The Effect of Pullet Rearing Environments on the Spatial Abilities of Laying Hens

By

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ABSTRACT

Laying hens are increasingly housed in systems that provide opportunities to perform highly motivated behaviors, such as perching. However, if laying hens do not traverse elevated structures successfully, they are at risk for falling from or colliding with perches and other features in their environment. Collisions and falls have been associated with keel bone fractures – a painful injury that results in reduced mobility and lower egg production. Promoting laying hens' spatial abilities, or how they orient in a three-dimensional environment, may improve movement amongst housing structures and offer a strategy to mitigate keel bone fractures. In Chapter 1, I reviewed the key components of spatial abilities and how they are susceptible to development during early life. This timeframe coincides with the portion of birds' lives when they are housed in pullet rearing environments, prior to the laying environment. However, the housing systems, timeframes, and metrics evaluated thus far leave limitations in our understanding of how rearing promotes spatial abilities and the consequences for keel bone fractures. Therefore, the objectives for my dissertation were 1) to determine if pullet rearing has a long-term effect on laying hen behavior, 2) to study a specific component of the rearing environment (height) and its influence on behavior and keel bone fractures, and 3) to assess a possible mechanism underlying behavioral differences via neural correlates of spatial cognition. Aviary-reared hens experienced fewer acceleration events and collisions at the keel through peak lay (35 weeks; wk) and displayed preferences for the highest perch through mid-lay (50 wk) compared to cage-reared hens in a laying environment of enriched colony cages (Chapter 2). These findings suggest that hens reared with less environmental complexity need more time to acclimate to layer housing containing structures and display different preferences for height. It was not clear which component of the rearing environment was responsible for these differences.

Cage-free rearing environments that primarily differed in available height (floor pen, single-tiered aviary, or two-tiered aviary) also resulted in hens displaying an acclimation period and different height preferences in a multi-tiered layer aviary (Chapter 4). Hens reared with minimal height (floor pen) were initially less active in using an aviary to find a nighttime roosting position, fewer hens used the higher zones of the aviary, and they also laid more eggs on the floor than hens reared with height (single-tiered and two-tiered pullet aviaries; Chapter 4). However, the acclimation period was relatively short as behavioral differences disappeared by the end of the first wk of housing and differences in floor egg prevalence disappeared by the second wk of lay (Chapter 4). Furthermore, cage-free rearing environments did not influence the rate of uncontrolled transitions or the prevalence and severity of keel bone fractures in aviaries (Chapter 4). Keel bone fracture prevalence was high, with ≥ 94% of hens experiencing fractures at 30 wk. The majority of fractures were minor in severity. I also aimed to determine if the acclimation period was a result of neurological differences related spatial cognition, as in whether birds reared with minimal height developed different neural capacities for processing spatial information. Neither dendritic morphology nor the gene expression of brain-derived neurotrophic factor in the hippocampus (two proxies for information processing in a brain region associated with spatial abilities) differed amongst cage-free rearing environments at 16 wk (Chapter 3). Taken together, these findings support previous hypotheses that rearing environments affect how hens acclimate to laying environments. The duration of the acclimation period varies by the behavioral, cognitive, and physical abilities promoted during rearing. Providing height during rearing is beneficial when hens are required to traverse height during lay. However, hens reared with minimal height are not neurologically impaired, in terms of the metrics we evaluated, and they acclimate within a few d or wk. The animal welfare implications

of this acclimation period need to be assessed on a commercial scale (e.g. accessing feed and water on elevated tiers). Although uncontrolled transitions and keel bone fractures were not affected in the present study, this may be related to the experimental setting of our research. Again, future work is needed in commercial environments where larger social groups and less space for take-offs and landings may exacerbate uncontrolled movements and injuries.

CHAPTER 1:

General Introduction

Spatial abilities of laying hens

Spatial ability is defined as an animal's ability to orient in the environment, particularly three-dimensional space that allows for horizontal and vertical movement (Wichman et al., 2007). Spatial ability is influenced by cognition, behavioral experience with three-dimensional space, and musculoskeletal coordination and strength. For chickens (*Gallus gallus domesticus*), these three aspects of spatial abilities are most sensitive to development early in life. I will review how each of these three aspects develop, specifically with regard to their influence on chickens' abilities to use and navigate vertical space. For the purposes of this review, the terms chick and pullet are used interchangeably to refer to sexually immature chickens.

Cognitive development. Input affecting cognitive components of spatial abilities begins during embryonic development. The embryo is situated such that the right eye of the chick faces outward towards the shell, while the left eye faces inward towards the body (reviewed by Daisley et al., 2009). The right eye thus receives light stimulation while the left eye remains in darkness, triggering forebrain development in the left hemisphere and resulting in brain lateralization prior to hatch (reviewed by Daisley et al., 2009). The resulting left hemisphere bias focuses chicks' visuo-spatial analysis on local features, allowing them to approach objects and discriminate food items while foraging (Vallortigara and Rogers, 2005). The chick's ability to perform this specialized visuo-spatial analysis is further complemented with a fully developed visual system by 2 days of age (Over and Moore, 1981; Schmid and Wildsoet, 1998). Chicks' behavior within

the first three days post-hatch reflect the effects of brain lateralization and visual abilities. Chicks imprint on and follow moving objects (e.g. maternal hen, other fowl, humans), locate their mother with auditory cues, navigate physical obstacles to regain contact with their mother, peck at moving objects similar to moving insects, and peck more at round objects as opposed to angular objects, suggesting a discrimination of objects that appear similar to the round shape of seeds (Spalding, 1954; Fantz, 1957). Additionally, chicks appear to have a possible genetic component of depth perception, such that depth perception was not affected by light or dark brooding (Kurke, 1955) or by altering the location of attachment shadows (Hershberger, 1970). Similarly, Tallarico and Farrell (1964) reared chicks on a visual cliff over a perceived shallow height or no height, and within 35 hours of hatch these chicks demonstrated a preference to stay away from a perceived deep height that they had no prior experience with. However, depth perception is also affected by early-life experience with kinesthetic cues (i.e. physical learning and movement that contribute to an animal's awareness of its body), suggesting an overlap between genetics and behavioral experience (Kurke, 1955).

At 11 days of age, chicks in natural settings begin to move out of sight of their mother, which coincides with a shift in brain lateralization towards right hemispheric dominance (Vallortigara et al., 1997; Freire and Cheng, 2004). Right hemisphere bias focuses chicks' visuo-spatial analysis on relational properties of the spatial layout, which contributes to fear and escape responses and allows chicks to broadly recognize global cues (Vallortigara and Rogers, 2005). The right hippocampus specifically is involved in processing and storage of spatial information (Freire and Cheng, 2004). Chickens with lesions in the right hippocampus are unable to find food using geometric information of an enclosure (e.g. a global cue) compared to chickens with

lesions in the left hippocampus or sham lesions, who's food search success was unaffected (Tommasi et al., 2003). The degree of development of the right hippocampus is affected by available spatial information in the environment. Freire and Cheng (2004) found that chicks who were unable to visually see their imprinting stimulus from 8 to 16 days of age had greater development of the right hippocampus than chicks that remained in visual contact with their imprinting stimulus. Similarly, Freire et al. (2004) found that chicks reared with visual barriers had better performance on spatial memory tests (i.e. visual displacement and detour tests) than chicks reared without visual barriers when tested at 11 days of age.

These findings suggest that the development of spatial cognition is most sensitive during the first 3 wk of life. There appears to be a genetic component to some elements of spatial cognition, such as visual acuity, discriminating local spatial cues, and some depth perception. However, after a shift in brain lateralization, other components of spatial cognition seem to be influenced by experience, such as interpreting global cues, spatial memory, and some depth perception. The extent to which experience alters spatial abilities can be further explained by reviewing bird's behavioral experience with three-dimensional space.

Behavioral experience with 3-D space. Interestingly, when chicks are provided opportunities to access vertical space, their behavioral development timeline with three-dimensional space coincides with the cognitive development timeline. For the purposes of this review, vertical space constitutes elevated perches, which birds can grip with their feet, or elevated platforms, where birds could perform horizontal locomotor activity in an elevated space.

Both perches and platforms require birds to engage in vertical locomotor activity first in order to access the space.

Some individual chicks were reported to utilize perches 10 to 40 cm from the floor as early as 8 days of age (Gunnarsson et al., 2000; Heikkilä et al., 2006; Wichman et al., 2007). However, multiple studies have reported that the majority of chicks begin to perch around 2 wk of age, similar to the timeline of brain lateralization (Heikkilä et al., 2006; Wichman et al., 2007; Kozak et al., 2016; Habinski et al., 2017). Chicks' vertical space use consistently increases during daytime observations throughout the first 6 to 9 wk of life, as high as 69 cm from the floor (Kozak et al., 2016; Habinski et al., 2017). There are mixed findings in the literature about the height that young birds are willing to go, with minimal vertical space use reported for 9-wk-old birds on platforms and perches higher than 70 cm from the floor (Kozak et al., 2016) while three-wk-old birds utilized 120 cm perch heights (Gunnarsson et al., 2000). Therefore, it seems that pullets will increasingly use vertical space if available, but the design of accessing the space is worth consideration.

Accessing the vertical space is also influenced by circadian rhythms and light. Pullets initially utilize perches more in the daytime hours than in the nighttime hours (Heikkilä et al. (2006): 21.3% vs 5.2% of 6-wk-old pullets in daytime and nighttime, respectively; Habinski et al. (2017): 46.7% vs 28.6% of 11-wk-old pullets in daytime and nighttime, respectively). Similarly, the first reported bout of perching for each individual pullet was observed during daytime hours as opposed to nighttime hours (Heikkilä et al., 2006). Furthermore, pullets appear to have a genetic ability to use light cues to orient within a maze, which suggests a critical role of light in

their perception of space (Zimmerman et al., 2003). These findings may indicate that pullets initially explore and learn about their spatial environment during the day (d), when it can be best perceived. Interestingly, as pullets grow older, activity and exploration declines (Heikkilä et al., 2006; Kozak et al., 2016) and nighttime vertical space use consistently increases (Habinski et al., 2017). This change in circadian rhythm of vertical space use represents the behavioral patterns observed in adult hens.

Adult hens display a circadian rhythm of nesting and egg laying in the morning, dust bathing and foraging in the afternoon, and roosting on perches at night (reviewed by Campbell et al., 2016). Nighttime roosting is hypothesized to derive from anti-predator behavior in the ancestral red junglefowl (*Gallus gallus*), such that vigilance on the highest perch would allow for maximal predator detection and reduce the likelihood of being caught by a predator (Newberry et al., 2001). This preference persists in domestic hens, despite the lack of predators in their environments. Schrader and Müller (2009) found that hens prefer perches over a plastic grid for roosting, but when offered a low perch versus a high grid for roosting, they will choose the high grid. These findings demonstrate that hens prefer height over material for nighttime roosting. Similarly, Brendler and Schrader (2016) observed perch use to be highest at nighttime compared to daytime in multi-tiered aviary designs. Specifically, birds were primarily utilizing perches within the highest tier of the system or the highest perch available in the low tier (Brendler and Schrader, 2016). Alternatively, during the d, hens use lower and non-elevated perches to enter and exit a multi-tiered aviary system to access various resources throughout the d, such as feed, nests, and scratch area (Campbell et al., 2016b).

Most studies reporting behavioral experience with three-dimensional space report averages at the flock level, but there is evidence that individual differences occur in vertical space use as well. For example, Heikkilä et al. (2006) found that individual chicks that spent more time underneath perches early in life started perching sooner than individual chicks that spent more time under the heating lamp (and consequently less time underneath perches) early in life. The authors proposed that these early-perching chicks may be more explorative and therefore find the perches earlier compared to chicks spending less time under the perches during the first wk. Interestingly, the early-perching chicks also had fewer social interactions with other chicks than later-perching chicks. These findings could be a result of early-perching chicks being on perches and therefore not on the floor to interact with other chicks, but also possibly that low-ranked chicks are the early-perching chicks in order to avoid dominant chicks on the floor (Heikkilä et al., 2006). Wichman et al. (2007) reported that the average percentage of time perching during the first 6 wk of life was 8.9 ± 0.5%, but the range across individuals was 0 to 25%, indicating a broad variability of individual perch use. This individual variation in space use may introduce follow up hypotheses about the role of personality in space use and how individuals influence the spatial distribution and clustering of birds.

Behavioral experience with three-dimensional space is dependent on the availability of vertical space, light, and circadian rhythms. Nonetheless, the majority of chickens will begin using vertical space by 2 wk of age, suggesting the importance of early-life experience. Space use does require activity and exercise, which facilitates musculoskeletal development – the last aspect to be considered for spatial abilities.

Musculoskeletal development and exercise. Bone development in chickens occurs in two phases – structural bone formation and medullary bone formation. For the purposes of this review, structural bone formation is primarily of interest as it occurs early in life, prior to the onset of increased estrogen that occurs when birds reach sexual maturity (reviewed by Casey-Trott et al., 2017b). During structural bone formation, bones grow longer and wider through the process of periosteal apposition, where osteoblasts add mineralized cortical tissue (i.e. compact structural bone) to the outside of the periosteal surface to increase cross-sectional area of the bone and make room for more development of trabecular tissue (i.e. spongy structural bone; reviewed by Casey-Trott et al., 2017b; Neijat et al., 2019). Structural bone formation is relevant to spatial abilities specifically because it is influenced by load-bearing exercise early in life. Load-bearing exercise is activity that works against gravity, such as accessing vertical space. Opportunities for load-bearing exercise are more common in a multi-tiered aviary, which provides opportunities for vertical and horizontal locomotor movement, as compared to a conventional cage. A recent study found that aviary-reared pullets had heavier breast and wing muscles compared to cage-reared pullets, but cage-reared pullets did have heavier leg muscles (Casey-Trott et al., 2017b). These findings demonstrate the differences in muscle use, and consequently muscle development, needed for horizontal movement (primarily leg) compared to vertical movement (primarily breast and wing to facilitate flight). The same study found that aviary-reared pullets had longer keel bones and greater bone density, mineral content, and cross-sectional area in the radius, humerus, and tibia compared to cage-reared pullets (Casey-Trott et al., 2017b). Another study also found that aviary-reared pullets had greater bone density, mineral content, and cross-sectional area in the tibia and humerus compared to cage-reared pullets (Regmi et al., 2015). These findings indicate that structural bone development is greater in

pullets when provided opportunities to exercise, which is further supported by aviary-reared pullets having stronger radius, humerus, and tibia bones compared to cage-reared pullets (indicated by higher bone breaking strength values; Casey-Trott et al., 2017b). When bone measurements were taken at an older age (73 wk), the effect of exercise early in life had a lasting effect with aviary-reared hens still having greater mineral content and cross-sectional areas of the radius, tibia, and humerus compared to cage-reared hens, regardless of the adult housing system they were placed into (conventional cage or enriched cage containing perches; Casey-Trott et al., 2017a). However, the enriched cage adult housing system did provide more opportunities for exercise than the conventional cage housing due to the provision of perches and more space per individual bird. As a result, enriched cage adult housing promoted or preserved the bone characteristics established by the rearing environment (Casey-Trott et al., 2017a).

Commercial laying hen housing: overview

Understanding the development of spatial abilities in chickens is crucial for considering how to house and manage them in a commercial setting. Over the last decade, American consumers have increasingly expressed concern for the well-being of commercially farmed chickens utilized for egg production (henceforth referred to as laying hens). This concern predominantly centers around the hens' housing system, specifically the amount of space and animal welfare afforded by the housing (Heng et al., 2013). Laying hens are commonly housed in one of two types of commercial housing systems: cages (e.g. conventional cages or enriched colony cages) or cage-free environments (e.g. multi-tiered aviaries). Currently, the majority of laying hens (i.e. 70.7%; United Egg Producers, 2021) are housed in cages. Conventional cages, the main type of cage housing in the United States, house hens in groups of 6 to 7 hens/cage (e.g. 432 to 555 cm^2/hen)

with access to feed and water (United Egg Producers, 2022a). However, key welfare concerns for this housing system are restricted movement (hens can turn around and sit down, but cannot fully extend their wings; Mench et al., 2011) and the inability for hens to perform highly motivated natural behaviors (e.g. nesting, foraging, dustbathing, and perching; Weeks and Nicol, 2006). Enriched colony cages aim to provide more space to each hen in larger groups (e.g. 748 cm^2/hen in a group of up to 60 birds), and the cage includes enrichments such as perches, nest areas with curtains, and scratch pads to promote natural behavioral expression (Coalition for Sustainable Egg Supply, 2016). However, this housing system was not widely implemented in the United States as animal protection groups, certification programs, and retail pledges strived to reduce all types of cage housing systems (Mench et al., 2011). Cage-free multi-tiered aviaries are non-cage, indoor houses where hens have more space per hen (e.g. 929 cm^2/hen) in groups of thousands, and they roam freely through horizontal and vertical space with perches, nest boxes, and scratch areas containing substrate for dustbathing and foraging (United Egg Producers, 2022b). Of the common housing systems, cage-free multi-tiered aviaries provide the greatest amount of space per individual hen and thus utilizing spatial abilities. As a result, consumer concern for animal welfare has led to recent state legislation and pledges from numerous food processors, restaurant chains, and supermarkets (e.g. McDonalds, Wal-Mart, Costco, etc.) to purchase only cage-free eggs. Currently, nearly 29.3% of hens are housed cage-free, but the shift in demand for cage-free eggs will require 66% of all hens to be housed in these systems by 2026 (United Egg Producers, 2021).

Animal welfare in cage-free aviary housing: keel bone damage. Despite opportunities to perform a wide range of species-typical behaviors in cage-free housing (e.g. nesting, foraging,

dustbathing, and perching; Weeks and Nicol, 2006), there are currently many disadvantages for welfare in these systems. To evaluate welfare, it is critical to understand the physical and mental state of an animal when coping with the conditions in which it lives and dies by evaluating its biological health and functioning, expression of (or lack thereof) normal behavior, and affective state (Fraser et al., 1997; OIE, 2005). Indeed, previous research has shown that cage-free systems have a greater incidence of injurious feather pecking, cannibalism, endo- and ectoparasites, piling and crowding behavior, bone fractures, and consequently overall greater rates of mortality than hens housed in conventional cages (reviewed by Lay et al., 2011).

The keel bone is the bird's sternum and is crucial for flight and general mobility as it serves as an attachment site for wing muscles (Casey-Trott et al., 2015). Damage to the keel bone manifests as deviations (bends) and/or fractures, and fractures specifically are associated with pain, reduced mobility, and reduced egg production efficiency and quality (Nasr et al., 2012, 2013). Keel bone damage is commonly considered most prevalent in cage-free multi-tiered aviaries (henceforth referred to as aviaries), with up to 73% of hens affected (Freire et al., 2003), as compared to enriched colony cages (31-33%; Vits et al., 2005; Sherwin et al., 2010) and conventional cages (16%; Clark et al., 2008). However, these estimates are nuanced considering that the criteria for the damage scoring system, reliability of the scoring system, genetic strain, and age of the hens can impede comparability of keel bone damage research (Rufener and Makagon, 2020). A recent review of keel bone damage work found that keel bone fractures specifically seem to be most prevalent in single-tiered aviaries (63%), followed by enriched colony cages (50.3%), floor housing (50.2%), and multi-tiered aviaries (38.2%), and conventional cages with the lowest fracture prevalence (23.5%; Rufener and Makagon, 2020).

Keel bone fractures are reported as early as 20 and 30 wk of age (Casey-Trott et al., 2017c; Stratmann et al., 2019). One of the key risk factors for this injury is pressure and trauma from structural enrichment, including collisions with perches and conspecifics or failed movements between levels of the aviary (Stratmann et al., 2015a). Providing ramps that facilitate vertical movement within aviaries has been associated with fewer collisions and falls, more controlled movements, and fewer palpated keel bone fractures (Stratmann et al., 2015a). Light may also facilitate vertical movement, with recent evidence showing falls and collisions being the most frequent during the dusk phase prior to lights off when compared to mid-day and mid-night (Stratmann et al., 2019). Additionally, the odds of vertical movement to access perches or nests decreases for hens with a severe keel fracture (visible gap in bone) compared to hens with healed fractures or no fractures (Rentsch et al., 2019). Interestingly, the same study showed that walking pace on ramps was not affected by keel fracture severity (Rentsch et al., 2019). This evidence suggests a link between keel bone fractures and spatial abilities, particularly the navigation and use of vertical space. However, the aforementioned aviary housing only comprises the adult phase of a hen's lifespan, generally 18-90 wk of age. Considering the critical development period during early life for spatial abilities, the type of rearing environment and whether it provides access to structural enrichment and exercise is worth consideration. Modifying the rearing environment may be more influential to aspects affecting spatial abilities and consequently keel bone fractures than an approach that only modifies adult housing.

Pullet rearing environment. The rearing environment constitutes the first 16 to 18 wk of life, when pullets are most sensitive to cognitive development, learning behaviors in three-dimensional space, and musculoskeletal development. There are limited data on the type of

housing used in American pullet rearing, but a recent report from the U.S. Department of Agriculture indicates that 91.3% of pullets are cage-reared while only 8.7% of pullets are floor-reared (USDA, 2014). The definition of floor-rearing and cage-rearing is not provided in the survey, but it is likely that the majority of pullets in cage-rearing do not experience vertical space and unclear as to whether or not the floor-rearing pullets do. A more detailed and recent survey of Canadian pullet rearing indicates that the majority of pullets are reared in conventional cages (42.4%) followed by single-tiered aviaries (33.3%) and multi-tiered aviaries (24.2%; Van Staaveren et al., 2019). It is generally understood that the pullet rearing environment should match the adult laying environment in order to facilitate the transition between housing facilities (Janczak and Riber, 2015), but no national guidelines, retail pledges, or legislation address pullet rearing in the United States.

A multitude of welfare components can be traced from the lay environment back to the rearing environment. For example, the use of vertical space, injurious feather pecking, floor eggs (i.e. eggs laid outside of the nest box), fearfulness, dustbathing, and bone strength are affected by the type of rearing environment (i.e. cage versus non-cage) and structural components within the environment (i.e. perches, vertical tiers, litter substrate; reviewed by Janczak and Riber, 2015). Recent research has demonstrated the relationship between rearing environment and keel bone fractures as well, where hens reared in conventional cages have greater keel bone fractures later in life than hens reared in aviaries when they are placed into enriched colony cages (Casey-Trott et al., 2017c). Pullets reared in aviaries have better working memory (Tahamtani et al., 2015), are less fearful and initially utilize three-dimensional space more (Brantsæter et al., 2016), and

have increased musculoskeletal development (reviewed in the "musculoskeletal development and exercise" section of this chapter) compared to pullets reared in conventional cages.

Based on the available literature, the opportunity to use and navigate vertical space during pullet rearing influences spatial abilities. Aviary-reared pullets have greater musculoskeletal development, greater spatial cognition (in terms of working memory), and possibly use vertical space more (limited evidence) than cage-reared pullets. These findings correspond with the developmental trajectory of spatial abilities, suggesting that modifying early life experiences will have impacts on the spatial abilities of pullets and hens. Additionally, aviary-reared pullets have less keel bone fractures than cage-reared pullets when placed into enriched colony cage layer housing. Considering the link between keel bone fractures and collisions and falls, these results suggest that aviary-reared hens may have improved spatial abilities compared to cage-reared hens. However, there are still notable gaps in the literature for understanding the relationship between rearing environment, spatial abilities in the laying environment, and consequently animal welfare outcomes.

Gaps in the literature

Spatial abilities have been recognized as a potential aspect linking keel bone fractures with rearing environments, but minimal studies have investigated this claim (Rufener and Makagon, 2020; Norman et al., 2021). The relationship between rearing environment and keel bone fractures has only been assessed in an adult enriched colony cage housing system, but the authors did not concurrently evaluate behavioral or cognitive metrics of spatial abilities. Therefore, the link between rearing and keel bone fractures is unclear, and these metrics need to be taken

simultaneously to understand their relationship. There is limited to no research connecting rearing environment to collisions and falls later in life, vertical space use past 23 wk of age (when keel bone fractures are known to increase), or neural correlates of spatial cognition that may provide insight into underlying mechanisms of behavioral differences. Multi-tiered aviary housing will become the predominant housing system in the United States, so evaluating the effect of rearing on keel bone fractures in this layer housing system is also needed. Additional pullet rearing environments need to be evaluated as previous research has predominantly evaluated a dichotomy of conventional cage versus aviary. These rearing environments differ in numerous ways, so studying specific components of rearing may provide insight into what features are most influential for pullet development. For example, changing structural enrichments within a rearing environment to alter the vertical space available (i.e. height) has not been evaluated.

Objective

The goal of my dissertation is to understand how rearing environments affect the development of laying hen spatial abilities. Specifically, I aim to understand the how pullet rearing environments influence the overall quantity and quality (i.e. skill) of vertical space use in the laying environment, the duration of that effect, neural metrics that may underly behavioral differences, and ultimately the implications for keel bone fractures. I will achieve this objective by evaluating: 1) the effect of rearing environment (cage vs aviary) on collisions and perch use in an enriched colony cage out to mid-production (50 wk of age); 2) the effect of providing height during rearing (floor vs singe-tiered aviary vs two-tiered aviary) on neural development in a region associated with spatial abilities; and 3) the effect of providing height during rearing (floor

vs singe-tiered aviary vs two-tiered aviary) on quantity and quality of vertical space use, eggs laid on the floor, and keel bone fractures in a multi-tiered layer aviary out to peak lay (27 to 30 wk of age). Ultimately my dissertation will provide insight into the design of rearing environments and their consequences for long-term behavior, neural correlates of spatial cognition, and keel bone fractures.

CHAPTER 2:

Pullet rearing affects collisions and perch use in enriched colony cage layer housing

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ABSTRACT

Hens reared in aviaries (AVI) as pullets have improved spatial abilities compared to hens reared in cages (CON). However, this effect on behavior has been shown only to 23 wk of age. Lohmann LSL-Lite hens were reared in either CON or AVI until 19 wk of age and then moved into enriched colony cages (ECC) containing two elevated perches of different heights (n=6 ECC/treatment). Focal hens (3 per ECC) were fitted with tri-axial accelerometers to record acceleration events at 21, 35, and 49 wk of age. Video recordings from each age were used to identify behaviors associated with acceleration events, as well as the proportion of hens utilizing perches. CON hens experienced more acceleration events ($p = 0.008$) and more collisions ($p = 0.04$) than AVI hens during the d at 21 and 35 wk of age. The total proportion of hens perching at night was similar between treatments across most time points, but fewer CON hens used the high perch compared to AVI hens throughout the study ($p = < 0.001$). Rearing in aviaries influences hen behavior out to peak lay for collisions and out to mid-lay for perch height preference in ECC.

INTRODUCTION

The pullet rearing environment, which constitutes the first 16 to 18 wk of life, can have long-term effects on laying hen welfare (Janczak and Riber, 2015). Studies of pullet housing have shown that the rearing environment affects cognition, behavior, as well as bone and muscle development. For example, compared to cage-reared hens, aviary-reared hens show improved working memory in the reversal phase of a holeboard task (Tahamtani et al., 2015). Birds reared with access to vertical space use perches and elevated platforms more at 19 wk of age than birds reared without elevated structures (Gunnarsson et al., 2000; Brantsæter et al., 2016). Aviary-reared pullets have stronger, more developed radius, humerus, and tibia bones, a larger keel bone, and heavier wing and breast muscles compared to cage-reared pullets at 16 wk of age (Casey-Trott et al., 2017b). Importantly, these effects on the skeletal system persist through end of the laying period. Aviary-reared hens have stronger bones with greater cross-sectional areas and higher mineral content compared to cage-reared hens at 73 wk of age (Casey-Trott et al., 2017c).

Rearing environments have also been shown to affect keel bone fracture development. Hens housed in enriched colony cages had a higher incidence of keel bone fractures at the end of the laying period if they were reared in cages versus aviaries (Casey-Trott et al., 2017a). The keel bone is crucial for flight and general mobility as it serves as an attachment site for wing muscles (Casey-Trott et al., 2015). Birds with fractured keels may experience pain (Nasr et al., 2012), reduced mobility (Nasr et al., 2012; Rentsch et al., 2019), and have reduced egg production (Nasr et al., 2013; Rufener et al., 2019b). Keel bone fractures are suspected to be partly attributed to

physical trauma to the bone (Stratmann et al., 2015a; Gebhardt-Henrich et al., 2017). Accordingly, previous research found the number of collisions that a hen experienced corresponded with the risk of keel fracture development (Makagon et al., 2017; Baker, 2018). Using tri-axial accelerometers, the potential causes of such trauma were examined (Baker et al., 2020). The keel-mounted sensors registered acceleration events experienced at the keel by hens as they navigated their enriched colony cage environments. Collisions with cage structures, and specifically the perch, caused most (~80%) of the acceleration events. However, the impact of the rearing environment on collisions was not explored.

The reported influence of rearing on keel bone fracture prevalence (Casey-Trott et al., 2017a) may reflect differences in the birds' abilities to navigate their adult environments. Therefore, the objective of this study was to investigate whether the early rearing environment (cage vs aviary housing) affects the behavior of hens after they are placed in their layer environment. Specifically, we aimed to determine whether pullet rearing affects the incidence of acceleration events recorded at the keel, and the behaviors associated with these events. We further explored actions and housing structures associated with observed collisions. Considering that the perch was the main structure associated with collisions in a previous study (Baker et al., 2020), we also investigated the effect of rearing on perching behavior focusing on perch use and the distribution of birds across the two available perches.

MATERIALS AND METHODS

Ethical Statement

All procedures were approved by the California Polytechnic State University Animal Care and Use Committee (Protocol #1613).

Animals

On farm data collection was conducted at the Cal Poly Poultry Center at California Polytechnic State University. A total of 301 pullets were used in this study. The pullets were selected from among 3,000 d-old Lohmann LSL-Lite chicks, with infra-red beak trim, that were obtained from Hy-Line North America, LLC (Burley, ID). All pullets were from the same parent flock.

Pullet Housing and Management

A total of 2,000 pullets were reared in a conventional cage system (CON; 98 x 69.5 x 41 cm, L x W x H; SALMET S1000 Rearing System, SALMET GmbH & Co. KG, Dietzenbach, Germany). The wire floored cages were stacked in three tiers of 36 cages/tier. All pullets were initially housed in the middle tier at 55 to 60 pullets/cage with a space allowance of 113.5 to 123.8 cm^2/pullet. Space per bird was increased during wk 6, at which time pullets were sorted among cages in the top and middle tiers. The pullets were redistributed among all three tiers at 12 wk of age, with 20 pullets/cage at 340.6 cm^2/pullet. Water was provided *ad libitum* via automatic water lines (Ziggity, Middlebury, IN) with 4 nipple drinkers/cage. An automated trough feeder provided hens with *ad libitum* access to a commercial diet.

A total of 1,000 pullets were reared in a multi-tiered aviary system (AVI; 16.5 x 4.7 x 2.5 m, L x W x H; SALMET Pedigrow 2 Rearing System, SALMET GmbH & Co. KG, Dietzenbach, Germany). The system contained three elevated tiers at 0.3 m, 1.2 m, and 1.6 m height from the floor. Pullets were enclosed into the middle tier for the first three wk (564.9 cm^2/pullet). After the first three wk, tier doors were opened and pullets could access the bottom and top tiers of the system, shelves on the outer edge of the middle and top tiers (147 x 18 cm/shelf, L x W), and a scratch area adjacent to the system containing pine wood shavings over a concrete floor (514,000 cm^2 scratch area). Considering all sections, shelves, and adjacent scratch area, the total available floor space was 2,267 cm^2/pullet for the remainder of the rearing period.

Each tier in AVI contained a wire mesh floor, manure belt, automatic water line, automatic feeder line, and square metal perch (2.5 cm diameter) above the feeder. The top tier contained an additional 11 square metal perches (27.7 cm/pullet of linear perch space). Wire mesh ramps were provided to facilitate access from the floor to the middle tier. Water was provided *ad libitum* with 132 nipple drinkers/tier (Ziggity, Middlebury, IN). AVI chicks were fed the same commercial diet as CON chicks.

For both rearing environments, temperature and artificial lighting were maintained according to the North American Edition of the LSL-Lite Layers Management Guide (Lohmann Tierzucht GmbH, Germany).

