region of interest tracings for the tibiotarsus in the proximal location, and (c) region of interest placement in the 3 rods of hydroxyapatite phantom using the oval tool.

Muscle deposition

After CT scanning, birds (n = 84) were prepared for dissection, and the separation of muscles was conducted following the procedures described by [18] and with the assistance of a veterinarian (A.A.) to ensure consistent muscle specimen collection. Birds were opened by cutting the skin on the caudal tip of the keel bone and peeling it back to expose the interior of the bird. To remove the right bicep and triceps brachii, the skin of the wing was peeled back, and a blunt dissection was made along the line of demarcation between the biceps and triceps. The bicep and triceps were gently freed from the bone, and the proximal and distal tendons were cut at the bone level. To separate the pectoralis muscles, fascia was cut along the line of demarcation, separating the fats from the pectoralis muscles and severing all the attachment at the origin (crania sternum, furcula, and sternal ribs), and at the insertion of the major (proximal ventral surface of the humerus) and of the minor (proximal dorsal surface of the humerus). The left leg muscles, tendons, and ligaments were detached from the bone, the Achilles tendon was severed, and the fascia along the synsacrum was detached. All muscles were immediately weighed upon removal. The left tibiae were frozen at −20 °C for ash percentage, and the right tibiae were frozen at −20 °C for breaking strength measures.

Tibia breaking strength

Mechanical properties of the right tibiotarsi were assessed using a three-point bending test as specified by the American National Standards Institute (ANSI) standards for the application of 3-point bending on animal bones [34]. Testing was performed using an Instron Dynamic and Static Material Test system (Model 5944, Instron Corp., Canton, MA, USA) equipped with a 500 N load cell and Automated Material Test System software (8800 MT Controller, Instron Corp., Canton, MA, USA). Prior to testing, previously frozen legs were thawed at refrigerator temperature.

Muscles surrounding the tibiotarsus were carefully dissected, tibiotarsal length and diameter at the midpoint were recorded, and the bones were wrapped in saline-soaked paper towels until testing to prevent the bones from drying out.

Rounded support pins and breaking blade were manufactured based on ANSI standards for the application of 3-point bending on animal bones [34]. A furculum width of 4 cm was used. This width did not adhere to the ANSI standards but was decided upon based on a consensus among co-authors. Due to the anatomy of the laying hen tibiotarsus, a 4 cm width ensured that the tibiotarsus was able to rest on the furculum in a manner in which the load would be applied to the midpoint of the bone evenly in the craniocaudal plane. The crosshead speed used was 3 mm/min, and the test was carried out to failure. Load and displacement data were collected and were used to obtain the breaking strength (N), stiffness (N/mm), and maximum bending moment (N/m).

Tibia ash percentage

The left tibiotarsi of euthanized birds was thawed approximately 24 h prior to data collection. The bones were cleaned from any surrounding muscles and soft tissues, and tibiae were separated from the fibula. The tibiae were cut into 3 pieces to fit into a Soxhlet chamber for ether extraction. Ceramic crucibles were air-dried for one hour and then placed in a desiccator for another hour. The weight of the dried crucibles was recorded. Left tibiae were dried at 100 °C for one hour, placed in a desiccator for another hour, and their weight was recorded. Tibiae were then placed inside the dried ceramic crucibles and ashed (ashing oven: Thermolyne 30400, Barnstead International, Dubuque, IA, USA) for 6 h at 600 °C. The ash was placed in a desiccator for one hour, and then the ash weight was recorded. The percentage of tibia ash was calculated by dividing the tibia ash weight by the tibia dry weight and multiplying by 100.

Bone resorption markers

During weeks 24, 36, and 40 of age, blood samples were collected from the brachial wing vein of 3 birds/pen (n = 84/week). Whole blood samples were transferred to 1.5 mL Eppendorf tubes, and serum was separated at 6000 rpm for 10 min at 4 °C. In order to test for the occurrence of bone resorption, serum samples were analyzed for levels of tartrate-resistant acid phosphatase 5b (TRACP-5b) and C-terminal telopeptide of type I collagen (CTX-I) using commercial ELISA kits Nanjing Jiancheng Institute of Bioengineering (Nanjing, China) and MyBioSource (San Diego, CA, USA), and according to manufacturer's instructions.

Data processing and statistical analysis

The raw accelerometer data, consisting of the date, time, and the related impulse in the X, Y, and Z dimensions, were downloaded from the devices (HOBOware Graphing & Analysis Software 001, Onset, Bourne, MA, USA) at the end of each 3D observation period. Data on hens' vertical (a_z: dorsoventral movement across vertical levels), horizontal (a_x: craniocaudal movement within the same vertical level), and lateral movement (a_y: mediolateral movement within the same vertical level) during light hours were obtained directly from loggers. Hens' triaxial movement (A_s) was calculated by summing and averaging raw movement data as follows.

$$A_s = \sqrt{a_x^2 + a_y^2 + a_z^2}$$

Acceleration data (gravity "g") were post-processed using MATLAB (MATLAB and Statistics Toolbox Release 2012, The MathWorks, Inc., Natick, MA, USA). In order to accurately calculate the incidence of massive acceleration shifts on the vertical (z) axis that represents perching, data were smoothed from noisy components by removing all minor acceleration fluctuations using a loop function.

$$A_i = \frac{1}{3}\sum_{j=i-1}^{i+1} A_j A_i' = \begin{cases} \mu, if\ |A_i - \mu| < t \\ \mu, if\ |A_i - \mu| \geq t \end{cases}$$

Data smoothing included the passing of the raw acceleration values (A_j) through an asymmetrical 3-point moving average low-pass filter (i = the middle point in the 3-point-moving average low-pass filter) and through a step function to define thresholds used to remove minor fluctuations (t = threshold values of minor fluctuations, i.e., between 0.001 and 0.043 g). After processing data, perching events were recognized by detecting massive shifts in acceleration in the *z*-axis of activity. which was defined as incidence (frequency "F") of perching or "vertical displacement". In order to precisely detect acceleration shifts due to perching and define thresholds for minor fluctuations in the *z*-axis, timestamped videos of birds while perching were obtained and compared with the corresponding activity data. Using the approach enabled us to locate shifts in *z*-axis acceleration mainly caused by perching and define the threshold cutoff points to remove minor fluctuation.

Data were analyzed using the R software (version 3.3.1) with the package "stats" (R Core Team, 2013). To test for the main effects of treatment (CP, EP, LP, and NP) and the age of the birds (activity and bone demineralization: 24, 36, and 40 weeks) on each variable, generalized linear mixed-effects models (GLMMs) were conducted using the "lme4" package [35]. In each GLMM, the interaction term between main effects was also tested as a fixed effect, and bird ID, pen, and day for activity were tested as random effects, with the family set to "Quasibinomial" for proportion data (ash %) and "Poisson" for the other data. Tukey's HSD multiple comparison procedure was used for post hoc comparisons using the "multcomp" package [36]. The "DHARMa" package was used for proportion data (ash%) to test residual distribution and assumptions for GLMM, while the Shapiro–Wilk test was utilized (i.e., activity (g), breaking strength (N), stiffness (N/mm)) for the normality analysis of the model residuals. Statistical

significance was set at $p < 0.05$. Descriptive statistics were calculated using the "psych package", and data are presented as mean ± standard error of the mean (SEM).

Results

Activity

During week 24, hens from CP, EP, and LP exhibited the greatest amount of overall activity compared to hens from NP pens ($p = 0.021, 0.033, 0.036$, respectively; Table 3.1). There were no differences between treatments for overall activity levels during weeks 36 or 40 (Table 3.1). During all observation weeks, hens from CP and LP pens showed greater vertical activity (week 24: (CP: $p = 0.019, 0.023$; LP: $p = 0.026, 0.031$); week 36: (CP: $p = 0.025, 0.0.35$; LP: $p = 0.038, 0.029$); week 40: (CP: $p = 0.028, 0.031$; LP: $p = 0.032, 0.028$); Table 3.1), and less horizontal activity (week 24: (CP: $p = 0.033, 0.029$; LP: $p = 0.034, 0.027$); week 36: (CP: $p = 0.028, 0.0.33$; LP: $p = 0.037, 0.039$); week 40: (CP: $p = 0.032, 0.027$; LP: $p = 0.033, 0.037$); Table 3.1) compared to hens from EP and NP pens. During all observation weeks, hens from CP and LP pens exhibited a higher average daily vertical displacement per bird compared to hens from EP and NP pens (week 24: (CP: $p = 0.018, 0.021$; LP: $p = 0.023, 0.019$); week 36: (CP: $p = 0.022, 0.027$; LP: $p = 0.023, 0.031$); week 40: (CP: $p = 0.022, 0.023$; LP: $p = 0.019, 0.027$); Table 3.1). There were no differences across weeks within the same treatment ($p > 0.05$).

Table 3.1. Overall, vertical, and horizontal activity levels, and average daily vertical displacement per bird (F) of laying hens housed in continuous perch (CP), early perch (EP), late perch (LP), and no perch (NP) pens at weeks 24, 36, and 40 of age (n = 84/week).

Week	Treatment	Overall Activity (g)	Vertical Activity (g)	Horizontal Activity (g)	Average Daily Vertical Displacement/Bird (F)
24	CP	1.42 ± 0.11 [a]	0.59 ± 0.06 [a]	0.83 ± 0.09 [a]	24.52 ± 2.96 [a]
	EP	1.33 ± 0.13 [a]	0.18 ± 0.01 [b]	1.15 ± 0.11 [b]	3.69 ± 0.69 [b]
	LP	1.39 ± 0.19 [a]	0.51 ± 0.07 [a]	0.88 ± 0.07 [a]	23.58 ± 4.58 [a]
	NP	1.29 ± 0.21 [b]	0.11 ± 0.03 [b]	1.18 ± 0.06 [b]	1.13 ± 0.96 [b]
	p-value	0.034	0.029	0.022	0.031
36	CP	1.44 ± 0.16 [a]	0.56 ± 0.06 [a]	0.88 ± 0.11 [a]	33.25 ± 4.21 [a]
	EP	1.45 ± 0.21 [a]	0.12 ± 0.03 [b]	1.33 ± 0.16 [b]	3.56 ± 1.25 [b]
	LP	1.41 ± 0.19 [a]	0.55 ± 0.09 [a]	0.86 ± 0.09 [a]	29.87 ± 5.25 [a]
	NP	1.31 ± 0.21 [a]	0.11 ± 0.01 [b]	1.20 ± 0.16 [b]	2.03 ± 1.03 [b]
	p-value	0.096	0.032	0.036	0.028
40	CP	1.35 ± 0.22 [a]	0.53 ± 0.07 [a]	0.82 ± 0.10 [a]	28.85 ± 6.69 [a]
	EP	1.39 ± 0.23 [a]	0.13 ± 0.06 [b]	1.26 ± 0.09 [b]	4.03 ± 2.36 [b]
	LP	1.36 ± 0.29 [a]	0.59 ± 0.03 [a]	0.77 ± 0.06 [a]	26.85 ± 4.52 [a]
	NP	1.32 ± 0.27 [a]	0.09 ± 0.01 [b]	1.23 ± 0.17 [b]	1.63 ± 0.85 [b]
	p-value	0.325	0.031	0.029	0.035

Treatments; CP: birds had continuous access to multi-tier perches from 0 to 40 weeks of age; NP: no access to perches from 0 to 40 weeks of age; EP: early access to perches during the rearing phase from 0 to 17 weeks of age; LP: Late access to perches during the laying phase from 17 to 40 weeks of age. [a,b] Means with differing superscripts indicate statistically significant differences within columns of the same week at $p < 0.05$.

Tibial bone mineral density (BMD) and cross-sectional area (CSA)

There were no differences between treatments for total CSA (Table 3.2). CP hens had greater cortical CSA and cortical BMD at all locations than other treatment groups (cortical CSA: proximal (p = 0.022, 0.018, 0.029), middle (p = 0.022, 0.031, 0.024), distal (p = 0.031, 0.028, 0.021); cortical BMD: proximal (p = 0.022, 0.027, 0.036), middle (p = 0.024, 0.023, 0.025), distal (p = 0.031, 0.019, 0.031); Table 3.2). EP and LP hens had greater cortical CSA at the proximal (p = 0.021, 0.023, respectively), middle locations (p = 0.032, 0.028, respectively), and greater cortical BMD values at all locations than NP hens (proximal (p = 0.019, 0.024), middle (p = 0.023, 0.025), distal (p = 0.027, 0.035); Table 3.2). However, CP hens had greater total BMD at all locations than NP hens (proximal: p = 0.013, middle: p = 0.009, distal: p = 0.012; Table 3.2), and at the middle (p = 0.029, 0.036, respectively) and distal (p = 0.035, 0.036, respectively) locations than EP and LP, while EP and LP had greater total BMD at all locations than NP (proximal: p = 0.019, 0.023; middle: p = 0.022, 0.031; distal: p = 0.029, 0.036, respectively; Table 3.2).

Table 3.2. Tibial total, medullary, and cortical bone mineral density (BMD; mg/cm3) and cross-sectional area (CSA; mm2) ±SEM for the proximal, middle, and distal regions of the right tibiotarsus of laying hens.

Parameter/ Treatment	Bone Cross-Sectional Area (mm^2)					
	Total			Cortical		
	Proximal	Middle	Distal	Proximal	Middle	Distal
CP	70.2 ± 1.1 [a]	53.8 ± 1.3 [a]	55.5 ± 0.7 [a]	37.1 ± 2.4 [a]	29.5 ± 2.1 [a]	29.2 ± 1.8 [a]
EP	70.1 ± 1.3 [a]	53.9 ± 1.0 [a]	55.3 ± 0.6 [a]	30.7 ± 2.1 [b]	23.9 ± 1.6 [b]	24.0 ± 1.5 [b]
LP	69.9 ± 1.1 [a]	54.5 ± 0.8 [a]	56.0 ± 0.7 [a]	31.5 ± 2.1 [b]	25.4 ± 1.6 [b]	24.2 ± 1.5 [b]
NP	69.9 ± 1.1 [a]	54.9 ± 0.7 [a]	56.0 ± 1.1 [a]	26.3 ± 1.7 [c]	21.6 ± 1.4 [c]	20.9 ± 1.4 [b]
p-value	0.235	0.185	0.635	0.021	0.019	0.024
Parameter/ Treatment	**Bone Mineral Density (mg/cm^3)**					
	Total			Cortical		
	Proximal	Middle	Distal	Proximal	Middle	Distal
CP	515.7 ± 13.7 [a]	730.6 ± 11.0 [a]	806.8 ± 11.0 [a]	1028.8 ± 23.6 [a]	1746.8 ± 16.9 [a]	1370.7 ± 889.5 [a]
EP	428.1 ± 15.4 [a]	591.8 ± 22.5 [b]	661.6 ± 14.6 [b]	740.7 ± 20.9 [b]	1257.7 ± 17.9 [b]	1000.6 ± 22.0 [b]
LP	438.4 ± 14.0 [a]	628.3 ± 18.8 [b]	669.7 ± 17.4 [b]	761.3 ± 21.0 [b]	1380.0 ± 22.6 [b]	1165.1 ± 19.6 [b]
NP	356.3 ± 11.4 [b]	502.6 ± 13.1 [c]	545.8 ± 20.7 [c]	726.9 ± 23.3 [c]	976.5 ± 19.1 [c]	889.5 ± 23.1 [c]
p-value	0.034	0.003	0.021	0.013	0.025	0.022

Treatments; CP: birds had continuous access to multi-tier perches from 0 to 40 weeks of age; NP: no access to perches from 0 to 40 weeks of age; EP: early access to perches during the rearing phase from 0 to 17 weeks of age; LP: Late access to perches during the laying phase from 17 to 40 weeks of age. [a–c] Means with differing superscripts indicate statistically significant differences within columns of the same week at $p < 0.05$.

Muscle deposition

Hens from CP and LP pens had heavier biceps brachii (CP: $p = 0.032, 0.025$; LP: $p = 0.036, 0.029$; Table 3.3), pectoralis majors (CP: $p = 0.0.026, 0.026$; LP: $p = 0.027, 0.037$; Table 3.3), and leg muscle groups (CP: $p = 0.031, 0.036$; LP: $p = 0.029, 0.027$; Table 3.3) compared to hens from EP and NP pens. There were no differences between treatments for weights of the triceps brachii or pectoralis minor ($p > 0.05$; Table 3.3).

Table 3.3. Mean weight (g) ±SEM of biceps brachii, triceps brachii, pectoralis major, pectoralis minor, and leg muscle group of laying hens.

Treatment	Biceps Brachii (g)	Triceps Brachii (g)	Pectoralis Major (g)	Pectoralis Minor (g)	Leg Muscle Group (g)
CP	4.25 ± 0.26 [a]	3.88 ± 0.31 [a]	124.58 ± 4.85 [a]	62.58 ± 5.55 [a]	141.85 ± 7.98 [a]
EP	3.65 ± 0.29 [b]	3.59 ± 0.22 [a]	112.55 ± 5.25 [b]	56.85 ± 1.14 [a]	124.55 ± 6.52 [b]
LP	4.18 ± 0.38 [a]	3.67 ± 0.29 [a]	119.93 ± 4.99 [a]	59.22 ± 3.55 [a]	138.57 ± 5.88 [a]
NP	3.52 ± 0.21 [b]	3.53 ± 0.25 [a]	107.58 ± 3.78 [b]	55.85 ± 5.03 [a]	120.79 ± 6.85 [b]
p-value	0.026	0.259	0.031	0.523	0.028

Treatments: CP: birds had continuous access to multi-tier perches from 0 to 40 weeks of age; NP: no access to perches from 0 to 40 weeks of age; EP: early access to perches during the rearing phase from 0 to 17 weeks of age; LP: Late access to perches during the laying phase from 17 to 40 weeks of age. [a,b] Means with differing superscripts indicate statistically significant differences within columns of the same week at $p < 0.05$.

Tibia breaking strength

At week 40 of age, housing hens in CP and LP pens resulted in greater tibia-breaking strengths (CP: $p = 0.019, 0.011$; LP: $p = 0.017, 0.009$, respectively) and stiffness (CP: $p = 0.021, 0.013$; LP: $p = 0.006, 0.019$, respectively) compared to housing hens in EP and NP pens (Figure 3.3).

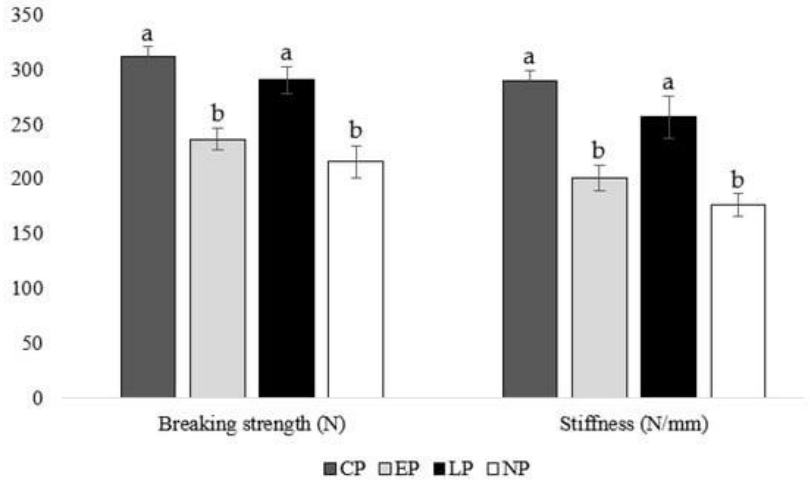

Figure 3.3. Mean tibia breaking strength (N) and stiffness (N/mm) of laying hens housed in continuous perch (CP), early perch (EP), late perch (LP), and no perch (NP) pens at 40 weeks of age (n = 84). a,b Means with differing superscripts indicate statistically significant differences between treatments within a parameter at $p < 0.05$.

Tibia ash percentage

At week 40 of age, the tibia of hens housed in CP pens contained a higher ash percentage compared to hens housed in EP, LP, and NP pens ($p = 0.003, 0.009, 0.012$, respectively; Figure 3.4).

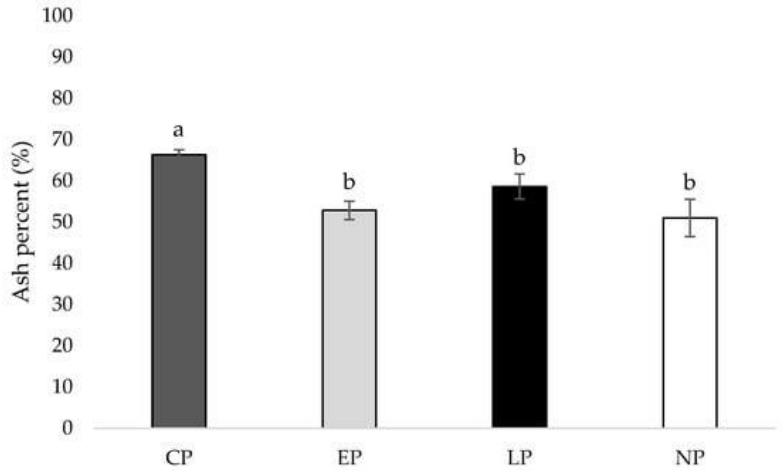

Figure 3.4. Mean tibia ash percent (%) of laying hens housed in continuous perch (CP), early perch (EP), late perch (LP), and no perch (NP) pens at 40 weeks of age (n = 84). a,b Means with differing superscripts indicate statistically significant differences between treatments at $p < 0.05$.

Bone demineralization

There were no differences in TRACP-5b or CTX-I concentrations between treatment groups at week 24 of age ($p > 0.05$; Figure 3.5a,b). At 36 weeks of age, hens from CP pens had the lowest TRACP-5b concentration compared to other groups (EP: $p = 0.039$, LP: $p = 0.023$, NP: $p = 0.013$), which was followed by hens from EP pens (LP: $p = 0.036$, NP: $p = 0.023$), then LP pens (NP: $p = 0.046$), with hens from NP pens having the highest concentration (Figure 3.5a). Furthermore, hens from CP and EP pens had the lowest CTX-I concentrations at week 36 of age compared to hens from LP and NP pens (CP: $p = 0.011$, 0.019; EP: $p = 0.036$, 0.037, respectively; Figure 3.5b). At week 40 of age, CP hens had the lowest TRACP-5b (EP: $p = 0.036$, LP: $p = 0.035$, NP: $p = 0.026$) and CTX-I (EP: $p = 0.029$, LP: $p = 0.031$, NP: $p = 0.036$) concentrations compared to EP and LP hens, with NP hens having the highest concentrations (CP: $p = 0.026$, EP: $p = 0.023$, LP: $p = 0.013$; Figure 3.5a,b).

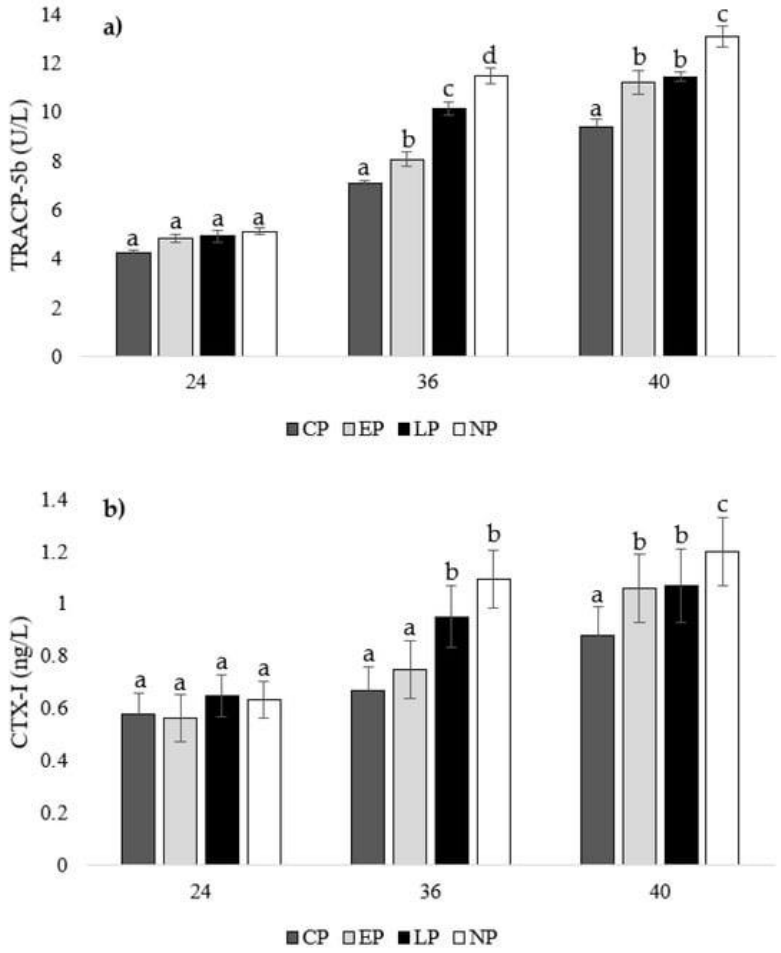

Figure 3.5. Mean serum concentrations of (a) tartrate-resistant acid phosphatase 5b (TRACP-5b; U/L) and (b) C-terminal telopeptide of type I collagen (CTX-I; ng/L) of laying hens housed in continuous perch (CP), early perch (EP), late perch (LP), and no perch (NP) pens at 24, 36, and 40 weeks of age (n = 84/week). [a-d]Means with differing superscripts indicate statistically significant differences between treatments within week at $p < 0.05$.

