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Comparison of corticosterone concentrations between serum and fecal samples as a measure of stress in male broilers.

A Thesis

Presented to

the Faculty of the Hutson School of Agriculture

Murray State University

Murray, Kentucky

In Partial Fulfillment

of the Requirements for the Degree

of Master of Science in Agriculture

by Kayla Weinacht

May 2023

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ABSTRACT

Stress has long been a topic of interest in the poultry industry due to its potential negative effects on chicken health and production of both eggs and meat. With increased global growth in egg and broiler production, more attention has been paid to the stressors that affect chickens in modern poultry operations, particularly environmental temperature and stocking density. Improving quantitative measurement of stress is always needed in poultry welfare research. In most studies, blood serum is measured for corticosterone, one of the biomarker hormones associated with stress in chickens. Methods of collecting blood serum are by their nature disruptive to the chicken and leave it with a higher level of stress than they previously experienced. Because of this, more research studies have been dedicated to evaluating different non-invasive ways to measure stress in poultry flocks. Fecal corticosterone levels are non-invasive and are used to quantify stress in wildlife. This method has been studied extensively in layer hens. Recent research has also focused on its use in broiler operations. This study investigated if fecal and serum corticosterone concentrations could be measured in a small flock of chickens managed with minimal control over environmental conditions. At 43 days of age (day 0), 24 male broilers were given either tap water or water treated with a corticosterone supplement (CORT, 20mg/L) for 48 hours. Blood was collected from one chicken per group (8 groups with 3 chickens per group) at -24, 6, 12, 24, 48, 72, and 96 h. Chickens were rotated between blood draws. Fecal samples were collected during the time of blood drawn from the bottom of each cage. Body weight was recorded for each group after blood was drawn. Data was analyzed using the Mixed procedure of SAS 9.4 with fixed effects of treatment and hour. CORT treatment reduced body weight at all time points.

H:L (heterophil to lymphocyte) ratio increased until 12 h and then decreased. Average serum CORT concentration increased to peak at 6 h and returned to baseline values by 24 h after onset of treatment. Average fecal CORT concentration increased to peak at 12 h and returned to baseline concentrations by 72 h after onset of treatment. The results of this study were compared to a previously published study on a larger flock in a more controlled environment. The results from this study indicate that fecal CORT concentrations reflect serum CORT concentrations with a 6 h delay, as compared to previously published research where there was a 12 h delay. Both sample methods allow for the measurement of stress in broilers.

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CHAPTER 1: INTRODUCTION

Chronic and acute stress are topics of interest in the poultry industry and research community, as it can have a negative impact on bird health and, ultimately, meat and egg production. Broilers can face many stressors. In today's intensive operations, if chickens are not managed properly, stressors could include over stocking, high temperature, or restriction of food or water Under poorly managed conditions, there can be detrimental effects on broiler production levels of eggs or meat. Foundational to welfare and stress, research is employing accurate measurements of stress conditions. Stress in poultry can be evaluated by monitoring corticosterone concentration (Najafi et al. 2015; Weimer et al. 2018; Trocino et al. 2020). The standard method to monitor this hormone in broilers is by using blood samples collected from the brachial vein (Weimer et al. 2018), though medial and metatarsal veins can also be utilized. The process is stressful for individual chickens and conducting this on a large sample of broilers in a production environment would be prohibitively time consuming. Simply attempting to monitor stress levels in chickens in this way would add to baseline levels of stress they are already experiencing.

Wildlife and biological communities collect fecal samples to measure stress levels in wild animals. The practice has become increasingly popular due to heightened research interest in wildlife stress and endocrine function (Palme 2009). By using this method there is no need to capture animals. Application of fecal analysis for corticosterone concentrations may present an opportunity in modern livestock and poultry operations to monitor corticosterone concentration without disrupting the animals.

Broiler research is typically done in large scale, flock-type settings that approximate commercial production conditions. These settings offer a real-world application to broiler production houses. While these studies are especially relevant to large scale producers, many universities do not have the facilities or budget to conduct research projects of this magnitude. Poultry research methods on a smaller scale should be explored too as a way of replicating and validating results of studies done in the larger scale settings.

The objective of this project was to determine if corticosterone levels could be measured in both blood and fecal samples from birds raised in an environment that was not tightly controlled and to compare the findings to previous large-scale studies. Additionally, the research was designed to fill a knowledge gap concerning the recovery of normal fecal corticosterone concentration after a stress event. Levels were monitored after the stress event to determine how quickly the concentration would return to normal. The brooder and growing environments in this study had very limited light and temperature controls. Chicks were brooded in a stock tank indoors in a standard science lab then transferred to metal cages in an outdoor shed where they remained for the duration of the study. Even with the use of heat lamps, temperature control in the brooder environment was subject to the building's centralized HVAC controls. In the grower stage, minimal heating and air circulation was possible. Because of this, temperature was monitored to evaluate how it affected study results. A corticosterone water supplement was used as indicated in recent similar studies. Since we were using limited numbers of chickens, fecal and blood samples were obtained in such a way that chickens did not have to be sacrificed or posted and repeat measures could be taken as needed.

Limitations & Assumptions

Part of the design of this experiment focused on limiting the number of birds required and evaluating a minimum number of samples to obtain significant results. Limits on environmental control as previously stated were in place. It was assumed that the birds would grow at a slower rate with lower feed efficiency than what would be expected in optimal commercial conditions, but that the results could still be applied as long as growth patterns were consistent with the norms.

CHAPTER 2: REVIEW OF LITERATURE

Overview of Stress in Poultry

Stress is the body's response to changes in the internal or external environment of an animal. These responses allow the animal to adapt and survive. It can lead to the "fight or flight" response, which allows the animal to react to perceived danger. When stressors impact the animal, many hormones are produced. The primary hormones involved in stress response in poultry include epinephrine, cortisol, and corticosterone. These hormones alter physiologic function, allowing the animal to adapt and to protect itself through increasing their heart rate, increased cardiac output, increased skeletal muscle blood flow, bronchiolar dilation, and increased respiration rate (Ranibar and Reetu 2011). In chickens, corticosterone is the primary hormone produced (Scanes 2016). While the immediate response to stress can be good, prolonged stress can result in negative physiologic changes, including decreased growth, immune function, and metabolism (Scanes 2016). Chronic and acute stress can lead to adverse health effects no matter what the cause is.

However, stress is more complex than just "fight or flight". Scanes and Christensen (2019) refer to stress as a "physiological, physical, or psychological tension or strain." Scanes (2016) noted a variety of stressors that can be used to stimulate stress responses in poultry. One category of stressors includes environmental changes, such as temperature and humidity adjustments (Jones et al. 2005), or high temperatures and high stocking densities (Najafi et al. 2015). One of the most common methods of inducing stress in poultry in a research setting involves altering environmental temperature. Changes in feed composition (Alm et al. 2014), feed withdrawal (Nwaigwe et al. 2020),

and dietary energy (Yang et al. 2015) also result in stressed chickens. Management factors, such as handling (Ericsson et al. 2016), housing in cages with spatial restriction (Shields and Greger 2013), overcrowding (Lokhande et al. 2009), and altered lighting (Huth and Archer 2015) can trigger negative changes in production and health as well.

Once an animal interprets something to be a potential threat, the stress response is triggered. No matter the cause of stress, the event will trigger the hypothalamic-pituitary-adrenal (HPA) axis (Ericcson 2016). The HPA axis is the relationship between the hypothalamus, pituitary gland, and adrenal glands, all of which work together when the body is stressed (Ericcson 2016). This system provides a negative feedback mechanism that regulates the physiological mechanisms of the stress response. When the body becomes stressed by an outside threat, the hypothalamus is activated. More specifically, the paraventricular nucleus, or nucleus of the hypothalamus, is stimulated to release corticotropin releasing hormone (CRH) and vasopressin (VP) (Mastorakos et al. 2006). These hormones work together to regulate adrenocorticotropic hormone (ACTH) production from the anterior pituitary (Ericsson 2016). When ACTH is released, it stimulates the release of glucocorticoids by promoting the uptake and conversion of cholesterol to cortisol, or in the case of avian species, corticosterone (Ericsson 2016). The HPA axis is controlled by negative feedback. In short, glucocorticoids inhibit more responses from the HPA axis by inhibiting the release of both CRH and ACTH. This allows the body to maintain a state of homeostasis. While the HPA axis is normally stimulated by outside stimuli, it can also be jump-started by two other hormones; adrenaline and oxytocin. These two hormones stimulate the production of ACTH (Scanes 2016). While healthy concentrations of glucocorticoids are needed to maintain

homeostasis, too much in the body can lead to protein catabolism, immune suppression, and increased susceptibility to infection (Scanes 2016).

Physiological Impact of Stress

Unintended or unavoidable stressors have many physiological impacts that must be evaluated to understand the health of an animal. Blood work of a stressed chicken can show signs of hypoglycemia, excess cytokines, and changes in plasma metabolites such as triglycerides (Mohan 2011). These can present physically in the retention of body fat, primarily in the abdomen of layer hens. Without running any blood tests, physical changes such as slow growth, decrease in muscle, and decreased appetite can indicate an animal experiencing excess stress.

These changes under any length of stress can weaken the chicken physically and suppress its immune system (Mohan 2011). It is important to note that all types of stress (i.e., chronic and acute) impact the immune system accordingly. Chronic stress, or stress that occurs over a long period of time, can affect the regulation and function of the immune system (Dhabhar 2009). This is commonly manifested as suppressed "immuno-protective parameters" such as antibody production (Dhabhar 2009). Because of this weakened immune function, chickens that undergo longer periods of stress can contract a variety of diseases. In contrast, acute stress, or stress that occurs in short durations, may actually enhance the immune system's response (Dhabhar 2009). This enhancement is dependent on the varying environmental and health conditions that the animal is in. Accurately determining if the animal has experienced chronic or acute stress can be crucial to understanding how stress has affected the body's immune system.

Impact of Stress on Production and Poultry Welfare

Production of poultry eggs and meat is contingent on having healthy animals. Without optimal health, production suffers, and unit costs of products increase. When specifically looking at the impacts of heat stress on poultry production it can significantly lower feed intake and body weight (Lara and Rostagno 2013). Lowered feed intake and body weight lowers the amount and quality of meat and eggs produced. Heat stress can negatively affect the fat deposition and quality of meat when experienced over a long period of time (Lara and Rostagno 2013). With chronic stress, fat deposition can increase while the quality of meat decreases. Without efforts to monitor and control stressors, production of eggs and meat can decrease, and flock mortality rates may increase (Elitok and Binguler 2018).

Stressors generally negatively impact both welfare and poultry production. An animal in good welfare standings is healthy, comfortable, and well-nourished (National Chicken Council 2022). Poultry production is the raising of poultry, such as broilers and layers, for products like meat and eggs. With the growing public awareness of animal welfare in animal production, more attention has been paid to addressing situations that impact the production of broiler chickens. Stressors that can impact broilers include temperature, lighting, litter management, and veterinary care (Vizzier Thaxton et al. 2016). Using modern equipment and technology has helped to make poultry housing environments as close to perfect as they can be. During a symposium reviewing animal welfare challenges, it was reported that average peak growth rates of chickens increased from 39 g per day to 59 g per day over the course of 29 years (Vizzier Thaxton et al. 2016). While much of the improvement in ADG has resulted from improved genetics and

nutritional management, improvements in housing and management also allow for better welfare resulting in greater production. Not only does addressing welfare concerns allow for chickens to grow in an environment free from stress but allows for long term sustainability of the flock.

