

Happy Chickens: Novel Physiological and Behavioral Measures of Cumulative Experience in Broilers and Laying Hens

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Academic Abstract

Conventional housing environments for broiler chickens and commercial laying hens are often barren, high-density environments with an emphasis on production efficiency.

These housing conditions limit birds' ability to display species-specific behaviors, can negatively impact health, and may contribute to negative cumulative experience.

Cumulative experience is the culmination of all positive and negative experienced during an animal's lifetime. However, cumulative experience is difficult to quantify, as no validated measures of cumulative experience exist. Additionally, existing measures of negative animal experience mostly rely on interpretations of animal behavior which can be subjective, time consuming, and difficult to interpret. Therefore, there is scientific need for objective measures that can detect cumulative experience in poultry. Secretory and plasma Immunoglobulin A (IgA), telomere length, feather corticosterone concentrations, and attention bias testing all seem to respond to positive and negative experiences in humans or other non-human animal species, indicating that they may be useful as measures for poultry. Therefore, the objective of this thesis was to determine if these novel measures could be used as indicators of cumulative experience in broiler chickens and laying hens.

In chapter 3, secretory and plasma IgA concentrations were measured in broilers raised in either high-complexity or low-complexity environments under either high or low

stocking density over three replicated experiments. Birds housed in highly complex environments showed higher concentrations of plasma IgA compared to birds housed in low-complexity environments at day 48 of age, indicating reduced chronic stress in the former. Additionally, day 48 secretory IgA concentrations were decreased in birds housed in high-density environments compared to birds housed in low density environments, indicating birds from high-density environments were more chronically stressed. In chapter 4, gonad and kidney telomere length was measured to determine cumulative experience in broilers raised in the same housing conditions and replicated experiments of chapter 3. Treatment did not impact gonad telomere length, in line with expectations as gonads contain stem cells which produce high concentrations of telomerase. Birds housed in high-complexity pens had longer kidney telomeres compared to birds in low-complexity pens, indicating high-complexity birds had more positive cumulative experience. Stocking density did not impact kidney telomere length. In chapter 5, attention bias, tonic immobility, plasma and secretory IgA concentrations, and feather corticosterone concentrations were determined in laying hens raised in conventional cages or enriched floor pens. Birds in enriched floor pens showed increased attention bias, decreased tonic immobility, increased secretory IgA concentrations at week 22 of age, and decreased feather corticosterone concentrations compared to caged hens. These results indicate that compared to conventional cages, enriched pens in this study improved immune systems, reduced chronic stress, reduced fear, but increased anxiety in hens.

In conclusion, secretory and plasma IgA and telomere length show appropriate contrast in response to broiler chicken housing conditions. However, additional work needs to be

done before these measures can be widely used as measures of cumulative experience in poultry. Furthermore, attention bias, secretory IgA, and feather corticosterone showed an appropriate contrast between chronic stress responses in laying hens, but confirmation is needed in other contexts. Overall, the results indicate a beneficial relationship between environmental complexity and poultry welfare physiology and affective state, with the exception for anxiety in laying hens. Thus, providing an enriched environment can improve the welfare of commercial poultry and result in positive cumulative experience in most situations. Additionally, these results indicate that stocking density is a negative environment in broilers but potentially less intense than previously thought under experimental conditions. The assessment of behavioral and physiological measures of cumulative and positive animal experience should be included in experiments seeking to determine the impacts of environmental or management conditions to determine the broader impacts on poultry welfare.

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General Audience Abstract

Conventional housing systems of broiler chickens (raised for meat) and laying hens (raised for egg production) can negatively impact their welfare. Animal welfare, defined as an animal's ability to interact with and cope with their environment, is an individual experience for each animal and fluctuates on a scale from very negative to very positive. Traditionally, measurements of animal welfare have focused only on avoiding the negative aspects of animal welfare such as fear, distress (negative stress), hunger, thirst, pain, and suffering. However, it is important that animals are provided opportunities to experience positive animal welfare to provide a life worth living. So, when measuring animal welfare, all positive and negative experiences (termed cumulative experience) should be included to form an accurate picture of an animal's welfare. However, no validated measures of cumulative experience exist in non-human animals. However, recently, several potential measures of cumulative experience have been proposed in human and non-human animals including secretory and plasma IgA, telomere length, feather corticosterone, and attention bias testing. So, the objective of this thesis was to determine if these proposed measures can be used to determine cumulative experience in commercial broilers and laying hens.

In chapters 3 and 4, we investigated if secretory and plasma IgA concentrations (measure of chronic stress; chapter 3) and telomere length (measure of cumulative experience; chapter 4) responded to environmental complexity (positive stimulus) and stocking density (negative stimulus) over three replicated experiments. Broilers were housed in a 2 × 2 factorial study of either high or low complexity or high or low density. This resulted in four treatment groups of high-complexity/high-density, low-complexity/low-density, high-complexity/low-density, and low-complexity/high-density. During chapter 3, environmental complexity increased concentrations of plasma IgA, indicating that birds from high-complexity pens were under less chronic stress compared to birds from low-complexity pens. Alternatively, high density decreased secretory IgA, indicating that birds from high-density pens were under a more chronic stress than birds from low density pens. In chapter 4, environmental complexity increased telomere length in broilers compared to low-complexity pens indicating that environmental complexity positively impacted cumulative experience. However, stocking density did not impact telomere length, indicating that high density did not negatively impact cumulative experience. In chapter 5, we investigated if attention bias (measure of anxiety), tonic immobility duration (measure of fear), plasma and secretory IgA (chronic stress), and feather corticosterone (chronic stress) responded to environmentally enriched floor pens (positive housing system) and conventional caging (negative housing system). We found that birds housed in enriched floor pens were more anxious (increased attention bias), less fearful (decreased tonic immobility duration), and less chronically stressed (increased SIgA concentrations at week 22 and increased feather corticosterone concentrations) compared to birds housed in conventional cages.

Overall, IgA concentrations and telomere length (broilers) and attention bias, secretory IgA concentration, and feather corticosterone concentrations (layers) seem useable as measures of animal experience in commercial poultry. Additionally, these results indicate that positive experience has a positive impact on cumulative experience in commercial poultry. Stocking density also seems to contribute to chronic stress in broilers, indicated by decreased SIgA concentrations, but only during the last few weeks of life. These findings should be confirmed by additional studies before common use as measures of cumulative experience in animals. However, the inclusion of measures of cumulative and positive animal experience should be included in experiment which wish to determine the broad impacts of housing system on non-human animals.

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Chapter 1. Introduction

Animal welfare can be defined as an animal's ability to interact with and cope with its environment¹. From a social and political perspective, animal welfare has become synonymous with animal well-being, or with humane treatment and care for animals within the agricultural industry. This has resulted in an increase in social interest in positive animal welfare in recent years². One of the first internationally recognized and accepted animal welfare frameworks was the Five Freedoms of Animal Welfare, established by Britain's Farm Animal Welfare Council in 1965³. The Five Freedoms focus on ensuring animals freedom from hunger/thirst, fear/distress, pain/injury/disease, and discomfort, and freedom to display natural behaviors³⁻⁵. These freedoms are widely used in policy statements, legislation, and company guidelines⁶. However, four of the five freedoms of animal welfare focus on freedom from negative states, rather than ensuring animals can thrive. Consensus is growing that true animal welfare is not just the avoidance of negative states, but also the induction of positive states to provide a life worth living². Additionally, it is assumed that animal welfare is influenced by the cumulative effects of both positive and negative events experienced by an individual^{7,8}. The Five Freedoms do not encompass 'cumulative experience' or cumulative animal welfare, as they are applicable to only some of the many experiences an animal will face in its lifetime. Therefore, a new animal welfare framework was developed to meet scientists' consensus. Dr. David Fraser (2008) proposed a framework where cumulative animal welfare is an interaction between three key components that ensure good animal

welfare: 1) biological functioning (physical and physiological health), 2) natural living (ability to display natural behaviors), and 3) affective states (long-term emotional mood state) (Figure 1.1)⁹.

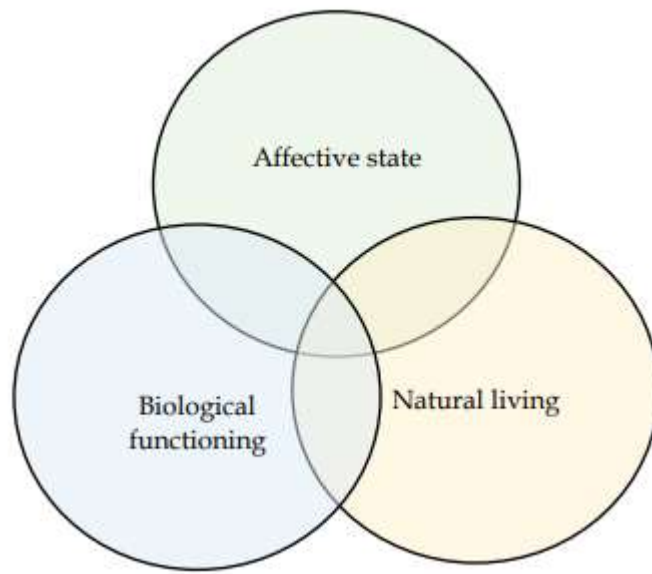


Figure 1.1. Three conceptions of animal welfare. Adapted from ⁹

However, when studying cumulative animal welfare focusing on any one aspect can completely change our perception of the animal's ability to thrive within its environment. For example, a focus strictly on basic health and functioning would state that domesticated animals should be physically and physiologically healthy. Under this view, the optimal housing system would be a total confinement system which limits the opportunity for an animal to become injured or be exposed to disease. However, total confinement systems are barren and can restrict the ability of an animal to display natural behaviors^{10,11} (decreased natural living) and often results in negative affective states

(anxiety or depression)^{12,13}, resulting in an environment that is suboptimal for positive cumulative animal welfare.

The least understood component of this animal welfare framework are affective states due to a lack of objective, validated measurements. Affective states are mood states which an animal experiences without any obvious outside stimuli and which results from the culmination of past life experiences⁷. To measure cumulative animal welfare, measurements of affective state must be included to ensure that production and research housing systems are eliciting positive affective states in production animals. To accomplish this, novel physiological and behavioral measurements of affective state are needed. Some behavioral measures of affective state exist, such as judgement bias and attention bias testing¹⁴⁻¹⁷, however, these tests need refinement for each species. Few physiological measures of affective state exist, and what measures do exist have severe limitations which diminish their viability for widespread use^{18,19}. Therefore, improved measures of affective state and cumulative experience are needed to ensure optimal cumulative welfare in production animals.

Chapter 2: Literature review

2.1 Broiler chickens

In the United States, broiler production was valued at over 21 billion USD in 2020, with a total of 9.22 billion broilers produced²⁰. Broiler chickens are a meat-type bird selected for fast growth and large body sizes²¹. Broilers are produced to efficiently provide meat for human consumption. Growth rates of broilers have steadily increased in the past 50 years, causing a decrease in marketing age and an increase in slaughter weight²². In 2022, broilers in the United States were raised to 47 days on average and weighed approximately 3.06 kg²³.

2.1.1 Housing conditions

Broilers are raised in relatively barren, large barn-like houses with large groups of conspecifics^{22,24}. These houses contain a litter substrate for bedding and ad libitum access to water and feed but limited to no access to complexity which may limit ability to display natural behaviors^{11,25}. Modern broiler houses can be entirely environmentally controlled via technology which maintains optimal temperature and humidity. Lighting programs differ between housing system dependent on if each house contains windows to allow natural light and to control growth and musculoskeletal development²⁶. However, the National Chicken Council (NCC) Animal Welfare Guidelines require 4 hours of darkness out of every 24 hours to allow for proper rest and circadian rhythms except during the first and last week of grow out²⁶. To maximize investment in a poultry house, producers will raise many birds in a single house, often at high stocking densities^{27,28}. This high stocking density and lack of environmental complexity can contribute to animal welfare concerns^{11,12,29-37}.

Stocking density is defined as the total amount of live bird weight per meter of usable floor space in a broiler house³⁸. Stocking densities are estimated using final bird weight to prevent stocking densities from exceeding legislative and industrial requirements during growth²⁶. However, these requirements are variable around the world. In Europe, legislative requirements exist which prevent broilers from being raised at stocking densities higher than 33kg/m², except for in accepted cases where producers have express documentation of good management practices and low mortality rates where birds can be raised at densities of up to 42kg/m². In the United States, no legislative requirements on broiler density exists, however, 95% of chicken raised in the U.S. are from companies who adhere to NCC guidelines of 36.6kg/m² for broilers between 2-2.5 kg and 41.5 kg/m² for birds from 2.5-3.4kg²⁶. Recently, the Better Chicken Commitment (BCC) has been gaining traction as a leading standard for broiler welfare parameters and has standards that all broilers be raised at less than 30 kg/m² by 2024³⁹.

High densities can result in poor leg health and lameness^{32,34,40}, disturbance of rest⁴¹⁻⁴⁶, and increased mortality⁴¹. General leg health and walkability are good estimators of general animal welfare and lameness is common welfare issue in broiler chickens^{41,47}. At high densities, the length of walking bouts decreases as birds age⁴², distance travelled decreases (42-46kg/m² compared to 30-34kg/m²)⁴³, gait scores are worsened (40kg/m² compared to 34 kg/m²)³⁵, and the severity of contact dermatitis increases (34-36kg/m² compared to 32 kg/m²)⁴⁸ when compared to lower stocking densities. Additionally, high-density environments can cause disturbance of rest⁴¹⁻⁴⁶. High-quality rest is important for basic psychological and physiological processes such as tissue repair and growth⁴⁵. Disturbance of rest occurs more often at high densities^{35,43,46} and can cause injuries⁴¹,

pain⁴¹, carcass damage⁴¹, and distress⁴⁹. High density can also help contribute to higher mortality⁴¹. Birds raised in high densities (20 birds/m²) had higher mortality due to heat stress than birds raised at low densities (10 birds/m²)⁵⁰.

In addition to stocking density, barren environments represent a significant risk factor for welfare concerns in broiler chickens. Barren environments limit broilers' ability to display highly motivated behaviors such as foraging⁵¹⁻⁵³, dust bathing^{54,55}, and perching⁵⁶. Foraging is a particularly highly motivated behavior in red jungle fowl, as regularly fed semi-wild birds have been observed spending 61% of their active time foraging⁵⁷. Similarly, chickens are highly motivated to forage and have been observed foraging in feces when no substrate is present^{52,53}. High litter moisture increases as birds age which can lead to low litter quality, limiting the appeal of litter as a foraging substrate^{53,58-61}. Additionally, low litter quality can limit the appeal of litter as a dustbathing substrate, diminishing dustbathing behavior^{53,58-62}. During dustbathing, birds will repeatedly rub their bodies in the substrate while rapidly flapping their wings and stretching their legs^{63,64}. Dust bathing has been shown to be highly motivated in broilers⁶⁴, laying hens^{65,66}, and red jungle fowl⁶⁶, with all showing dustbathing motivation in suboptimal substrates or without substrates^{3,54,55}. Similar to foraging and dustbathing, perching is a highly motivated behavior in poultry and is thought to be an anti-predatory behavior which provides protection while resting^{45,67}. Broilers continue to show this motivation and will perch if given easily accessible perches⁵⁶ or platforms⁶⁸. However, perch design is important as perch usage is low in broilers if the perches have small diameter (<11.3 cm circumference for females or 13.4 cm circumference for males) or

are difficult to access^{68,69}. In broilers, platforms seem to be preferred to traditional perches likely due to ease of use and accessibility^{68,70}.

2.2 Laying Hens

Modern commercial laying hens are genetically selected for reproductive rate and large egg production. This has resulted in a steady increase in egg production in the US since 1950. For example, in 1950, 56.9% of hens produced one egg per day in commercial facilities, while in 2000 82.0% of hens produced one egg per day⁷¹. In the United States in 2020, 325 million laying hens produced over 96 billion eggs⁷². The most common genetic strains used are White Leghorns which produce white eggs or brown hybrid birds which resemble New Hampshire Reds and Barred Plymouth Rocks⁷³. Unlike broilers, laying hens go through multiple production phases in different housing environments. In general, birds are hatched in a commercial hatchery and then housed in a pullet barn from day of hatch to ~18 weeks of age⁷³. To prevent premature stimulation of egg lay, pullets are light restricted to no more than 10 hours of light daily⁷³. At 18 weeks of age, birds are moved to a layer house where they remain for the duration of the production cycle. Production cycles begin at first lay, typically ~20 weeks of age and continue until ~60-65 weeks of age with egg production peaking at ~week 30⁷³. At ~week 60-65, birds will be rested, or molted⁷³. During molting, producers will provide a feed suitable for non-production hens and reduce the lighting period for a short period which halts egg production and causes loss of primary feathers⁷⁴. Following this period, producers will restrict lighting and provide a low protein feed during a resting period⁷⁵. This period of time allows the hens to rebuild calcium stores and body condition before the next production cycle begins⁷³. Following molting, hens are brought back into production by

increasing light and protein content in the feed. Hens will be kept from one to three production cycles depending on economic factors and each subsequent cycle will be shorter and produce lower-quality eggs⁷³.

2.2.1 Housing conditions

Laying hen housing systems are varied, with the most common housing types in the US being conventional battery caging, and cage-free multi-level aviary systems⁷².

Conventional battery cage systems consist of rowed wire cages with feed trough and water line access. While dimensions and number of birds per cage can vary between producers, the United Egg Producers guidelines require a minimum space allowance of 20.83x20.83 cm, or ~433cm² per bird⁷⁶. These same guidelines recommend a minimum of 5 lux of light intensity throughout the house⁷⁶. Cage-free multi-tiered aviary systems are open house systems which consist of an open floor with litter, nest boxes, and elevated platforms which allow free movement of birds throughout the house⁷⁷. Both systems are completely environmentally controlled, with technology that maintains optimal temperature and humidity.

Conventional caging systems can negatively impact the welfare of laying hens⁷⁸⁻⁸⁴.

Conventional cages severely limit the space per bird and provide no access to the outdoors, foraging substrate, or any form of environmental complexity, therefore limiting the ability to display natural behaviors such as perching, nesting, and foraging^{63,82-87}.

Inability to display natural behaviors can lead to boredom and frustration, increased stress, and adverse behaviors^{10,11,88-90}. Provision of a complex environment via access to environmental enrichments such as perches, nesting areas, and foraging substrates are imperative for laying hen welfare¹⁰. Perching is a highly motivated behavior in laying

hens and can help improve bone health^{82,91}, reduce fearfulness and aggression⁹², provide escape from aggressors^{82,91,93}, and lower risks of piling or smothering⁸². In conventional cage housing systems, access to perches is non-existent as cages lack space for perches to be installed. In cage-free systems, perches are provided, which have resulted in unintended animal welfare concerns. Perches and platforms in aviary systems require jumping and flying which can lead to keel bone fractures and deformities, with prevalence of up to 80% of birds, if the birds do not land successfully^{82,94,95}. While perch designs with soft surfaces and larger surface areas have been shown to help reduce risks to keel bones and leg health, keel bone injuries can still occur⁹⁶. Providing perches during the pullet phase also seems to increase muscle strength, motor skills, balance, and cognitive spatial skills in laying hens^{91,97}. While the provision of perches has a positive impact on the welfare of laying hens^{91,92,97,98}, additional work needs to be done to understand and mitigate keel bone damage and deformities in environments with access to platforms and perches.

Another highly-motivated behavior in laying hens is nesting^{83,85-87}. Laying hens prefer to lay their eggs in secluded nests^{79,85-87,99,100}. The strong motivation is illustrated by the hens' willingness to work for nest access. Hens will work harder to find a secluded nesting space 20 minutes before egg-laying than they will to find feed 4 hours after food deprivation¹⁰⁰. Similarly, hens experience more frustration, quantified by the number of gavel-calls, due to denial of nesting space than they do from food and water deprivation⁹⁹. Conventional caging does not provide a dedicated nesting space and birds housing in cages are forced to lay eggs on the sloped wire floor which has been shown to

lead to repetitive, stereotyped pacing in some, but not all, laying hens^{82,101}, and the retention of eggs^{83,101,102}

Foraging is a third highly motivated behavior that birds will display even when feed is provided ad libitum^{82,83}. Termed ‘contra-freeloading’, this behavior indicates a strong behavioral motivation to forage even if food is freely available¹⁰³. Foraging requires a form of friable substrate, which is not provided in conventional cages. Birds housed in cages prefer litter to wire flooring when given the choice¹⁰⁴. In cage free systems, birds are provided a substrate for foraging via litter flooring. Access to foraging substrate can have positive impacts on severe feather pecking behavior¹⁰. Severe feather pecking is a behavior where a hen will pull or pluck the feathers of other laying hens¹⁰⁵. This behavior is undesirable and can have negative impacts on laying hen health, egg production, and mortality^{10,106}. The addition of a foraging substrate such as litter, rice hulls, and oat husks can decrease severe feather pecking and increase foraging behavior in adult layers^{10,105,107–109}. However, the addition of foraging substrate and an increase in foraging behavior will not completely stop severe feather pecking behavior, leading producers to seek other solutions such as beak trimming^{10,105}.