Discussion

Activity

Perch access did impact overall hen activity level at week 24 of age but not at weeks 36 and 40, with hens from CP, EP, and LP pens showing the greatest amount of overall activity

compared to hens from NP pens. Interestingly, access to perches from 0 to 17 weeks of age resulted in increased overall activity at 24 weeks of age. However, we did not see an effect of perch access on overall activity at 36 and 40 weeks of age. During weeks 24, 36, and 40 of age, hens from CP and LP pens performed more vertical activity, less horizontal activity, and had a higher average daily vertical displacement per bird compared to hens from EP and NP pens. This is likely due to the fact that hens from CP and LP pens had access to an appropriate perching structure and thus more opportunities to move vertically compared to EP and NP hens who had no access to perches and could not physically move vertically to the same extent. Because hens are highly motivated to perch on high areas of their home pen, it follows that the hens with access to multi-tier perches showed more vertical activity, as they were likely jumping to reach elevated surfaces within the pen [14,15,17,37]. Furthermore, EP hens exhibited a greater proclivity to perch on elevated structures, such as feeders and nest boxes, within their surroundings in comparison to NP hens. This anecdotal observation suggests an increased inclination to perch among EP hens, which was potentially attributed to their early exposure to perching experiences during the rearing phase.

Tibial bone mineral density (BMD) and cross-sectional area (CSA)

Perch access influenced the tibial bone mineral density at 40 weeks of age, with birds from CP pens having a greater total BMD content compared to hens from NP pens, indicating access to perches beneficially impacted tibial bone mineral density. Furthermore, CP hens had greater cortical CSA and BMD at all locations than the other treatment groups with EP and LP hens having greater CSA and BMD values than NP hens. This finding is in line with previous studies that found access to perches increases bone strength in laying hens [16,38,39]. Ours results align with previous research and suggest that load-bearing exercise from continual perch use improves bone characteristics with early or late perch access having intermediate responses compared to no perch

access at all. However, one previous study found that the addition of perches to conventional cages did not increase the tibial BMD of 71-week-old hens [15]. This could be due to differences in the design of perches or that White Leghorns were the strain of the birds used in the previous study compared to Hy-Line Brown in the current study. The minimal differences noted in tibial BMD and (CSA) between EP and LP hens are surprising. This suggests that even in the later stages of the laying cycle, hens can derive benefits from perch provisions, thereby enhancing their bone health. On the other hand, early provision of perches during the rearing phase appears to have a positive impact on bone health throughout the laying phase. However, it is important to note that neither scenario is directly compared with the potential benefits of providing perches during both rearing and laying phases.

Muscle deposition

By the end of the study at 40 weeks of age, hens from CP and LP pens had heavier biceps brachii, pectoralis majors, and leg muscle groups compared to hens from EP and NP pens with no differences for the weights of triceps brachii or pectoralis minors. Providing continuous access to perches resulted in heavier muscles compared to not providing perches at all due to higher activity levels during rearing and laying. Perching is considered a form of load-bearing exercise that has previously shown to increase muscle deposition in poultry [14,15]. By the end of the study, hens with late access to perches had heavier muscles than hens with access to perches during the rearing phase. This was contrary to some previous work, as early access to perches has been shown to increase muscle deposition in adults due to there being more opportunities for exercise during development [15,40]. However, hens with access to perches during the lay phase performed more vertical activity and jumped more frequently than hens without access to perches, suggesting these activities beneficially impacted muscle growth even after puberty. Access to perches during the

lay phase ultimately had a more beneficial effect on muscle deposition at 40 weeks of age compared to access to perches during the rearing phase.

Tibia breaking strength

Timing of perch access impacted tibia strength with CP and LP hens having a higher breaking strength and stiffness at 40 weeks of age compared to EP and NP hens. Some previous studies found no difference in tibia breaking strength between housing systems with or without perches [41,42]. However, other studies found that access to perches as an adult improves tibia strength: for example, hens housed with perches from 19 weeks of age had stronger bones and better preserved cortical bone than hens housed without perches at 65 weeks of age [16]. Furthermore, hens housed with perches from 16 weeks of age had a higher tibia breaking strength than hens in conventional cages at 73 weeks of age [21]. In agreement with our results, the previous study found no effect of rearing environment on adult bone breaking strength. However, in its companion study, they discovered a greater beneficial effect of rearing pullets with perches on breaking strength at 16 weeks of age than what was discovered for adult hens, highlighting the importance of providing opportunities for exercise during bone development [18]. Although we did not find an effect of providing perches during rearing on adult bone breaking strength, numerical differences between the CP (breaking strength: 311.02 N; stiffness: 289.88 N/mm) and LP (breaking strength: 289.96 N; stiffness: 256.26 N/mm) groups suggest that providing perches during the rearing (i.e., bone development) and lay phase may be more beneficial to bone strength than providing perches during the lay phase alone. Our results suggest that providing perches either continuously or at the beginning of the lay phase permits sufficient opportunity for exercise to improve breaking strength by week 40 of age compared to hens not provided perches at all or only during the rearing period.

Tibia ash percentage

Perch access impacted tibia ash percent at 40 weeks of age, where the tibia of hens housed in CP pens contained a higher ash percentage compared to hens housed in EP, LP, and NP pens, suggesting that continuous perch access (perch access from 0 to 40 weeks of age) beneficially impacted bone mineral content. One prior study found that free range hens with access to perches had a greater tibia ash percent at 38 and 45 weeks of age compared to hens in conventional cages with or without access to perches, indicating that a greater freedom of movement and more opportunities for exercise improve tibia mineral content compared to providing simple perches in a caged environment alone [43]. In agreement, hens housed in floor pens with perches had higher tibia ash percentages compared to hens housed in conventional cages [44]. However, a couple previous studies found no relationship between housing type and tibia ash percent [45,46]. In our study, continuous perch access improved tibia mineral content compared to early, late, or no access to perches.

Bone resorption

In our study at 24 weeks of age, there were no differences in TRACP-5b and CTX-I levels between treatment groups, indicating all treatments started at similar levels of bone resorption. We observed differences in bone resorption at 36 weeks of age, with the lowest TRACP-5b concentrations found in hens from CP pens, which was followed by hens from EP pens, then LP pens, with hens from NP pens having the highest concentrations. Furthermore, hens from CP and EP pens had the lowest CTX-I concentrations at week 36 of age compared to hens from LP and NP pens. Our results indicate that hens from CP pens showed mild bone resorption compared to

hens from NP pens, which showed the highest levels due to an absence of bone reservoirs. The lack of activity during rearing and laying does not improve bone characteristics and leaves adult laying hens at risk for increased bone resorption and ultimately weakened bones. Hens from EP pens showed low levels of bone resorption, which was comparable to hens from CP pens at week 24 of age. However, at week 36 of age, bone resorption increased to a level slightly higher than hens from CP pens, but it was still less than hens from LP and NP pens, which is an effect that can be contributed to a higher bone reserve due to increased perching activity during rearing. At week 40 of age, CP hens had the lowest TRACP-5b and CTX-I concentrations compared to EP and LP hens with NP hens having the highest concentrations. Both EP and LP hens showed similar bone resorption levels, as the effect of early perch access dissipated and the effect of later perch access slowed bone resorption levels compared to hens from NP pens.

Conclusions

The outcomes derived from our investigation indicate that the continuous provision of multi-tier perch access throughout the rearing and early lay phase (0–40 weeks of age) exerts a favorable influence on activity level and thus the musculoskeletal health of laying hens at 40 weeks, thereby contributing to an improvement in overall hen welfare when compared to the absence of perch access. Similarly, the availability of perches during the early lay period (17–40 weeks of age) demonstrates positive effects on activity, muscle deposition, and bone strength; however, these benefits are not as pronounced as those observed with continuous perch access. Moreover, the introduction of perches during the rearing phase (0–17 weeks of age) is associated with a deceleration in bone demineralization, aligning with the outcomes observed in hens with access to

perches during the laying phase. Nevertheless, early perch access does not manifest an overarching positive impact on the musculoskeletal health or activity levels of laying hens at 40 weeks of age, suggesting that early exposure during developmental stages does not confer long-term benefits in these aspects. The findings underscore the need for further research to elucidate the effects of early exercise during the rearing phase on bone demineralization in adult laying hens, providing a more comprehensive understanding of the nuanced relationships between developmental experiences and musculoskeletal health.

Author Contributions

Conceptualization, M.G.A. and A.A.; methodology, M.G.A., C.H. and A.A.; formal analysis, M.G.A. and A.A.; investigation, M.G.A., A.M.J., C.H., M.A.-R. and A.A.; data curation, M.G.A., A.M.J., C.H. and A.A.; writing—original draft preparation, M.G.A. and A.A.; writing—review and editing, M.G.A., M.A.-R. and A.A.; supervision, M.A.-R. and A.A.; project administration, M.G.A. and A.M.J.; funding acquisition, M.A.-R. and A.A. All authors have read and agreed to the published version of the manuscript.

Funding

This study was funded by the United Sorghum Checkoff Program (project # RG002-21). Technical Contribution No. 7252 of the Clemson University Experiment Station, this material is based upon work supported by NIFA/USDA, under project number SC-1029. Any opinions, findings, conclusions, or recommendations expressed in this publication are those of the authors and do not necessarily reflect the views of the USDA.

Institutional Review Board Statement

The study was conducted in accordance with the Declaration of Helsinki and approved by Clemson University's Institutional Animal Care and Use Committee (protocol #: AUP2021-0068; November 2021).

Informed Consent Statement

Not applicable.

Data Availability Statement

For access to data from the study, please contact the corresponding author.

Acknowledgments

We would like to thank the employees and student workers at the Morgan Poultry Center for their help, care, and troubleshooting. We would also like to thank our undergraduate volunteers, especially Bailey Ward, Christianna Hoshko, and Isabella Raymond, for their help during the trial.

Conflicts of Interest

The authors declare no conflicts of interest.

References

1. Whitehead, C.C. Overview of Bone Biology in the Egg-Laying Hen. *Poult. Sci.* 2004, *83*, 193–199. [Google Scholar] [CrossRef] [PubMed]
2. Whitehead, C.C.; Fleming, R.H. Osteoporosis in Cage Layers. *Poult. Sci.* 2000, *79*, 1033–1041. [Google Scholar] [CrossRef] [PubMed]
3. Nasr, M.; Murrell, J.; Wilkins, L.; Nicol, C. The Effect of Keel Fractures on Egg-Production Parameters, Mobility and Behaviour in Individual Laying Hens. *Anim. Welf.* 2012, *21*, 127–135. [Google Scholar] [CrossRef]
4. Nasr, M.A.F.; Nicol, C.J.; Murrell, J.C. Do Laying Hens with Keel Bone Fractures Experience Pain? *PLoS ONE* 2012, *7*, e42420. [Google Scholar] [CrossRef] [PubMed]
5. Cufadar, Y.; Olgun, O.; Yildiz, A.Ö. The Effect of Dietary Calcium Concentration and Particle Size on Performance, Eggshell Quality, Bone Mechanical Properties and Tibia Mineral Contents in Moulted Laying Hens. *Br. Poult. Sci.* 2011, *52*, 761–768. [Google Scholar] [CrossRef] [PubMed]
6. Saunders-Blades, J.L.; MacIsaac, J.L.; Korver, D.R.; Anderson, D.M. The Effect of Calcium Source and Particle Size on the Production Performance and Bone Quality of Laying Hens. *Poult. Sci.* 2009, *88*, 338–353. [Google Scholar] [CrossRef]
7. Koutoulis, K.C.; Kyriazakis, I.; Perry, G.C.; Lewis, P.D. Effect of Different Calcium Sources and Calcium Intake on Shell Quality and Bone Characteristics of Laying Hens at Sexual Maturity and End of Lay. *Int. J. Poult. Sci.* 2009, *8*, 342–348. [Google Scholar] [CrossRef]
8. Fleming, R.H.; McCormack, H.A.; Whitehead, C.C. Bone Structure and Strength at Different Ages in Laying Hens and Effects of Dietary Particulate Limestone, Vitamin K and Ascorbic Acid. *Br. Poult. Sci.* 1998, *39*, 434–440. [Google Scholar] [CrossRef]
9. Rennie, J.S.; Fleming, R.H.; McCormack, H.A.; McCorquodale, C.C.; Whitehead, C.C. Studies on Effects of Nutritional Factors on Bone Structure and Osteoporosis in Laying Hens. *Br. Poult. Sci.* 1997, *38*, 417–424. [Google Scholar] [CrossRef]
10. Bishop, S.C.; Fleming, R.H.; McCormack, H.A.; Flock, D.K.; Whitehead, C.C. Inheritance of Bone Characteristics Affecting Osteoporosis in Laying Hens. *Br. Poult. Sci.* 2000, *41*, 33–40. [Google Scholar] [CrossRef]
11. Hocking, P.M.; Bain, M.; Channing, C.E.; Fleming, R.; Wilson, S. Genetic Variation for Egg Production, Egg Quality and Bone Strength in Selected and Traditional Breeds of Laying Fowl. *Br. Poult. Sci.* 2003, *44*, 365–373. [Google Scholar] [CrossRef]
12. Fleming, R.H.; McCormack, H.A.; McTeir, L.; Whitehead, C.C. Relationships between Genetic, Environmental and Nutritional Factors Influencing Osteoporosis in Laying Hens. *Br. Poult. Sci.* 2006, *47*, 742–755. [Google Scholar] [CrossRef]
13. Podisi, B.K.; Knott, S.A.; Dunn, I.C.; Burt, D.W.; Hocking, P.M. Bone Mineral Density QTL at Sexual Maturity and End of Lay. *Br. Poult. Sci.* 2012, *53*, 763–769. [Google Scholar] [CrossRef] [PubMed]
14. Enneking, S.A.; Cheng, H.W.; Jefferson-Moore, K.Y.; Einstein, M.E.; Rubin, D.A.; Hester, P.Y. Early Access to Perches in Caged White Leghorn Pullets. *Poult. Sci.* 2012, *91*, 2114–2120. [Google Scholar] [CrossRef] [PubMed]
15. Hester, P.Y.; Enneking, S.A.; Haley, B.K.; Cheng, H.W.; Einstein, M.E.; Rubin, D.A. The Effect of Perch Availability during Pullet Rearing and Egg Laying on

Musculoskeletal Health of Caged White Leghorn Hens. *Poult. Sci.* 2013, *92*, 1972–1980. [Google Scholar] [CrossRef] [PubMed]
16. Jendral, M.J.; Korver, D.R.; Church, J.S.; Feddes, J.J.R. Bone Mineral Density and Breaking Strength of White Leghorns Housed in Conventional, Modified, and Commercially Available Colony Battery Cages. *Poult. Sci.* 2008, *87*, 828–837. [Google Scholar] [CrossRef]
17. Gunnarsson, S.; Yngvesson, J.; Keeling, L.J.; Forkman, B. Rearing without Early Access to Perches Impairs the Spatial Skills of Laying Hens. *Appl. Anim. Behav. Sci.* 2000, *67*, 217–228. [Google Scholar] [CrossRef]
18. Casey-Trott, T.M.; Korver, D.R.; Guerin, M.T.; Sandilands, V.; Torrey, S.; Widowski, T.M. Opportunities for Exercise during Pullet Rearing, Part I: Effect on the Musculoskeletal Characteristics of Pullets. *Poult. Sci.* 2017, *96*, 2509–2517. [Google Scholar] [CrossRef]
19. Casey-Trott, T.M.; Korver, D.R.; Guerin, M.T.; Sandilands, V.; Torrey, S.; Widowski, T.M. Opportunities for Exercise during Pullet Rearing, Part II: Long-Term Effects on Bone Characteristics of Adult Laying Hens at the End-of-Lay. *Poult. Sci.* 2017, *96*, 2518–2527. [Google Scholar] [CrossRef]
20. Wood-Gush, D.G.M. *The Behaviour of the Domestic Fowl*; Heinemann Educational Books Ltd.: Portsmouth, NW, USA, 1971; ISBN 9780435. [Google Scholar]
21. Olsson, I.A.S.; Keeling, L.J. Night-Time Roosting in Laying Hens and the Effect of Thwarting Access to Perches. *Appl. Anim. Behav. Sci.* 2000, *68*, 243–256. [Google Scholar] [CrossRef]
22. Olsson, I.A.S.A.S.; Keeling, L.J.J. The Push-Door for Measuring Motivation in Hens: Laying Hens Are Motivated to Perch at Night. *Anim. Welf.* 2002, *11*, 11–19. [Google Scholar] [CrossRef]
23. Wilkins, L.J.; Brown, S.N.; Zimmerman, P.H.; Leeb, C.; Nicol, C.J. Investigation of Palpation as a Method for Determining the Prevalence of Keel and Furculum Damage in Laying Hens. *Vet. Rec.* 2004, *155*, 547–549. [Google Scholar] [CrossRef]
24. Harlander-Matauschek, A.; Rodenburg, T.B.; Sandilands, V.; Tobalske, B.W.; Toscano, M.J. Causes of Keel Bone Damage and Their Solutions in Laying Hens. *Worlds Poult. Sci. J.* 2015, *71*, 461–472. [Google Scholar] [CrossRef]
25. Whitehead, C.C. *Bone Biology and Skeletal Disorders in Poultry*; Poultry Science Symposium Series; Carfax Publishing Co.: Centreville, VA, USA, 1992. [Google Scholar]
26. Moinard, C.; Statham, P.; Haskell, M.J.; McCorquodale, C.; Jones, R.B.; Green, P.R. Accuracy of Laying Hens in Jumping Upwards and Downwards between Perches in Different Light Environments. *Appl. Anim. Behav. Sci.* 2004, *85*, 77–92. [Google Scholar] [CrossRef]
27. Appleby, M.C.; Duncan, I.J.H. Development of Perching in Hens. *Biol. Behav.* 1989, *14*, 157–168. [Google Scholar]
28. Regmi, P.; Deland, T.S.; Steibel, J.P.; Robison, C.I.; Haut, R.C.; Orth, M.W.; Karcher, D.M. Effect of Rearing Environment on Bone Growth of Pullets. *Poult. Sci.* 2015, *94*, 502–511. [Google Scholar] [CrossRef]
29. Regmi, P.; Nelson, N.; Steibel, J.P.; Anderson, K.E.; Karcher, D.M. Comparisons of Bone Properties and Keel Deformities between Strains and Housing Systems in End-of-Lay Hens. *Poult. Sci.* 2016, *95*, 2225–2234. [Google Scholar] [CrossRef] [PubMed]

30. Casey-Trott, T.M.; Guerin, M.T.; Sandilands, V.; Torrey, S.; Widowski, T.M. Rearing System Affects Prevalence of Keel-Bone Damage in Laying Hens: A Longitudinal Study of Four Consecutive Flocks. *Poult. Sci.* 2017, *96*, 2029–2039. [Google Scholar] [CrossRef]
31. Vits, A.; Weitzenbürger, D.; Hamann, H.; Distl, O. Production, Egg Quality, Bone Strength, Claw Length, and Keel Bone Deformities of Laying Hens Housed in Furnished Cages with Different Group Sizes. *Poult. Sci.* 2005, *84*, 1511–1519. [Google Scholar] [CrossRef]
32. Hy-Line Brown. Hy-Line Management Guide. Available online: https://www.hyline.com/filesimages/Hy-Line-Products/Hy-Line-Product-PDFs/Brown/BRN%20COM%20ENG.pdf (accessed on 25 September 2023).
33. Harrison, C.; Jones, J.; Bridges, W.; Anderson, G.; Ali, A.; Mercuri, J. Associations among Computed Tomographic Measures of Bone and Muscle Quality and Biomechanical Measures of Tibiotarsal Bone Quality in Laying Hens. *Am. J. Vet. Res.* 2023, *84*, 1–9. [Google Scholar] [CrossRef]
34. ANSI/ASAE S459 MAR1992 (R2017). Shear and Three-Point Bending Test of Animal Bone. Available online: https://webstore.ansi.org/preview-pages/ASABE/preview_ANSI+ASAE+S459+MAR1992+(R2017).pdf (accessed on 25 September 2023).
35. Bates, D.; Mächler, M.; Bolker, B.; Walker, S. Fitting linear mixed-effects models using lme4. *arXiv* 2014, arXiv:1406.5823. [Google Scholar] [CrossRef]
36. Hothorn, T.; Bretz, F.; Westfall, P. Simultaneous inference in general parametric models. *Biom. J. J. Math. Methods Biosci.* 2008, *50*, 346–363. [Google Scholar] [CrossRef]
37. Brantsæter, M.; Nordgreen, J.; Rodenburg, T.B.; Tahamtani, F.M.; Popova, A.; Janczak, A.M. Exposure to Increased Environmental Complexity during Rearing Reduces Fearfulness and Increases Use of Three-Dimensional Space in Laying Hens (*Gallus gallus domesticus*). *Front. Vet. Sci.* 2016, *3*, 14. [Google Scholar] [CrossRef]
38. Tactacan, G.B.; Guenter, W.; Lewis, N.J.; Rodriguez-Lecompte, J.C.; House, J.D. Performance and Welfare of Laying Hens in Conventional and Enriched Cages. *Poult. Sci.* 2009, *88*, 698–707. [Google Scholar] [CrossRef] [PubMed]
39. Regmi, P.; Smith, N.; Nelson, N.; Haut, R.C.; Orth, M.W.; Karcher, D.M. Housing Conditions Alter Properties of the Tibia and Humerus during the Laying Phase in Lohmann White Leghorn Hens. *Poult. Sci.* 2016, *95*, 198–206. [Google Scholar] [CrossRef]
40. Hester, P.Y.; Enneking, S.A.; Jefferson-Moore, K.Y.; Einstein, M.E.; Cheng, H.W.; Rubin, D.A. The Effect of Perches in Cages during Pullet Rearing and Egg Laying on Hen Performance, Foot Health, and Plumage. *Poult. Sci.* 2013, *92*, 310–320. [Google Scholar] [CrossRef] [PubMed]
41. Moinard, C.; Morisse, J.P.; Faure, J.M. Effect of Cage Area, Cage Height and Perches on Feather Condition, Bone Breakage and Mortality of Laying Hens. *Br Poult. Sci.* 1998, *39*, 198–202. [Google Scholar] [CrossRef]
42. Widowski, T.M.; Caston, L.J.; Hunniford, M.E.; Cooley, L.; Torrey, S. Effect of Space Allowance and Cage Size on Laying Hens Housed in Furnished Cages, Part I: Performance and Well-Being. *Poult. Sci.* 2017, *96*, 3805–3815. [Google Scholar] [CrossRef]

43. Sharma, M.K.; White, D.; Chen, C.; Kim, W.K.; Adhikari, P. Effects of the Housing Environment and Laying Hen Strain on Tibia and Femur Bone Properties of Different Laying Phases of Hy-Line Hens. *Poult. Sci.* 2021, *100*, 100933. [Google Scholar] [CrossRef]
44. Silversides, F.G.; Singh, R.; Cheng, K.M.; Korver, D.R. Comparison of Bones of 4 Strains of Laying Hens Kept in Conventional Cages and Floor Pens. *Poult. Sci.* 2012, *91*, 1–7. [Google Scholar] [CrossRef]
45. Yilmaz Dikmen, B.; İpek, A.; Şahan, Ü.; Petek, M.; Sözcü, A. Egg Production and Welfare of Laying Hens Kept in Different Housing Systems (Conventional, Enriched Cage, and Free Range). *Poult. Sci.* 2016, *95*, 1564–1572. [Google Scholar] [CrossRef] [PubMed]
46. Neijat, M.; Casey-Trott, T.M.; Robinson, S.; Widowski, T.M.; Kiarie, E. Effects of Rearing and Adult Laying Housing Systems on Medullary, Pneumatic and Radius Bone Attributes in 73-Wk Old Lohmann LSL Lite Hens. *Poult. Sci.* 2019, *98*, 2840–2845. [Google Scholar] [CrossRef] [PubMed]

Chapter 4

INFLUENCE OF PERCH-PROVISION TIMING ON ANXIETY AND FEARFULNESS IN LAYING HENS[3]

[3] Anderson MG, Johnson AM, Jacobs L, Ali ABA. Influence of Perch-Provision Timing on Anxiety and Fearfulness in Laying Hens. *Animals*. 2023; 13(19):3003. https://doi.org/10.3390/ani13193003

Simple Summary

Perch access and age during access to perches may impact laying hen welfare. Our study aimed to determine the effects of early or late access to perches on behavioral measures of anxiety (AB: attention bias test) and fearfulness (TI: tonic immobility test) in laying hens. Pullets were housed in pens with or without access to perches until 17 weeks of age, at which point perch access either continued or was removed until 37 weeks of age, resulting in four treatments: continuous perch access (CP: 0–37 weeks), early perch access (EP: 0–17 weeks), late perch access (LP: 17–37 weeks), no perch access (NP). AB was performed at 21 and 37 weeks of age, and TI was performed at 20, 25, and 37 weeks of age. CP hens showed reduced anxiety and fearfulness, benefiting animal welfare, while NP hens showed increased anxiety and fearfulness. LP hens required around 16 weeks to adapt to the addition of perches in their environment, indicated by increased anxiety and fearfulness at 20 weeks of age that dissipated by week 37 of age. Removing perches in the EP pens resulted in increased fear and anxiety, which also disappeared by week 37 of age. Perch access benefits animal welfare, and removing or preventing access should be avoided.