Economic Impact of Stress

Stress-related economic losses in animal agriculture, however unavoidable they may be, can be lessened through proper care of the animals. This is an incentive for producers to control the stress on their animals so that they produce at their highest productivity rate. Stress in broilers affects both their physical condition and their productivity. It is estimated that approximately $51 million is lost in the broiler production industry and $126 to $165 million in total loss in the poultry industry due to heat stress alone (St-Pierre et al. 2003). This is due to less food intake by the chickens, leading to lower body weights. While this is a small margin in an industry that grosses over $20 billion, on a producer level this could mean the difference between breaking even or losing money for the year. Najafi et al. (2015) investigated the effects of stocking densities and environmental heat on performance in broilers. Heat had negative effects on body weight, weight gain, and feed conversion ratios. Respiratory alkalosis, resulting from hyperventilation, was also seen as it reduced blood flow to the gastrointestinal tract (Najafi et al. 2015). This may also contribute to declines in growth performance. The study, conducted under tropical conditions, noted that lower stocking density of 0.063 m^2/chicken had no detrimental effect on body weight, but stocking densities of 0.1 and 0.067 m^2/chicken had a negative effect on weight gain (Najafi et al. 2015). This implies that stocking density's effect on growth is dependent on the system the animals are raised

in. Rosales (1994) found that overcrowding of chickens can also lead to a less uniform flock. When a flock is raised under stressful conditions it can lead to higher rates of culling, diseases, and less than optimal reproductive performance (Rosales 1994). To avoid preventable losses, chickens need to be kept in low stress environments.

Measuring Stress through Poultry Behavior

In a production setting, it is not feasible to draw blood from every chicken, so producers watch for abnormal behavior in their flocks. Behavior is the most non-invasive way to effectively monitor stress provided that the individuals involved in behavior monitoring are well trained in flock assessment. In one published study, it was noted that under heat stress, broilers were more prone to increased standing, walking, and panting (Mahmoud et al. 2015). Stress from food or water restriction commonly causes aggression between chickens as they compete for limited feed and water (Rosales 1994). In a similar study conducted by Estevez et al. (2002) chickens became increasingly aggressive towards each other as the food became depleted and the number of competitors for this food increased. While some aggression between chickens is normal for a pecking order, this does not usually become established until six to ten weeks of age (Rosales 1994). Aggression during feeding can start before this age when chickens are stressed due to a restriction. Estevez et al. (2002) suggested that aggression observed when competing for food is determined by the amount of energy required and risk of injury related to the potential benefit. While this is not to say that some chickens will not become aggressive when food is limited, it shows that the amount of aggression shown will vary between individual chickens based on how much the chicken values the food over injury.

Feather preening activity is known to increase as stress increases. One study contributed this increased activity to the chicken's desire to reduce stress (Kozak et al. 2019). It can often be the first sign of disturbance in a flock. Grooming behaviors (i.e., preening, flapping wings, or shaking) are common in chickens that are maintaining their hygiene. These behaviors can also be seen in chickens trying to reduce agitation. Kozak et al. (2019) confirmed that preening is a displacement behavior that allowed the hens to reduce stress. This was observed mostly in the hens with elevated corticosterone levels. In severe cases of feather preening and pecking, cannibalism can occur. Costa et al. (2012) explained that this is a result of selective breeding in commercial layer breeds. While most commercial breeds do not carry a wild genotype, some chickens can express this behavioral gene when put in certain environmental conditions. Cannibalism can lead to increased injury and mortality in flocks. It is important to note that while these signs are the most common symptoms of stress, the severity will vary between chickens.

Physiologic Evaluation of Stress

Monitoring behavior can show the first effects of stress, but some physiologic evaluations can also reveal important information. Another common way to evaluate stress in broilers is through drawing blood to measure corticosterone levels in serum or plasma. As previously noted, increased stress in poultry caused by higher stocking density coincides with increased corticosterone, as well as serum sodium, eosinophil, and heterophil/lymphocyte ratio (H:L ratio) (Nwaigwe et al. 2020). These are significant biomarkers of stress in broilers. When stressed, the nervous system stimulates the release of hormones into the bloodstream. One response is to trigger the bone marrow to produce blood cells, specifically white blood cells (WBC). This accounts for the increased WBC

count often seen in hematological analysis of stressed broilers. While elevated WBC counts are common in stress of any kind, heterophil and lymphocyte counts can depend on the stressor the chicken is exposed to. Nwaigwe et al. (2020) noted that in poultry exposed to heat and food withdrawal stress, there were increased H:L ratios, but that under high stocking density stress alone, the H:L ratios were low. It is important to note that while corticosterone does show in the blood when an animal is stressed, it is not the only hormone to increase H:L ratio (Scanes and Christensen 2019).

While blood serum or plasma can show elevated hormone levels it only provides an accurate indication of the chicken's stress level at the time of drawing. The handling method, time it takes to capture the chicken, and time drawing blood can increase the CORT levels (Weimer et al. 2018). For this reason, it was important for past researchers to develop different non-invasive methods to measure stress. Feather health and quality, along with behavior, are important factors in stress assessment. Chickens under increased stress can have feathers of lower structural quality. High corticosterone levels can cause low feather mass, altered micro-structures, and shorter lengths than those not exposed to stress (Lattin et al. 2011). Not only does this affect the feathers already present on the chicken but can affect the quality of feathers that will grow in the future. Bortolotti et al. (2008) was one of the first to report that feathers could be used to measure endocrine levels in chickens. Feathers were plucked from the wing of the chicken before being minced and soaked in methanol. The remnants were filtered, dried, and reconstituted before being measured for corticosterone with a radioimmunoassay (Bortolotti et al. 2008). This study showed that feathers provided a way to non-invasively measure long term stress in chickens. Most importantly, it allowed for CORT to be measured at various

times due to new feather growth showing variation in the chicken's hormone levels (Bortolotti et al. 2008). It was noted that while there was a correlation between feather CORT and plasma CORT there may not always be such a strong correlation in animals not under controlled stress.

When measuring stress in feathers it is also important which part of the feather is used. Lattin et al. (2011) showed that CORT levels were different throughout the feather. This study showed that the highest CORT deposition was towards the more distal portions of the feather with the lowest deposition levels at the most proximal portion (Lattin et al. 2011). More recently, Weimer et al. (2018) conducted a study to evaluate different methods to measure stress in broilers. In this study, both body and primary feathers were collected to identify which type of feather gives accurate readings of stress. This showed that there is a strong correlation between CORT measured in both types of feathers. It was noted that while body and primary feathers both increased in CORT concentration over time in experimental broilers, primary feathers also showed an increase in CORT in control broilers (Weimer et al. 2008). While this could be due to stress of humans and handling, it was unclear as to why there was an increase in control broilers. Because of this inconsistency, there is still a need for further research on variation of corticosterone levels in feather types.

Another non-invasive method to assess stress, particularly through corticosterone concentrations, is through evaluation of fecal samples. This was first seen in 1977, when Czekala and Lasley (1977) published “Technical Note on Sex Determination in Monomorphic Birds using Faecal Steroid Analysis”. This technical note led to a study by Bercovitz, Czekala, and Lasley (1978) that used endocrine levels to determine sex in

birds. While this study didn't test specifically for stress in fecal samples, it showed that fecal samples can be evaluated for steroid metabolite levels. Since then, it has become increasingly popular in the wildlife and animal welfare communities to use fecal samples to test for different fecal steroid metabolites. Until recently, it was mostly used in wildlife endocrinology, welfare, and ecology research (Palme et al. 2013). Because of differences in metabolism and excretion rates of steroids between species it is important that the technique is validated in each species before being used to accurately measure steroid metabolites in feces. Numerous studies have been performed on a variety of different species, both domesticated and wild, to validate the use of fecal samples to measure cortisol.

The first to examine the relationship between fecal corticoid metabolites and ovulation activity in cheetahs was Jurke et al. in 1997. Results showed that fecal samples collected from male cheetahs showed an increase in cortisol over the two days that the animal was stressed (Jurke et al. 1997). With these findings they continued to compare the increase in stress to the female cheetah's reproductive cycle. For this, fecal samples were collected from the female population over the period of a year. Cyclicity of the females was tracked through the fecal samples collected. When comparing cortisol levels to the regular cycling pattern, females in the high cortisol group (cortisol ranging from 190 to 210 ng) showed major deviations from the regular pattern (Jurke et al. 1997). In one specific female, there was no evidence of estrus for the two years the study was conducted. While noting that further testing of this endocrine correlation was required, these findings both validated fecal cortisol metabolite sampling and related cortisol measures to complications with reproduction.

More recently, a 2012 review by Palme evaluated published studies in livestock species to validate measuring stress hormone metabolites in fecal samples when exposed to different stressors. This study showed that, if used correctly, non-invasive techniques to monitor glucocorticoid metabolites in fecal samples were useful in assessing animal welfare (Palme 2012). Most recently, a study was conducted on female Greater Sage-Grouse in 2021 that addressed the relationship between habitat and fecal corticosterone levels. It was found that CORT concentrations varied between individuals based on their reproductive status (Rabon et al. 2021). The above studies validated the method of measuring corticosterone or cortisol metabolites in fecal samples of the respective species and allowed for further research into welfare of that species. Even though this is a good way to measure CORT in poultry, there is approximately a four-hour delay from the time of stressor to the time CORT was able to be measured in fecal samples (Weimer et al. 2018). While fecal steroid metabolite assays are mostly seen in the wildlife and ecology industries, it has made an appearance within the animal agriculture industry in the last decade.

Fecal Assay Use

While fecal assays can be performed in many species, the actual process to extract the steroid metabolites can be complicated. It is important to select the proper extraction procedure for the species of choice. Part of this process involves choosing the correct solvent. The most common procedure is to centrifuge feces suspended in an alcohol and water mixture. When dealing with mammalian species most studies use methanol, while avian studies tend to use ethanol suspensions (Palme et al. 2013). Recovery of the steroid metabolites is dependent on the polarity of the mixture and the concentration of the

solvent. In cases of low concentrations of fecal steroids, it can be useful to combine two extraction steps to increase recovery (Palme et al. 2013). This allows for measurement of small amounts of cortisol. Both wet and dry samples can be used for analysis and there is no evidence that one is better than the other. Wet samples are preferred by many researchers due to the drying process being time consuming. Dry samples are preferred when samples are small and dry or when undigested materials need to be removed (Palme et al. 2013). When using wet samples, drying must take place. This should be performed at either very high or very low temperatures so that bacterial enzymes do not metabolize the steroids further. As a general rule, it is suggested to keep the extraction method as simple as possible unless the samples need more sophisticated procedures. Choosing a procedure that is more complicated than necessary introduces more variation when running an assay.

Managing Poultry Stress

As public interest in animal welfare has increased, it has become more important for producers to directly address welfare-related stressors. The National Chicken Council (NCC) published the “Animal Welfare Guidelines and Audit Checklist" for any facility involved in breeding or raising chickens. Although the NCC was originally founded to increase consumer demand in the 1950s (National Chicken Council 2022), it quickly expanded into all aspects of the poultry industry. Now, it helps to advocate for the broiler industry and influences policies and legislation that affects the poultry industry. It lists policies in a variety of poultry topics including animal welfare, food safety, nutrition, and worker safety. The NCC welfare guidelines state that an animal in good welfare standing is considered to be “healthy, comfortable, well nourished, safe, able to express innate

behavior, and not suffering from unpleasant states." (National Chicken Council: Animal Welfare Guidelines and Audit Checklist 2022). There are many different guidelines for hatching, raising, and breeding broilers. Not only is the importance of the physiological well-being of the chickens emphasized, mental and emotional state are also recognized as being important. Additionally, the guidelines stress that poultry should always be cared for in ways to minimize stress and pain. In order to ensure this, personnel are required to be trained annually in different handling practices. This training emphasizes the specific procedures used in each employee's job, but also emphasizes that abuse or neglect is not tolerated (National Chicken Council: Animal Welfare Guidelines and Audit Checklist 2022). Even if handlers do follow these guidelines exactly, handling procedures can be stressful for the chicken. To help minimize stress broilers experience in their lives, producers have constantly improved their facilities and handling practices based on current research available. Key factors in facilities to limit environmental stress include good ventilation and temperature control (Scanes and Christensen 2019). Without these key factors stress can increase leading to a rapid decrease in food and water intake and growth of the chickens.