2.3 Affective states

Although housing conditions for broiler chickens and laying hens can have impacts on bird health^{32–34,94–96,110,111} and behavior^{30,106,112,113}, we know little about how these systems impact emotions and affective states. Emotions and affective states are important aspects of animal welfare and should be measured and understood to ensure that animal welfare is optimized. Emotions can be defined as “short-term functional states produced by stimuli that animals work to either gain (reward) or avoid

(punishment), which help the animal to appropriately respond to environmental changes”¹⁴. This definition highlights that emotions are relatively short-term states which can be positively valenced (reward), negatively valenced (punishment), or somewhere in between (neutral valence). Emotions help animals make decisions about their environment¹⁴. Emotions are biologically comprehensive meaning they impact physiology, subjectivity, and behavior, and occur in response to any stimulus (person, object, event)¹⁴. In contrast, affective states are long-term mood states which occur without any obvious outside stimuli and which results from the culmination of emotions over time⁷. Both emotions and affective states can be modeled according to their valence and arousal level or intensity^{7,115}. For emotions, arousal level and intensity depend on the stimulus that elicits the response. For example, a prey animal under attack would likely experience fear, a high-intensity, negatively-valenced emotion. For affective states, the intensity and valence do not depend on a specific stimulus, but rather the culmination of arousal level and valence of experiences over time^{7,116,117}. For example, an animal continuously under attack from predators will experience multiple bouts of fear, potentially resulting in the animal experiencing a high-arousal, negatively-valenced affective state, even when the predator is not present.

2.3.1 Behavioral measures of affective state: Cognitive bias testing

Behavioral measurements of affective state rely on the idea that an animal’s prolonged mood state will impact how it makes decisions within its environment, or its ‘bias’. This bias impacts an animal’s cognition, defined here as the mechanism by which an animal acquires, processes, stores, and acts on information in its environment¹¹⁸. The association between affective state, cognitive bias, and information processing has been well documented, especially in humans. Humans in a negative affective state will more

readily bias their judgement and judge ambiguous stimuli as negative, be more vigilant to potentially threatening situations, and will remember negative experiences more than humans in a positive affective state¹¹⁹⁻¹²¹. This results in three common biases which could be used to develop affective state testing in non-human animals which are judgement bias, attention bias, and memory bias^{17,122}. Several studies have shown that judgement bias and attention bias can be used as measures of cognitive bias in poultry, swine, sheep, cattle, and companion animals^{15,17,118,123-136}. However, to date, there are no studies investigating memory bias in non-human animals. Additionally, as the attention bias test requires no training and is less time consuming than judgement bias¹⁴ this thesis



will focus on the attention bias test for measurements of cognitive bias in commercial poultry.

Fig 2.1. Illustration of how pinpoint emotional stimuli can elicit prolonged mood states which impact how animals process information. This information processing leads to a cognitive bias which can be used to measure animal affective states. Adapted from ¹³⁷

Cognitive bias testing is a novel, objective measure of animal affective state. A cognitive bias test allows for evaluation of both valence and arousal level of an animal's emotional state. This is an advantage over traditional behavioral measures of animal welfare which can be subjective to interpret, focus primarily on negative states, and potentially indicate arousal rather than valence^{14,18,138-141}. Additionally, cognitive bias testing allows for

measurement following manipulation of affective state, such as via changes in environmental conditions or pharmaceutical manipulation, allowing for *a priori* hypothesis testing¹³⁹.

2.3.2 Attention bias

To measure attention bias, the test measures how much focus or attention animals devote to a specific stimulus¹⁴. Attention bias can be described as, “the differential allocation of attentional resources towards one stimulus compared to others”, and has been well used in cognitive bias testing in humans, pigs, cattle, laying hens, sheep, and broilers^{12,14,16,123–126,128,130–132,134,135,142,143}. Attention bias is related to anxiety, with anxious individuals allocating more attention to negative stimuli than calm individuals^{144,145}. This anxiety can be quantified by measuring vigilance behaviors (alertness, freezing) in response to threatening and non-threatening situations¹⁴. At least 13 studies have investigated attention bias, and of those, 12 reported an effect of treatment on attention bias in animals^{12,16,123,124,128,130,131,135,136,142,143,146}.

The most common attention bias test approach involves presenting a positive and negative stimulus simultaneously^{14,147}. This method allows the tester to determine if the animal’s attention is biased towards the positive or negative stimulus¹⁴⁷. For poultry, this task would usually occur in an enclosed arena, with feed as a positive stimulus and a conspecific alarm call in response to a predator as the negative stimulus¹². The latency for a bird to begin feeding following the alarm call is indicative of their attention bias, with longer latencies and more frequent or longer vigilance behaviors indicating a greater level of anxiety, thus more a negative affective state^{12,14}. Alternatively, a shorter latency to

begin feeding and/or decreased vigilance behaviors, indicates a greater level of calmness, thus a more positive affective state^{12,14}.

2.4 Physiological measures of animal experience

Animal welfare is influenced by the cumulative positive and negative events experienced by an animal^{7,8}. This ‘cumulative experience’ is therefore the sum of all positive and negative experiences over an animal’s lifetime^{8,148}. Physiological measures can provide more insights on cumulative experience and several such measures have been proposed with varying levels of success^{8,18,149}. Of these, the most used and tested are circulating glucocorticoids. However, concentrations of brain neurotransmitters such as serotonin, dopamine, and oxytocin have been investigated, as have immune system measurements such as heterophil/lymphocyte ratio¹⁵⁰. However, all previous physiological measures of animal welfare have weaknesses which limit their viability for widespread use as measures of cumulative animal experience. For physiological measures of cumulative experience to be viable and objective they must be valence- and dose-dependent⁸. Valence-dependence suggests the measure must increase or decrease in response to the valence of an animal’s experience. Dose-dependence refers to the ability of a measure to respond to the intensity of a stimulus. Additionally, measures of cumulative experience should respond to chronic and acute stimuli which refers to long term and short-term experiences of an animal. To date, no physiological measures of animal welfare fulfill all of these requirements for use as measures of cumulative experience.

The most commonly used physiological measure is glucocorticoids released by the hypothalamic-pituitary-adrenal (**HPA**) axis. Glucocorticoids are the most common stress

hormone in vertebrate species and have long been used to quantify stress in production animals. Yet, glucocorticoids indicate arousal or intensity of a stimulus, but not the valence of a stimulus^{18,149} and both positive and negative stimuli of equal intensities can result in identical glucocorticoid responses. For example, pigs subjected to a dominance intruder test showed nearly identical peripheral HPA responses between winners and losers¹⁵¹. In this instance, the dominance test is a negative experience for the losers and a positive experience for the winners, however, this is impossible to determine from HPA axis responses alone¹⁵¹.

There is scientific need for validated objective physiological measures of animal cumulative experience. Recently, several measures have been proposed as potential indicators of animal experience in human and non-human animals^{8,18,149,152}. For the purposes of this thesis, the focus will be on telomere length, secretory and plasma Immunoglobulin A (IgA), and feather corticosterone concentrations.

2.4.1 Telomere length: A measure of cumulative experience

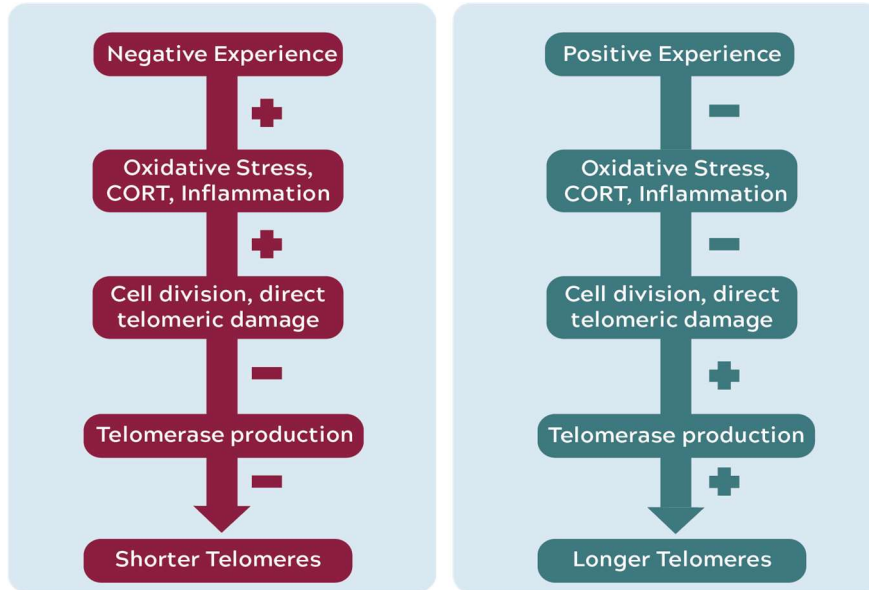
Telomere length, or more specifically telomere shortening, may be a potential biomarker for the cumulative experience in agricultural animals⁸. Telomeres are protein-DNA complexes and telomeric DNA is comprised of repeats of a nucleotide sextet of TTAGGG. Telomeres occur in all eukaryotic cells, and the sequence, TTAGGG, is conserved in all vertebrate species. Telomeres serve to protect gene-encoding DNA from the “end chain replication problem”, which is the idea that the entire chromosome cannot be copied every time a cell divides due to the need for an RNA primer to begin DNA replication. Therefore, telomeres shorten every cellular division, and this shortening, or telomere attrition, is a primary contributor to cellular aging and apoptosis of somatic

cells^{8,153}. This has led to a distinction between chronological age and biological age, where chronological age is the time proportional shortening of telomeres in response to mitotic divisions and biological age is the combination of chronological age and telomeric shortening due to stressful life events^{8,154}.

Telomere length and shortening rate is not consistent between animal species, or even between tissue types within single animals. Humans have, on average, telomere lengths between 8-20 kilobases (Kb) at birth, while chickens can have telomere lengths of greater than 1 megabase (Mb) at birth¹⁵⁵. The rate of shortening is increased in tissues that have high rates of cellular division. For example, leukocyte (high rate of division) telomere attrition is very high during early life and slows in adulthood, where muscle (low rate or no cellular division) telomere attrition rate is consistent throughout life^{8,156-159}. Therefore, tissue selection should be biologically relevant for the animal being sampled.

Telomere attrition may be a quantitative measure of animal welfare and cumulative experience. Humans that have been exposed to negative stressors, such as anxiety¹⁶⁰, depression¹⁶¹, neuroticism¹⁶², pessimism¹⁶³, childhood exposure to violence¹⁶⁴, family disruption¹⁶⁵, and chronic pain^{166,167}, have shorter telomere lengths than age-matched controls⁸. Additionally, telomere attrition seems to slow or potentially reverse in human who undergo positive lifestyle changes such as exercise, healthy diet, healthy sleep schedules, and positive mental interventions (meditation/mindfulness)¹⁶⁸⁻¹⁷¹. Telomere length in women who were caregivers to disabled children was negatively correlated to both years as a caregiver and their perceived level of stress¹⁷². The latter indicates that Telomere length is dose-dependent, showing responsiveness to the duration of the stressor (years as a caregiver). Additionally, Telomere length seems to be valence

dependent as it responds to both positive experience (mindfulness, exercise, healthy diet, healthy sleep, etc.¹⁶⁸⁻¹⁷⁰) and negative experience (anxiety, depression, neuroticisms,



pessimism, etc.¹⁶²⁻¹⁶⁷). Telomere length of healthy, middle-aged women was 35 bp shorter after a major stressful life event (divorce, loss of household, death of a relative, unemployment, etc.)¹⁶⁸.

Fig 2.2. Illustration of how positive and negative experience impact telomere length via causing fluctuations in oxidative stress, corticosterone production (CORT) and inflammation. In a situation of negative experience, such as distress, oxidative stress, corticosterone concentrations, and inflammation will increase which in turn will cause increases in cell division, direct telomeric damage by free radicals, and decreased telomerase production eventually leading to shorter telomeres. Adapted from ⁸

Table 2.1. Studies investigating telomere length in relation to animal welfare variables. Adapted from ⁸

Stressor	Species	Effect on telomere length	Reference
Rapid reproduction	Wild house mouse	↓	173
Overcrowding	Wild house mouse	↓	174

Repeated salmonella infection	Wild house mouse	↓	175
Repeated forced participation in acutely stressful events	Laboratory mouse	↓	173
Daily injection of corticosterone	Laboratory mouse	↓	173
High stocking density	Broiler chicken	↓	31
High stocking density	Laying hen chicken	↓	176
Acute and chronic infection	Great reed warbler	↓	177
Expanded brood sizes	Zebra finch	↓	178
Genetically high growth rate	Ocho Salmon	↓	179

Birds have telomere maintenance where telomerase is suppressed in somatic cells following hatching¹⁵⁵, however, some bird species have evolved excessively long telomeres both at the chromosome end and in interstitial repeats. Chickens can have telomeres of over 1 mb in length, which is much longer than the humans average 8-20 Kb average length^{8,180}. Similar to humans, chicken somatic tissues display division-dependent telomeric shortening, correlating with age, a phenomenon not seen in rodents who maintain long telomeres throughout life^{8,155}. Additionally, chicken somatic cells are seemingly devoid of telomerase, resulting in permanent and progressive telomeric shortening¹⁵⁵.

Avian telomeres are classified into three different categories (class I, II, and III arrays) which denote their length and location¹⁵⁵. Class I arrays are interstitial arrays ranging from 0.5-10 Kb in length and are highly variable between species¹⁸¹. Class II arrays are 10-40 Kb in length and are terminally located resulting in division-dependent telomeric shortening, Class III arrays are also terminal arrays but range in size from 200 kb-4 Mb,

earning this class the title “mega-telomeres”^{180,182–184}. Chickens have mega-telomeres; however, the distribution of these class III arrays is widely variable between strain, genetic line, and even individual^{180,182–184}. These mega-telomeres could represent a problem for quantification of telomere length via PCR, as PCR may lack the resolution to determine differences in length of excessively long telomeres, although this has not been confirmed.

A few studies have established an association between animal welfare status and telomere length in commercial chickens^{176,185}. Cobb 500 broilers fed corticosterone showed shorter telomere length when compared to the control¹⁸⁵. Similarly, single comb White Leghorn layers raised under high stocking density and feed restriction showed shorter telomere length and an increased rate of telomeric shortening when compared to Leghorns raised under lower densities and without feed restriction¹⁷⁶. Ross 308 broilers raised under high-density environments had a lower percentage of telomeric DNA when compared to birds raised under low-density environments¹⁷⁶. Otherwise, no research on telomere dynamics in relation to poultry welfare status has been done. As telomere length shows dose- and valence-dependence in humans, it could be a valuable biomarker for animal welfare status in commercially produced chickens. Therefore, more work is needed to determine how positive and negative environmental factors impact telomere length and telomere shortening in commercial chickens.

2.4.2 Secretory and plasma IgA: A measure of chronic stress

Immunoglobulin A (**IgA**) is the most common antibody found on mucosal surfaces in birds¹⁴⁹. IgA functions primarily as part of the innate immune system to neutralize viruses, toxins, and bacteria before they cross mucosal epithelium and cause

disease^{149,186–188}. IgA can also be present in blood to help control inflammation¹⁴⁹. Physiological and physical stress can impact IgA dynamics¹⁴⁹. At mucosal surfaces, IgA is produced by resident IgA-secreting plasma cells (mature B-cells). Therefore, IgA concentrations are independent between different mucosal surfaces and circulating IgA concentrations^{149,186,189}. Additionally, circulating IgA is monomeric while secretory IgA is dimeric and characterized by the addition of a secretory component which stabilizes IgA against degradation by host and microbial proteases alike^{149,186,190,191}. The mechanism which could impact IgA production which is thought to be relevant from a welfare perspective is through activation of the physiological stress response. Stress activates the physiological stress response which causes activation of the hypothalamic-pituitary-adrenal axis (**HPA**) and sympathetic-adrenal-medullary (**SAM**) axis to produce glucocorticoids and catecholamines^{149,192,193}. These outputs bind to glucocorticoid and B2-adrenergic receptors on B-cells to modulate IgA production on mucosal surfaces^{149,194–196}.

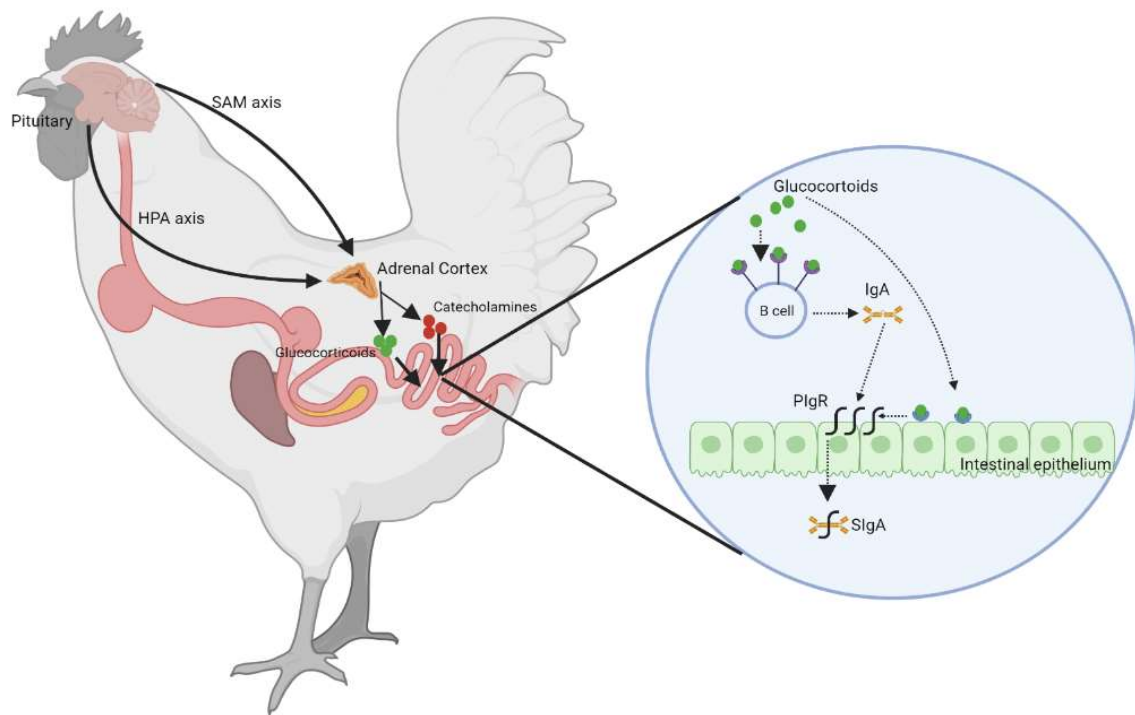


Figure 2.3. Mechanism by which the stress response can influence Immunoglobulin A concentrations in commercial poultry. The hypothalamic-pituitary-adrenal axis and sympathetic-adrenal-medullary axis produce glucocorticoids and catecholamines, which can interact with B cells to influence IgA production, or with intestinal epithelium to influence PIGR production, with increased PIGR production being associated with increased SIgA production. Diagram created in BioRender and adapted from ¹⁴⁹.

Acute and chronic stress responses negatively impact an animal's immune function^{29,149,197–199}. therefore, immune responses, including IgA concentrations, could potentially provide insights in animal welfare status. For example, an association between affective states and IgA concentration was found in cats. Individual shelter cats rated as frustrated or anxious had lower fecal SIgA concentrations than cats rated as content²⁰⁰. Similarly, anxious cats had increased fecal IgA following petting for 10 minutes, four

times daily, for 10 days and were less likely to exhibit behaviors related to frustration or anxiety^{201,202}.