Abstract

Perches can enhance laying hen welfare, but their effectiveness might be age-dependent. We investigated early and late perch access effects on anxiety and fear in pullets through attention bias (AB) and tonic immobility (TI) tests. Pullets (n = 728) were raised with or without multi-level perches: CP (continuous perch access: 0–37 weeks), EP (early perch access: 0–17 weeks), LP (late perch access: 17–37 weeks), and NP (no perch access). AB was conducted in weeks 21 and 37 (n = 84/week), and TI was performed in weeks 20, 25, and 37 (n = 112/week). CP hens fed quicker

than EP, LP, and NP in AB at weeks 21 and 37 ($p \leq 0.05$). CP and NP feeding latencies were stable, while EP and LP fed faster at week 37 ($p \leq 0.05$). CP had the shortest TI at week 20 ($p < 0.05$). CP and LP had the shortest TI in weeks 25 and 37 (all $p \leq 0.05$). Unlike NP, CP reduced anxiety and fear. Adding perches during laying (LP) raised anxiety at week 21, adapting by week 37, and removing pre-laying perches (EP) worsened fear at weeks 20 and 25 and anxiety at week 21, recovering by week 37. Adding or removing perches prior to the lay phase increased fear and anxiety, an effect that disappeared by week 37 of age. Our study indicates that continuous perch access benefits animal welfare compared to no perch access at all.

Keywords: laying hen; behavior; attention bias; tonic immobility; perch

Introduction

The evaluation of affective states can be used to improve animal welfare. Negative affective states, such as chronic anxiety and fear, raise major welfare concerns because while adaptive to survival, these negative affective states can lead to excessive responses to routine husbandry practices and a decreased ability to cope with environmental change in production settings. For example, extreme fear can result in panicked behavioral responses in laying hens in response to an unusual stockperson behavior, which in turn can lead to piling and suffocation [1]. Furthermore, excessive fear can cause increased sensitivity to stress, poor feed intake, low body weight, and decreased production [2,3]. Although difficult to distinguish, excessive anxiety may have similar negative consequences, as anxiety itself has been defined as a persistent, excessive, and inappropriate emotional state that triggers physiological and behavioral responses lacking adaptive value [4]. Therefore, it is important for animal welfare and productivity to keep anxiety and fear low.

One approach to limit negative affective states is by providing housing conditions that meet motivational needs. For example, laying hens are highly motivated to perch even early in life, and fulfilling this motivation likely improves their affective states, although not directly assessed [5,6]. Laying hens housed in conventional cages were more fearful and showed lower antibody levels early in life compared to hens in enriched, cage-free housing environments [7]. Inducing positive experiences during the pullet phase is important, as behaviors become more rigid later in life after the ontogenetic period has passed [8]. For instance, pullets reared in complex aviary systems that were subsequently transitioned to barren cage environments at a reproductive age were less fearful than those reared in barren cage environments throughout, indicating that environmental complexity during rearing can reduce fearfulness later in life [9]. In addition, providing

environmental complexity (i.e., perches) during rearing can improve musculoskeletal health [10], which in turn could benefit affective states due to their better physical ability to perch [11,12].

Adverse early life experiences, such as the inability to perform highly motivated behaviors, could have long-term negative impacts on laying hen cognition and behavior. Rearing pullets without perches impaired the spatial cognitive skills of the adult hen in a spatial cognition test compared to those reared with perches [13]. Hens refine their spatial skills as young pullets through practice, so preventing this could lead to impaired spatial cognition and an inability to successfully navigate their environment as they are moved to the laying hen facility [13,14]. Pullets given access to perches from 0 to 8 weeks of age jumped to higher perches compared to those not given access to perches until 8 weeks of age [13]. Although effects on cognition and behavior are determined, it is unknown whether early access to perches impacts pullet and laying hen emotion and affect.

The loss or gain of perches when transitioning from the pullet to the layer phase may impact anxiety and fearfulness. The removal of perches after the pullet phase leads to changes in behavior related to frustration and boredom because behavioral needs are not met [15,16,17], which could increase levels of anxiety and fearfulness. Laying hens show frustration-related behaviors, such as increased restlessness and attempted take-offs when access to perches is prevented compared to hens allowed access to perches [18]. It is possible that the effects of losing perch access may be more detrimental to animal welfare than not having any perches at all, but this has not been previously tested.

Anxiety levels can be assessed through attention bias (AB) testing, with AB referring to the differential allocation of attentional resources towards one stimulus compared to others [19]. For example, animals in anxious states exhibit increased attentional bias towards a potential threat,

where more time spent focusing on a perceived threat compared to neutral or positive stimuli indicates increased anxiousness [20]. While anxiety is an affect-mediated response to potentially dangerous situations influenced by previous life experiences, fear is a short-term response to an immediate threat [21]. The behavioral responses of fear and their intensity, either rational or irrational, result from gene–environment interactions during the animal's development and provide insight into their ability to cope with presently dangerous stimuli [21]. To measure fearfulness in poultry, a tonic immobility (TI) test is often used, which uses the prey species' freezing response [22]. Longer tonic immobility durations positively correlate with increased fearfulness [23,24,25,26]. Although anxiety and fear responses can look similar, the two emotions can be opposing and are not always aligned [3,7,27]. Therefore, AB and TI tests could provide valuable insights into laying hen anxiety and fear levels in response to housing environments, as well as giving insight to the distinct emotional states.

The use of AB and TI tests to evaluate anxiety and fearfulness could provide a better understanding of the impacts of perch provision and its timing on laying hen affective state and welfare. Our objective was to investigate the effects of early and late access to perches on anxiety and fearfulness in laying hens. Pullets were housed either with or without multi-tier perches from 0–37 weeks of age, and half of them experienced a loss or gain of perches at 17 weeks of age. We hypothesized that birds housed without any perch access would have the highest levels of anxiety and fearfulness, followed by those reared with perches that were subsequently taken away during the laying phase, then birds reared without perches that were later added to the environment, with birds housed with perches throughout the entire trial having the lowest levels of anxiety and fearfulness.

Materials and Methods

Ethics

This experiment was approved by Clemson University's Institutional Animal Care and Use Committee (protocol #: AUP2021-0068).

Animal and housing

This experiment was conducted in a ventilation- and temperature-controlled poultry house at the Morgan Poultry Center, Clemson, South Carolina, USA, from December 2021 to August 2022. Day-old Hy-Line® brown chicks (n = 728) were randomly allocated across 28 pens (26 birds/pen). Each pen was 5.04 m^2 with approximately 7.6 cm deep clean pine wood shavings covering the floor. For the first 3 weeks, the heat was provided by a focal electric brooder per pen and a gas-fired brooder for the entire house. The temperature was initially set at 35–36.1 °C at day 0, then progressively reduced by 3–4 °C every week until 3 weeks of age, when brooders were removed. The temperature was reduced weekly until 6 weeks of age to 21.1°F, then maintained until the end of the study, following the standard breed guidelines [28]. Feed and water were provided ad libitum. From 0 to 3 weeks, the feed was provided in tube feeders and water in gallon drinkers. For the first week of life, supplementary feed trays were provided. After 3 weeks, feed was provided in circular hanging feeders, and water was available in automatic cup drinkers. The light was provided by a single 60-watt incandescent overhead lightbulb per pen, and pens were kept on a decreasing lighting schedule starting at 20 L:4 D cycle at 1 week old and decreased by increments of either 1.5 or 2 h until 10 L:14 D from 7 weeks of age to the end of the study. During week 6 of age, all birds were neck tagged (GST15, Ketchum Manufacturing INC. ON, Canada) for individual identification.

Treatments

During the rearing phase (0–17 weeks of age), pullets were either housed in pens with multi-tier perches (n = 14 pens) or without perches (n = 14 pens). At 17 weeks of age, birds within a pen were moved to a new pen so that their access to perches during the lay phase (17–37 weeks of age) was either removed or remained the same. Thus, all birds were exposed to the same level of stress from placement into a new setting simulating the pullet transfer from the rearing to the laying facility in the industry. This resulted in four treatments: continuous perch access from 0–37 weeks of age (CP; n = 7 pens); early perch access only during the rearing phase from 0–17 weeks of age (EP; n = 7 pens); late perch access only during lay phase from 17–37 weeks of age (LP; n = 7 pens); and no perch access from 0–37 weeks of age (NP; n = 7 pens). The adjustable perches were built from 5 × 5cm pressure-treated wooden lumber. Each perch structure contained 3 rungs of varying height, each 165.1 cm in length, resulting in 495.3cm of total perching space and approximately 19 cm of perch space per bird. The rungs were 38.1 cm, 62.2 cm, and 88.4 cm high, with a 12.7 cm distance between each perch rung.

Attention bias test

The AB test followed a group testing approach described by Campbell et al. and Anderson et al. [7,29] on three randomly selected birds per pen at "onset of lay" weeks 21 (n = 84; hen-day% = 81.85 ± 4.68%) and "peak-lay" 37 (n = 84; hen-day% = 94.52 ± 1.12%) of age. All 3 birds per pen were tested simultaneously. Two observers performed the AB test in a room adjacent to the main poultry house in a testing arena constructed of wire fencing (140 L × 132 W × 94 H cm) with pine shavings on the floor and a feeder containing poultry feed. Once the three birds were placed in the arena, a conspecific alarm call signaling a ground predator was played for 8s. Immediately

following the alarm call, latencies to begin feeding (s) and the occurrence of vigilance behaviors during the first 30 s were recorded. Four vigilance behaviors were recorded (freezing, neck stretching, looking around, and erect posture) as either observed (1) or not observed (0) within the first 30 s of testing and summed to obtain a vigilance score for each individual bird ranging from 0 (no vigilance behavior observed) to 4 (all vigilance behaviors observed at least once), as previously described by [7,29]. Latencies to begin and resume feeding were recorded following the methodologies described by [7]. Birds from the first round of AB testing were identified by neck tag number and not tested again during the second round of AB testing. Individual identification between the birds during the AB test was possible by marking the birds with livestock spray (Quik Shot Livestock Marker, LA-CO Industries Inc., IL, USA). Table 4.1 summarizes the AB testing method and is adapted from Campbell et al. [7]. For more details on the attention bias testing methods, see [7,29].

Table 4.1. Summary of the attention bias (AB) testing methodology adapted from Campbell et al. [7]. Birds were tested in groups of three at 21 and 37 weeks of age.

Scenario	Procedure	Test Duration	Variables collected
Test begins	Play first alarm call	300 s	Not applicable
No birds begin feeding	Test runs for 300 s	300 s	All birds receive a maximum latency to begin feeding score of 300 s
One bird begins feeding	Test runs for 300 s	300 s	Latency to begin feeding for bird that began feeding Other two birds receive a maximum latency score of 300 s
Two birds begin feeding	Test runs for 300 s. Play second alarm call at 300 s and test runs for an extra 120 s.	420 s	Latencies to begin feeding for the two birds that began feeding Third bird receives a maximum latency score of 300 s Latencies to resume feeding for two birds that began feeding if they resume feeding before test ends
Three birds begin feeding before 270 s	Test runs until the last bird begins feeding. Allow birds 5 s to feed, then play second alarm call. Test runs until 300 s.	300 s	Latencies to begin and resume feeding for all three birds if they resume feeding before the test ends
Three birds begin feeding between 270–300 s	Test runs until the last bird begins feeding. Allow birds 5 s to feed, then play second alarm call. Test runs an extra 120 s.	420 s	Latencies to begin and resume feeding for all three birds if they resume feeding before the test ends

Tonic immobility test

Tonic immobility (TI) was performed by two observers in the center area of the poultry house. At weeks 20 (onset of lay; hen-day% = 80.23 ± 5.85%), 25 (early-lay; hen-day% = 90.89 ± 3.47%), and 37 of age (peak-lay; hen-day% = 94.52 ± 1.12%), four randomly selected birds per pen (n = 128) were tested for TI as described by [29,30]. The birds selected for the TI test were not the same

as those selected for the AB test. Similar to AB testing, individual birds were TI tested only once during the trial. TI was induced by the handler placing the bird on its back into a V-shaped cradle, then placing one hand over the sternum and the other over the head. After 15 s, the handler removed their hands from the bird, stepped out of its line of sight, and recorded latency until the righting response (TI duration [s]). If the bird attempted to right itself within 10 s of the handler removing their hands, the handler attempted to induce TI again by repeating the technique, with a maximum of three induction attempts. If TI could not be induced, the bird received a minimum latency score of 0 s. If the bird remained in TI for the full testing period (5 min), the bird received a maximum latency score of 300 s. Inter-observer reliability was calculated during a 3-day training period when the two observers performed AB and TI alternatively on the same 40 birds that were not included in the current study. Inter-observer reliability was calculated using Cohen's kappa agreement coefficient (κ), following [31], using the "cohen.kappa" function in the "psych" package, and intra-observer agreement was considered good when Kappa exceeded 0.90 [Kappa = 0.96 ($p < 0.001$); 95% CI (0.90, 0.99)].

Statistical analysis

Data were analyzed using the R software (version 3.3.1) with the package "stats" (R Core Team, 2013). To test for the main effects of treatment (CP, EP, LP, and NP) and the age of the birds (TI: 20, 25, and 37 weeks; AB: 21 and 37 weeks) on each variable, generalized linear mixed-effects models (GLMMs) were conducted using the "lme4" package (Bates, et al., 2014). In each GLMM, the interaction term between main effects was also tested as fixed effects, and bird ID and pen as random effects, with the family set to "Quasibinomial" for proportion data and "Poisson" for the other data. Tukey's HSD multiple comparison procedure was used for post-hoc comparisons using the "multcomp" package [32]. The "DHARMa" package was used for

proportion data (i.e., percentage of birds feeding and resumed feeding) to test residual distribution and assumptions for GLMM, while the Shapiro–Wilk test was utilized (i.e., TI duration (s) and time to begin and resume feeding (s)) for the normality analysis of the model residuals. Statistical significance was set at $p < 0.05$. Descriptive statistics were calculated using the "psych package", and data are presented as mean ± standard error of the mean (SEM).

Results

Attention bias test

Latency to begin feeding

At the onset of lay (week 21 of age), CP hens began feeding faster than EP, LP, and NP hens ($F_{3,80} = 235.23$; $p = 0.003$; Figure 4.1). At peak-lay (week 37 of age; $F_{3,80} = 544.19$; $p = 0.001$), CP hens began feeding faster than EP hens ($p = 0.021$), while the latter fed faster than LP hens ($p = 0.016$), and LP hens faster than NP hens ($p = 0.017$; Figure 4.1). EP and LP hens ($F_{1,40} = 196.85$; $p = 0.023$) began feeding faster at week 37 compared to week 21 ($p = 0.021$ and 0.031, respectively; Figure 4.1), but no other post-hoc differences within treatment were observed.

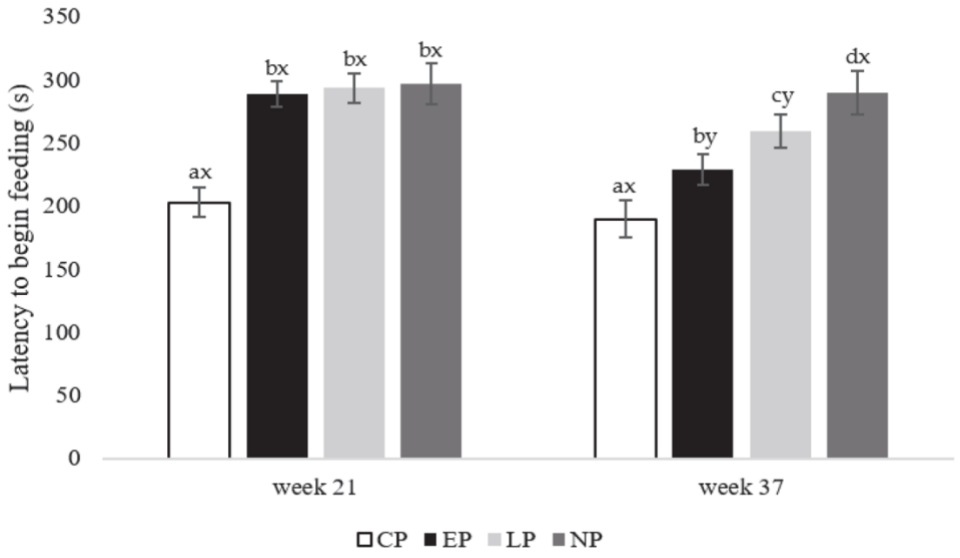

Figure 4.1. Latency to begin feeding (0–300 s) for laying hens in CP (continuous perch), EP (early perch), LP (later perch), and NP (no perch) housing environments during the attention bias test at onset of lay at week 21 and peak-lay at 37 of age (n = 112 hens/week). [a-c]Different superscripts indicate statistically significant differences between treatments within the same week at $p < 0.05$. [x-z]Different superscripts indicate statistically significant differences between weeks within the same treatment at $p < 0.05$.

Latency to resume feeding

At week 21 of age (onset of lay), CP hens resumed feeding faster than EP and LP hens, with the longest latency to resume feeding observed in NP hens ($F_{3,80} = 463.85$; $p = 0.001$; Figure 4.2). Peak-lay at week 37 of age ($F_{3,80} = 301.85$; $p = 0.002$; Figure 4.2), CP and LP hens resumed feeding faster compared to EP hens (p= 0.021 and 0.032, respectively), with EP hens resuming feeding faster than NP hens ($p = 0.029$; Figure 4.2). Within treatment ($F_{1, 40} = 124.46$; $p = 0.031$; Figure 4.2), EP hens resumed feeding faster at week 21 compared to week 37 ($p = 0.026$), and LP hens resumed feeding faster at peak-lay compared to during the onset of lay ($p = 0.019$; Figure 4.2); however, no differences were observed between weeks 21 and 37 in latency to resume feeding for CP and NP hens.

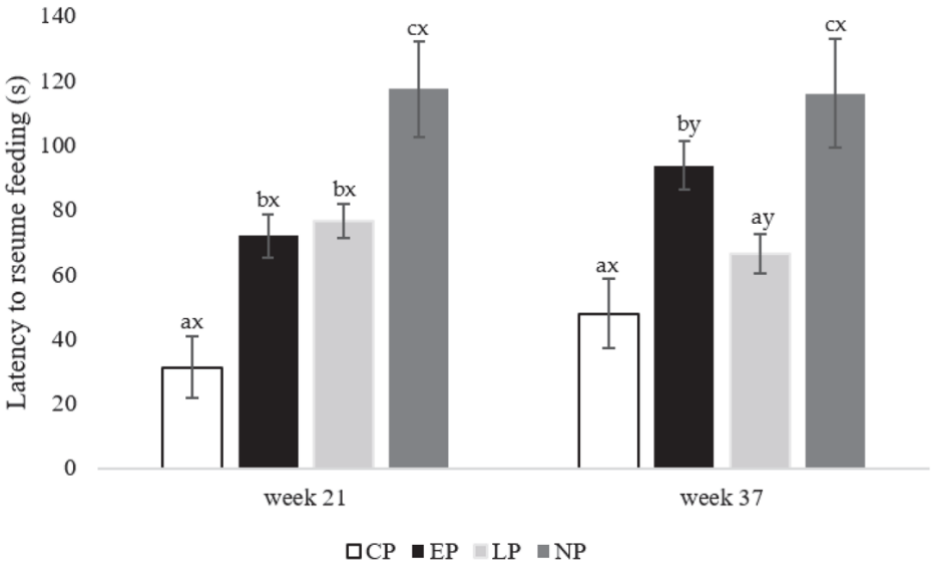

Figure 4.2. Latency to resume feeding (0–120 s) expressed as (mean ± SEM) for laying hens in CP (continuous perch), EP (early perch), LP (late perch), and NP (no perch) housing environments during the attention bias test at the onset of lay at week 21 and peak-lay at week 37 of age (n = 112 hens/week). [a–c] Different superscripts indicate statistically significant differences between treatments within the same week at $p < 0.05$. [x–z] Different superscripts indicate statistically significant differences between weeks within the same treatment at $p < 0.05$

Percentage of birds to begin and resume feeding

More birds from CP and LP pens began feeding compared to EP ($F_{3,80}$ = 399.23; p = 0.021; Figure 4.3) and NP birds the onset of lay at week 21 (p = 0.016, and 0.023, respectively; Figure 4.3). While at peak-lay in week 37 ($F_{3,80}$ = 423.26; p = 0.026; Figure 4.3), more CP birds began feeding than EP (p = 0.001), NP (p = 0.001), and LP birds (p= 0.026), more LP birds were observed to begin feeding than EP and NP (p = 0.033, and 0.036, respectively; Figure 4.3). EP pens had more birds feeding at week 21 compared to week 37 ($F_{1, 40}$ = 99.56; p = 0.036; Figure 4.3), with no observed differences between weeks for other treatments.