Layer Housing and Management

At 19 wk of age, all birds were moved into their adult housing system – a two-story barn containing 128 enriched colony cages (ECC; 64 cages/floor; 25 ± 2 hens/ECC). Birds were placed in ECC with other birds from the same rearing treatment, and treatments were distributed randomly across each story of the barn. A total of 12 ECC were chosen as focal cages (n=6 ECC/rearing treatment). Each ECC (360 x 63 x 56 cm, L x W x H; SALMET AGK 3600 Enrichable Colony System, SALMET GmbH & Co. KG, Dietzenbach, Germany) contained two elevated round metal perches (9 and 25 cm from cage floor) that were each 225 cm in length, as well as an enclosed nest area (51 x 30 x 52 cm, L x W x H) and scratch pad (35 x 13 cm, L x W) over a wire mesh floor. The hens had access to 907.2 cm^2/hen of floor space and 18 cm/hen of perch space. Water was provided *ad libitum* with 8 nipple drinkers/cage (Ziggity, Middlebury, IN). A commercial diet was provided *ad libitum* in automatic feeders which ran along each cage row. The artificial lighting program started at 12 h/d at initial placement and was subsequently stepped up by 1 h/wk to reach 16 h/d.

Behaviors Associated with Acceleration Events at the Keel

At 21, 35 and 49 wk, three randomly selected focal hens/ECC (36 total hens/age) were fitted with a custom jacket containing a tri-axial accelerometer, with a sensor positioned over the keel bone (Custom Idea Ltd, Shepton Mallet, UK; for more information on jackets and accelerometers, see Baker et al., 2020). A different set of focal hens was selected at each age. Jackets were fitted to hens during nighttime hours in order to minimize hen disturbance. Each white jacket contained a unique black symbol, which allowed us to individually identify the focal hens during recording. Jackets and accelerometers were worn continuously for 11 d at each age.

Hens habituated to the jackets during the first d, and data were recorded for the remaining 10 d. Accelerometers were programmed to record events at the keel with a combined magnitude of acceleration >12 G-Units (G) to avoid the inclusion of minor body movements (Baker et al., 2020). The combined magnitude of acceleration was calculated as the square root of the sum of squared acceleration in three dimensions (X, Y, and Z; Baker et al., 2020). At the conclusion of each age trial, a log containing date, time, and the maximum combined magnitude of acceleration for each event was generated for each hen. This log was used for video analysis.

ECC were video recorded continuously during each of the three 10 d trials using two video cameras per cage (Veilux 700 Line Day Night IR Bullet Camera, Veilux Inc., Grand Prairie, TX, USA) connected to a digital video recorder with surveillance software (GeoVision Digital Surveillance System version 8.5, GeoVision Inc., Taipei, Taiwan). After the end of each age trial, trained observers used the accelerometer log with the video recordings to identify the behavior associated with each acceleration event (Table 2.1; Baker et al., 2020). Observers were blind to rearing treatment, randomly assigned to video from both rearing treatments, and analyzed all videos using GeoVision Software (GeoVision Digital Surveillance System, ViewLogEx v.8.5.6.0). Inter-observer reliability was high (≥ 90% inter-observer agreement between trainer and each trainee of approximately 20 acceleration events).

Perching Behavior

Hen perching behavior was evaluated from video on the second and sixth d of each 10-d trial. Using instantaneous scan sampling with 5-min intervals, the number of hens using the low and high perch were observed. It was previously reported that 5-min interval sampling was

representative of perching behavior throughout a 15 h d (Daigle and Siegford, 2014). A total of 5 hours of video was reviewed on each d: three hours while the house lights were on (06:00 to 06:55, 12:00 to 12:55, 16:00 to 16:55; "Day") and 2 h while lights were off (23:00 to 23:55, 02:00 to 02:55; "Night"). Preliminary analysis (a review of 24 hours of video data for five ECC) found this sampling scheme to be representative of the entire d for perching behavior.

Data Analysis

For all data analyses, R statistical software (version 3.5.2; R Core Team, 2021) and linear mixed effect models (lme4 package; Bates et al., 2015) were used. Model assumptions were checked for normality of residuals by visually evaluating Q-Q plots, residual vs fitted values plots, and homoscedasticity of independent variables. As models did not meet one or more normality criteria, generalized linear mixed effect models with a Poisson distribution (lme4 package; Bates et al., 2015) were used to analyze the total number of acceleration events and total number of collisions sustained by each hen across the 10 d at each age. Model assumptions for generalized linear mixed effects models were checked using package DHARMa, version 0.2.2 (Hartig, 2022). Following a previous study (Baker et al., 2020), only acceleration events and collisions associated with accelerations >20 G were included in the statistical analyses. The effect of treatment (factor with 2 levels: CON, AVI), age (factor with 3 levels: 21 to 22, 35 to 36, and 49 to 50 wk), Day/Night (factor with 2 levels: Day, Night), and their interactions (treatment x age, treatment x Day/Night, age x Day/Night, treatment x age x Day/Night) were assessed, with hen ID nested in age nested in ECC as a random effect. The final models were obtained by a stepwise backwards reduction, using ANOVA for model comparison with a p-value of >0.05 as the criterion of exclusion.

Within each 5-min observation, we calculated the average proportion of hens from each ECC that utilized either of the two perches. The numerator was the total number of hens on the low perch and high perch, and the denominator was the total number of hens in the ECC. The data were analyzed using a zero-inflated beta regression model (glmmTMB package; Brooks et al., 2017). The effect of treatment (factor with 2 levels: CON, AVI), age (factor with 3 levels: 21 to 22, 35 to 36, and 49 to 50 wk), Day/Night (factor with 2 levels: Day, Night), and their interactions (treatment x age, treatment x Day/Night, age x Day/Night, treatment x age x Day/Night) were assessed, with d nested in age nested in cage as a random effect.

We also calculated the average proportion of perching hens from each ECC that utilized the high perch during nighttime hours. The numerator was the total number of hens on the high perch, and the denominator was the total number of hens on the low perch and high perch. The same zero-inflated beta regression model described previously was used, excluding the effect of light.

RESULTS

All Acceleration Events: Descriptive Summary

At 21 to 22 wk of age, we obtained accelerometer data for 31 hens (16 CON hens across 6 ECC; 15 AVI hens across 6 ECC). Data from 5 of the 36 focal hens were excluded as their accelerometers failed to record or store data properly. A total of 470 acceleration events were recorded during this 10-d data collection period. Of the 470 recorded acceleration events, 393 (83.6%) were successfully matched to video. The remaining 16.4% fell into the Cannot ID

category (Table 2.1) and were excluded from further analysis. A previous study using the same accelerometers identified acceleration events 12 to 20 G to be associated mainly with general hen movements (e.g. 45.8% grooming; Baker et al., 2020). Similarly, in our study acceleration events of 12 to 20 G were mainly general movement events (Fig 2.1). Therefore, statistical analysis focused on the 209 acceleration events >20 G, which represented 16 CON and 13 AVI hens from the 12 ECC. Fig 2.1 shows the behavioral categories associated with these events. Collisions were the most frequent cause of acceleration events >20 G (59.3%), followed by aggression (19.1%), mass-scattering (12.4%), grooming (6.2%), and wing-flapping (3.0%).

For 35 to 36 wk of age, a total of 267 acceleration events were recorded from 28 hens (14 CON hens across 6 ECC; 14 AVI hens across 6 ECC; 8 accelerometers failed to record properly). Of the 211 acceleration events that were matched to video, 70 had accelerations >20 G. Six of the hens only experienced collisions with accelerations 12 to 20 G. As a result, the analyzed data represented 13 CON and 9 AVI hens (from all 12 replicate ECC). Collisions were the most frequent cause of acceleration events >20 G (44.3%), followed by aggression (18.6%), grooming (18.6%), wing-flapping (14.3%), and mass-scattering (4.2%).

Lastly, for 49 to 50 wk of age, a total of 172 acceleration events were recorded from a total of 26 hens over the 10-d data collection period (14 CON hens across 6 ECC; 12 AVI hens across 6 ECC). Ten of the initial 36 accelerometers failed during the course of data collection and their data were not included in analysis. Of the 153 identifiable acceleration events, 52 acceleration events had accelerations >20 G from 11 CON (6 ECC) and 8 AVI hens (5 ECC). Aggression was the most frequent cause of acceleration events >20 G (40.4%), followed by grooming (32.7%),

and collisions (26.9%). Mass-scattering and wing-flapping were not associated with any acceleration events >20 G for this timepoint.

Incidence of Acceleration Events > 20 G

Rearing treatment, age, and Day/Night had a three-way interactive effect on the incidence of acceleration events > 20 G ($\chi 2$=9.59, df=2, P=0.008; Fig 2.2). During the d, both rearing treatments sustained the most acceleration events at 21 wk of age. However, we observed a reduction in the number of acceleration events sustained by AVI hens at 35 wk of age that remained fairly constant to 49 wk of age. For CON hens, we observed that the number of acceleration events sustained decreased between 21, 35, and 49 wk of age. Daytime observations also revealed that CON hens sustained more acceleration events than aviary-reared hens at 21 and 35 wk of age (estimated means [95% CI]: 4.3 [2.8, 6.7], 2.8 [1.7, 4.7], 1.2 [0.6, 2.1] acceleration events/hen/10 d in CON birds vs. 3.4 [2.1, 5.5], 0.7 [0.3, 1.4], 1.3 [0.7, 2.6] acceleration events/hen/10 d in AVI birds at 21, 35 and 49 wk of age, respectively). At night, AVI hens had a consistently low number of acceleration events sustained across all ages (0.6 [0.3, 1.3], 0.2 [0.08, 0.7], 0.1 [0.04, 0.5] acceleration events/hen/10 d at 21, 35 and 49 wk of age). Moreover, CON hens sustained the greatest number of nighttime acceleration events at 21 wk of age, and then the acceleration events decreased and remained low at 35 and 49 wk of age (1.8 [1.1, 2.9], 0.2 [0.05, 0.6], 0.06 [0.008, 0.4] acceleration events/hen/10 d at 21, 35 and 49 wk of age, respectively).

Incidence of Collisions > 20 G

Rearing treatment, age, and Day/Night had a three-way interactive effect on the incidence of collisions that were associated with acceleration events > 20 G ($\chi 2=6.34$, df=2, P=0.04; Fig 2.3). The patterns of the interaction were similar to the patterns reported for all acceleration events. During the d, AVI and CON hens experienced the most collisions at 21 wk of age. However, the number of collisions experienced by AVI hens decreased by 35 wk of age and stayed consistent at 49 wk of age. In CON hens, the number of collisions sustained over time decreased across subsequent time points, and CON hens experienced more collisions than AVI hens at 21 and 35 wk of age (estimated means [95% CI]: 2.0 [1.1, 3.6], 1.0 [0.5, 2.1], 0.2 [0.08, 0.7] collisions/hen/10 d in CON birds vs. 1.3 [0.7, 2.6], 0.2 [0.08, 0.7], 0.3 [0.09, 0.8] collisions/hen/10 d in AVI birds at 21, 35 and 49 wk of age, respectively). During the night, AVI hens experienced minimal collisions across all ages (0.4 [0.2, 0.9], 0.1 [0.02, 0.4], 0.2 [0.05, 0.6] collisions/hen/10 d at 21, 35 and 49 wk of age). CON hens experienced the most collisions during lights off at 21 wk of age, but collisions decreased by 35 wk of age and remained low when assessed at the end of the trial (1.2 [0.6, 2.3], 0.04 [0.006, 0.3], 0.04 [0.006, 0.4] collisions/hen/10 d at 21, 35 and 49 wk of age).

Collision Objects and Actions: Descriptive Summary

Over the course of the study, a total of 117 collisions > 20 G were recorded for CON hens across all cages and all time points. The majority of these were with the high (34.2%) and low perch (29.1%), followed by the floor (20.5%), other birds (14.5%), and the cage-wall (1.7%). CON hens experienced collisions by running into the object or conspecific (31.6%), slipping on a perch (27.4%), being pushed by another conspecific (23.1%), and ascending onto (15.4%) and

descending from (2.5%) perches. For AVI hens, a total of 52 collisions with accelerations > 20 G were recorded across all cages and all time points. The highest proportion of collisions were with the high perch (40.4%), followed by the floor (26.9%), the low perch (17.3%), other birds (11.5%), and the cage-wall (3.9%). The actions associated with collisions for AVI hens were slipping on a perch (34.6%), ascending to perches (23.1%), being pushed by another conspecific (21.2%), running into the object or conspecific (17.3%), and descending from perches (3.8%).

Perching Behavior

Rearing, age, and Day/Night had a three-way interactive effect on perch use ($\chi2$=8.57, df=2, P=0.01; Fig 2.4). As indicated in Fig 2.4, daytime perch use was low in both treatments. At night, the proportion of birds perching was comparable across treatments at 21 and 35 wk of age. At 49 wk of age, a higher proportion of AVI hens were observed perching as compared to CON hens (0.317 [0.249, 0.393], 0.322 [0.254, 0.400], 0.352 [0.280, 0.432] proportion of CON hens perching/ECC/5-min observation vs. 0.290 [0.226, 0.363], 0.331 [0.261, 0.409], 0.489 [0.407, 0.572] proportion of AVI hens perching/ECC/5-min observation at 21, 35, and 49 wk, respectively; estimated means [95% CI]).

Of the hens that were using perches, treatment and age affected the proportion of hens using the high perch specifically. More perching AVI hens utilized the high perch than perching CON hens throughout lay ($\chi2$=11.1, df=1, P=<0.001; Fig 2.5). Regardless of rearing treatment, all hens increased use of the high perch at 49 wk of age compared to 21 and 35 wk ($\chi2$=6.40, df=2, P=0.04).

DISCUSSION

The objective of this study was to determine whether pullet rearing conditions affect the incidence of acceleration events and collisions at the keel, as well as perching behavior, for ECC housed laying hens. We predicted that AVI hens would sustain fewer acceleration events and fewer collisions than CON hens, and we predicted that AVI hens would perch more overall, and specifically on the high perch, than CON hens. We found that both AVI and CON hens experienced fewer acceleration events at the keel and collided with features of their environment less frequently as they aged. However, AVI hens sustained fewer acceleration events and fewer collisions than CON hens at initial and peak phase of the laying period (21 and 35 wk of age), suggesting that AVI hens were navigating their laying environment with more ease from an earlier age than CON hens.

Previous research has demonstrated that rearing environments contribute to learning ability, such that birds reared in enriched environments learn faster than birds reared in non-enriched environments. Floor-reared pullets with 1 wk of exposure to outdoor range were faster to learn a Y-maze task compared to floor-reared pullets with no other structural or sensory enrichment (Krause et al., 2006). Similarly, floor-reared pullets with three wk of sensory enrichment were faster to learn a T-maze task than floor-reared pullets without enrichment (Campbell et al., 2018). During the reversal phase of a holeboard task, pullets reared in a multi-tiered aviary for 12 wk were faster to complete the task than pullets reared in a conventional cage for 16 wk (Tahamtani et al., 2015). These previous findings suggest that enriched (AVI) rearing environments improve birds' abilities to learn compared to non-enriched (CON) rearing environments, which may have contributed to AVI hens experiencing fewer acceleration events

and collisions than CON hens at earlier time points. Cognitive testing was not performed in our experiment, but our findings indicate that more cognitive tests and potential brain analyses are needed to assess the effect of rearing on long-term learning in novel environments.

It is worth noting that the magnitude of difference between rearing treatments' estimated means was low, with CON hens experiencing about 1 to 2 acceleration events/hen/10 d and about 1 collision/hen/10 d more than AVI hens at initial and peak lay. However, assuming that these numbers remained consistent in the 14 wk between initial and peak lay, CON hens could have cumulatively experienced up to 10 collisions more per hen than AVI hens between 21 to 35 wk of age. Previous work found that birds with a higher number of collisions were at an increased risk for sustaining keel bone fractures (Makagon et al., 2017; Baker, 2018), and other researchers reported that CON hens have a higher incidence of keel bone fractures than AVI hens at 73 wk of age in ECC (Casey-Trott et al., 2017a). Therefore, considering our data with these previous two studies, it is possible that CON hens cumulatively experience more collisions and thus are at a greater risk for injury. Future research should evaluate the effect of rearing on both collisions and keel bone fractures concurrently for further evaluation. As mentioned, the number of collisions experienced across the 10-d trials could be considered low, with estimated means ranging from 0 to 2 collisions/hen/10 d >20 G across rearing treatments and ages during the d. Our study found fewer collisions on average than previous work using the same accelerometers, which evaluated the number of collisions per hen across two 3-wk timepoints/ECC (Baker et al., 2020). Baker et al. (2020) reported an average of 12.0 and 12.6 collisions/hen/3 wk >20 G during the d (estimated 5.7 and 6.0 collisions/hen/10 d). The hens used in this previous work were floor-reared, older, of a different strain (Hy-line W36), and housed in ECC with a different spatial

layout of perches than those in our study. Some or all of these factors could have contributed to the discrepancy in the number of collisions reported in our study and the previous one (Baker et al., 2020). Overall, it is important to consider that acceleration events at the keel are experienced by individuals. The spread of the interquartile range of acceleration event data, particularly at 35 wk age (Fig 2.2), suggests that some CON individuals experienced more acceleration events than the majority of AVI hens, which could further increase their risk for keel bone fractures during peak lay.

Collisions constituted the main cause of acceleration events experienced at the keel bone for initial and peak lay. This finding confirms previous results obtained using the same accelerometers that collisions constitute a majority of acceleration events >20 G (Baker et al., 2020). However, the number of collisions remained consistent over two age groups (52 to 60 and 74 to 83 wk) in the previous results (Baker et al., 2020), whereas the collisions in our study decreased as birds aged. Other researchers have also found hens to experience fewer collisions with increasing age (Stratmann et al., 2015a, 2019). The inverse relationship between collisions and age has been attributed to hens being the most active earlier in the laying period and reducing activity over time (Campbell et al., 2016b; Kozak et al., 2016; Rufener et al., 2019a; Stratmann et al., 2019). An alternative explanation for our study could be that hens from both rearing treatments were experiencing a novel environment at initial lay. Neither of the rearing environments matched the laying environment in terms of group size, space allowance, and the design and spatial layout of physical structures. A mismatch between the rearing and laying environment is attributed to behavioral challenges when transitioning into the laying environment (e.g. aggression, space perception; Janczak and Riber, 2015). As a result, having the

most acceleration events and collisions at the initial phase of the laying period could also be attributed to adapting to a novel environment.

We descriptively evaluated the objects that birds collided with, as well as the actions that birds performed at the time of collision, to provide further detail into how birds utilized their space. Collisions with either of the perches constituted the greatest proportion of collision objects for both treatments (63.3% of collisions with low perch and high perch for CON hens vs 57.7% of collisions with low perch and high perch for AVI hens). These findings are in agreement with previous research (Baker et al., 2020), who also found perches to be the main objects that birds collided with in ECC (71.5% of collisions with perches). The authors noted that collisions with perches occurred mainly as hens ascended onto them (Baker et al., 2020). Along with slipping while perching, ascending onto perch was also among the top two actions associated with perch collisions by AVI hens in this study. Meanwhile, the top two collision action categories for CON hens were running into an object or a conspecific while locomoting across the floor and slipping on a perch.

We predicted that more AVI hens would be observed perching than CON hens at all ages. If true, this finding would explain why perch ascent may have been a leading source of collisions for AVI hens, but not CON hens. Previous research found that pullets reared with access to vertical space utilized perches and elevated platforms more than birds reared without perches (Gunnarsson et al., 2000; Brantsæter et al., 2016). However, in our study, the total proportion of birds perching was similar across rearing treatments and for most time points. Although CON hens were not raised with perches, they may have perched on the water lines as pullets, offering

one possible explanation for the similar perch use, including during the earliest observation time point. It is also possible that the 2 wk that the birds spent in the ECC system before the 21 wk data collection provided the chicks with sufficient time to learn to use the perches. This is particularly likely since the low perch was located only 9 cm from the cage floor and ran the length of the cage, making it easily accessible for the birds.

Daytime perch use was low, with only 1 to 8% of birds observed perching, regardless of perch height or rearing treatment. A previous study reported a range of 17 to 30% of birds perching during lights on in groups of 33 to 50 hens per ECC (Louton et al., 2016). Others reported birds spending 7 to 25% of their time perching during the d in groups of 14 hens per ECC (Struelens et al., 2008). In these studies, daytime perch use was attributed to short bouts of grooming, standing, sleeping, and facilitating access to another resource (e.g. water line). In our study, the perches did not facilitate access to feed or water, so it is possible that they were utilized less during the daytime while birds were active and using other resources in the ECC. On the other hand, laying hens are highly motivated to access perches at night for roosting, which is attributed to an antipredator defense strategy (Olsson and Keeling, 2000). At least one-third of birds, regardless of rearing treatment, were observed roosting on perches at night at all three ages in our study. The remaining proportion of birds were commonly observed huddled together in one or two large groups on the floor of the ECC. The proportion of roosting birds in our study is lower than reported in several previous studies of nighttime perching among cage housed hens (53 to 72% of hens roosting, Louton et al., 2016; 85 to 100% of hens roosting, Hester, 2014). However, other researchers have reported that the majority of hens in ECC sleep huddled on the floor during lights off as opposed to roosting on perches (Casey-Trott and Widowski, 2016).

Birds in our study had sufficient perch space for all hens to be perching simultaneously on either perch (at least 15 cm per hen, Hester, 2014), but possibly different designs and layout of perches in ECC contributed to the behavioral differences in our study compared to others.

We evaluated perching birds' preference for the low versus high perch. Given the low daytime perch use observed, our analysis focused on nighttime observations. While both AVI and CON hens utilized perches at night, more AVI hens used the high perch. Previous studies that evaluated the effect of rearing on perch and platform use collected data only at 16 and 19 wk of age (Gunnarsson et al., 2000; Brantsæter et al., 2016). Our work demonstrates that rearing influences height preference into mid-lay (49 wk of age). It is not clear from our study whether the influence of rearing on roost height preference reflects differences in spatial cognition, perception, learning, or physical abilities. It is also unclear whether it was the height or other aspects of perch design that were preferred by the AVI birds, or that may have dissuaded CON birds from mounting it. Unlike the low perch, the high perch in this study was suspended from the cage ceiling and consequently could sway back and forth. This instability of the perch could have impacted the birds' preference for it.

In addition to perch use, the lighting period, whether lights were on or off, also had an effect on collisions. All birds, regardless of rearing treatment, experienced more collisions during the d versus during the night. Similarly, previous studies found that the majority of collisions took place during lights on, and specifically during the dusk and dawn phases when hens were moving to and from roosting (Stratmann et al., 2019; Baker et al., 2020). In our study, we did not observe a pattern of collisions during dawn and dusk specifically. Instead, the higher prevalence of

collisions during lights on could be attributed to hens being generally more active during the d than during the night. In laying hens, more illuminance promotes more physical activity (Boshouwers and Nicaise, 1993), and, as a result, hens have more opportunities for collisions during lights on. Furthermore, the lights on period is twice as long as the lights off period in our study (16 h vs 8 h, respectively), so hens also had more total time to sustain collisions during the lights on period as opposed to lights off.

Lastly, we found social interactions to be an important contributing factor to keel acceleration events, such that aggression was the second-most cause of acceleration events in another study and in our study (Baker et al., 2020). Coupled with the fact that a notable collision object category was conspecifics and a notable collision action category was birds being pushed by conspecifics, these findings warrant further investigation into the relationship of social behavior and collisions. In the context of early experiences, it is important to consider that rearing environments differ in the group sizes allowance, availability of space for hiding or retreat, and other aspects that influence social experiences and development of chicks. Research into the interactions between rearing, social behavior, space use, and collisions is needed.

We proposed that the reported influence of rearing on keel bone fracture prevalence may be due to differences in birds' abilities to move through their adult environment. Our study found that rearing environment does affect the number of acceleration events experienced at the keel, collisions associated with these acceleration events, and perch use. Collisions have been linked to keel bone fractures in prior research, and these behavioral effects seem to persist through peak lay for acceleration events and collisions and through mid-lay for perching. Keel bone fracture

assessment was outside the scope of this study. However, higher keel bone fracture incidences for cage-reared vs aviary-reared hens in ECC systems were found during peak lay (Casey-Trott et al., 2017a). It is possible that experiencing more collisions through peak lay, as demonstrated in our study, may explain these results. Future studies should evaluate collisions and keel bone fractures from the same individual hens to evaluate the link between rearing environment, collisions, and keel bone fractures.

ACKNOLWEDGEMENTS

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DISCLOSURES

This research received no external funding.

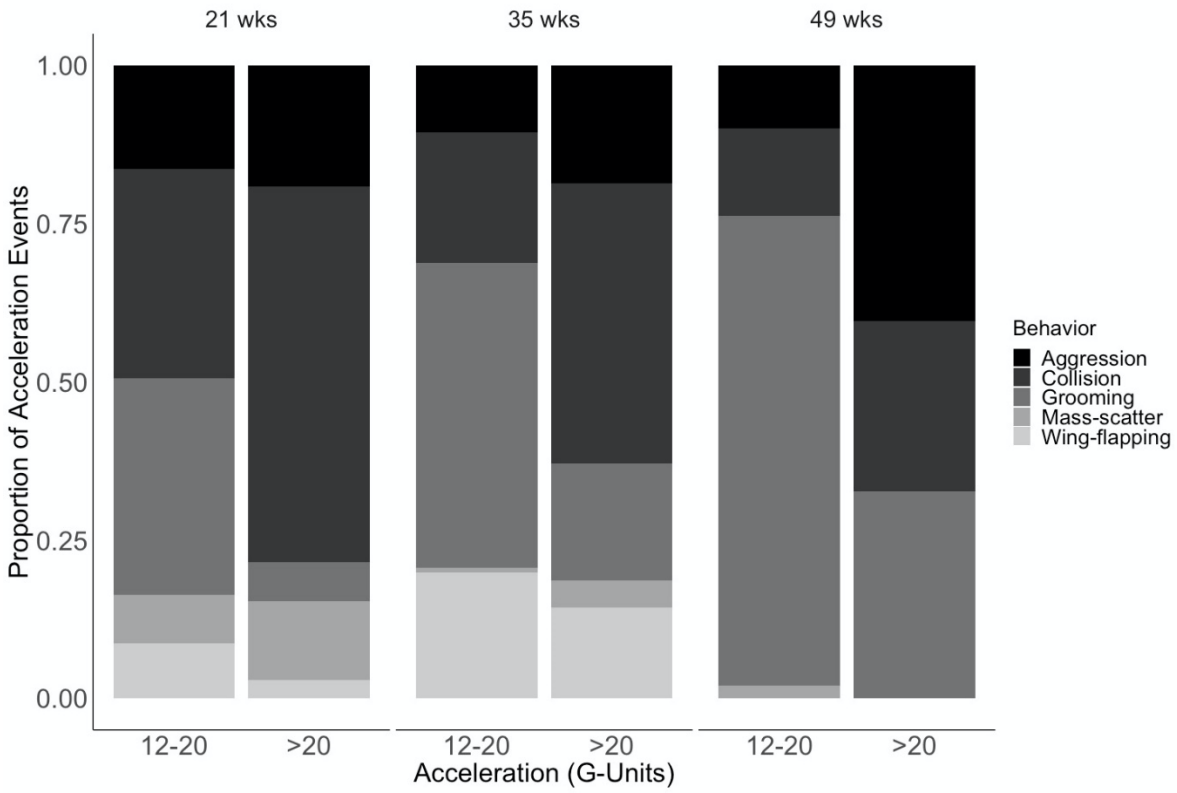

Figure 2.1. All identifiable acceleration events sustained by all hens at each age, grouped by behavioral category and acceleration category. At 21 wk, the 12 to 20 G acceleration category is based on 184 events and the >20 G category is based on 209 events. At 35 wk, 12 to 20 G represents 141 events while >20 G represents 70 events. Lastly, at 49 wk, 12 to 20 G contains 101 events and >20 G contains 52 events.

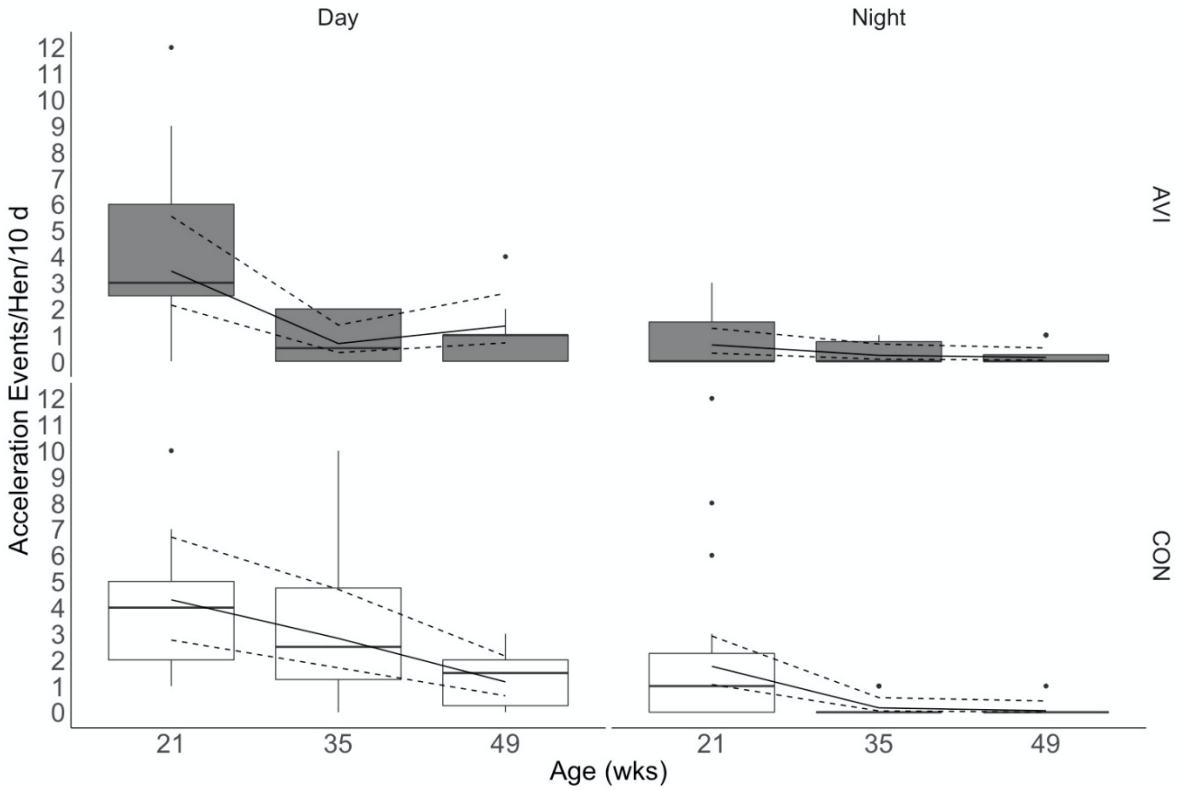

Figure 2.2. Total acceleration events >20 G sustained per hen over 10 d at each age during d (16 h/d) and night (8 h/d) in enriched colony cages (ECC). Hens were reared in either conventional cages (CON) or a multi-tiered aviary (AVI) until 19 wk of age before being moved into ECC. Acceleration events consisted of collision, aggression, grooming, wing-flapping, and mass scattering events. Boxes represent the interquartile range, where the black line represents the median, the top of the box represents the 75th quartile, and the bottom of the box represents the 25th quartile. Whiskers represent the minimum and maximum values, while black dots indicate outliers. The solid line overlaying the box plots is the estimated mean values from the generalized linear mixed effects model, and the dashed lines are the 95% confidence intervals of the estimated means.