Additionally, high-anxiety mice have lower mean serum IgA concentrations than low-anxiety mice²⁰³. In contrast, mice exposed to acute immobilization stress showed increased nasal IgA concentrations compared to the control group¹⁹². Acute stress activates the sympathetic nervous system, activating the HPA and SAM axis which release glucocorticoids and catecholamines that interact with B cells to increase short-term IgA concentrations^{149,204}. This effect is seen with both positive and negative acute stressors, suggesting IgA responds to arousal rather than valence of acute stressors¹⁴⁹. In chronically stressful situations, repetitive activation of the acute stress response causes prolonged exposure of mucosal epithelium to glucocorticoids and catecholamines^{149,204}. When chronically stressed, the immune system is consistently downregulated, leading to decreased IgA concentrations^{149,205}. Long-term acoustic noise exposure in shelter dogs reduced salivary IgA concentrations compared to dogs not subjected to noise²⁰⁶. Chronic negatively-valenced stressors often cannot be avoided (such as high stocking density, noise, high ammonia levels, barren environments, social isolation), resulting in repeated, long-term activation of the acute stress response, followed by immunosuppression, reflected in decreased IgA concentrations.

IgA as a measure of animal welfare may be more useful when applied to chronically negatively-valenced situations or situations where chronic stress is mediated through environmental interventions. One approach to mediate chronic stress is through the provision of environmental enrichments to create a complex environment. Environmental enrichments allow animals to display species-specific natural behaviors resulting in

decreased stress^{10,11,25,88,90,199,207}, but also provide choice of whether to interact with positively stressful enrichments. Long term, the choice provided by environmental enrichment likely decreases the chronic stress experienced, resulting in increases in IgA concentrations. This is consistent with previous studies which show that positive stimuli increase secretory and circulating IgA concentrations^{149,189,208,209}. The choice provided by environmental enrichment is important, as a positive stimulus can become negative when the choice to interact with the stimulus is removed. For example, voluntary exercise and exercise training programs (generally positive stimuli) in rats increased salivary and submandibular SIgA concentrations indicating positive stress, however, forced intense exercise (running to exhaustion) decreased small intestine SIgA indicating chronic negative stress^{189,208,210}. Intensive, forced exercise in elite athlete horses lowered serum IgA compared to horses subjected to less intensive exercise indicating that elite athlete horses are under chronic negative stress²¹¹. Lack of choice associated with barren housing systems negatively impacted IgA concentrations in rats and pigs. Transition to metabolic housing, a barren and isolated housing system, decreased fecal SIgA^{212,213}. However, the effect of social isolation on IgA concentrations are not consistent across species. Fecal SIgA concentrations in shelter dogs increased following social isolation from a conspecific partner, even when stereotypic behaviors increased²¹⁴.

Table 2.2. Studies investigating IgA in relation to welfare variables. Adapted from ¹⁴⁹

Welfare-associated variable	Species	Welfare variable	Effect on IgA concentrations	Reference
Choice in exercise	Rat	Voluntary exercise vs. control	↑ ↑ ↓	189
	Rat	Exercise training		208
	Rat	Forced intense exercise		210

	Horse	Intensive exercise	↓	211
Acute and chronic stress	Dog	Acoustic stress	↓	206
	Mouse	Repeated restraint stress	↓	215
	Mouse	Acute restraint stress	↑	216
Environmental stress	Mouse	Movement to novel cage	↑	209
	Rat	Movement to metabolic housing	↓	212
	Pig	Movement to metabolic housing	↓	213

The relationship between IgA concentrations, animal welfare, and housing conditions have not been studied in commercial poultry. One stressor related to IgA concentrations in commercial poultry is heat stress. Heat stress in commercial broilers (Cobb, Qing Yuan Ma) and commercial laying hens (Hy-line Brown) decreased IgA concentrations in serum and plasma^{217,218}. Additionally, lacrimal IgA concentrations in caged laying hens decreased in conjunction with high levels of plasma corticosteroids²¹⁹.

The research in other species, plus the research related to heat stress and HPA-axis outputs in poultry, suggest a potential value of IgA concentrations as a physiological measure of animal welfare in poultry.

2.4.3 Feather corticosterone: a measure of chronic stress

The HPA axis releases circulating glucocorticoids in response to perceived or actual acute stressors^{220,221}. This release of glucocorticoids causes a variety of physiological and behavioral changes that allow the animal to react to and overcome the stimulus in order to re-establish homeostasis^{152,220,222–225}. In the short-term, this glucocorticoid release is well-adaptive, allowing the animals to respond to a suite of

environmental stressors¹⁵². However, in the long-term, repeated activation of the HPA axis and consistent high concentrations of glucocorticoids can negatively impact cognitive ability, growth, immune health, reproduction, and survival^{152,220,226}.

Circulating glucocorticoid concentrations are commonly used as a measure to quantify acute stress^{152,221,227–229}. However, these concentrations are susceptible to sampling-induced acute stress, fail to respond to chronic stressors, and often indicate arousal of the system rather than the valence of a stimulus^{152,221,230}. While limited, circulating glucocorticoids can be useful in situations of acute stress when stimuli are known to be negative, such as transport of broiler chickens^{231–233}. In these instances, measurement of valence is not needed and circulating glucocorticoids are viable for quantification of the intensity of stress caused by the stimulus.

Corticosterone can be quantified in feathers providing insights in chronic rather than acute stress experiences¹⁵². Feathers are highly vascularized, allowing for incidental deposition of circulating corticosterone in the keratin matrix¹⁵². Corticosterone can be deposited throughout the feather growth period (days or weeks), providing the ability to quantify HPA activity over a prolonged period of time¹⁵². Feathers can be collected painlessly and quickly, minimizing acute stress caused by sampling¹⁵².

Feather corticosterone is used as a measure for chronic stress in avian ecological studies. Feather corticosterone concentrations in wild bird populations respond to differences in management^{234,235}, reproductive status²³⁶, immune status²³⁷ and body condition²³⁸. Few studies of feather corticosterone dynamics have occurred in commercial poultry^{111,239}. High stocking density increased feather corticosterone concentrations in Brown Nick laying hens when compared to birds housed in lower densities¹¹¹. In turkey hens, feather

corticosterone was higher during egg lay (week 30-week 45) in a high production line when compared to a low production line²³⁹.

2.5 Dissertation focus

This dissertation investigates novel behavioral and physiological measures of animal affective state, cumulative experience, and chronic stress and how they fluctuate depending on environment in commercial poultry. Chapter 3 is focused on plasma and secretory IgA, a novel physiological measure of chronic stress (IgA concentrations and telomere length) in three replicated experiments in broiler chickens housed under two stocking densities and two environmental complexity conditions. Chapter 4 investigates how these same stocking density and environmental complexity treatments impact telomere length, a novel physiological measure of cumulative experience. Chapter 5 is focused on laying hens housed in either conventional battery cages or enriched floor pens and the impact of these housing conditions on novel behavioral and physiological measures of affective state (attention bias) and chronic stress (secretory/plasma IgA and feather corticosterone). Finally, chapter 6 will provide a general discussion and conclusion and highlight areas of potential future research. The overall objective of this dissertation is to determine if these novel indicators are viable measures affective state and animal experience to better measure cumulative animal welfare.

Chapter 3: Plasma and fecal Immunoglobulin-A as measures of chronic stress in broiler chickens in response to environmental complexity and stocking density

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[3.1 Abstract](#)

Commercial housing systems may contribute to chronic negative stress in broiler chickens, reducing their animal welfare. The objective was to determine how secretory (fecal) and plasma immunoglobulin-A (IgA) levels in fast-growing broilers respond to positive and negative housing conditions. In three replicated experiments, male Ross 708 broilers (N=1,650/experiment) were housed in a 2×2 factorial study of high or low environmental complexity and high or low stocking density. In experiments 1 and 3, but not in 2, high complexity tended to positively impact day 48 plasma IgA concentrations. When three experiments were combined, high-complexity positively impacted day 48 plasma IgA concentrations. Stocking density and the complexity × density interaction did not impact day 48 plasma IgA concentrations. Environmental complexity and the complexity × density interaction did not impact day 48 secretory IgA concentrations. High density negatively impacted day 48 secretory IgA concentrations overall, but not in individual experiments. These results indicate that environmental complexity decreased chronic stress, while high stocking density increased chronic stress. Thus, plasma IgA levels increased under complex housing conditions (at day 48) and secretory IgA levels (at day 48) decreased under high-density conditions, suggesting that chronic stress differed among treatments. Therefore, these measures may be useful to quantify chronic

stress, but only if statistical power is high. Future research should replicate these findings under similar and different housing conditions to confirm the suitability of IgA as a chronic stress measure for broiler chickens.

Keywords: Secretory IgA, Plasma IgA, broiler, environmental complexity, stocking density

3.2 Introduction

The World Health Organization defines stress as any change which causes physical, emotional, or physiological strain to an animal²⁴⁰. Stress activates the Hypothalamus-Pituitary-Adrenal (HPA) axis, which starts a chain of signaling that culminates in the release of glucocorticoids²⁴¹. Stress can be positive (eustress) or negative (distress) and short-term (acute) or long-term (chronic). From an animal-welfare perspective, it is desirable to avoid acute and chronic distress and promote opportunities for acute and chronic eustress.

Associations between environmental factors and stress are well-established in broiler chickens, where barren housing systems^{51-53,55,56,64}, high stocking density^{30,32,34,40-43,45,46} and excessive heat²⁴² may potentially cause acute and chronic physical or emotional distress. For example, barren housing conditions can lead to negative affective states, reduced frequencies of natural behaviors such as perching or dustbathing, and frustration and boredom^{11-13,243}. High stocking density can lead to decreased foot and leg health^{33-35,40,42,43}, frustration and boredom^{11,243}, and increased acute distress reflected in increased circulating glucocorticoid and heat shock protein 70 concentrations^{244,245}.

Stress in production animals is often quantified using circulating glucocorticoids^{244,245}, which are steroid hormones produced in the adrenal gland. Circulating glucocorticoids

are reflective of experienced acute stress and indicate arousal (intensity) rather than the valence (positivity/negativity) associated with a stimulus^{7,246}. Therefore, interpretations of the impacts of housing conditions on circulating glucocorticoids are difficult, as similar increases in glucocorticoid concentrations could occur from both positive and negative stimuli as long as the intensity is comparable. For example, pigs subjected to a dominance intruder test showed nearly identical glucocorticoid responses between winners and losers¹⁵¹. In this instance, the dominance test is a negative experience for the losers and a positive experience for the winners, however, this difference is impossible to judge from glucocorticoid concentrations alone. Therefore, it is not possible to evaluate the valence of stimuli when only assessing the glucocorticoid response. This limits the usefulness of glucocorticoid measures to only inherently negative stimuli, such as catching and loading for transportation in broiler chickens, as the valence of the stimuli is known, and glucocorticoids can be used to measure the intensity^{229,247}. Additionally, circulating glucocorticoids are only suitable as measures of acute rather than chronic stress, as concentrations of blood glucocorticoids can change within minutes²²¹. Therefore, there is need for indicators for chronic stress which can reflect the valence of stimuli.

Secretory and plasma immunoglobulin-A (IgA) concentrations show potential as a measure of chronic stress. IgA is the most common antibody found on mucosal surfaces and functions as a first line of defense against inhaled and ingested pathogens^{149,186,187}. While both secretory IgA (SIgA) and plasma IgA (PIgA) function primarily as part of the innate immune system, concentrations of SIgA and PIgA are independent due to differences in structure and production. SIgA is dimeric and produced by resident mature

B-cells at mucosal surfaces, while PIgA is monomeric and produced by B-cells in bone marrow²⁴⁸. Additionally, SIgA contains the polymeric immunoglobulin receptor (PIgR) as a secretory component that could control SIgA production^{149,186,189}.

Both SIgA and PIgA concentrations are downregulated in response to physiological or physical stress and upregulated in situations of positive experience^{123,149,203,215,249–251}. In rats and pigs, transfer to metabolic housing systems decreased SIgA concentrations compared to animals in non-metabolic housing systems^{209,212,213}. In rats^{189,210}, mice²⁰⁸, and horses²¹¹, forced intense exercise decreased SIgA and serum IgA concentrations, while voluntary exercise had the opposite effect. When a male rat is paired with a female, male SIgA concentrations initially decrease before steadily increasing, indicating that male/female pairing is a positive stimulus²⁵¹. The opposite occurred when male rats were grouped with five other males, with SIgA concentrations steadily decreasing, indicating that male-only group housing is a negative stimulus²⁵¹.

Few studies investigated SIgA concentrations' response to environmental stimuli in chickens. Chronic heat stress decreased SIgA concentrations in Cobb and Qing Yuan Ma broilers and Hy-line Brown laying hens^{217,242}. Additionally, Bovar Brown laying hens housed in enriched floor pens showed increased SIgA concentrations compared to hens raised in conventional battery caging²⁴⁹. The impact of chronic housing stressors other than heat stress on broiler chickens' IgA concentrations is unclear.

Environmental complexity can improve broiler chicken welfare outcomes, such as improving affective states¹², health^{11,252,253}, and occurrences of species-specific behaviors¹¹. Environmental complexity decreased anxiety and fear, and increased optimism in Ross 708 broiler chickens compared to birds raised in more barren housing

conditions^{12,13}. Environmental complexity benefitted leg and bone health, decreasing lameness as broilers aged^{252,253}. In Japanese quail, environmental enrichments decreased the negative impacts of a chronic stressor (repeated restraint stress) on the immune system¹⁹⁹. While most studies report a positive impact of environmental complexity on broiler chicken welfare outcomes, some studies report no impact^{25,37,47,254}.

High stocking density is a well-studied housing condition for broiler chickens that can negatively impact welfare outcomes including behavior, performance, and leg and foot health^{30,32,34,40-43,45,46}. High densities (40 kg/m²) increased gait scores (more severe lameness) compared to birds raised at lower densities (34 kg/m²)³⁵. Additionally, the severity of contact dermatitis increased in high densities (34-36kg/m²) compared to lower densities (32 kg/m²)⁴⁸. High densities can cause disturbance of rest^{35,43,46}, injuries⁴¹, pain⁴¹, distress⁴⁹, mortality⁴¹, and carcass damage⁴¹.

The sensitivity and response of SIgA and PIgA concentrations to positive and negative stimuli in chickens and other species indicate a potential application as measures for chronic stress in broiler chickens. However, impacts of housing conditions other than heat stress, such as complexity and stocking density, are not yet examined. Additionally, the potential combined impact of environmental complexity and stocking density on PIgA and SIgA are unknown. Therefore, the objective of this study was to determine how environmental complexity and stocking density impacted SIgA and PIgA concentrations in fast-growing broiler chickens. We hypothesized that birds housed in high-complexity environments would have increased IgA concentrations and birds housed in high-density environments would have decreased IgA concentrations. We hypothesized that broilers housed in highly complex, low-density environments would show higher IgA

concentrations indicating lower chronic distress compared to broilers from low-complexity, high-density environments.

3.3 Materials and methods

3.3.1 Animals and housing

This experiment was approved by the Virginia Tech Institutional Care and Use Committee (IACUC protocol 19-175). In three separate replicated experiments, day-old Ross 708 male broilers (N=1,650 birds/experiment) were sourced from a commercial hatchery (Elizabethtown, PA, USA). Birds were vaccinated for Marek's disease at the hatchery. The experiments consisted of a 2×2 factorial design comparing environmental complexity (high complexity [HC] vs. low complexity [LC]) and stocking density (high density [HD] vs. low density [LD]) as factors. At d1 of age, birds were randomly allocated into 12 pens, each containing one of four treatments (high-complexity/high density [HC/HD], high-complexity/low-density [HC/LD], low-complexity/high-density [LC/HD], and low-complexity/low-density [LC/LD]), with three replicates per treatment, in a randomized block design.

Each pen (14.5 m²) contained clean pine wood shavings (~10 cm depth), 4 steel galvanized feeders, and 3 nipple water lines (3 nipples/line). Birds were phase-fed a commercial corn-soy broiler diet which was formulated to meet their nutritional requirements and included a starter phase (d1-d14), a grower phase (d15-d28), and a finisher phase (d29-d48). Birds had *ad libitum* access to feed and water. During the first week, pens contained 3 heat lamps and were under continuous lighting. Due to a technical problem in experiment 1, birds received 24h light for an additional week from d7-14. Thereafter, the lighting schedule was 18L:6D with a light intensity of ~15 lux

during light hours. Temperature was 35°C on d1 and gradually reduced to 21°C on d24 and maintained until d50. Due to a pathogen exposure in experiment 1, birds were given a therapeutic dose of antibiotics from d33 until d40 of age via the water lines.

3.3.1.1 Environmental Complexity

The HC pens contained four functional spaces, with a “feeding” area (~3.2m²), a “comfort” area (~3.2m²), a “resting” area (~3.2m²), and an “exploration” area (~4.3m²). The feeding area contained all feeders (~12kg volume) and 1/3 of mineral pecking stone broken into smaller pieces (Proteka, Inc., Lucknow, ON, Canada). The comfort area contained a wooden dust bath (~2m²) with ~68kg playground sand (QUIKRETE, Atlanta, GA, USA), which was raked and partially replaced during rearing. The resting area contained three perching structures¹². In experiment 1, perches (183 cm L × 31 cm W × 9 cm H) were built out of 1.9-cm diameter PVC pipe and were treated with textured black spray paint (Rust-Oleum, Vernon Hills, IL, USA). In experiments 2 and 3, perching platforms (122 cm L × 46 cm W × 8 cm H) were constructed from 10-cm wide wooden boards. In experiment 1, linear perching space per bird was 15.2 cm in the LD pens and 7.6 cm in the HD pens. In experiments 2 and 3, perching space per bird was 76 cm² in the LD pens and 39 cm² in the HD pens. In the exploration area, six temporary enrichments were paired and rotated on a three-day schedule. These enrichments included four colored plastic balls (Clink N’ Play, USA), four yellow treat dispenser balls with oats (Lixit Corp., Napa, CA, USA), four polyethylene string bundles suspended at bird level, iceberg lettuce (experiment 1: chopped and placed in red rubber Kong toy (KONG, Golden, CO, USA) on litter) or cabbage (experiment 2 and 3: half a head suspended at bird level), four metal wire balls filled with alfalfa hay (Darice, Strongsville, OH, USA), and laser lights manually projected in the pens two times a day for 5 min. The

enrichments were paired so that each set contained a nutritional and occupational enrichment: hay balls/hanging strings, yellow oat balls/plastic balls, and lettuce or cabbage/laser lights.

The LC pens were also divided into four functional areas but contained no enrichments.

Four galvanized steel feeders were dispersed throughout the pen.

3.3.1.2 Stocking Density

HD pens contained 180 birds and LD pens contained 90 birds per pen for a final targeted stocking density of 40-42 kg/m² or 20-22 kg/m² respectively. Final stocking density at d50 was 42.1 kg/m² (experiment 1), 42.6 kg/m² (experiment 2), and 42.1 kg/m² (experiment 3) in HD pens and 23.8 kg/m² (experiment 1), 23.3 kg/m² (experiment 2), and 22.1 kg/m² (experiment 3) in LD pens.

3.3.2 Measurements

Blood samples were collected at d28 and d48 of age in all three experiments. Blood was collected from five birds per pen (15 samples/treatment/experiment) via the brachial vein and transferred to glass collection tubes coated with 0.5% EDTA solution, gently inverted a few times, placed on ice, and then transported to the lab for further processing.

Following transport, blood samples were centrifuged at 10,000 × g for 5 min. Plasma was separated from red blood cells before storage at -20°C. During blood collection, sample times were recorded, from start of handling to removal of the needle from the bird, to ensure all samples were collected in under two minutes.

Fresh fecal samples were collected from pen floors on d48 of age in all three experiments following observation of defecation, and stored in microcentrifuge tubes, placed on ice, then transported to the lab for storage at -80°C until further processing. In the first two

experiments, fecal samples were pooled at pen level following collection resulting in 12 samples collected per experiment (3 samples/treatment/experiment). In experiment 3, individual fecal samples were collected (N=5/pen) from arbitrary individuals following visual confirmation of defecation to increase sample size.

For quantification of SIgA, total protein content of fecal samples was extracted via a saline extraction method^{213,249,255,256}. In short, samples were weighed and suspended in an extraction buffer of 0.01 phosphate buffered saline, 0.5% tween (Sigma-Aldrich, St. Louis, MO, USA), and 0.05% sodium azide at a ratio of 10 mL buffer to 1 g sample. Following suspension, samples were centrifuged at $1,500 \times g$ for 20 min at 5°C. Sample supernatant was then separated from solids, transferred into a microcentrifuge tube with 20 μ L protease inhibitor cocktail (Sigma Aldrich., St. Louis, MO, USA), and homogenized before storage at -20°C.