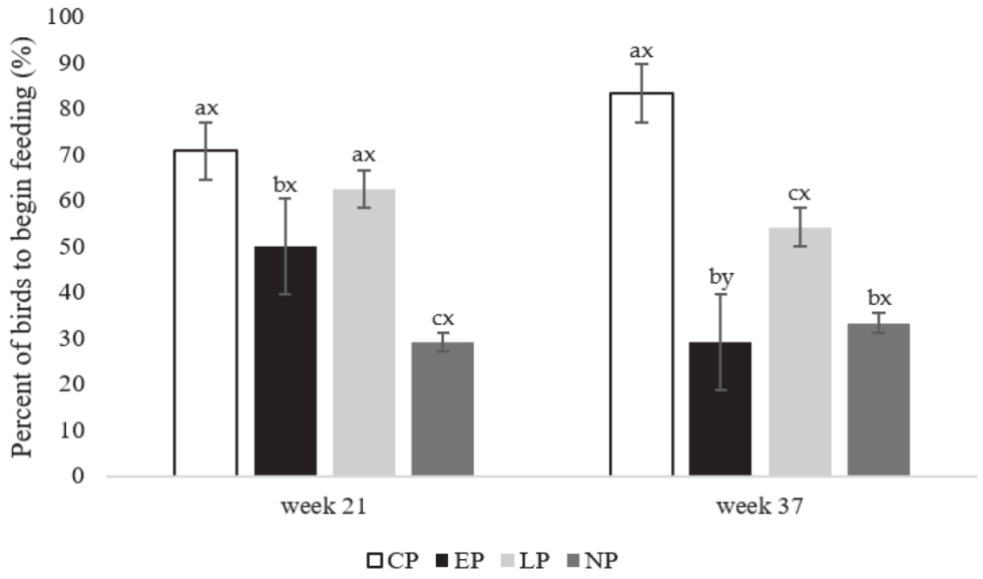

Figure 4.3. Percentage (%) of laying hens (expressed as mean ± SEM) observed to begin feeding from CP (continuous perch), EP (early perch), LP (late perch), and NP (no perch) housing environments during the attention bias test at onset of lay at week 21 and peak-l lay at week 37 of age (n = 112 hens/week). $^{a–c}$ Different superscripts indicate statistically significant differences between treatments within the same week at $p < 0.05$. $^{x–z}$ Different superscripts indicate statistically significant differences between weeks within the same treatment at $p < 0.05$.

More birds from CP pens resumed feeding ($F_{3,80} = 248.52$; $p = 0.019$) compared to birds from LP, EP, and NP pens in week 21 ($p = 0.026$, 0.021, and 0.017, respectively; Figure 4.4). Similarly, more CP birds resumed feeding ($F_{3,80} = 301.26$; $p = 0.023$) than EP, NP, and LP pens in week 37 ($p = 0.013$, 0.019, and 0.029, respectively; Figure 4.4), while the LP group showed more birds resuming feeding than EP (p= 0.032) and NP ($p = 0.029$) pens. Within treatment ($F_{1, 40} = 108.32$; $p = 0.031$), more birds from LP pens resumed feeding at week 37 compared to week 21 ($p = 0.027$), with no observed differences between weeks for other treatments (Figure 4.4).

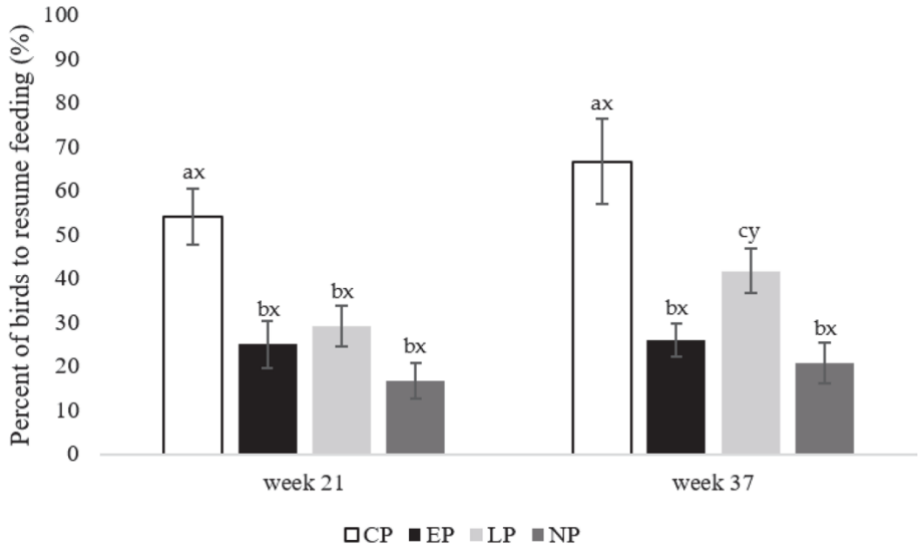

Figure 4.4. Percentage (%) of laying hens (expressed as mean ± SEM) observed to resume feeding from CP (continuous perch), EP (early perch), LP (late perch), and NP (no perch) housing environments during the attention bias test at onset of lay at week 21 and peak-lay at week 37 of age (n = 112 hens/week). The timer was reset to zero after the second alarm call was played to record latency to resume feeding. a–c Different superscripts indicate statistically significant differences between treatments within the same week at $p < 0.05$. x–z Different superscripts indicate statistically significant differences between weeks within the same treatment at $p < 0.05$.

-

Vigilance behavior

Vigilance behavior scores differed between treatments at the onset of lay in week 21 ($F_{3,80}$ = 98.36; p = 0.033), with NP and EP hens having the highest scores compared to LP hens (p = 0.036 and 0.032, respectively), with the lowest vigilance score seen in CP hens (p = 0.023 and 0.021, respectively; Figure 4.5). At peak-lay in week 37 ($F_{3,80}$ = 89.58; p = 0.026), NP hens had the highest vigilance score compared to the other treatment groups (p = 0.019 (CP), 0.022 (EP), 0.031 (LP); Figure 4.5). Between weeks ($F_{1, 40}$ = 112.69; p = 0.029), EP and LP hens had the highest vigilance scores at week 21 compared to 37 (p = 0.021 and 0.036, respectively), with no differences in vigilance scores between weeks in the other treatment groups (Figure 4.5).

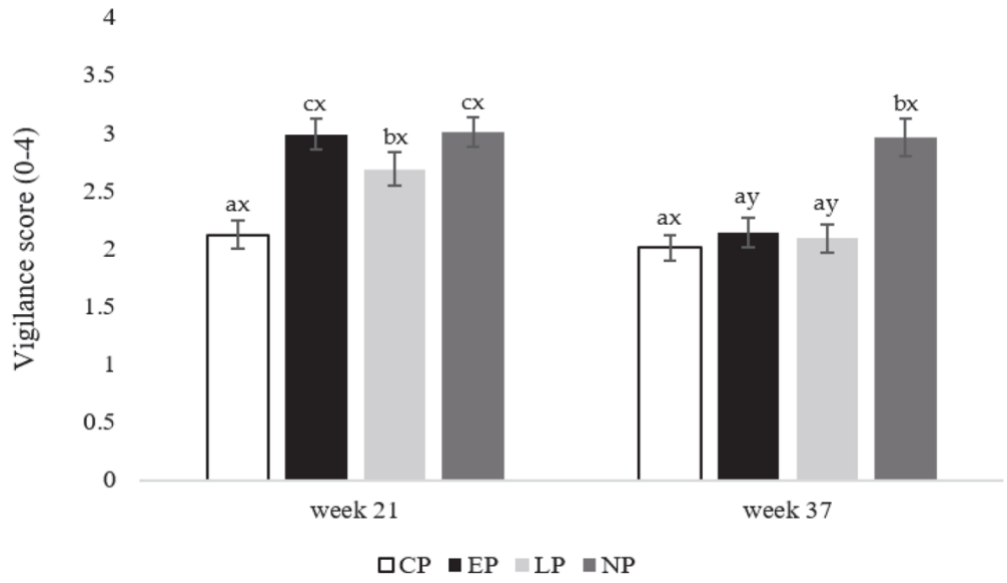

Figure 4.5. Vigilance behavior scores (expressed as means ± SEM) for laying hens in CP (continuous perch), EP (early perch), LP (late perch), and NP (no perch) housing environments during AB testing at onset of lay at week 21 and peak-lay at week 37 of age (n = 84 hens/week). [a–c] Different superscripts indicate statistically significant differences between treatments within the same week at $p < 0.05$. [x–z] Different superscripts indicate statistically significant differences between weeks within the same treatment at $p < 0.05$.

Tonic immobility test

Tonic immobility duration

CP hens had the shortest TI duration compared to EP, LP, and NP hens at the onset of lay in week 20 ($F_{3,108} = 385.99$; $p = 0.026$, 0.016, and 0.011, respectively; Figure 4.6). For early-lay at week 25, CP and LP hens had the shortest TI durations compared to EP and NP hens ($F_{3,108} = 246.36$; $p = 0.011$), while at peak-lay in week 37, CP and LP hens had the shortest TI durations compared to EP hens ($F_{3,108} = 222.58$; $p = 0.031$), with NP hens having longer TI durations than EP hens ($p = 0.037$; Figure 4.6). By treatment per week, CP and EP hens showed shorter TI durations at week 37 compared to 20 and 25 ($F_{2,81} = 126.89$; $p = 0.031$), and LP hens showed

shorter TI durations at week 25 and 37 compared to 20 (p = 0.033, and 0.027, respectively; Figure 4.6). No differences were found between weeks of testing for NP hens (p > 0.05; Figure 5.6).

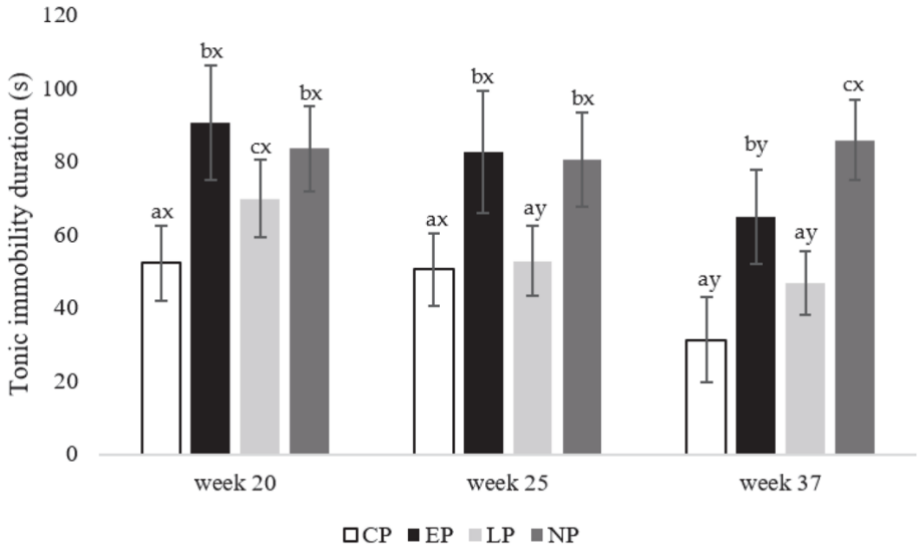

Figure 4.6. Tonic immobility duration (0–300 s) expressed as (mean ± SEM) for laying hens in CP (continuous perch), EP (early perch), LP (late perch), and NP (no perch) housing environments at the onset of lay at week 21, early-lay at week 25, and peak-lay at 37 w weeks of age (n = 112 hens/week). a–c Different superscripts indicate statistically significant differences between treatments within the same week at $p < 0.05$. x–z Different superscripts indicate statistically significant differences between weeks within the same treatment at $p < 0.05$.

Tonic immobility induction attempts

At the onset of lay in week 20, attempts to induce TI were higher in CP and LP hens compared to EP and NP hens ($F_{3,108}$ = 97.55; p = 0.029), with the lowest number of attempts to induce TI recorded in NP hens at week 20 (p = 0.036 (CP), 0.019 (EP), 0.027 (LP); Figure 4.7). At early-lay in week 25 ($F_{3,108}$ = 88.59; p = 0.022) and peak-lay at week 37 ($F_{3,108}$ = 102.95; p = 0.017), induction attempts were lowest in NP hens compared to other treatment groups; however,

there were no observed differences in induction attempts between weeks within any treatment group (Figure 4.7).

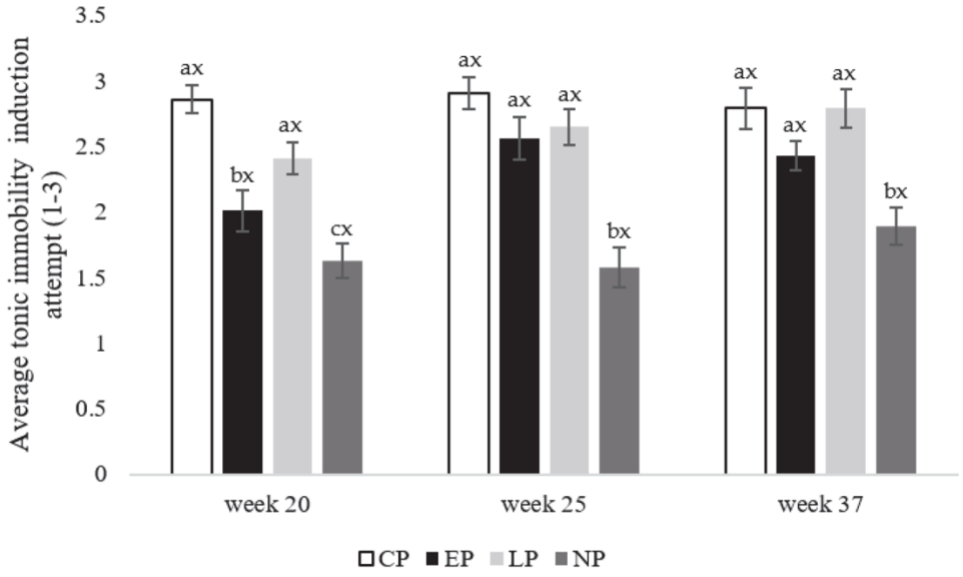

Figure 4.7. Tonic immobility induction attempts (1–3) expressed as (mean ± SEM) for laying hens in CP (continuous perch), EP (early perch), LP (late perch), and NP (no perch) housing environments during the onset of lay at week 21, early-lay at week 25, and peak- lay at 37 weeks of age (n = 112 hens/week). $^{a–c}$ Different superscripts indicate statistically significant differences between treatments within the same week at $p < 0.05$. $^{x–z}$ Different superscripts indicate statistically significant differences between weeks within the same treatment at $p < 0.05$.

General summary of results

Table 4.2 summarizes the current study's AB and TI test results.

Table 4.2. Simple summary of attention bias (AB) and tonic immobility (TI) results. Hens were kept in continuous perch (CP), early perch (EP), late perch (LP), or no perch (NP) housing environments.

Attention Bias Test Measure					
Between Treatments	**Latency to Begin Feeding (s)**	**Percent of Birds to Begin Feeding (%)**	**Latency to Resume Feeding (s)**	**Percent of Birds to Resume Feeding (%)**	**Vigilance Behaviors**
Week 21	CP < EP, LP, NP	NP, EP < CP, LP	CP < EP, LP < NP	EP, LP, NP < CP	CP < LP < EP, NP
Week 37	CP < EP < LP < NP	NP, EP < LP < CP	CP, LP < EP < NP	NP, EP < LP < CP	CP, EP, LP < NP
Between weeks					
CP	Not sig. between weeks	Not sig. between weeks	Not sig. between weeks	Not sig. between weeks	Not sig. between weeks
EP	Week 37 < week 21	Week 37 < week 21	Week 21 < week 37	Not sig. between weeks	Week 37 < week 21
LP	Week 37 < week 21	Not sig. between weeks	Week 37 < week 21	Week 21 < week 37	Not sig. between weeks
NP	Not sig. between weeks	Not sig. between weeks	Not sig. between weeks	Not sig. between weeks	Not sig. between weeks
Tonic Immobility Measure					
Between treatments	Duration (s)			Induction attempts	
Week 20	CP < EP, LP, NP			NP < EP < CP, LP	
Week 25	CP, LP < EP, NP			NP < LP, EP, CP	
Week 37	CP, LP < EP < NP			NP < LP, EP, CP	
Between weeks					

CP	Week 37 < week 20, 25	Not sig. between weeks
EP	Week 37 < week 20, 25	Not sig. between weeks
LP	Week 25, 37 < week 20	Not sig. between weeks
NP	Not sig. between weeks	Not sig. between weeks

Discussion

The objective of this study was to investigate the effects of early and late access to perches on anxiety and fearfulness in laying hens. Attention bias tests evaluate an animal's level of anxiety, where shorter latencies to begin and resume feeding coupled with fewer vigilance behaviors indicate decreased anxiety compared to longer latencies to begin and resume feeding coupled with a greater occurrence of vigilance behaviors [33,34,35]. Tonic immobility tests can be used as a tool to measure fearfulness in poultry [25,36,37], where shorter TI durations and more induction attempts indicate decreased fearfulness compared to longer TI durations and fewer induction attempts. Birds housed with continuous access to perches showed responses consistent with decreased anxiousness and fearfulness compared to the other treatment groups. Birds without access to perches consistently exhibited responses suggesting increased anxiousness and fearfulness compared to birds with access to perches. We observed a negative impact of removing perches in the EP pens on fearfulness at the onset of lay in week 20 and the early-lay period in week 25 of age and on anxiety at the onset of lay in week 21 of age. Lastly, there was a negative impact of adding perches in the LP pens on anxiety at 21 weeks of age and fearfulness at 20 weeks of age.

Attention bias

CP hens showed the shortest latencies to begin and resume feeding regardless of age, showed the greatest percentages of birds that began and resumed feeding at weeks 21 (onset of lay) and 37 (peak-lay), and exhibited the lowest vigilance behavior scores at the onset of lay. This suggests that birds from CP pens showed less bias towards the perceived threat and more attention to the positive stimulus, indicating a lower anxiety level compared to birds from other treatment groups. The exceptions were that at peak-lay, CP and LP hens had similar latencies to resume feeding and that at week 21, CP and LP had a similar percentage of birds begin feeding. The NP hens showed increased anxiety based on longer latencies to begin feeding at peak-lay and resume feeding at weeks 21 and 37 compared to all other treatment groups, a lower percentage of birds to begin feeding at week 21, and the highest vigilance behaviors scores at weeks 21 and 37. These longer latencies and increased vigilance behaviors indicate greater attention allocated toward the perceived threat (conspecific alarm call), suggesting a higher anxiety level than the other treatment groups. However, some results do not fully align with this statement. At the onset of lay, EP and LP birds had similar latencies to begin feeding and similar percentages of birds to resume feeding, and EP birds had a similar vigilance behavior score as birds from NP pens. At peak-lay, EP and NP pens had similar percentages of birds to begin and resume feeding.

Providing laying hens with perches throughout their life offers birds the opportunity to fulfill a strong motivation to perch. Laying hens are highly motivated to perch, which is reflected in their willingness to push open heavier doors in order to gain access to a perch than to gain access to a sham perch that could not be used for perching [38]. Our results align with previous findings that providing complex environmental conditions reduces anxiety in laying hens [7], broiler chickens

[29], and starlings [39]. Broilers housed in complex pens with perches, dust baths, and temporary enrichments showed shorter latencies to begin and resume feeding in an AB test compared to broilers housed in monotonous environments, indicating reduced anxiousness in the former [29]. In contrast, laying hens housed in conventional cages showed responses indicating reduced anxiety compared to laying hens housed in floor pens with perches [7]. Although the methodologies were similar, latencies to begin and resume feeding were much lower in the previous study (54-100s for conventional cage and 54-146s for enriched floor pen in the previous study compared to 290–297 s for NP and 190–203 s for CP in the present study). This could be due to strain differences [40], test age differences (30 weeks of age compared to 21 and 37 weeks during the present study) [7], or inherent differences in husbandry. Ultimately, our results are the first to suggest that Hy-Line Brown hens housed with access to multi-tier perches throughout their lifetime are less anxious at weeks 21 and 37 of age than those housed without perches.

During the AB test in week 21 (onset of lay), hens from the EP group showed similar latencies to begin feeding to hens from LP and NP pens, similar latencies to resume feeding to hens from LP pens, a comparable percentage of birds to resume feeding to those observed in LP and NP pens, and a similar vigilance score to hens from NP pens. This could indicate that the removal of perches increased anxiousness in hens from EP pens comparable to the addition of a novel object within the environment or having no perches at all. Previous research has established hens' strong motivation to perch [18,38,41,42,43]. Chicks begin to perch between 7 and 10 days of age [43] and the amount of time spent perching increases with age [41]. By preventing access to perches during the lay phase, for which hens have an inelastic demand (they will work for access to perches despite increasing costs), hens may suffer and experience elevated levels of anxiety [44].

Depriving hens of the opportunity to perch after access to perches during rearing (0–17 weeks of age) can increase anxiety at the onset of lay (21 weeks of age).

At peak-lay, birds from EP pens exhibited greater anxiety (longer latencies to resume feeding and fewer birds that began and resumed feeding) compared to birds from LP pens. However, some behavioral responses indicate decreased anxiety in the EP group compared to LP birds and NP birds (latency to feed), or similar levels of anxiety to LP birds (vigilance) and NP birds (percent of birds feeding). We would expect birds from EP pens to show increased anxiety at week 37 of age compared to birds from LP pens because they lack access to an appropriate environmental structure to exhibit perching behavior. Preventing the expression of this highly motivated behavior likely influences anxiety because hens do not have access to appropriate elevated surfaces which they perceive as a safe space, increasing the occurrence of negative states [45,46]. Furthermore, birds from EP pens showed decreased anxiety at peak-lay compared to birds from NP pens (latency to begin and resume feeding, vigilance behavior), suggesting that perch access, even when removed at 17 weeks of age, is more beneficial to anxiousness at 37 weeks of age than not having access to perches at all.

Late access to perches (LP) resulted in longer latencies to begin and resume feeding, greater vigilance, and fewer birds resuming feeding in week 21 compared to peak-lay. These responses indicate greater anxiousness at the onset of lay, when birds recently gained access to perches, compared to peak-lay when birds had prolonged perch access. Furthermore, providing late access to perches (LP) resulted in almost equally negative affective states compared to hens reared without perch access (NP) at week 21 of age (onset of lay), indicated by similar percentages and latencies of birds to begin feeding. The LP hens may still be adapting to their new environment,

contributing to the responses consistent with increased anxiousness during week 21 (i.e., after the perches were added to the pens). Without any prior exposure to multi-tier perches during development, the hens may have experienced reduced spatial navigation skills, impairing their ability to successfully utilize the perches. For example, pullets reared with perches from 0-8 weeks of age were able to jump to higher perches compared to those without access to perches until after 8 weeks of age [13]. Additionally, there are concerns about transferring cage-reared pullets to aviaries due to their lack of navigational practice in a setting with greater vertical space [47]. Accidents during take-off to perch or landing are more common in birds reared without perches, which could increase the occurrence of keel bone fractures or collisions with pen mates resulting in aggressive interactions [47]. Furthermore, hens that did not receive enrichment in floor pens during rearing and were moved into an aviary at 25 weeks of age did not occupy the upper tiers of the aviary and took 20 weeks to adapt to the system [48]. Ultimately, pullets should be reared in conditions similar to their adult environment, likely also because this reduces behavior-related problems [47]. Although we did not measure perching behavior in the current study, hen responses during the attention bias test following the addition of perches within the environment indicated that hens took at least 16 weeks to adapt to their new environment, as they had no prior experience with perches. However, as we did not test between 21 and 37 weeks of age, future studies should focus on this period to discover the true adaptation period of hens to new objects within their home environment.

Adding perches later in life did not improve affect but rather had a varied result on behavioral responses during the AB test. At week 37 of age, birds from LP pens exhibited latencies to resume feeding that were comparable to hens from the CP group (LP: 66 s; CP: 48 s). However, LP hens began feeding later and had fewer birds begin and resume feeding than the CP treatment group at

week 37. This could suggest that the addition of perches did not completely improve the affective state to the standard found in hens from the CP group, possibly because hens from LP pens did not have access to perches during musculoskeletal development, as did hens from CP pens. In other words, the quality of perch use was maybe insufficient as in the CP group because learning to use perches after the pullet phase takes longer due to low muscle strength, a lack of motor skills, and an inability to keep balance [13]. When looking at the within-treatment differences across weeks 21 (onset of lay) and 37 (peak-lay) for LP birds, there is a decrease in latencies to begin and resume feeding, as well as an increase in the percentage of birds to resume feeding, suggesting that the addition of perches did reduce anxiousness within the LP treatment group at peak-lay in week 37 of age. Another explanation may be based on the affective state as an accumulation of experiences. Affective states are the result of cumulative life experiences, ranging from positive to negative, and this can impact how animals respond to certain situations, specifically how anxiously an animal responds to perceived threats [34,49,50]. Hens from LP pens inherently had fewer positive experiences as they had fewer opportunities to express highly-motivated perching behavior than hens from CP pens that had perches their entire life. Subsequently, hens from LP hens were likely in a more negative affective state compared to hens from CP pens, inducing the bias towards potentially threatening stimuli during the AB test [34,50,51].