Based on the literature review, the objectives of this study were to evaluate if CORT concentrations could be measured in a small flock of chickens, using both blood and fecal samples from chickens housed in a caged environment where temperature and humidity were not controlled, and compare the results to previously published studies. Additionally, normalization of CORT concentrations following the end of a stress event were explored. It was hypothesized that CORT concentrations in blood would increase

more quickly than in feces in response to a stress event and would normalize more quickly afterward.

CHAPTER 3. MATERIALS AND METHODS

This study was approved by the Murray State University Institutional Animal Care and Use Committee (protocol #2023-021; Appendix A). A detailed list of materials used are included in Appendix B. Documentation of events for the study started 40 days before treatments were initiated (Table 1), to include monitoring growth of young chicks as well as responses during and after treatment.

Chick Housing

Twenty-nine male Ross 308 three-day old broiler chicks were delivered from Moyer's Chicks, Inc. in Quakertown, Pennsylvania. Chicks were brooded in a single stock tank (1.4m x 0.5m) (Tuff Stuff Products, Terra Bella, CA) bedded with Country Lane Premium 100% Pine Shavings (Orscheln Farm and Home LLC, Moberly, MO 65270) in the animal science laboratory on the Murray State University campus. Chicks were stocked at a density of 0.01 m^2 per chick during the brooding period. On their last day in the brooder the stocking rate on a weight basis was 46 kg per m^2. A heat lamp (250w, 120v), placed approximately 24 inches from the floor, was used at all times while in the brooder. Ambient temperature in the laboratory was subject to changes due to sunlight from south facing windows and changes in the HVAC system for the building. The HVAC system for this building is not room specific. lights were not specifically controlled but were usually on during working hours and off otherwise.

Table 1. Timeline for chicken management and data collection events in project assessing serum and fecal corticosterone as an indicator of stress in chickens.

Date	Event	Day of Study[1]	Age of Chick (d)
Sept 28	Chick arrival, placement in brooder	-40	3
Oct 10	First colored bands placed[2]	-28	15
Oct 11	Began collecting group weights daily	-27	16
Oct 20	Changed colored bands	-18	25
	Began collecting individual weights daily		
Oct 24	Moved to study location	-14	29
	Placed 3 chicks/cage, by group		
Oct 30	(YEL) Blood collection for baseline	-8	35
	Fecal collection, all groups		
Nov 7	First CORT treatment administered	0	43
	Feed change		
	(BLU) Blood collection, 6 h		
	Fecal collection, 6 h, all groups		
	(PUR) Blood collection, 12 h		
	Fecal collection, 12 h, all groups		
Nov 8	(YEL) Blood collection, 24 h	1	44
	Fecal collection, all groups		
	Second CORT treatment administered		
Nov 9	(BLU) Blood collection, 48 h	2	45
	Fecal collection, all groups		
	Removed CORT treatment from all TXT groups		
Nov 10	(PUR) Blood collection, 72 h	3	46
	Fecal collection, all groups		
Nov 11	(YEL) Blood collection, 96 h	4	47
	Fecal collection, all groups		

[1]Day 0 in this column marks the start of treatment with the induction of stress in treatment chickens.
[2]Colored bands (YEL=yellow, BLU=blue, and PUR=purple) were used to track which chickens in each group were used for blood collection at each time point.
[3]Delay in study start date due to delay in arrival of corticosterone.

On d-14 of the study, chicks were moved to an outdoor shed located at the university's Hutson Farm and placed into cages (0.45m x 0.61m x 0.97m) by group. There were 3 chickens per cage, resulting in a density of 0.19 m^2 per chicken. At the time of treatment (d0), the stocking density on a weight basis was 11 kg per m^2. The photoperiod was controlled to provide 18 hours light and 6 hours dark. The lights were turned on at 5 am and turned off at 11 pm. Ambient temperature was recorded twice daily starting when the chickens were moved into the cages. Minimal heating was available in the facility.

Group Selection and Identification

On d-28, colored leg bands (Chicken Hill, Horseshoe Bend, ID) were placed on the chick's legs to separate them into 9 groups of three chicks each, and 1 group of 2 chicks. Chicks were randomly allocated to groups. Chick weights were taken daily as a group until d-18. On d-18, the initial, colored bands were replaced with larger, numbered bands (Patelai) colored either yellow, blue, or purple. There were 2 groups of 10 chicks and 1 group of 9. Groups were separated by number (Table 2), and the first 8 chicks in each group were designated as study animals. Individual weights were taken once bands were switched, and both individual and calculated group weights were recorded through the end of the study. Groups 2, 4, 6, and 8 were designated control (CON), while groups 1, 3, 5, and 7 were designated treatment (TRT). Groups 9 and 10 were designated as alternate animals to be used as replacement chickens, and so were managed the same as other groups until the treatment was administered.

On d-5, chick BLU-025 was isolated due to health concerns and was ultimately euthanized. Chick YEL-010, which was closest in weight to BLU-025, was used as a

replacement. All weights for that group for the remainder of the study were recorded using YEL-010 in place of BLU-025.

Table 2. Chicken band color, number, and cage designations.

Cage Number	Band Color	Band Number	Band Color	Band Number	Band Color	Band Number
1	YEL	001	BLU	021	PUR	041
2	YEL	002	BLU	022	PUR	042
3	YEL	003	BLU	023	PUR	043
4	YEL	004	BLU	024	PUR	044
5	YEL	005	BLU	025	PUR	045
6	YEL	006	BLU	026	PUR	046
7	YEL	007	BLU	027	PUR	047
8	YEL	008	BLU	028	PUR	048
9	YEL	009	BLU	029	PUR	049
10	YEL	010	BLU	030		

Note. Chickens in cages 9 and 10 (YEL-009, YEL-010, BLU-029, BLU-030, and PUR-049) were designated as replacement animals.

Chicken Diet- Brooding

Chicks were fed commercially formulated 18% Chick Starter & Grower Medicated Crumbles by Country Lane (Orscheln Farm and Home LLC, Moberly, MO 65270). Chicks were fed approximately 450 g twice daily. Towards the end of the brooding period chicks did occasionally run out of food and water due to spillage or need for more food or water.

Chicken Diet- Barn Setting

On d-0, feed was changed to Purina Start & Grow 18% Protein Medicated Crumbles (Purina Animal Nutrition LLC, Arden Hills MN 55126). The change was necessitated by the local vendor discontinuing the original feed. Chicks were given ad libitum access to food and water during the entire study.

Corticosterone Treatment

Treatment consisted of supplying water mixed with a corticosterone/ethanol mixture as developed by Weimer (2018). The mixture consisted of 160 mg of corticosterone powder (product number C2505, Sigma-Aldrich, St. Louis, MO 63103) mixed with 80 mL of 190 proof ethyl alcohol (PHARMCO-AAPER, Shelbyville, KY 40065). This mixture was added to 8 L of tap water. Corticosterone water (CORT) was mixed the night before treatments were given to chicks. The treatment was stored in two Igloo 2-gallon sport stackable beverage coolers (Igloo, Katy, TX 77494) until it was given to each TRT group. Treatment was administered one hour after the start of the photoperiod (6am). Two liters of CORT were given to each TRT group on d0 and again on d1. Unconsumed CORT was disposed of before adding new CORT on d1. On d2, unconsumed CORT water was disposed of, and normal tap water provided to TRT groups. The CON groups were given ad libitum access to normal tap water for the duration of the study.

Blood and Fecal Collection

Feces and blood were collected to determine corticosterone concentrations. On d -8, baseline samples were collected. Multipurpose Absorbent Pads (Sam's West Inc, Bentonville, AR) were placed below each group's cage to collect feces one and a half hours before each blood draw. Immediately before blood collection, a fecal sample was collected from the pad for each, placed into a one-quart resealable bag, and stored in a -20°C freezer. Fecal sample collection weights varied between each collection. At the baseline collection an average of 86 g was collected from each cage. At 6 hours an average of 79 g was collected from each cage. At 12 hours an average of 57 g was

collected from each cage. At 24 hours an average of 35 g was collected from each cage. At 48 hours an average of 68 g was collected from each cage. At 72 hours an average of 41 g was collected from each cage. At 96 hours an average of 36 g was collected from each cage.

Blood was collected from the brachial or medial metatarsal vein. Collections were made from alternate sides, right and left. On d-8, blood was collected from the left brachial vein of YEL chicks to determine baseline corticosterone concentrations. Prior to drawing blood Lidocaine and Prilocaine Cream, 2.5%/ 2.5% (Fougera, Melville, NY 11747) was placed on the area to help with pain management during the blood draw. Blood was collected from the left brachial vein with a 25Gx ⅝" Hypodermic Needle (Exelint, Redondo Beach, CA 90278) and either a 3 mL disposable syringe without needle (Exelint, Redondo Beach, CA 90278) or 1 mL disposable syringe without needle (Exelint, Redondo Beach, CA 90278). Blood was then placed into a 3 mL Monoject blood collection tube with no additive (Coviden, Mansfield MA 02048). Blood was collected by a university veterinary technology faculty member.

On d0, at 6:00 am, CORT was administered to TRT animals. Fecal and blood sampling occurred at 6, 12, 24, 48, 72, and 96 h post CORT administration (Table 3). Data collection was conducted as described previously, except that a 23Gx1" disposable needle (Exelint, Redondo Beach, CA 90278) was used due to an increase in chicken size.

At 12 h post CORT administration, the amount of blood collected from the left brachial vein was insufficient for analysis for 3 animals, so additional blood was drawn from the right medial metatarsal vein.

Table 3. Treatment and sample collection events

Day	Time	Event	Group
d-8	4:30 am	Absorbent pads placed	All study groups
	6:00 am	Fecal sample collection	All study groups
		Blood collection	YEL group
d0	6:00 am	CORT administration	All TRT groups
	10:30 am	Absorbent pads placed	All study groups
	12:00 pm	Fecal sample collection	All study groups
		Blood collection	BLU group
	4:30 pm	Absorbent pads placed	All study groups
	6:00 pm	Fecal sample collection	All study groups
		Blood collection	PUR group
d1	4:30 am	Absorbent pads placed	All study groups
	6:00 am	CORT administration	All TRT groups
		Fecal sample collection	All study groups
		Blood collection	YEL group
d2	4:30 am	Absorbent pads placed	All study groups
	6:00 am	CORT water replaced with tap water	All TRT groups
		Fecal sample collection	All study groups
		Blood collection	BLU group
d3	4:30 am	Absorbent pads placed	All study groups
	6:00 am	Fecal sample collection	All study groups
		Blood collection	PUR group
d4	4:30 am	Absorbent pads placed	All study groups
	6:00 am	Fecal sample collection	All study groups
		Blood collection	YEL group

At 24 h post CORT administration, blood was initially collected from the right brachial vein. In 5 animals, insufficient blood was collected for analysis, so additional blood was drawn from the right medial metatarsal vein. In 2 of those 5 animals, a third collection was conducted on the left brachial vein.

At 48 h post CORT administration, blood was initially collected from the right brachial vein. In 3 animals, insufficient blood was collected for analysis, so additional blood was drawn from the right medial metatarsal vein. In 1 of those 3 animals, a third collection was conducted on the left brachial vein.

At 72 h post CORT administration, blood was initially collected from the left brachial vein. In 3 animals, insufficient blood was collected for analysis, so additional blood was drawn from the right brachial vein. In 1 of those 3 animals, a third collection was conducted on the right medial metatarsal vein.

At 96 h post CORT administration, blood was initially collected from the right brachial vein. In 2 animals, insufficient blood was collected for analysis, so additional blood was drawn from the right medial metatarsal vein. In 1 of those 2 animals, a third collection was conducted on the left brachial vein.