Concentrations of SIgA and PIgA (μ g/mL) were determined using a commercial enzyme-linked immunosorbent assay (ELISA; Abcam, Cambridge, MA, USA) following manufactures instructions. Intra-assay CV% were below 2% for all samples (min 0.2%; max 2%). During sampling, it was impossible to blind sample collectors for treatment, as samples were collected in the poultry facility. However, during sample analysis, laboratory technicians were blinded for treatment.

3.3.3 Statistical analysis

All statistical analyses were done in JMP Pro 16 (SAS Institute Inc., Cary, NC, USA).

Pen was considered the experimental unit and bird the observational unit for PIgA analysis. In experiments 1 and 2, pen was the experimental and observational unit for SIgA analysis, while in experiment 3 pen was the experimental unit and sample (N=5)

the observational unit. Data residuals for all response variables were normally distributed based on visual inspection of normal quantile plots. Data from each experiment were analyzed by experiment using linear mixed models with environmental complexity, stocking density, and their interactions as fixed factors. Pen number was included as a random factor. In addition, data from all three experiments were combined and analyzed using linear mixed models with environmental complexity, stocking density, and their interactions as fixed factors. Pen number and experiment number were included as random factors. Post-hoc analysis was performed with Tukey HSD corrections. Associations were considered significant at $P \leq 0.05$ and a trend at $P \leq 0.1$. All data are presented as LSmeans \pm SEM.

3.4 Results

3.4.1 Day 28 plasma IgA concentrations

3.1 Day 28 plasma IgA concentrations

D28 PIgA concentrations were impacted by a complexity \times density interaction ($F_{1,59}=6.20$; $P=0.037$) in experiment 2 and tended to be impacted by the interaction when all three trials were combined ($F_{1,179}=1.93$; $P=0.055$; Table 1), but not in experiment 1 ($F_{1,59}=-0.14$, $P=0.894$;) or 3 ($F_{1,59}=0.549$, $P=0.479$). Pairwise comparisons in experiment 2 and overall were not significant ($P > 0.1$). Complexity or stocking density treatments did not impact d28 PIgA concentrations in experiment 1 (Complexity: $F_{1,59}=-0.5$, $P=0.574$ Density: $F_{1,59}=-0.69$, $P=0.494$), experiment 2 (Complexity: $F_{1,59}=1.33$; $P=0.276$; Density: $F_{1,59}=0.08$; $P=0.789$) or experiment 3 (Complexity: $F_{1,59}=0.04$, $P=0.845$ Density: $F_{1,59}=0.234$, $P=0.642$; Table 1). Overall, d28 PIgA did not differ due to complexity ($F_{1,179}=0.41$; $P=0.525$) or stocking density treatments ($F_{1,179}=0.289$; $P=0.591$; Table 1).

Table 3.1. LSmean±SEM estimates of day 28 plasma immunoglobulin-A ($\mu\text{g}/\mu\text{L}$ plasma) concentrations in experiment 1 (N=60), experiment 2 (N=60), experiment 3 (N=60) and overall (N=180).

Treatment	Experiment 1	Experiment 2	Experiment 3	Overall
High complexity (HC)	139±12	265±31	213±19	208±29
Low complexity (LC)	156±12	215±31	219±19	196±29
High density (HD)	155±12	246±31	223±19	207±29
Low density (LD)	141±12	234±31	209±19	197±29
HC/HD	145±18	327±44	210±28	231±22
HC/LD	134±18	204±44	217±28	184±21
LC/HD	164±18	165±44	236±28	176±21
LC/LD	148±18	264±44	202±28	208±22

3.4.2 Day 48 plasma IgA concentrations

48 PIgA concentrations were not impacted by the complexity \times density interaction during experiment 1 ($F_{1,59}=1.00$; $P=0.347$), experiment 2 ($F_{1,59}=0.17$; $P=0.695$), experiment 3 ($F_{1,59}=0.234$; $P=0.6414$), or overall ($F_{1,179}<0.1$; $P=0.997$).

D48 PIgA concentrations tended to be increased in the HC treatment during experiment 1 ($F_{1,59}=4.49$; $P=0.068$) and experiment 3 ($F_{1,59}=3.52$; $P=0.098$), but not experiment 2 ($F_{1,59}=0.74$; $P=0.420$; Figure 1). Across three experiments, d48 PIgA concentrations were increased in the HC treatment compared to the LC treatment ($F_{1,179}=6.87$; $P=0.011$; Figure 2).

Stocking density did not impact d48 PIgA concentrations in individual experiments (1: $F_{1,59}<0.01$; $P=0.991$, 2: $F_{1,59}=0.34$; $P=0.579$, 3: $F_{1,59}=0.22$; $P=0.646$; Figure 1) or overall ($F_{1,179}=0.19$; $P=0.664$; Figure 2).

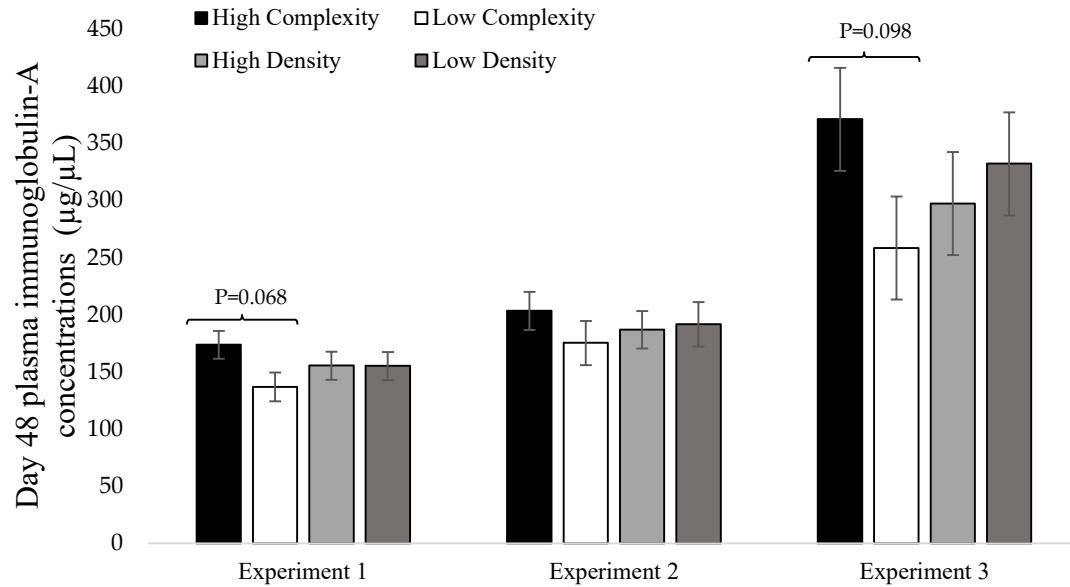


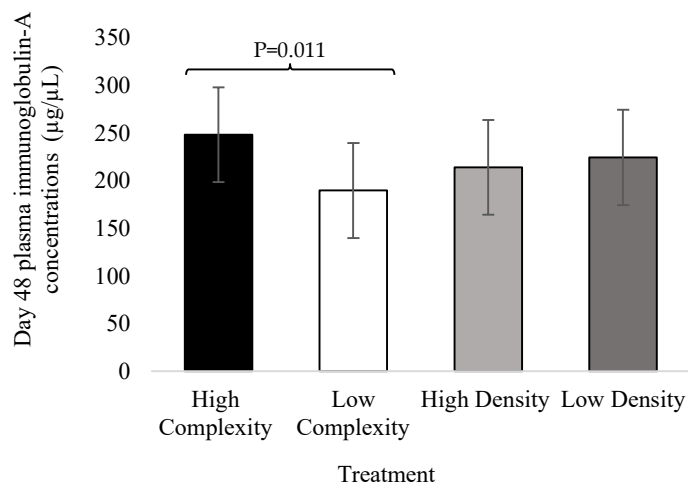
Figure 1. Least square mean estimates (\pm SEM) of plasma immunoglobulin-A concentrations in broilers on day 48 of age housed at high or low complexity and at high or low stocking density in experiment 1 ($n=60$), experiment 2 ($N=60$) and experiment 3 ($N=60$).

Figure 2. Least squared mean estimates (\pm SEM) of plasma immunoglobulin-A concentrations in broilers on day 48 of age housed at high or low complexity and at high or low stocking density across all three experiments ($N=180$).

3.4.3 Day 48 fecal IgA concentrations

No complexity \times density interaction on d48 SIgA was observed during experiment 1

($F_{1,11}=0.40$; $P=0.544$), experiment 2 ($F_{1,11}=0.056$; $P=0.819$), experiment 3 ($F_{1,59}=0.96$;



P=0.357), or overall ($F_{1,59}=-1.11$; $P=0.298$). D48 SIgA concentrations were not impacted by environmental complexity or stocking density treatments in experiment 1 ($F_{1,8}=0.41$; $P=0.694$; $F_{1,8}=-1.26$; $P=0.247$), experiment 2 ($F_{1,8}=2.29$; $P=0.168$; $F_{1,8}=-1.44$; $P=0.187$), or experiment 3 ($F_{1,59}=-0.06$; $P=0.957$; $F_{1,59}=0.897$; $P=0.371$).

Overall, d48 SIgA concentrations were decreased in the HD treatment compared to the LD treatment ($F_{1,83}=4.69$; $P=0.033$; Figure 3). Environmental complexity did not impact d48 SIgA concentrations across all three experiments ($F_{1,59}=-0.06$; $P=0.957$; Figure 3).

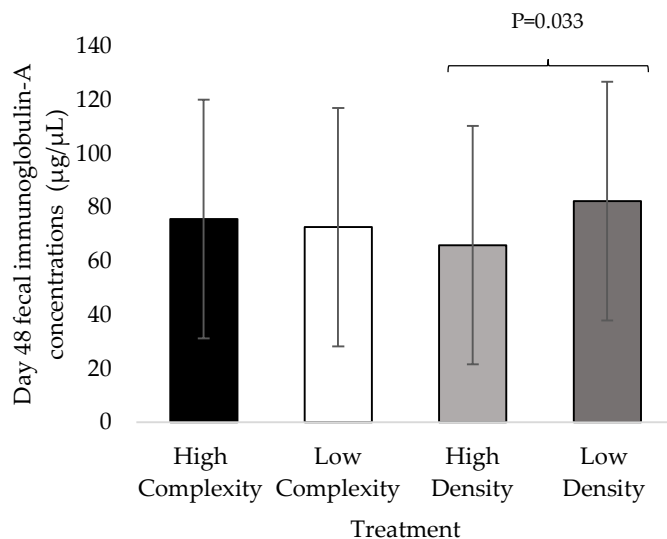


Figure 3. Least squared mean estimates (\pm SEM) of secretory (fecal) immunoglobulin-A concentrations in broilers on day 48 of age housed at high or low complexity and at high or low stocking density across all three experiments ($N=90$).

3.5 Discussion

This study investigated the impacts of positive (environmental complexity) and negative (stocking density) housing conditions on broiler chicken PIgA and SIgA concentrations in three replicated experiments. We did not confirm the hypothesis that broilers housed in

highly complex, low-density (HC/LD) environments would show higher IgA concentrations, indicative of lower chronic stress compared to broilers in low-complexity, high-density (LC/HD) environments. We were able to confirm our hypothesis that IgA concentrations increase in response to high-complexity environments and decrease in response to high-density environments, as pIgA and sIgA concentrations at day 48 differed in response to environmental complexity and stocking density, respectively. This is in line with previous studies where IgA concentrations responded to positive and negative experience in mice, rats, dogs, horses, and laying hens^{189,203,206,208–214,249,251}. The current study is the first to detect an impact of environmental complexity on pIgA concentrations and an impact of stocking density on sIgA concentrations in broiler chickens.

At d28 of age, an interaction effect between complexity and stocking density on pIgA concentrations was found in experiment 2 and across all experiments, however no pairwise differences were found, making interpretation of this interaction difficult. Sample sizes for this experiment were relatively low (N=15 samples/treatment/experiment; N=45 samples/treatment total). It is possible that this interaction would have resulted in interpretable results with a greater sample size, thus with a higher statistical power.

Neither environmental complexity or stocking density impacted d28 pIgA concentrations in any of the three experiments or overall, which was unexpected. High stocking density is considered a negative stimulus for broiler chickens^{30,32,34,40–43,45,46}. The negative impacts of high stocking density generally do not occur until densities reach over 30 kg/m²^{32,33}. This higher density is reached when birds reach a certain weight (as birds

grow and space availability remains the same), which in our study was at 33 days of age in HD pens. This means that the HD birds in our study were exposed to high densities and the associated potential distress for approximately two weeks of life (day 33-day 48). At d28 of age, actual stocking density was still relatively low (~22-24 kg/m² in HD pens) so it may not be experienced as a negative stressor in young broilers. In line, birds were less fearful when raised in HD pens when compared to birds raised in LD pens at d26 of age¹². While stocking density is generally considered a negative stimulus^{30,32,34,40-43,45,46}, other studies do not report a negative impact of stocking density on broiler affective state, and acute distress^{12,13,257}. Future studies should investigate the impacts of stocking density on birds at different ages to determine if the length of exposure to high density impacts welfare outcomes and IgA concentrations.

We observed a positive impact of a complex environment on d48 PIgA concentrations in experiments 1 (trend), 3 (trend), and overall, although no association was found in experiment 2. The tendencies observed in experiments 1 and 3 may be due to a relatively low sample size, which is supported by the statistical association found when combining the three experiments, and the numerical (non-significant) differences observed in experiment 2. Therefore, future experiments investigating PIgA concentrations in relation to housing conditions should ensure higher statistical power than in the current study.

Overall, the positive effect of a complex environment on d48 PIgA concentrations is replicable across experiments if experimental conditions are similar. Our results are consistent with findings in other species^{189,200,202,208,214}. A complex environment allows broilers to express species-specific behaviors, which can induce a positive affective state compared to broilers raised in low-complexity environments^{11-13,25,258}. Additionally, low-

complexity environments can result in chronic and acute distress due to the inability to display these natural behaviors. This distress could increase circulating glucocorticoid concentrations and HPA axis activity. Physiological stress responses can impact IgA concentrations via glucocorticoid signaling^{149,192,193}. Glucocorticoids bind to IgA-secreting B-cells and reduce the expression of IgA-encoding mRNA^{149,194-196}. Decreased expression of IgA-encoding mRNA could decrease the transcription of IgA proteins. Reduced transcription of IgA proteins leads to decreased concentrations of functional IgA. Low-complexity and barren environments can cause distress^{10,11,25,88,259}, thus birds raised in these environments could have higher concentrations of circulating glucocorticoids in comparison to high-complexity environments. Increased glucocorticoid concentrations in low-complexity birds results in increased interactions between glucocorticoids and IgA-secreting B-cells thereby decreasing expression of IgA mRNA and transcription of IgA proteins in low-complexity birds, resulting in lower pIgA concentrations. Our results indicate that d48 pIgA is responsive to positive housing conditions (environmental complexity) and could be useful as a measure of chronic stress in broilers. Yet, it is still not fully understood what role glucocorticoids play in pIgA production, especially when broilers are kept in positive conditions.

Environmental complexity did not impact d48 sIgA concentrations. The lack of impact may indicate that environmental complexity was not a positive housing stimulus for broiler chickens. However, this is contradictory to our d48 pIgA results and previous studies which indicate that environmental complexity reduced distress and improved affect in broilers^{11-13,25,90,253}. For example, broilers housed in high-complexity environments were less fearful, less anxious, and less fearful following acute stressors

than broilers housed in low-complexity environments^{12,90}. Additionally, this is contradictory to previous studies which show that SIgA responds to positive experience in other species^{189,208}. Broiler chicken SIgA may be insensitive to positive experience, which cannot be confirmed by previous research. It is also possible that we did not detect an impact of environmental complexity due to measurement error. Fecal IgA can be difficult to quantify as fecal proteases can breakdown SIgA quickly following defecation^{213,249,255,256}. We ensured sample freshness by visual confirmation of defecation and freezing the samples quickly after collection. Currently we have no evidence supporting that d48 SIgA is a useful indicator of positive experience in broilers.

High stocking density resulted in low d48 SIgA concentrations when all experiments were combined. High stocking density can negatively impact broiler health^{34,260}, behavior^{35,42}, and can cause distress^{31,36,257}. In this context, decreased SIgA concentrations indicate increased chronic stress in broilers housed in HD pens compared to broilers in LD pens. One proposed mechanism for this is that high levels of circulating glucocorticoids in distressed animals interact with PIgR-secreting epithelial cells at mucosal surfaces and decrease PIgR concentrations in the mucus^{192,193,196,208,215,261,262}. PIgR is the secretory component of SIgA and is required for SIgA to cross epithelial barriers and enter the mucosal lumen^{149,263}. If PIgR concentrations are decreased in distressed animals, SIgA concentrations are also decreased, as less SIgA can be released into mucosal lumens. D48 SIgA concentrations respond to high stocking density as hypothesized, therefore can be a viable measure of chronic negative stress (high stocking density) in broiler chickens. We recommend replication under similar and different conditions to confirm the viability of this chronic distress measure in broilers.

The negative impact of HD was not reflected in d48 pIgA concentrations, where no impact of HD was observed. This result may be due to the different production mechanisms for pIgA and sIgA. pIgA is produced by B-cells in the bone marrow²⁶³, which could limit the ability of circulating glucocorticoids to access these B-cells when animals experience chronic distress. Therefore, glucocorticoids may not reduce pIgA production in response to chronic stress. Circulating pIgA is monomeric and does not bind with pIgR to enter circulation, so glucocorticoids can only reduce pIgA production via direct interactions with IgA secreting B-cells in bone marrow²⁶³, further limiting the impact of these hormones on pIgA. However, glucocorticoids can easily interact with sIgA secreting B-cells on intestinal surfaces, decreasing sIgA production under chronic stress. Additionally, sIgA is dimeric and requires the inclusion of pIgR to enter the mucosal lumen (). pIgR-secreting epithelial cells can also interact with glucocorticoids under chronic stress, decreasing pIgR production and indirectly decreasing sIgA concentrations. These mechanisms suggest that sIgA concentrations may decrease more quickly in response to increased HPA axis activity than pIgA concentrations which are less accessible to circulating glucocorticoids. This explains how sIgA concentrations were lowered in response to high density but pIgA concentrations were not. Overall, these results indicate that d48 sIgA is sensitive to chronic stress caused by high density while pIgA concentrations were not.

To our knowledge, no studies have compared how pIgA and sIgA respond to circulating glucocorticoid concentrations in poultry. This is the first study to assess both pIgA and sIgA in response to housing conditions in non-human animals, which makes it difficult to confirm our proposed theories. Therefore, we recommend further investigation into the

differences in production mechanisms between pIgA and sIgA to determine how they respond to circulating glucocorticoid concentrations under similar and different housing conditions.

D48 pIgA concentrations varied largely between experiments, which suggests that pIgA concentrations may not be consistent between flocks. Concentrations of physiological measures of chronic stress should ideally be consistent between flocks, so that the welfare measure would allow for direct comparisons between flocks and studies. The variation in d48 pIgA concentrations suggests it may not be possible to determine 'normal' pIgA concentrations. Instead, pIgA may be more appropriate to be used as a relative comparative indicator between treatments in a single experiment or between similar husbandry conditions in non-experimental contexts.

This is the first study to investigate the impacts of housing conditions on pIgA and sIgA concentrations in broiler chickens at 48 days of age. While sample sizes were relatively low, especially for sIgA during experiments 1 and 2 (N=12/experiment), we were able to detect differences between complexity treatments for pIgA and stocking density treatments for sIgA. This indicates that pIgA at day 48 concentrations show promise as a biomarker for positive experience and sIgA concentrations as a biomarker for negative experience in broiler chickens.