Overall, hens from CP pens showed decreased anxiety compared to other treatment groups at weeks 21 and 37 of age. Hens from NP pens consistently showed increased anxiousness at weeks 21 and 37 of age compared to hens from other treatment groups. Removing perches from the environment increased anxiety levels at 21 weeks of age; however, the effect of removing perches on anxiety levels at week 37 of age remains unclear. Adding perches to the environment (LP)

resulted in slightly increased anxiety at 21 weeks of age; however, at week 37 of age, the anxiety level had decreased.

Tonic immobility

CP hens exhibited the shortest TI durations compared to other treatments across all weeks of testing, with the exception of early-lay at week 25 and peak-lay at 37 weeks of age, when durations did not differ from LP hens. Additionally, CP hens had the highest number of attempts to induce TI at the onset of lay in week 20 compared to EP and NP hens and at weeks 25 and 37 compared to NP hens. These results indicate that hens from the CP pens were the least fearful at week 20 of age and that hens from CP and LP hens were least fearful at weeks 25 and 37 of age, in alignment with some previous studies [7,52]. Laying hens in enriched pens with access to perches exhibited reduced TI durations compared to hens housed in conventional cages, suggesting they were less fearful [7]. Additionally, hens with access to perches from 16 to 74 weeks of age had a reduced flight distance compared to those without perches, indicating reduced fearfulness in the former and supporting the idea that access to perches improves the birds' sense of security [52]. Other studies found no relationship between perch access and fearfulness [53,54]. For example, TI durations for laying hens housed with or without perches did not differ (232 s vs. 304 s) at 36 weeks of age [54]. These TI durations were longer than those observed in the current study at week 37 of age (CP: 31s vs. NP: 86 s), which could be attributed to genetic strain differences, different environments, or a different level of human interaction, as the TI methodology was comparable between studies. Domestic fowl selected for specific traits typically possess different temperaments, which can be shown through their level of fear or flightiness [54,55,56,57]. Our results suggest that providing Hy-Line Brown hens with multi-tier perches throughout their

lifetime reduces fearfulness compared to all treatments at the onset of lay and compared to EP and NP treatments at early-lay and peak-lay.

Hens without perch access had longer TI durations and fewer induction attempts than hens with continuous or late perch access across all testing weeks, indicating they were more fearful. Perching is a natural behavior seen in domestic hens' wild ancestors to avoid predation and remains a highly motivated behavior in laying hens even after years of domestication [38,58]. Allowing access to perches can reduce fearfulness, as birds gain a feeling of security from perching because they provide an unobstructed view of their surroundings [59,60]. Environments that do not provide appropriate perching structures may subject hens to increased fearfulness, as they may feel less secure due to their reduced surveillance of the surrounding area [58]. In line, laying hens on a low perch were quicker to escape due to an approaching ground predator than laying hens on an elevated perch, indicating that hens on higher perches have a better sense of security [46]. Our results suggest that hens without access to perches had a reduced sense of security and were more fearful than hens from CP and LP groups at weeks 20 and 25 and all treatment groups at peak-lay; however, no differences in fearfulness were found between EP and NP groups at the onset of lay in week 20 and early-lay at 25 weeks of age.

After the removal of perches, TI durations for EP hens did not differ from NP hens at weeks 20 (EP: 91 s; NP: 84 s) or 25 (EP: 83 s; NP: 81 s) of age, suggesting that EP hens had similar levels of fear as hens without perches. This result could indicate the negative impact of the removal of perches at weeks 20 and 25 of age; however, by week 25, EP hens had a similar number of induction attempts as CP and EP hens. The removal of environmental structures important for performing highly motivated behaviors can have detrimental effects on animal welfare. For

example, removing environmental enrichment resulted in a pessimistic judgment bias in starlings [61]. However, by week 37, EP hens had shorter TI durations compared to NP hens (EP: 65 s; NP: 86 s), indicating that EP hens adapted to the loss of resources by 37 weeks of age. This is further supported by the shorter TI durations with increasing age (week 37 vs. 20 and 25) in the EP treatment. While we did not observe differences in TI duration between EP and NP treatment groups during weeks 20 and 25, hens from the EP groups required a consistently higher number of attempts to induce tonic immobility compared to hens from NP groups, and thus it was more difficult to generate the anti-predator freezing response in hens from EP pens. This finding could indicate that providing perches only during rearing impacts fearfulness slightly less negatively than not providing perches at all.

Hens from LP pens were less fearful than hens from EP and NP pens at 20 (onset of lay), 25 (early-lay), and 37 (peak-lay) weeks of age but showed comparable fear to CP hens at weeks 25 and 37, suggesting that current perch access is more important than past access and better than no access to perches. Within the LP treatment group, TI durations were longer during week 20 compared to weeks 25 and 37. This indicates that adding perches early or late in life reduces fear, when fear is measured concurrent with perch access. However, this reduction in fear over time may also be due to repeated exposure to human presence. Although previous studies recommend rearing pullets in the same environment that they are destined for in the lay phase because of the influence that perch access during rearing has on adult behavior and spatial navigation skills while using perches [13,14], our results suggest that current access to perches reduces fear. We did not evaluate perching behavior in the current study, but whether the hens utilized the perches successfully or not, we still observed the beneficial effect of adding perches at 17 weeks of age on fearfulness at 20, 25, and 37 weeks of age.

Fear was greater in CP hens at the onset of lay and early-lay compared to peak-lay at week 37 of age. We argue that repeated exposure to humans that is inherent with husbandry conditions reduces fear as hens aged. While domesticated poultry are inherently afraid of humans [37,62], repeated exposure can reduce this fearfulness, especially when the interaction is considered to be positive [63,64,65]. Birds in the current study were exposed to human presence on a daily basis, and on many occasions, workers were inside the pens multiple times per day. It is possible that as the birds aged, they became increasingly habituated to human presence and handling, resulting in reduced fearfulness during the TI test. Although all hens were exposed to the same level of human interaction, CP, EP, and LP hens showed decreased fear responses as they aged, while NP hens did not. Hens from NP pens showed consistently longer TI durations compared to the other treatment groups. This could suggest that no access to perches: 1) was so impactful on the level of fear that habituation to human exposure made no difference, or 2) the inability to escape to a safe area hindered their ability to cope with human interaction. In line, laying hens seek out perches as a safe space from predators or aggressive pen mates, especially at night, for resting and to monitor their surroundings [18,58]. So, preventing access to perches negatively impacted fear in Hy-Line Brown laying hens. Overall, our results indicate that perch provision, either continuous or later in life, reduces fear when measured during perch access in Hy-Line Brown laying hens at early-lay and peak-lay.

Some previous work supports that anxiety and fear can be opposing and are different emotional experiences [3,7,27]. Where anxiety is a "coherent cognitive-affective structure" ultimately centered around the uncontrollability of possible future negative events, fear is an emotional response to presently dangerous negative events [66]. However, the behavioral responses to each can appear similar [67] and some studies have found anxiety and fear to be positively correlated

as they are both coping strategies to escape from threats [40,68] This could be because there is overlap within the brain mechanisms controlling fear and anxiety, leading to the idea that anxiety is an exaggerated form of fear that allows the animal to prepare for future events [21]. In the current study, behavioral responses of anxiety and fearfulness did align with one another, although they likely produced different emotional experiences.

Our study is limited by our solely behavioral measures of affective state. A truly well-rounded evaluation of affective state and animal welfare includes not only behavioral but also physiological (i.e., heart rate, blood pressure, heterophil lymphocyte ratio) measures. Future research should be conducted to confirm our findings that perch access can reduce both fear and anxiety in behavioral and physiological measures, including in different genetic strains.

Conclusions

The current study implies that providing laying hens with multi-tier perches throughout their lifetime can improve emotion and affective state by reducing fearfulness and anxiety, whereas no access to perches negatively impacted emotion and affective state. The addition of perches to the environment at 17 weeks of age resulted in greater anxiety at 21 weeks of age, but this effect decreased by 37 weeks of age, indicating that adaptation to a new adult environment requires at least 16 weeks. Furthermore, adding perches reduced fearfulness by week 20 of age compared to hens that lost their perch access or never had perch access. At weeks 25 and 37 of age, late access to perches resulted in similar fear levels as in hens with perch access their entire life, suggesting that current perch access reduces fearfulness. Removing perches from the environment at 17 weeks of age resulted in increased anxiety at 21 weeks of age and increased fearfulness at weeks 20 and

25 of age, which dissipated by week 37 of age. Furthermore, birds from EP pens showed decreased anxiety at week 37 compared to birds from NP pens, suggesting that perch access, even when removed at 17 weeks of age, is more beneficial to anxiousness at 37 weeks of age than not having access to perches at all. Our results indicate that continuous access to perches or access to perches at the time of assessment (for late access) resulted in the best outcomes for fear and anxiety in these laying hens.

Author Contributions

Conceptualization, M.G.A. and A.B.A.A.; methodology, M.G.A. and A.B.A.A.; formal analysis, A.B.A.A.; investigation, M.G.A. and A.M.J.; data curation, M.G.A. and A.M.J.; writing—original draft preparation, M.G.A. and L.J.; writing—review and editing, M.G.A., L.J. and A.B.A.A.; supervision, A.B.A.A.; project administration, M.G.A., A.M.J. and A.B.A.A. All authors have read and agreed to the published version of the manuscript.

Funding

This study was funded by the United Sorghum Checkoff Program (project # RG002-21). Technical Contribution No. 7219 of the Clemson University Experiment Station, this material is based upon work supported by NIFA/USDA, under project number SC-1029. Any opinions, findings, conclusions, or recommendations expressed in this publication are those of the authors and do not necessarily reflect the views of the USDA.

Institutional Review Board Statement

The study was conducted in accordance with the Declaration of Helsinki, and approved by Clemson University's Institutional Animal Care and Use Committee (protocol #: AUP2021-0068; November 2021).

Informed Consent Statement

Not applicable.

Data Availability Statement

For access to data from the study, please contact the corresponding author.

Acknowledgments

We would like to thank the employees and student workers at the Morgan Poultry Center for their help, care, and troubleshooting. We would also like to thank our undergraduate volunteers, especially Bailey Ward, Christianna Hoshko, and Isabella Raymond, for their help during the trial.

Conflicts of Interest

The authors declare no conflict of interest.

References

1. Gray, H.; Davies, R.; Bright, A.; Rayner, A.; Asher, L. Why Do Hens Pile? Hypothesizing the Causes and Consequences. *Front. Vet. Sci.* 2020, *7*, 1047. [Google Scholar] [CrossRef] [PubMed]
2. De Haas, E.N.; Kemp, B.; Bolhuis, J.E.; Groothuis, T.G.G.; Rodenburg, T.B. Fear, Stress, and Feather Pecking in Commercial White and Brown Laying Hen Parent-Stock Flocks and Their Relationships with Production Parameters. *Poult. Sci.* 2013, *92*, 2259–2269. [Google Scholar] [CrossRef] [PubMed]
3. de Haas, E.N.; Kops, M.S.; Bolhuis, J.E.; Groothuis, T.G.G.; Ellen, E.D.; Rodenburg, T.B. The Relation between Fearfulness in Young and Stress-Response in Adult Laying Hens, on Individual and Group Level. *Physiol. Behav.* 2012, *107*, 433–439. [Google Scholar] [CrossRef]
4. Ohl, F.; Arndt, S.S.; van der Staay, F.J. Pathological Anxiety in Animals. *Vet. J.* 2008, *175*, 18–26. [Google Scholar] [CrossRef]
5. Kozak, M.; Tobalske, B.; Martins, C.; Bowley, S.; Wuerbel, H.; Harlander-Matauschek, A. Use of Space by Domestic Chicks Housed in Complex Aviaries. *Appl. Anim. Behav. Sci.* 2016, *181*, 115–121. [Google Scholar] [CrossRef]
6. Weeks, C.A.; Nicol, C.J. Behavioural Needs, Priorities and Preferences of Laying Hens. *Worlds Poult. Sci. J.* 2006, *62*, 296–307. [Google Scholar] [CrossRef]
7. Campbell, A.M.A.M.; Johnson, A.M.A.M.; Persia, M.E.M.E.; Jacobs, L. Effects of Housing System on Anxiety, Chronic Stress, Fear, and Immune Function in Bovan Brown Laying Hens. *Animals* 2022, *12*, 1803. [Google Scholar] [CrossRef]
8. Newberry, R.C. Environmental Enrichment: Increasing the Biological Relevance of Captive Environments. *Appl. Anim. Behav. Sci.* 1995, *44*, 229–243. [Google Scholar] [CrossRef]
9. Brantsæter, M.; Nordgreen, J.; Rodenburg, T.B.; Tahamtani, F.M.; Popova, A.; Janczak, A.M. Exposure to Increased Environmental Complexity during Rearing Reduces Fearfulness and Increases Use of Three-Dimensional Space in Laying Hens (Gallus Gallus Domesticus). *Front. Vet. Sci.* 2016, *3*, 14. [Google Scholar] [CrossRef]
10. Campbell, D.L.M.; de Haas, E.N.; Lee, C. A Review of Environmental Enrichment for Laying Hens during Rearing in Relation to Their Behavioral and Physiological Development. *Poult. Sci.* 2019, *98*, 9–28. [Google Scholar] [CrossRef]
11. Campbell, D.L.M.; Makagon, M.M.; Swanson, J.C.; Siegford, J.M. Perch Use by Laying Hens in a Commercial Aviary. *Poult. Sci.* 2016, *95*, 1736–1742. [Google Scholar] [CrossRef] [PubMed]
12. Hester, P.Y.; Enneking, S.A.; Haley, B.K.; Cheng, H.W.; Einstein, M.E.; Rubin, D.A. The Effect of Perch Availability during Pullet Rearing and Egg Laying on Musculoskeletal Health of Caged White Leghorn Hens. *Poult. Sci.* 2013, *92*, 1972–1980. [Google Scholar] [CrossRef]
13. Gunnarsson, S.; Yngvesson, J.; Keeling, L.J.; Forkman, B. Rearing without Early Access to Perches Impairs the Spatial Skills of Laying Hens. *Appl. Anim. Behav. Sci.* 2000, *67*, 217–228. [Google Scholar] [CrossRef]
14. Tahamtani, F.M.; Nordgreen, J.; Nordquist, R.E.; Janczak, A.M. Early Life in a Barren Environment Adversely Affects Spatial Cognition in Laying Hens (Gallus Gallus Domesticus). *Front. Vet. Sci.* 2015, *2*, 3. [Google Scholar] [CrossRef]

15. Duncan, I.J.H. Frustration in the Fowl. In *British Poultry Science*; Freeman, B.M., Gordon, R.F., Eds.; Taylor & Francis: Abingdon, UK, 1970; pp. 15–30. [Google Scholar]
16. Duncan, I.J.H.; Wood-Gush, D.G.M. Thwarting of Feeding Behaviour in the Domestic Fowl. *Anim. Behav.* 1972, *20*, 444–451. [Google Scholar] [CrossRef]
17. Duncan, I.J.H.; Wood-Gush, D.G.M. Frustration and Aggression in the Domestic Fowl. *Anim. Behav.* 1971, *19*, 500–504. [Google Scholar] [CrossRef] [PubMed]
18. Olsson, I.A.S.; Keeling, L.J. Night-Time Roosting in Laying Hens and the Effect of Thwarting Access to Perches. *Appl. Anim. Behav. Sci.* 2000, *68*, 243–256. [Google Scholar] [CrossRef]
19. Crump, A.; Arnott, F.; Bethell, E.J. Affect-Driven Biases as Animal Welfare Indicators: Review and Methods. *Animals* 2018, *8*, 136. [Google Scholar] [CrossRef] [PubMed]
20. Lee, C.; Verbeek, E.; Doyle, R.; Bateson, M. Attention Bias to Threat Indicates Anxiety Differences in Sheep. *Biol. Lett.* 2016, *12*, 20150977. [Google Scholar] [CrossRef]
21. Steimer, T. The Biology of Fear- and Anxiety-Related Behaviors. *Dialogues Clin. Neurosci.* 2002, *4*, 231–249. [Google Scholar] [CrossRef]
22. Hébert, M.; Versace, E.; Vallortigara, G. Inexperienced Preys Know When to Flee or to Freeze in Front of a Threat. *Proc. Natl. Acad. Sci. USA* 2019, *116*, 22918–22920. [Google Scholar] [CrossRef]
23. Campbell, D.L.M.; Dickson, E.J.; Lee, C. Application of Open Field, Tonic Immobility, and Attention Bias Tests to Hens with Different Ranging Patterns. *PeerJ* 2019, *7*, e8122. [Google Scholar] [CrossRef]
24. Hansen, I.; Braastad, B.O.; Storbraten, J.; Tofastrud, M. Differences in Fearfulness Indicated by Tonic Immobility between Laying Hens in Aviaries and in Cages. *Anim. Welf.* 1993, *2*, 105–112. [Google Scholar] [CrossRef]
25. Jones, R.; Faure, J. Tonic Immobility in the Domestic Fowl as a Function of Social Rank Hens, Behavior, Dominance. *Biol. Behav.* 1982, *7*, 27–32. [Google Scholar]
26. Milošević, N.; Perić, L.; Strugar, V. Duration of Tonic Immobility in Laying Hens Affected by Different Housing Systems. In Proceedings of the EPC 2006-12th European Poultry Conference, Verona, Italy, 10–14 September 2006. [Google Scholar]
27. Nordquist, R.E.; Heerkens, J.L.T.; Rodenburg, T.B.; Boks, S.; Ellen, E.D.; van der Staay, F.J. Laying Hens Selected for Low Mortality: Behaviour in Tests of Fearfulness, Anxiety and Cognition. *Appl. Anim. Behav. Sci.* 2011, *131*, 110–122. [Google Scholar] [CrossRef]
28. Hy-Line Brown Management Guide. Available online: https://www.hyline.com/filesimages/Hy-Line-Products/Hy-Line-Product-PDFs/Brown/BRN%20COM%20ENG.pdf (accessed on 2 February 2023).
29. Anderson, M.G.; Campbell, A.M.; Crump, A.; Arnott, G.; Newberry, R.C.; Jacobs, L. Effect of Environmental Complexity and Stocking Density on Fear and Anxiety in Broiler Chickens. *Animals* 2021, *11*, 2383. [Google Scholar] [CrossRef]
30. Pichova, K.; Nordgreen, J.; Leterrier, C.; Kostal, L.; Moe, R.O. The Effects of Food-Related Environmental Complexity on Litter Directed Behaviour, Fear and Exploration of Novel Stimuli in Young Broiler Chickens. *Appl. Anim. Behav. Sci.* 2016, *174*, 83–89. [Google Scholar] [CrossRef]
31. Landis, J.R.; Koch, G.G. The Measurement of Observer Agreement for Categorical Data. *Biometrics* 1977, *33*, 159. [Google Scholar] [CrossRef]
32. Hothorn, T.; Bretz, F.; Westfall, P. Simultaneous Inference in General Parametric Models. *Biom. J.* 2008, *50*, 346–363. [Google Scholar] [CrossRef] [PubMed]

33. Campbell, D.L.M.; Taylor, P.S.; Hernandez, C.E.; Stewart, M.; Belson, S.; Lee, C. An Attention Bias Test to Assess Anxiety States in Laying Hens. *PeerJ* 2019, *2019*, e7303. [Google Scholar] [CrossRef]
34. Paul, E.S.; Harding, E.J.; Mendl, M. Measuring Emotional Processes in Animals: The Utility of a Cognitive Approach. *Neurosci. Biobehav. Rev.* 2005, *29*, 469–491. [Google Scholar] [CrossRef]
35. Yiend, J. The Effects of Emotion on Attention: A Review of Attentional Processing of Emotional Information. *Cogn Emot* 2010, *24*, 3–47. [Google Scholar] [CrossRef]
36. Gallup, G.G.; Rager, D.R. Tonic Immobility as a Model of Extreme States of Behavioral Inhibition. In *Motor Activity and Movement Disorders*; Humana Press: Totowa, NJ, USA, 1996; pp. 57–80. [Google Scholar] [CrossRef]
37. Jones, R.B. Fear and Adaptability in Poultry: Insights, Implications and Imperatives. *Worlds Poult Sci. J.* 1996, *52*, 131–174. [Google Scholar] [CrossRef]
38. Olsson, I.A.S.A.S.; Keeling, L.J.J. The Push-Door for Measuring Motivation in Hens: Laying Hens Are Motivated to Perch at Night. *Anim. Welf.* 2002, *11*, 11–19. [Google Scholar] [CrossRef]
39. Brilot, B.O.; Bateson, M. Water Bathing Alters Threat Perception in Starlings. *Anim. Behav.* 2012, *8*, 379–381. [Google Scholar] [CrossRef]
40. Peixoto, M.R.L.V.; Karrow, N.A.; Newman, A.; Widowski, T.M. Effects of Maternal Stress on Measures of Anxiety and Fearfulness in Different Strains of Laying Hens. *Front. Vet. Sci.* 2020, *7*, 128. [Google Scholar] [CrossRef]
41. Heikkilä, M.; Wichman, A.; Gunnarsson, S.; Valros, A. Development of Perching Behaviour in Chicks Reared in Enriched Environment. *Appl. Anim. Behav. Sci.* 2006, *99*, 145–156. [Google Scholar] [CrossRef]
42. Appleby, M.C.; Duncan, I.J.H. Development of Perching in Hens. *Biol. Behav.* 1989, *14*, 157–168. [Google Scholar]
43. Riber, A.B.; Wichman, A.; Braastad, B.O.; Forkman, B. Effects of Broody Hens on Perch Use, Ground Pecking, Feather Pecking and Cannibalism in Domestic Fowl (Gallus Gallus Domesticus). *Appl. Anim. Behav. Sci.* 2007, *106*, 39–51. [Google Scholar] [CrossRef]
44. Dawkins, M.S. From an Animal's Point of View: Motivation, Fitness, and Animal Welfare. *Behav. Brain Sci.* 1990, *13*, 1–9. [Google Scholar] [CrossRef]
45. Jacobs, L.; Blatchford, R.A.; de Jong, I.C.; Erasmus, M.A.; Levengood, M.; Newberry, R.C.; Regmi, P.; Riber, A.B.; Weimer, S.L. Enhancing Their Quality of Life: Environmental Enrichment for Poultry. *Poult. Sci.* 2023, *102*, 102233. [Google Scholar] [CrossRef]
46. Keeling, L.J. A Comparison of Two Basic Characteristics of a Perch for Laying Hens. In Proceedings of the Proceedings of the 31st International Congress of the ISAE; Hemsworth, P.H., Spinka, M., Kostal, L., Eds.; Research Institute of Animal Production: Prague, Czech Republic, 1997; p. 83. [Google Scholar]
47. Janczak, A.M.; Riber, A.B. Review of Rearing-Related Factors Affecting the Welfare of Laying Hens. *Poult. Sci.* 2015, *94*, 1454–1469. [Google Scholar] [CrossRef] [PubMed]
48. MacLachlan, S.S.; Ali, A.B.A.; Toscano, M.J.; Siegford, J.M. Influence of Later Exposure to Perches and Nests on Flock Level Distribution of Hens in an Aviary System during Lay. *Poult. Sci.* 2020, *99*, 30–38. [Google Scholar] [CrossRef] [PubMed]