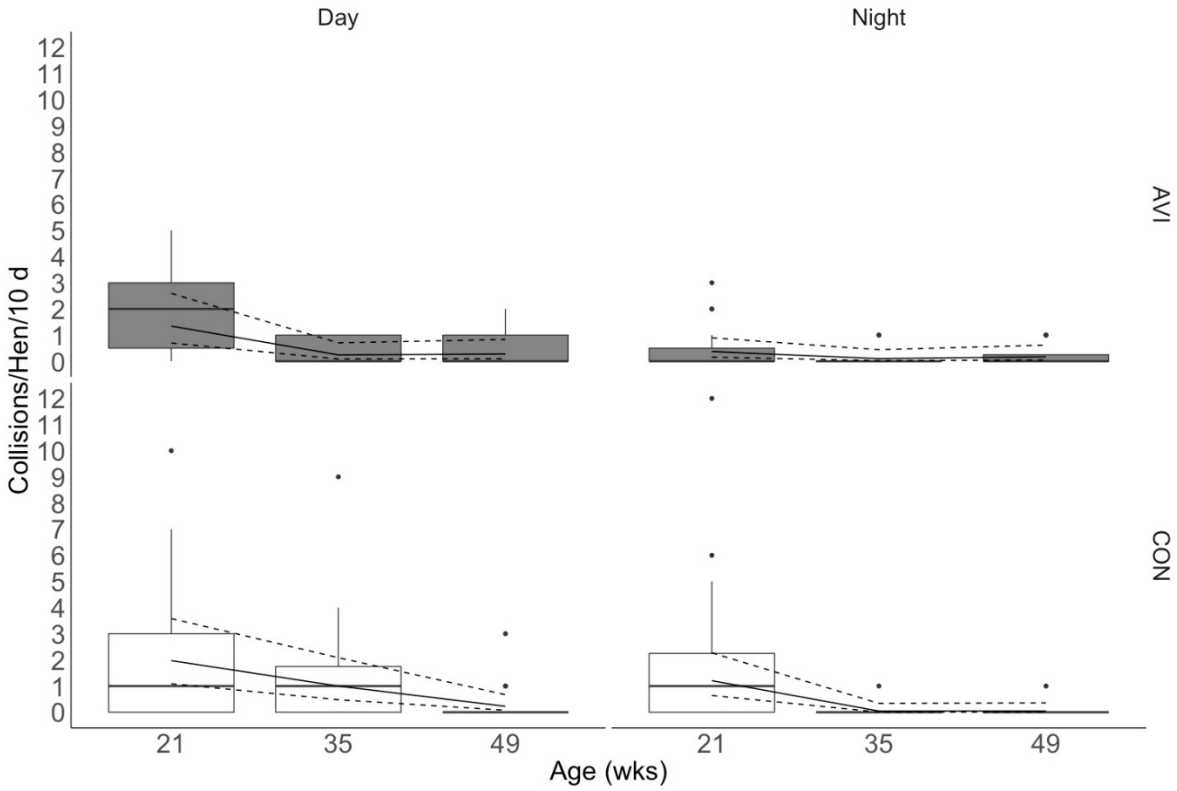

Figure 2.3. Total collisions > 20 G sustained per hen over 10 d at each age during lights on (16 h/d) and lights off (8 h/d) in enriched colony cages (ECC). Hens were reared in either conventional cages (CON) or a multi-tiered aviary (AVI) until 19 wk of age before being moved into ECC. Collisions were with structural objects or conspecifics. Boxes represent the interquartile range, where the black line represents the median, the top of the box represents the 75th quartile, and the bottom of the box represents the 25th quartile. Whiskers represent the minimum and maximum values, while black dots indicate outliers. The solid line overlaying the box plots is the estimated mean values from the generalized linear mixed effects model, and the dashed lines represent the 95% confidence intervals of the estimated means.

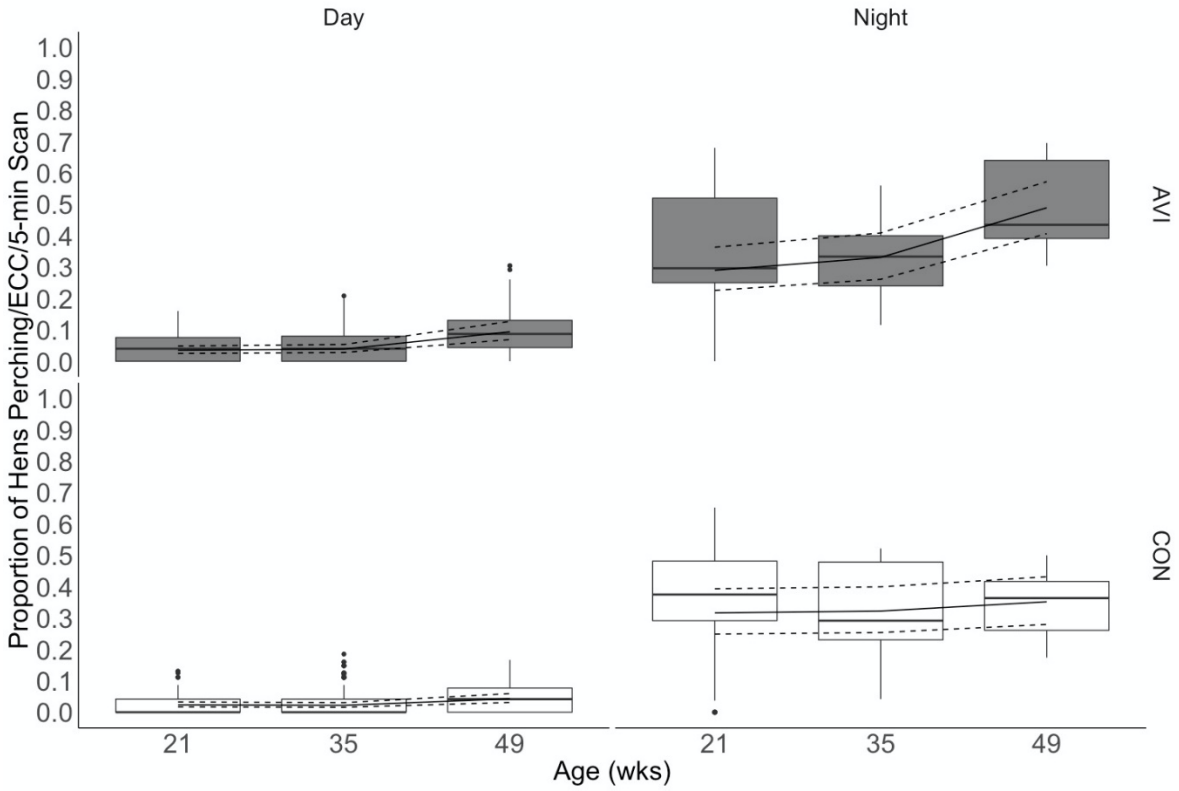

Figure 2.4. The proportion of birds perching per enriched colony cage at each age. Hens were reared in either conventional cages (CON) or a multi-tiered aviary (AVI) until 19 wk of age before being moved into enriched colony cages. Boxes represent the interquartile range, where the black line represents the median, the top of the box represents the 75th quartile, and the bottom of the box represents the 25th quartile. Whiskers represent the minimum and maximum values, while black dots indicate outliers. The solid line overlaying the box plots is the estimated mean values from the generalized linear mixed effects model, and the dashed lines are the 95% confidence intervals of the estimated means.

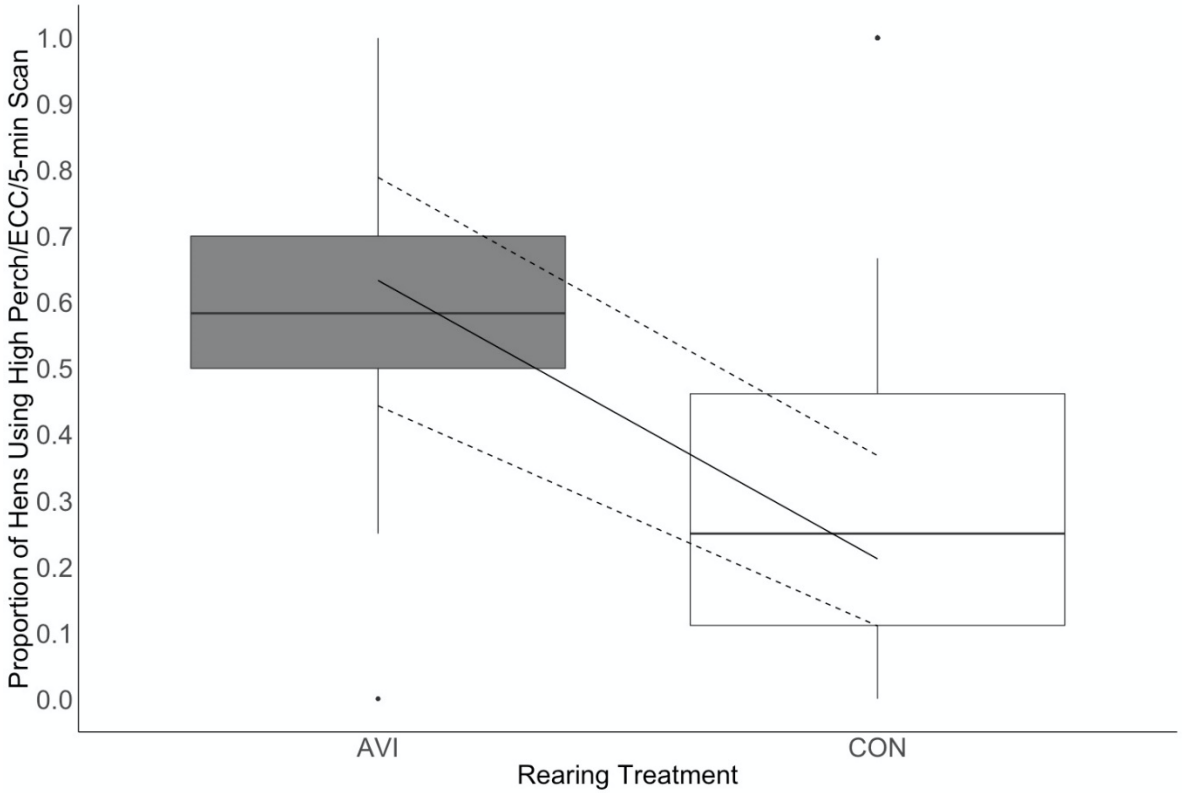

Figure 2.5. The proportion of perching birds on the high perch by rearing treatment throughout lay (21 to 49 wk). Hens were reared in either conventional cages (CON) or a multi-tiered aviary (AVI) until 19 wk of age before being moved into enriched colony cages. Boxes represent the interquartile range, where the black line represents the median, the top of the box represents the 75th quartile, and the bottom of the box represents the 25th quartile. Whiskers represent the minimum and maximum values, while black dots indicate outliers. The solid line overlaying the box plots is the estimated mean values from the generalized linear mixed effects model, and the dashed lines are the 95% confidence intervals of the estimated means.

Table 2.1. Definitions of behaviors associated with acceleration events.

Behavior	Definition
Collision	Bird hits or moves into an object at moment of event. The object that the bird collided with is identified: high perch, low perch, floor, cage wall, or bird. The action that the bird performed immediately preceding the collision is also identified: ascent towards perch, descent from perch, slipping while walking on top of a perch, physical push from conspecific, or running into an object on the bird's own.
Aggression	Bird fights with or confronts other birds, but no clear collision event is observed. Aggressive behavior typically involves pushing out chest or standing taller while orienting towards each other, flapping wings, chasing and jumping, and aggressive pecking directed at another bird, typically at the head.
Grooming	Bird is preening, cleaning and aligning feathers using own beak, or ruffles feathers.
Wing flapping	Bird opens and extends both wings, but not due to collision event or aggressive interaction.
Mass scattering	All or large group of birds in cage move to one side of the cage (cage disturbance), with no clear collision observed for focal hen.
Cannot ID	Cannot identify acceleration event because hen behavior was obstructed from view, no clear behavior could be determined, or a video error occurred.

CHAPTER 3:

Providing height to pullets does not influence hippocampal dendritic morphology or brain-derived neurotrophic factor at the end of the rearing period

Manuscript in preparation for *Poultry Science*

ABSTRACT

Pullets reared with diverse behavioral experiences are faster to learn spatial cognition tasks and acclimate more successfully to laying environments with elevated structures. However, the neural underpinnings of the improved spatial abilities are unclear. The objective of this study was to determine whether providing structural height in the rearing environment affected the development of the hippocampus and whether hippocampal neural metrics correlated with individual behavior on spatial cognition tasks. Female Dekalb White pullets were reared in a floor pen (FL), single-tiered aviary (ST), or two-tiered aviary (TT). Pullets completed floor-based Y-maze and elevated visual cliff tasks to evaluate depth perception at 15 and 16 wk, respectively. At 16 wk, brains were removed for Golgi-Cox staining (n = 12 for FL, 13 for ST, 13 pullets for TT) and qPCR to measure gene expression of brain-derived neurotrophic factor (*BDNF*; n = 10 for FL, 11 for ST, and 9 pullets for TT). Rearing environment did not affect various morphometric outcomes of dendritic arborization, including Sholl profiles; mean dendritic length; sum dendritic length; number of dendrites, terminal tips, or nodes; soma size; or *BDNF* mRNA expression ($p > 0.05$). Hippocampal subregion did affect dendritic morphology, with multipolar neurons from the ventral subregion differing in several characteristics from multipolar neurons in the dorsomedial or dorsolateral subregions ($p < 0.05$). Generally, neural

metrics did not correlate with individual differences in behavior during the spatial cognition tasks, except for the positive correlation between the expression of *BDNF* and the number of times individual pullets looked down at the edge of a 30 cm high visual cliff ($r_s = 0.42$, $p = 0.03$). Overall, providing height during rearing did not affect dendritic morphology or *BDNF* at 16 wk of age, but other metrics in the hippocampus or other brain regions warrant further investigation. Additionally, other structural or social components or the role of animal personality are areas of future interest for how rearing environments influence pullet behavior.

Key words: brain-derived neurotrophic factor, chicken, Golgi stain, hippocampus, rearing environment

INTRODUCTION

Pullets (*Gallus gallus domesticus*) grow and develop in rearing environments until just prior to the onset of egg laying, approximately 16 to 18 wk of age, at which time they are transitioned into their laying environments. During this early period of life, pullets are sensitive to environmental factors that influence cognitive, behavioral, and physical development (reviewed in Campbell et al., 2019). Multiple studies have linked rearing environments to pullet and hen spatial abilities. Pullets reared with elevated structures, access to outdoor range, or novel sensory enrichment were faster to learn spatial cognition tasks (i.e. Y-maze, holeboard task, T-maze, and detour task; Krause et al., 2006; Tahamtani et al., 2015; Campbell et al., 2018; Norman et al., 2019) than pullets reared without these environmental components. Pullets reared with access to elevated space also acclimated faster to novel environments as they used elevated structures more and had fewer collisions compared to pullets reared on the floor or in conventional cages

(Gunnarsson et al., 2000; Brantsæter et al., 2016; Pullin et al., 2020). While it is not possible to disentangle whether the early-life experiences influenced cognition or physical ability, these findings suggest that environments offering more diverse behavioral experiences during rearing modify pullet development such that they can more effectively process spatial information. However, the neural basis of these developmental changes has yet to be described.

The hippocampus brain region is an area of interest for studying the neural underpinnings of spatial abilities in chickens, as it is associated with spatial orientation, navigation, and memory in avian species (Krebs et al., 1989; Bingman and Mench, 1990; Tommasi et al., 2003). Previous research failed to find a link between pullet rearing environment and hippocampal volume or tyrosine hydroxylase, an enzyme involved in dopamine synthesis (Tahamtani et al., 2016; Campbell et al., 2018). However, hippocampal volume is a coarse metric of brain function, whereas finer measurements of neuronal cytoarchitecture can offer biologically relevant insight into the cellular and synaptic mechanisms underlying spatial cognition (Roth et al., 2010). Further investigation into neural correlates of plasticity is, therefore, warranted.

Brain function is determined in large part by the pattern of synaptic connections, which is strongly influenced by experience-dependent changes in the complexity of dendritic branching (Nithianantharajah and Hannan, 2006). Golgi staining is a technique used to visualize and quantify the dendritic arbors of individual neurons in the intact brain by stochastically filling intact cell bodies and their axonal and dendritic processes (Carter and Shieh, 2015). In studies modeling experience-induced plasticity in mammals, the technique has been used to evaluate the effects of environmental enrichment on neuronal cytoarchitecture. Several studies have reported

increased hippocampal dendritic arborization as a result of environmental enrichment (Berman et al., 1996; Faherty et al., 2003; Darmopil et al., 2009; Nithianantharajah et al., 2009). However, we are aware of only one study using Golgi staining to assess experience-induced plasticity in the chicks' hippocampal cytoarchitecture (Freire and Cheng, 2004). Chicks reared with visual barriers to their imprinting stimulus experienced changes in perception (visual occlusion) and physical access to the stimulus. Those chicks had longer dendrites and more dendritic spines in the hippocampus at 16 d of age than chicks reared without visual barriers, suggesting that the former had an advantage at processing spatial information. The authors concluded that these changes were hippocampal-specific, after finding no changes in the neostriatum of the brain. Within the hippocampus, the right hemisphere had a higher degree of dendritic development than the left hemisphere, suggesting that the right hemisphere was more involved in spatial processing than the left (Freire and Cheng, 2004). Therefore, the right hemisphere hippocampus is an ideal candidate for investigating how pullet rearing environments modify neuronal cytoarchitecture and consequently birds' abilities to process spatial information.

Brain function is also influenced by the prevalence of certain molecules that are involved in regulating neuronal survival. Brain-derived neurotrophic factor (*BDNF*) is a growth factor implicated in activity-dependent plasticity, learning, and memory due to its roles in cell survival, cell proliferation, and synaptic transmission regulation (Miranda et al., 2019). The highest levels of *BDNF* gene expression were found in the hippocampus of adult mice (Hofer et al., 1990). In mammals, higher expression of hippocampal *BDNF* has been linked with environmental enrichment that promotes exercise, socialization, novelty, or a combination (reviewed in van Praag et al., 2000; Rossi et al., 2006; Cao et al., 2017). We are aware of only one study

measuring the expression of *BDNF* as an outcome of providing pullets with different rearing environments. Pullets reared with perches were less fearful, had lower levels of plasma corticosterone, and higher levels of *BDNF* gene expression in the hypothalamus compared to pullets reared without elevated structures, suggesting that rearing environment influences neural correlates for stress mediation in the hypothalamus (Yan et al., 2020). However, investigating the influence of the rearing environment on hippocampal *BDNF* as a metric of spatial cognition is understudied in pullets.

If neuronal cytoarchitecture and gene expression of *BDNF* are successful neural metrics for chicken spatial cognition, then correlating these metrics to individual pullet behavior on spatial cognition tasks could provide more insight into neural underpinnings of individual differences in behavior. The relationship between brain function and performance on spatial cognition tasks has yet to be described in pullets. In humans, brain image analyses revealed that activity in the hippocampus correlated with individual differences in spatial learning strategies (Shelton and Gabrieli, 2004), and hippocampal volume correlated with individual differences in sense-of-direction (Burte et al., 2018). Depth perception is an aspect of spatial cognition that can be measured utilizing tasks such as a visual cliff (Shinkman, 1962; Jones, 2021) or Y-maze with asymmetrical arms (Jones, 2021). The visual cliff task evaluates the birds' willingness to move across a perceived vertical gap set to various depths, whereas the Y-maze requires the birds to discriminate depth in the horizontal plane. Individual birds differ in their strategies to complete these tasks. Individual differences in the number of times pullets look down at the edge of the visual cliff to gather information prior to crossing, latency to cross the visual cliff, latency to exit the Y-maze, and choice of Y-maze exit arm have been reported (Jones, 2021). These individual

behavioral differences could be correlated with individual differences in measures of brain function within the hippocampus.

The primary aim of this study was to determine whether providing height in the rearing environment affected the development of multipolar neurons in the hippocampus, specifically regarding neuronal cytoarchitecture and *BDNF*. We predicted that pullets reared with the most height would have a higher degree of dendritic arborization in hippocampal multipolar neurons as indicated by Sholl profiles with more intersections at any given distance from the soma, higher values for non-Sholl dendritic metrics, and higher gene expression of hippocampal *BDNF* compared to pullets reared with intermediate or minimal height. This study also aimed to determine whether hippocampal neural metrics correlated with individual behavior in spatial cognition tasks. We hypothesized that hippocampal metrics would explain individual differences in behavior in Y-maze and visual cliff spatial cognition tasks. We predicted that some or all of the non-Sholl metrics and gene expression of *BDNF* would correlate with the number of times looking down, crossing, and latency to cross a visual cliff at 30 and 90 cm heights, as well as choosing the short arm and latency to exit the arena in a Y-maze task.

MATERIALS AND METHODS

All experimental procedures were reviewed and approved by the University of California, Davis Institutional Animal Care and Use Committee (Protocol #20307).

Pullet Housing and Management

Dekalb White pullets (N=835) were raised in 15 pens (3.05 x 3.05 x 2.74 m, L x W x H) in one building at the Hopkins Avian Facility at University of California, Davis (Davis, CA). The pullets were obtained at 1 d of age from a commercial hatchery and randomly assigned to one of three rearing environments (55 to 56 pullets/pen, 5 pens/rearing environment): floor (**FL**), single-tiered aviary (**ST**), or two-tiered aviary (**TT**; Fig 3.1). The building was divided into five blocks of 3 pens/block to ensure that rearing environments were distributed throughout the building. Rearing environments were randomly assigned to pens within each block. At 8 wk of age, a portion of pullets were removed from each pen as part of data collection procedures for another component of this project, resulting in a density of 45 pullets/pen until data collection at 16 wk.

From 1 d of age, pullets were bedded with pine wood shavings (Mallard Creek Inc., Rocklin, CA). Water was provided *ad libitum* via automatic water lines (Lubing USA, Cleveland, TN) with 12 nipples /pen. A start and grow diet (Purina Start and Grow Medicated Crumbles, Purina Animal Nutrition LLC, Gray Summit, MO) was provided *ad libitum* using two 13.6 kg round feeders/pen (52 cm circumference/feeder). Temperature and artificial lighting were maintained according to the Dekalb White Product Guide (Dekalb, The Netherlands). Husbandry personnel entered each pen one time daily to monitor and make adjustments for feed, water, litter, lights, ventilation, and animal health.

In FL pens, pullets had access to four metal floor perches (121.9 x 3.8 x 10.5 cm, L x W x H). In ST pens, pullets could access an aviary structure containing three elevated metal perches (121.9 x 3.8 cm, L x W) installed at the heights of 35.4 cm (two perches) and 64.7 cm from the floor

(third perch), as well as one elevated tier with a plastic slatted surface (61.0 x 121.9 L x H; Dura-Slat Poultry and Kennel Flooring, Southwest Agri-Plastics, Inc., Addison, TX) located 62.9 cm off the floor. A wire mesh ramp was provided to facilitate access to the tier (96.5 x 31.8 cm, 40-degree angle; McNichols Wire Mesh, McNichols Co., Inc., Livermore, CA). A single metal floor perch, identical to the ones used in FL pens, was placed adjacent to the structure. The aviary structure in the TT pens contained three elevated metal perches (121.9 x 3.8 cm, L x W) located 29.4, 89.9, and 125.7 cm from the floor, as well as two elevated tiers with plastic slatted surfaces (30.5 x 121.9, L x W) located 62.9 cm and 123.8 cm from the floor. A wire mesh ramp facilitated access to both tiers (190.5 x 31.8 cm, 40-degree angle). A metal floor perch was provided next to the aviary structure. In ST and TT, chicken wire was used to prevent pullets from accessing floor space directly underneath the tiers of the aviary in order to keep stocking density constant across all three rearing environments.

To ensure that birds' visual experiences were limited to their own rearing environments, heavy-duty, plastic tarps (3.05 x 1.83 m, L x W; Everbilt, The Home Depot, Inc., Atlanta, GA) were hung on pen walls and doors to reduce visibility into adjacent pens and the hallway used by human caretakers and researchers. The top half of the pen (0.91 m between where the tarp ended and the ceiling) was not covered so that ventilation through the building was not obstructed. As a result, TT birds standing on the highest tier could see over the tarp at approximately 8 wk of age, gaining additional visual cues that pullets in ST and FL rearing environments did not have.

Individual Behavior during Spatial Cognition Tasks

At 15 wk of age, two to three pullets were randomly selected from each pen, received a leg band for individual identification, and tested individually in a Y-maze (n = 14 pullets/rearing environment; N = 42 pullets total). At 16 wk of age, the same pullets were tested individually on a visual cliff. Testing at each age occurred across 5 d, where one pen from each rearing environment was tested/d (3 pens total tested/d). The order of rearing environment tested was randomized daily to prevent order bias. Construction of the tasks, procedure used during the testing, as well as methods for behavioral observations are described in detail in Jones (2021). In brief, the Y-maze task required individual birds to choose one arm of the maze to exit into an arena in two consecutive trials. In one trial, the Y-maze arms were equal length (1:1 ratio; 90 cm each), and in another trial the arms were unequal length (1:3 ratio; 30 and 90 cm). The order of trials was randomized across birds to prevent order bias, and the side of the longer arm (right or left) was also randomized across trials to prevent side bias. Trials were considered complete after the bird had exited one of the arms or after 150 sec had elapsed, whichever came first. For the scope of the present study, we aimed to correlate neural metrics with the most spatially challenging tasks; therefore, we evaluated individual pullet behavior only during the unequal length trial. We recorded which arm the pullet exited from during the unequal length trial (short or long) as well as the latency to complete the trial.

The visual cliff task required individual birds to cross over a table that was fitted with checkerboard material and topped with plexiglass, a design that created illusions of depth without risk of the bird falling (e.g. Shinkman, 1962). Pullets were tested in three trials of different heights in a randomized order: 15, 30, and 90 cm, where the latter two heights created

perceived depth. Trials were considered complete after the bird had crossed over the cliff to a platform at the end of the table or after 90 sec had elapsed, whichever came first. Similar to the Y-maze, we aimed to correlate neural metrics with the most spatially challenging tasks; therefore, we evaluated individual pullet behavior only during the 30 and 90 cm height trials. We recorded the number of times the bird looked down during the trial, whether or not the pullet crossed the table, and the latency to complete the trial.

Brain Tissue Collection

Immediately after completing the visual cliff task at 16 wk of age, each pullet was euthanized using an overdose of isoflurane inhalant, followed by rapid decapitation for brain tissue collection (n = 14 pullets/rearing environment). Brains were removed in less than three minutes from decapitation and hemisected. The right hemisphere was immediately placed into impregnation solution for Golgi-Cox methods described below. The left hemisphere was flash frozen on dry ice and stored in a -80°C freezer until quantitative polymerase chain reaction (qPCR) analysis described below.

Golgi-Cox Staining

Golgi-Cox staining was performed on the right hemisphere tissue using the FD Rapid GolgiStain Kit (FD NeuroTechnologies, Inc., Columbia, MD) following modified manufacturer's instructions and as previously described (Lein et al., 2007; Keil et al., 2017). During impregnation in Solutions A and B, instructions were modified for pullets by using an estimated hemispheric brain volume of 2 cm^3 (estimate based on whole chicken brain volumes that were previously reported as 3.3 to 3.8 cm^3 for Brown Leghorn, Frahm and Rehkämper, 1998, and 4.3

cm^3 for *Gallus gallus domesticus* broadly, Kawabe et al., 2009). Further modifications were made after immersion in Solution C, where tissue was submerged in 10% sucrose in PBS for 4 h at 4 °C, then stored in 30% sucrose in PBS at 4 °C for approximately 7 mo until sectioning. Prior to sectioning, the right hemisphere tissue was transferred to 70% ethanol in distilled water for 15 min at 4 °C, then sectioned coronally at 100 μm on a vibratome set to a frequency of 70 Hz and speed of 0.40 mm/s (VT-1000, Leica Biosystems Inc., Buffalo Grove, IL). Sections (interaural 0.40 to 3.76 mm, Puelles et al., 2007) were mounted on gelatin-subbed slides and dried for a maximum of 2 wk at room temperature in the dark. The final stain was applied to sections with Solutions D and E following the manufacturer's instructions, then sections were dehydrated and topped with a coverslip using Permount™ Mounting Medium (Thermo Fisher Scientific, Waltham, MA). A portion of pullets were excluded from further analysis due to errors that occurred during sectioning or the final stain steps that made imaging neurons in the hippocampus impossible (n = 2 pullets for FL, 1 pullet for ST, and 1 pullet for TT).

Brightfield image stacks of hippocampal multipolar neurons were taken at 20x magnification in 0.2 μm steps using an IX-81 inverted microscope (Olympus, Shinjuku, Japan) and MetaMorph Image Analysis Software (version 7.1, Molecular Devices, Sunnyvale, CA). One to five neurons per animal were imaged from the hippocampal coronal slices of individual pullets, ranging from interaural 1.12 to 3.76 mm (n = 12 pullets for FL, 51 total neurons; n = 13 pullets for ST, 54 total neurons; n = 13 pullets for TT, 52 total neurons; see Fig 3.2 for representative images). Criteria for consistent and accurate selection of neurons included three conditions previously described (Lein et al., 2007): 1) well-impregnated neurons with no evidence of incomplete or artificial staining, 2) blood vessels, glia, or non-descript precipitate do not obscure neuron or branches,

and 3) the cell body is located in the middle third of the thickness of the section. Additional criteria were incorporated for pullets, including 4) neurons are multipolar projection neurons and have at least four thick spinous dendrites coming out around an ovoid or spherical-shaped soma (Tömböl et al., 2000) and 5) hippocampal subregion will be identified for the multipolar neuron at time of imaging (Fig 3.2). The ventral subregion (**V**) is defined as the V-shaped region at the caudal end of the hippocampus. Once the hippocampus begins to widen, the dorsomedial region (**DM**) begins. This region widens and then narrows again. Once the region narrows into a consistent width for the remainder of the hippocampus, it is the dorsolateral region (**DL**). These distinctions align with previous work evaluating avian hippocampal subregions (Atoji and Wild, 2004; Smulders, 2017). One pullet may have neurons imaged from more than one subregion, so the final sample size based on subregion was n = 36 pullets for the largest region DL, 109 total neurons; n = 20 pullets for DM, 35 total neurons; n = 9 pullets for V, 13 total neurons. At least two neurons from each rearing environment were represented in each subregion.

Dendrites and somas from all imaged neurons were manually traced by one experimenter using NeuroLucida (version 11, MBF Bioscience, Williston, VT). Arbor complexity was quantified by automated Sholl analysis (NeuroLucida Explorer, version 11, MBF Bioscience), where the sum of intersections are reported between neuronal dendrites and 10-μm Sholl rings centered on the neuronal soma. Area under the curve (AUC, 0 to 260 μm) was calculated for the Sholl curve using Prism (version 9, GraphPad Software, San Diego, CA), including comparisons between proximal (0 to 40 μm) versus distal AUC (40 to 260 μm). The 40 μm Sholl ring was used to separate proximal versus distal because it was the peak for all three Sholl curves for the rearing environments. Non-Sholl neuronal metrics included mean dendritic length, sum dendritic length,

number of dendrites, number of terminal tips, number of nodes, and soma size. The experimenter was not completely blind to rearing environment during staining, imaging, or tracing because pen numbers were associated with sample identification. However, the experimenter was not able to readily identify the rearing environment associated with pen numbers for the majority of the pens.

qPCR Analysis

The left hemisphere was sectioned coronally at 100 μm on a cryostat (CM 1860, Leica Biosystems Inc., Buffalo Grove, IL) for microdissection of the hippocampus using a 3mm punch (interaural -0.08 to 3.76 mm, Puelles et al., 2007). Punched tissue (mean ± SD punch weight: 19.8 ± 5.2 mg) was stored in a -80°C freezer until RNA extraction. To extract total RNA and run real-time qPCR reactions, we followed the methods previously described for avian brain nuclei tissue in Farrar et al., (2022). Briefly, RNA was extracted using a modified Direct-zol RNA Miniprep kit (Zymo Research, Irvine, CA) and RNA quality was evaluated using a Nanodrop ND-1000 spectrophotometer (Thermo Fisher Scientific, Wilmington, DE). Samples were excluded from analysis if their 260/280 and 260/230 ratios were < 1.8 or if their RNA concentration was low (< 100 ng/μL), which resulted in the exclusion of n = 4 pullets for FL, 3 pullets for ST, and 5 pullets for TT. The final sample size for qPCR was n = 10 for FL, n = 11 for ST, and n = 9 pullets for TT. RNA was then treated with DNase I (Invitrogen, Waltham, MA) to remove any remaining genomic DNA, and converted to complementary DNA (cDNA) using qScript cDNA Supermix (Quantabio, Beverly, MA). cDNA was diluted 5-fold prior to qPCR analysis. qPCR for each sample was run in triplicate on a CFX384 Touch Real-time PCR detection system (Bio-Rad Laboratories, Hercules, CA) using the reaction mix and cycling

protocol described in Farrar et al., (2022). All samples were run on a single qPCR plate, including negative controls (i.e. H_2O) to verify that there was no contamination. Similar to the Golgi-Cox methods, the experimenter was not completely blind to rearing environment during all stages for qPCR analysis due to sample identification being associated with the pen number.