Blood Handling and Analysis

During blood collection, blood smears were made using frosted microscope slides (Jorgensen Laboratories Inc, Loveland, CO 80538). One drop of blood was placed on a microscope slide near the frosted edge. Another slide was used to create a monolayer with a smooth feathered edge. Each slide was marked with the time period and group number using a Fisher finest fine tip marking pen (Fisher HealthCare, Pittsburgh, PN). Slides were allowed to air dry and then stained using Pivetal Rapid Differential 3- Step Stain Kit (Aspen Veterinary Resources LTD, Liberty, MO 64068). Slides were dipped five times into the fixative solution. Slides were then blotted and then dipped five times into solution 2. Slides were blotted again and dipped into the last solution, a counterstain, five times, blotted, and rinsed under tap water until the water ran clear. The pH of the tap water was 7.0. Once the slides were stained, they were placed at an angle to dry. Once the slides were dried, Heterophil: Lymphocyte ratio was counted by a university veterinary technology faculty member. Slides were viewed at 1,000x multiplication using the 100x oil immersion lens. Slides were evaluated twice, by the same person, to verify counts.

Blood samples were allowed to clot for a minimum of 30 min before serum was collected. To prepare each sample for centrifugation, the blood clot was rimmed with a wooden applicator stick. Tubes were placed in a C5 centrifuge (LW Scientific, Lawrenceville, GA 30046) at 4000 rpm for 10 min. Serum was pipetted using 3 ml plastic transfer pipettes (Next Generation, York, PN 17406-9200) and serum placed into 1.5 ml microcentrifuge tubes with a cap (Simport Scientific, Beloeil, QC, Canada). If more serum was needed, tubes with blood clots were centrifuged again at 4000 rpm for 10 min. Additional serum was pipetted and placed into the corresponding microcentrifuge tube. Serum samples were then frozen at -20°C until analyzed.

Blood serum samples were taken to Breathitt Veterinary Center (Hopkinsville, KY) for analysis. Samples were run according to Arbor Assays' DetectX Corticosterone Enzyme Immunoassay Kit© manual (2009). Samples were prepared by adding five microliters of the dissociation reagent was added to a one milliliter test tube. Five microliters of serum were added to this tube and then vortexed and allowed to incubate at room temperature for five or more minutes. This mixture was then diluted with 490 microliters of assay buffer. Assays were run using a 1x8 well strip plate. Fifty microliters of either prepared samples or standards were pipetted into each well. Seventy-five microliters of assay buffer were pipetted into the non-specific binding (NSB) well. Fifty microliters of assay buffer were pipetted into the maximum binding (B0) well. Twenty-five microliters of the DetectX corticosterone conjugate were added to each well. Twenty-five microliters of the DetectX corticosterone antibody were added to each well except for the NSB well. The plate was covered with a plate sealer and shaken at room temperature for one hour. The plate was aspirated and washed four times with 300

microliters of wash buffer then dried on a clean towel. One hundred microliters of the TMB substrate were added to each well. The plate was then incubated for thirty minutes at room temperature. Fifty microliters of the stop solution were added to each well. The optical density for each well was generated using a BioTek ELx808 plate reader and VMRD ELISAWare 1.5.5 software. Optical density values were then copied into MyAssays to create a standard curve. Concentration, concentration average, coefficient variation, and standard deviation were also calculated using MyAssays.

Fecal Handling and Analysis

The following analysis is based on extraction methods outlined in Alm (2019). It has been modified in that 0.4 g dried fecal material was used instead of 0.2 g. Frozen fecal samples were allowed to thaw on the bench top until they could be easily broken apart. Samples were broken into 10-to-12-gram pieces and weighed in aluminum drying pans (VWR, Randor, PA 19087). Pans were labeled by group number using permanent markers. Samples were then placed into a convection oven (Avantco Equipment, Meridian, ID, 83646) and dried for approximately 21 h. Samples were removed, weighed again to assess moisture loss, and placed into new quart sized resealable freezer bags labeled with the sample time, group number, and date dried. Samples were again frozen until being processed further.

Frozen, dried fecal samples were allowed to thaw on the bench top for approximately 30 minutes. Each sample was crushed into smaller pieces. Approximately 1.4 g of each sample was measured and weighed in aluminum drying pans. Pans were labeled by group number and time period and placed into a convection oven. Samples were dried a second time for approximately 16 h. Samples were removed and weighed

again to assess moisture loss. Using weighing paper, 0.4 g of each sample was weighed and placed into glass test tubes labeled with the group number and time period. Using a pipette, 2 mL of methyl alcohol was added to each test tube. Test tubes were covered with parafilm (Pechiney Plastic Packaging, Menasha, WI). Each test tube was vortexed for 10s and then shaken for 30 min. Samples were then centrifuged for 30 min at 4350 rpm. The supernatant was poured off into a plastic test tube labeled with the sample number, group number, and time drawn and frozen at -20°C until analysis.

Extracted fecal samples were taken to Breathitt Veterinary Center (Hopkinsville, KY) for analysis. Samples were run according to Arbor Assays' DetectX Corticosterone Enzyme Immunoassay Kit© manual (2009). Samples were prepared by adding 480 microliters of diluted assay buffer was added to twenty microliters of the fecal sample to create a 4% dilution. These samples were then analyzed the same as the serum samples in the previous section.

Statistical Analysis

Data were analyzed using the Mixed procedure of SAS 9.4 (SAS Institute Inc., Cary, NC) with fixed effects of treatment and hour. Each cage was considered a single experimental unit. Significance was declared at $P < 0.05$. Treatment and hour interactions were evaluated where appropriate. Time by treatment LSMean comparison contrasts were analyzed post hoc to compare Control and CORT group measures within each sampling time point. Results are reported as LSMean contrasts within each time point. Charts were prepared using Microsoft Excel version 2302.

CHAPTER 4. RESULTS AND DISCUSSION

One animal, chicken BLU-025, was removed from the study on d-5 due to health concerns and was ultimately euthanized. It was replaced by a healthy chicken, chicken YEL-010, from the reserve group. All other animals in the study remained in good health. Interactions between treatment and hour were not all statistically significant ($P<0.05$). Values that were significant include body weight at 45, 46, and 47 days of age, H:L Ratio at 6, 12, and 24 hours, serum corticosterone at 6 and 12 hours, and fecal corticosterone at 6, 12, 24, and 48 hours.

Performance of Chickens Compared to Industry Standard

Control chickens were compared to the Ross 308 male performance objectives (Figure 1) to observe how chickens grew throughout the study. Overall, performance was lower than expected but trended in the same direction as the standard for the breed. Broilers did not consistently gain weight as expected, either daily or relative to average daily gain (ADG) (Figure 2; Figure 3). Average daily gains were measured as a rolling six-day average. When observing daily weights, performance increased similarly to the Ross performance objectives until 26 days of age there was a decrease in weight average from 657.5 to 537.5 g. After that chicken performance increased until another small decrease at 32 days of age where the weight decreased from 870.8g to 858.3 g. Performance continued to increase at the same rate until slowing from 40 days of age to 41 days of age where it continued to increase. When observing ADG, control chickens varied from Ross performance objective ADG (Figure 2; Figure 3). From 20 days of age to 26 days of age ADG decreased from 50 g per d to -6 g per d. From 26 days of age ADG increased drastically to surpass the ADG standard from the Ross performance

objectives. Peak ADG occurred at 144 g at 36 days of age. At that time the Ross standard ADG was 110 g. ADG of control chickens then decreased until 42 days of age where ADG was 85 g per d. Control chickens ADG rapidly increased once again until peaking at 161 g per day at 46 days of age.

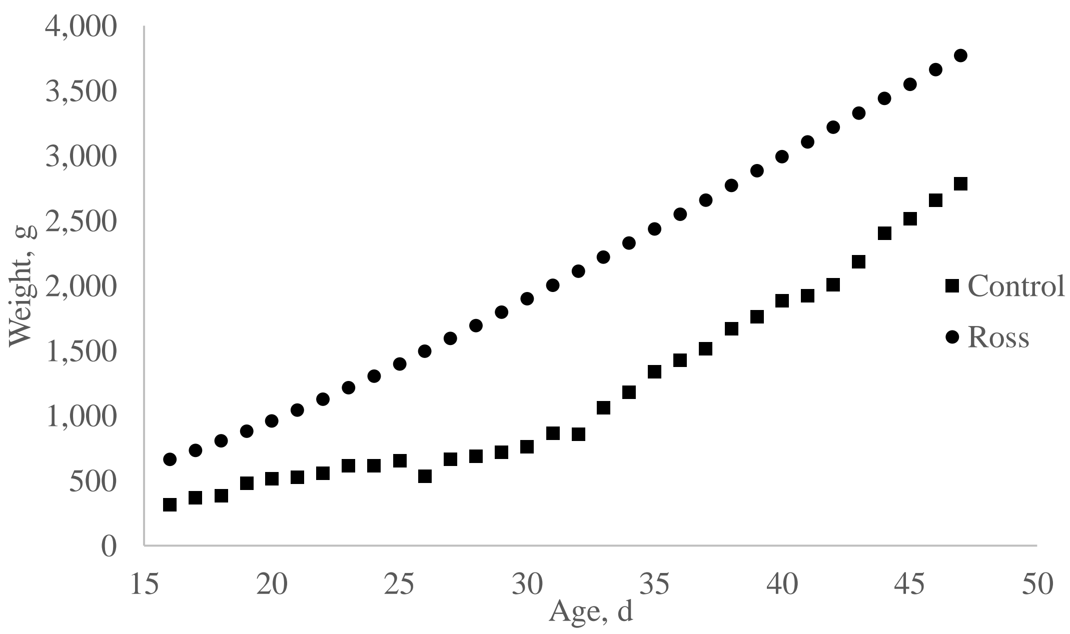

Figure 1. Average daily weight, g, of control broiler groups compared to Ross 308 Male Performance Objectives over a 32-day period.

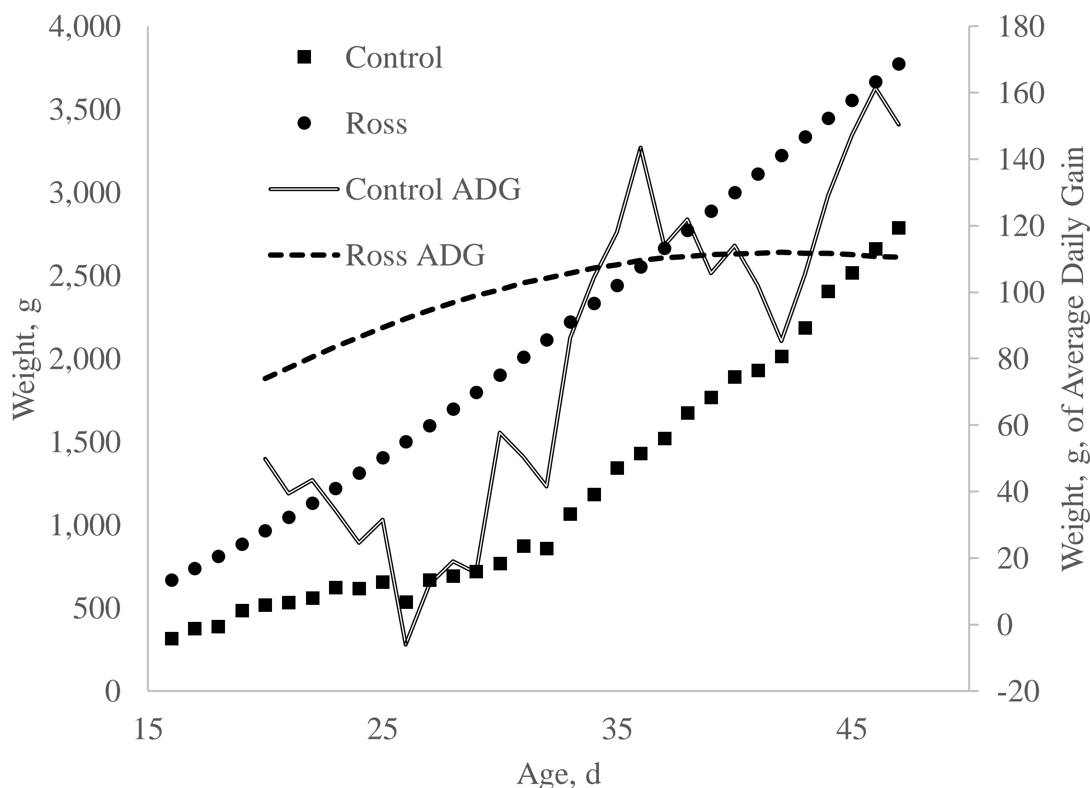

Figure 2. Comparison of rate of weight gained and average daily gain between Ross 308 Male Performance Objectives to control broilers over a 32-day period.