3.6 Conclusion

Environmental complexity positively impacted plasma immunoglobulin-A concentrations in broiler chickens at day 48 of age, suggesting reduced chronic stress when housed in highly complex environments compared to simple environments. This difference was not observed in plasma immunoglobulin-A at day 28 of age or for secretory (fecal)

immunoglobulin-A concentrations at day 48. High stocking density negatively impacted secretory (fecal) immunoglobulin-A concentrations in broiler chickens at day 48 of age across all three experiments, suggesting increased chronic stress when housed at high-density conditions compared to low-density conditions. This difference was not observed for plasma immunoglobulin-A concentrations at day 28 or day 48. Our results show the potential of day-48 plasma immunoglobulin-A concentration as an indicator of positive welfare, as it increased in response to complexity which is considered a positively-valenced stimulus. Furthermore, our results show the potential of day-48 secretory (fecal) immunoglobulin-A concentration as an indicator for chronic distress, as it decreased in response to high stocking density, which is considered a negatively-valenced stimulus. Future research should attempt to replicate outcomes under similar and different conditions to confirm these as animal welfare measures for broiler chickens.

Chapter 4: Cumulative experience in broiler chickens: Telomere length as a biomarker for good animal welfare

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4.1 Abstract

Cumulative experience can be defined as the sum of all positive and negative experiences during an animal's lifetime. No physiological measures of cumulative experience in production animals exist. Telomere length shows promise as a biomarker of cumulative experience in humans and non-human animals but is not yet assessed for broiler chickens. Therefore, our objective was to determine the telomere length responses to positive and negative experience in fast-growing broiler chickens. In three replicated experiments, male Ross-708 broilers were housed in a 2×2 factorial study investigating high environmental complexity as a positive environment (vs. low-complexity; 6 pens/treatment) and high stocking density as a negative environment (vs. low density; 6 pens/treatment). Telomere length was quantified at day 48 of age via RT-qPCR from gonad and kidney samples (N=9 samples/treatment/tissue/experiment). Data were analyzed for experiments separately and all combined using mixed models with complexity, density, and their interactions as fixed factor and pen as random factor. Treatments did not impact gonad telomere length. Environmental complexity tended to positively impact kidney telomere length in experiment 2, and positively impacted kidney telomere length (experiment 1-3 combined). High stocking positively impacted kidney telomere length (experiment 3). No interaction between environmental complexity and

stocking density was found. These results indicate that telomeres are longer in response to positive experience (environmental complexity) when compared to low-complexity environments. As few quantitative measures for good animal welfare status exist, this could be valuable for application in future research and commercial applications. these findings should be confirmed prior to a broader application of the measure as a biomarker of good broiler chicken welfare.

Keywords: Animal welfare, Broiler, Cumulative Experience, Environmental Enrichment, Stocking Density, Telomere length

4.2 Introduction

Ethical concerns over production animal treatment highlights our responsibility to optimize animal welfare outcomes. This includes ensuring that production animals have healthy biological functioning, the ability to display natural behaviors, and experience positive emotional states⁹. Animals in our care undergo positive and negative events which contribute to their cumulative welfare status^{7,8}. Therefore, the term ‘cumulative experience’ is used to refer to the total sum of all positive and negative impacts on the health, behavior, and emotional states of production animals during their lifetime^{8,148}. High stocking density (**HD**) is an example of a negative condition that may impact cumulative experience in broiler chickens, while environmental complexity (**EC**) is a positive stimulus that may impact cumulative experience in broilers^{10,11,30}. However, it is difficult to determine how HD and EC impact cumulative experience as no measures of cumulative experience exist for broiler chickens. What measures of animal experience do exist focus on negative experience and affect, such as fear, distress, and anxiety^{18,138,140,141}. As nearly all measures of broiler experience rely on interpretations of

behavior, a physiological measure of cumulative experience in broiler chickens can be valuable.

Recently, telomere length has gained attention as a potential marker of cumulative experience in humans^{160–163,170}, laboratory animals^{173–175}, poultry^{31,176}, and undomesticated birds^{177,178,264–267}. Telomeres are protein-DNA complexes which end-cap chromosomes and protect gene encoding DNA from the ‘end-chain replication problem’⁸. Telomeres shorten every time a cell divides, which is a primary contributor to cellular aging and apoptosis in somatic cells^{8,268}. These end-cap telomeres protect against cellular aging and apoptosis. However, telomeres do not only shorten during mitosis, but also in response to oxidative stress^{8,153}. Associations between aging and stress have long been established^{269–274} and have led to a distinction between chronological age and biological age^{8,154}. In the context of telomeric shortening, or telomeric attrition, chronological age is the time-proportional shortening of telomeres in response to the number of mitotic divisions of a cell. Biological age is the combination of chronological age and changes in TA caused by lifetime events.

Telomere length in humans has been proposed as a ‘psychobiomarker’ of cumulative lifetime stress and negative experience^{8,172}. For example, shorter telomeres and increased biological age have been associated with anxiety¹⁶⁰, depression¹⁶¹, neuroticism¹⁶², pessimism¹⁶³, family disruption¹⁶⁵, chronic pain^{166,167}, and childhood exposure to violence¹⁶⁴. Additionally, telomere length in humans seems responsive to positive experience. Healthy lifestyle choices decrease the rate of telomere shortening in response to stressful life events^{168,169} and increase concentrations of telomerase¹⁷⁰ which should decrease biological age.

Similar results were found in non-human animals. Telomere length in mice is negatively impacted overcrowding¹⁷⁴, excessive reproduction¹⁷⁴, forced participation in stressful challenges¹⁷³, and salmonella infection¹⁷⁵. Telomere length was decreased in laying hens housed at high stocking density and after a 14-day feed restriction¹⁷⁶. In broilers, telomere length could be used to determine cumulative experience and biological age in relation to housing conditions such as HD or EC. However, to date, there is only one study investigating telomere length in relation to broiler chicken housing conditions³¹. Ross 308 broilers housed in high-density environments showed decreased telomere length compared to birds housed in low-density environments³¹. In broilers, high stocking density is generally considered a negative housing stimulus as broilers raised in high stocking density environments can display increased lameness^{34,260}, decreased ability to display natural behaviors^{35,42} and increased distress^{31,36,257}.

Broilers are often raised in low-complexity environments which restrict broilers' ability to display species-specific behaviors, leads to boredom and frustration, and negative affective states^{12,13,25,275}. Broilers raised in low-complexity environments show increased fear and anxiety and decreased optimism compared to broilers raised in high-complexity environments^{12,13}, indicating that birds from low-complexity environments were in a worse affective state. The provision of environmental enrichment, thus a more complex environment, has positive impacts on broiler health^{11,252,253}, behavior¹¹, affective state¹³, stress⁹⁰ and can make broilers more resistant to stressful events⁹⁰. An EC-induced distress reduction could slow the rate of telomere shortening in those broilers. Additionally, the positive experience provided by EC could mirror the effects seen in humans where

positive experience increased telomerase activity and reversed the impacts of stress on TA^{160,168–171}.

Terminal restriction fragment (**TRF**) analysis has long been considered the ‘gold standard’ for telomere length quantification^{276–279}. However, TRF is expensive and had low throughput, thus requiring a more affordable, higher throughput option^{276,277}. Real-time quantitative polymerase chain reaction (RT-qPCR) assays are suitable alternatives to TRF for telomere length quantification^{276,280–283}. Additionally, care should be taken when selecting sample tissues from each species, as telomere dynamics differ from tissue to tissue¹⁸². For example, gonadal tissue is primarily comprised of stem cells which produce a high amount of telomerase^{284–286}. Telomerase is the only enzyme capable of repairing telomeric DNA and is expressed in very low quantities in somatic cells and high quantities in stem cells. Therefore, gonadal telomere length does not decrease due to cell division due to high concentrations of telomerase and should not decrease due to cumulative experience, however, this is not confirmed. In contrast, kidney cells are primarily somatic cells with low concentrations of telomerase²⁸⁷. In these cells, telomere length may be susceptible to cumulative experience.

The responsiveness of telomere length to positive and negative experience in previous work indicates that it could be used as a biomarker for cumulative experience in broiler chickens. However, the impacts of environmental complexity and high stocking density on telomere length in broilers is mostly unknown³¹. Therefore, the objective of this study was to determine how telomere length is impacted by EC (positive experience) and HD (negative experience) compared to opposing conditions. We hypothesized that relative telomere length (rTL) values from broiler chicken gonadal tissue would be unaffected by

experimental treatments. Additionally, we hypothesized that rTL values would be increased in broilers housed in high-complexity environments and shorter in birds housed in high-density environments compared to birds housed in low-complexity and low density, respectively. Finally, we hypothesized that birds housed in high-complexity, low-density environments would show longer telomeres than birds housing in low-complexity, high-density environments.

4.3 Methods

4.3.1 Experimental design

All procedures in this study were approved by the Virginia Tech Institutional Animal Care and Use Committee (IACUC 19-175). This study was designed using a 2×2 factorial approach with environmental complexity (high or low) and stocking density, resulting in four treatment groups: low-density/low-complexity (LD/LC), low density/high-complexity (LD/HC), high-density/low-complexity (HD/LC), high-density/high-complexity (HE/HC).

4.3.2 Birds and housing

In three replicated experiments, 1-day-old male Ross 708 chicks ($n=1,620/\text{experiment}$) were randomly sorted into four treatment groups, three replicate pens per treatment, with 12 pens per experiment. Twenty additional chicks were euthanized upon arrival to collect gold standard DNA samples. Experimental design is described in detail in ^{12,13}. Pens (14.5 m^2) contained clean pine wood shavings, four galvanized steel feeders, and 3 nipple water lines (3 nipples/line). Birds had ad libitum access to feed and water and were phase fed a commercial corn/soy diet which was formulated to meet their nutritional needs. This diet included a starter (d1-d14), grower (d15-d28), and finisher phase (d28-d50). Pens contained three heat lamps and 24h continuous lighting during the first week of life,

and 18h light:6h dark after the first week with a light intensity of ~15 lux during light hours. In experiment 1, chicks unintendedly received 24h light for an extra week (until d14 of age). On d1, temperatures within the house were 35°C and gradually decreased to 21°C on d24, which was maintained until d50. During experiment 1, birds were given a therapeutic dose of antibiotics from d33 to d40 of age in response to a pathogen exposure.

4.3.2.1 Environmental complexity

HC pens were divided into four functional areas with permanent and temporary enrichments. These areas were a feeding area (~3.2 m²) with 4 galvanized steel feeders and 1/3 of a mineral pecking stone broken into smaller pieces (Proteka, Inc., Lucknow, ON, Canada), a comfort area (~3.2 m²) with a 2m² wooden dust bath with playground sand, a play and exploration area (~4.3m²) with paired sets of temporary enrichments, and a rest area (~3.2 m²) containing three sets of perching structures. In experiment 1, PVC pipe perching structures (183 cm L × 31 cm W × 9 cm H × 1.9 cm diameter) provided 15.2 cm of linear perching space per bird in LD pens and 7.6 cm in HD pens. In experiments 2 and 3, wooden boards were provided as the perching structures (122 cm L × 46 cm W × 8 cm H) and provided 76 cm² of perching space in LD pens and 39 cm² of perching space in HD pens. Temporary enrichments were grouped in pairs and rotated every 3d. Pairs were (1) hanging bundles of white polypropylene string and metal wire balls with alfalfa hay (Darice, Strongsville, OH, USA), (2) plastic balls (5.8 cm diameter; Click N Play, USA) and treat dispensers (7.6 cm diameter; Lixit Corp., Napa, CA, USA) filled with oats, (3) red laser light (5 min; 2×day; Ethical Products, Inc., Bloomfield, NJ, USA) and Kong toy (5.6 cm diameter; KONG, Golden, CO, USA) with iceberg lettuce (experiment 1), or half a head of cabbage hung at bird height (experiment 2 and 3). The

LC pens were also divided into four areas but contained no enrichments. The four galvanized steel feeders were distributed in three areas of the pen.

4.3.2.2 Stocking density

HD pens contained 180 birds and LD pens contained 90 birds to target a final stocking density of 40-42 kg/m² in HD pens and 20-22 kg/m² in LD pens. Final stocking densities at d50 were 42.1 kg/m² (experiment 1), 42.6 kg/m² (experiment 2), and 42.1 kg/m² (experiment 3) in HD pens and 23.8 kg/m² (experiment 1), 23.3 kg/m² (experiment 2), and 22.1 kg/m² (experiment 3) in LD pens.

4.3.3 Measurements

At d1 of age, 10 birds from each experiment were euthanized via cervical dislocation and kidney and gonad samples were collected to serve as gold standard DNA samples. Three birds/pen were euthanized via cervical dislocation on d48 of age and kidney and gonad samples were collected for quantification of telomere length. This resulted in 36 kidney and 36 gonad samples per experiment (9 samples/tissue/treatment) for a total sample number of 216 for all three experiments (N=72 kidney and gonad samples/experiment). Kidney and gonad samples in 1.5 mL microcentrifuge tubes were placed on dry ice before transport and storage at -80°C.

Kidney and gonad samples were thawed, and DNA was extracted using the QIAamp DNA mini kit (Qiagen, Hilden, Germany) following the manufacturer protocol. Purified DNA samples were then nano-dropped and diluted using molecular-grade water to standardize the amount of DNA in each sample (10 µg/µL). DNA integrity gels were used to ensure good quality DNA for RT-qPCR analysis, and all samples were of high quality as they yielded a single high molecular weight band.

Telomere length was determined using an RT-qPCR method described by Pfaffl²⁸² and Eastwood et al.²⁸³. In short, the proportion of telomeric DNA is calculated relative to the quantity of DNA from a single copy gene, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), in the same DNA sample to form a ‘relative telomere length’ value, or rTL. This proportion reflects the length differences between telomeres and the constant GAPDH amplicon. Telomere primers were Tel1b 5’—CGG TTT GTT TGG GTT TGG GTT TGG GTT TGG GTT TGG GTT—3’ AND Tel2b 5—GGC TTG CCT TAC CCT TAC CCT TAC CCT TAC CCT TAC CCT—3’. The control GAPDH primers were GAPDHF 5’—CCT AGG ATA CAC AGA GGA CCA GGTT—3’ and GAPDHR 5’—GGT GGA GGA ATG GCT GTCA—3’. Reactions were set up on 96-well plates (Qiagen) with a total reaction volume of 15 μ L, containing 3.6 μ L molecular grade water, 0.15 μ L (1 μ M) forward primer, 0.15 μ L (1 μ M) reverse primer, 7.5 μ L SybrGreen (Qiagen), and 3.6 μ L sample. RT-qPCR plates were covered using plastic adhesive plate covers and reactions were analyzed using a 7500 Fast Real-Time PCR machine (Applied Biosystems, Beverly, MA, USA). A standard curve was created for each plate using a “golden sample” of DNA extracted using the QIAamp DNA mini kit from 20 1-day-old broiler chicks. These golden sample extractions were pooled and a two-fold serial dilution of 40, 20, 10, 5, and 2.5 ng/ μ L was used on each plate. All controls and samples were run in triplicate. All standard curves were used for quality control and all plates had standard curves within acceptable ranges with efficiencies within $100\pm 15\%$ and $R^2 > 0.98$. Individual well efficiencies and quantitation cycle (CQ) values were calculated in LinregPCR²⁸³. rTL was calculated by using equation 1. In short, individual well efficiencies are raised to the power of delta CQ (interplate control CQ minus individual

sample CQ) (Equation 1). The resulting triplicate telomere and GAPDH values are then averaged and divided by each other to create a single rTL value (Equation 1)^{282,283}.

$$rTL = \frac{(E_{telomere})^{\Delta Cq_{telomere}}}{(E_{GAPDH})^{\Delta Cq_{GAPDH}}}$$

Equation 4.1. Equation used to calculate relative telomere length (rTL) of kidney or gonad samples from broiler chickens. Utilizing this equation, the individual well efficiencies (E) of telomere or GAPDH are raised to the power of the inter plate control Cq – target Cq (delta CQ). This equation is previously described and adapted from²⁸³

4.3.4 Statistical analysis

Statistical analyses were done in JMP Pro 16 (SAS Institute Inc., Cary, NC, USA). Pen was considered the experimental unit (N=12/experiment) and bird the observational unit (N=36/experiment). Data residuals were normally distributed based on visual inspection of normal quantile plots. Data from each experiment were analyzed by experiment ('experiment 1-3') using linear mixed models with environmental complexity, stocking density, and their interactions as fixed factors. Pen number was included as a random factor. In addition, data from all three experiments were combined ('overall') and analyzed using linear mixed models with environmental complexity, stocking density, and their interactions as fixed factors. Pen number and experiment number were included as random factors. Post-hoc analysis was performed with Tukey HSD corrections. Associations were considered significant at $P \leq 0.05$ and a trend at $P \leq 0.1$. All data are presented as LSmeans \pm SEM.

4.4 Results

4.4.1 Gonad rTL measurements

EC or SD did not impact gonad rTL in experiment 1-3 or overall (Table 4.1).

There was no interaction effect between EC and SD on gonad rTL in experiment 1 ($F_{1,35}=0.03$; $P=0.872$), experiment 2 ($F_{1,35}=0.19$; $P=0.676$), experiment 3 ($F_{1,35}=0.21$; $P=0.870$), or overall ($F_{1,107}=0.26$; $P=0.614$).

Table 4.1. Least squared means estimates \pm SEM of gonad relative telomere length measurements in experiments 1- 3, and overall (data of 3 experiments combined). Birds were housed in either high-complexity (HC) or low-complexity (LC) and high-density (HD) or low-density (LD) pens. $N=108$

Experiment	HC	LC	EC test statistics
Experiment 1	1.19 \pm 0.12	1.13 \pm 0.15	$F_{1,35} = 0.09$; $P = 0.776$
Experiment 2	1.37 \pm 0.17	1.44 \pm 0.17	$F_{1,35} = 0.09$; $P = 0.773$
Experiment 3	1.34 \pm 0.09	1.40 \pm 0.09	$F_{1,35} = 0.28$; $P = 0.609$
Overall	1.31 \pm 0.10	1.31 \pm 0.10	$F_{1,107} < 0.01$; $P = 0.987$
	HD	LD	SD test statistics
Experiment 1	1.10 \pm 0.12	1.22 \pm 0.15	$F_{1,35} = 0.35$; $P = 0.572$
Experiment 2	1.40 \pm 0.17	1.42 \pm 0.17	$F_{1,35} < 0.01$; $P = 0.924$
Experiment 3	1.48 \pm 0.09	1.26 \pm 0.09	$F_{1,35} = 3.15$; $P = 0.113$
Overall	1.34 \pm 0.10	1.29 \pm 0.10	$F_{1,107} = 0.48$; $P = 0.493$

4.4.2 Kidney rTL measurements

EC did not impact kidney rTL during experiment 1 ($F_{1,35}=0.31$; $P=0.59$), or 3 ($F_{1,35}=1.33$; $P=0.286$) (Figure 4.1). In experiment 2, kidney rTL tended to be higher ($F_{1,35}=5.11$; $P=0.051$) in HC pens compared to LC pens (Figure 4.1). Overall, kidney rTL was longer ($F_{1,107}=4.74$; $P=0.033$) in birds housed in HC pens compared to LC pens (Figure 4.2). SD did not impact kidney rTL during experiment 1 ($F_{1,35}=0.50$; $P=0.50$), or experiment 2 ($F_{1,35}=1.84$; $P=0.21$) (Figure 4.1). However, kidney rTL was higher in birds housed in HD pens ($F_{1,35}=6.07$; $P=0.044$) compared to birds housed in LD pens during experiment 3 (Figure 4.1). Kidney rTL was not impacted by SD ($F_{1,107}=0.23$; $P=0.63$) over all three trials (Figure 4.2). The EC \times SD interaction did not impact kidney rTL values during

experiment 1 ($F_{1,35}=0.58$; $P=0.47$), 2 ($F_{1,35}<0.01$; $P=0.95$), 3 ($F_{1,35}=0.13$; $P=0.730$), or overall ($F_{1,107}=0.03$; $P=0.86$).