We ran qPCR for the gene of interest, brain-derived neurotrophic factor (*BDNF*), in addition to two reference genes, beta-actin (*ACTB*) and peptidylprolyl isomerase A (*PPIA*). These reference genes have previously been shown to be stable in avian neural tissue (Zinzow-Kramer et al., 2014). We verified that rearing environment did not have a significant effect on mean reference gene expression for *ACTB* ($F_{2,11} = 0.44$, $p = 0.66$) or *PPIA* ($F_{2,11} = 1.33$, $p = 0.31$). Primers used for qPCR were designed on species-specific gene sequences for *Gallus gallus* using the NCBI Primer-BLAST tool (Table 3.1). Primers were then validated by running a 10-fold serial dilution to determine amplification efficiencies (mean ± SD: 96.2 ± 3.0 %; Table 3.1) and confirmed single amplicons via melt curve analysis.

We determined relative expression for *BDNF* using the ddCt method (Livak and Schmittgen, 2001). Briefly, *BDNF* expression for each sample was normalized to the geometric mean of *ACTB* and *PPIA (*dCt*),* then calculated relative to the reference treatment group (ddCt). Here, the FL rearing environment was used as a reference because those pullets did not have access to elevated space. We report relative expression as fold change (2^{-ddCt}).

Statistical Analysis

A completely randomized design with three treatments was used, where pen was the experimental unit. For statistical analysis of gene expression, R statistical software (version 4.0.4, R Core Team, 2021) was used. We evaluated the effect of rearing environment (factor with three levels: FL, ST, and TT) on mean reference gene expression (*ACTB, PPIA*) or fold change for the gene of interest (*BDNF*, where FL was the reference group) with linear mixed effect models (nlme package, version 3.1.152; Pinheiro et al., 2021), with pen as a random effect. Model assumptions were checked by visually evaluating the Q–Q plot, distribution of residuals, and homoscedasticity (sjPlot package, version 2.8.10, Lüdecke, 2021). Fold change values were log transformed to meet model assumptions.

Golgi-Cox statistical analyses were conducted in JMP Pro (version 16, SAS Institute, Cary, NC). For the Sholl analysis, AUC, and non-Sholl neuronal metrics, a mixed model was used with rearing environment (factor with 3 levels: FL, ST, and TT) and hippocampal subregion (factor with 3 levels: V, DM, and DL) as fixed effects. An interaction of rearing environment and hippocampal subregion was not significant, and its exclusion resulted in a lower AIC for all models, indicating a better model fit. For the Sholl analysis model, random effects included Sholl radius nested within neuron ID, neuron ID nested within pullet ID, and pullet ID nested within pen. The model also used a Repeated Covariance Structure with a first-order autoregression structure (AR(1)) that held pullet ID as the subject (described in Wilson et al., 2017). For AUC and non-Sholl neuronal metrics, a Residual structure was specified for the Repeated Covariance Structure, and pullet ID nested within pen was used as a random effect. AUC and non-Sholl neuronal metrics did not appear to be normally distributed based on histograms and residual

plots, so they were natural log transformed to achieve normality. If a fixed effect was statistically significant, posthoc pairwise comparisons were made between levels using Tukey-Kramer HSD tests.

R statistical software (version 4.0.4, R Core Team) was also used to analyze correlations between individual pullet behavior on cognitive tasks and the individual's neural outcomes. The number of pullets used in each correlation analysis was determined by the individuals included for each brain metric and their performance on the cognition task. A total of 38 pullets were included in the Golgi-Cox analysis. To evaluate the relationship between the six non-Sholl neuronal metrics and behaviors on the visual cliff task, the number of times each individual looked down and whether the pullet crossed the visual cliff at 30 and 90 cm were evaluated for all 38 pullets. The relationship between non-Sholl neuronal metrics and the latency to cross the visual cliff was evaluated only for the pullets that crossed (N = 14 and N = 11 pullets at 30 and 90 cm, respectively). For the Y-maze task, non-Sholl neuronal metrics were correlated with the latency to exit the maze and the choice of the short arm when exiting only for pullets that exited the maze (N = 22 pullets).

A total of 30 pullets were included in the *BDNF* gene expression analysis. Due to errors in sample labeling, the log fold change of *BDNF* expression was not able to be successfully matched to the individual behavior for four pullets; therefore, *BDNF* was correlated with 26 pullets for the number of times looking down and whether the pullet crossed the visual cliff at 30 and 90 cm. Of those 26 pullets, the relationship between the log fold change of *BDNF* and latency to cross was analyzed for the 8 pullets that crossed the cliff. Latency to exit the Y-maze

and the choice of the short arm when exiting were correlated with *BDNF* only for pullets that exited the maze (N = 15 pullets).

A Spearman's rank correlation was utilized to evaluate the relationships between neural outcomes and continuous behaviors (ggpubr package, version 0.4.0; Kassambara, 2020). Continuous behaviors on the visual cliff at 30 and 90 cm were the number of times looking down and latency to cross the cliff, and continuous behavior in the Y-maze was latency to exit the maze. A Mann-Whitney U test ("wilcox.test" function in base R, stats package, version 0.4.0) was used to evaluate the relationships between neural outcomes and binary behaviors. Binary behaviors included crossing the visual cliff at 30 and 90 cm and choosing the short arm of the Y-maze.

RESULTS

Dendritic Morphology

The Sholl analysis indicated that rearing environment did not affect the dendritic arborization for multipolar hippocampal neurons ($F_{2,26} = 0.069$, $p = 0.93$; Fig 3.3), and no other morphological differences were noted between rearing environments (Table 3.2). However, the Sholl analysis showed that there are differences in dendritic arborization amongst subregions of the hippocampus ($F_{2,50} = 4.05$, $p = 0.03$; Fig 3.4). Specifically, V multipolar neurons have a higher number of intersections between dendrites and Sholl rings compared to DM and DL multipolar neurons overall and a higher AUC in the distal arbor than DL (Table 3.3). Other morphological differences were noted between the hippocampal subregions, such that V multipolar neurons also

have longer dendrites on average ($F_{2,144.9} = 5.62$, $p = 0.005$) compared to DM and DL multipolar neurons. V neurons also had longer dendrites in sum ($F_{2,150.5} = 7.20$, $p = 0.001$), more terminal tips ($F_{2,138} = 3.34$, $p = 0.04$) and more branching nodes than DL neurons ($F_{2,135.2} = 3.66$, $p = 0.03$; Table 3.3). DL multipolar neurons have a smaller area of the soma compared to DM and V multipolar neurons ($F_{2,137.3} = 9.94$, $p < 0.0001$).

BDNF Gene Expression

Rearing environment did not have a significant effect on relative *BDNF* expression ($F_{2,11} = 1.16$, $p = 0.35$; Fig 3.5).

Neural Outcomes Correlated with Individual Behavior

BDNF gene expression was positively correlated with the number of times that birds looked down when crossing the visual cliff at a height of 30 cm ($r_s = 0.42$, $N = 26$, $p = 0.03$). *BDNF* gene expression did not significantly correlate with any other behavior on the visual cliff or Y-maze cognitive tasks ($p > 0.05$; Table 3.4). None of the six non-Sholl neuronal metrics significantly correlated with individual pullet behavior on either cognitive task ($p > 0.05$; Table 4).

DISCUSSION

The objectives of this study were 1) to determine whether manipulating height provided to pullets during the first 16 wk of life affected hippocampal development, and 2) to assess if hippocampal metrics correlated with individual behavior in spatial cognition tasks. We found that rearing environment did not affect dendritic morphology of multipolar neurons in the right

hemisphere hippocampus, but morphology differed across hippocampal subregions. Similarly, providing height to pullets did not affect *BDNF* gene expression in the left hemisphere hippocampus. The hippocampal metrics measured in this study did not correlate with the majority of behaviors associated with the cognitive tasks, except for a positive correlation of *BDNF* with looking down at 30 cm in the visual cliff task.

Dendritic Morphology

In a previous study, chicks reared with visual barriers to their imprinting stimulus had longer dendrites and more dendritic spines in the right hemisphere hippocampus at 16 d of age than chicks reared without visual barriers (Freire and Cheng, 2004). Key methodological differences between the previous study and our study, including those related to chick socialization, space allowance, and timing of sample collection, may explain why we did not observe an effect of rearing environment on hippocampal dendritic morphology. Specifically, Freire and Cheng (2004) socially isolated chicks until 7 d of age, then paired chicks in groups of two until 16 d of age. Birds in the present study did not experience social isolation as they were housed in groups of 55 to 56 birds already at 1 d of age, then in groups of 45 birds between 8 to 16 wk of age. Social isolation is a known stressor for chicks and used as a model for the anxiety-depression continuum (Warnick et al., 2009). There is limited research on isolation-induced hippocampal changes in chicks, but multiple studies in mammals have shown that stressful social experiences early in life affects a variety of neurological measurements in multiple brain regions, including hippocampal dendritic morphology (reviewed by Davidson and Mcewen, 2012). Rearing chicks in larger social groups as we did may buffer any changes to hippocampal dendritic morphology that might have occurred from structural differences in the physical environment.

Space allowance is another methodological consideration. Freire and Cheng (2004) reared chicks in a cardboard box measuring 0.30 x 0.25 x 0.30 m (l x w x h; 0.075 m^2 area) for the first 7 d, followed by a larger box from 8 to 16 d of age (0.55 x 0.40 x 0.60 m; 0.22 m^2 area). Chicks in the present study were reared in a pen with an area 124 and 42 times larger than the first and second boxes used in the previous study, respectively (3.05 x 3.05 x 2.74 m; 9.30 m^2 area). More space likely provided more opportunities for physical activity, and higher rates of physical activity in mammals are associated with changes in hippocampal dendritic morphology, cell proliferation, and cell survival (reviewed by van Praag et al., 2000). Therefore, the limited social interactions and presumably lower rates of physical activity in the previous study may have created different neural conditions that were more susceptible to morphological changes induced by environmental structures. Accordingly, the range of length of hippocampal multipolar dendrites was previously reported as 85 to 115 μm (Freire and Cheng, 2004), which is about half of the average dendrite length found in our study. These differences in dendrite length support the notion of rearing environment cultivating different neural conditions between the two studies. Variation in dendrite length may have also been related to tissue thickness, with the former study assessing dendrites from 200 μm sections compared to 100 μm sections utilized in the present study. Dendrites commonly become shorter with age, so it is unlikely that the difference in length is due to different sampling time points (16 d of age vs 16 wk of age; Pannese, 2011).

Age at the time of analysis likely also influences whether morphological changes are detectable in the brain. The previous study analyzed dendritic morphology at 16 d of age, shortly after a known shift in brain lateralization (Vallortigara et al., 1997). We evaluated dendritic morphology

at 16 wk of age to capture the end of the rearing period and just prior to sexual maturity. Brain maturation reportedly persists through 8 to 10 wk of age for chickens (Rogers, 1995), so we anticipated that morphological changes would still be detectable after maturation was complete. A recent study investigating the influence of early-life environment on neuronal morphogenesis also failed to find morphological changes at 21 wk of age. Those researchers evaluated pullets reared in groups of 145 birds, either in a floor pen with no enrichments or in a pen where visual, auditory, and physical enrichments were exchanged or moved every 2 or 3 d for the first 3 wk of life (Campbell et al., 2018). The size of the telencephalon and hippocampus were measured using cresyl violet staining at 21 wk of age, and there were no significant differences between the two rearing environments. Therefore, measuring morphology at earlier time points may be a future methodological consideration, pending the research question. It is worth noting that other studies found morphological differences in older hens. At 48 wk of age, hens that had spent 32 wk in different adult environments showed changes in hippocampal soma size and tyrosine hydroxylase innervation patterns (Patzke et al., 2009). At 52 wk of age, birds that were reared with a foster hen for the first 7 wk of life had a greater degree of lateralization in hippocampal soma size compared to birds reared without a foster hen (Nordquist et al., 2013). These studies reflect additional methodological considerations. Birds may need longer experiences with their environment to show morphological changes in the hippocampus, and social influences during rearing may have more of an effect on hippocampal morphology than physical structures.

Golgi-Cox methodology revealed differences in morphology between hippocampal subregions, where V multipolar neurons notably differed on most dendritic characteristics from one or both of the other subregions examined in this study. Dendritic characteristics differentiated by the

three hippocampal subregions have not been previously reported, and this variation can inform future methodological considerations for quantifying dendritic morphology in multipolar neurons. However, the results should be interpreted with caution considering that a limitation of this study is a low representation of neurons in the V subregion. Across subregions, we found an average of 8 dendrites per multipolar neuron, which is comparable to the range of 4 to 6 dendrites per multipolar neuron described previously (Tömböl et al., 2000). Our study appears to be the first to report the Sholl profile, number of nodes, and number of tips for chicken hippocampal multipolar neurons. Previous studies using Nissl (Nordquist et al., 2013) or cresyl violet (Patzke et al., 2009) staining techniques to quantify soma size in the chicken hippocampus have distinguished between DM and V subregions, where DM cells were found to be larger than V cells. Our findings differed in that DM and V cells were statistically similar, but cells from both regions were larger than DL cells. The discrepancies between our study and others for soma size may reflect staining technique differences between studies. The soma area for multipolar neurons across subregions in our study aligns with the multipolar soma diameter previously reported for 28-d-old chicks using Golgi-Cox methods (e.g. Tömböl et al., 2000 reported 20 μm cell diameter, which is an estimated soma area of 314 μm^2). However, the hippocampal soma area reported with Nissl and cresyl violet techniques appears to be half of this size (91.8 to 127.2 μm^2 for neurons with cresyl violet in 48 wk old hens in Patzke et al., 2009; 130.8 to 169.3 μm^2 for neurons with Nissl in 52 wk old hens in Nordquist et al., 2013). Research in rodents comparing Golgi-Cox stain to Nissl and cresyl violet also observed the latter stains to underestimate soma size, a notable methodological consideration for future work (Pilati et al., 2008). Taken together, our findings highlight the importance of distinguishing between hippocampal subregions to account for morphological variation, which may provide insight

regarding functional differences homologous to the mammalian hippocampal subregions (Gupta et al., 2012; Atoji et al., 2016).

BDNF Gene Expression

Environmental enrichment that promotes physical activity, learning, and memory has been linked with higher *BDNF* expression by providing more opportunities for social interactions and engaging with novel objects (van Praag et al., 2000). We theorized that varying the height of structures provided to pullets would increase load bearing exercise to access elevated structures, as well as foster novel learning through the cognitive and visual experience of accessing the height. However, rearing environment did not impact hippocampal *BDNF* gene expression, which may be due to methodological differences in providing sensorimotor stimulation between our study and earlier research. Previous studies were successful in modifying *BDNF* levels by giving animals larger enclosures, social groups, and changing or moving objects in the environment frequently when compared to animals experiencing social isolation, a barren environment, or both (van Praag et al., 2000; Rossi et al., 2006; Cao et al., 2017). The size of social groups and space allowance were kept constant across rearing environments in the present study, so there may be either a similar level of barrenness, or alternatively a similar level of complexity, perceived by birds across the rearing environments tested. Despite birds using elevated structures by the second wk of rearing (M. Makagon, unpublished data), the load bearing exercise and cognitive and visual experience of accessing different levels of height were not sufficient to trigger changes in hippocampal *BDNF*.

A recent study on pullet rearing evaluated environments where social groups and barrenness differed significantly by comparing birds reared in conventional cages (groups of approximately 25 birds/cage until 16 wk of age) or multi-tiered aviaries (groups of approximately 25 birds/cage for the first 4 wk of life, then a group of thousands of birds in an aviary until 16 wk of age; Tahamtani et al., 2016). Pullet rearing environment did not affect hippocampal tyrosine hydroxylase at 20 or 24 wk of age. Tyrosine hydroxylase is a rate-limiting enzyme in dopamine biosynthesis that could have a downstream effect on *BDNF*, as dopamine release can induce *BDNF* expression (Tahamtani et al., 2016). In combination with our study, this recent research suggests that the pullet rearing environment may have minimal effects on hippocampal *BDNF* and dopaminergic pathways regardless of social or physical features. Future research should investigate other pathways in the hippocampus (e.g. markers of neurogenesis, Armstrong et al., 2020) or alternative brain regions that have been understudied for experience-induced changes in chickens, such as the cerebellum, forebrain (e.g. hypothalamus), nucleus rotundus, or visual Wulst. Likewise, future research could evaluate neurological outcomes after birds have experienced a challenge. For example, pullets reared with access to perches had higher hypothalamic *BDNF* gene expression at 53 d of age compared to pullets reared on a litter floor without perches when they were exposed to an environmental challenge (Yan et al., 2020). Even though our work found similar neural metrics across rearing environments at the end of the rearing period, the brains may respond differently once they are challenged with a stressor, like being moved into the novel adult laying hen environment. Taking more neurological measurements after challenges and into the laying period are areas of future research consideration.

Furthermore, the physical structures in the pens remained unchanged and in the same location from 1 d of age. Commercial housing manufacturers promote management practices where height does change throughout the rearing period. For example, pullets are released from various aviary enclosures at different heights and different ages, or pullets are exposed to structures that gradually rise in height throughout the rearing period (Chore-Time, 2019; Big Dutchman, 2022; Vencomatic Group, 2022). The timing of exposure to staged increases in height may trigger neurological changes that stationary environments do not influence, which has not been studied to our knowledge and warrants further investigation.

Neural Outcomes Correlated with Individual Behavior

Neural correlates to individual differences in behavior have been identified across a range of species for a variety of behaviors, including hippocampal metrics linked to spatial orientation and spatial learning strategies in humans (Shelton and Gabrieli, 2004; Burte et al., 2018). Previous work in farmed chickens did not evaluate neural correlates with individual behavior differences, but researchers found no relationships between hippocampal measurements and performance in spatial cognition tasks averaged at the group level. For example, chickens reared in conventional cages took, on average, longer to complete a holeboard task and demonstrated poorer working memory during the reversal phase of the task than chickens reared in multi-tiered aviaries (Tahamtani et al., 2015). However, those same bird groups did not differ between rearing environments in staining intensity for tyrosine hydroxylase in the hippocampus and the caudolateral nidopallium (Tahamtani et al., 2016). Similarly, chickens reared with sensorimotor enrichment were, on average, faster to learn a T-maze task than chickens reared without enrichment, but there were no differences between groups for volume of the hippocampus or

telencephalon (Campbell et al., 2018). In the present study, birds were subsampled from a different experiment as part of a larger project that demonstrated that TT and ST pullets were more likely to cross, crossed faster, and looked down less in a visual cliff task than FL pullets (Jones, 2021). We theorized that dendritic morphology and *BDNF* in the hippocampus would correlate with these individual differences. *BDNF* positively correlated with looking down behavior on the visual cliff at the 30 cm height only. *BDNF* is associated with learning and memory (Miranda et al., 2019), which could include processing and learning spatial information gathered by looking down. However, it is not clear why this correlation would not also be present at 90 cm, a presumably more challenging height for gathering information. The relationship between *BDNF* and information processing about height needs to be further explored.

Previous work in chickens has commonly evaluated two brain regions of interest with regard to spatial cognition, and the present study evaluated one region. Research in humans showed how interactions amongst a network of brain regions explained individual differences in spatial orientation ability (Arnold et al., 2014). Future studies investigating neural correlates for spatial behavior in individual birds should consider a similar network approach. For example, visual processing of impending collisions with looming objects involves the thalamofugal and tectofugal pathways in pigeons (Wu et al., 2005; Liu et al., 2008). These pathways may also be relevant in a network of processing spatial information in chickens, or different networks may be more significant for chickens considering that they are terrestrial and pigeons are arboreal in their spatial abilities. Finally, some individual differences in behavior may not have notable neural underpinnings and are instead behavioral strategies associated with personality (i.e.

consistent inter-individual variation in behavior; Dall et al., 2004). Future research aiming to explain individual differences in spatial behavior may consider correlations across multiple behaviors that categorize personality type, such as exploration, activity, social interactions, and fearfulness (reviewed in Garnham and Løvlie, 2018; Campbell et al., 2021).

In conclusion, modifying only height in the pullet rearing environment did not affect hippocampal dendritic morphology or hippocampal *BDNF*. Additionally, the majority of these hippocampal measurements did not correlate with individual differences in behavior on visual cliff or Y-maze tasks. Future work evaluating the relationship between pullet rearing environments and neural outcomes should consider measuring other pathways in the hippocampus (e.g. neurogenesis) or investigating a network of other brain regions. If the hippocampus is pursued further, the subregion should be identified as we found significant morphological differences for multipolar neurons amongst the subregion. The age at which height is introduced, the duration of exposure to height, and possible social influences should also be considered when designing the rearing environment. Finally, there is much evidence that the rearing environment does modify bird behavior. If neural correlates are not able to be identified to explain the behavior changes, then other approaches to investigate the underlying cause are warranted (e.g. animal personality).

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DISCLOSURES

The authors have no conflicts of interest to declare.

A)

B)

C)

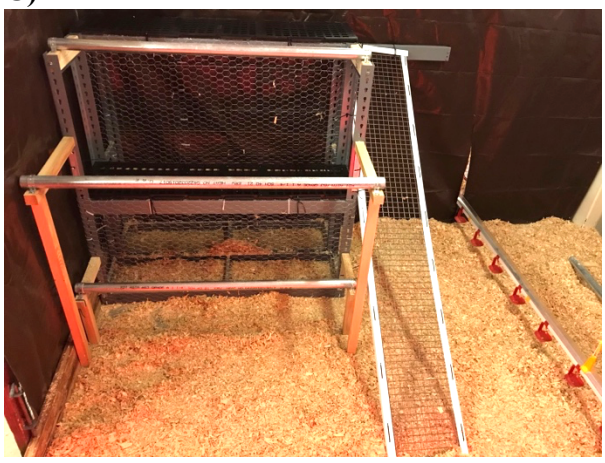

Figure 3.1. Female Dekalb White pullets were reared in one of three environments for the first 16 wk of life: floor rearing environment (A), a single-tiered aviary (B), and two-tiered aviary (C).

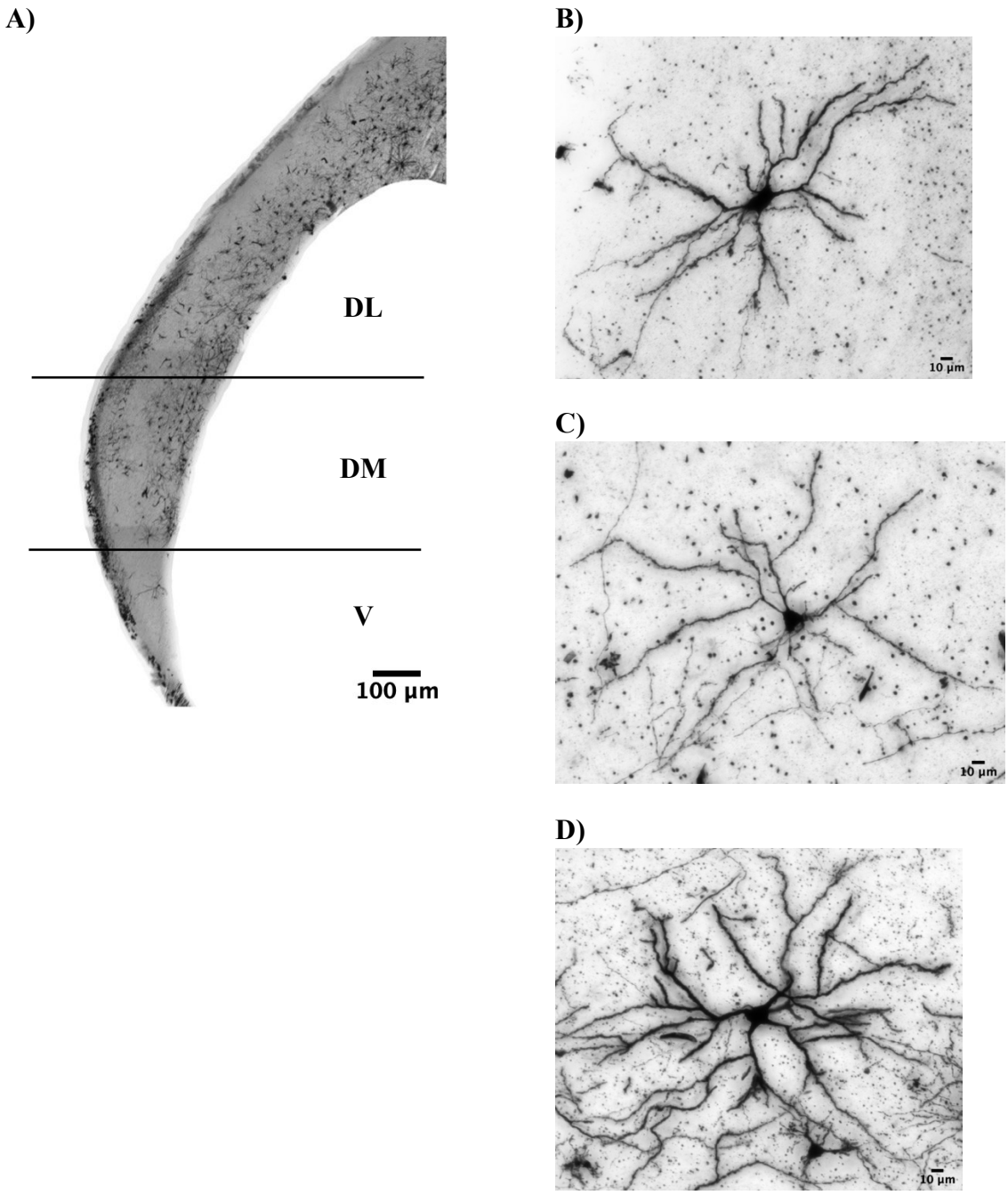

Figure 3.2. A pullet hippocampus was imaged with three images at 4X, then fused together with ImageJ software to create a representative image of the brain region (A). Subregions of the hippocampus were distinguished for ventral, V, dorsomedial, DM, and dorsolateral, DL for neuron imaging. Neurons were imaged at 20X for female Dekalb White pullets at 16 wk of age that were raised in one of three rearing environments. Representative photomicrographs of

Golgi-stained hippocampal multipolar neurons at 20X magnification from the floor rearing environment (B), a single-tiered aviary (C), and two-tiered aviary (D).

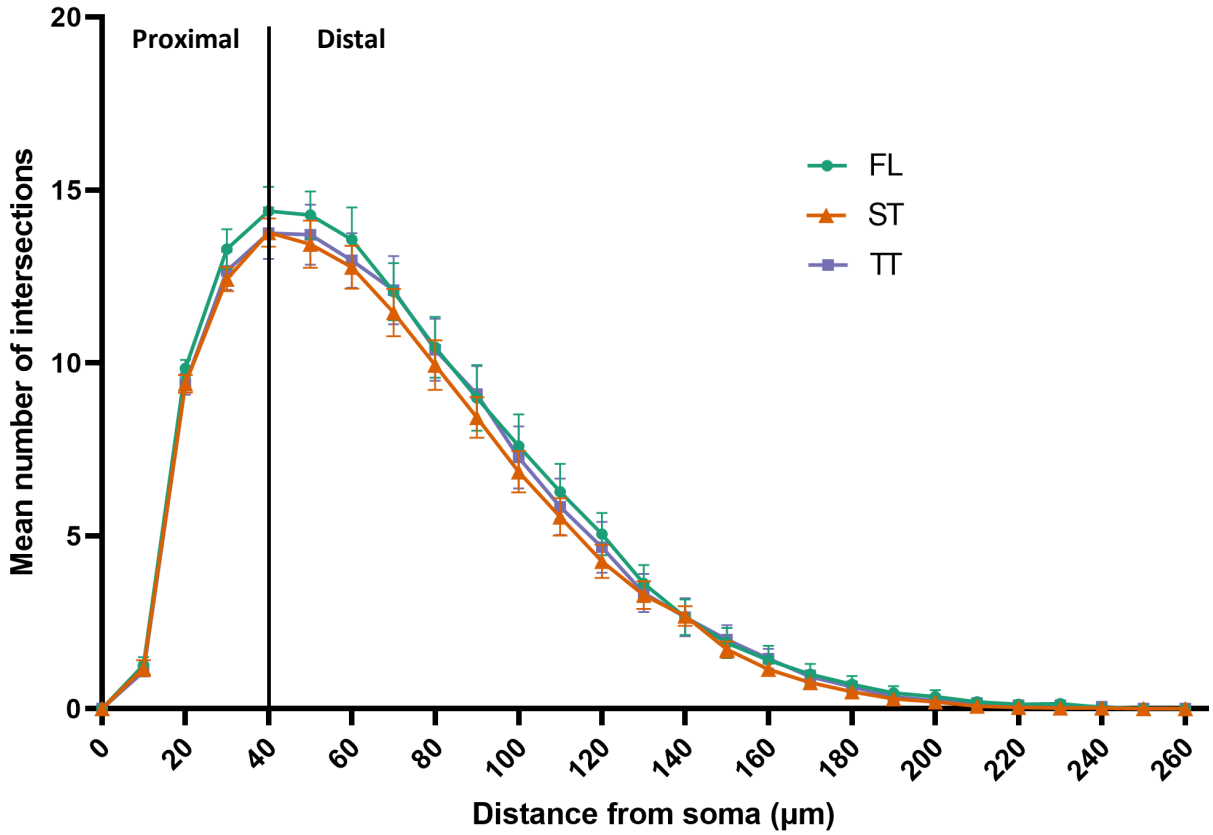

Figure 3.3. Sholl plots of the dendritic arbors from right hemisphere hippocampal multipolar neurons in female Dekalb White pullets at 16 wk of age. Pullets were reared on the floor (FL; n = 12 pullets, 51 total neurons), in a single-tiered aviary (ST; n = 13 pullets, 54 total neurons), or in a two-tiered aviary (TT; n = 13 pullets, 52 total neurons). Values are presented as mean ± SEM of the number of dendritic intersections at concentric rings in 10 µm segments from the soma (green circle for FL, orange triangle for ST, and purple square for TT). A black line at 40 µm represents the cutoff for proximal versus distal area under the curve analysis.

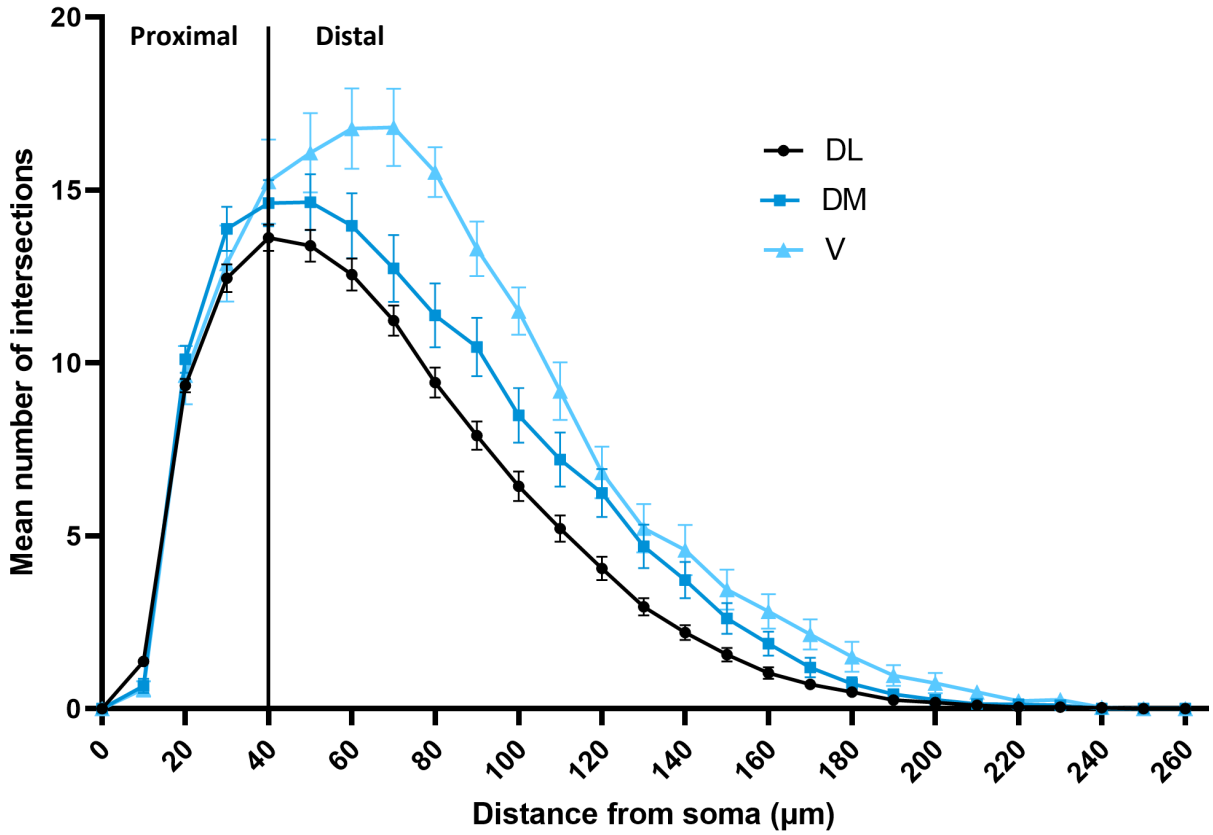

Figure 3.4. Sholl plots of the dendritic arbors from right hemisphere hippocampal multipolar neurons in female Dekalb White pullets at 16 wk of age. Neurons were sampled from hippocampal subregions dorsolateral (DL; n = 36 pullets, 109 total neurons), dorsomedial (n = 20 pullets, 35 total neurons), and ventral (V; n = 9 pullets, 13 total neurons). Values are presented as mean ± SEM of the number of dendritic intersections at concentric rings in 10 µm segments from the soma (black circle for DL, blue square for DM, and light blue triangle for V). A black line at 40 µm represents the cutoff for proximal versus distal area under the curve analysis.