Differences between Ross 308 male performance standards and control chickens were largely due to environmental and management constraints of the set up. During brooding, chicken performance was constrained by inconsistency of feed and water supply and lack of adequate temperature control. Chicks ran out of food during brooding due to spillage or inability to reach feed due to shavings in their feeder. In one instance, the water source had been knocked over during the night and chicks were left without water until the morning. Even a couple of hours of not having access to feed or water can stunt growth significantly for that day. Slower weight gain in study chickens could also be due to higher stocking densities during the brooding period. Chicks were stocked at 44 kg per m^2 during brooding. The recommendation from the National Chicken Council

(2022) for chicks below 2.04 kg is 32 kg per m^2. Between 2.04 kg and 2.49 kg the recommendation is for chicks to be stocked at 37 kg per m^2. Normal weight gain is very dependent on the type of system the chickens are raised in. Higher stocking density can negatively affect growth of broilers especially in the brooding stages. Najafi et al. (2015) suggested that a stocking density of 0.063 m^2 per chicken, or 16 chickens per m^2, would have no negative impact on weight of cages birds. In this study, chickens at the brooding stage did not have as much area as they needed causing their AGD to decrease until being moved into a larger environment allowing them to increase in AGD above performance standards for the breed. Without a lower stocking density, chickens cannot grow to their full capabilities. Once moved to cages, there were less constraints on chicken performance. At that time, chickens were stocked at 0.19 m^2 per chicken, or 11 kg per m^2. The recommendation for chickens of this weight is 37 kg per m^2 (National Chicken Council: Animal Welfare Guidelines and Audit Checklist 2022). Chickens had ample space to grow in cages. Without the constraint of stocking density, ADG increased to higher than production standards for this breed. Data from this study, along with previous studies to validate impacts of stocking density, has shown that it plays a significant role in poultry production. Chickens that do not have the area necessary for growth will not produce as much, leading to lower profits for the producer.

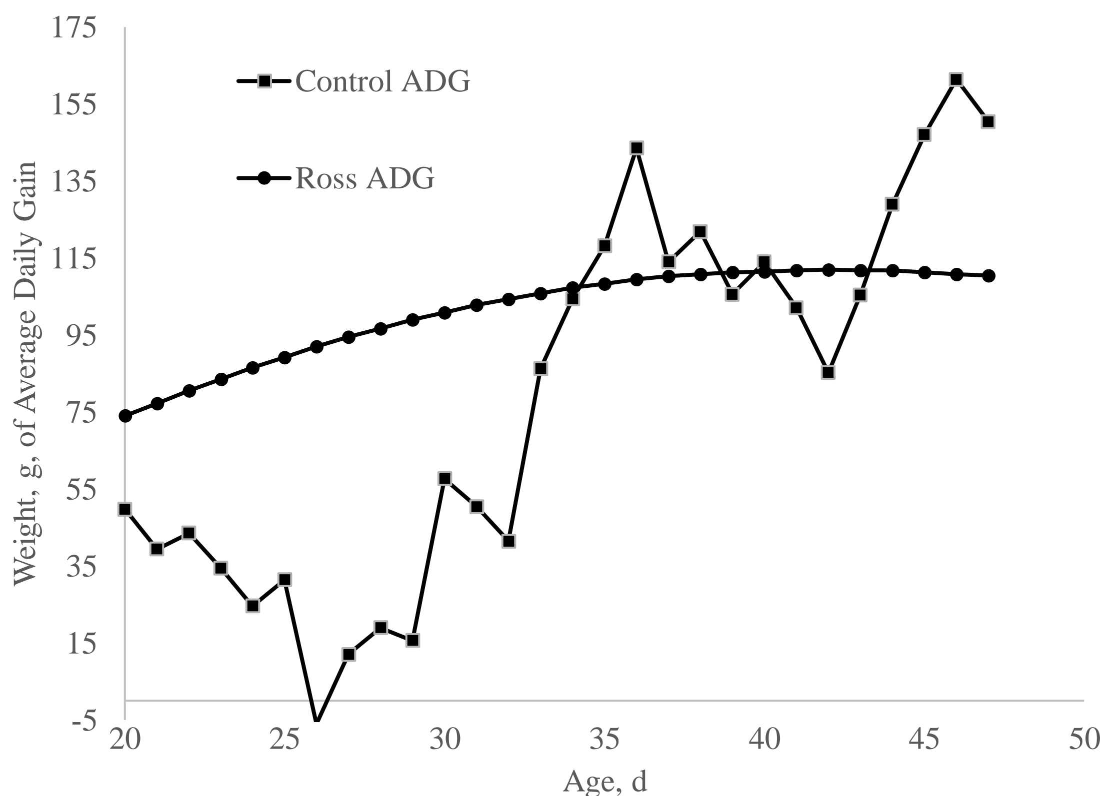

Figure 3. Average daily gain of control chickens compared to Ross 308 Male Performance Objectives over a 28-day period.

From the first day weight was taken ADG consistently decreased until 30 days of age where weights increased. At 29 days of age chicks were moved out of the brooder and into a larger cage in the barn. Once moved, AGD and daily weights increased quickly. Both control and treatment groups surpassed Ross 308 performance objectives in ADG until there was a drop in temperature (Figure 4). Weight of chickens mirrored temperature shifts of the barn. Figure 4 shows the comparison between control chicken's ADG and the average ambient temperature in the barn. While the temperature did not have a lot of drastic changes, when it did shift so did weights. When first moving the chickens to the barn there was an initial drop in temperature from 21°C at 30 days of age

to 13°C at 31 days of age. ADG of control chickens dropped from 58 g to 50 g during this time. The most drastic change of weights when comparing these two measurements is when temperature dropped from 19°C at 41 days of age to 15°C at 42 days of age. This caused a decrease in ADG by 17 g. Once the temperature increased to 24°C at 44 days of age ADG increased once again to peak at 161 g at 46 days of age.

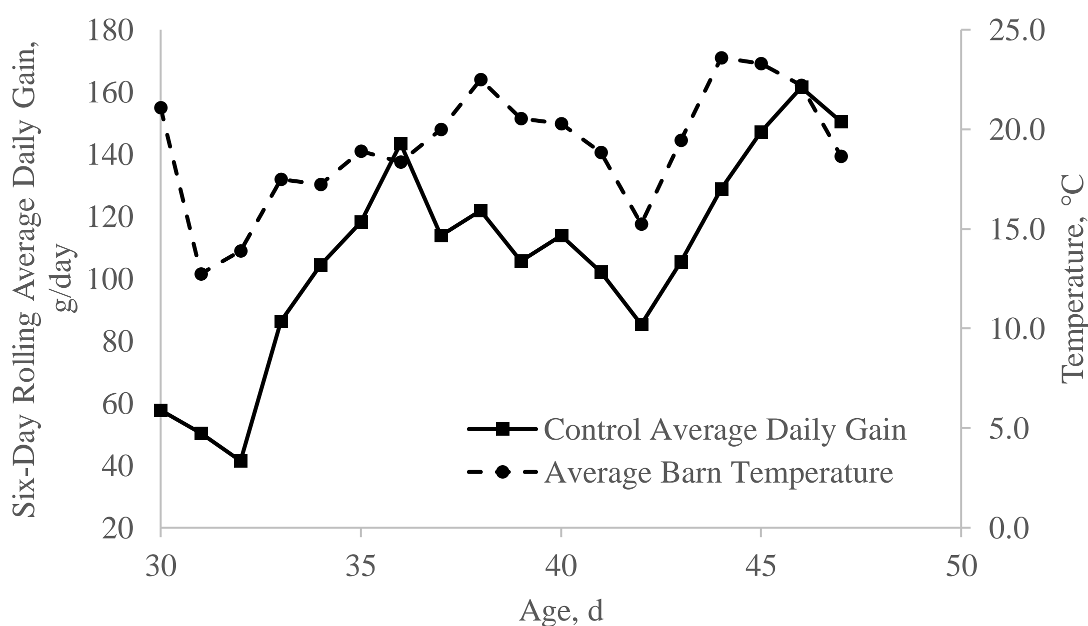

Figure 4. Average daily gain of control chickens compared to the average ambient temperature, °C, in the barn over an 18-day period.

Aside from higher stocking densities, temperature can have a significant impact on body weight. Chickens can be extremely sensitive to changes in temperature which often show in weight fluctuations. It can significantly lower body weight by up to 32.6% when chickens are exposed to excess heat (Lara and Rostagno 2013). Though the temperature was inconsistent at brooding, it was not tracked throughout the brooding period. Temperature influenced weight and ADG of chickens. During the post-brooding

growing period when temperature decreased so did weight and ADG. When temperature increased so did weight and ADG. Chickens are normally raised at temperatures starting at 32°C at one day old and decreased to 21°C by 27 days (Aviagen 2022). Throughout this study chickens were exposed to temperatures that were too low for optimal weight gain. When exposed to colder temperatures chicken performance decreased. This is due to energy being used by the chicken being used to keep itself warm when temperatures drop below 20°C (Osti et al. 2017). At the point in which ADG peaked (161 g at 46 days of age) chickens were within the correct temperature range for optimal performance. At both high and low temperature chickens cannot be expected to grow at a normal rate and would not produce as much meat or eggs as chickens with adequate temperature control.

Performance Effect of Stress on Chickens

Trends in weight between control and stressed chickens were similar, however the chickens in this study underperformed throughout the entire study. There was a small decrease in weight at 26 days of age from 637.5 g to 537.5 g. After this initial decrease, weight continued to increase (Figure 5). Weight increased slowly until decreasing at 32 days of age from 816.67 g to 799.17 g. This could have been due to moving chickens from the brooding area to the barn. After this decrease, groups continued to increase in weight until 43 days of age. At this point, control chickens continued to gain weight normally, but stressed chickens gained weight at a slower rate (Table 4; Figure 5). At 43 days of age, the first CORT treatment was given to stress the chickens in the treatment group. While this did not affect the average weight at 43 days of age (2105 g) it can explain the slow growth from 43 days of age to 44 days of age. The treatment lasted for 48 hours until it was replaced with normal tap water. At 45 days of age (48-hours after

the treatment) stressed chickens weighed an average of 2236 g (*P*<0.0001), showing that chickens gained only 22.5 g. This slow gain can be due to the stressed chickens having diarrhea starting at12 hours after the CORT treatment. The diarrhea did not resolve but did get better throughout the remainder of the study. On average stressed chickens gained 22.5 g from 44 days of age to 45 days of age, 115 g from 45 days of age to 46 days of age, and 85 g from 46 days of age to 47 days of age (Figure 6). In comparison, control chickens at this time gained 113.3 g, 142.5 g, and 129.16 g between those days. Compared to the Ross performance objectives chickens should have been gaining 111 g per day on those days.

Table 4. Body weight (g) means and standard errors by age for broiler chicks stressed with CORT and unstressed (control).