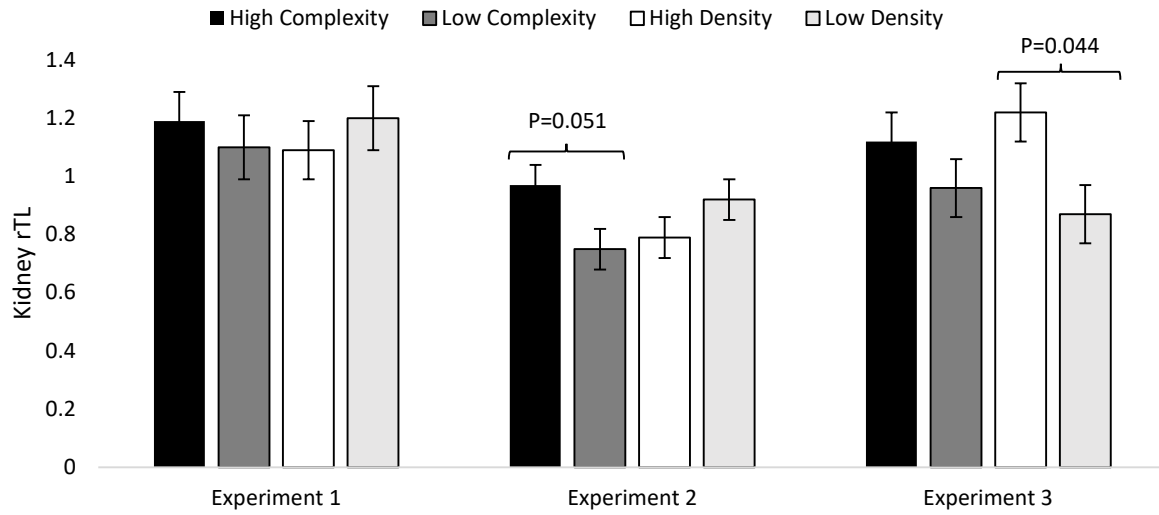


Figure 4.1. Least squared means estimates of kidney relative telomere length (rTL) measurements of birds from high-complexity, low-complexity, high-density, and low-density pens from experiments 1, 2, and 3.

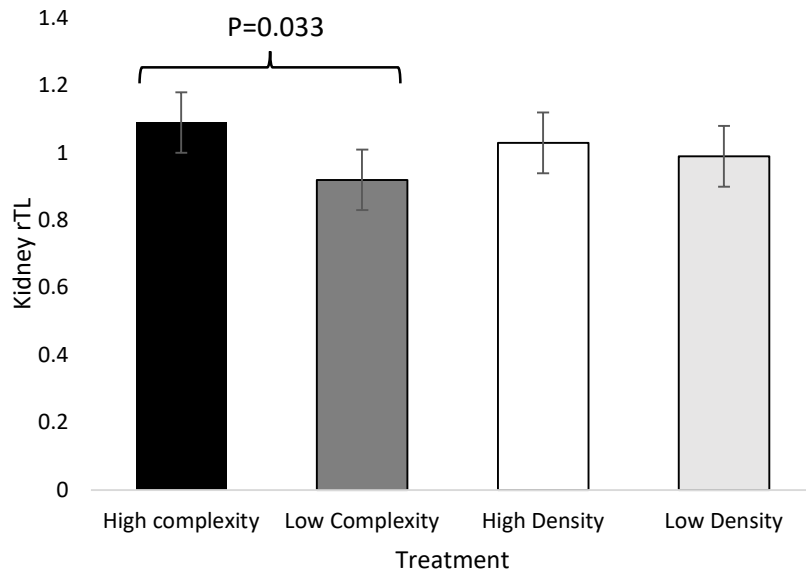


Figure 4.2. Least squared means estimates \pm SEM of kidney relative telomere length (rTL) measurements of birds from high-complexity, low-complexity, high-density, and low-density pens from all three experiments.

4.5 Discussion

This study investigated the effects of environmental complexity (EC) and stocking density (SD) on telomere length as a measure of cumulative experience in broiler chickens. We observed no effect of treatments on gonadal rTL values, and depending on the experiment, a positive effect of EC (experiment 2 tendency and overall) and SD (experiment 3) on kidney rTL. No interaction effects between EC and SD were observed. Overall, these results indicate that rTL seems able to detect an impact of cumulative positive experience related to complexity.

We hypothesized that broiler gonadal rTL values would not be impacted by housing treatments due to high populations of stem cells and production of telomerase, and results are aligned with this hypothesis. Gonadal tissue is primarily comprised of stem cells which produce high amounts of telomerase²⁸⁴⁻²⁸⁷. Contrarily, telomeres in certain tissues do not shorten due to mitosis, such as tissues with high populations of stem cells (bone marrow, germinal tissues)^{284,287}. It was previously unclear if these types of tissues are resistant to the impacts of cumulative experience on telomere length, as no studies have investigated the impacts of cumulative experience on telomere length of these cell populations. The high concentrations of telomerase in (gonadal) stem cells repair any shortening that occurs due to cell division²⁸⁴, but also likely repair damage caused by negative experiences. Thus, if LC and HD (assumed negative experiences) sped up biological aging, telomerase likely prevented or repaired that impact. Our study shows that gonadal rTL values are not impacted by positive or negative experience associated with EC or SD in broiler chickens, indicating that gonadal tissue may not

show increased biological aging in response to cumulative experience. Therefore, studies should avoid use of these tissues (bone marrow, gonads) when investigating the impact of environmental conditions on telomere length.

Over all three trials (and as a tendency in Experiment 2), we were able to confirm our hypothesis that birds housed in HC pens would show higher rTL values, indicating longer telomeres, than birds housed in LC pens. This implies that the birds housed in complex environments showed slower biological aging than birds in environments with little complexity, thus a more positive cumulative experience for the former. The effect was not observed in experiments 1 and 3, although birds in HC pens showed numerically higher rTL values compared to LC pens, aligned with Experiment 2 and overall results. Longer rTL in HC birds is consistent with previous work in humans that underwent positive experiences^{160,168-170}. In broilers, provision of environmental enrichment to create a complex environment is considered to contribute to positive experience^{11,13,252,253}. Therefore, we consider two potential mechanisms for the higher rTL values in HC birds compared to LC birds. Firstly, positive experience in response to a complex environment reduced the number of distressing events experienced by HC birds and provided opportunities for positive lifetime experiences. This decreased oxidative stress and telomeric DNA damage, reducing or mitigating telomere shortening due to stressful life events. Chronic or acute stress was not assessed in this study; thus, the reduced distress in the HC treatment cannot be confirmed. Yet, EC can decrease distress in birds^{88-90,275,288}. Broilers housed in high-complexity pens were less fearful following three acute stressors (sound stress, heath stress, and crating stress) compared to broilers housed in low-complexity environments⁹⁰. Laying hens housed in high-

complexity pens had a reduced startle reflex and comb temperature in response to acute stressors (light flash, restraint, and sudden appearance of a novel object) compared to low-complexity pens, indicating that those birds were more resistant to distress compared to birds from low-complexity environments⁸⁸. Based on these and other findings, we can infer that the positive impacts of HC on kidney telomere length are at least in part due to a reduction and mitigation of stressful situations.

Secondly, positive experience in response to a complex environment may have increased telomerase production in kidneys, repairing and lengthening telomeres. As telomerase production was not quantified in the current study, and no studies have investigated telomerase production in non-human animals, this mechanism is not yet confirmed. These mechanisms are not mutually exclusive, but rather could have interacted to result in longer kidney telomeres in HC birds compared to LC birds. Future research should investigate how EC impacts telomerase production and distress responses in relation to telomere length to better determine how positive experience impacts telomere length in broiler chickens.

Contrary to our hypothesis, birds housed in HD pens had higher kidney rTL values in experiment 3, indicating those birds had longer telomeres and a more positive cumulative experience than birds housed in LD pens. Results in experiment 1, 2 (and overall) do not align with these findings in experiment 3, as the numeric rTL values were lower for HD compared to LD pens. These inconsistent results contradict a previous study that showed that Ross 308 broilers telomere length was shorter in birds raised under HD cages (0.058 m²/bird) compared to LD cages (0.116 m²/bird)³¹. Space allowance in our experiments was approximately 0.081 m²/bird in HD pens and 0.161 m²/bird in LD

pens. Therefore, our HD treatment provided birds with more space than the HD treatment in³¹ and our LD treatment provides more space than the LD treatment in³¹. It is possible that the stocking density over 42 kg/m² as tested in³¹ caused excess distress, in turn leading to shortened telomeres, which could be an effect not yet reflected at the HD in the current study. However, the methodology for telomere length quantification used in³¹ is fluorescent in situ hybridization (FISH) making direct comparisons between their results and the current study impossible. Few production systems exceed stocking densities of 42 kg/m² (0.081 m²/bird). For example, the United Kingdom Code of Practice allows a maximum stocking density of 39 kg/m² with stringent requirements²⁸⁹. The European Council Directive (2007/43/EC) similarly restricts the maximum density to 42 kg/m². In the United States no legislation limits the stocking density for broiler production, however, 95% of broilers are produced by companies following the National Chicken Council Animal Welfare Guidelines, which recommends a maximum stocking density of 41.5 kg/m² for broilers with target weights of 2.5 to 3.4 kg²⁶. In general, HD is considered a negative stimulus which can lead to decreased foot and leg health^{33–35,40,42,43}, and increased distress compared to LD^{33,36}. However, there are studies that do not observe a negative impact of HD on broiler affective state^{12,13} and distress²⁵⁷. For example, Ross 708 broilers raised in HD pens showed decreased fear when compared to birds raised in LD pens¹². Maximum stocking density determines the number of birds placed based on birds' average weight at the end of production. This means that stocking density is much lower in the early stages of production and density will steadily increase as birds grow. Negative impacts of stocking density are observed at densities over 30 kg/m²^{32,33}. A stocking density of 30 kg/m² was approximately reached in the HD pens at

d36 of age²⁹⁰. Therefore, it is possible that the negative effects of HD only impacted the birds from d36-d50 of life. In this case, there may not be enough time for sufficient telomere shortening to occur and be detected. Additionally, stocking densities up to 42 kg/m² may not be severe enough negative environments to cause telomeric DNA damage. Therefore, stocking densities of 42 kg/m² might not elicit negative cumulative experience in broilers. In fact, results from experiment 3 would indicate that high-density positively impacted cumulative experience when compared to low density environments. This finding could indicate that, in certain flocks, densities of 42 kg/m² may be perceived by the birds as a positive housing system, reducing distress. This could be due to a perception of safety in numbers among broilers, where larger group sizes allow for increase protection from threats and decreased distress due to startling production stimuli. However, this finding is unique and has little previous support. Additionally, animal welfare is an individual experience²⁹¹. Each animal perceives situations, stimuli, and housing systems differently and these differences are reflected in how an animal interacts with, responds to, and thrives within their environment²⁹¹. It is possible that, when arbitrarily selecting birds for sampling during experiment 3, we selected birds which had personalities that perceive HD environments as positive. In this case, the sampled birds may not have been representative of the flock. This would help to explain the differences in findings between the three experiments as numerical rTL values from birds raised in HD pens were lower than birds raised in LD pens during experiments 1 and 2. We recommend further investigation into the impacts positive and negative stimuli on broiler chicken kidney telomere length to determine the viability of telomere length as a biomarker of cumulative experience in broilers. One negative stimulus that could be

studied with prolonged 'exposure' could be feed restriction²⁹² in broiler or broiler breeder flocks.

We were unable to confirm our hypothesis that birds from HC/LD environments would show a decreased rate of TA (longer rTL) compared to birds from LC/HD environments. This is potentially due to low sample sizes for individual treatments (N=9 samples/treatment/experiment). It is possible that if sample sizes were increased, differences between treatments would have been observed. However, as previously mentioned, it is also possible that stocking densities of 42 kg/m² do not impact telomere length in broiler chickens. If true, no differences between interaction treatments would be expected as only environmental complexity would impact telomere length. This is supported by the observed results in kidney samples which show that high-complexity positively impacted telomere length. Future studies should investigate this interaction at higher sample sizes to determine if individual treatments impact telomere length.

This is the first experiment to investigate telomere length in broilers using this RT-qPCR method. To confirm these results and ensure that RT-qPCR is an appropriate measure for the quantification of telomere length in broilers it should be validated using TRF analysis. Additionally, even with low sample sizes we were able to detect differences between complexity treatments, and for one experiment between density treatments. This suggests that telomere length quantification via RT-qPCR is a promising as a high throughput and economically practical biomarker of cumulative experience in broiler chickens.

4.6 Conclusion

Environmental complexity and stocking density did not impact gonadal telomere length of male broiler chickens. Following expectations, environmental complexity positively impacted kidney telomere length over all three trials, indicating that the broilers raised in highly complex environments had a more positive cumulative experience and a lower biological age than broilers raised in low-complexity environments. High stocking density positively impacted telomere length during experiment 3, indicating that birds raised in high-density environments had more positive cumulative experience than birds raised in low density environments. This finding is contradictory to experiments 1, 2, and overall, where no impact of density was found. However, it does indicate that the impacts of stocking density on animal experience may not be consistent between flocks or birds. Our results are the first to show the potential of telomere length as a measure of positive experience in broilers as relative telomere length increased in response to a positive environment. However, we were unable to confirm its potential as an indicator of the negative impact of high stocking density in broilers. Future studies should consider telomerase production and more prolonged negative stimuli when researching telomere length in broiler chickens. Finally, future studies should validate the RT-qPCR assay using TRF analysis.

Chapter 5: Effects of Housing System on Anxiety, Chronic Stress, Fear, and Immune Function in Bovan Brown Laying Hens

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5.1 Abstract

The scientific community needs objective measures to appropriately assess animal welfare. The study objective was to assess the impact of housing system on novel physiological and behavioral measurements of animal welfare for laying hens, including secretory and plasma Immunoglobulin (IgA; immune function), feather corticosterone (chronic stress), and attention bias testing (ABT; anxiety), in addition to the well-validated tonic immobility test (TI; fearfulness). To test this, 184 Bovan brown hens were housed in 28 conventional cages (3 birds/cage) and 4 enriched pens (25 birds/pen). Feces, blood, and feathers were collected 4 times between week 22 and 43 to quantify secretory and plasma IgA and feather corticosterone concentrations. TI tests and ABT were performed once. Hens that were from cages tended to show longer TI, had increased feather corticosterone, and decreased secretory IgA at 22 weeks of age. The caged hens fed quicker, and more hens fed during the ABT compared to the penned hens. Hens that were in conventional cages showed somewhat poorer welfare outcomes than the hens in enriched pens, as indicated by increased chronic stress, decreased immune function at 22 weeks of age but no other ages, somewhat increased fear, but reduced anxiety. Overall, these novel markers show some appropriate contrast between housing treatments and

may be useful in an animal welfare assessment context for laying hens. More research is needed to confirm these findings.

5.2 Introduction

Animal welfare is a multifaceted concept that involves an animal's ability to interact and cope with its environment. Good animal welfare would be achieved when an animal is allowed to display natural behaviors (natural living), be healthy and function normally (basic health and functioning) and experience a generally positive emotional state (affective state)⁹. For laying hens, commercial housing systems such as conventional cages can negatively impact animal welfare by restricting natural behaviors, causing health and functioning concerns such as cage layer fatigue, and likely results in worse affective states (anxiety and fear)⁸⁴. While aspects of natural living and basic health and functioning are relatively easily measured and well-studied, few studies investigate the effects of housing system on the affective states of laying hens. Additionally, as nearly all measures of affective state rely on interpretations of animal behavior, there is need for additional physiological measurements to allow for a more cumulative assessment of animal affective states.

With limited physiological measures of emotion and affective state available, most insight comes from behavioral assessments of negative emotions such as fear. Fear is a short-term emotional response to a current threat and elicits either a freeze, fight, or flight response^{12,293,294}. A common assessment of animal emotion is performed using fear tests, such as a novel object test, human approach test, or tonic immobility (TI) test²⁹⁵.

Measuring fear using TI is well-documented in poultry and utilizes their natural prey-predator behavioral response. TI is a type of freezing behavior where birds will feign

death and is used when captured by a predator²⁹⁶. Previous investigations of TI in relation to housing systems had variable results. For example, barren caging conditions have resulted in longer TI durations, thus greater levels of fear, in Hyline Brown laying hens when compared to more enriched conditions^{112,297}. However, other studies have found no effect of housing system on TI duration in laying hens^{298,299}. These variable results highlight the need for further investigations of TI and other measures to elucidate the effects of housing system on emotion and affect.

Affective state is a long-term mood state which comes from the culmination of experiences and emotional responses. An animal's life experiences elicit short-term emotional responses³⁰⁰. These responses culminate to form a mood, which can range from positive to negative in valence, and shapes the animal's affective state. Affective states influence how animals make decisions and can bias their cognitive reasoning, which can then be used to infer the affective state based on behaviors that indicate information-processing^{14,17,300}. One cognitive bias test that was previously applied in chickens is the attention bias test (ABT). The ABT is a validated and well-used method to measure affective states, more specifically anxiety, in agricultural animals¹⁴. In an ABT, the level of vigilance or attention an animal allocates to a perceived threat is quantified¹⁴. This allocation of vigilance is differential and affect-mediated, with more anxious affective states resulting in more vigilance towards a threatening stimulus^{12,14}. The ABT was validated in laying hens, with birds that were given anxiogenic drugs displaying increased vigilance compared to control birds¹²⁶. Excess anxiety decreases the ability of commercial poultry to cope with changes in their environment such as transport, handling, and loud noises. In laying hens, the ABT reflected anxiety that was related to

range usage^{126,132}. Determining anxiety via the ABT was also successful in broilers¹², sheep^{123,143}, and pigs¹³⁵. However, ABT has not yet been applied to assess the impact of housing system (cage vs. cage-free) environments on laying hen anxiety.

Feather corticosterone (CORT) is a potential promising physiological biomarker for chronic stress¹⁵². Concentrations of feather CORT can provide a retrospective view on stressful experiences during feather growth³⁰¹. The calamus of a feather is highly vascularized which allows for the deposition of circulating CORT into the feather as it grows, allowing for quantification of hypothalamic-pituitary-adrenal (HPA) axis activity over a prolonged period of time¹⁵². The extraction and quantification of feather CORT has been validated for use in layers, broilers, turkeys, and non-domesticated bird species and requires no invasive sampling procedures^{152,236,301,302}. Feather CORT could provide insight in chronic stress that is caused by housing systems in laying hens.

Secretory Immunoglobulin-A (IgA) is the most abundant antibody on mucosal surfaces including the intestinal tract and has an important role in mediating the adaptive humoral immune defense^{149,186,187}. In addition, IgA circulates in the blood, yet the role is less understood and likely serves to help reduce inflammation¹⁴⁹. Concentrations of IgA also seem to reflect the valence (positivity or negativity) of environmental stimuli¹⁴⁹. For example, IgA concentrations are downregulated in response to physical or psychological stress^{203,215,250,251}. Broiler chickens and laying hens under prolonged periods of heat stress (chronic negative stressor) showed decreased concentrations of plasma IgA when compared to the control treatment^{217,242}. Mice that were exposed to restraint stress over four days (negative stressor) showed decreased concentrations of intestinal IgA compared to the control²¹⁵. Shelter cats with access to enrichment (positive stimuli) had higher

levels of secretory (fecal) IgA than cats without access to enrichments²⁰⁰. In mice, prolonged voluntary exercise (a high arousal-positive-valence activity) increased salivary IgA concentrations after 3 weeks, indicating that IgA concentrations can increase in response to positive activities¹⁸⁹. In addition, forced prolonged exercise (a high arousal-negative-valence activity) had the opposite effect in horses, rats, and humans^{149,198,210,211}. The valence-dependent response of circulating and secretory IgA indicates the potential for use as a marker for the affective state.

The combined use of novel and well-validated measures for emotion and affective state could provide a better understanding of the impacts of different housing conditions on laying hen welfare. In addition, it allows further confirmation of novel measures as we can compare novel test outcomes with well-validated test responses, such as the TI test. Therefore, the objectives of this study are to (1) determine if the housing system impacts laying hen welfare outcomes that are related to the affective state and emotion and (2) determine if these novel measures can be used to assess laying hen welfare. We hypothesized that birds that were housed in enriched floor pens (pen) would show decreased fear and anxiety, increased IgA concentrations, and decreased feather CORT concentrations than birds that were housed in traditional conventional caging, indicating that these novel measures can be used to assess laying hen welfare and that enriched housing systems contribute to positive affective states in pen laying hens.

5.3 Materials and methods

5.3.1 Birds and housing treatments

This experiment was approved by the Virginia Tech Institutional Animal Care and Use Committee (IACUC protocol 18-205). Day-old Bovar Brown chicks (n = 184) were

sourced from a commercial hatchery (Blackstone, VA, USA) where they were beak trimmed after hatch. The birds received a Salmonella vaccination at 16 weeks of age. From day one to week six of age, 84 chicks were reared in conventional cages and 100 chicks in floor pens as part of an unrelated study that was investigating the impact of dietary phosphorous on egg production.