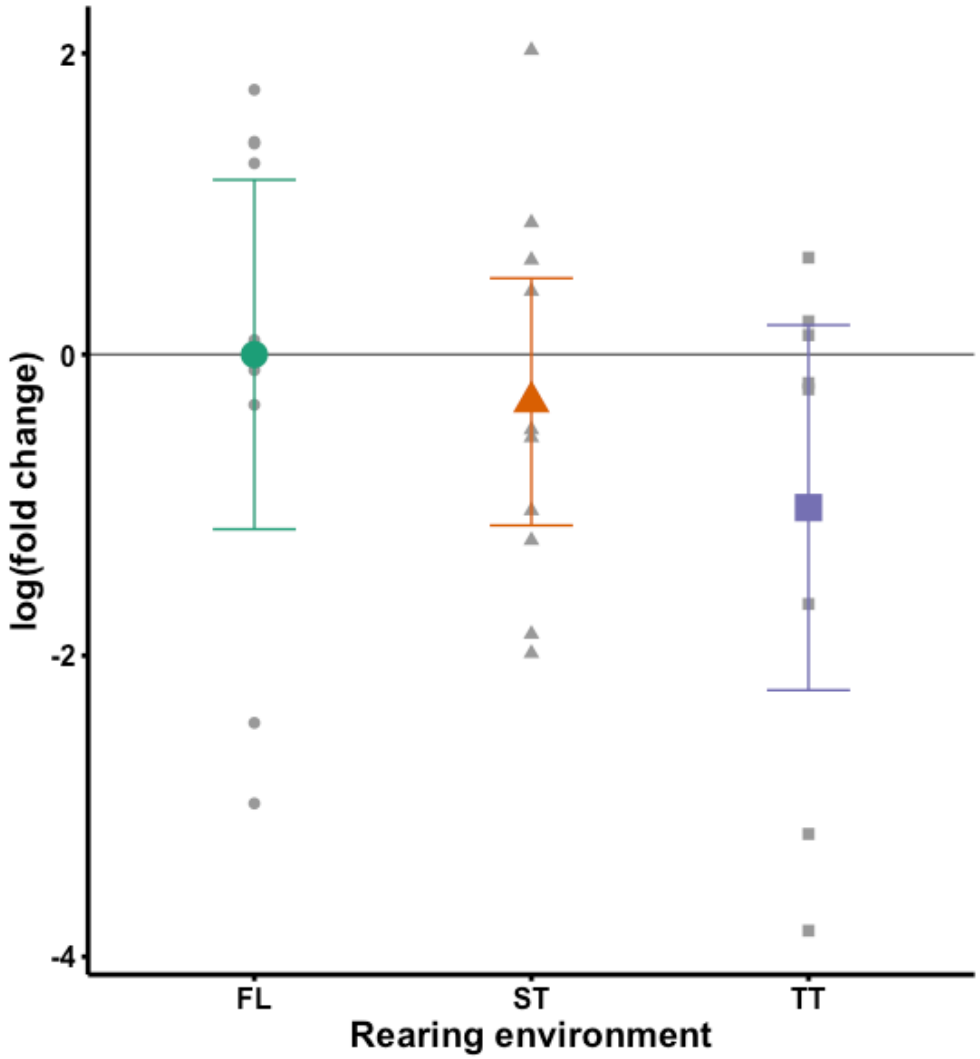

Figure 3.5. Log-transformed fold change of brain-derived neurotrophic factor (*BDNF*) gene expression in the hippocampus. Female Dekalb White pullets were reared on the floor (FL), in a single-tiered aviary (ST), or in a two-tiered aviary (TT) until 16 wk of age. Left hemisphere hippocampus was biopsied for real-time qPCR analysis of *BDNF* expression. Values are presented as mean ± 95% CI of the log-transformed fold change in colored shapes (green circle for FL, orange triangle for ST, and purple square for TT). FL served as the reference group because pullets in this rearing environment did not have access to elevated structures. Data points for individual animals are displayed in gray shapes corresponding to rearing environmen

Table 3.1. **Primer sequences used in qPCR**. Primers for each gene were designed on gene sequences specific to *Gallus gallus* (see NCBI Accession number) using the NCBI Primer-BLAST tool. Primer efficiency was determined by running a standard curve consisting of five 10-fold serial dilutions of purified PCR product. All primers were designed to be exon-spanning.

Gene (Abbreviation)	NCBI Accession number	Amplicon length (base pairs)	Efficiency (%)		Primer sequence
Beta actin (*ACTB*)	NM_205518.1	172	97.8	F	CTGACTGACCGCGTTACTCC
				R	CATACCAACCATCACACCCTGA
Brain-derived neurotropic factor (*BDNF*)	NM_001031616.1	147	92.8	F	TGAGACCAAATGCAACCCCA
				R	ATAAACCGCCAGCCAACTCT
Peptidylprolyl isomerase A (*PPIA*)	NM_001166326.1	122	98.1	F	GGGATTTGGCTACAAGGGCT
				R	CGGCAAACTTCTCCCCGTAA

Table 3.2. **Dendritic morphology parameters of hippocampal multipolar neurons from female Dekalb white pullets at 16 wk of age after being reared in different environments.** Values were analyzed with a natural log transformation and are presented as back-transformed estimates for each rearing environment: floor (FL; n = 12 pullets, 51 total neurons), single-tiered aviary (ST; n = 13 pullets, 54 total neurons), and two-tiered aviary (TT; n = 13 pullets, 52 total neurons). There were no significant differences for any morphology parameter across rearing environments (P > 0.05).

Morphology parameter	FL Estimate (95% CI)	ST Estimate (95% CI)	TT Estimate (95% CI)	P-value
Area under Sholl curve (AUC)	1342.6 (1184.3, 1521.9)	1329.2 (1171.3, 1508.4)	1335.2 (1180.0, 1510.9)	0.99
Proximal AUC (0 to 40 µm)	307.4 (288.8, 327.2)	298.4 (279.8, 318.2)	295.9 (278.1, 314.9)	0.59
Distal AUC (40 to 260 µm)	1004.8 (854.0, 1182.3)	1011.6 (858.5, 1192.0)	1017.1 (866.4, 1194.0)	0.99
Mean dendritic length (µm)	197.2 (174.8, 222.5)	207.9 (183.9, 235.0)	211.4 (187.6, 238.3)	0.64
Sum dendritic length (µm)	1686.7 (1488.9, 1910.9)	1668.8 (1472.0, 1892.0)	1684.2 (1489.5, 1904.3)	0.99
Dendrites, *n*	8.5 (8.0, 9.0)	8.1 (7.6, 8.6)	8.0 (7.5, 8.5)	0.23
Terminal tips, *n*	26.6 (24.3, 29.2)	24.9 (22.6, 27.3)	26.2 (24.0, 28.7)	0.44
Branching nodes, *n*	17.3 (15.1, 19.8)	16.1 (14.0, 18.5)	16.7 (14.6, 19.1)	0.68
Soma size (μm^2)	301.6 (282.1, 322.3)	306.5 (286.2, 328.2)	312.5 (292.5, 333.9)	0.69

Table 3.3. **Dendritic morphology parameters of hippocampal multipolar neurons from different hippocampal subregions from female Dekalb white pullets at 16 wk of age.** Values were analyzed with a natural log transformation and are presented as back-transformed estimates from subregions within the hippocampus: ventral (V, n = 9 pullets, 13 total neurons), dorsomedial (DM, n = 20 pullets, 35 total neurons), and dorsolateral (DL, n = 36 pullets, 109 total neurons). Within a row, back-transformed estimates having different superscripts are significantly different ($P < 0.05$).

Morphology parameter	V Estimate (95% CI)	DM Estimate (95% CI)	DL Estimate (95% CI)	P-value
Area under Sholl curve (AUC)	1671.0 (1373.2, 2033.5)^{A}	1290.3 (1140.3, 1460.1)^{ab}	1105.1 (1026.3, 1189.9)^{b}	0.0002
Proximal AUC (0 to 40 µm)	304.3 (272.0, 340.4)	304.4 (284.3, 325.9)	293.0 (282.2, 304.2)	0.56
Distal AUC (40 to 260 µm)	1358.6 (1051.8, 1754.8)^{A}	963.0 (819.9, 1131.2)^{ab}	790.2 (717.8, 869.8)^{b}	0.0002
Mean dendritic length (µm)	259.8 (212.9, 317.1)^{A}	182.5 (161.2, 206.6)^{b}	182.8 (170.1, 196.4)^{b}	0.005
Sum dendritic length (µm)	2061.3 (1701.5, 2497.1)^{A}	1607.9 (1424.3, 1815.3)^{ab}	1430.4 (1329.1, 1539.3)^{b}	0.001
Dendrites, *n*	8.1 (7.2, 9.0)	8.6 (8.1, 9.2)	7.9 (7.6, 8.1)	0.06
Tips, *n*	29.5 (25.2, 34.6)^{A}	24.7 (22.4, 27.2)^{ab}	23.8 (22.5, 25.1)^{b}	0.04
Branching nodes, *n*	20.8 (16.5, 26.3)^{A}	15.1 (13.0, 17.4)^{ab}	14.9 (13.7, 16.1)^{b}	0.03
Soma size (μm^2)	331.5 (295.0, 372.5)^{A}	318.4 (296.4, 342.0)^{a}	273.6 (262.8, 284.7)^{b}	<0.0001

Table 3.4. **Spearman's rho correlation coefficients or Mann-Whitney W-statistics for pairwise comparisons between neural outcomes (*BDNF* expression or morphology parameters from Golgi-Cox staining) and behaviors on a visual cliff or Y-maze cognitive task.** Values are for 16-wk-old female Dekalb white pullets.

Behavior	*BDNF* Expression	Mean dendritic length (μm)	Sum dendritic length (μm)	Dendrites (n)	Terminal tips (n)	Branching nodes (n)	Soma size (μm^2)
Visual Cliff							
Looking down at 30 cm (n)	r_s = 0.42 N = 26 p = 0.03*	r_s = -0.030 N = 38 p = 0.86	r_s = -0.0061 N = 38 p = 0.97	r_s = 0.082 N = 38 p =0.62	r_s = -0.029 N = 38 p = 0.86	r_s = 0.021 N = 38 p = 0.90	r_s = 0.034 N = 38 p = 0.84
Looking down at 90 cm (n)	r_s = 0.18 N = 26 p = 0.37	r_s = 0.28 N = 38 p = 0.09	r_s = 0.29 N = 38 p = 0.07	r_s = 0.051 N = 38 p = 0.76	r_s = 0.23 N = 38 p = 0.16	r_s = 0.26 N = 38 p = 0.11	r_s = -0.28 N = 38 p = 0.09
Crossing at 30 cm (yes/no)	W = 103 N = 26 p = 0.09	W = 173 N = 38 p = 0.76	W = 177 N = 38 p = 0.67	W = 164 N = 38 p = 0.98	W = 169.5 N = 38 p = 0.84	W = 176 N = 38 p = 0.69	W = 131 N = 38 p = 0.34
Crossing at 90 cm (yes/no)	W = 95 N = 26 p = 0.22	W = 137 N = 38 p = 0.84	W = 140 N = 38 p = 0.76	W = 111.5 N = 38 p = 0.52	W = 142 N = 38 p = 0.71	W = 153 N = 38 p = 0.45	W = 98 N = 38 p = 0.28
Latency to cross at 30 cm (s)	r_s = 0.24 N = 8 p = 0.58	r_s = 0.28 N = 14 p = 0.33	r_s = 0.36 N = 14 p = 0.21	r_s = -0.022 N = 14 p = 0.94	r_s = 0.40 N = 14 p = 0.16	r_s = 0.39 N = 14 p = 0.17	r_s = -0.35 N = 14 p = 0.22
Latency to cross at 90 cm (s)	r_s = -0.26 N = 8 p = 0.54	r_s = -0.15 N = 11 p = 0.65	r_s = -0.26 N = 11 p = 0.43	r_s = 0.032 N = 11 p = 0.93	r_s = -0.073 N = 11 p = 0.84	r_s = -0.046 N = 11 p = 0.89	r_s = 0.036 N = 11 p = 0.92
Y-maze							
Latency to exit maze (s)	r_s = 0.021 N = 15 p = 0.94	r_s = -0.29 N = 22 p = 0.20	r_s = 0.0096 N = 22 p = 0.97	r_s = 0.37 N = 22 p = 0.09	r_s = 0.014 N = 22 p = 0.95	r_s = -0.086 N = 22 p = 0.70	r_s = -0.10 N = 22 p = 0.65
Choice of short arm (yes/no)	W = 17 N = 15 p = 0.95	W = 37 N = 22 p = 0.97	W = 33 N = 22 p = 0.84	W = 42 N = 22 p = 0.64	W = 36 N = 22 p = 1	W = 32 N = 22 p = 0.77	W = 36 N = 22 p = 1

CHAPTER 4:

Providing height in the pullet rearing environment affects behavior during initial acclimation to a layer aviary, but not keel bone fractures, in an experimental setting

Manuscript in preparation for *Poultry Science*

ABSTRACT

Hens are increasingly housed in multi-tiered layer aviaries where they are at risk for falling from aviary structures and incurring keel bone fractures (KBF). Prior to moving into laying environments, birds are reared in pullet environments where spatial abilities are sensitive to development. We aimed to determine if providing height during rearing improved how hens used elevated space in layer aviaries, resulting in fewer KBF. Female Dekalb White pullets were reared in a floor pen (FL), single-tiered aviary (ST), or two-tiered aviary (TT; n = 5 pens/environment) through 16 wk of age. At 17 wk, rearing structures were replaced with layer multi-tiered aviaries. The location of hens and number and quality of vertical transitions were analyzed from video at 17 wk on the first and seventh d of aviary access (D1, D7) and 19, 23, and 27 wk. Eggs laid on the floor were collected weekly from 17 to 28 wk, and KBF prevalence and severity were scored from radiographs at 18, 26, 28, and 30 wk. On D1, more ST and TT hens utilized the aviary during the daytime ($p = 0.0077$), particularly higher zones ($p = 0.0019$), and were more active when searching for a roosting spot ($p < 0.0001$) than FL hens. FL hens made longer transitions during the evening and shorter transitions during the morning ($p = 0.02$) on D1. By D7, all hens were behaving similarly on most metrics, and this persisted to 27 wk. FL hens laid more floor eggs than ST and TT hens for the first 2 wk of lay ($p < 0.0001$).

Uncontrolled transitions were rare overall and not affected by rearing environment ($p > 0.05$). Similarly, rearing environment did not influence KBF prevalence or severity ($p > 0.05$). The experimental setting may have contributed to the rarity of uncontrolled transitions, so commercial trials are needed in future work. Overall, floor-reared hens needed a longer time to acclimate to layer aviaries than hens reared with height.
Key words: aviary, floor eggs, pullet, rearing environment, spatial abilities

INTRODUCTION

The majority of laying hens in the United States will need to be housed in cage-free environments by 2026 to meet requirements of recent state legislation as well as product demand from retailers, food service providers, and food manufacturers (United Egg Producers, 2021). Multi-tiered aviaries, which require hens to traverse elevated tiers and perches to access resources (e.g. feed, water, nests, roosting positions), are commonly used in cage-free housing. As hens move amongst the varying heights, they are at risk for falling from structures and colliding with objects or conspecifics, which could result in keel bone fractures (Stratmann et al., 2015a, 2019). Keel bone fractures are an animal welfare concern as they have been linked with reduced mobility, pain, neurological correlates for negative affective state, and lower egg production (Riber et al., 2018; Rufener et al., 2019a,b; Armstrong et al., 2020). Improving hens' movement in multi-tiered aviaries may be a strategy to mitigate the occurrence of keel bone fractures.

As the industry shifts towards cage-free housing, the legislative requirements and purchasing demands currently only apply to the adult layer environment. Before hens are moved into their

layer housing, they are reared in pullet environments for the first 16 to 18 wk of life. Hens' spatial abilities, meaning the way in which they orient in a three-dimensional environment (Wichman et al., 2007), are sensitive to development early in life. Experience-induced changes in visual, cognitive, behavioral, and musculoskeletal development in the pullet environment can modify how birds perceive and process spatial information and how they physically access different heights (Campbell et al., 2019). Hens reared in aviaries demonstrated improved working memory on a holeboard task (Tahamtani et al., 2015), utilized elevated structures more (Brantsæter et al., 2016), and developed better quality bones (Regmi et al., 2015; Casey-Trott et al., 2017b) than hens reared in cages. Aviary-reared hens also experienced fewer keel bone fractures throughout lay compared to cage-reared hens in layer enriched colony cages, which was attributed to the improved qualities of spatial abilities from rearing (Casey-Trott et al., 2017a).

However, considering that aviaries and cages represent vastly different environments, it is not clear which components of the rearing environment (e.g. structural enrichment, space allowance, group size, etc.) are most influential. Similar interpretation limitations apply to research comparing the effects of different types of cage-free rearing on behavior in layer environments. Colson et al. (2008) reported that hens reared in floor pens furnished with elevated platforms and perches used the highest levels of a multi-tiered layer aviary less, performed shorter flights, and laid more eggs outside of the nest than hens reared in multi-tiered pullet aviaries. The specific aspect of rearing design contributing to these outcomes cannot be identified as the three rearing treatments used in the previous study (enriched floor pens and two aviary designs) differed in floor and perch space allowances, group size, location of feed, and available height.

Research comparing the effects of specific features of rearing housing and their long-term implications for laying hen spatial abilities and keel bone integrity has focused mainly on perch availability and provision of ramps. For example, pullets reared in cages furnished with perches had larger muscles at the end of lay and higher keel mineral density during mid-lay than pullets reared in cages without perches, but keel bone fractures were not affected (Hester et al., 2013). Floor-reared pullets that had perches for the entire 16 wk rearing period were compared with floor-reared pullets that did not receive perches until 8 wk of age in a testing arena, where pullets jumped to platforms of different heights to obtain a food reward (Gunnarsson et al., 2000). Pullets reared without early access to perches were less likely to access the highest platforms, but neither behavior in aviaries nor keel bone integrity were evaluated (Gunnarsson et al., 2000). Hens reared with ramps to facilitate access to elevated structures in a single-tiered pullet aviary used elevated structures more, paced and crouched less before jumping, and experienced a lower prevalence of keel bone damage in a single-tiered layer aviary than hens reared without ramps (Norman et al., 2021). These studies offer promising, yet limited, findings that controlling for specific attributes of the rearing environment affects spatial behavior and keel bone integrity later in life. More research is warranted to evaluate specific components of cage-free rearing environments and their influence on spatial abilities and keel bone fractures in order to best prepare hens for multi-tiered aviary layer housing.

Our objective was to evaluate how experience with height in the pullet environment influenced the overall utilization (how much movement and use) and skill of using (quality of movements) elevated space, the prevalence of laying eggs on the floor instead of in the nest box, and the prevalence and severity of keel bone fractures. We were interested to explore if there was a

threshold of height that would best prepare birds for their layer aviaries and whether increasing the amount of height provided (i.e. single-tiered vs two-tiered pullet housing) would yield increasing benefits for the birds. As a result, we used three pullet rearing environment treatments: floor (minimal height), single-tiered pullet aviary (intermediate height), or two-tiered pullet aviary (more height than the single-tiered aviary). Building on previous research, we hypothesized that opportunities to interact with elevated structures during early life would improve spatial abilities of hens, leading to increased use of elevated resources and reduced keel injuries. We predicted that hens reared with access to elevated perches and tiers would utilize the layer aviary, particularly the highest structures, more than hens reared with minimal height, as indicated by the proportion of hens on the system and the number of vertical transitions. We further predicted that hens reared with access to elevated structures would experience fewer uncontrolled vertical transitions and make transitions that spanned larger amounts of height. We predicted that hens reared with elevated structures would lay fewer floor eggs (nest boxes were located within the aviary), experience a lower prevalence of keel bone fractures, and incur less severe fractures than hens reared with minimal height due to their patterns of layer aviary use. Lastly, we were interested in exploring the effects of time of d and age on the these behavioral, physiological, and production variables.

MATERIALS AND METHODS

All experimental procedures were reviewed and approved by the University of California, Davis Institutional Animal Care and Use Committee (Protocol #20307).

Pullet Housing and Management

A total of 835 Dekalb White pullets were obtained at 1 d of age from a commercial hatchery and randomly distributed across 15 pens (3.05 x 3.05 x 2.74 m, L x W x H) with 55 to 56 pullets/pen. Pens were located in one building at the Hopkins Avian Facility at University of California, Davis (Davis, CA) and contained structures for one of three rearing environment treatments (n = 5 pens/rearing environment): floor (**FL**), single-tiered aviary (**ST**), or two-tiered aviary (**TT**; Fig 1). To ensure that rearing environment treatments were distributed throughout the building, it was divided into five blocks of 3 pens/block. Treatments were balanced across blocks, such that one replicate of each treatment was randomly assigned to a pen location within each block.

Pullet environments (FL, ST, and TT) varied primarily in the amount of height available, such that perching availability, space allowance, and group sizes were consistent across pens. All pullets had access to four round metal perches, each with a 3.8 cm diameter and measuring 121.9 cm in length. The top of the four perches in FL pens were 10.5 cm from the litter to provide pullets with the experience of gripping a perch but minimal height. ST pens contained a single-tiered aviary structure, where three of the perches were elevated (35.4 cm height for two perches and 64.7 cm height for one perch) alongside one elevated tier with a plastic slatted surface (61.0 x 121.9 x 62.9 L x W x H; Dura-Slat Poultry and Kennel Flooring, Southwest Agri-Plastics, Inc., Addison, TX). A wire mesh ramp was provided to facilitate access to the tier (96.5 x 31.8 cm, 40-degree angle; McNichols Wire Mesh, McNichols Co., Inc., Livermore, CA). The fourth perch was placed adjacent to the structure at 10.5 cm height, identical to FL pens. The two-tiered aviary structure in the TT pens contained three elevated perches located at heights of 29.4, 89.9, and 125.7 cm, as well as two elevated tiers with plastic slatted surfaces (30.5 x 121.9, L x W)

located 62.9 cm and 123.8 cm high. A wire mesh ramp facilitated access to both tiers (190.5 x 31.8 cm, 40-degree angle). A 10.5 cm high perch was provided next to the two-tiered aviary structure. In order to keep floor space allowance constant across all three rearing environments, chicken wire was used to prevent pullets from accessing the litter area directly underneath the tiers of the aviary (9.3 m^2 total floor space/pen).

The group size of 55 to 56 pullets/pen resulted in 8.9 cm/pullet of perch space and 0.17 m^2/pullet of floor space from 0 to 8 wk of age. During the 8th wk of age, approximately 10 pullets/pen were removed as part of data collection procedures for another component of this project (Lu et al., 2021) to create a group size of 45 pullets/pen until 16 wk. From 8 to 16 wk, pullets had 10.8 cm/pullet of perch space and 0.21 m^2/pullet of floor space. During the 16th wk of age, another 10 pullets/pen were removed resulting in a group size of 28 to 30 pullets/pen at the end of the pullet housing period (16.2 to 17.4 cm/pullet of perch space and 0.31 to 0.33 m^2/pullet of floor space).

To control for visual experiences with rearing environments, heavy-duty, plastic tarps (3.05 x 1.83 m, L x W; Everbilt, The Home Depot, Inc., Atlanta, GA) were hung on pen walls and doors to reduce visibility into adjacent pens and the hallway used by husbandry personnel and researchers. We could not cover the top half of the pen (0.91 m between where the tarp ended and the ceiling) so that ventilation through the building was not obstructed. At approximately 8 wk of age, TT birds standing on the highest tier in their pen could see over the tarp, experiencing additional visual cues that pullets in ST and FL rearing environments did not have.

All pens contained pine wood shavings for litter substrate (Mallard Creek Inc., Rocklin, CA), one automatic water line (Lubing USA, Cleveland, TN; 12 nipples/pen) for *ad libitum* water access, and two 13.6 kg round feeders/pen (52 cm circumference/feeder) for *ad libitum* feed access (Purina Start and Grow Medicated Crumbles, Purina Animal Nutrition LLC, Gray Summit, MO). An electric wire was attached to the top of the water line to prevent perching on the line. The artificial lighting schedule was maintained according to the Dekalb White Product Guide (Dekalb, The Netherlands) using lights directly above pens and in the adjacent hallway. Lights in the hallway were turned off 30 min prior to lights above the pen to create a dimming effect. Natural lighting was also available through the translucent plastic curtains permanently fitted to the sides of the building. Husbandry personnel adjusted pen resources if needed during their routine daily check.

Layer Housing and Management

At 17 wk of age, birds from each pen were caught and placed into transportation coops (Transportation Game Bird and Poultry Coop-10, Kuhl Corporation, Flemington, NJ) and all rearing structures were removed from the pen and replaced with a multi-tiered layer aviary (Fig 4.2). The birds were then released back into their home pen (28 to 30 birds/pen). Changing the housing structures took place over a period of two consecutive days by block, such that 3 pens/rearing environment were changed on the first d and 2 pens/rearing environment were changed on the second d.

Regardless of rearing treatment, all hens had access to the same style of layer aviary. The aviary contained five metal perches (121.9 cm L, 3.8 cm DIA) installed at four different heights (one

perch at 50.0, 125.7, and 181.1 cm and two perches at 245.3 cm from the floor) and three elevated tiers at 69.2, 137.2, and 198.2 cm from the floor. Taken together, hens had access to 20 cm/hen of perch space. The bottom tier contained a plastic slat area (121.9 x 30.5 cm, L x W; Dura-Slat Poultry and Kennel Flooring, Southwest Agri-Plastics, Inc., Addison, TX) in front of a colony nest (121.9 x 30.5 cm, L x W; Large Reversible Roll Out Chicken Nest Box, Best Nest Box, Hudson, OH). The middle and top tier contained plastic slats only (121.9 x 61 cm, L x W; Lubing USA, Cleveland, TN). Considering tiers and the litter floor, hens had access to a total floor space of 11.2 m^2 (0.37 to 0.40 m^2/hen for 28 to 30 hens/pen).

As was the case in pullet housing, pine wood shavings covered the entire floor area (4 cm depth; Mallard Creek Inc., Rocklin, CA). Water and feed (16% Protein Layer Mini-Pellet, Bar Ale Inc., Williams, CA) were provided with the same equipment previously described. Lighting schedule was initially maintained with artificial and natural lighting according to the Dekalb White Product Guide, but the way in which light was delivered was modified to manage aggressive pecking behavior. Aggression-related wounds on combs, wattles, and feet were first noted at 18 wk of age. Artificial lights directly above pens were turned off at 19 wk, 5 d prior to video recording for this age, to dim light intensity. Natural light and artificial lights in the hallways maintained the schedule recommended by the Dekalb White Product Guide. Pecking blocks (11.3 kg Flock Block Premium Poultry Supplement, Purina Animal Nutrition LLC, Gray Summit, MO) were added to each pen at 20 wk of age to provide additional foraging opportunities. At 25 wk of age, artificial lighting in the hallway was turned off and only natural lighting was used via the translucent plastic curtains permanently fitted to the sides of the

building (14 h light, 10 hr dark; https://gml.noaa.gov/grad/solcalc/). Husbandry procedures were the same as for pullets with the addition of daily egg collection.

Behavior Data Collection

Each pen was video recorded continuously across 2 d at 17, 19, 23, and 27 wk of age using three video cameras/pen (4K Ultra HD IP Security Camera, Lorex Corporation, Irvine, CA) connected to a network video recorder with pentaplex operation (4K Ultra HD Security NVR, Lorex Corporation, Irvine, CA). At 17 wk of age, we recorded video on the first (D1) and seventh (D7) full d after all aviaries were installed with the goal of assessing acclimation during the first wk post-transfer. At 19, 23, and 27 wk, video was recorded on 2 consecutive d.

Focal Hen Vertical Transitions. When pullets were placed at 1 d of age, 10 focal pullets/pen (N = 150 focal pullets) were randomly selected and individually marked with non-toxic food coloring (Student Kit Soft Gel Paste Food Color, AmeriColor Corp., Placentia, CA). The markings were touched up as needed, with non-toxic food coloring for down feathers and non-toxic livestock marker (Markal® All-Weather Paintstik Livestock Marker, LA-CO Industries, Inc., Elk Grove Village, IL) once primary feathers developed. A single focal pullet (FL treatment) died during the rearing period; therefore, there were 149 total focal hens when the layer period began. The behavior of focal hens was assessed based on continuous sampling of 3 h of video/d (2 d/age), including a morning h (immediately after artificial lights turned on), midday h (1 to 2 PM at 17 and 19 wk, 12 to 1 PM at 23 and 27 wk), and evening h (immediately before artificial lights turned off). At 27 wk, natural light was used instead of artificial light, as described above. Therefore, morning was coded as 1 h immediately after cameras across all pens

changed from black and white to color (> 1 lux), and evening was coded as 1 h immediately before cameras across all pens changed from color to black and white (≤ 1 lux).

Focal hen observations focused on the occurrence of vertical transitions. Following Rufener et al. (2019a), we defined a vertical transition as a movement that results in the hen's location changing from one zone to another and staying in the destination zone for 1 second or longer. Based on the locations of perches and tiers, we specified five distinct zones within the multi-tiered layer aviary (Fig 4.2). The litter zone delineated the floor area. Zone A included the bottom tier, nest box, and lowest elevated perch. Zone B contained the middle tier and associated elevated perch. Zone C contained the top tier and associated elevated perch. Finally, Zone D contained the two perches located at the top of the aviary. When a focal hen made a vertical transition, the following criteria were identified: hen ID, origin and destination zone, destination surface (system perch, nest box perch, tier, or litter), and whether the transition was controlled or uncontrolled. A transition was considered controlled if a focal hen landed on both feet in an upright body posture without excessive wing flapping (Stratmann et al., 2015a). If her feet were not visible, then landing in an upright body position with her body centered over her feet was also considered controlled. An uncontrolled transition occurred when a focal hen landed on one foot, contacted the landing surface with another body part other than her feet (most commonly the chest, abdomen, or a wing), performed excessive wing flapping, made physical contact with a pen structure or another hen during the transition (for less than 1 sec) or at the end of the transition, or various combinations of these characteristics. If the landing was out of view due to the camera angle, then the transition control was marked as unidentifiable but origin and destination zones were still recorded, if visible. A total of 18 observers were trained to identify

focal hen vertical transitions and score their criteria on 2 h of video (N = 100 focal hen transitions). Inter-observer agreement was high for identifying the number of focal hen transitions and their criteria (≥ 90%). Uncontrolled transitions were rare, so observer reliability for this criteria was also assessed using a 55-question video test (24 controlled, 31 uncontrolled) for 8 of the 18 observers. Uncontrolled transition reliability was ≥ 0.82 (Cohen's kappa; irr package, version 0.84.1, Gamer et al., 2019) for this subsample of observers. They also spot-checked 1 h of video for each of the observers that did not complete the test for additional verification of agreement on uncontrolled transitions. Trained observers were blind to rearing environment treatment, randomly assigned video from different rearing environments and ages, viewed videos for analysis using either IINA video player (version 1.1.2, https://iina.io/) or Behavioral Observation Research Interactive Software (BORIS, version 7.10.7, http://www.boris.unito.it/, Friard and Gamba, 2016), and entered data into a Microsoft Excel Workbook (version 16.61.1, Microsoft Corporation, Redmond, WA). Inter-observer agreement was spot-checked to ensure ≥ 90% throughout the observers' tenure in the data collection.