Age, d	Control	Stressed	*P* Value
42	2014 ± 44	1923 ± 38	0.074
43	2188 ± 51	2105 ± 11	0.103
44	2404 ± 45	2213 ± 147	0.0005
45	2518 ± 67	2236 ± 51	<.0001
46	2660 ± 68	2351 ± 78	<.0001
47	2789 ± 64	2436 ± 79	<.0001

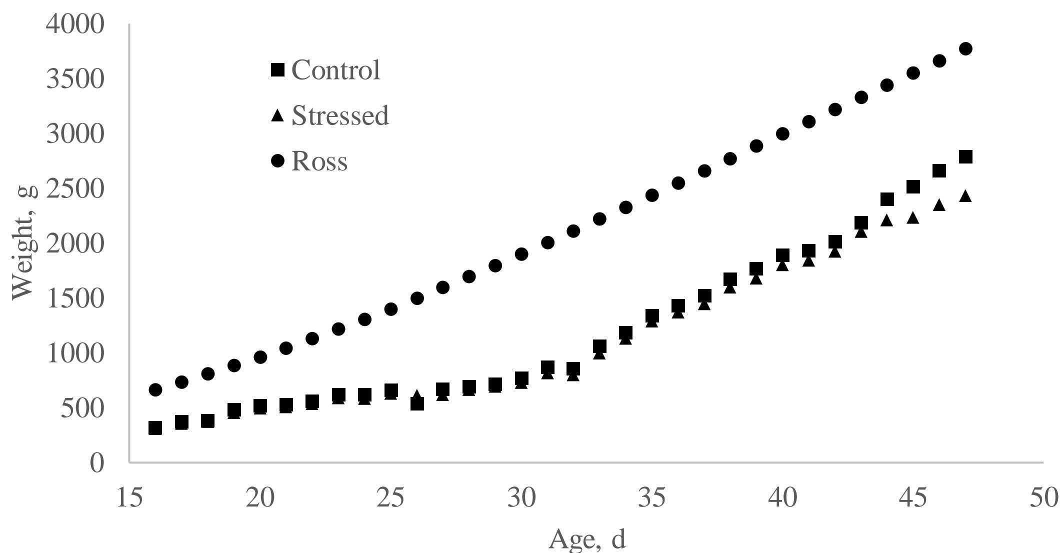

Figure 5. Effect of corticosterone (CORT) treatment in water on daily weights of stressed chickens, given CORT water, compared to control chickens, given normal water, and Ross 308 Male Performance Objectives over a 32-day period. Data shown are average weights, g.

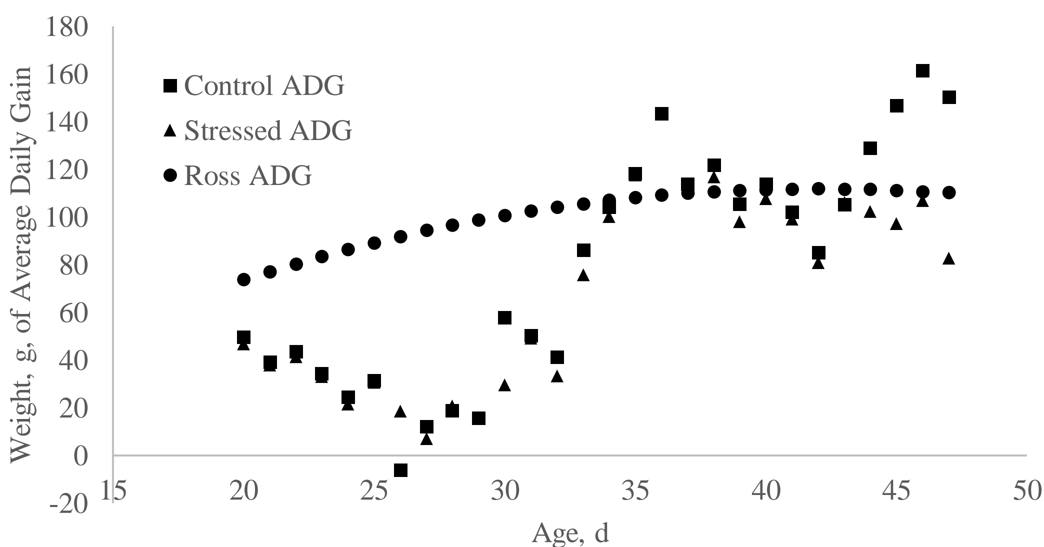

Figure 6. Effect of corticosterone (CORT) treatment on average daily gain of stressed chickens, given CORT water, to control chickens, given normal water, and Ross 308 Male Performance Objectives over a 31-day period.

The relationships between body weight and high levels of stress were evaluated. In this study, stress had a significant effect on body weight (Table 5, Figure 5). While performance was low throughout the study, body weight and ADG were affected once CORT treatment was administered. It is common to see body weights decrease as chickens become stressed (Weimer et al. 2018, Lokhande et al. 2009). Body weight decreased slightly and then continued to increase at a slow rate during the stress treatment. Some studies reported that growth ceased during high levels of stress (Puvadolpirod and Thaxton 2000).

Both stressed and control chickens responded similarly to temperature fluctuations (Figure 7). The only noticeable difference is 44 and 45 days of age in stressed chickens when ADG dropped from 106 g at 43 days of age to 103g at 44 days of age and 97 g at 45 days of age instead of increasing with the temperature increase. There was also a drastic decrease at 47 days of age from 107 g to 83 g. This decrease does seem to mirror a temperature drop from 22°C to 19°C.

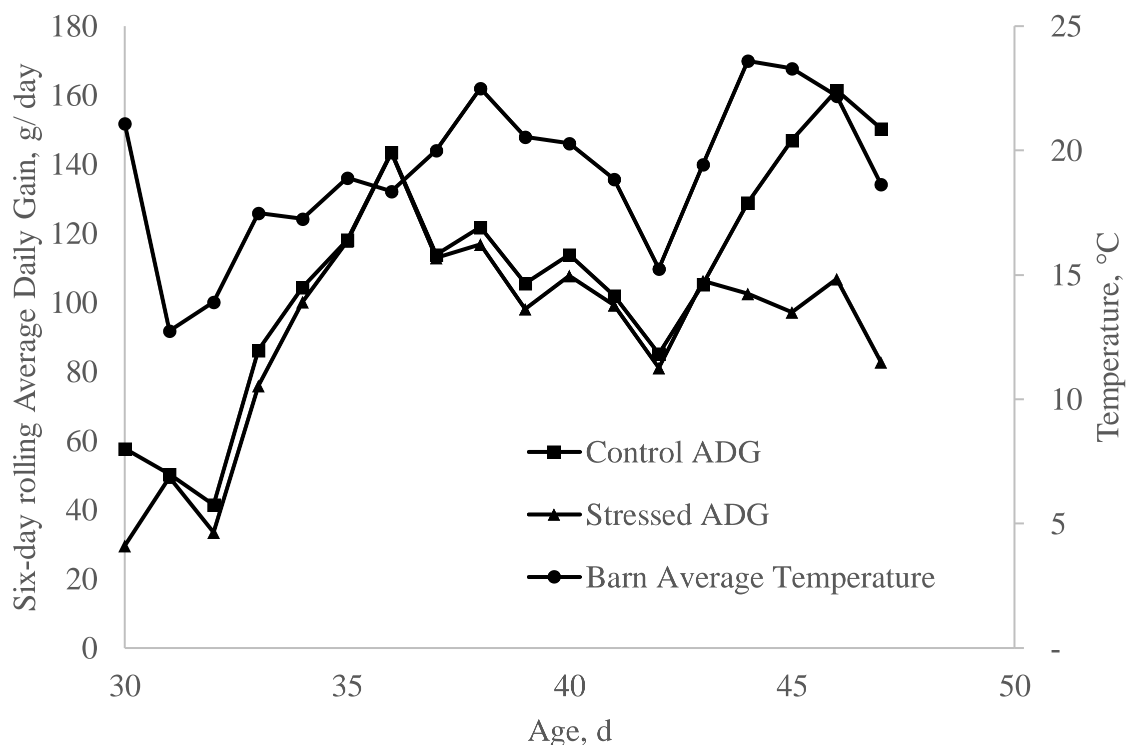

Figure 7. Effect of corticosterone (CORT) treatment on average daily gain (ADG) of stressed chickens, given CORT water, and control chickens, given normal water, compared to the average ambient barn temperature, °C, over an 18-day period.

Effect of Stress on Serum and H:L Ratio

There was a significant increase in CORT concentrations at 6 hours after onset of CORT treatment (Table 5, Figure 8, Figure 9). Serum CORT concentrations were elevated at 6 (53.38 ng/mL, $P<0.0001$) and 12 hours (29.82 ng/mL, $P<0.0001$) after the addition of CORT treatment to the drinking water. Serum CORT concentrations returned to normal concentrations by 24 hours (5.29 ng/mL, $P=0.77$) after onset of treatment and remained low until the end of the study. Throughout the course of CORT treatment, the average CORT concentrations of the chickens in the stressed group were 53.38 ng/mL at 6 hours, 29.82 ng/mL at 12 hours, 5.29 ng/mL at 24 hours, and 5.38 ng/mL at 48 hours

(Table 5). At these times the control chickens averaged CORT concentrations were at 3.37 ng/mL, 3.84 ng/mL, 3.85 ng/mL, and 5.95 ng/mL respectively. At 72 hours after CORT treatment all chickens were similar in CORT concentrations with control chickens averaging 7.93 ng/mL while stressed chickens averaged 7.74 ng/mL (P=0.97). Chickens stayed at similar averages at 96 hours after onset of CORT treatment. Control chickens averaged CORT concentration was 7.48 ng/mL, and the stressed chickens average CORT concentration was 6.34 ng/mL (P=0.803). Over time variability and concentrations lessened as CORT levels normalized and treatment stopped.

Average H:L ratio were consistant with results of serum CORT concentrations (Table 5). H:L ratios at the baseline measurement were considered normal as the control average was 0.51 and stressed was 0.75 (P=0.70). This changed once CORT was administered. At 6 hours after administration stressed H:L ratio increased to 1.98 (P=0.04) while control only increased to 0.64. H:L ratio spiked to 6.73 (P<0.0001) in stressed chickens at 12 hours while control increased once again to 0.8. Both control and stressed groups decreased slightly at 24 hours. Stressed groups had a ratio average of 2.86 (P=0.002) while control groups averaed 0.78. H:L ratio continued to decrease at 48 hours where stressed groups had an average of 1.17 (P=0.40) while control groups averaged 0.63. At 72 hours both stressed and control groups increased to 0.82 and 1.87 (P=0.10) respectively. At 96 hours both groups decreased to almost normal H:L ratio averages with control groups averaging 0.73 and stressed groups averaging 0.84 (P=0.87).

Serum CORT and H:L ratio are frequently used biomarkers used to identify periods of stress in poultry research. As chickens become stressed, serum CORT and H:L ratio increase (Weimer et al 2018, Gross and Siegel 1983, Chikumba et al. 2013). This

was shown throughout the CORT treatment by a peak in H:L ratio at 12 hours after treatment and a peak in serum CORT concentration 6 hours after treatment. Though both are good measures of stress, H:L ratio is typically a better measure of long-term stress while serum CORT concentration is used as a short-term measure of stress (Gross and Siegel 1983). When comparing the results of the present study to data from a previous study CORT concentrations and H:L ratios were different.Weimer et al (2018) reported high serum CORT concentrations from 6 hours after onset of CORT treatment until a decrease after 48 hours. In the present study there was a peak in CORT concentrations at 6 hours, a small decrease at 12 hours, and concentrations returned to normal at 24 hours after onset of CORT treatment. Average CORT concentrations were similar at 6 hours after onset of treament but the return to baseline was faster in the present study. Weimer et al. (2018) reported an average serum CORT concentration of 54.5 ng/mL compared to the present study of serum CORT concentration of 53.38 ng/mL. H:L ratios were higher than in Weimer et al. (2018). They reported that H:L ratios increased throughout the duration of the three day study even after removing CORT treatment. H:L ratio started at 0.44 at the beginning of the study and increased to 4.96 at the end of 72 hours. Findings from this study showed that H:L ratios increased until 12 hours after onset of CORT treatment and then decreased until returning to almost baseline values. High baseline H:L ratios in both control and treatment chickens in this study as compared to Weimer et al. (2018) could be due to stress from low environmental temperatures.