49. Mendl, M.; Burman, O.H.; Paul, E.S. An Integrative and Functional Framework for the Study of Animal Emotion and Mood. *Proc. R. Soc. B* 2010, *277*, 2895–2904. [Google Scholar] [CrossRef] [PubMed]
50. Harding, E.J.; Paul, E.S.; Mendl, M. Animal Behaviour: Cognitive Bias and Affective State. *Nature* 2004, *427*, 312. [Google Scholar] [CrossRef]
51. Mendl, M.; Paul, E.S. Animal Affect and Decision-Making. *Neurosci. Biobehav. Rev.* 2020, *112*, 144–163. [Google Scholar] [CrossRef]
52. Donaldson, C.J.; O'Connell, N.E.; O'Connell, N.E. The Influence of Access to Aerial Perches on Fearfulness, Social Behaviour and Production Parameters in Free-Range Laying Hens. *Appl. Anim. Behav. Sci.* 2012, *142*, 51–60. [Google Scholar] [CrossRef]
53. Brake, J.; Keeley, T.P.; Jones, R.B. Effect of Age and Presence of Perches During Rearing on Tonic Immobility Fear Reactions of Broiler Breeder Pullets. *Poult. Sci.* 1994, *73*, 1470–1474. [Google Scholar] [CrossRef]
54. Campo, J.L.; Gil, M.G.; Dávila, S.G.; Muñoz, I. Influence of Perches and Footpad Dermatitis on Tonic Immobility and Heterophil to Lymphocyte Ratio of Chickens. *Poult. Sci.* 2005, *84*, 1004–1009. [Google Scholar] [CrossRef]
55. Duncan, I.J.H. How Do Fearful Birds Respond? In *Second European Symposium on Poultry Welfare*; German Branch of the World's Poultry Science Association: Celle, Germany, 1985; pp. 96–106. [Google Scholar]
56. Jones, R.B. Social and Environmental Aspects of Fear in the Domestic Fowl. In *Cognitive Aspects of Social Behaviour in the Domestic Fowl*; Zayan, R., Duncan, I.J.H., Eds.; Elsevier: Amsterdam, The Netherlands, 1987; pp. 82–149. [Google Scholar]
57. Keer-Keer, S.; Hughes, B.O.; Hocking, P.M.; Jones, R.B. Behavioural Comparison of Layer and Broiler Fowl: Measuring Fear Responses. *Appl. Anim. Behav. Sci.* 1996, *49*, 321–333. [Google Scholar] [CrossRef]
58. Newberry, R.C.; Estevez, I.; Keeling, L.J. Group Size and Perching Behaviour in Young Domestic Fowl. *Appl. Anim. Behav. Sci.* 2001, *73*, 117–129. [Google Scholar] [CrossRef]
59. EFSA Panel on Animal Health and Animal Welfare (AHAW). Scientific Opinion on Welfare Aspects of the Use of Perches for Laying Hens. *EFSA J.* 2015, *13*, 4131. [Google Scholar] [CrossRef]
60. Bist, R.B.; Subedi, S.; Chai, L.; Regmi, P.; Ritz, C.W.; Kim, W.K.; Yang, X. Effects of Perching on Poultry Welfare and Production: A Review. *Poultry* 2023, *2*, 134–157. [Google Scholar] [CrossRef]
61. Bateson, M.; Matheson, S.M. Performance on a Categorisation Task Suggests That Removal of Environmental Enrichment Induces "pessimism" in Captive European Starlings (Sturnus Vulgaris). *Anim. Welf.* 2007, *16*, 33–36. [Google Scholar] [CrossRef]
62. Jones, B.; Boissy, A. Fear and Other Negative Emotions. In *Animal welfare*; CABI: Wallingford, UK, 2011; pp. 78–97. [Google Scholar]
63. Cransberg, P.H.; Hemsworth, P.H.; Coleman, G.J. Human Factors Affecting the Behaviour and Productivity of Commercial Broiler Chickens. *Br. Poult. Sci.* 2000, *41*, 272–279. [Google Scholar] [CrossRef]
64. Graml, C.; Niebuhr, K.; Waiblinger, S. Reaction of Laying Hens to Humans in the Home or a Novel Environment. *Appl. Anim. Behav. Sci.* 2008, *113*, 98–109. [Google Scholar] [CrossRef]

65. Jones, R.B.; Waddington, D. Modification of Fear in Domestic Chicks, Gallus Gallus Domesticus, via Regular Handling and Early Environmental Enrichment. *Anim. Behav.* 1992, *43*, 1021–1033. [Google Scholar] [CrossRef]
66. Barlow, D.H. Unraveling the Mysteries of Anxiety and Its Disorders from the Perspective of Emotion Theory. *Am. Psychol.* 2000, *55*, 1247–1263. [Google Scholar] [CrossRef] [PubMed]
67. Davis, M.; Walker, D.L.; Miles, L.; Grillon, C. Phasic vs Sustained Fear in Rats and Humans: Role of the Extended Amygdala in Fear vs Anxiety. *Neuropsychopharmacology* 2010, *35*, 105–135. [Google Scholar] [CrossRef] [PubMed]
68. Engel, O.; Müller, H.W.; Klee, R.; Francke, B.; Mills, D.S. Effectiveness of Imepitoin for the Control of Anxiety and Fear Associated with Noise Phobia in Dogs. *J. Vet. Intern. Med.* 2019, *33*, 2675–2684. [Google Scholar] [CrossRef]

Chapter 5

EVALUATION OF BORON AS A FEED ADDITIVE TO IMPROVE MUSCULOSKELETAL HEALTH OF HY-LINE W-36 PULLETS[4]

[4] Anderson MG, Johnson AM, Clark A, Harrison C, Arguelles-Ramos M, Ali, A. Evaluation of boron as a feed additive to improve musculoskeletal healht of Hy-Line W-36 pullets. Accepted in *Poultry*.

Simple Summary

In this study, we used a total of 528 Hy-Line W-36 pullets to investigate the effects of boron supplementation (0mg/kg, 50mg/kg, 100mg/kg, and 150mg/kg) over 17 weeks. Performance parameters remained unaffected. Pullets receiving 150mg/kg demonstrated higher pectoralis major weights at 11 weeks and increased muscle weights at 17 weeks. The 150mg/kg group exhibited larger cortical cross-sectional areas at both 11 and 17 weeks. Moreover, higher bone mineral density (BMD), tibia ash percentages, and bone-specific alkaline phosphatase and pro-collagen type 1 n-terminal propeptide concentrations were observed. Pullets in the 150mg/kg group displayed greater failure load and maximum bending moment, indicating improved bone strength. These findings suggest that providing 150mg/kg of boron enhances musculoskeletal characteristics in Hy-Line W-36 pullets up to 17 weeks without impacting performance parameters.

Abstract

Boron supplementation may improve the musculoskeletal health of pullets before entering the lay phase. This study aimed to evaluate different boron amounts on performance, muscle deposition, tibia cross-sectional area (CSA) and mineral density (BMD), ash percent, breaking strength, and bone mineralization (bone-specific alkaline phosphatase [BALP] and pro-collagen type 1 n-terminal propeptide [P1NP]) of a white-feathered strain of pullets. A total of 528 Hy-Line W-36 pullets were distributed across 24 pens and fed basal diets containing varying amounts of boron (C: 0mg/kg; L: 50mg/kg; M: 100mg/kg; H: 150mg/kg) for 17 weeks. Performance parameters (body weight, average daily weight gain/bird, and average daily feed intake/bird) were measured at weeks 4, 7, 10, 13, and 16, while all other measures were taken at 11 and 17 weeks of

age. Performance was not impacted by boron supplementation. Pectoralis major weights were higher in H pullets at 11 weeks of age, and we also observed higher pectoralis major, minor, and leg muscle weights in H pullets at 17 weeks of age. Pullets fed the H diet had larger cortical CSA than the other treatment groups at 11 weeks of age. At 17 weeks of age, both H and M groups had larger cortical CSA than L and C groups, but the M group had slightly smaller cortical CSA. Pullets fed the H diet had higher BMD values than other treatment groups at 11 weeks of age. At 17 weeks of age, pullets fed the H diet had the highest total BMD values compared to the other treatment groups, and cortical BMD increased with increasing boron inclusion. Pullets fed the H diet had the highest tibia ash percentages and concentrations of BALP and P1NP. Pullets fed the M and H diets had greater failure load and maximum bending moment than pullets fed the L or C diet at 11 weeks of age, with H pullets having greater stiffness values than other groups. At 17 weeks of age, pullets fed the H diet had greater failure load and maximum bending moment compared to all other treatment groups. Our results suggest providing boron within the diet at 150mg/kg improves musculoskeletal characteristics of Hy-Line W-36 pullets up to 17 weeks of age, without impacting performance parameters.

Keywords: boron, pullet, musculoskeletal health, performance

Introduction

Laying hens are prone to bone weakness and osteoporosis due to the intense demand for calcium for eggshell production [1]. Around 2.2 grams of calcium is required to form just one eggshell, and a majority of this calcium is mobilized from the skeleton by osteoclasts [1]. Medullary bone is primarily made up of calcium and acts as a reservoir for the purpose of eggshell formation. However, osteoclasts do not discriminate, so some structural (i.e., cortical) bone is mobilized alongside the medullary bone [1,2]. Progressive loss of cortical bone is the main contributing factor to bone fracture and osteoporosis later in life [2,3]. Osteoporosis is an animal welfare concern because it can cause acute and chronic pain, reduced mobility, and reduced production.

Various nutritional interventions have been investigated as a solution to prevent osteoporosis. For example, supplementing the diet with vitamin D can facilitate intestinal absorption of calcium and phosphorus and maintain circulating calcium blood levels, which may prevent bone loss [4–6]. Furthermore, it is essential to provide nutritional supplementation for bone health during the pullet phase when the skeleton is still developing. This allows for the development of optimal bone quality before calcium resorption from the bone reserves begins during the laying phase.

Although boron is not an essential nutrient for poultry, it may present some benefits to laying hen musculoskeletal health. Older studies suggest boron may play a role in the metabolism of calcium, which helps improve bone strength and prevent fractures [7,8]. However, a majority of studies focus on broiler chicken health. For example, some studies indicate that boron improves growth rate, nutritional efficiency, calcium and phosphorus retention and reduces the effects of vitamin D deficiency in broiler chickens [8–12]. Furthermore, a deficiency

in boron may impact normal development of bone and cartilage, bone ash content, and concentrations of plasma calcium, phosphorus, and magnesium [12–14]. In laying hens, supplementing with boron has been shown to increase tibia calcium content [15], calcium retention [16], shear stress and ash content of the tibia [17], serum calcium concentration [18], bone resistance [19], femur bone strength, and tibia ash and calcium content [20]. In recent years, boron has not been evaluated as a proactive method to aid the bone health of pullets. To our knowledge, the only previous study performed in pullets was published in 1997, with inclusion rates of 50, 100, and 200mg/kg boron [17]. This may be because some previous studies discovered negative effects of boron supplementation on measures of laying hen health and performance. For example, body weight was lower for Barred Rock hens fed 50, 100, and 200mg/kg boron compared to the basal diet [20]. Also, egg production, feed consumption, and body weight of White Leghorn hens decreased when fed 400mg/kg compared to 50, 100, or 200mg/kg boron [21,22]. However, in another study, boron did not affect the same measures at a lower inclusion rate of up to 250mg/kg in Hisex-Brown hybrids [18]. Because differing results indicate that boron could negatively affect certain aspects of laying hen health, our objective was to establish a recent and relevant foundation for future research to determine appropriate boron inclusion rates. Furthermore, the limited availability of recent studies on boron supplementation in pullets warranted a cautious approach to incorporating boron into the diet due to uncertain outcomes. This comprehensive study is the first to evaluate the effect of boron as a feed supplement to improve the musculoskeletal health of Hy-Line W-36 pullets prior to entering the lay phase.

We hypothesized that pullets fed a diet supplemented with boron would show improved musculoskeletal health compared to pullets fed a control diet. The current study aimed to determine

the optimal inclusion rate of boron within a commercial pullet diet and its effects on performance and musculoskeletal health.

Materials and Methods

Ethics

This project was approved by Clemson University's Institutional Animal Care and Use Committee (protocol #AUP2021-0068).

Animals and housing

This experiment was conducted in a ventilation and temperature-controlled poultry house at the Morgan Poultry Center, Clemson, South Carolina, USA. Day-old white Hy-Line W-36 chicks (n = 528) were randomly allocated across 24 pens (22 bird/pen) until 17 weeks of age. Each pen was 5.04 m^2 with approximately 3 7.6 cm clean pine wood shavings covering the floor. For the first 3 weeks, the heat was provided by a focal electric brooder per pen in addition to a gas-fired brooder for the entire house.. From 0 to 3 weeks of age, feed was provided in tube feeders and water in gallon drinkers, and for the first week of life, supplementary feed trays were provided. After 3 weeks, feed was provided in circular adjustable hanging feeders and water was available in automatic cup drinkers. Feed and water were provided ad libitum. For the first 3 weeks, heat was provided by one focal electric brooder per pen and a gas-fired brooder for the entire house. The temperature was initially set at 35-36°C at day 0, then reduced by 2-3°C every week until 3 weeks of age when brooders were removed. Temperature was reduced weekly until 6 weeks of age to 21°C, then maintained until the end of the study following standard breed guidelines [23]. Chicks underwent vaccination against Marek's disease, Newcastle disease

(NDV), infectious bronchitis (IB), infectious bursal disease (IBD or Gumboro), avian encephalomyelitis (AE), and fowl pox according to standard breed guidelines [23] at the hatchery and throughout the trial period. The light was provided by one 60-watt incandescent overhead lightbulb per pen, and each pen was kept on a decreasing light schedule starting at 20L:4D during the first week and was decreased by increments of either 1.5 or 3 hours until 10L:14D from 7 weeks of age until the end of the study when birds were 17 weeks old following standard breed guidelines [23].

Treatments

From 0 to 17 weeks of age, birds were phase-fed commercial mash pullet diets to meet the bird's nutritional needs and correspond to the average bird body weight. The basal diet was formulated to meet or exceed requirements (Table 5.1), following the standard breed guidelines [23]. The starter 1 diet was given from 0-3 weeks old, the starter 2 diet from 4-6 weeks old, the grower diet from 7-15 weeks old, and the pre-lay diet from 15-17 weeks of age. Diets were supplemented with varying levels of boron in the form of boric acid (Sigma-Aldrich Boric Acid B0394, containing 16.2% boron), resulting in four treatment groups (6 pens/treatment): control (C; 0mg/kg boron), low (L; 50mg/kg boron), medium (M; 100mg/kg boron), and high (H; 150mg/kg boron).

Table 5.1. Ingredient percentage and calculated nutrient analysis of 4 basal diets used in the current experiment.

Ingredient	**Starter 1 (%)**	**Starter 2 (%)**	**Grower (%)**	**Pre-lay (%)**
Corn	58.5	61.9	61.9	61.8
45% Soybean Meal	35.7	27.8	22.0	21.7
Mono-dicalcium Phosphate	1.45	1.41	1.38	2.19
Wheat Middlings	1.31	6.42	12.4	9.68
Calcium Carbonate	1.24	1.26	1.36	2.88
Soybean Oil	0.50	0.00	0.00	0.00
Salt	0.45	0.45	0.45	0.45
Choline Chloride 60%	0.45	0.40	0.40	0.40
DL-Methionine	0.27	0.22	0.19	0.19
Vitamin/Mineral Premix*	0.15	0.15	0.15	0.15
L-Threonine	0.04	0.04	0.05	0.06
L-Lysine	0.00	0.03	0.05	0.53
Calculated analysis				
Crude Protein	20.0	18.3	17.5	16.5
Crude Fat	1.89	1.66	1.90	1.95
Crude Fiber	4.04	3.87	2.91	2.65
Calcium	1.05	1.00	0.95	2.50
Phosphorus	0.35	0.34	0.74	0.81
Methionine	0.46	0.40	0.40	0.35
Threonine	0.72	0.66	0.63	0.57
Lysine	1.01	0.85	0.75	0.75
Metabolizable Energy (kcal/kg)	2926	2906	2882	2893

Samples of all diets were analyzed to confirm nutrient composition. *Provimi Corporate Layer 2 with phytase (Lewisburg, OH, USA) composed of: selenium 255ppm, zinc 6.5%, vitamin A 8294000 IU/kg, phytase activity 399166.2 FTU/kg.

Performance

Body weight (BW) and average daily feed intake per bird (ADFI) were calculated weekly at weeks 4, 7, 10, 13, and 16 of age. Feed offered and refused were recorded weekly, and ADFI was calculated; similarly, birds' body weight was calculated using the following formulas to calculate the average daily body weight gain per bird (ADWG).

$$ADF = \frac{Feed\ offered\ - feed\ refused}{\#days\ \times \#birds} \qquad ADWG = \frac{Finish\ weight\ - start\ weight}{\#days}$$

Computed tomography (CT) image acquisition

At 11 and 17 weeks of age, 2 birds per pen per week (n = 48 birds/week) were euthanized on-farm by CO_2 inhalation, placed in a cooler of ice, and immediately transported to Godley-Snell Research Center on Clemson University's campus. Birds were individually placed inside a V-shaped foam cradle in a dorsal recumbent position atop a hydroxyapatite calibration phantom (QRM Quality Assurance in Radiology and Medicine, Möhrendorf Germany). The head and legs were stretched in opposite directions and taped to maintain this position in the cradle during image acquisition, following methodology described by Harrison et al. [24] and Anderson et al. [25]. CT images were acquired using a helical mode, head 0-10kg protocol, 0.5mm slice thickness, and bone and soft tissue reconstruction algorithms. CT images were acquired using a Toshiba Aquilion TSX-101A, 16-slice scanner (GE Healthcare, Chicago IL, USA). Birds were dissected immediately after CT scanning and frozen at -29°C for further testing.

Bone cross-sectional area (CSA) and bone mineral density (BMD)

For each CT study, the right tibiotarsal bone and muscle measurements were made using a standardized CT image analysis protocol previously published by Harrison et al. (2023) [24]. Cross-sectional density (HU) and area (mm) of the total and medullary components of the tibiotarsal bone were recorded at predefined proximal, middle, and distal transverse slice locations using hand-traced regions of interest. The cross-sectional area (CSA) of the muscle group surrounding the tibiotarsus at each predefined proximal, middle, and distal location was

also measured. The CT densities for each of the rods in the bone calibration phantom were recorded using the oval ROI tool. The CT densities in HU were then converted to hydroxyapatite values using graphical analysis techniques described in Harrison et al. [24].

Muscle deposition

Birds were removed from the -29°C freezer and allowed to thaw at refrigerated temperature for 24 hours prior to dissection, which included obtaining weights of the biceps brachii, triceps brachii, pectoralis major, pectoralis minor, and leg muscle group. The separation of muscles followed procedures described by Anderson et al. [25] and Casey-Trott et al. [26] and with the assistance of a veterinarian (A.A) to ensure consistent muscle specimen collection. The left tibiae were frozen at -29°C for ash percentage, and the right tibiae were frozen at -20°C for breaking strength measures.

Ash percentage

Left tibiotarsi of euthanized birds were thawed approximately 24 hours prior to data collection. The bones were cleaned from any surrounding muscles and soft tissues, and tibiae were ashed according to the methods described by Anderson et al. [25].

Breaking strength

Mechanical properties of the right tibiotarsi were assessed using a three-point bending test as specified by the American National Standards Institute (ANSI) standards for the application of 3 point bending on animal bones [27]. Testing was performed using an Instron Dynamic and Static Material Test system (Model 5944, Instron Corp., Canton, MA, USA) equipped with a 500N load cell and Automated Material Test System software. Tibiae breaking

strength was measured according to methods described by Anderson et al. [25]. Load and displacement data were collected and were used to obtain the breaking strength (N), stiffness (N/mm), and maximum bending moment (N/m).

Bone mineralization

During weeks 11 and 17 of age, blood samples were collected from the brachial wing vein of 3 birds per pen per week (n = 72). Whole blood samples were transferred to 1.5mL Eppendorf tubes, and serum was separated at 6000 rpm for 10 minutes at 4˚C. Serum samples were analyzed for levels of bone-specific alkaline phosphatase (BALP) and procollagen type 1 N-terminal propeptide (P1NP) using commercial ELISA kits Nanjing Jiancheng Institute of Bioengineering (Nanjing, China) and MyBioSource (San Diego, CA, USA), respectively.

Statistical analysis

Statistical analyses were performed using the R software 'stats' package (version 4.3.2, R Core Team, 2023). Descriptive statistics were calculated using the "psych" package. Evaluation of data with a Shapiro-Wilk's test (p>0.05) using "shapiro.test" package and a visual inspection of histograms using "hist." package revealed that data from all measurements were normally distributed. Generalized linear mixed models were developed with family set to "Poisson," using the lme4 package to describe the influence of boron supplementation on performance parameters, CT parameters, muscle deposition, breaking strength, ash%, and bone mineralization, across weeks of age, and all possible interactions [28]. Dietary treatment and week of age were included as main effects and unit and individual birds where possible, as random effects, $p \leq 0.05$ was considered significant, using the following model:

$$Y_{ijkl} = \mu + B_i + T_j + BT_{ij} + C_{kl} + e_{ijkl}$$

where Y_{ijkl} is the dependent variable, μ is the overall mean, B_i is the effect of the dietary treatment, T_j is the effect of week of age, BT_{ij} is the interaction effect between B_i dietary treatment and Tj week of age, C_{kl} is the effect of individual birds within the unit of B_i, and across T_j weeks of age, and e_{ijkl} is the residual error.

Statistically significant effects were further analyzed using Tukey's honestly significant difference (HSD) multiple comparison procedure using the "multcomp" package [29]. Tukey's HSD, significant differences between pairwise comparisons, are indicated in figures or tables by different superscript letters. Data are presented as mean ± standard error of the mean (SEM) with P values of the pairwise comparisons.

Results

Performance

At weeks 4, 7, 10, 13, and 16 weeks of age, there were no differences in individual body weight, average daily weight gain per bird, or average daily feed intake per bird between treatment groups ($p > 0.05$; Table 5.2).

Table 5.2. Body weight, average daily weight gain, and average daily feed intake per bird (g) fed (mean ± SEM) a control diet (C), or a basal diet supplemented with low (L; 50mg/kg), medium (M; 100mg/kg), and high (H; 150mg/kg) boron at weeks 4, 7, 10, 13, and 16 weeks of age.

Body weight/bird					
Week	4	7	10	13	16
C	332.7±13.5	581.2±25.6	787.6±30.0	1192.5±37.0	1306.5±43.6
L	339.9±13.0	588.7±23.6	792.6±25.6	1196.6±41.2	1352.7±44.5
M	345.5±14.0	583.5±24.6	796.9±31.5	1232.4±32.0	1299.4±49.3
H	350.0±16.2	592.6±30.5	802.4±38.3	1203.7±39.0	1367.0±56.2
Average daily weight gain/bird					
C	12.3±1.2	79.9±9.6	80.4±8.3	72.5±11.0	49.2±6.7
L	12.4±1.0	77.0±6.6	82.3±9.6	76.6±12.3	48.3±5.9
M	13.5±1.5	81.0±8.3	86.2±10.3	81.3±10.3	50.5±9.9
H	14.0±1.2	86.6±5.6	89.9±5.9	79.7±9.6	50.9±10.6
Average daily feed intake/bird					
C	31.1±4.9	48.3±6.6	68.3±6.0	80.0±11.3	84.3±6.7
L	30.2±5.2	49.3±10.0	68.1±6.9	79.6±9.6	83.7±5.3
M	30.5±6.3	48.1±8.6	67.3±10.6	78.9±10.7	82.6±7.0
H	31.0±5.7	48.0±6.0	66.2±12.0	78.0±13.0	81.0±3.6

Bone cross-sectional area (CSA) and bone mineral density (BMD)

At week 11 of age, pullets from H pens had the highest cortical CSA compared to other treatment groups (proximal: M = 0.036, L = 0.026, C = 0.019; middle: M = 0.031, L = 0.022, C = 0.016; Table 5.3), except at the distal location. There were no differences between treatments for total CSA ($p > 0.05$; Table 5.3). Also at week 11 of age, hens from H pens had consistently higher total BMD than other treatment groups (middle: M = 0.031, L = 0.021, C = 0.011; distal: M = 0.026, L = 0.016, C = 0.001), except for the proximal location where C pens had the lowest total BMD compared to other treatments (proximal: H = 0.003, M = 0.016, L = 0.026; Table 5.3). Hens from H pens had higher cortical BMD than other treatments (proximal: M = 0.031, L = 0.024, C = 0.021; distal: M = 0.013, L = 0.029, C = 0.041), except at the middle location (middle: L = 0.025, C = 0.016; Table 5.3), where it was not significantly different from M pens.

Table 5.3. Tibia cross-sectional area (CSA; mm^2) and bone mineral density (BMD; mg/cm^3) of pullets (mean ± SEM) fed a control diet (C), or a basal diet supplemented with low (L; 50mg/kg), medium (M; 100mg/kg), and high (H; 150mg/kg) boron at week 11 of age (n = 48). [a-d] Means with different superscripts within columns differ at $p < 0.05$.