Location of Hens at the Group Level. We used group level observations (including focal and non-focal hens) to quantify the distribution of all birds within each aviary. We counted the number of hens within each zone using instantaneous scan sampling. Scans occurred at 30-min intervals during the d. Due to change in the length of the photoperiod, there were 27 intervals at 17 wk and 29 intervals at 19, 23, and 27 wk to represent daytime use. Preliminary video analysis indicated that, once birds settled into a zone for their nighttime roosting position, they did not move from that zone until the lights came on. Therefore, only two scan samples, taken 4 h apart, were completed during the nighttime. A hen was counted as being in a zone when the majority

(over half) of her body was in the zone at the time of the scan, regardless of whether she was stationary or moving within or across zones. The number of birds in each zone were counted by two trained observers. Inter-observer reliability was determined from both observers scoring 1 d of video (all daytime and nighttime intervals) for 3 pens (1 pen/rearing environment), and they achieved 0.99 reliability (intraclass correlation coefficient; irr package, version 0.84.1). One of the observers, who was blind to rearing treatment, analyzed all 19, 23, and 27 wk video in a randomized order for rearing environment and age to prevent order bias. The second observer was not completely blind to rearing environment as they were involved in the experimental design, but they could not readily identify the rearing environment associated with the majority of pen numbers. This observer analyzed all 17 wk video in a randomized order for rearing environment. Both observers viewed videos for analysis using VLC media player (version 3.0.16, VideoLAN, Paris, France) and entered data into a Microsoft Excel Workbook (version 16.61.1, Microsoft Corporation, Redmond, WA).

Floor Eggs

Eggs were collected once daily in the afternoon by husbandry personnel from 17 to 28 wk of age. The first eggs were laid during wk 17 (1 egg from a FL pen and 1 egg from a ST pen), and hens across all pens were laying eggs during wk 18. Egg location data was recorded on 4 d/wk from 18 to 27 wk and 3 d during wk 28, for a total of 47 d/pen. For each pen, the number of eggs collected from the litter area (i.e. floor eggs), the nest box, and the aviary tiers were recorded. Days were excluded from the dataset if the nest box perch was left up from egg collection on the previous d and consequently prevented hens from accessing the nest box (N = 4 d for 1 FL pen, 2 d for 1 ST and 1 TT pen, 1 d for 1 ST pen); if the husbandry personnel forgot to fill out the

datasheet (N = 1 d for 3 FL, 1 ST, and 2 TT pens, 2 d for 1 ST pen); as well as 2 d during 23 wk for all pens when datasheets were not available in the barn.

Keel Bone Fracture Assessment

At 18, 26, 28, and 30 wk, all of the focal hens were captured by pen for radiography, which was conducted in the same building where hens were housed. The radiograph was taken using a portable X-ray unit (Citation Digital Radiography, MyVet Imaging, Inc., Closter, NJ; Battery Powered Portable X-ray System, Poskom, Goyang, South Korea; Xmaru 1012 WCC/WGC Flat Panel Detector, Rayence, Closter, NJ) at a film-focus distance of 60 cm and voltage of 50 kV/2.5 mAs. Each focal hen was briefly inverted to place her feet into a poultry shackle, inducing immobility in order to capture the radiograph (Širovnik and Toscano, 2017). This procedure took approximately 10 sec/hen. Once all focal hens from a pen were radiographed, they were released back into their home pen. The radiography procedure was repeated for all pens on a single d at 18, 26, and 28 wk. At 30 wk, focal hens were radiographed over the course of 9 d to accommodate data collection for another portion of the larger project involving the same flock of hens (Jones, 2021; Rufener et al., 2021). The order of rearing environment was randomized within a d at all ages to prevent order bias, as well as across d at 30 wk. Due to focal hen mortality, the number of focal hens slightly varied at each age (18 wk, N = 149; 26 wk, N = 145; 28 and 30 wk, N = 143 focal hens).

Radiographs were downloaded as DICOM files (N = 580 radiographs), converted to JPEG files, then scored by one trained observer for the presence (binary variable; fracture, no fracture) and severity of fractures (continuous variable) as described in Rufener et al. (2018). Briefly, a tagged

visual analogue scale (tVAS) was used to quantify aggregate fracture severity, which was defined as the total amount of bone affected by fractures. The tVAS scores ranged from 0.0 to 10.0 to correspond six visual analogue tags ranging from no fracture to extremely severe. Reliability between the observer and the trainer on 80 radiographs was 0.96 (intraclass correlation coefficient; irr package, version 0.84.1). The observer was blind to rearing environment and scored radiographs in an order randomized by rearing environment and age to prevent order bias.

Statistical Analysis

All statistical analyses were conducted in R statistical software (version 4.2.0; R Core Team, 2021) using RStudio (version 2022.02.2+485) for macOS Catalina 10.15.7. All model fits were assessed for deviations from their expected distribution, overdispersion, outliers, and homogeneity of variance via plot and test functions in the DHARMa package (version 0.4.5; Hartig, 2022) for generalized linear mixed models or sjPlot package (version 2.8.10; Lüdecke, 2021) for linear mixed models. For all analyses, the final models were obtained by a stepwise backwards reduction using ANOVA for model comparison with a p-value of > 0.05 as the criterion of exclusion. The significance of rearing environment and age will only be reported if their interaction term was not significant. Model estimates and 95% confidence intervals were obtained with the effects package (version 4.2.1, Fox and Hong, 2009).

Number of Focal Hen Vertical Transitions. The total number of vertical transitions/h/d was summarized for each focal hen and analyzed with generalized linear mixed models (glmmTMB package, version 1.1.3, Brooks et al., 2017) with a negative binomial distribution. At

17 wk, separate models were run for each h (morning, afternoon, and evening) where rearing environment (factor with 3 levels: FL, ST, and TT), days in the aviary (factor with 2 levels: D1, D7), and their interaction were included as fixed effects, with focal hen ID nested in pen as a random effect. For the remaining ages, separate models were also run for each h with rearing environment (factor with 3 levels: FL, ST, and TT), age (factor with 3 levels: 19, 23, and 27 wk), and their interaction included as fixed effects and focal hen ID nested in pen as a random effect.

Uncontrolled Transitions. Data were summarized as the number of uncontrolled transitions/d/pen. A generalized linear mixed model (glmmTMB package, version 1.1.3, Brooks et al., 2017) with a negative binomial distribution was used with separate models for 17 and 19 to 27 wk. Rearing environment (factor with 3 levels: FL, ST, and TT), age (17 wk model: days in the aviary as a factor with 2 levels, D1 and D7; 19 to 27 wk model: age as a factor with 3 levels, 19, 23, and 27 wk), and their interaction were assessed as fixed effects. Two random effects were included: the pen and the log of the total number of transitions/d/pen with an identifiable landing as an offset.

Number of Zones Crossed during a Vertical Transition. For each vertical transition (N = 19,709 total focal hen vertical transitions across all ages), the number of zones crossed was calculated by subtracting the destination zone number from the origin zone number, resulting in 1, 2, 3, or 4 zones crossed during a vertical transition. Vertical transitions were also classified as ascending (N = 12,499 focal hen vertical transitions) or descending (N = 7,210 focal hen vertical transitions). During data exploration, we found that 99.7% of ascending transitions crossed 1 zone and the remaining 0.3% crossed 2 zones. Descending transitions had a greater distribution

of transitions over the number of zones crossed (1 zone: 57.0%, 2 zones: 21.5%, 3 zones: 20.5%, and 4 zones: 1.0% of descending transitions). Therefore, ascending transitions were excluded from further analysis. For descending transitions, data were dichotomized as crossing 1 zone vs. crossing > 1 zone and analyzed using two generalized linear mixed models (lme4 package, version 1.1.29; Bates et al., 2015) with a binomial distribution. The two models represented 17 wk and 19 to 27 wk data, where the effect of rearing environment (factor with 3 levels: FL, ST, and TT), age (17 wk model: days in the aviary as a factor with 2 levels, D1 and D7; 19 to 27 wk model: age as a factor with 3 levels, 19, 23, and 27 wk), h (factor with 3 levels: morning, afternoon, evening), and their interactions were assessed. Focal hen ID nested in pen was included as a random effect.

Location of Hens on the Aviary. We calculated the total number and average proportion of hens utilizing the aviary/d/pen during daytime and nighttime observations. Of the hens utilizing the aviary, we also calculated the proportion of hens in each zone. The proportion of hens utilizing the aviary and proportion of aviary-using hens in each zone were analyzed with generalized linear mixed models (glmmTMB package, version 1.1.3, Brooks et al., 2017) with a β distribution. Separate models were fitted for daytime vs. nighttime data. Within each time of d, separate models were also used for each proportion (total, Zone A, Zone B, Zone C, and Zone D proportions). Each model assessed the effect of rearing environment (factor with 3 levels: FL, ST, and TT), age (17 wk model: days in the aviary as a factor with 2 levels, D1 and D7; 19 to 27 wk model: age as a factor with 3 levels, 19, 23, and 27 wk), and their interaction, with pen included as a random effect.

Floor Eggs. For each pen, the number of floor eggs and the total number of eggs laid were summarized across days for each wk in order to calculate one proportion of floor eggs laid/wk/pen. The prevalence of system eggs (i.e. eggs laid on aviary tiers) was rare (0.15% of all eggs collected throughout the study); these were not included in the count of floor eggs. The proportion of floor eggs were analyzed using a generalized linear mixed model (glmmTMB package, version 1.1.3, Brooks et al., 2017) with a β distribution that included rearing environment (factor with 3 levels: FL, ST, and TT), age (continuous, 11 timepoints from 18 to 28 wk), and their interaction, with pen included as a random effect.

Keel Bone Fractures. Keel bone fractures were not detected on any of the focal hens at 18 wk, therefore this age was excluded from the analysis to improve the model fit. Keel bone fracture prevalence was analyzed with a generalized linear mixed model (lme4 package, version 1.1.29; Bates et al., 2015) with a binomial distribution. For keel bone fracture severity, radiographs that did not have a fracture (score of 0) were excluded, and a linear mixed model (lme 4 package, version 1.1.29; Bates et al., 2015) was used. Both models included rearing environment (factor with 3 levels: FL, ST, and TT), age (factor with 3 levels: 26, 28, and 30 wk), and their interaction, with focal hen nested within pen as a random effect.

RESULTS

Number of Focal Hen Vertical Transitions

During initial acclimation at 17 wk, rearing environment did not affect the number of focal hen vertical transitions in the morning ($\chi^2 = 4.99$, df = 2, $p = 0.083$), but it did affect the number of

transitions that occurred in the afternoon and evening (Fig 4.3). In the afternoon, TT focal hens made approximately 2 fewer vertical transitions/h than ST and FL hens across days ($\chi^2 = 10.52$, df = 2, $p = 0.0052$). In the evening, rearing environment and days in the aviary had an interactive effect on the number of focal hen vertical transitions ($\chi^2 = 22.46$, df = 2, $p < 0.0001$). ST and TT hens made 3 times more vertical transitions than FL hens on D1, then decreased by D7 to a similar number as FL hens. Between D1 and D7, all hens slightly decreased their activity by 1 vertical transition/h in the morning ($\chi^2 = 4.23$, df = 1, $p = 0.04$), and all hens slightly increased their activity by a similar magnitude in the afternoon ($\chi^2 = 12.52$, df = 1, $p = 0.0004$). There was no interactive effect between days in the aviary and rearing environment in the morning ($\chi^2 = 2.07$, df = 2, $p = 0.36$) or afternoon ($\chi^2 = 2.38$, df = 2, $p = 0.30$).

From 19 to 27 wk, the number of vertical transitions during morning and afternoon hours were not affected by rearing environment (morning: $\chi^2 = 2.65$, df = 2, $p = 0.27$; afternoon: $\chi^2 = 2.99$, df = 2, $p = 0.22$) or an interaction between rearing environment and age (morning: $\chi^2 = 2.36$, df = 4, $p = 0.67$; afternoon: $\chi^2 = 7.98$, df = 4, $p = 0.092$; Fig 4.4). However, in the evening, ST and TT hens performed nearly twice the number of vertical transitions as FL hens at 23 wk only ($\chi^2 = 12.50$, df = 4, $p = 0.014$). In the morning, all hens slightly decreased their activity by approximately 1 transition/h from 19 to 23 wk, then increased their activity by the same magnitude between 23 to 27 wk ($\chi^2 = 33.55$, df = 2, $p < 0.0001$). In the afternoon, all hens slightly decreased their activity over time ($\chi^2 = 18.75$, df = 2, $p < 0.0001$), such that hens performed 1 transition/h less by 27 wk than they performed at 19 wk.

Uncontrolled Transitions

Uncontrolled transitions were rare (N = 404 uncontrolled transitions out of 19,512 focal hen vertical transitions with an identifiable landing across all ages, or 2.07% of transitions). At 17 wk, neither rearing environment ($\chi^2 = 2.23$, df = 2, $p = 0.33$) nor its interaction with days in the aviary ($\chi^2 = 1.78$, df = 2, $p = 0.41$) affected the rate of uncontrolled transitions/d/pen. Uncontrolled transitions decreased between D1 and D7 ($\chi^2 = 10.95$, df = 1, $p = 0.00093$; estimated mean [95% CI]; D1: 8.03 [6.22, 10.36], D7: 3.99 [2.81, 5.67] uncontrolled transitions/d/pen). From 19 to 27 wk, TT hens displayed approximately 1 more uncontrolled transition/d/pen than ST hens ($\chi^2 = 6.42$, df = 2, $p = 0.04$; FL: 2.87 [2.11, 3.89], ST: 2.21 [1.63, 2.98], TT: 3.69 [2.89, 4.72] uncontrolled transitions/d/pen). All hens experienced 1 fewer uncontrolled transitions/d/pen between 19 to 23 wk and maintained a similar level to 27 wk ($\chi^2 = 7.17$, df = 2, $p = 0.028$; 19: 3.77 [2.98, 4.78], 23: 2.26 [1.65, 3.10], 27: 2.74 [2.01, 3.72] uncontrolled transitions/d/pen). There was no interactive effect between rearing environment and age on uncontrolled transitions ($\chi^2 = 8.43$, df = 4, $p = 0.077$).

Descending Transitions Crossing More than One Zone

There was a three-way interactive effect of rearing environment, days in the aviary, and h of the d on the proportion of descending transitions crossing more than one zone during the first wk of layer aviary housing ($\chi^2 = 11.64$, df = 4, $p = 0.02$; Table 4.1). Between D1 and D7, FL hens increased their proportion of morning transitions crossing more than one zone and decreased their proportion of evening transitions crossing more than one zone. FL hens were consistent across days in the afternoon, and ST and TT hens remained consistent across days for all hours.

From 19 to 27 wk, rearing environment had a main effect on the proportion of descending transitions crossing more than 1 zone, such that FL hens displayed a higher proportion of these transitions than ST hens across ages ($\chi^2 = 7.05$, df = 2, $p = 0.029$; Table 4.1). Age and h of the d had an interactive effect ($\chi^2 = 75.67$, df = 4, $p < 0.0001$), where the proportion of descending transitions crossing more than 1 zone remained consistent across ages for afternoon and evening but sharply decreased at 27 wk in the morning. Furthermore, the hens made lowest proportion of transitions crossing more than 1 zone in the morning compared to the afternoon and evening. There was no interactive effect between rearing environment and age ($\chi^2 = 4.27$, df = 4, $p = 0.37$), rearing environment and h of the d ($\chi^2 = 6.12$, df = 4, $p = 0.16$), nor a three-way effect amongst rearing environment, age, and h of the d ($\chi^2 = 7.09$, df = 8, $p = 0.53$).

Destination Surface of Descending Transitions: Descriptive Summary

At 17 wk, there was a total of 1,839 descending transitions across all pens (n = 481 for FL, 721 for ST, and 637 descending transitions for TT). For descending transitions crossing 1 zone, the litter area was the landing surface for the majority of movements (96.7 for FL, 83.5 for ST, and 78.5 % of descending transitions for TT). However, hens also landed on the tier (3.3 for FL, 11.9 for ST, and 19.4 % of descending transitions for TT), and only ST and TT hens landed on perches (4.6 for ST and 2.1 % of descending transitions for TT). For descending transitions crossing more than 1 zone, the landing surface was overwhelmingly the litter area for all rearing environments (99.6 for FL, 99.4 for ST, and 100 % for TT). FL and ST hens had 1 and 2 descending transitions crossing more than 1 zone, respectively, landing on a tier (0.4 for FL and 0.6 % of descending transitions for ST).

From 19 to 27 wk, there were 5,371 total descending transitions (n = 1,444 for FL, 2,163 for ST, and 1,764 for TT). For descending transitions crossing 1 zone, the litter continued to be the main landing surface (FL: 86.2, ST: 73.5, TT: 67.4 % of descending transitions), followed by the tier (FL: 13.3, ST: 23.2, TT: 30.0 %). Perches as a landing surface accounted for 0.1% of FL descending transitions but represented 3.2% and 2.4% of ST and TT descending transitions, respectively. The nest box perch as a landing surface also accounted for a small proportion of transitions for all treatments (FL: 0.4, ST: 0.1, and TT: 0.2 %). Descending transitions crossing more than 1 zone mostly landed on the floor, similar to 17 wk (FL: 99.6, ST: 99.9, and TT: 99.7 %). The tier accounted for a small proportion of landings for these transitions spanning more zones (FL: 0.4, ST: 0.1, and TT: 0.3 %).

Location of Hens on the Aviary

In the first wk of aviary access, there was an interactive effect between rearing environment and days in the aviary for the proportion of hens using the aviary during the daytime ($\chi^2 = 9.72$, df = 2, $p = 0.0077$). More ST and TT hens were observed on the aviary than FL hens on D1 and D7, but FL hens increasingly used the aviary by D7 (Fig 4.5). Of the hens using the aviary in the daytime, there were interactive effects between rearing environment and days in the aviary for Zones A and C (Fig 4.6). A higher proportion of FL aviary-using hens were observed on Zone A on D1 compared to ST and TT hens, but FL hens reduced use of Zone A by D7 ($\chi^2 = 6.30$, df = 2, $p = 0.043$). FL aviary-using hens had the lowest proportion of birds in Zone C on D1 compared to ST and TT hens, but FL hens increased use of Zone C by D7 ($\chi^2 = 12.55$, df = 2, $p = 0.0019$). Across days, there was a higher proportion of FL aviary-using hens in Zone B than ST and TT aviary-using hens ($\chi^2 = 7.57$, df = 2, $p = 0.023$). Across all rearing environments, fewer hens were

observed on Zone B by D7 ($\chi^2 = 13.86$, df = 1, $p = 0.0002$). There was no interactive effect between rearing environment and days in the aviary for Zone B ($\chi^2 = 2.69$, df = 2, $p = 0.26$). Zone D use was minimal, and there was no effect of rearing environment ($\chi^2 = 3.45$, df = 2, $p = 0.18$), days in the aviary ($\chi^2 = 2.30$, df = 1, $p = 0.13$), or their interaction ($\chi^2 = 0.048$, df = 2, $p = 0.98$) on the proportion of aviary-using hens observed in this zone.

For nighttime aviary use during the first wk of placement, there was an interactive effect between rearing environment and days in the aviary for the proportion of hens using the aviary ($\chi^2 = 9.68$, df = 2, $p = 0.0079$). FL and ST hens increasingly used the aviary for nighttime roosting by the end of the first wk, and TT hens remained consistent (Fig 4.5). There was also an interactive effect between rearing environment and days in the aviary for the proportion of aviary-using hens observed roosting in Zone B and Zone C (Fig 4.7). A higher proportion of FL aviary-using hens roosted in Zone B on D1 than ST and TT hens, but fewer FL hens used Zone B by D7 ($\chi^2 = 9.55$, df = 2, $p = 0.0084$). For Zone C, FL hens increasingly used the zone by the end of the first wk, but ST and TT hens remained consistent ($\chi^2 = 7.17$, df = 2, $p = 0.028$). Across days, a higher proportion of aviary-using TT hens roosted in Zone D compared to FL hens, with ST hens intermediate ($\chi^2 = 14.54$, df = 2, $p = 0.0007$). There was no effect of days in the aviary ($\chi^2 = 3.35$, df = 1, $p = 0.067$) or their interaction with rearing environment ($\chi^2 = 4.52$, df = 2, $p = 0.10$) for hens using Zone D. The proportion of aviary-using hens in Zone A was not influenced by rearing environment ($\chi^2 = 0.87$, df = 2, $p = 0.65$), days in the aviary ($\chi^2 = 1.09$, df = 1, $p = 0.30$), or their interaction ($\chi^2 = 3.71$, df = 2, $p = 0.16$).

From 19 to 27 wk, there was no effect of rearing environment or its interaction with age on the proportion of hens using the aviary during the d (rearing: $\chi^2 = 4.35$, df = 2, $p = 0.11$; interaction: $\chi^2 = 7.96$, df = 4, $p = 0.093$) or for aviary-using hens found in Zone A (rearing: $\chi^2 = 4.32$, df = 2, $p = 0.12$; interaction: $\chi^2 = 2.48$, df = 4, $p = 0.65$), Zone C (rearing: $\chi^2 = 5.88$, df = 2, $p = 0.053$; interaction: $\chi^2 = 6.98$, df = 4, $p = 0.14$), and Zone D (rearing: $\chi^2 = 2.82$, df = 2, $p = 0.24$; interaction: $\chi^2 = 5.00$, df = 4, $p = 0.29$). As hens aged, fewer hens were observed on the aviary during the daytime ($\chi^2 = 105.44$, df = 2, $p < 0.0001$; Fig 4.8), and aviary-using hens increased use of Zone A ($\chi^2 = 61.64$, df = 2, $p < 0.0001$) and Zone D ($\chi^2 = 23.39$, df = 2, $p < 0.0001$) but decreased use of Zone C ($\chi^2 = 14.53$, df = 2, $p = 0.0007$; Fig 4.9). For Zone B, there was an interactive effect between rearing environment and age ($\chi^2 = 14.58$, df = 4, $p = 0.0056$), such that generally fewer aviary-using hens used this zone over time, but the proportion of ST hens remained consistent between 23 and 27 wk and the proportion of FL and TT hens continued to decrease.

At nighttime, the interaction between rearing environment and age ($\chi^2 = 7.10$, df = 4, $p = 0.13$), as well as the main effect of rearing environment ($\chi^2 = 2.18$, df = 2, $p = 0.34$) did not affect the proportion of hens using the aviary. As they aged from 19 to 27 wk, a greater proportion of hens were observed using the aviary for nighttime roosting ($\chi^2 = 98.06$, df = 2, $p < 0.0001$; Fig 4.8). Fewer hens were observed in Zone A (interaction: $\chi^2 = 11.54$, df = 4, $p = 0.021$) and Zone B (age: $\chi^2 = 82.21$, df = 2, $p < 0.0001$) over time, but ST and TT hens showed the steepest drop in using Zone A (Fig 4.10). More hens were observed roosting in Zone C between 23 to 27 wk ($\chi^2 = 32.78$, df = 2, $p < 0.0001$), but there were more FL aviary-using hens in Zone C than ST and TT hens ($\chi^2 = 7.68$, df = 2, $p = 0.021$). For Zone D, there was an interactive effect ($\chi^2 = 12.55$, df = 4, $p = 0.014$), such that more FL hens increasingly used this zone, but they used it the least compared to

ST and TT hens at 19 wk and compared to TT hens at 23 wk. There was no interaction between rearing environment and age for Zone B ($\chi^2 = 9.28$, df = 4, $p = 0.054$) or Zone C ($\chi^2 = 3.31$, df = 4, $p = 0.51$), as well as no main effect of rearing environment for Zone B ($\chi^2 = 3.32$, df = 2, $p = 0.19$).

Floor Eggs

Rearing environment and age had an interactive influence on the proportion of floor eggs ($\chi^2 = 24.83$, df = 2, $p < 0.0001$), such that FL pens laid a higher proportion of floor eggs than ST and TT pens for the first 2 wk of lay (Fig 4.11).

Keel Bone Fractures

The prevalence of keel bone fractures increased between 26 to 28 wk, then remained consistently high to 30 wk ($\chi^2 = 70.91$, df = 2, $p < 0.0001$; Table 4.2). There was no effect of rearing environment ($\chi^2 = 0.70$, df = 2, $p = 0.70$) or its interaction with age ($\chi^2 = 2.54$, df = 4, $p = 0.64$) on the prevalence of keel bone fractures. Similarly, the severity of keel bone fractures slightly increased with age ($\chi^2 = 22.32$, df = 2, $p < 0.0001$; Fig 4.12). Neither rearing environment ($\chi^2 = 3.00$, df = 2, $p = 0.22$) nor its interaction with age ($\chi^2 = 3.26$, df = 4, $p = 0.52$) influenced keel bone fracture severity.

DISCUSSION

The objective of this study was to determine if providing height during pullet rearing influenced hens' overall utilization and skill in using elevated space, the prevalence of floor eggs, and the prevalence and severity of keel bone fractures in a layer multi-tiered aviary. We found that hens

reared with access to height (ST and TT hens) utilized height more initially than FL hens based on increased focal hen activity levels in the evening, higher group level use of the aviary during the daytime, and preferences for higher zones during the daytime and nighttime. However, hens from FL systems acclimated to the layer environment quickly, with most differences in aviary use resolving by the end of the first wk. Some differences in zone preferences for nighttime roosting persisted to 23 wk. Rearing environment did not affect skill based on the prevalence of observed uncontrolled transitions, but FL hens differed in the number of zones crossed in a transition in the morning and evening during the first wk. This difference in strategy persisted between FL and ST hens throughout the trial. Floor eggs were higher for FL hens than ST and TT hens for the first 2 wk of lay, perhaps reflecting observed differences in system use. Contrary to our predictions, overall keel bone fracture prevalence and severity was not affected by pullet rearing environment. Fracture prevalence was high but fracture severity was low, suggesting that the radiography method used offers a high degree of sensitivity in detecting fractures.

Utilizing height

Inspective exploration occurs when animals are introduced to new environments and gather information that may or may not have biological significance (Wood-Gush and Vestergaard, 1989). During the daytime on the first d of observations (D1 at 17 wk), a higher proportion of hens reared with height used the aviary overall, especially the second-highest zone. These initial differences in daytime aviary use may reflect differences in inspective exploration promoted by the rearing environment to gather information about the new housing structure. The alternative to inspective exploration is fear (Wood-Gush and Vestergaard, 1989). Previous research found that hens reared without height, as a result of being in cages, utilized elevated platforms and structures

less during initial lay and scored higher on a fearfulness-related principal component analysis than aviary-reared hens (Brantsæter et al., 2016). Fearfulness was not measured in the present study, but it is possible that FL hens may have initially been more apprehensive in using the multi-tiered aviary structure due to their lack of familiarity with height. Exploratory behavior is also used to satisfy biologically relevant motivations because it increases the likelihood of finding the stimulus (Nicol and Guilford, 1991; Olsson and Keeling, 2000). In the evening prior to the lights going off, activity within the aviary is typically high as hens are motivated to find a preferred roosting spot before eventually settling for the night (Blokhuis, 1984; Stratmann et al., 2019). Displaying higher activity in the evening on D1 suggests that focal hens reared with height initially explored the aviary more than FL hens to find a nighttime roosting spot.

Alternatively, FL hens may not necessarily be more fearful but rather display different preferences for height initially. Indeed, FL hens do not avoid height entirely during the d. They used the bottom two zones of the aviary on D1, then increased their overall daytime aviary use and shifted to the second highest zone by the end of the first wk. Rearing-induced preferences and acclimation periods in the laying environment have been previously reported. Hens from caged rearing environments used elevated structures in their layer pens less than aviary-reared hens after 3 wk in their adult housing (19 wk of age); however, this effect disappeared 2 wk later (23 wk of age; Brantsæter et al., 2016). Hens reared in floor pens furnished with elevated perches and platforms preferred to use the lower tiers of a layer aviary more than aviary-reared hens during initial lay (Colson et al., 2008). Furthermore, cage-reared hens experienced more daytime acceleration events and collisions at the keel with perches (as measured by tri-axial accelerometers) than aviary-reared hens through peak lay (35 wk) in layer enriched colony cages, but this effect disappeared by mid-

lay (50 wk; Pullin et al., 2020). Taken together with the present study, these findings demonstrate that hens reared with less environmental complexity, particularly with less height, acclimate to elevated structures, but they need more time to do so than hens reared with height. The length of acclimation time is likely influenced by a combination of factors from the rearing and laying environments that promote different physical, cognitive, and behavioral experiences, but the more similar the two environments, the more successful the acclimation is likely to be (reviewed by Janczak and Riber, 2015; Campbell et al., 2019).

The acclimation period of lower daytime aviary use resulted more floor eggs for the first 2 wk of lay. Previous research in experimental and commercial layer flocks reported that rearing pullets with access to perches by 4 wk of age reduced the prevalence of floor eggs through peak lay in aviaries (30 to 35 wk of age; Appleby et al., 1988; Gunnarsson et al., 1999). Another study found that hens reared in enriched floor pens laid a higher proportion of floor eggs than aviary-reared hens from 17 to 27 wk of age in their layer aviaries (Colson et al., 2008). Higher rates of floor eggs in these studies were attributed to hens being unable to reach the nest due to assumed poorer physical abilities from the quantity or timing that height was provided in their rearing environment. In the present study, FL hens utilized the zone containing the nest box (Zone A) from D1. They could physically access the nest but used it less often, which may be associated with initial fear or preferences described above. Floor eggs were often found clustered together in the litter in a darkened corner underneath Zone A, suggesting that hens seemed to prefer this nest site over the nest boxes provided to them in the aviary. As more FL hens used the aviary structure over time, the prevalence of floor eggs decreased, suggesting that they modified their preferences after gaining more experience. Kruschwitz et al. (2008) reported that litter can be preferred over nest

boxes by some individual hens for egg laying, but nest boxes become more attractive as hens gain experience using them. More floor eggs, even for a temporary period, increases labor costs for caretakers that have to spend more time collecting eggs (Matthews and Sumner, 2015). Therefore, promoting physical ability and exploratory behavior by the hens, particularly in the initial wk of aviary housing, could have practical benefits for the farm as well.

The prevalence of floor eggs in the present study was high (weekly rates ranging from 39.4 to 85.1 %) compared to several other studies (1.1 to 28.7 % cumulative from start of lay to peak lay in commercial flocks, Gunnarsson et al., 1999; 3.5 to 6.6 % cumulative from 17 to 27 wk in experimental flocks, Colson et al., 2008; 0.3 to 4.2 % range of weekly rates in commercial flocks, Oliveira et al., 2019); although one experimental study reported a cumulative range of 0 to 100 % across treatment groups (Appleby et al., 1988). We did not train hens to use the nests so that the influence of rearing environment would not be altered, but this is a common strategy utilized on commercial farms. Future research on floor eggs in multi-tiered aviaries should investigate the efficacy of various nest training programs to facilitate hens utilizing the nest and how hens from different rearing environments respond to training.

The highest proportion of hens were observed on the aviary during the nighttime compared to the daytime throughout the study, and hens from all rearing environments increasingly used the aviary for nighttime roosting as they aged. Previous work in multi-tiered aviaries also found that the highest proportion of hens utilized the aviary at night and sought the highest structures available for roosting (Odén et al., 2002; Brendler and Schrader, 2016; Campbell et al., 2016c). Similarly in the present study, hens from all rearing environments increasingly shifted to higher zones as they

aged, but there were some effects of rearing environment on zone preference. Hens typically prefer to roost on perches more than flat plastic grids or flat shelves, but roosting on all types of surfaces has been observed (Brendler and Schrader, 2016; Campbell et al., 2016c). Preferences for roosting surface in multi-tiered aviaries have previously been associated with height, age, and available space (Brendler and Schrader, 2016; Campbell et al., 2016c). However, rearing environment appears to influence roosting surface preference as well. From 19 to 27 wk, FL hens maintained the highest proportion of aviary-using hens on Zone C (flat tier and one perch); meanwhile, more ST and TT hens moved from Zone C up to Zone D (additional perches). The link between pullet rearing environment and preference for roosting surface has not been previously studied, but broiler breeder hens preferred the shape of the roost they had previous experience with over other shapes they were naïve to (Muiruri et al., 1990). Perches were included in the FL pens so that pullets had experience gripping the same perches they received in the layer aviary, but these perches were not elevated beyond 10.5 cm and rarely used (Graham, 2022). Consequently, FL hens were sleeping on the flat surface of the litter floor during pullet rearing. Even though they display the species-specific motivation to move high for nighttime roosting in the laying environment, they use the highest zone the least that is only perches. Their lack of early experience with perches as a roosting surface may contribute to their preference of Zone C throughout the end of the trial, which offers a flat plastic grid for roosting in addition to a perch.