Table 5. Effect of synthetic corticosterone administration on heterophil to lymphocyte (H:L) ratio, serum corticosterone concentration, and fecal corticosterone concentration of broiler chickens.

Sampling hour[1]	H:L Ratio			Serum Corticosterone (ng/mL)			Fecal Corticosterone (ng/g)		
	Control	Stressed	*P* value	Control	Stressed	*P* value	Control	Stressed	*P* value
B	0.51 ± 0.18	0.75 ± 0.34[a]	0.70	2.63 ± 0.37	3.39 ± 1.58[a]	0.86	3.49 ± 1.04	8.51 ± 4.61[a]	0.72
6	0.64 ± 0.31	1.98 ± 0.63[abc]	0.04	3.37 ± 0.87	53.38 ± 22.35[c]	<0.0001	4.54 ± 1.44	47.66 ± 24.8[b]	0.0085
12	0.80 ± 0.57	6.73 ± 2.75[d]	<0.0001	3.84 ± 0.69	29.82 ± 6.17[b]	<0.0001	4.72 ± 1.25	114.56 ± 29.04[d]	<0.0001
24	0.78 ± 0.78	2.86 ± 1.09[bc]	0.002	3.85 ± 0.80	5.29 ± 2.29[a]	0.77	7.86 ± 3.47	74.47 ± 39.64[bc]	<0.0001
48	0.63 ± 0.16	1.17 ± 0.62[a]	0.40	5.95 ± 2.34	5.38 ± 3.05[a]	0.902	4.06 ± 1.33	88.14 ± 39.06[c]	<0.0001
72	0.82 ± 0.34	1.87 ± 0.56[ab]	0.10	7.93 ± 0.74	7.74 ± 0.7[a]	0.97	3.91 ± 1.51	8.15 ± 2.67[a]	0.74
96	0.73 ± 0.25	0.84 ± 0.10[a]	0.87	7.48 ± 0.25	6.34 ± 1.0[a]	0.803	2.76 ± 0.42	2.01 ± 0.87[a]	0.95

[1]Six-week-old broilers administered normal tap water (Control) or a corticosterone water treatment (Stressed) measured at baseline (B), seven days prior to treatment, and 6 treatment time points.

[abcd]LSMean comparisons within a column lacking a common superscript differ at $P < 0.05$

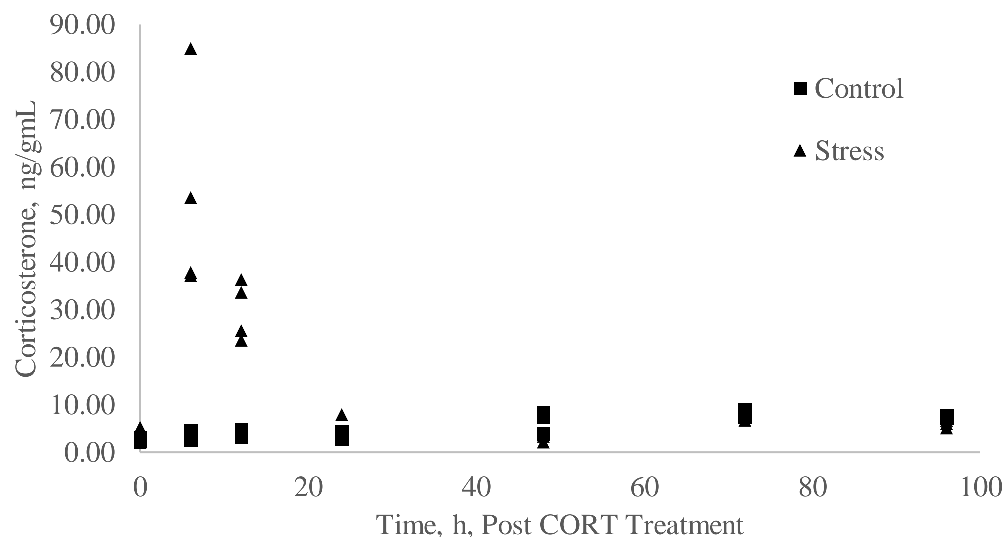

Figure 8. Effect of corticosterone (CORT) treatment on serum corticosterone concentrations in serum samples of stressed chickens, given CORT water, compared to control chickens, given normal water, from -24 h to 96 h with 7 time points. Data shown are average serum CORT concentrations, ng/mL.

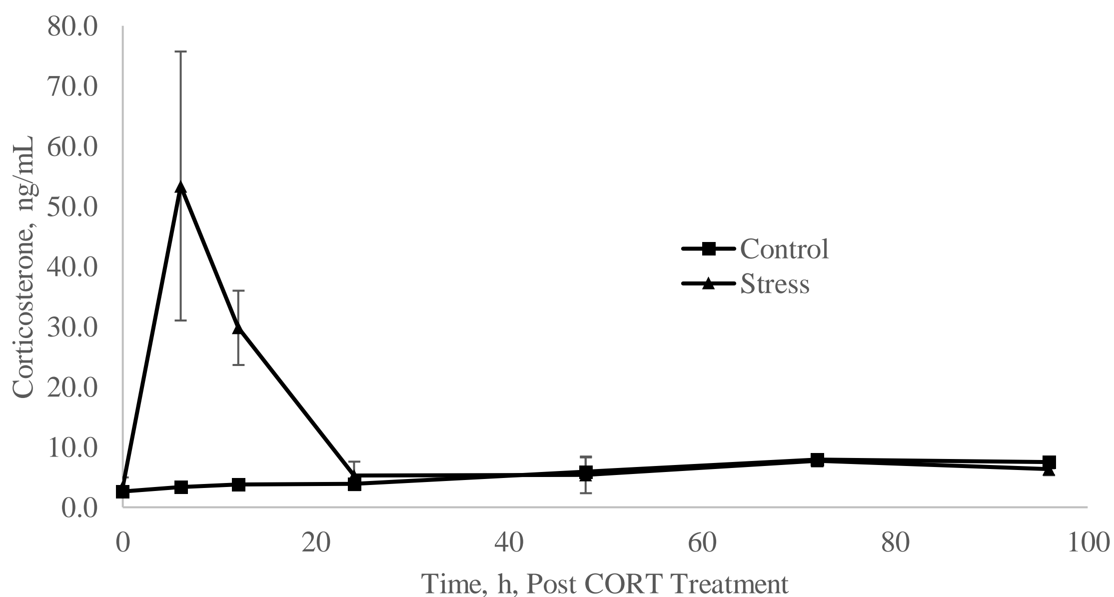

Figure 9. Effect of corticosterone (CORT) treatment in drinking water on serum corticosterone concentrations in stressed chickens, given CORT water, versus control chickens, given normal water, measured from -24 h to 96 h with 7 time points. Data shown are average serum CORT concentrations, ng/mL, ± SEM.

Fecal Corticosterone Concentrations

Fecal corticosterone concentrations varied greatly between control and stressed groups (Table 5). From baseline measurement to 72 hours after CORT treatment averate CORT concentrations for control chickens averaged 3.49 ng/g. There was a large spike in CORT concentration for stressed chickens. At baseline measurement stressed groups averaged 8.51 ng/g of CORT (P=0.72). At this time there was little variation between individual groups. At 6 hours after CORT treatment there was a small increase in average CORT concentration in stressed chickens (47.66 ng/g, P=0.0085) and there started to be a larger variation between individual groups (Figure 10). The largest concentration of CORT in stressed chickens can be seen at 12 hours post CORT treatment (Figure 11). At this time the CORT average for was 114.56 ng/g (P<0.0001). After this spike, there was a decrease to 74.47 ng/g (P<0.0001) average CORT concentration at 24 hours and then another increase of CORT concentration at 48 hours (88.14 ng/g, P<0.0001). At 72 hours post CORT treatment CORT concentration started to return to normal values lowering to an average of 8.15 ng/g (P=0.74) in stressed chickens and ultimately measuring at an average of 2.01 ng/g (P=0.95) at 96 hours. At this time (96 hours) control chicken groups averaged 2.76 ng/g of CORT.

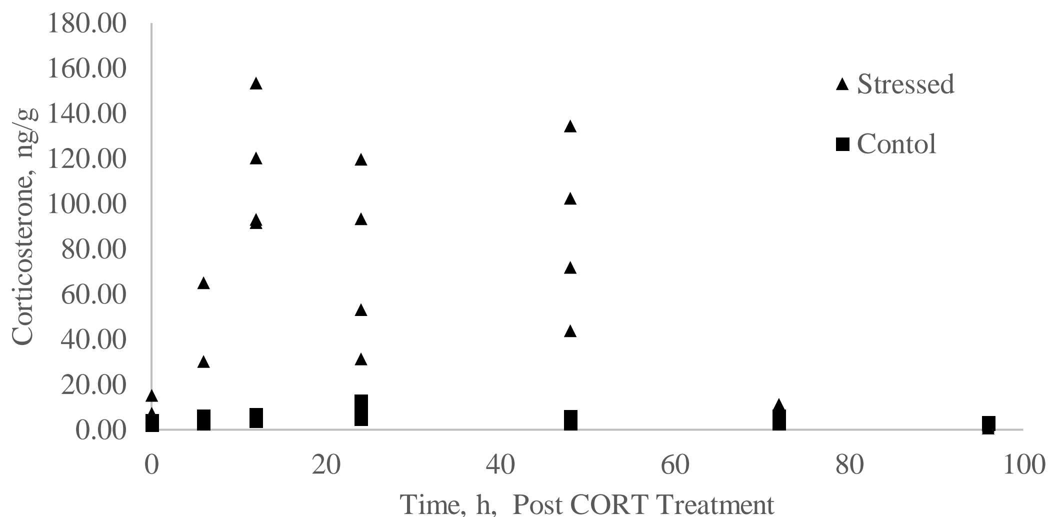

Figure 10. Effect of corticosterone (CORT) treatment on fecal corticosterone concentrations in fecal samples of stressed chickens, given CORT water, compared to control chickens, given normal water, from -24 h to 96 h with 7 time points. Data shown are average fecal CORT concentrations, ng/g.

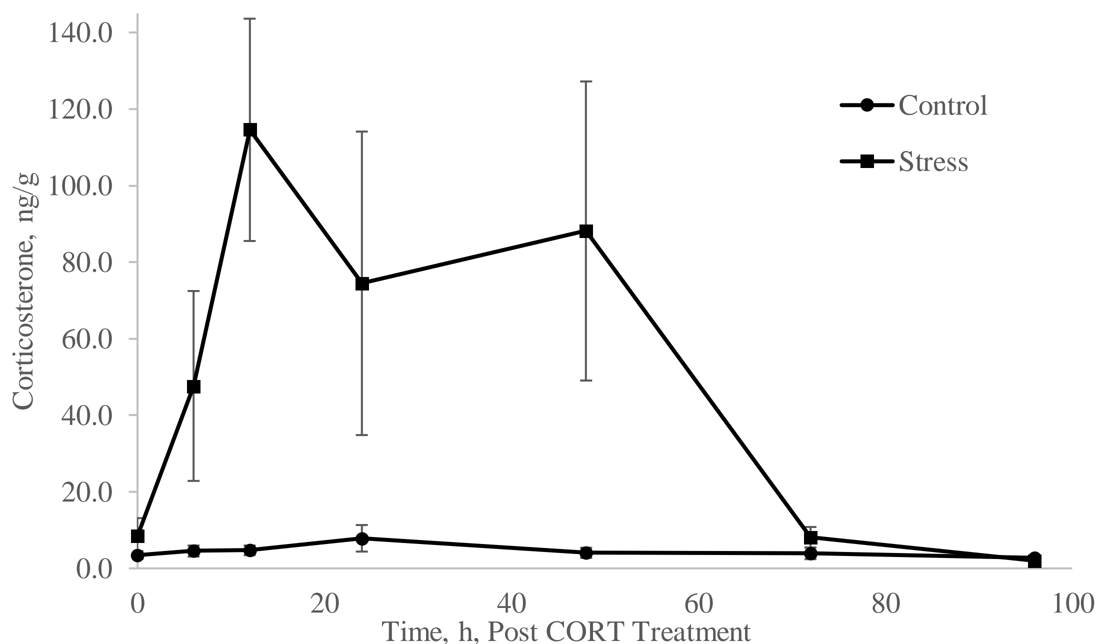

Figure 11. Effect of corticosterone (CORT) treatment on fecal corticosterone concentrations in fecal samples of stressed chickens, given CORT water, compared to control chickens, given normal water, from -24 h to 96 h with 7 time points. Data shown are average fecal CORT concentrations, ng/g, ± SEM.