In week six, all the birds were wing banded for individual identification, relocated to another facility, and regrouped, yet housing treatments (either cage or floor pen) remained consistent. The chicks from floor pens were randomly distributed over four enriched floor pens (pen of 16.7 m²), with 25 birds per pen. The chicks from cages were distributed over 14 conventional cages (cage) of 0.093 m², with six birds per cage until week 12 of age. At week 12, three birds from each cage were moved to 14 unoccupied cages to reduce the stocking density, resulting in 28 cages total with three birds per cage. The penned birds were not regrouped at 12 weeks of age. All the pens contained pine wood shavings (7 cm depth), one trough feeder (92.7 cm²/bird) and bell drinker (Plasson, Ma'agan Michael, Israel), 10 galvanized steel nest boxes that were arranged in two tiers of five boxes (30.5 cm long × 30.5 cm wide × 35.6 cm high), one hay bale (replaced as needed), and a head of cabbage that was provided twice per week, which was suspended from the ceiling at bird level. The birds had access to perches (5.49 m or 0.22 m/bird perch space) consisting of pressure-treated 5 cm × 10 cm boards for the frame and three 2.5 cm diameter PVC pipes that were mounted at 45 cm, 60 cm, and 90 cm heights.

There were four rows of stacked conventional cages (38 cm wide × 38 cm long × 46 cm high) with a sloped wire floor that were located in the same room as the pens, and contained two nipple drinkers per cage, a feed trough that was suspended outside the cage

with a gap in the wire for birds to access feed, and an egg collection trough on the opposite side. The pen space allowance was 0.668 m²/bird, compared to 0.031 m²/bird in the cage housing after week 12. The birds had ad libitum access to feed and water. From hatch to week six, the pullets were fed an experimental diet as part of the phosphorous experiment. Following week six, the birds were phase fed diets that were formulated to meet their nutritional needs that were appropriate for their age and developmental status. Lighting included 12 h light and 12 h dark, with windows allowing for natural light exposure during the daytime. Daylight exposure was equal between the treatments. Temperatures within the house were managed by assessing bird comfort based on behavioral responses (huddling when cold/panting when warm), however, a cold period in the winter reduced the in-house temperatures to a minimum of 5 °C when the birds were 33–37 weeks of age.

5.3.2 Behavioral measurements

A TI test was performed to assess fearfulness on six arbitrarily selected hens per pen (total of 24 birds) and on one hen per cage from 24 randomly selected cages (total of 24 hens) at 23 weeks of age. The test was performed by a single researcher in the hallway of the room in which the hens were housed as described in ¹². The hens were placed on their backs in a V-shaped wooden cradle and restrained by the researcher by placing one hand on the sternum and cupping the head with the other hand. After 15 s of restraint, the researcher stepped away without making eye contact. Following the induction of TI, the duration (s) was recorded to determine fearfulness. If the induction of TI was unsuccessful, the researcher attempted to induce TI again for a maximum of three times.

If TI was not induced in three attempts, the latency to rightening was scored as 0 s. The maximum duration of TI was 300 s.

An attention bias test (ABT) was performed by two observers to assess anxiety using a modified method as described in ¹² on nine hens per pen and three hens per cage for 12 arbitrarily selected cages at 30 weeks of age. One bird from each cage that was tested during ABT was also tested for TI. The inter-observer agreement was tested for latency to start feeding of 12 hens and was good among the two observers (Cronbach's α of 0.841). The test arena consisted of plastic paneling and rubber flooring (76.2 cm \times 76.2 cm), and contained a trough feeder with feed, mealworms, and oats. The arena was located in a separate room near the hens' room but far enough to block out the alarm call in the hens' room. The birds were tested in familiar groups of three hens. After placement of three hens in the arena, a conspecific ground predator alarm call was played for 8 s. Following the alarm call, the number of birds and latencies to begin feeding (s) were recorded. Video recordings (EOS Rebel T7 DSLR Camera, Canon, Tokyo, Japan) were used to determine the occurrence (yes/no) of vigilant behaviors (freeze, neck stretches, looking around, and erect posture) within the first 30 s of testing. Each of the four vigilance behaviors

Table 5.1. Method that was used during attention bias (ABT) testing. The birds were tested in groups of three and the testing procedure differed depending on the number of birds who began feeding following the first alarm call.

Scenario	Procedure	Total test duration	Data recorded
Testing begins	Play first alarm call	300 s	n/a
No birds begin feeding	Allow test to run 300 s	300 s	All birds receive 300 s maximum latency to begin feeding
One bird begins feeding	Play first alarm call and allow test to run for 300 s	300 s	Latency to begin feeding for bird that began feeding. Other two birds receive

			maximum latency of 300 s
Two birds begin feeding	Play first alarm call and allow test to run for 300 s. Play second alarm call at 300 s and allow test to run until 420 s.	420 s	Latencies to begin feeding for two birds. Third bird receives maximum latency of 300 s. Latencies to resume feeding for two birds that began feeding if they feed before 420 s.
All three birds begin feeding before 270 s	Play first alarm call and allow test to run until the third bird begins feeding. Allow birds to feed for 5 s and play second alarm call. Allow test to run until 300 s	300 s	Latencies to begin feeding. Latencies to resume feeding for all three birds if they resume feeding prior to 300s.
All three birds begin feeding between 270-300 s	Play first alarm call and allow test to run until the third bird eats. Allow birds to feed for 5 s and play second alarm call. Extend testing duration to 420 s.	420 s	Latencies to begin feeding. Latencies to resume feeding for all three birds if they resume feeding before 420 s.

n/a: not applicable

were scored as a 1 (yes) or 0 (no) and combined to obtain a vigilance score from 0 to 4^{12,132}. Scores of >0 indicate that the test was significantly threatening to achieve vigilance, however, higher scores do not necessarily indicate more vigilance behavior. The alarm call was replayed for 8 s if one of four scenarios occurred (Table 1). Birds that did not start feeding after the first alarm call received a maximum latency of 300 s. After the second alarm call, the number of birds that were feeding and the latency to resume feeding (s) were recorded.

5.3.3 Molecular measurements

5.3.3.1 Feces and blood

A total of 4 fecal samples and 12 plasma samples per treatment per time point were collected during weeks 22, 25, 29, and week 43 of age to determine the fecal and plasma

IgA concentrations. Fresh fecal samples were collected from the cage or pen floors and pooled in microcentrifuge tubes. The birds were arbitrarily selected from floor pens (n = 3/pen) or cages (n = 1 bird/cage) for blood sampling during each time point. Across ages, nine birds were selected twice, and 30 birds were selected once.

For fecal samples, visual inspection and observation of defecation were used to ensure the freshness of the samples and to prevent degradation of fecal IgA by fecal proteases. Following collection, the fecal samples were placed on ice and then stored at -80°C . Fecal IgA was quantified using the total protein extraction with a saline extraction method that was similar to that described in ^{212,255,256}. A total of 10 mL of a saline extraction buffer (0.01 M phosphate-buffered saline, 0.5% Tween (Sigma-Aldrich, St. Louis, MO, USA), and 0.05% sodium azide) was added to each 1 g fecal sample, followed by homogenization. Fecal suspensions were centrifuged at $1500\times g$ for 20 min at 5°C and the supernatant was removed and placed in microcentrifuge tubes. Then, 20 μL of protease inhibitor cocktail (Sigma-Aldrich, St. Louis, MO, USA) was added to the supernatant and homogenized before storage at -20°C until analysis.

Blood (1 mL) was drawn from the brachial vein and collected in glass tubes containing 0.05% EDTA for anticoagulation. The sample collection times (s) were recorded from start of handling until the removal of the needle, to ensure that all the samplings occurred in under two minutes to minimize the potential effects of sampling stress. Prolonged handling can impact acute-stress-related blood parameters [41], although it is unknown whether this is the case for IgA. The mean sample collection time ($\pm\text{SD}$) was 77 ± 30 s and IgA concentrations showed no correlation with collection times ($R^2 = 0.0007$; $p = 0.80$). The sample vials were then lightly mixed via inversion before storage on ice. Then,

the blood samples were centrifuged at 10,000× g for 10 min at room temperature, after which the plasma was removed and aliquoted into sterile microcentrifuge tubes and stored at −20 °C until analysis. The plasma and fecal samples were analyzed for IgA concentrations [$\mu\text{g}/\mu\text{L}$] via a commercial ELISA kit (Abcam, Cambridge, MA, USA) following the manufacturer instructions. The intra-assay CV% were below 2% for all the samples (min: 0.005; max: 1.1%).

5.3.3.2 Feathers

Tail feathers ($n = 6/\text{treatment per timepoint}$; 48 samples total) were collected during weeks 22, 25, 29, and 43 of age to determine the feather CORT concentrations. The tail feather samples were collected by cutting the calamus as close to the skin as possible without contacting or damaging the skin. At each sampling timepoint, different tail feathers were collected, thus the same tail feather was never collected more than once. The birds were arbitrarily selected from floor pens (3 birds/pen) and conventional cages (1 bird/cage) for feather sampling at each timepoint. Across ages, six birds were selected twice, and 36 birds were selected once. Following collection, the feathers were stored in Whirl Pac bags (Nasco, Fort Atkinson, WI, USA) and stored at −20 °C until assay. Visual inspection ensured that the least damaged feathers were selected for the assay. The feather CORT concentrations were determined following an extraction procedure that was described in ¹⁵². First, the feathers were weighed (mg) to standardize the feather CORT concentrations by feather weight. The feathers were finely minced (including vane and rachis) using surgical scissors (<5 mm sections) into 20 mL scintillation vials. Following mincing, 1 mL of methanol was added, and the vials were placed in a sonicating water bath at room temperature for 30 min. The samples were then placed in a

shaking hot water bath (Jouan Inc., Precision Sci. Div. Chicago, IL, USA) at 56 °C overnight. The samples were filtered to remove feather material and the filtrate was transferred to scintillation vials. The methanol was allowed to evaporate completely under a fume hood and CORT was reconstituted in 1 mL ELISA buffer. Reconstituted CORT concentrations were assayed using a commercial ELISA kit (Abcam, Cambridge, MA, USA) following the manufacturers protocol. The intra-assay CV% were below 13% for all the feather CORT samples (range: 0.15–12.50%).

5.3.4 Statistical analysis

All statistical analyses were performed in JMP Pro 15 (SAS institute, Cary, NC, USA). Pen or cage was considered the experimental unit for all the response variables. Bird was considered the observational unit for all response variables besides fecal IgA where pen or cage was the observational unit due to pooling of the samples. The distribution of residuals of all the dependent variables were visually inspected using a normal quantile plot to determine normalcy. Normally distributed dependent variables included TI duration (s), ABT latency to begin feeding (s), latency to resume feeding (s), plasma and fecal IgA concentrations (ng/mL), and feather CORT concentrations (ng/mg). Normally distributed data were analyzed using general linear mixed models. Fixed effects were housing system (pen or cage), age (weeks 22, 25, 29, 43) and their interaction. For all the response variables besides fecal IgA, mixed models were used with pen or cage number random effects so that the model identifies the unit (cage or pen) to which the treatment was randomly assigned and independently applied. Non-significant interactions ($p > 0.1$) were removed from the model. Post-hoc analysis was done using Tukey HSD testing. Dependent variables without normally distributed residuals were tested using a

nonparametric Wilcoxon Rank sum test. These variables included ABT birds (%) which began and resumed feeding, total vigilance scores (1–4), and birds (%) displaying vigilance behaviors. Associations were deemed significant at $p \leq 0.05$ and trends at $p \leq 0.1$. The data are presented as LS means \pm SEM unless otherwise noted.

5.4 Results

5.4.1 Behavioral measures

TI duration (Figure 5.1) tended to be shorter for the pen hens (82.88 ± 35.86 s) compared to the caged hens (135.70 ± 21.78 s; $F_{1,46} = 3.16$; $P = 0.080$). During ABT, 92.1% of birds (35/38) showed ≥ 1 vigilance behaviors. After the first alarm call, more cage hens tended to begin feeding compared to the pen birds ($\chi^2 = 3.55$; $P = 0.058$; Table 5.2). Latencies to begin feeding were shorter in the cage hens compared to the pen hens ($F_{1,70} = -2.33$; $P = 0.022$; Table 5.2). Following the second alarm call, more cage birds resumed feeding compared to the pen birds ($\chi^2 = 5.28$; $P = 0.020$; Table 5.2). The latency to resume feeding did not differ between the cage and pen hens ($F_{1,50} = 1.20$; $P = 0.279$; Table 5.2). The total vigilance behavior scores ($\chi^2 = 0.01$; $P = 0.967$) and the frequency of observed vigilance behaviors (all $P > 0.200$) did not differ between the treatments (Table 5.2).

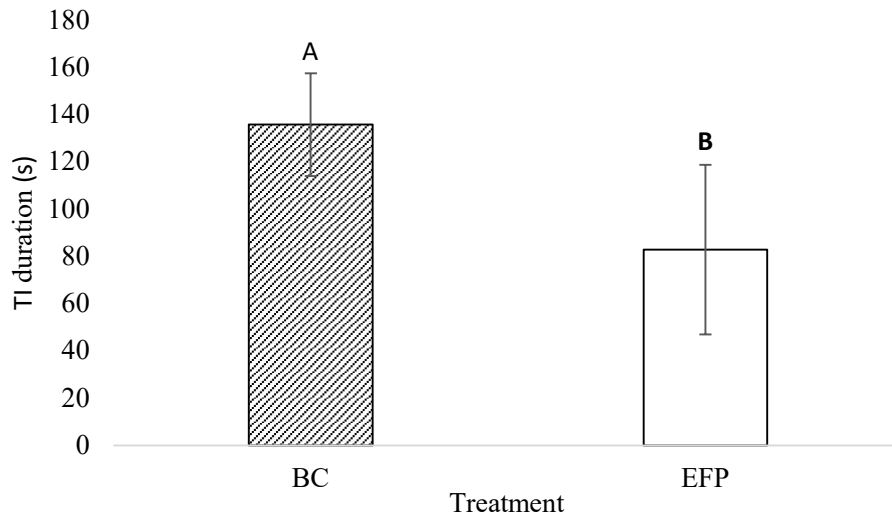


Figure 5.1. Tonic immobility (TI) duration at week 23 of age ($N=24$ birds/treatment) for hens housed in conventional battery cages (BC) and hens housed in enriched floor pens (EFP). Bars lacking a common superscript tend to differ at $P<0.1$. Data is presented as $LS\text{means}\pm SEM$.

Table 5.2. Responses in the attention bias test at 30 weeks of age for laying hens housed in conventional battery cages ($N=36$) or enriched floor pens ($N=36$).

Measure	Battery cage	Enriched floor pen
Latency to begin feeding (s)	99.2±24.6 ^a	145.9±24.6 ^b
Latency to resume feeding (s)	54.2±13.6	54.1±13.9
Birds begin feeding (%)	91.7 ^A	77.7 ^B
Birds resume feeding (%)	87.9 ^a	82.1 ^b
Vigilance behavior score (0-4 score) ¹	2.4±0.4	2.3±0.3
Freeze (% birds)	55	35
Erect (% birds)	55	44
Neck stretch (% birds)	65	55
Look (% birds)	80	72

¹ Birds were scored either 0 (not observed) or 1 (observed) for each of four vigilance behavior characteristics (erect posture, neck stretching, freezing, and looking around),

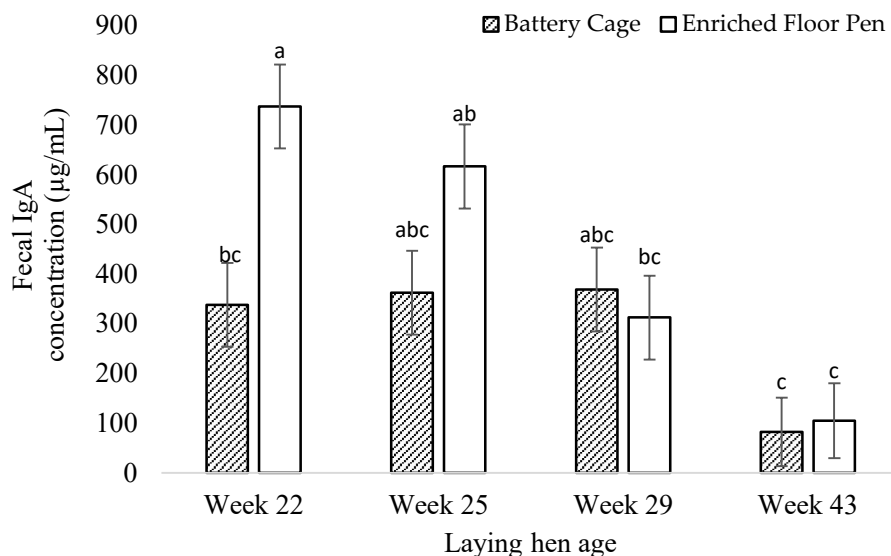
resulting in a vigilance score between 0 (no vigilance behavior observed) and 4 (all vigilance behaviors observed). Data are displayed as raw means \pm SEM

a-b Row values lacking a common superscript differ at $p < 0.05$.

A-B Row values lacking a common superscript tend to differ at $p < 0.10$.

5.4.2 Molecular measures

There was a treatment by age interaction effect on the fecal IgA concentrations ($F_{1,27} = 3.51$; $P = 0.035$; Figure 5.2). The fecal IgA concentrations were lower in the cage hens in week 22 ($F_{1,27} = -3.38$; $P = 0.049$) compared to the pen hens in that week (Figure 5.2). The fecal IgA concentrations did not differ between the treatments in weeks 25 ($F_{1,27} = -1.94$; $P = 0.540$), 29 ($F_{1,27} = 0.23$; $P = 0.999$), and 43 ($F_{1,27} = -0.37$; $P = 0.999$). The fecal IgA concentrations in the pen layers were higher in weeks 22 compared to week 29 ($F_{1,27} = 4.23$; $P = 0.008$) and week 43 ($F_{1,27} = 6.06$; $P = 0.002$). The fecal IgA concentrations were higher in the pen hens in week 25 when compared to week 43 ($F_{1,27} = 4.85$; $P = 0.002$). No difference in the fecal IgA concentrations were found in the cage



hens over time ($P > 0.1$).

Figure 5.2. Least square mean estimates (\pm SEM) of fecal (secretory) immunoglobulin-A (IgA) concentrations in hens housed in conventional battery

cages or enriched floor pens during weeks 22, 25, 29, and 43 of life. Bars lacking a common superscript differ at $P < 0.05$.

Plasma IgA concentrations were not impacted by treatment ($F_{1,88} = 0.66$; $P = 0.419$) or treatment by week interaction ($F_{1,48} = -1.63$; $P = 0.110$). The plasma IgA concentrations were lower in week 22 ($87.25 \pm 15.65 \mu\text{g/mL}$) compared to week 29 ($260.90 \pm 15.65 \mu\text{g/mL}$; $F_{1,88} = -7.69$; $P < 0.001$) and 43 ($201.80 \pm 14.45 \mu\text{g/mL}$; $F_{1,88} = -5.36$; $P < 0.001$), and lower in week 25 ($104.55 \pm 16.45 \mu\text{g/mL}$) compared to week 29 ($F_{1,88} = -7.00$; $P < 0.001$) and week 43 ($F_{1,88} = -4.44$; $P < 0.001$). The plasma IgA concentrations in week 29 were higher than in week 43 ($F_{1,88} = 2.82$; $P = 0.030$).

The feather CORT concentrations by feather weight (ng/mg) were higher in the cage hens compared to the pen hens ($F_{1,43} = 2.18$; $P = 0.004$; Figure 5.3). The feather CORT concentrations were higher in week 22 and 25 compared to week 29 ($F_{1,43} = 6.07$; $P = 0.015$; Figure 5.4), but lower in week 29 compared to week 43 ($F_{1,20} = -3.30$; $P = 0.012$; Figure 5.4). There was no treatment by week interaction effect on the feather CORT ($F_{1,37} = 1.43$; $P = 0.251$).