Skill and strategy in using height

Uncontrolled movements and collisions have been reported to account for up to 32% of descending transitions (Stratmann et al., 2015a) and up to 21% of flights (Campbell et al., 2016a) observed in aviary settings. Experiencing more collisions, a characteristic of uncontrolled

movements, has furthermore been associated with a higher risk for keel bone fractures (Baker, 2018). We anticipated that experience with height early in life would result in improved spatial skills, and thus fewer of these uncontrolled movements and improved keel integrity. In the current study, rearing environment did not influence the number of uncontrolled transitions during the first wk. Contrary to our predictions, TT hens experienced more uncontrolled transitions between 19 and 27 wk of age than ST hens while maintaining a similar rate of total transitions. However, the magnitude of this difference was low (1 uncontrolled transition/d/pen), and uncontrolled transitions were rare across all rearing treatments. Previous work, which compared cage versus aviary rearing on the rate of collisions recorded in enriched colony cage layer housing, also reported differences of low magnitude between treatment groups (1 to 2 collisions/pen/10 d; Pullin et al., 2020). The previous study did not quantify keel fracture prevalence, so it was originally not known if the low magnitude would have implications for injuries. If collisions play a primary role in keel bone fracture formation, the rarity of these movements in the present study (2% of all vertical transitions) gives context to our finding that keel bone fracture prevalence and severity did not differ among rearing treatments.

Uncontrolled transitions decreased across the earliest timepoints in our study. Falls and collisions in enriched colony cages and aviaries have also reportedly decreased with age as hens acclimate to their new housing, become less active with age, or both (Stratmann et al., 2015a, 2019; Pullin et al., 2020). However, as previously stated, the incidence of uncontrolled transitions in the present study was low overall compared to previous research evaluating uncontrolled movements in multi-tiered aviaries (13 to 32% of all descending transitions classified as falls and collisions, Stratmann et al., 2015a; 9 to 21% of all flights classified as failed landings, Campbell et al., 2016a).

Differences in stocking density and group size between the previous studies and our work may partially account for these disparities. The current study housed hens in groups of 28 to 30 hens/pen (0.37 m^2/hen), whereas the other two studies took place in a small-scale commercial setting with 225 hens/pen (0.14 m^2/hen, Stratmann et al., 2015a) and a large-scale commercial setting with 49,677 and 49,842 hens/house (0.13 m^2/hen, Zhao et al., 2015; Campbell et al., 2016a). In the larger environments, individual hens have less space for take-offs and landings and are negotiating those movements amongst a larger flock than in an experimental environment, making the larger environments more complicated to traverse. Correspondingly, research evaluating controlled movements and falls in commercial multi-tiered aviaries (4,468 hens mean flock size/barn, space allowance not provided) found that the rate of falls was related to the times of d when the greatest number of hens were most active (dusk and dawn; Stratmann et al., 2019). As birds enter and exit the aviary pre- and post-roosting during dusk and dawn, respectively, fall risk is higher in larger groups as up to 90% of hens could be moving simultaneously (Stratmann et al., 2015a, 2019; Campbell et al., 2016b). Space allowance and social dynamics are some additional factors in commercial environments that differ from experimental settings, which may exacerbate the rate of uncontrolled transitions.

Downward movements spanning longer distances are more difficult for hens to make than shorter distances or upward movements generally (Scott et al., 1997; Moinard et al., 2004a; Moinard et al., 2004b). Studies have shown that hens displayed less control, refused to jump, or missed the perch entirely when moving downward between two perches at longer distances and steeper angles (Scott et al., 1997; Moinard et al., 2004a; Moinard et al., 2004b). In the current study, we observed greater variation for distance traveled during descending transitions when compared to ascending

transitions. Over the first wk, FL hens increased their proportion of descending transitions crossing more than 1 zone in the morning, and ST and TT hens remained consistent. This finding corresponds with our nighttime aviary use data, such that more FL hens roosted on the aviary, particularly Zone C, by D7; therefore, they would be at a higher location to descend from in the morning. In the evening on D1, FL hens made a higher proportion of these longer descending transitions than ST and TT hens but decreased by D7. This finding contradicts previous work. In multi-tiered aviaries, hens reared in enriched floor pens made fewer long flights (defined as > 1 m; ascending or descending) than hens reared in aviaries 3 wk after placement (Colson et al., 2008). We propose that the distance traveled during descending transitions is nuanced and may be more reflective of strategy rather than skill, and additional parameters are needed to quantify skill of descents. For example, the research associating downward transitions with skill evaluated movements between two perches, where the landing surface is small (approximately 3 to 4 cm) so accuracy is higher stakes (Scott et al., 1997; Moinard et al., 2004a; Moinard et al., 2004b). Accuracy and skill are unlikely to be as relevant for landing surfaces with a greater area (e.g. floor or tier). Lower rates of failed landings have been reported on the litter floor compared to perches in aviaries (Campbell et al., 2016a). Furthermore, aviaries modified with platforms or ramps provide greater surface area for hens to use during vertical movement, and these aviaries have higher rates of controlled movements than standard aviaries (Stratmann et al., 2015a). In the present study, the landing surface for the vast majority of descending transitions crossing more than 1 zone was the litter floor for all rearing environments. For transitions crossing only 1 zone, there is more variation in landing surfaces for ST and TT hens, particularly at 17 wk. Landing surfaces, rather than distance traveled, may be more representative of skill. It is notable that

perches remain the least used surface during a descending transition, providing support that descents to perches may be more challenging.

FL hens maintained a different strategy for descending transitions throughout the end of the trial than ST hens, such that FL hens displayed a higher proportion of descending transitions crossing more than 1 zone than ST hens. However, TT hens did not differ from either group. It is difficult to determine what characteristics separate FL and ST hens for this metric but does not separate them from TT. Focal hens were used to collect data on vertical transition behavior, and notable individual variation was observed amongst focal hens within the same rearing environment (as seen in the box range and whiskers spanning most of the y-axis in boxplots in Fig 4.3 and 4.4), sometimes resulting in wide 95% confidence intervals from the statistical model. This individual variation in behavior may drive differences between rearing environments, or even mask some of the effects of rearing environment. All hens displayed a lower proportion of transitions crossing more than 1 zone during the morning at 27 wk. This finding was an artefact of shifting to natural lighting at this age to manage aggressive behavior in the flock. Once all cameras in the barn turned to color from the natural light stimulus to begin recording behavior in the morning h, the majority of hens had already descended from their nighttime roosting spot, thus missing the bulk of transitions that crossed more than 1 zone at this timepoint.

Keel bone fractures

Rearing environment did not influence the prevalence or severity of keel bone fractures in the present study. Other researchers found keel bone fractures in enriched colony cages to be linked to pullet rearing, such that cage-reared hens experienced a higher prevalence of fractures than

aviary-reared hens from 30 to 70 wk of age (Casey-Trott et al., 2017a). They attributed this effect to increased keel bone growth promoted by the load-bearing exercise opportunities in the aviary rearing environment (Casey-Trott et al., 2017b). Using space in the layer housing more skillfully was also hypothesized to be linked to the reduction in fractures, considering that a higher prevalence of keel bone fractures has been linked with higher rates of collisions (Stratmann et al., 2015a). Rearing environment did not affect skill of using space in the present study and did not affect fracture prevalence. It primarily affected the utilization of elevated space during 17 wk of age, but these differences in behavior did not affect keel bone fractures because all hens had 0% prevalence at 18 wk of age. Although we cannot make a connection amongst rearing environment, uncontrolled movements, and keel bone fracture prevalence, this connection will need to be evaluated in commercial facilities to account for other features of housing that may exacerbate uncontrolled movements. Other methodological and environmental characteristics may explain the high fracture prevalence in our study.

Palpation, or manually feeling for the presence of callouses and fractures, has historically been the most common technique for keel bone fracture assessment (Casey-Trott et al., 2015; Rufener and Makagon, 2020). However, the method is associated with low inter-observer reliability and low sensitivity for identifying fractures on the caudal tip or dorsal surface of the bone, minor fractures, or new fractures (Casey-Trott et al., 2015; Tracy et al., 2019). Radiography was used as the assessment method in in the present study to provide a higher degree of sensitivity for these fracture characteristics and ensure inter-observer reliability using a tagged visual analogue scale (Rufener et al., 2018). Our fracture prevalence was over 90% for hens across rearing environments at 30 wk of age, which is higher than the average prevalence reported for white laying hen strains

at this age (23.1%) or hens in aviary housing over 49 wk of age (37 to 39%; reviewed by Rufener and Makagon, 2020). These previously reported prevalence rates were likely estimated from palpation as only 6.1% of keel bone fracture studies have used radiography (Rufener and Makagon, 2020). A recent study using radiography on hens from 22 to 61 wk found that 97% of hens experienced at least one keel bone fracture, and most fractures occur at the caudal tip of the keel (Baur et al., 2020). Finding a higher prevalence of fractures with radiography is due to the detailed characteristics that can be collected on fractures with this method as opposed to palpation. The majority of fractures in the present study were identified as minor in severity, falling within the first or second visual analogue tag of fractured keels (Rufener et al, 2018). Further research is needed to examine the causes and animal welfare outcomes for minor fractures.

In conclusion, rearing hens with minimal height temporarily reduces their utilization of height, modifies their strategy for accessing height, and increases the prevalence of floor eggs. There were minimal differences between ST and TT hens, suggesting that more height did not result in better consequences for the hens. Although keel bone fracture prevalence and severity were not affected by rearing environment in the present study, the sensitivity of radiography for detecting fractures provides insight to the large scale of this injury. The period of acclimation displayed by floor-reared hens may have greater consequences in a commercial-scale environment with larger flocks and less space per individual hen compared to an experimental setting. Therefore, the objectives of the present study should be replicated in a commercial setting.

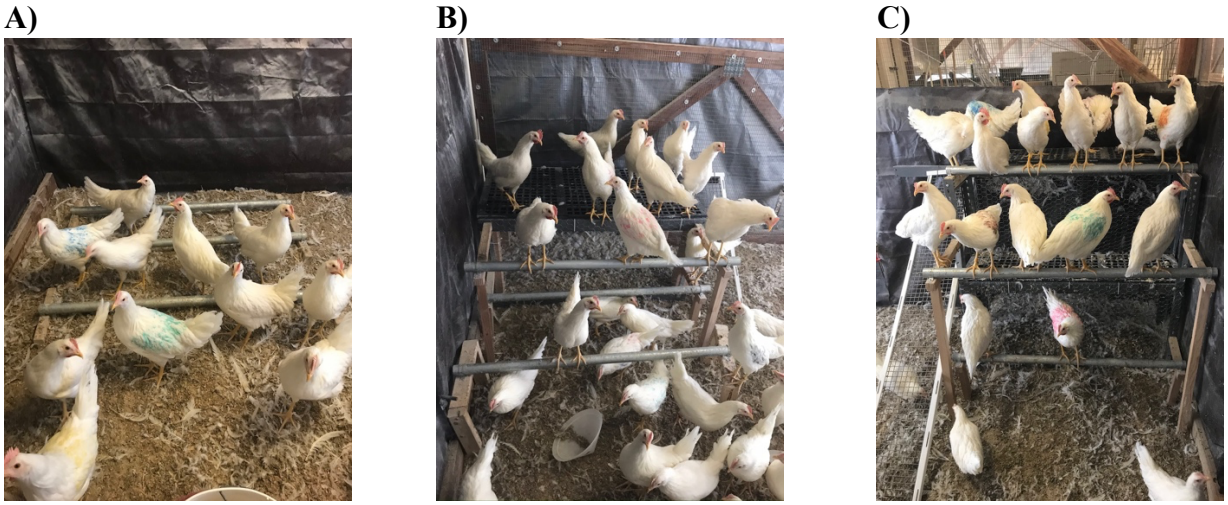

Figure 4.1. Female Dekalb White pullets were reared in one of three environments for the first 16 wk of life: floor rearing environment with 10 cm high floor perches (A), a single-tiered aviary with three elevated perches and one elevated tier (B), and two-tiered aviary with three elevated perches and two elevated tiers (C). Pullets were reared in groups of 55 to 56 pullets/pen from 1 d to 8 wk of age, then 45 pullets/pen from 8 to 16 wk of age (n = 5 pens/rearing environment). Pullets were 16 wk of age at the time of the photographs.

Figure 4.2. At 17 wk, rearing environment structures were removed from pens and replaced with a layer multi-tiered aviary for female Dekalb White hens (28 to 30 hens/pen; n = 5 pens/rearing environment). The aviary was divided into five zones to record vertical transition behavior between zones during the d and the proportion of hens within each zone across the daytime and nighttime. Hens were photographed at 17 wk of age.

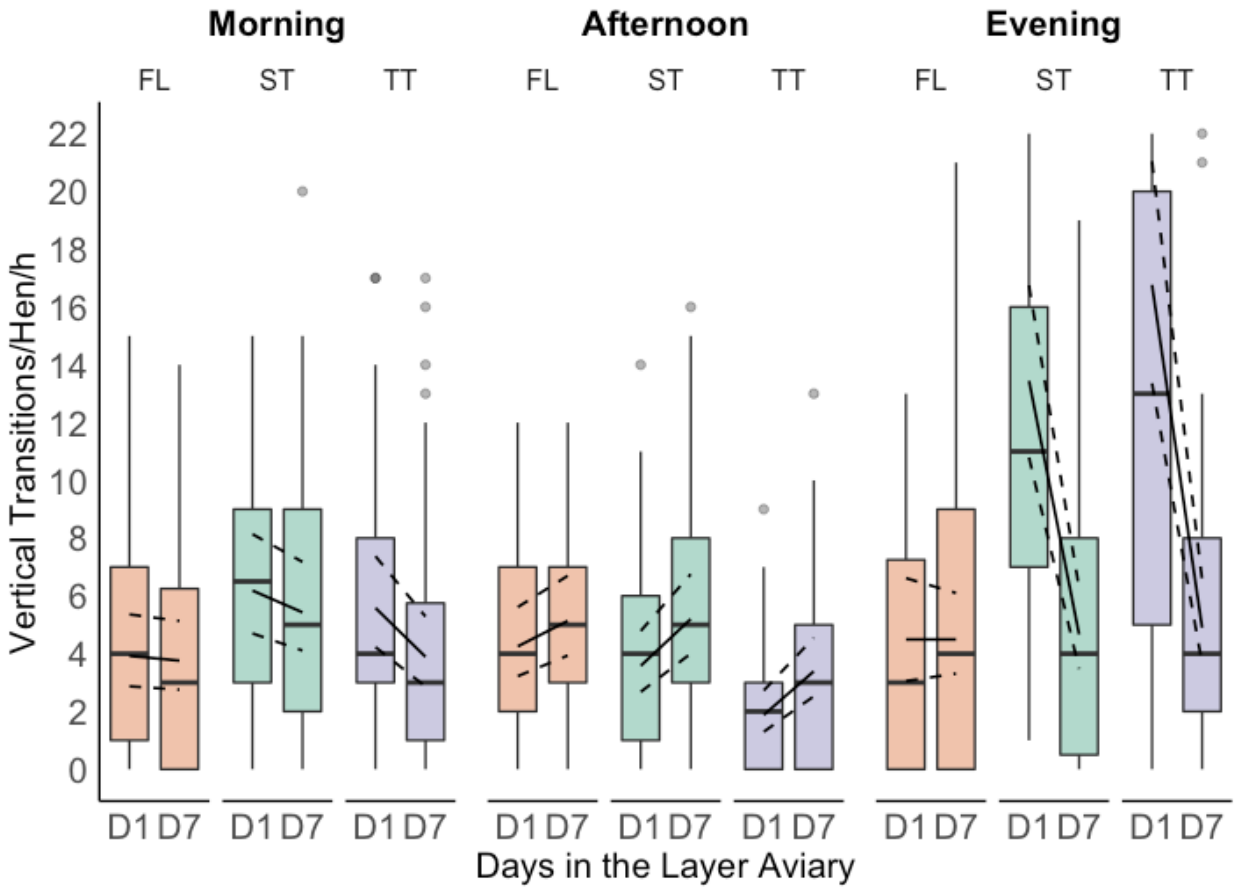

Figure 4.3. The number of vertical transitions made by Dekalb White focal hens at 3 h (morning, afternoon, and evening) during the first wk of housing in a multi-tiered layer aviary. Hens were reared in one of three pullet rearing environments: floor (FL), single-tiered aviary (ST), or a two-tiered aviary (TT; 10 focal hens/pen, n = 5 pens/rearing environment). The first full d that hens were housed in the layer aviary without atypical human disturbance is D1, followed by the seventh full d (D7). Raw data are displayed in box plots, where boxes represent the interquartile range, where the black line represents the median, the top of the box represents the 75th quartile, and the bottom of the box represents the 25th quartile. Whiskers represent the minimum and maximum values, while grey dots indicate outliers. The solid line overlaying the box plots is the estimated mean values from the generalized linear mixed effects model and the dashed lines are the 95% confidence intervals of the estimated means.

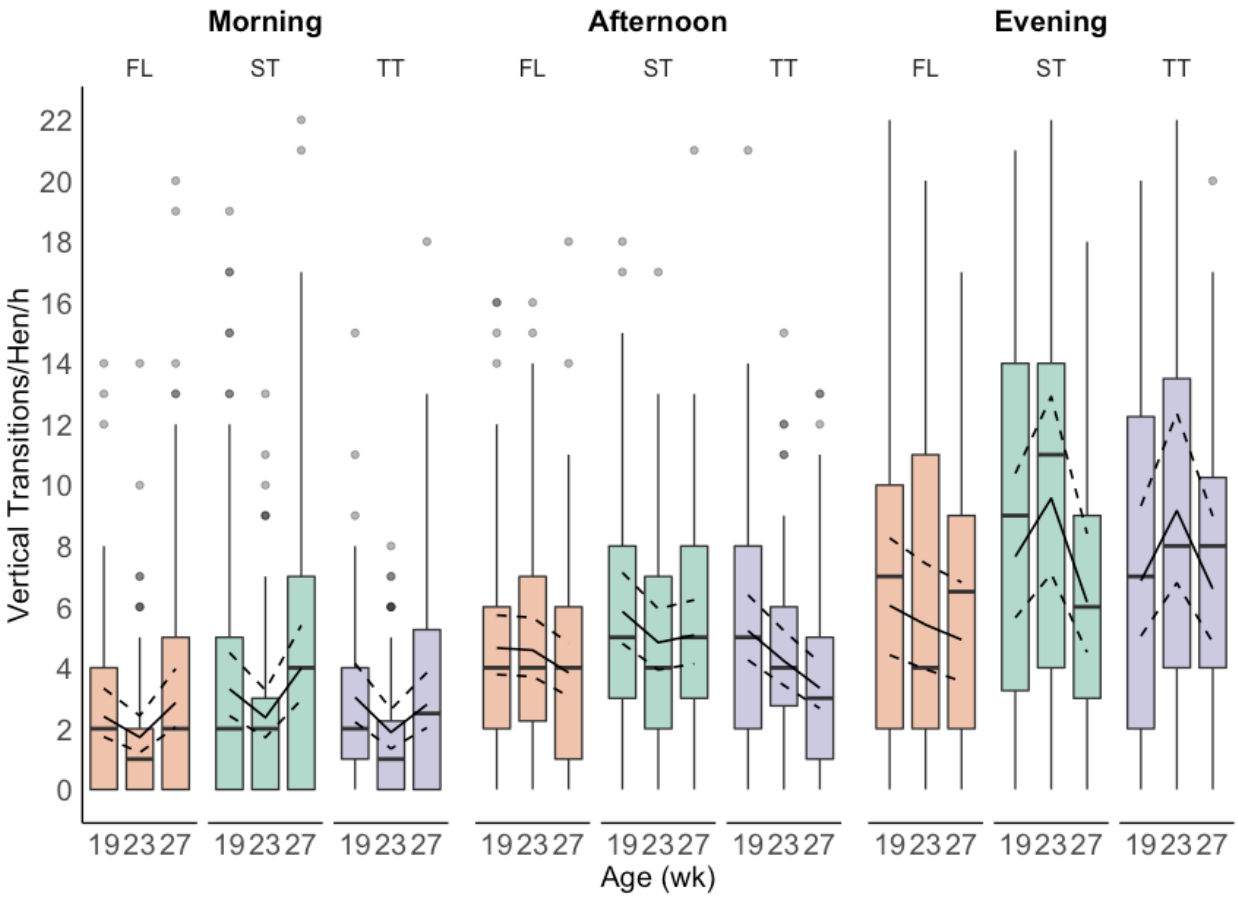

Figure 4.4. The number of vertical transitions made by Dekalb White focal hens at 3 h (morning, afternoon, and evening) in a multi-tiered layer aviary at 19, 23, and 27 wk of age. Hens were reared in one of three pullet rearing environments: floor (FL), single-tiered aviary (ST), or a two-tiered aviary (TT; 10 focal hens/pen, n = 5 pens/rearing environment). Boxplots display raw data. Boxes represent the interquartile range, where the black line represents the median, the top of the box represents the 75th quartile, and the bottom of the box represents the 25th quartile. Whiskers represent the minimum and maximum values, while grey dots indicate outliers. The solid line overlaying the box plots is the estimated mean values from the generalized linear mixed effects model and the dashed lines are the 95% confidence intervals of the estimated means.

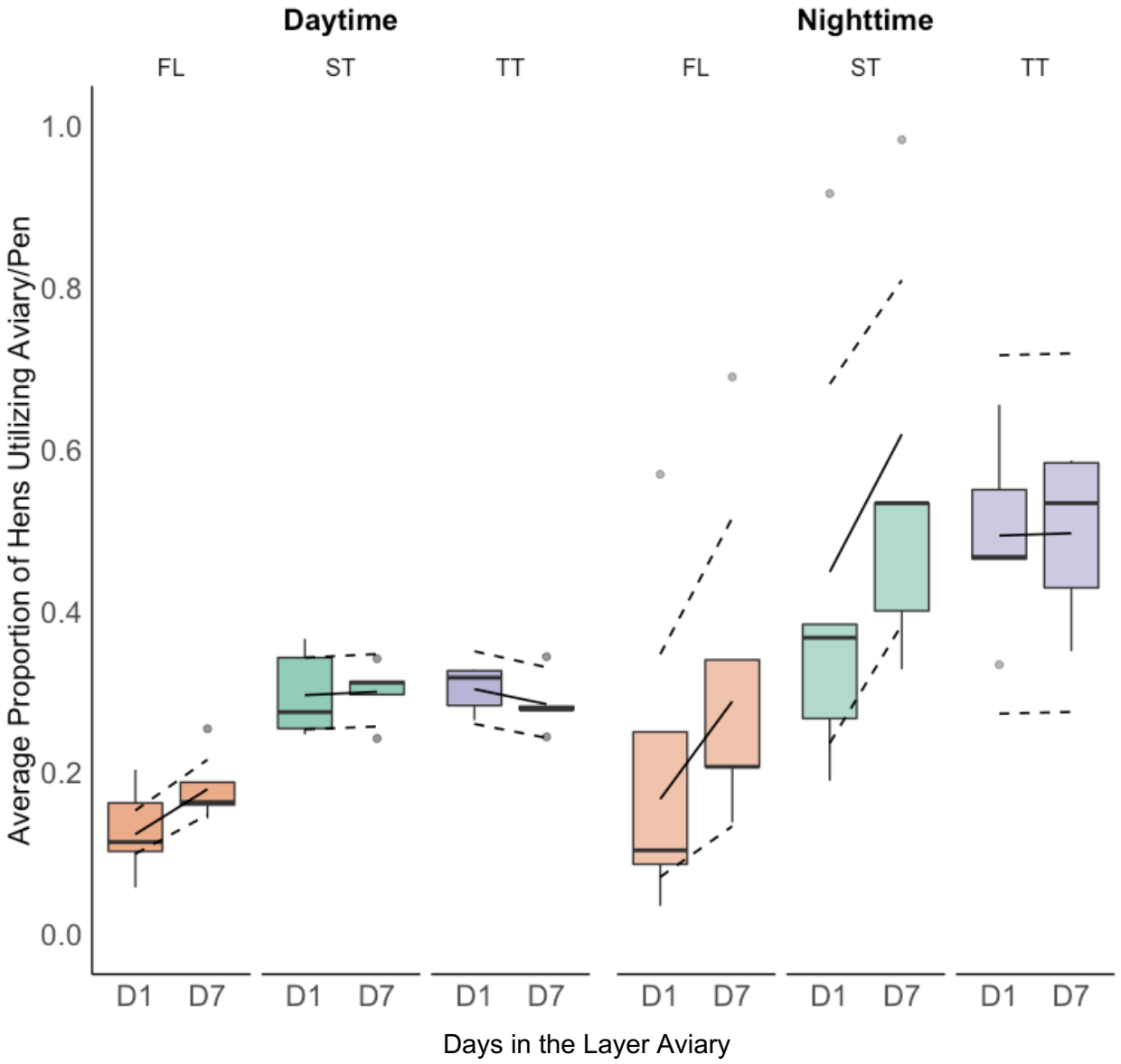

Figure 4.5. The average proportion of laying hens utilizing a layer multi-tiered aviary structure during the first wk of housing. Dekalb White hens (28 to 30 hens/pen; n = 5 pens/rearing environment) were reared in one of three pullet rearing environments through 16 wk of age: floor (FL), single-tiered aviary (ST), or a two-tiered aviary (TT). The first full d that hens were housed in the layer aviary without atypical human disturbance is D1, followed by the seventh full d (D7). Raw data are displayed in box plots, where boxes represent the interquartile range, where the black line represents the median, the top of the box represents the 75th quartile, and the bottom of the box represents the 25th quartile. Whiskers represent the minimum and maximum values, while grey dots indicate outliers. The solid line overlaying the box plots is the estimated mean values from the generalized linear mixed effects model and the dashed lines are the 95% confidence intervals of the estimated means.

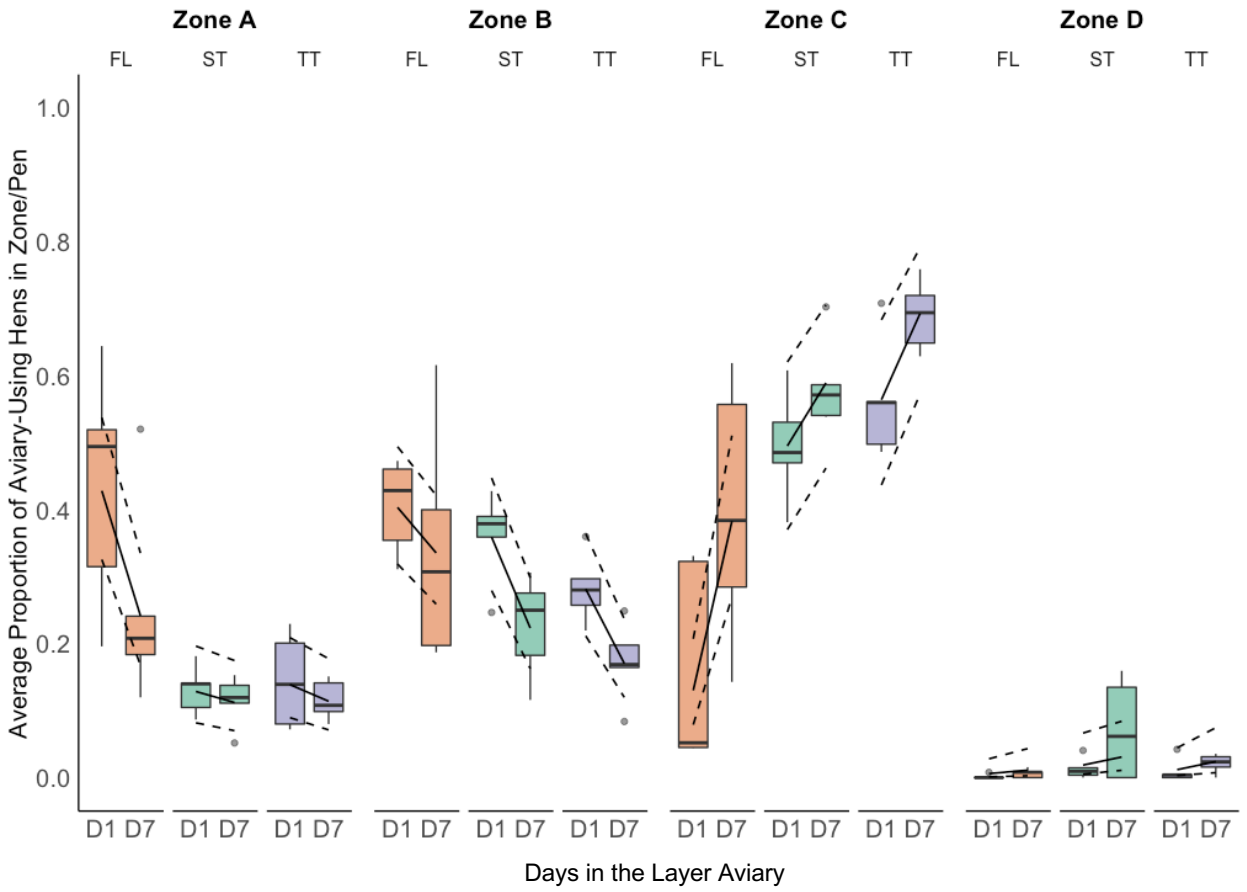

Figure 4.6. Of hens using a layer multi-tiered aviary structure, the average proportion of hens in each zone of the aviary during the daytime is displayed for the first wk of housing. Dekalb White hens (28 to 30 hens/pen; n = 5 pens/rearing environment) were reared in one of three pullet rearing environments through 16 wk of age: floor (FL), single-tiered aviary (ST), or a two-tiered aviary (TT). The first full d that hens were housed in the layer aviary without atypical human disturbance is D1, followed by the seventh full d (D7). Raw data are displayed in box plots, where boxes represent the interquartile range, where the black line represents the median, the top of the box represents the 75th quartile, and the bottom of the box represents the 25th quartile. Whiskers represent the minimum and maximum values, while grey dots indicate outliers. The solid line overlaying the box plots is the estimated mean values from the generalized linear mixed effects model and the dashed lines are the 95% confidence intervals of the estimated means.

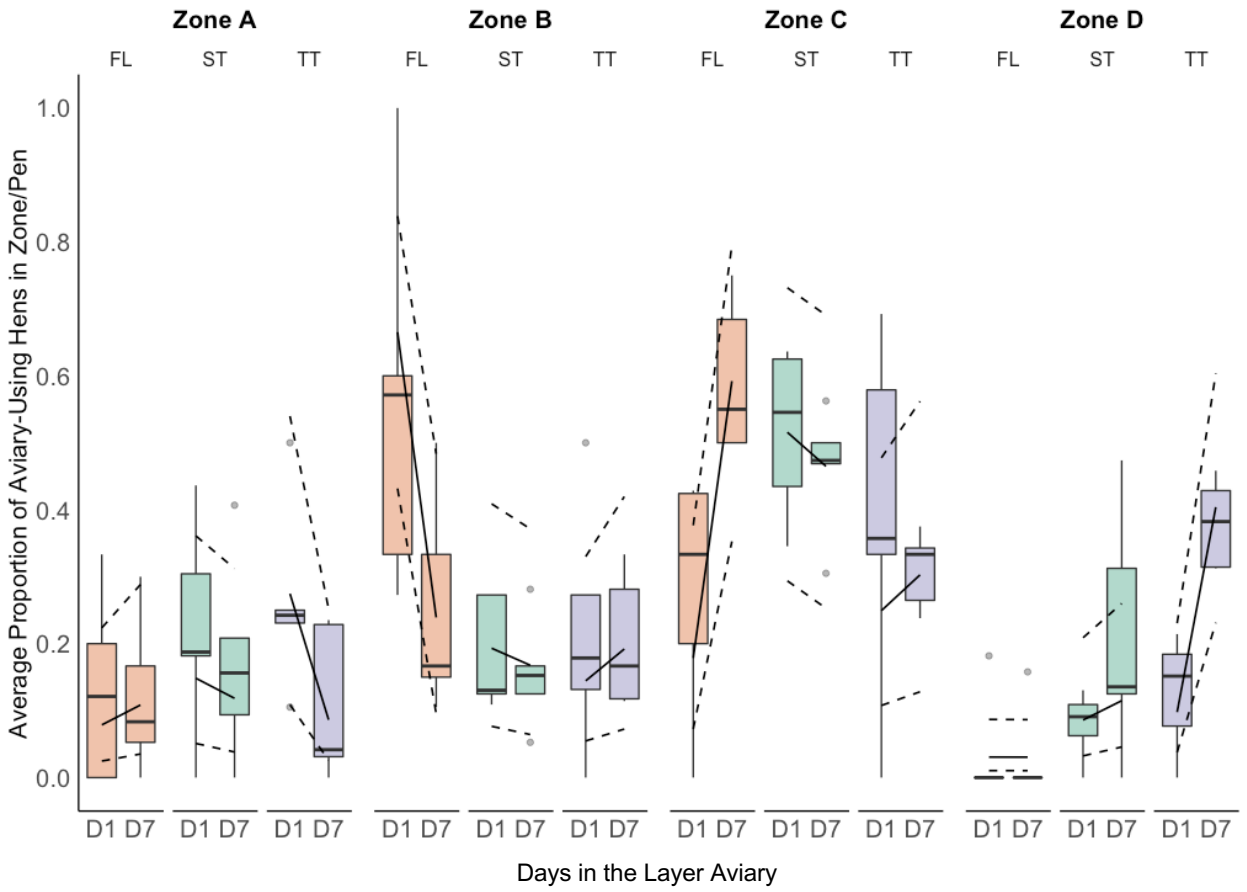

Figure 4.7. Of hens using a layer multi-tiered aviary structure, the average proportion of hens in each zone of the aviary during the nighttime is displayed for the first wk of housing. Dekalb White hens (28 to 30 hens/pen; n = 5 pens/rearing environment) were reared in one of three pullet rearing environments through 16 wk of age: floor (FL), single-tiered aviary (ST), or a two-tiered aviary (TT). The first full d that hens were housed in the layer aviary without atypical human disturbance is D1, followed by the seventh full d (D7). Raw data are displayed in box plots, where boxes represent the interquartile range, where the black line represents the median, the top of the box represents the 75th quartile, and the bottom of the box represents the 25th quartile. Whiskers represent the minimum and maximum values, while grey dots indicate outliers. The solid line overlaying the box plots is the estimated mean values from the generalized linear mixed effects model and the dashed lines are the 95% confidence intervals of the estimated means.