Week 11						
CSA						
	Total			Cortical		
	Proximal	Middle	Distal	Proximal	Middle	Distal
C	46.7±0.8[a]	35.1±0.9[a]	36.9±0.9[a]	31.0±0.8[a]	25.5±0.7[a]	26.0±0.7[a]
L	47.2±0.9[a]	35.7±0.6[a]	37.3±0.8[a]	31.3±0.7[a]	25.8±0.7[a]	26.5±0.8[a]
M	47.3±0.9[a]	36.2±0.8[a]	37.5±0.8[a]	33.2±0.6[a]	26.7±0.9[a]	27.2±0.5[a]
H	47.8±0.8[a]	36.5±0.8[a]	38.0±1.2[a]	34.2±0.8[b]	27.5±0.9[b]	27.6±0.4[a]
BMD						
	Total			Cortical		
	Proximal	Middle	Distal	Proximal	Middle	Distal
C	378.9±7.1[a]	539.3±12.1[a]	496.9±11.0[a]	665.0±13.2[a]	927.6±54.4[a]	778.4±49.8[a]
L	401.3±9.0[b]	553.8±8.0[b]	541.1±11.0[b]	681.4±11.0[a]	1208.0±74.9[b]	896.4±62.2[b]
M	415.4±8.5[b]	568.2±11.0[b]	570.2±11.2[c]	789.0±11.3[b]	1418.3±105.9[c]	1095.3±50.4[c]
H	429.7±8.0[b]	616.1±11.4[c]	673.0±118.6[d]	856.5±16.8[c]	1516.3±114.2[c]	1273.3±49.2[d]

At week 17 of age, pullets from H pens had the highest cortical CSA compared to other treatment groups (middle: M = 0.029, L = 0.016, C = 0.021; distal: M = 0.031, L = 0.036, C = 0.027), except at the proximal location (proximal: L = 0.025, C = 0.019; Table 5.4), where it was not significantly different from M pens. There were no differences between treatments for total CSA ($p > 0.05$; Table 5.4). Similarly, hens from H pens had higher total BMD than other treatments (proximal: M = 0.023, L = 0.019, C = 0.009; middle: M = 0.026, L = 0.021, C = 0.005; distal: M = 0.036, L = 0.019, C = 0.026; Table 5.4), and higher cortical BMD (middle: M = 0.041, L = 0.026, C = 0.021; distal: M = 0.043, L = 0.039, C =0.024), except at the proximal location (proximal: L = 0.023, C = 0.036; Table 5.4), where it was not significantly different from M pens.

Table 5.4. Tibia cross-sectional area (CSA; mm^2) and bone mineral density (BMD; mg/cm^3) of pullets (mean ± SEM) fed a control diet (C), or a basal diet supplemented with low (L; 50mg/kg), medium (M; 100mg/kg), and high (H; 150mg/kg) boron at week 17 of age (n = 48). [a-d] Means with different superscripts within columns differ at $p < 0.05$.

	Week 17					
	CSA					
	Total			Cortical		
	Proximal	Middle	Distal	Proximal	Middle	Distal
C	61.5±1.0[a]	46.2±1.2[a]	48.6±1.2[a]	40.9±1.1[a]	33.5±0.9[a]	34.2±1.0[a]
L	62.0±1.2[a]	47.0±0.8[a]	49.0±1.1[a]	41.1±0.9[a]	33.9±0.9[a]	34.9±1.0[a]
M	62.1±1.1[a]	47.6±1.1[a]	49.2±1.1[a]	43.6±0.8[b]	35.0±1.1[b]	35.7±0.7[a]
H	62.7±1.0[a]	47.9±1.0[a]	49.9±1.5[a]	44.9±1.1[b]	36.0±1.1[c]	36.1±0.6[b]
	BMD					
	Total			Cortical		
	Proximal	Middle	Distal	Proximal	Middle	Distal
C	533.6±10.0[a]	759.6±17.0[a]	699.9±15.5 [a]	936.7±18.6[a]	1306.5±76.6[a]	1096.3±70.2[a]
L	563.6±12.6[a]	777.9±11.2[a]	760.0±15.5[b]	957.0±15.5[a]	1696.6±105.2[b]	1259.0±87.3[b]
M	582.6±11.9[a]	796.9±15.4[a]	799.7±15.7[c]	1106.6±15.9[b]	1989.3±148.5[c]	1536.3±70.7[c]
H	601.9±11.1[b]	862.9±16.0[b]	942.6±26.0[d]	1199.6±23.6[b]	2123.6±160.0[d]	1783.4±69.0[d]

Muscle deposition

At week 11 of age, pullets fed the H diet had heavier pectoralis major muscles compared to all other treatment groups (M = 0.039, L = 0.031, C = 0.028; Figure 5.1), with no other observed differences. At week 17 of age, pullets fed the H diet had heavier pectoralis major (M = 0.043, L = 0.036, C = 0.034, pectoralis minor (M = 0.044, L = 0.040, C = 0.037, and leg muscle groups (M = 0.041, L = 0.033, C = 0.036) compared to all other treatment groups (Figure 5.2).

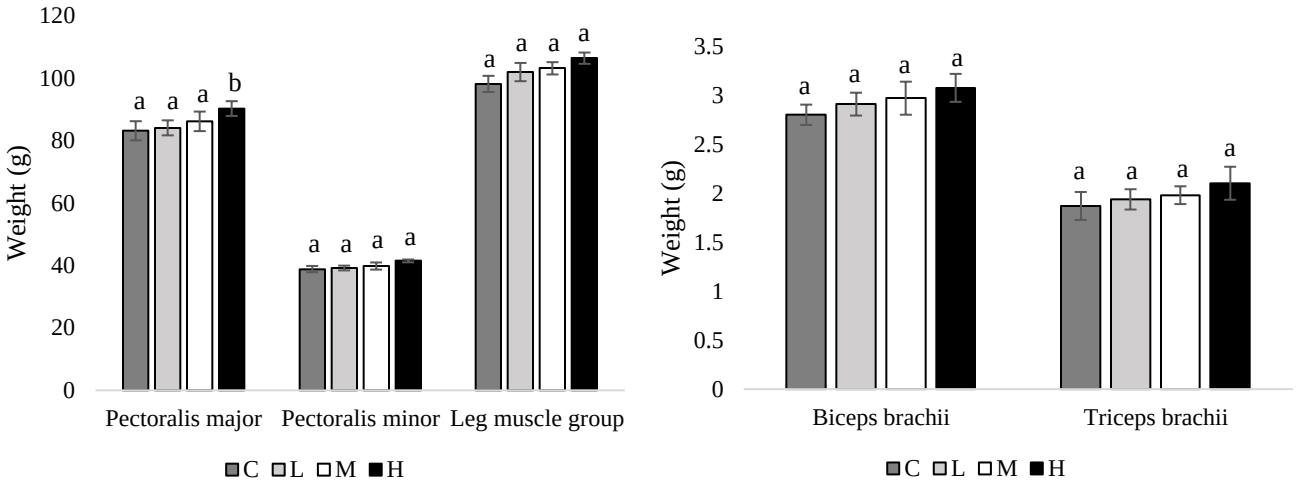

Figure 5.1. Muscle mean weight (g) ±SEM of pullets fed a control diet (C), or a basal diet supplemented with low (L; 50mg/kg), medium (M; 100mg/kg), and high (H; 150mg/kg) boron at week 11 of age (n = 48 birds). [a-b] Means with different superscripts differ at $p < 0.05$.

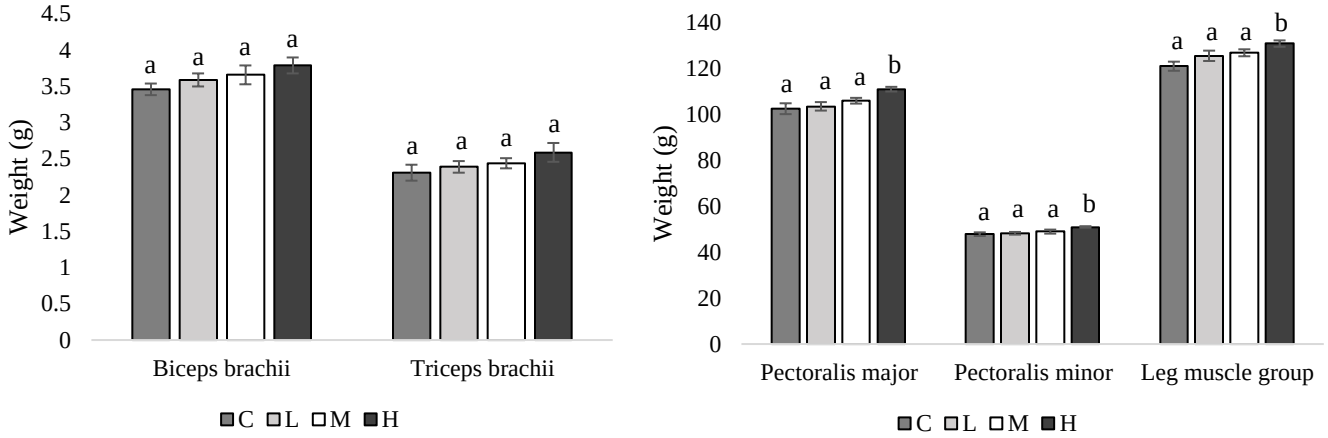

Figure 5.2. Muscle mean weight (g) ±SEM of pullets fed a control diet (C), or a basal diet supplemented with low (L; 50mg/kg), medium (M; 100mg/kg), and high (H; 150mg/kg) boron at week 17 of age (n = 48 birds). [a-b] Means with different superscripts differ at $p < 0.05$.

Ash percentage

At 11 and 17 weeks of age, pullets fed the H diet had greater tibia ash percentages compared to all other treatment groups (Week 11; M = 0.013, L = 0.009, C = 0.001, Week 17; M = 0.021, L = 0.015, C = 0.009; Figure 5.3).

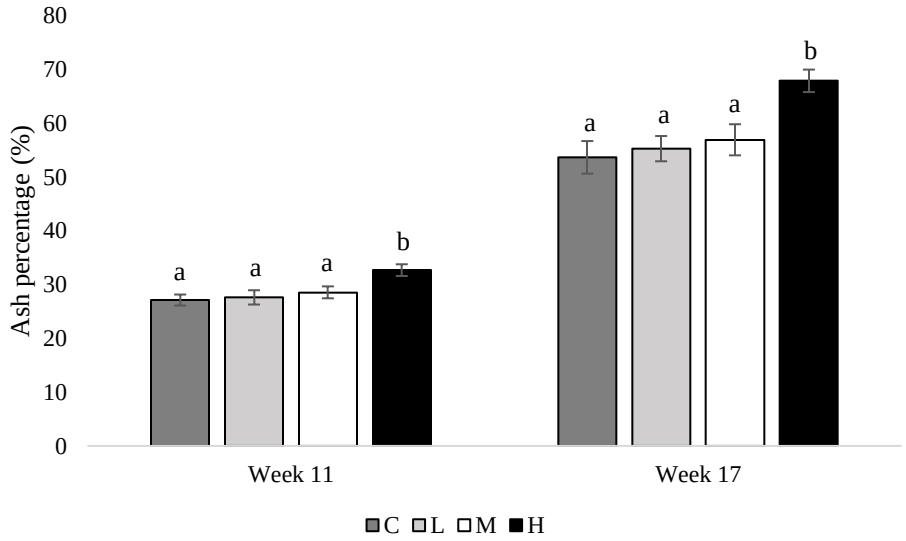

Figure 5.3. Tibia ash percentage of pullets fed a control diet (C), or a basal diet supplemented with low (L; 50mg/kg), medium (M; 100mg/kg), and high (H; 150mg/kg) boron at weeks 11 and 17 of age (n = 48 birds/week). [a-b] Means with different superscripts differ at $p < 0.05$.

Breaking strength

The tibiae of pullets fed the M or H diets had greater failure load (M; L = 0.032, C = 0.025, H; L = 0.012, C = 0.009) and maximum bending moment (M; L = 0.033, C = 0.012, H; L = 0.008, C = 0.002) compared to pullets fed the L or C diet at 11 weeks of age (Table 5.5). Furthermore, pullets fed the H diet had greater stiffness values compared to pullets fed the M diet (week 11; 0.036, week 17; 0.029), with the lowest stiffness in L and C pullets at both 11 and 17 weeks of age (week 11; 0.031, 0.023, week 17; 0.011, 0.021, respectively; Table 5.5). At 17 weeks of age, pullets fed the H diet had greater failure load (M = 0.033, L = 0.019, C = 0.003) and maximum bending moment (M = 0.029, L = 0.009, C = 0.001) compared to all other treatment groups (Table 5.5).

Table 5.5. Tibia breaking strength (N), stiffness (N/mm), and maximum bending moment (N/m) of pullets fed a control diet (C), or a basal diet supplemented with low (L; 50mg/kg), medium (M; 100mg/kg), and high (H; 150mg/kg) boron at weeks 11 and 17 of age (n = 48 birds/week). [a-c] Means with different superscripts within columns differ at $p < 0.05$.

	Week 11		
	Failure load	**Stiffness**	**Maximum bending moment**
C	178.2±5.4[a]	174.6±3.6[a]	471.3±13.6[a]
L	177.4±6.7[a]	183.6±6.9[a]	485.1±12.1[a]
M	202.8±7.3[b]	220.9±2.6[b]	541.2±15.7[b]
H	213.3±5.7[b]	236.2±3.6[c]	566.5±21.3[b]
	Week 17		
C	225.6±6.9[a]	245.9±4.6[a]	645.6±16.6[a]
L	236.6±9.0[a]	251.5±8.6[a]	655.5±14.5[a]
M	256.7±9.2[a]	279.6±3.7[b]	660.0±13.6[a]
H	288.3±7.6[b]	291.6±4.9[c]	682.5±12.0[b]

Bone mineralization

At weeks 11 and 17 of age, pullets fed the H diet had the greatest concentrations of BALP, compared to pullets fed the M diet (week 11 = 0.026, week17 = 0.033), followed by pullets fed the L diet (week 11 = 0.013, week 17 = 0.027), and pullets fed the C diet had the least BALP concentration (week 11 = 0.008, week17 = 0.020; Figure 5.4). Also, at both weeks of age, pullets fed the H diet had the greatest P1NP concentrations compared to pullets fed the M diet (week 11 = 0.036, week 17 = 0.021), with pullets fed the L (week 11 = 0.023, week 17 = 0.019) and C diets having the least P1NP concentrations (week 11 = 0.009, week 17 = 0.001; Figure 5.4).

c

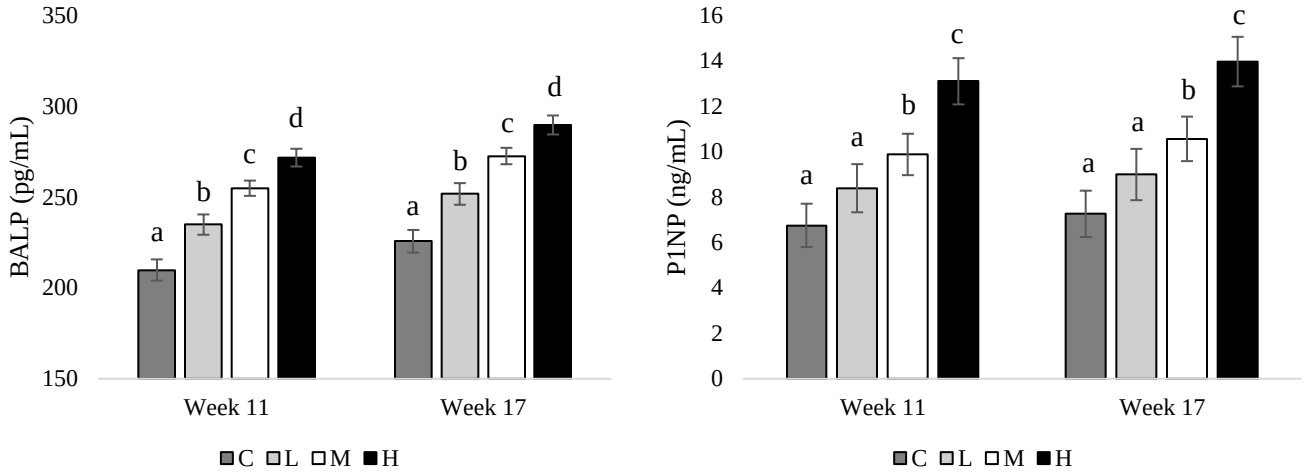

Figure 5.4. Serum concentrations of bone-specific alkaline phosphatase (BALP) and procollagen type 1 N-terminal propeptide (P1NP) in pullets fed a control diet (C), or a basal diet supplemented with low (L; 50mg/kg), medium (M; 100mg/kg), and high (H; 150mg/kg) boron at weeks 11 and 17 of age (n = 72 birds/week). [a-d] Means with different superscripts differ at $p < 0.05$.

Discussion

There were no differences in individual body weight, average daily weight gain per bird, or average daily feed intake per bird between the control diet and any of the diets supplemented with boron. This indicates that boron supplementation has no effect on pullet performance parameters. Previous studies in pullets and laying hens are in agreement with the current results, as they report no difference in body weight as a result of boron supplementation [17,18]. However, a previous study reported that 64-week-old laying hens fed dietary boron had lower body weights than the control group [20]. Moreover, results from the current study contrast with some previous work in broiler chickens, where male broilers supplemented with 5 ppm boron resulted in heavier broilers [8]. Similarly, broilers fed 37.4 ppm boron increased weight gain

from 1 to 21 days of age without impacting the feed conversion ratio [10]. Perhaps the difference in weight gain of broilers in previous studies and the lack of difference in pullets in our study is due to broilers' intense genetic selection for feed consumption and conversion into body mass. Since broilers gain a large amount of weight in a small amount of time, we may observe greater performance differences compared to pullets that do not gain weight rapidly and are not highly motivated to feed compared to broilers. Our results suggest that boron supplementation at 50, 100, or 150mg/kg does not impact pullet performance parameters.

This is the first study to apply computed tomography to evaluate bone cross-sectional area and mineral density as a result of dietary boron supplementation in pullets. There were no differences between any of the treatment groups in total CSA at week 11 or week 17 of age, indicating that adding boron to the diet does not impact the overall size of the tibia. However, it was observed that pullets fed the H diet exhibited larger cortical CSA, and higher total and cortical BMD than the other treatment groups at 11 weeks of age. By 17 weeks of age, both H and M treatment groups had larger cortical CSA than the L and C groups, albeit the M group had slightly smaller cortical CSA than the H group. Additionally, pullets fed the H diet exhibited the highest total BMD values compared to the other treatment groups. Notably, cortical BMD increased with increasing boron inclusion, peaking in the H group and declining in the C group. Previous studies have suggested that boron supplementation can enhance cortical bone area and strength, as evidenced by ostrich chicks supplemented with 400mg/l boron in drinking water showing significantly higher tibial cross-sectional areas compared to the control group or those given lower doses [30]. Also, in that study, ostrich chicks supplemented with 200mg/l boron in the drinking water displayed higher tibial bone mineral densities and stronger bones compared to the other treatment groups [30]. Boron is known to facilitate the resorption of minerals such as

calcium and phosphorus, which are found in the bone in large quantities [16]. Moreover, boron acts with vitamin D, calcium, and magnesium, all of which are vital for bone metabolism [31]. For example, a deficiency of boron in the diet diminishes bone development and can cause bone irregularities in chicks that are deficient in vitamin D [32]. Supplementing the diet with boric acid and vitamin D has shown to elevate tibia calcium levels in laying hens [15], suggesting a higher mineral content within the bone. Trace element distribution in laying hens is promoted with boron supplementation at 60, 120, or 240mg/kg without negatively impacting bone calcium, phosphorus, and magnesium [19]. It is believed that boron influences bone metabolism by affecting bone composition and physical attributes through its stimulation of the formation of the organic matrix, which serves as the foundation for the calcification of bone [15,33]. Building up cortical bone reserves in pullets before they enter reproductive activity is especially important, as they draw calcium from bones for eggshell formation [3,34]. So, these findings, alongside previous studies, support the notion that adding boron to the diet at 100 or 150mg/kg is beneficial in enhancing bone characteristics, particularly mineral content and cortical area of the tibia, potentially reducing the risk of bone fractures later in life.

The current study found that pullets fed the H diet had higher tibia ash percentages than the other treatment groups. Similarly, in a previous study with White Leghorn pullets, tibia ash content increased at 50, 100, and 200mg/kg boron supplementation [17]. Also, laying hen femur and tibia ash content increased at 25 and 50mg/kg boron [20]. Boron has been suggested to play a role in the metabolism of certain minerals, such as calcium and phosphorus [7,8]. These macro-minerals are known to play integral roles in normal skeletal development and functioning [35]. The higher tibia ash percentages and BMD values found in pullets fed the H diet indicate that boron supplementation aids in retaining macro-minerals within the tibia of 11- and 17-week-old

Hy-Line W-36 pullets. This indicates that 150mg/kg boron supplementation increases tibia mineral content at 11 and 17 weeks of age, which is in agreement with the CSA and BMD results obtained from CT scans. We also observed greater concentrations of BALP and P1NP, which are indicators of bone formation and indicate higher rates of bone mineralization by osteoblasts [36,37]. Although these are novel biomarkers of bone mineralization in poultry and have not previously been tested, the higher concentrations of BALP and P1NP, together with the bone mineral density and ash percent data, indicate that boron supplementation at 150mg/kg increases osteoblast activity and therefore, bone mineralization.

Although body weights and feed efficiency did not differ between treatment groups, we did observe differences between pectoralis major muscle weights at 11 weeks of age, with pullets fed the H diet having heavier muscles compared to all other treatment groups. Also, at week 17 of age, pullets fed the H diet had heavier pectoralis major muscles, as well as heavier pectoralis minor and leg muscle groups than the other treatment groups. The primary factors influencing adaptations to bone are locally acting stressors and strains generated by intrinsic muscle forces, as well as external loads [38]. The physical loading of bones directs the deposition of bone materials to areas experiencing the highest physical stress [38,39]. White-feathered strains are known to be flighty [40,41] and anecdotal observations indicate that pullets used in this study frequently flew to areas at the top of the pens. Almost all pullets roosted on elevated structures within the pen at night, which were at least 2 meters from the ground. Reaching such high areas within the pen would have required pullets to jump and use their wings to fly the distance to the elevated surface, imposing mechanical load on the breast and leg muscles [42]. This may explain why we observed an increase in breast muscle mass, as this is where the flight muscles attach, and leg muscles, as the legs are used in flight, take off, and landing [43,44]. However, this effect

on muscle deposition was only observed in pullets fed the H diet. It is possible that the reciprocal relationship between bone density and muscle mass could explain this difference, together with the beneficial effect of boron supplementation on bone characteristics in a compounding manner [39]. Weight-bearing activity can increase bone density and muscle mass [45]. When muscles exert force on the bones during this activity, bone formation is stimulated, leading to increased bone density alongside the increased bone mineralization, strength, and cross-sectional area observed as a result of boron supplementation [45]. Casey-Trott et al. [26] hypothesized that the increased keel bone size of pullets reared in an aviary system was due to the increased wing-assisted activity. Wing use involves the breast muscle (i.e., pectoralis major and minor), which are attached to the keel. Therefore, increased wing use increases pectoralis muscle contraction against the keel, which could induce keel bone formation. Alternatively, the increased muscle deposition seen in pullets fed the H diet may be a compensatory mechanism due to the denser bones. The denser tibiotarsal bones observed in pullets fed the H diet may direct the deposition of tissues in those areas, such as the leg muscle group, by 17 weeks of age as a compensatory mechanism since all birds were housed in identical environments, with access to the same resources and space per bird.