Evaluation of fecal CORT concentration has become a more popular way to evaluate stress levels in poultry. Fecal CORT concentration took longer to increase than serum CORT concentration and peaked at 12 hours after onset of CORT treatment. There is a range of concentrations throughout the stressed groups possibly due to diarrhea starting at 12 hours after onset of treatment that continued until improving at 72 hours after onset of treatment. CORT concentrations took longer than serum concentrations to decrease. In fecal samples, CORT remained increased until returning to a lower average at 72 hours. This delay in concentration increase can be contributed to metabolism and excretion rates of corticosterone. In a published study, injected corticosterone was excreted in fecal material within 8 hours of injection. However, over two thirds of the hormone could be seen at one hour after injection (Rettenbacher et al. 2006). Since CORT in the present study was ingested and not injected intravenously it seems that the hormone took 12 hours to be metabolized.

In comparison to previous studies that have evaluated fecal CORT concentrations, concentrations were higher in this study. At 6 hours after treatment the previous study's average CORT concentration was approximately 12 ng/g in stressed chickens. At 6 hours after treatment in this study the average CORT concentration was 47.66 ng/g in stressed chickens. Control chickens at this time had much lower concentrations. In the previous study control concentration at 6 hours was approximately 5 ng/g compared to control chickens in this study where concentration average for control chickens was 4.54 ng/g. At 12 hours, the peak of this study, average CORT concentration was 114.56 ng/g compared to approximately 25 ng/g in the previous study.

Serum CORT Concentration Compared to Fecal CORT Concentration

Serum CORT concentration and fecal CORT concentration were similar to each other however fecal CORT concentration took longer to return to lower concentration. Average fecal concentrations were delayed by at least 6 hours compared to average serum concentration. This can be seen in Figure 12 where serum peaks in concentration at 6 hours after treatment as compared to fecal concentration that peaks at 12 hours. Fecal CORT concentration also took longer than serum to return to lower concentrations. Serum CORT concentration returned to lower concentrations at 24 hours where fecal CORT concentration did not return to lower concentrations until 72 hours after onset of treatment. This is because serum CORT offers a concentration value at the time the sample is drawn. Fecal CORT shows concentrations after CORT has been metabolized. This can be affected by how long it takes the individual chicken to release the hormone (Weimer et al. 2018).

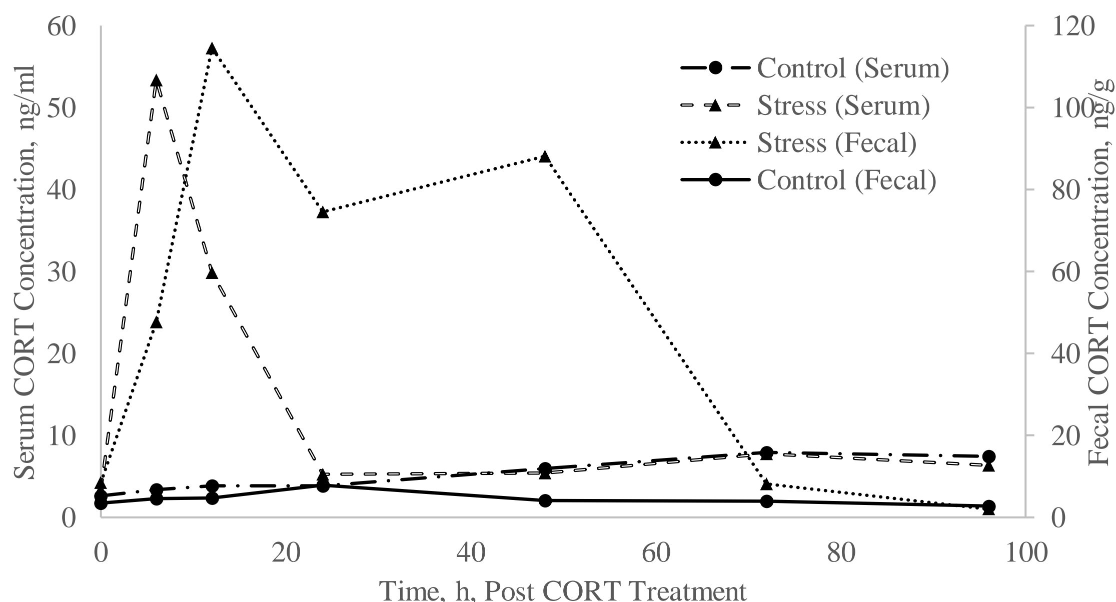

Figure 12. Effect of corticosterone (CORT) treatment in drinking water on average serum and fecal corticosterone concentrations in chickens given CORT water, compared to chickens given normal water, measured from -24 h to 96 h.

CHAPTER 5. CONCLUSIONS

Measuring both fecal and serum CORT concentrations as a way to evaluate stress in broilers has been validated through many previous studies. Administration of CORT in water for 48 h resulted in increased H:L ratios, serum CORT concentrations, fecal CORT concentrations, and a decrease in body weight. Each biomarker measured showed a significant difference at 12 h after onset of treatment. These results indicate that each biomarker is a reliable marker of stress in broilers. When done properly evaluating fecal CORT concentrations allows producers an opportunity to monitor corticosterone without disturbing their flocks.

Unlike previous studies, this study has shown that these methods can be used in smaller, caged flocks of broilers. This study shows that significant research can be conducted by universities that do not have access to large, environmentally controlled facilities. Further research should be conducted in different areas of this study to further validate these methods. Before being applied to commercial poultry producers, research needs to be conducted to simplify assay methods and standardize sampling methods. Fecal extraction methods also need to be standardized. This could include further research into whether methanol or ethanol should be used. Continued collection needs to be done on fecal and serum samples to assess how corticosterone concentrations respond to chronic and acute stress. This way a baseline can be established in a flock to monitor for spikes in corticosterone to control for stressors that may not be obvious to producers. Absorption of corticosterone in poultry should be researched to assess how the body absorbs this hormone and how much of it will pass through the digestive system when orally ingested. Research also needs to be conducted on other breeds of poultry. Other

breeds could respond differently to high stress situations. This method should be validated in breeds popular in both production and backyard settings. Lastly, research in other subjects in animal and poultry science should be conducted on a smaller scale than normal to validate the use of small scale studies in that area.

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APPENDIX A. IACUC APPROVAL LETTER

MURRAY STATE UNIVERSITY
Institutional Animal Care and Use Committee

April 25, 2022

Dr. Thomas Powell
Animal and Equine Science
Murray State University
Murray, KY 42071

Dear Dr. Powell:

The Murray State University Institutional Animal Care and Use Committee (IACUC) has approved your research protocol for the project titled, "Evaluation of fecal samples as a measure of physiological stress in poultry – pilot study."

The protocol timeline is approved through May 31, 2022. Please use the Animal Use Report (attached) to keep up-to-date information about the animals. At the termination of the protocol, you will need to complete the Conclusion Report (attached) and list final information concerning the animals.

If you have any questions, please contact me at (270) 809-3534.

Sincerely,

Kristi Stockdale

Kristi Stockdale IACUC Coordinator
cc:
IACUC File

We are Racers. murraystate.edu

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Equal education and employment opportunities M/F/D, AA employer. Murray State University supports a clean and healthy campus. Please refrain from personal tobacco use.

APPENDIX B. MATERIALS LIST

- Lidocaine and Prilocaine Cream, 2.5%/ 2.5% (Fougera, Melville, NY 11747)
- 3 mL Disposable Syringes without needle (Exelint, Redondo Beach, CA 90278)
- 1 mL Disposable Syringes without needle (Exelint, Redondo Beach, CA 90278)
- 25Gx ⅝” Hypodermic Needle (Exelint, Redondo Beach, CA 90278)
- 23Gx1” Hypodermic Disposable Needle (Exelint, Redondo Beach, CA 90278)
- Monoject Blood Collection Tube 10.25mm x 64mm Silicone Coated Tube, Glycerin Coated Red Stopper, No Additive, 3mL Draw (Coviden, Mansfield MA 02048)
- Microscope Slides, Frosted Edge (Jorgensen Laboratories Inc, Loveland, CO 80538)
- Country Land 18% Chick Starter Crumbles (Orscheln LLC, Moberly, MO 65270)
- Baby Poultry Leg Bands Large, Size 5 (Chicken Hill, Horseshoe Bend, ID)
- Multipurpose Absorbent Pads (Sam’s West Inc, Bentonville, AR 72716)
- Great Value Gallon Freezer Bags (Walmart Inc, Bentonville, AR 72716)
- Great Value Quart Freezer Bags (Walmart Inc, Bentonville, AR 72716)
- Corticosterone (C2505) (Sigma-Aldrich, St. Louis, MO 63103)
- Purina Start & Grow 18% Protein Medicated Crumbles (Purina Animal Nutrition LLC, Arden Hills MN 55126)
- Igloo 2 Gallon Sport Stackable Beverage Cooler (Igloo, Katy, TX 77494)
- Pivetal Rapid Differential 3- Step Stain Kit (Aspen Veterinary Resources LTD, Liberty, MO 64068)
- C5 Centrifuge (LW Scientific, Lawrenceville, GA 30046)
- Country Lane Premium 100% Pine Shavings (Orscheln Farm and Home LLC, Moberly, MO 65270)
- Plastic Test Tube (Animal Reproduction Systems, Chino, CA 91710)
- Test Tube Cap (Animal Reproduction Systems, Chino, CA 91710)
- Disposable Culture Tube (RTU, Rutherford, NJ)
- Single Channel Pipettor (SCHEER, Serial Number #YE17BAG0063505)
- 3 ml Plastic Transfer Pipettes (Next Generation, York, PN 17406-9200)
- Fisher Vortex Genie 2 (Fisher Scientific, Cohemia, NY)
- 1.5 ml Microcentrifuge Tube with Cap (Simport Scientific, Beloeil, QC, Canada)
- Fisher Finest Fine Tip Marking Pen (Fisher HealthCare, Pittsburgh, PN)
- Parafilm (Pechiney Plastic Packaging, Menasha, WI 54952)
- VWR Aluminum Drying Pan W/Hole 30mL (VWR, Randor, PA 19087)
- Avantco CO-16 Half Size Countertop Convection Oven 1.5 cubic feet, 120V, 1600W (Avantco Equipment, Meridian, ID, 83646)
- Methyl Alcohol (Azer Scientific Inc, Morgantown, PA)
- Ethyl Alcohol 190 Proof (PHARMCO-AAPER, Shelbyville, KY 40065)
- Colored Leg Rings with Numbers (Patelai)
- Corticosterone 5-Pack Enzyme Immunoassay Kit (Catalog Number: K014-H5, Lot Number:22CS034a) (Arbor Assays, Ann Arbor, MI 48108)
- 140-gallon Super Duty Oval Tank (Tuff Stuff Products, Terra Bella, CA)
- Carolina Immersion Oil for Microscopy (Carolina Biological Supply Company Burlington, NC)