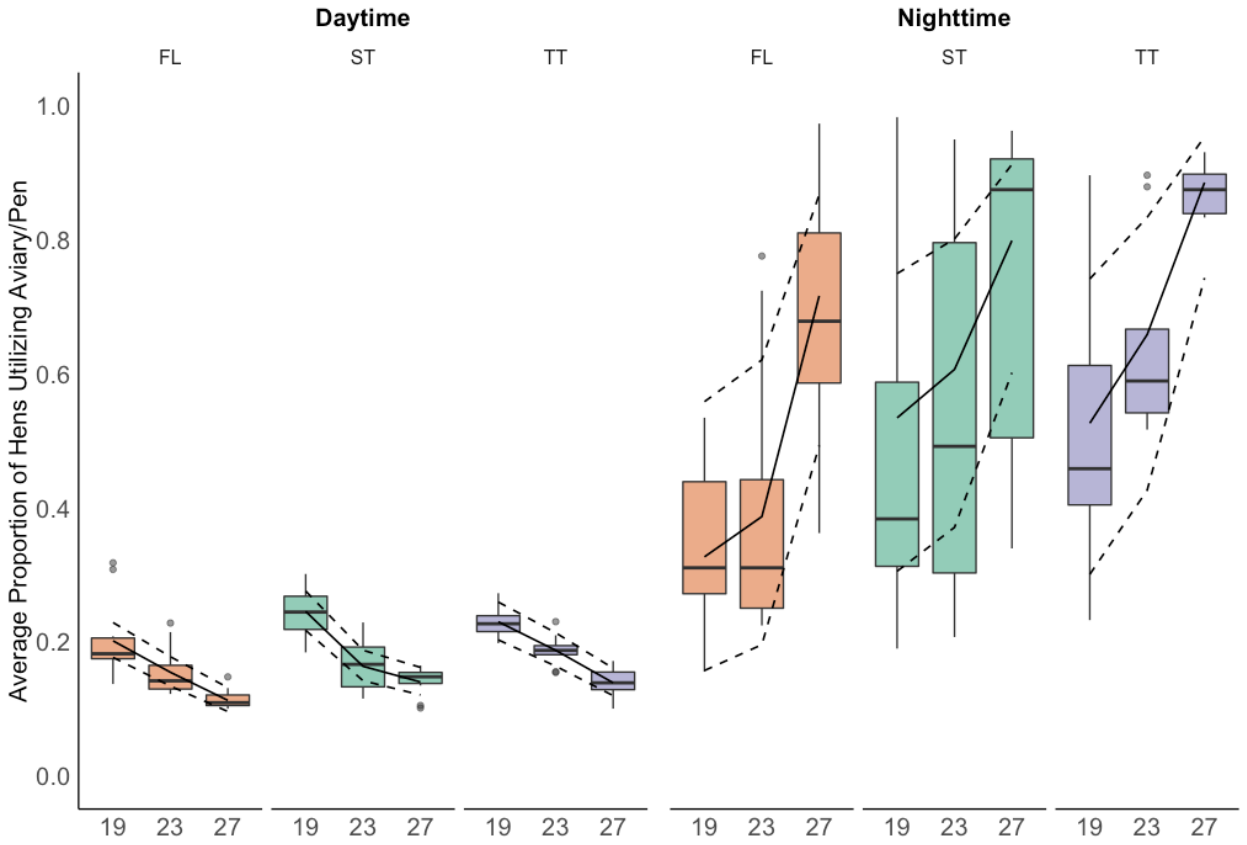

Figure 4.8. The average proportion of laying hens utilizing a layer multi-tiered aviary structure from 19 to 27 wk of age Dekalb White hens (28 to 30 hens/pen; n = 5 pens/rearing environment) were reared in one of three pullet rearing environments through 16 wk of age: floor (FL), single-tiered aviary (ST), or a two-tiered aviary (TT). Raw data are displayed in box plots, where boxes represent the interquartile range, where the black line represents the median, the top of the box represents the 75th quartile, and the bottom of the box represents the 25th quartile. Whiskers represent the minimum and maximum values, while grey dots indicate outliers. The solid line overlaying the box plots is the estimated mean values from the generalized linear mixed effects model and the dashed lines are the 95% confidence intervals of the estimated means.

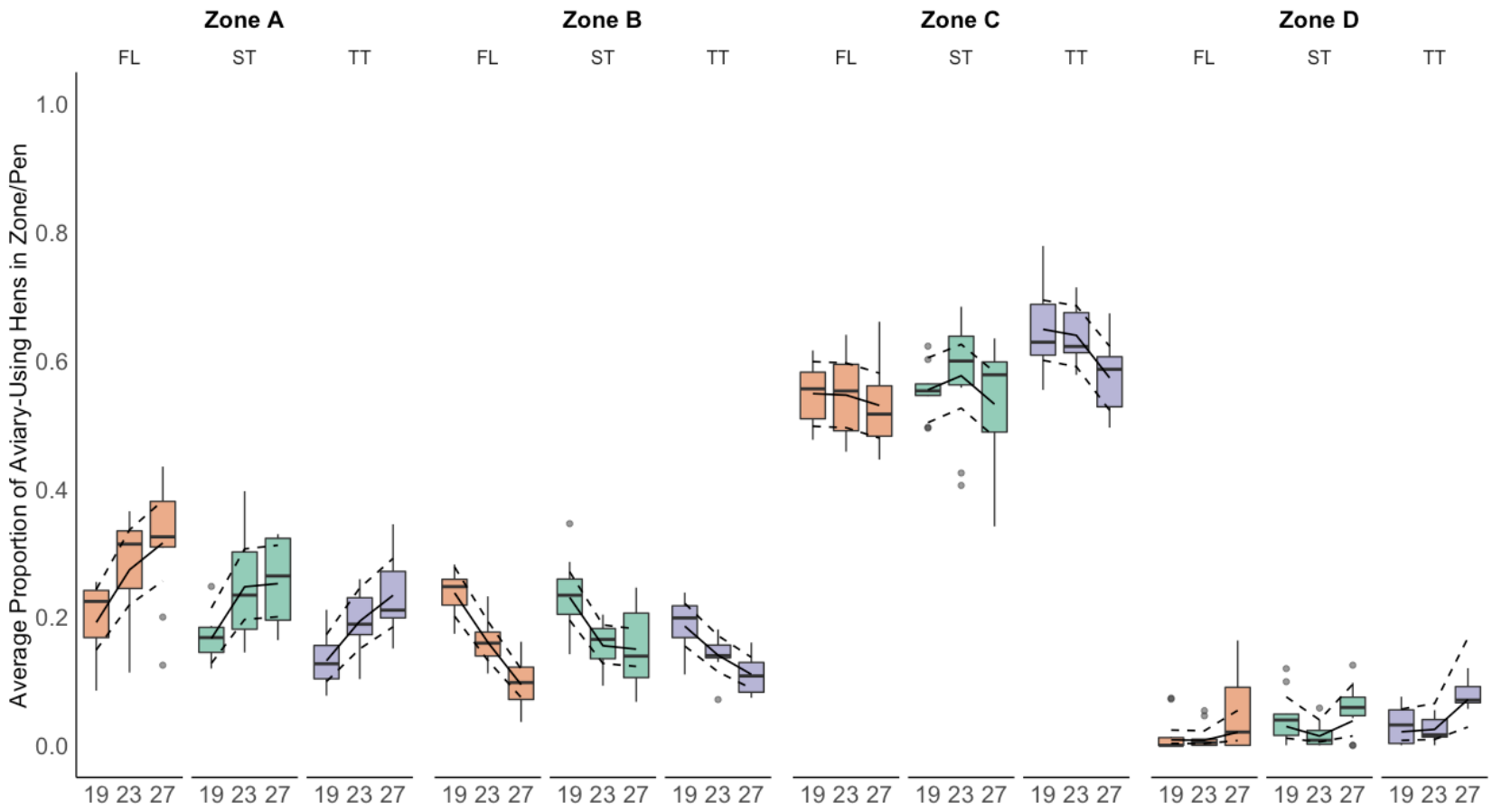

Figure 4.9. Of hens using a layer multi-tiered aviary structure, the average proportion of hens in each zone of the aviary during the daytime is displayed for 19 to 27 wk. Dekalb White hens (28 to 30 hens/pen; n = 5 pens/rearing environment) were reared in one of three pullet rearing environments through 16 wk of age: floor (FL), single-tiered aviary (ST), or a two-tiered aviary (TT). Raw data are displayed in box plots, where boxes represent the interquartile range, where the black line represents the median, the top of the box represents the 75th quartile, and the bottom of the box represents the 25th quartile. Whiskers represent the minimum and maximum values, while grey dots indicate outliers. The solid line overlaying the box plots is the estimated mean values from the generalized linear mixed effects model and the dashed lines are the 95% confidence intervals of the estimated means.

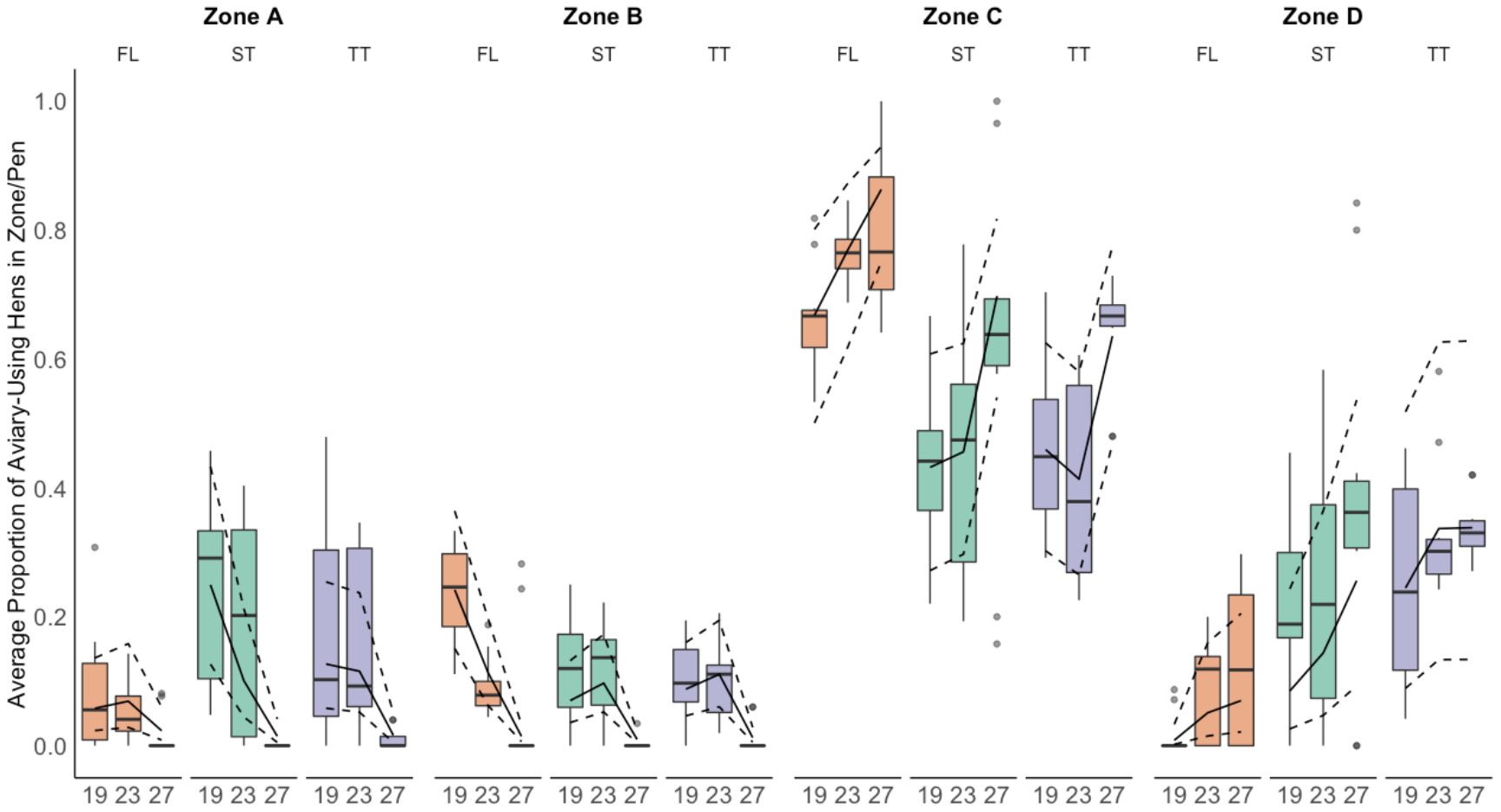

Figure 4.10. Of hens using a layer multi-tiered aviary structure, the average proportion of hens in each zone of the aviary during the nighttime is displayed for 19 to 27 wk. Dekalb White hens (28 to 30 hens/pen; n = 5 pens/rearing environment) were reared in one of three pullet rearing environments through 16 wk of age: floor (FL), single-tiered aviary (ST), or a two-tiered aviary (TT). Raw data are displayed in box plots, where boxes represent the interquartile range, where the black line represents the median, the top of the box represents the 75th quartile, and the bottom of the box represents the 25th quartile. Whiskers represent the minimum and maximum values, while grey dots indicate outliers. The solid line overlaying the box plots is the estimated mean values from the generalized linear mixed effects model and the dashed lines are the 95% confidence intervals of the estimated means.

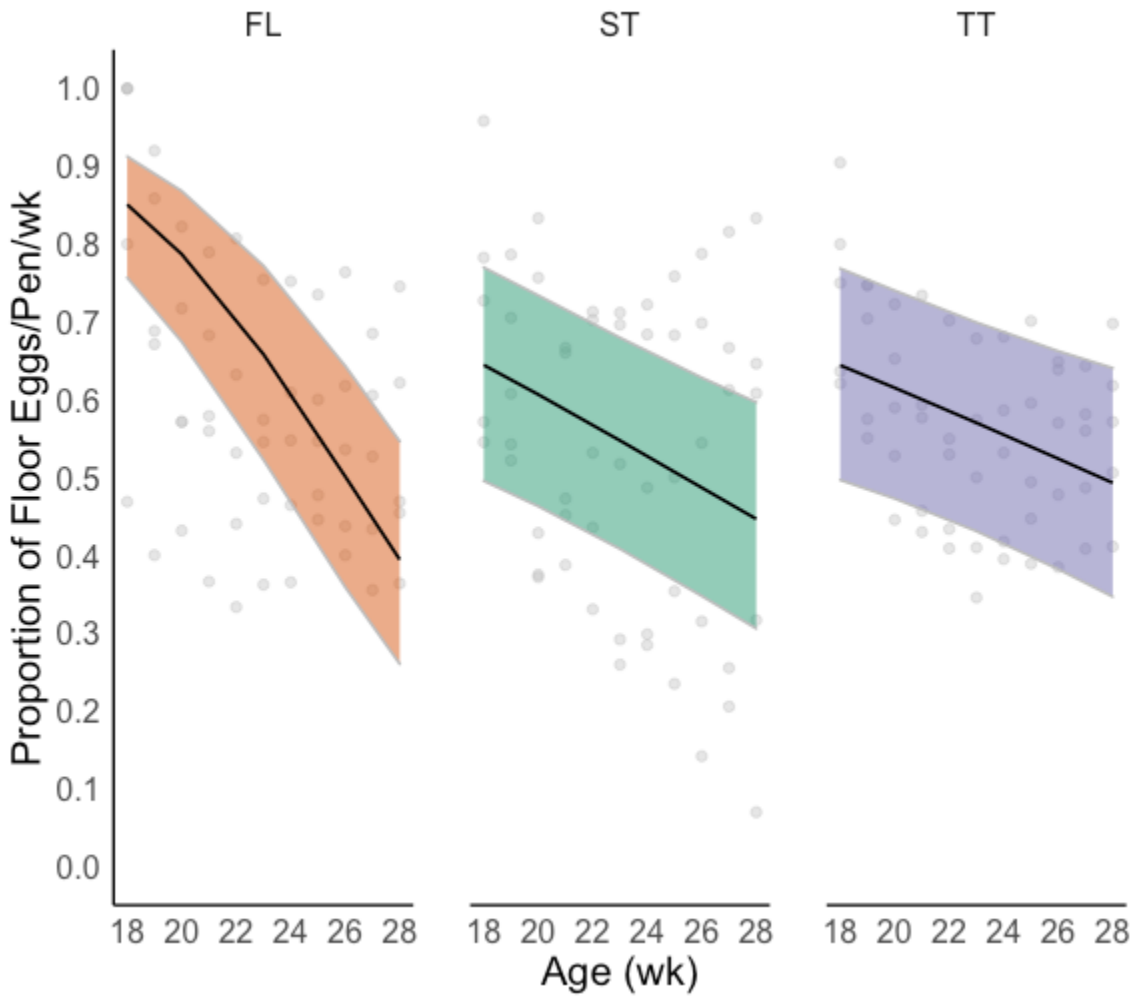

Figure 4.11. The proportion of floor eggs laid/pen by Dekalb White hens on a weekly basis from 18 to 28 wk of age. Hens were reared in one of three pullet rearing environments: floor (FL), single-tiered aviary (ST), or a two-tiered aviary (TT). Raw data points for each rearing environment appear in grey. The solid black line overlaying the raw data points corresponds to the estimated mean values from the generalized linear mixed effects model and the colored ribbon represents the 95% confidence intervals of the estimated means.

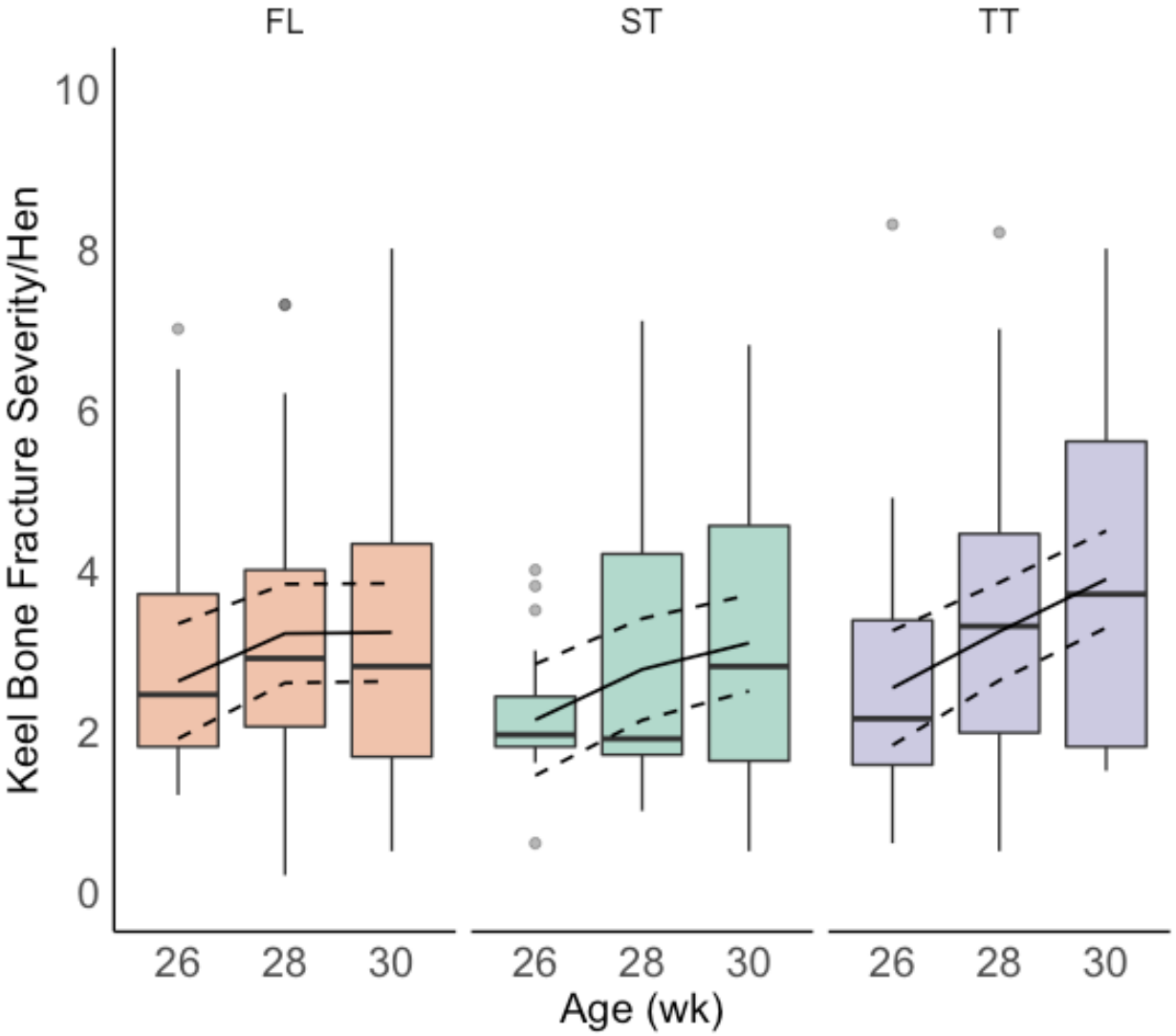

Figure 4.12. Keel bone fracture severity as assessed by a tagged visual analogue scale that quantified the total amount of bone affected by fractures, where scores ranged from 0 to 10.0 to correspond to no fracture to extremely severe. Radiographs from the same Dekalb White focal hens were evaluated longitudinally after they were reared in one of three pullet rearing environments through 16 wk of age: floor (FL), single-tiered aviary (ST), or a two-tiered aviary (TT). Raw data points are represented by boxplots. Boxes represent the interquartile range, where the black line represents the median, the top of the box represents the 75th quartile, and the bottom of the box represents the 25th quartile. Whiskers represent the minimum and maximum values, while grey dots indicate outliers. The solid line overlaying the box plots is the estimated mean values from the generalized linear mixed effects model and the dashed lines are the 95% confidence intervals of the estimated means.

Table 4.1. **Proportion of descending transitions crossing more than 1 zone by Dekalb White hens.** Hens were reared in either a floor (FL), single-tiered aviary (ST), or two-tiered aviary (TT) pullet rearing environment through 16 wk, then housed in a multi-tiered layer aviary where descending transitions were recorded.

Time of d and Age (wk)	FL Estimate (95% CI)	ST Estimate (95% CI)	TT Estimate (95% CI)
Morning			
17, D1	0.32 (0.23, 0.43)	0.41 (0.32, 0.52)	0.40 (0.30, 0.51)
17, D7	0.56 (0.44, 0.68)	0.45 (0.35 0.55)	0.41 (0.30, 0.53)
19	0.46 (0.35, 0.57)	0.34 (0.26, 0.42)	0.35 (0.27, 0.44)
23	0.41 (0.31, 0.53)	0.29 (0.21, 0.39)	0.43 (0.33, 0.54)
27	0.095 (0.054, 0.16)	0.11 (0.072, 0.17)	0.12 (0.075, 0.19)
Afternoon			
17, D1	0.41 (0.30, 0.53)	0.44 (0.33, 0.56)	0.43 (0.26, 0.61)
17, D7	0.59 (0.47, 0.69)	0.49 (0.38, 0.60)	0.55 (0.43, 0.67)
19	0.56 (0.47, 0.65)	0.50 (0.42, 0.59)	0.60 (0.52, 0.68)
23	0.58 (0.49, 0.67)	0.43 (0.35, 0.53)	0.54 (0.44, 0.63)
27	0.48 (0.38, 0.59)	0.42 (0.34, 0.51)	0.59 (0.49, 0.69)
Evening			
17, D1	0.71 (0.56, 0.83)	0.53 (0.46, 0.61)	0.51 (0.43, 0.58)
17, D7	0.46 (0.35, 0.57)	0.51 (0.40, 0.62)	0.50 (0.38, 0.61)
19	0.54 (0.45, 0.62)	0.45 (0.37, 0.52)	0.47 (0.39, 0.55)
23	0.48 (0.39, 0.57)	0.32 (0.26, 0.39)	0.40 (0.33, 0.47)
27	0.52 (0.42, 0.62)	0.37 (0.29, 0.46)	0.42 (0.33, 0.51)

Table 4.2. **Prevalence of keel bone fractures for Dekalb White laying hens that were reared either on the floor (FL), with a single-tiered aviary (ST), or with a two-tiered aviary (TT) through 16 wk of age.** The same focal hens were radiographed at each age for longitudinal keel bone fracture assessment.

Age (wk)	FL Estimate (95% CI)	ST Estimate (95% CI)	TT Estimate (95% CI)
18	0.0 (0.0, 0.0)	0.0 (0.0, 0.0)	0.0 (0.0, 0.0)
26	0.46 (0.14, 0.82)	0.43 (0.13, 0.79)	0.25 (0.063, 0.62)
28	0.95 (0.74, 0.99)	0.82 (0.46, 0.96)	0.88 (0.57, 0.98)
30	0.97 (0.78, 1.00)	0.95 (0.75, 0.99)	0.94 (0.71, 0.99)

CHAPTER 5:

General Discussion and Conclusions

The objectives for my dissertation were 1) to determine if the pullet rearing environment has a long-term effect on laying hen behavior (out to mid-lay), 2) to study how providing height specifically during rearing affected the quantity and quality of vertical space use and keel bone fractures, and 3) to assess a possible neurological mechanism underlying behavioral differences. I found that rearing environment affected hen collision behavior and perch use out to peak and mid-lay, respectively, in enriched colony cages. Aviary-reared hens reduced their acceleration events and collisions at the keel sooner than cage-reared hens, thus acclimating faster to the new layer environment (Chapter 2). Aviary-reared hens also preferred to use the highest perch available throughout the duration of the study (Chapter 2). Rearing hens with height also resulted in a faster acclimation timeline in multi-tiered layer aviaries. Hens from single-tiered and two-tiered pullet aviaries were more active when finding a nighttime roosting position, more hens used the highest zones of the aviary, and they laid fewer floor eggs than floor-reared hens (Chapter 4). However, these differences disappeared quickly (within 1 wk of housing for most behavior metrics and within 2 wk of lay for floor eggs). The fast acclimation time coupled with the lack of neurological differences at the end of rearing (Chapter 3) suggest that floor-reared hens were not seriously impaired from their rearing environment. They demonstrated different preferences and were initially less exploratory of the aviary, but ultimately they behaved similarly to the other rearing environments after gaining experience. Although this acclimation period did not affect the skill of vertical movements (uncontrolled transitions and the amount of

height crossed during a transition) or the prevalence and severity of keel injuries, it may have consequences for animal welfare and labor of caretakers in commercial settings.

Based on the findings of the present research, it is not clear how the acclimation period affects hen welfare. In Chapter 2, keel bone fractures were not assessed, so the implications of the acclimation period for injury are unknown. However, considering our similar experimental design to previous work where cage-reared hens sustained more fractures than aviary-reared hens (Casey-Trott et al., 2017a), it is likely that the poorer quality of vertical space use (i.e. prolonged rates of collisions) by cage-reared hens were associated with injuries. The delayed use of the aviary, particularly the higher zones, by FL hens in Chapter 4 did not have consequences for keel bone fractures. In the experimental pens, accessing height offered additional floor space, nests, and roosting positions, which may have satisfied motivations for some hens (Weeks and Nicol, 2006). However, these opportunities were not preferred by all hens as some demonstrated a preference for laying eggs and sleeping on the floor (as noted by the high prevalence of floor eggs and the lack of instances where 100% of hens roosted on the aviary at night). In commercial settings, feed and water are also provided on some or all elevated tiers of the aviary, so it is necessary for all hens to acclimate quickly to utilize height to obtain vital resources. Alternatively, hens are at risk of dehydration and malnourishment if they do not find feed and water (Tauson, 2005). If rearing-induced preferences for lower tiers persist in commercial environments, crowding may result and effectively reduce feeder space, which may increase aggression (Sirovnik et al., 2018). Additional labor of caretakers could be used to encourage hens to use height (e.g. walking houses more often or placing individual hens onto tiers, involving human-animal interactions) or modifications of the aviary to promote learning the

location of resources (e.g. enclosing hens into tiers or restricting access to segments of the litter area for a temporary period of time, temporarily reducing available floor space and increasing stocking density; Tauson, 2005). These additional factors of feed and water access, social dynamics, human-animal interactions, or increased stocking density during acclimation in commercial environments may pose animal welfare concerns and warrant future research.

Honing our understanding of what specific components of the rearing environment most improve acclimation is imperative for providing recommendations to equipment manufacturers and animal auditing programs for the design of pullet housing. Alternatively, comparing systems that differ in numerous facets (e.g. cage vs aviary rearing) or utilizing broad terms like "complex environments" (Du et al., 2022) do not provide specific context for what pullets need to cope with the transition to layer housing. Providing height in some capacity appears to be beneficial for hens when they are destined for a layer system involving height. Increasing height between ST and TT treatments (Chapter 4) did not provide increasing benefits to the hens, suggesting that the distance of height may be less important than simply the availability of height. It is a common practice in commercial environments to provide height at different ages in the pullet aviary to coincide with pullet growth and vaccination schedules, either by releasing pullets from enclosed tiers or gradually raising structures to increase height over time (discussed in Chapter 3). Future investigations into height during rearing should explore the age at which height is introduced and its implications for spatial abilities.

Regardless of rearing environment, keel bone fracture prevalence was high in the present work, capturing a large scale of minor fractures with a radiography technique (Chapter 4). It is not

readily clear what is causing the high prevalence of fractures, but the metal perches commonly utilized in aviaries may have contributed (Baur et al., 2020). Indeed, lower keel bone fractures have been reported when metal perches are covered with a soft polyurethane material to reduce pressure on the keels during perching (Stratmann et al., 2015b). In addition to evaluating the effect of rearing environment on uncontrolled transitions and keel bone fractures in a commercial setting (discussed in Chapter 4), future research could also consider honing environmental components that are contributing to injuries regardless of how well the hens are prepared to use them.

In conclusion, providing height during rearing environment promotes more efficient acclimation to layer housing in terms of the quantity and, for enriched colony cages, quality of vertical space use. Although we did not find a link between rearing environment and keel bone fractures, the work requires replication in a commercial setting. Furthermore, the acclimation period necessitates more research with regard to animal welfare implications considering its possible association with feed and water access, social dynamics, human-animal interactions, and stocking density. Environmental components in the layer aviary contributed to keel bone fractures moreso than the rearing environment in the present study, so the design of aviaries and the welfare implications of minor fractures (e.g. pain, mobility, egg laying) require attention.

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