The present study utilized a three-point bend test to measure failure load, stiffness, and maximum bending moment. Failure load indicates the bone's breaking strength, with a higher failure load indicating a stronger bone that requires more force to break [46]. Stiffness refers to the resistance of a bone to an applied force during elastic deformation (i.e., deformation disappears when the external force is removed), where a stiffer bone is more rigid and able to withstand increased force without permanently deforming [47]. In this study, tibiae of 11-week-old pullets fed the M and H diets had greater failure load and maximum bending moment

compared to pullets fed the L and C diet, indicating the tibiae were stronger and more elastic in 11-week-old pullets fed a diet supplemented with 100 or 150mg/kg boron. At 17 weeks of age, only pullets fed the H diet had greater failure load and maximum bending moment compared to all other treatment groups. This may suggest that only the highest level of boron inclusion sustained the beneficial results observed on tibia strength and elasticity beyond 11 and up to 17 weeks of age. At 11 and 17 weeks of age, the tibiae of pullets fed the H diet showed greater stiffness values than those fed the M diet, with the lowest stiffness values in L and C pullets. Our results are in line with previous studies, which noted that boron supplementation has a beneficial impact on bone strength characteristics via optimal calcium absorption [30,31,48]. For example, shear stress of the tibia and shear fracture of the femur in White Leghorn pullets increased when fed 50 and 100mg/kg boron [17], boron supplementation at 60, 120, or 240mg/kg for 16 weeks increased shear force, stress, and fracture energy of the tibia in 26-week-old Lohmann hens[19], and femur bone strength increased in 64-week-old Barred Rock hens fed 25 and 50mg/kg boron for 60 weeks [20]. One study investigating the long-term impact of boron supplementation found that shear fracture energy of the tibia and radius increased in 72-week-old White Leghorns fed 200mg/kg boron starting at 32 weeks of age, indicating a decrease in bone brittleness by the end of the lay period [22]. However, one study found no impact of boron on bone strength characteristics [49]. The lack of difference may be because this study was performed in the last 28 days of production when hens were around 60 weeks of age. At this late point in production when the bone calcium reserves are already quite depleted, as well as providing the nutritional intervention for only 28 days, would make it increasingly difficult to observe any beneficial impact of boron on skeletal health. Our results suggest that providing boron at 150mg/kg improves bone strength characteristics in 11- and 17-week-old Hy-Line W-36 pullets.

Conclusions

Measures of musculoskeletal health in Hy-Line W-36 pullets were beneficially altered up to 17 weeks of age as a result of supplementing the diet with 150mg/kg boron. Our results highlight the beneficial effect of boron supplementation on muscle deposition, tibia ash content and breaking strength, and bone mineralization, without impacting performance parameters. Providing boron at 100mg/kg showed some beneficial effects, such as improved bone-cross sectional area and bone mineral density, although this effect was not as pronounced as what was observed in pullets fed 150mg/kg boron. We also observed enhanced bone strength characteristics in pullets fed 100mg/kg boron at 11 weeks of age, however this effect dissipated by 17 weeks of age. Future studies should focus on investigating the long-term impact of feeding dietary boron to pullets on the musculoskeletal health, as well as egg production and quality, of laying hens.

Author Contributions

Conceptualization, M.G.A. and A.A.; methodology, M.G.A., A.C., C.H., and A.A.; formal analysis, M.G.A., A.C., and A.A.; investigation, M.G.A., A.M.J., C.H., M.A.R, and A.A.; data curation, M.G.A., A.M.J., C.H., and A.A.; writing—original draft preparation, M.G.A. and A.A.; writing—review and editing, M.G.A., M.A.R, and A.A.; supervision, M.A.R., and A.A.; project ad-ministration, M.G.A., A.C., and A.M.J.; funding acquisition, M.A.R., and A.A. All authors have read and agreed to the published version of the manuscript.

Funding

This study was funded by the United Sorghum Checkoff Program (project # RG002-21). Technical Contribution No. 7279 of the Clemson University Experiment Station, this material is based upon work supported by NIFA/USDA, under project number SC-1029. Any opinions, findings, conclusions, or recommendations expressed in this publication are those of the authors and do not necessarily reflect the views of the USDA.

Institutional Review Board Statement

The study was conducted in accordance with the Declaration of Helsinki, and approved by Clemson University's Institutional Animal Care and Use Committee (protocol #: AUP2021-0068; November 2021).

Informed Consent Statement

Not applicable.

Data Availability Statement

For access to data from the study, please contact the corresponding author.

Acknowledgments

We would like to thank the employees and student workers at the Morgan Poultry Center for their help, care, and troubleshooting. We would also like to thank our undergraduate volunteers, especially Bailey Ward, Christianna Hoshko, and Isabella Raymond, for their help during the trial.

Conflicts of Interest

The authors declare no conflict of interest.

References

1. Whitehead, C.C. Overview of Bone Biology in the Egg-Laying Hen. *Poult Sci* **2004**, *83*, 193–199, doi:10.1093/PS/83.2.193.
2. Whitehead, C.C. *Bone Biology and Skeletal Disorders in Poultry*; Poultry science symposium series; Carfax Publishing Co, 1992;
3. Whitehead, C.C.; Fleming, R.H. Osteoporosis in Cage Layers. *Poult Sci* **2000**, *79*, 1033–1041, doi:10.1093/PS/79.7.1033.
4. Chen, C.; Turner, B.; Applegate, T.J.; Litta, G.; Kim, W.K. Role of Long-Term Supplementation of 25-Hydroxyvitamin D3 on Egg Production and Egg Quality of Laying Hen. *Poult Sci* **2020**, *99*, 6899–6906, doi:10.1016/j.psj.2020.09.020.
5. Liu, D.; Veit, H.; Wilson, J.; Denbow, D. Long-Term Supplementation of Various Dietary Lipids Alters Bone Mineral Content, Mechanical Properties and Histological Characteristics of Japanese Quail. *Poult Sci* **2003**, *82*, 831–839, doi:10.1093/ps/82.5.831.
6. Bhattarai, H.K.; Shrestha, S.; Rokka, K.; Shakya, R. Vitamin D, Calcium, Parathyroid Hormone, and Sex Steroids in Bone Health and Effects of Aging. *J Osteoporos* **2020**, *2020*, 1–10, doi:10.1155/2020/9324505.
7. Elliot, M.A.; Edwards, H.M. Studies to Determine Whether an Interaction Exists Among Boron, Calcium, and Cholecalciferol on the Skeletal Development of Broiler Chickens. *Poult Sci* **1992**, *71*, 677–690, doi:10.3382/ps.0710677.
8. Rossi, A.F.; Miles, R.D.; Damron, B.L.; Flunker, L.K. Effects of Dietary Boron Supplementation on Broilers. *Poult Sci* **1993**, *72*, 2124–2130, doi:10.3382/ps.0722124.
9. Hunt, C.D. Dietary Boron Modified the Effects of Magnesium and Molybdenum on Mineral Metabolism in the Cholecalciferol-Deficient Chick. *Biol Trace Elem Res* **1989**, *22*, 201–220, doi:10.1007/BF02916650.
10. Fassani, E.; Bertechini, A.; Brito, J.; Kato, R.; Fialho, E.; Geraldo, A. Boron Supplementation in Broiler Diets. *Rev Bras Cienc Avic* **2004**, *6*, 213–217, doi:10.1590/S1516-635X2004000400004.
11. Kurtoğlu, F.; Kurtoğlu, V.; Çelik, İ.; Keçeci, T.; Nizamlioğlu, M. Effects of Dietary Boron Supplementation on Some Biochemical Parameters, Peripheral Blood Lymphocytes, Splenic Plasma Cells and Bone Characteristics of Broiler Chicks given Diets with Adequate or Inadequate Cholecalciferol (Vitamin D). *Br Poult Sci* **2005**, *46*, 87–96, doi:10.1080/00071660400024001.
12. Kurtoĝlu, V.; Kurtoĝlu, F.; Coşkun, B. Effects of Boron Supplementation of Adequate and Inadequate Vitamin D3-Containing Diet on Performance and Serum Biochemical Characters of Broiler Chickens. *Res Vet Sci* **2001**, *71*, 183–187, doi:10.1053/rvsc.2001.0517.
13. Nielsen, F.H.; Shuler, T.R. Studies of the Interaction between Boron and Calcium, and Its Modification by Magnesium and Potassium, in Rats. *Biol Trace Elem Res* **1992**, *35*, 225–237, doi:10.1007/BF02783768.
14. Nielson, F. *Nutrition Today*. 1992, pp. 6–12.
15. Sizmaz, O.; Yildiz, G. Influence of Dietary Boric Acid and Ascorbic Acid on Performance, Egg Traits, Cholesterol and Bone Parameters of Laying Hens. *Ankara Üniv Vet Fak Derg* **2016**, *63*, 151–156.

16. Adarsh, V.; Dintaran, P.; Shivakumar, G.N.K.; Vijayarangam, E.A.; Kumar, D.D.; Nagaraj, K.; Eknath, J.S. Effect of Boron Supplementation on Laying Performance of White Leghorn Hens Fed Diet with and without Adequate Level of Calcium. *Trop Anim Health Prod* **2021**, *53*, 444, doi:10.1007/s11250-021-02878-x.
17. Wilson, J.H.; Ruszler, P.L. Effects of Boron on Growing Pullets. *Biol Trace Elem Res* **1997**, *56*, 287–294.
18. Kurtoğlu, V.; Kurtoğlu, F.; Coşkun, B. Effects of Boron Supplementation on Performance and Some Serum Biochemical Parameters in Laying Hens. **2006**.
19. Olgun, O.; Yazgan, O.; Cufadar, Y. Effects of Boron and Copper Dietary Supplementation in Laying Hens on Egg Shell Quality, Plasma and Tibia Mineral Concentrations and Bone Biomechanical Properties. *Rev Med Vet (Toulouse)* **2012**, *163*, 335–342.
20. Mizrak, C.; Yenice, E.; Can, M.; Yildririm, U.; Atik, Z. Effects of Dietary Boron on Performance, Egg Production, Egg Quality, and Some Bone Parameters in Layer Hens. *S Afr J Anim Sci* **2010**, *40*.
21. Wilson, J.H.; Ruszler, P.L. Effects of Dietary Boron Supplementation on Laying Hens. *Br Poult Sci* **1996**, *37*, 723–729, doi:10.1080/00071669608417902.
22. Wilson, J.H.; Ruszler, P.L. Long Term Effects of Boron on Layer Bone Strength and Production Parameters. *Br Poult Sci* **1998**, *39*, 11–15, doi:10.1080/00071669889312.
23. *Hy-Line W-36 Management Guide*; 2020;
24. Harrison, C.; Jones, J.; Bridges, W.; Anderson, G.; Ali, A.; Mercuri, J. Associations among Computed Tomographic Measures of Bone and Muscle Quality and Biomechanical Measures of Tibiotarsal Bone Quality in Laying Hens. *Am J Vet Res* **2023**, 1–9, doi:10.2460/ajvr.23.05.0109.
25. Anderson, M.G.; Johnson, A.M.; Harrison, C.; Arguelles-Ramos, M.; Ali, A. Impact of Perch Provision Timing on Activity and Musculoskeletal Health of Laying Hens. *Animals* **2024**, *14*, 265, doi:10.3390/ani14020265.
26. Casey-Trott, T.M.; Korver, D.R.; Guerin, M.T.; Sandilands, V.; Torrey, S.; Widowski, T.M. Opportunities for Exercise during Pullet Rearing, Part I: Effect on the Musculoskeletal Characteristics of Pullets. *Poult Sci* **2017**, *96*, 2509–2517, doi:10.3382/PS/PEX059.
27. ANSI/ASAE *ANSI/ASAE S459 MAR1992 (R2017) Shear and Three-Point Bending Test of Animal Bone*; St. Joseph, 2017;
28. Bates, D.; Mächler, M.; Bolker, B.; Walker, S. Fitting Linear Mixed-Effects Models Using Lme4. *J Stat Softw* **2015**, *67*, doi:10.18637/jss.v067.i01.
29. Hothorn, T.; Bretz, F.; Westfall, P. Simultaneous Inference in General Parametric Models. *Biometrical Journal* **2008**, *50*, 346–363, doi:10.1002/BIMJ.200810425.
30. Cheng, J.; Peng, K.; Jin, E.; Zhang, Y.; Liu, Y.; Zhang, N.; Song, H.; Liu, H.; Tang, Z. Effect of Additional Boron on Tibias of African Ostrich Chicks. *Biol Trace Elem Res* **2011**, *144*, 538–549, doi:10.1007/s12011-011-9024-y.
31. Devirian, T.A.; Volpe, S.L. The Physiological Effects of Dietary Boron. *Crit Rev Food Sci Nutr* **2003**, *43*, 219–231, doi:10.1080/10408690390826491.
32. Bai, Y.; Hunt, C.D. Dietary Boron Enhances Efficacy of Cholecalciferol in Broiler Chicks. *The Journal of Trace Elements in Experimental Medicine* **1996**, *9*, 117–132, doi:10.1002/(SICI)1520-670X(1996)9:3<117::AID-JTRA4>3.0.CO;2-P.

33. Nielsen, F.H. Dietary Fat Composition Modifies the Effect of Boron on Bone Characteristics and Plasma Lipids in Rats. *BioFactors* **2004**, *20*, 161–171, doi:10.1002/biof.5520200305.
34. Whitehead, C.C.; Fleming, R.H.; Julian, R.J.; Sørensen, P. Skeletal Problems Associated with Selection for Increased Production. *Poultry Genetics, Breeding, and Biotechnology* **2003**, *3*, 29–52.
35. Bozkurt, M.; Küçükyilmaz, K.; Çath, A.U.; Çinar, M.; Çabuk, M.; Bintaş, E. Effects of Boron Supplementation to Diets Deficient in Calcium and Phosphorus on Performance with Some Serum, Bone and Fecal Characteristics of Broiler Chickens. *Asian-Australas J Anim Sci* **2012**, *25*, 248, doi:10.5713/AJAS.2011.11211.
36. Magnusson, P.; Arlestig, L.; Paus, E.; Di Mauro, S.; Testa, M.P.; Stigbrand, T.; Farley, J.R.; Nustad, K.; Millan, J.L. Monoclonal Antibodies against Tissue-Nonspecific Alkaline Phosphatase. *Tumor Biology* **2002**, *23*, 228–248, doi:10.1159/000067254.
37. Chavassieux, P.; Portero-Muzy, N.; Roux, J.-P.; Garnero, P.; Chapurlat, R. Are Biochemical Markers of Bone Turnover Representative of Bone Histomorphometry in 370 Postmenopausal Women? *J Clin Endocrinol Metab* **2015**, *100*, 4662–4668, doi:10.1210/jc.2015-2957.
38. Shipov, A.; Sharir, A.; Zelzer, E.; Milgram, J.; Monsonego-Ornan, E.; Shahar, R. The Influence of Severe Prolonged Exercise Restriction on the Mechanical and Structural Properties of Bone in an Avian Model. *The Veterinary Journal* **2010**, *183*, 153–160, doi:10.1016/j.tvjl.2008.11.015.
39. Kolakshyapati, M.; Flavel, R.J.; Sibanda, T.Z.; Schneider, D.; Welch, M.C.; Ruhnke, I. Various Bone Parameters Are Positively Correlated with Hen Body Weight While Range Access Has No Beneficial Effect on Tibia Health of Free-Range Layers. *Poult Sci* **2019**, *98*, 6241–6250, doi:10.3382/ps/pez487.
40. Manet, M.W.E.; Kliphuis, S.; Nordquist, R.E.; Goerlich, V.C.; Tuyttens, F.A.M.; Rodenburg, T.B. Brown and White Layer Pullet Hybrids Show Different Fear Responses towards Humans, but What Role Does Light during Incubation Play in That? *Appl Anim Behav Sci* **2023**, *267*, 106056, doi:10.1016/j.applanim.2023.106056.
41. De Haas, E.N.; Bolhuis, J.E.; de Jong, I.C.; Kemp, B.; Janczak, A.M.; Rodenburg, T.B. Predicting Feather Damage in Laying Hens during the Laying Period. Is It the Past or Is It the Present? *Appl Anim Behav Sci* **2014**, *160*, 75–85, doi:10.1016/j.applanim.2014.08.009.
42. Moinard, C.; Statham, P.; Green, P.R. Control of Landing Flight by Laying Hens: Implications for the Design of Extensive Housing Systems. *Br Poult Sci* **2004**, *45*, 578–584, doi:10.1080/00071660400006321.
43. Harlander-Matauschek, A.; Rodenburg, T.B.; Sandilands, V.; Tobalske, B.W.; Toscano, M.J. Causes of Keel Bone Damage and Their Solutions in Laying Hens. *Worlds Poult Sci J* **2015**, *71*, 461–472, doi:10.1017/S0043933915002135.
44. Jackson, B.E.; Tobalske, B.W.; Dial, K.P. The Broad Range of Contractile Behaviour of the Avian Pectoralis: Functional and Evolutionary Implications. *Journal of Experimental Biology* **2011**, *214*, 2354–2361, doi:10.1242/jeb.052829.
45. Pufall, A.; Harlander-Matauschek, A.; Hunniford, M.; Widowski, T.M. Effects of Rearing Aviary Style and Genetic Strain on the Locomotion and Musculoskeletal Characteristics of Layer Pullets. *Animals* **2021**, *11*, 634, doi:10.3390/ani11030634.

46. Kim, W.K.; Donalson, L.M.; Herrera, P.; Woodward, C.L.; Kubena, L.F.; Nisbet, D.J.; Ricke, S.C. Research Note: Effects of Different Bone Preparation Methods (Fresh, Dry, and Fat-Free Dry) on Bone Parameters and the Correlations Between Bone Breaking Strength and the Other Bone Parameters. *Poult Sci* **2004**, *83*, 1663–1666, doi:10.1093/ps/83.10.1663.
47. Silva, M.J. *Bone Mechanical Testing by Three-Point Bending*; 2016;
48. McCoy, H.; Kenney, M.A.; Montgomery, C.; Irwin, A.; Williams, L.; Orrell, R. Relation of Boron to the Composition and Mechanical Properties of Bone. *Environ Health Perspect* **1994**, *102*, 49–53, doi:10.1289/ehp.94102s749.
49. Wilson, J.H.; Ruszler, P.L. Effects of Dietary Boron on Poultry Bone Strength. *Transactions of the ASAE* **1995**, *38*, 167–170.

Chapter 6

OVERALL CONCLUSIONS

This chapter provides a summary of the findings from earlier chapters presented in this dissertation. Additionally, it outlines potential future research directions aimed at expanding upon the conclusions drawn from the previous chapters. In Chapter 2 we discovered apparent enhancements in pullets at both 11 and 17 weeks of age that suggested that weight-bearing exercise resulting from interaction with perches exerted a beneficial impact on musculoskeletal properties. Providing pullets with multi-tier perches during development promoted exercise, improved musculoskeletal health, and stimulated vertical activity, resulting in pullets that were better prepared for the lay phase and potentially reducing the risk of bone fractures in the future.

We aimed to enhance our understanding of the long-term impacts of perching interventions on laying hen welfare and bone health in Chapter 3. We found that the continuous provision of multi-tier perches throughout the rearing and lay phase resulted in a beneficial impact on activity level and thus the musculoskeletal health of laying hens at 40 weeks of age. This contributed to an improvement in overall hen welfare compared to not providing access to perches at any point during hens' lifetime. Providing perches during only the laying phase had positive effects on activity, muscle deposition, and bone strength, but these benefits were not as pronounced as those observed with continuous perch access throughout their lifetime. We also discovered that providing perches only during the rearing phase did not have a long-term positive impact on musculoskeletal health or activity levels of adult laying hens at 40 weeks of age. We concluded that early exposure to perches during the developmental stages does not confer long-term benefits in these aspects of welfare and health.

Because hens are highly motivated to perch, we aimed to investigate the effects of perch provision timing on hen affective state in Chapter 4. From this study, we were able to conclude that providing laying hens with multi-tier perches throughout their lifetime improves emotion and affective state by reducing fearfulness and anxiety, whereas no access to perches negatively impacted emotion and affective state. Adding perches to the environment during the laying phase resulted in greater anxiety at 21 weeks of age, but this dissipated by 37 weeks of age, indicating that adaptation to a new adult environment requires at least 16 weeks. Furthermore, late access to perches resulted in similar fear levels as hens with access to perches for their entire life, suggesting that current perch access reduces fearfulness. We also discovered that perch access, even when removed before the lay phase, is more beneficial to anxiousness at 37 weeks of age than not having perches at all. We were able to conclude that continuous access to perches or access to perches at the time of assessment (for late access) resulted in the best outcomes for fear and anxiety in laying hens.

Within Chapter 5, we elucidated the effects of a nutritional enrichment on musculoskeletal health and performance of pullets. Boron supplementation at 150mg/kg improved musculoskeletal characteristics of pullets up to 17 weeks of age, without impacting performance parameters. Enriching the diet with boron at 100mg/kg showed some beneficial effects, although this effect was not as pronounced as what was observed in pullets fed 150mg/kg boron.

Ultimately, we conclude that providing continuous perch access to laying hens from 0 to 40 weeks of age has the greatest beneficial impact on musculoskeletal health and activity compared to not providing perches at all, followed by giving access to perches during just the lay phase (from 17-40 weeks of age), with some beneficial effects observed when providing perches during the rearing phase. Additionally, supplementing the diet with 150mg/kg boron has positive impacts on

the musculoskeletal health of pullets at 11 and 17 weeks of age, without compromising performance parameters.

FUTURE RESEARCH

After investigating the impact of perch provision, timing of perch access, and nutritional supplement on the behavior, bone health, and welfare of laying hens, some conclusions remain unclear and could be explained with further research. We discovered that early access to perches (i.e., during the rearing phase from 0-17 weeks of age) slowed bone demineralization at 36 weeks of age. Then, levels of bone demineralization became similar to what was observed in hens with access to perches only during the laying phase (i.e., 17-40 weeks of age) at 40 weeks of age. Further research would be advantageous to understanding the effects of exercise during the rearing phase on bone demineralization in adult laying hens. This would provide a more comprehensive understanding of the nuanced relationships between developmental experiences and musculoskeletal health of adults laying hens. Furthermore, determining strain-specific outcomes of providing opportunities for exercise on musculoskeletal health is imperative. Various strains are known to have different physiological and psychological responses to the same environmental conditions, so determining how specific laying hen strains respond to management interventions meant to improve bone health would help optimize and improve welfare. In Chapter 4, we concluded that at least a 16 week period is required for hens to adjust to a new environment. Evaluating the timeline for hens to adapt to a new adult environment would assist in improving animal welfare by reducing stress. Finally, future investigations should focus on assessing the enduring effects of administering dietary boron to pullets on musculoskeletal health, egg

production, and egg quality of laying hens. Additionally, research should delve into the precise mechanism through which boron influences bone metabolism and health.

APPENDEX A

Table 6.1. Performance of laying hens housed in continuous perch (CP; perch access from 0-40 weeks of age), early perch (EP; perch access from 0-17 weeks of age), late perch (LP; perch access from 17-40 weeks of age), and no perch (NP; no perch access) groups during the experiment described in Chapter 3. FCR = feed conversion ratio; ADFI = average daily feed intake; HDEP = hen-day egg production across weeks (week 24, 30, 36, 40). There were no significant differences for hen performance between treatments ($p < 0.05$).

		Week 24	**Week 30**	**Week 36**	**Week 40**
CP	ADFI (g/hen)	108.9 ± 10.3	107.6 ± 11.3	108.6 ± 10.6	106.6 ± 11.8
	FCR (g feed/g egg)	2.1 ± 0.65	2.1 ± 0.96	2.1 ± 1.03	2.1 ± 0.32
	HDEP	94.8 ± 5.23	95.9 ± 2.84	96.9 ± 3.73	98.04 ± 2.09
EP	ADFI (g/hen)	116.2 ± 11.6	114.3 ± 14.2	112.1 ± 11.5	109.9 ± 10.5
	FCR (g feed/g egg)	2.0 ± 0.63	2.0 ± 0.85	2.1 ± 1.01	2.1 ± 0.36
	HDEP	94.9 ± 3.28	95.7 ± 3.41	96.4 ± 3.48	98.0 ± 1.79
LP	ADFI (g/hen)	105.9 ± 11.5	102.6 ± 10.8	104.3 ± 11.5	103.8 ± 10.6
	FCR (g feed/g egg)	1.94 ± 0.23	1.99 ± 0.42	2.03 ± 0.45	1.89 ± 0.52
	HDEP	95.1 ± 4.30	96.3 ± 4.36	96.2 ± 4.63	98.6 ± 1.31
NP	ADFI (g/hen)	106.2 ± 11.8	103.3 ± 10.8	104.8 ± 11.8	104.39 ± 11.7
	FCR (g feed/g egg)	1.9 ± 0.42	2.0 ± 0.56	2.0 ± 0.45	1.9 ± 0.85
	HDEP	95.7 ± 3.63	96.6 ± 3.56	96.4 ± 3.52	98.7 ± 1.90