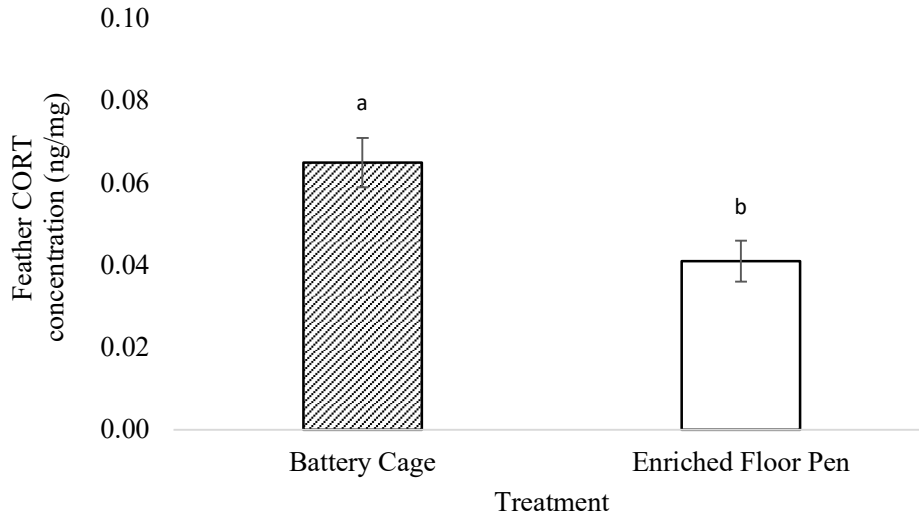


Figure 5.3. Least square means estimates (\pm SEM) of total feather corticosterone (CORT) concentrations for laying hens housed in conventional battery cages or in enriched floor pens. Bars lacking a common superscript differ at $P < 0.05$. $N = 12$ samples/timepoint (6 samples/treatment) from weeks 22, 25, 29, and 42 days of age.

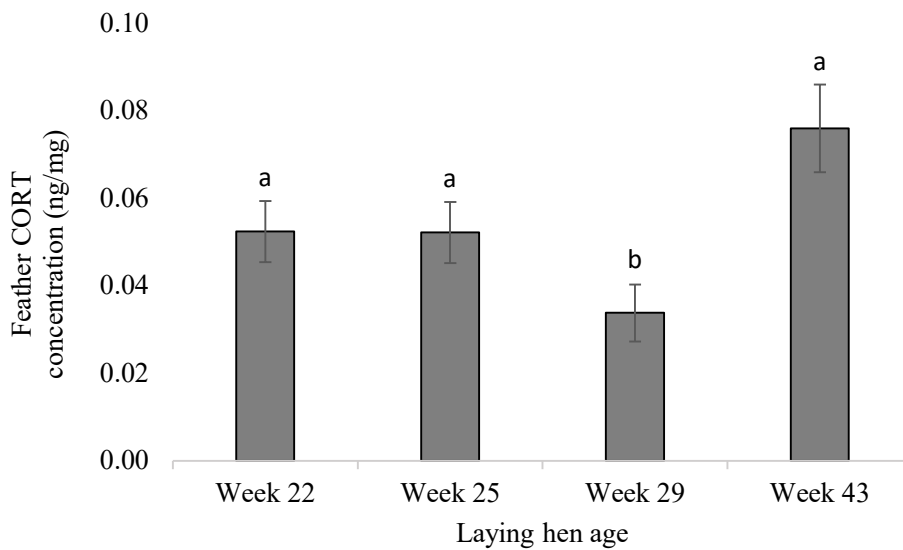


Figure 5.4. Least square means estimates (\pm SEM) of total feather corticosterone (CORT) concentrations by age. Bars lacking a common superscript differ at $P < 0.05$.

5.5 Discussion

This study investigated the effects of housing system (enriched floor pens vs. conventional caging) on behavioral and molecular markers for laying hen welfare including TI, attention bias, fecal and plasma IgA concentrations, and feather CORT concentrations. Birds in conventional cages tended to be more fearful based on TI responses, were less anxious based on ABT responses, and experienced more chronic stress based on low IgA levels and high feather CORT levels. Overall, these results suggest that the cage layers experienced a decreased welfare status when compared to pen layers, except in terms of anxiety. This is the first study to show a relationship between housing conditions and IgA and feather CORT responses in laying hens.

5.5.1 Behavioral measures

Cage housing tended to result in more fearful but less anxious birds compared to pen housing, which is in line with earlier findings^{303,304}. Fearfulness is considered a negative emotion and frequent bouts of fear can be indicative of negative affective states in animals^{12,128,305}. Similar to our results, cage housing increased the TI duration in 18-week-old (cages: 519 s vs. pens: 471 s) and 1-year-old (cages: 443 s; pens: 189 s) white leghorn hens when compared to floor pen housing¹²⁸. However, these reported durations were longer than those in the current study in Bovan brown laying hens (cages: 136 s vs. pens: 88 s), potentially due to strain differences^{228,306}. Although the difference in the current study was only a tendency, we suspect this is due to limited statistical power because of the relatively small sample size, as a large numerical difference in TI durations was observed. Previous studies have found no difference in fear levels related to housing system in commercial laying hens^{299,307}. In ideal situations, fear-associated

behavior is adaptive and aids in helping animals prevent injury during threatening encounters. However, fear-associated behavior in production housing systems is generally maladaptive, with animals having no ability to escape or appropriately respond to fearful stimuli³⁰⁸. Over time, this excessive fear response could lead to frustration, learned helplessness, and eventually negative affective states which can impact productivity. Increased fear can lead to decreased egg production^{309,310}, increased feather pecking³¹⁰⁻³¹³, and increased injury rate³¹⁴. In systems that have high fear levels, these impacts could erase, or reverse production gains that are generally attributed to intensive housing systems. The increased level of fear in cages compared to pens could have contributed to a negative affective state in hens in cages.

Cage housing conditions reduced anxiety in laying hens compared to pen housing conditions. Attention bias testing was validated for use in laying hens^{126,132}, and has been applied to test the effect of environmental conditions on anxiety in starlings³¹⁵ and broilers¹². During the test, 92.1% of birds showed some form of vigilance behavior, indicating that the ABT was sufficiently threatening to achieve anxiety. Contrary to expectations, cage housing decreased the latency to begin feeding and resulted in a higher percentage of hens feeding after the first and second alarm call compared to pen housing. These results indicate that hens that were housed in cages did not bias their attention towards the threat, thus were less anxious than hens that were housed in pens, which is somewhat contradictory to earlier studies^{12,14}. Broilers from high-complexity pens were quicker to begin feeding following an alarm call compared to broilers from low-complexity pens (high-complexity: 160 s vs. low-complexity 214 s;¹²). Outside-ranging laying hens showed shorter latencies to feed compared to hens that never went outside

(outdoor: 86 s vs. indoor: 170 s¹³²). Although the treatment conditions were different in both studies compared to the current study, they both involve a level of environmental complexity. In the current study, as in Anderson et al. 2021¹², the test was performed in groups of three birds, rather than testing birds individually (as in ¹²⁶). The cage hens were tested with their cage conspecifics, and pen hens with 3 out of 25 pen conspecifics. It is possible that pen hens experienced this temporary regrouping as more negative than the cage hens, depending on the social hierarchy within the pen. Further testing is needed to confirm the effect individual versus group testing of anxiety, and of these housing conditions on anxiety in laying hens.

Our results support earlier findings that fear and anxiety can be opposing^{294,316} although some studies show they can be positively associated^{317,318}. Fear is a generally fast adaptive state of vigilance to a present, negatively valenced stimulus which activates a defensive response such as fight, flight, or freeze²⁹³. Anxiety elicits vigilance and apprehension to non-existing or ambiguous threats²⁹³. Although the symptoms of fear and anxiety are similar²⁹³, evidence suggests that they are distinct emotional experiences. Research in rodents shows that three stages of defense exist, which include the pre-encounter defense (apprehension to a place where a predator has been seen), a circa-strike defense (physical contact with a predator), and a post-encounter defense (predator is identified at a distance)^{293,319-321}. Anxiety has been associated with the pre- and post-encounter stages, but not with the circa-strike phase²⁹³. As the TI test is performed, the researcher acts as a predator coming in physical contact with the hen, inducing a catatonic state. If similar stages of defense exist in chickens, the TI test most likely simulates the circa-strike defense phase. This suggests that the emotional experiences that

are tested in the ABT and the TI test are associated with distinct phases of defense. Cage birds may be more fearful in the circa-strike phase and less anxious in the pre- and post-encounter stages compared to the pen birds. Thus, this could explain why the outcomes are opposing. Although these defense phases have not been confirmed in chickens, it is possible that a similar distinction exists. Therefore, it is possible that housing systems impact hen fear and anxiety differently. Overall, the hens' behavioral responses indicate that anxiety and fear are affected by housing system, with pens tending to reduce fear but not anxiety compared to cages.

5.5.2 Molecular measures

The housing treatments impacted fecal IgA concentrations during week 22, resulting in decreased concentrations in the cage layers compared to the pen layers. Our results indicate that birds that are housed in complex, low density environments (enriched floor pens) are under less chronic stress (reflected by immune status) than birds that are housed in barren, highly confined environments (conventional cages) at 22 weeks of age.

Additionally, our results show that fecal IgA could be a potential physiological indicator of animal welfare status. This is in line with past findings in swine, rodents, laying hens, and broilers^{149,212,213,217,242}. Prolonged heat stress decreased plasma IgA concentrations in broilers and layers^{217,242}, which showed plasma IgA concentrations in similar ranges as in the current study (previous study: 0.162–0.290 mg/mL vs. current study: 0.087–0.260 mg/mL).

Secretory and plasma IgA concentrations are promising valence-dependent indicators of stress in certain mammals^{213,215,251}. We did not find an impact of housing conditions on plasma IgA concentrations, showing at least in similar contexts, this measure could be

less relevant for laying hen welfare assessments. However, to our knowledge this study is the first to investigate secretory and plasma IgA in an animal welfare context in laying hens. Access to litter, containing microorganisms and fecal material, could have impacted the higher secretory IgA concentrations in pens compared to the cage hens at 22 weeks of age. Alternative housing systems (aviary, pens) result in higher concentrations of bacteria and fungi in the air than conventional cage housing^{322,323}. Cages are considered more hygienic than litter systems due to the separation of waste and animals^{29,322}. However, all the birds were housed in the same space, so airborne bacteria and fungi could reach the cage hens too. Nevertheless, ingestion of litter and fecal material could have exposed the hens' intestinal tracts to pathogens and initiated an immune response. In turn, the pen hens could have had increased fecal IgA concentrations to mediate an immune response against those intestinal pathogens. Further research should determine the impact of litter access on intestinal immune challenges and include a chronic stressor that is unrelated to housing conditions. Then, the impact of litter access and chronic stress can be separated and the use of secretory IgA as a biomarker for animal welfare confirmed.

Even though housing conditions impacted the fecal IgA concentrations at 22 weeks of age, the plasma IgA concentrations did not differ. Secretory IgA may provide more insight in stress-induced immune system disruptions because IgA are most prominently active and present on mucosal surfaces including the intestinal tract²⁵¹. IgA originating from the intestinal tract will be deposited in fecal matter as it passes through. Negative stressors, including heat stress^{197,324-326} and stocking density³²⁷⁻³³⁰ negatively impact the intestinal tract via damaged intestinal morphology and decreased microbiota diversity in commercial broilers and laying hens. These impacts on intestinal health could also impact

the immune system, and, therefore, intestinal IgA via a reduction in IgA-secreting plasma cells (mature B-cells) or via direct interactions with intestinal glucocorticoids¹⁴⁹ indicating multiple possible mechanisms for the observed impacts of welfare status on IgA concentrations. Our results are the first to suggest an association between housing conditions and fecal IgA responses in laying hens, albeit only at 22 weeks of age. These results are a first step towards using secretory IgA as an indicator of welfare status in laying hens. Further research is needed to elucidate the impact of litter access and bird age on IgA concentrations.

Feather CORT assays have been used in several species including wild birds, broilers, and laying hens^{152,236,301,302}. The feather CORT concentrations were higher in cage hens compared to the pen hens overall, however, feather CORT concentrations did not differ between the housing treatments at any individual timepoints. The latter is likely due to the low sample volume at individual timepoints. Our CORT concentrations indicate that layers from cages experienced more chronic stress than layers from pens. This difference possibly occurred due to repeated bouts of frustration or negative stress contributing to negative affective states. During these bouts of frustration or negative stress, the HPA axis releases glucocorticoids into the bloodstream, which are partially deposited into the feathers as they grow. The sum of these deposits is used to quantify stress over the whole feather growth period. Brown Nick laying hens that were housed at high stocking density, which is largely considered a chronic stressor in commercial poultry^{227,330–333} had increased feather CORT at 10 weeks of age, when compared to birds that were housed at a lower stocking density¹¹¹. In the current study, the stocking density differed considerably between the treatments, which could have contributed to the low feather

CORT concentrations in pens compared to cage hens. While feather cover was not quantified as part of this experiment, birds that were housed in cages showed poorer feather coverage than birds that were housed in pen housing. It is possible that this poor feather coverage in cage birds would have made them more susceptible to cold stress during the cold period in the winter months (weeks 33–37) thereby increasing their feather CORT concentrations. Additional research should be done to determine if cold stress will impact feather CORT concentrations. Feather CORT concentrations also significantly decreased at week 29 of age compared to week 25 regardless of treatment. This was unexpected as feather CORT should increase continuously throughout life (as CORT keeps being deposited) unless the feather is regrown or replaced. It is possible that this observed decrease is methodologically related, however, all analyses were performed by the same researcher and the same protocol was followed. Feather CORT concentrations do rebound and reach a peak in week 43, which coincides with the cold spell that was observed from weeks 33 to 37 and likely reflects chronic stress that was caused during this period to birds in both housing systems. Overall, these results indicate that feather CORT shows promise as a viable physiological biomarker for chronic stress in laying hens. However, additional research is needed to confirm these results in commercial production systems and in other genetic strains.

5.6 Conclusions

The birds' responses in the current study suggest that conventional cages may induce negative affective states and emotions, reflected in somewhat increased fear and chronic stress responses, compared to enriched floor pens. However, anxiety was reduced in hens that were housed in conventional cages compared to the enriched pens. Similar to

previous research, these results highlight the importance of including a range of measures when assessing the impact of housing conditions on animal welfare. The novel measures of animal welfare that were tested in this study (Immunoglobulin A and feather corticosterone) indicate a downregulated immune response at 22 weeks of age (decreased fecal Immunoglobulin A) and increased chronic stress response (increased feather corticosterone) in conventionally caged hens compared to hens that were housed in enriched floor pens. However, fecal Immunoglobulin A concentrations require more research to confirm that the difference was not because of a mounted immune response due to exposure to litter. Overall, enriched pens resulted in improved laying hen immune responses at 22 weeks of age, decreased chronic stress, somewhat decreased fear, but increased anxiety indicating potential improvements in affective states (fear and chronic stress) in some respects, but worsening in others (anxiety), and an improvement in the basic health and functioning (immune responses) when compared to conventionally housed birds. Fecal (secretory) Immunoglobulin A and feather corticosterone quantification show some contrasting responses in line with expectations and behavioral outcomes yet need further confirmation before application as a routine measure for emotion and affective state in laying hens.

Chapter 6: General Conclusions

6.1. Environmental complexity and stocking density impacts on secretory and plasma IgA, and telomere length (chapter 3, 4)

To our knowledge this is the first research to indicate a positive impact of environmental complexity on cumulative experience in broiler chickens. Complex environments increased plasma IgA concentrations and telomere length in broilers (chapter 3, 4), indicating that complex environments reduced distress and/or caused positive experience. Complex environments provide broilers with choice between multiple positive, meaningful activities which allow them to display natural, species-specific behaviors which can contribute to physical and mental health. Contrarily, no impact of environmental complexity was found on secretory IgA concentrations. However, it is possible that plasma IgA and secretory IgA react differently to differently valenced environments. Only secretory IgA concentrations showed appropriate contrast associated with stocking density. After replication of results in other research, secretory IgA may be the more responsive measure for detecting chronic stress in broiler chickens and could be more useful than plasma IgA and telomere length for detecting negative experience related to high stocking density. Additionally, plasma IgA concentrations, secretory IgA concentrations, and telomere length all show appropriate contrast in response to housing conditions in broiler chickens, thus seem to be useful as measures of cumulative poultry experience. However, results need to be replicated before these measures can be used more broadly as indicators for cumulative experience in poultry in research and industry.

6.2. Housing impact on novel behavioral (attention bias) and physiological (IgA and feather corticosterone concentrations) measures of laying hen welfare

Chapter 5 showed that birds from conventional caging were less anxious but more fearful than birds from environmentally enriched floor pens. Anxiety and fear can seemingly be

opposing, and the impact of housing system on affective states and emotions, respectively, may not always be correlated. Additionally, birds raised in environmentally enriched floor pens showed decreased chronic stress and better immune responses at 22 weeks of age when compared to birds housed in conventional battery caging. These results further contribute to the body of literature that states that battery caging limits the ability of birds to display natural, species-specific behaviors potentially leading to increased chronic stress and immunosuppression. Additionally, we showed the first evidence of a relationship between housing conditions and IgA and feather CORT responses in laying hens. However, while these results establish that battery caging contributes to increased chronic stress in laying hens, additional confirmation is needed to ensure these impacts are not due to access to litter in enriched floor pens or a cold period experienced by the birds. Overall, these results show that IgA and feather CORT concentrations show promise as measures of chronic stress in laying hens.

6.3. Recommendations for future research

- Determine if the production mechanisms of plasma IgA and secretory IgA are responsible for different responses to positive and negative stimuli.

In chapter 3, concentrations of plasma IgA and secretory IgA showed contrasted responses between housing treatments indicating they could be appropriate measures of chronic stress in broiler chickens. However, the results between plasma IgA and secretory IgA were not consistent. Plasma IgA responded to only positive housing stimuli (environmental complexity) and secretory IgA responded only to negative housing stimuli (high stocking density). We propose that this differential response could be due to differences in production mechanisms between plasma IgA and secretory IgA. Future

research should investigate these production mechanisms and determine if they impact the direction and speed of response of plasma and secretory IgA to housing systems in broiler chickens.

- Determine how environmental complexity increases telomere length in broilers

In chapter 4, kidney telomeres were longer in birds in highly complex conditions compared to birds in low-complexity conditions. We theorize that longer telomeres were at least in part due to a reduction in stressful events and a mitigation of negative effects of stressful events in complex environments. Additionally, increased telomerase production in response to a complex environment could have contributed to longer kidney telomeres. However, telomerase production has not been investigated in broiler chickens. Therefore, future studies should investigate how environmental complexity impacts telomerase production and the role telomerase plays in increasing telomere length in animals under positive experience.

- Investigate the impacts of severe chronic negative experience on telomere length in broilers

Stocking density did not impact telomere overall telomere length in broiler chickens and positively impacted telomere length during experiment 3. It is possible that telomere length is not a viable biomarker for negative experience in broilers. It is possible that a chronic, more severe negative stimulus would result in telomeric shortening, for example feed restriction in broiler breeders and broiler breeders. Future research should investigate the impacts of more severe negative stimuli on telomere length in birds to determine if telomere length responds to higher intensity negative stimuli.

- Validate telomere measurements via RT-qPCR by comparisons to terminal restriction fragment analysis

The RT-qPCR assay used to determine relative telomere length needs to be validated via comparisons with terminal restriction fragment analysis, as this is considered the “golden standard” of telomere length measurements^{8,283}. Future research should compare telomere length measurements from terminal restriction fragment analysis to measurements from RT-qPCR on the same samples to determine correlation values between the assays.

- Determine if access to litter can impact secretory IgA concentrations in commercial laying hens

Secretory IgA concentrations were lower in hens housed in conventional caging compared to birds in enriched floor pens. Future research needs to determine the impact of litter access alone on these response variables. The birds housed in enriched floor pens could have consumed their feces and potentially exposed their digestive tract to fecal pathogens, changing their intestinal immune response and thus secretory IgA concentrations. However, the direct impact of litter access on intestinal antibody concentrations has not been investigated. This impact should be determined before widespread use of secretory IgA as a measure of animal welfare, at least in studies comparing housing systems without equal access to a foraging substrate.

Chapter 7: Final Conclusions

Overall, we were able to determine that telomere length, secretory and plasma IgA concentrations, attention bias testing, and feather corticosterone concentrations are

appropriate measures of positive or negative experience in broiler chickens or laying hens. However, the majority of these measures were much better at determining positive experience in relation to environmental complexity than negative experience related to stocking density or cage housing. Therefore, it is unclear based on the results from this dissertation if these are true measures of cumulative experience in poultry, or rather simply measures of either positive or negative experience. This dissertation contributes valuable information to the growing body of literature on how to measure the impacts of housing conditions on poultry welfare.

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