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Investigating Growth Performance and Intestinal Barrier Integrity in Heat-stressed Modern Broilers and Their Ancestor Jungle Fowl

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Investigating Growth Performance and Intestinal Barrier Integrity in Heat-stressed Modern Broilers and Their Ancestor Jungle Fowl

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ABSTRACT

Heat stress (HS) has a negative effect on poultry production sustainability due to its adverse consequence on bird welfare, health, growth, and mortality. Although modern broilers have greater gut mass and higher energy use efficiency than unselected birds, they are more vulnerable to HS that induces "leaky gut syndrome," or increased intestinal permeability. The aim of the current study was to determine the effect of HS on growth performance and gut barrier integrity in three modern broiler lines and their ancestor the Jungle Fowl. Four chicken populations including Giant Jungle Fowl (JF), Athens Canadian Random Bred (ACRB), 1995 Arkansas Random Bred (95RAN), and Modern Random Bred (MRB) were studied. Day-old male broiler chicks from each population were raised under thermoneutral (TN) conditions with feed intake, water intake, and temperature measured daily. On day 28 the birds were subjected to one of two environment conditions: TN (24°C) or acute HS (2 hrs at 36°C). After two hours, samples from each section of the small intestine were harvested from two birds per line per treatment and flash frozen in liquid nitrogen. Following 28, the remaining birds were grown out to 56, during which birds were subjected to chronic cyclic HS (8 hrs a day at 36ºC). Growth performance, metabolite and blood hormone concentrations, and molecular data were analyzed by two-way ANOVA. These data show the significant effect HS had on growth performance and intestinal barrier integrity of the studied modern broilers. Acute HS was shown to decrease performance in the modern broilers and had significant effect on mRNA and protein expression of heat shock, tight junction, gap junction, and other intestinal barrier associated proteins. These data provide evidence for a mechanistic understanding of gut barrier physiology and how it can be influenced by growth-rate and heat stress.

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CHAPTER 1. REVIEW OF THE LITERATURE

1.1 INTRODUCTION

As poultry continues to be a major commodity in the world market, the rising price of feed, reduction of subclinical antibiotic use, and environmental challenges have re-centered industry focus on the efficiency of rearing broilers in imperfect conditions (Nawab et al., 2018). The environment in which the poultry industry strives to raise their stock is still subject to disease, calling for more in-depth research to improve feed efficiency and poultry production sustainability, despite environmental challenges. An important factor in the overall performance, health, and general welfare of poultry is the gastrointestinal tract (GI). The GI functions to convert feed into nutrients for growth, and to serve as a primary defense against enteric disease (Broom and Kogut, 2018). With the poultry industry's decision to minimize the use of sub therapeutic antibiotics as a result of consumer pressure the GI now operates at a disadvantage. When damaged, the specific, selective intestinal barrier becomes more permeable leading to a condition called "leaky gut syndrome," a major contributor to poor gut health (Galarza-Seeber et al., 2016). One estimation places the cost of poor gut health at 11 cents per bird (Elvidge, 2016), or roughly $128 to $165 million for the U.S. poultry industry. Worldwide, poultry production is impacted by an increase in global temperatures and by inefficient housing conditions common to developing nations (Glatz and Pym, 2013). These environmental conditions are subjecting the world's poultry to a condition known and documented as 'heat stress' (HS). HS has been shown to affect the health and well-being of poultry by causing metabolic disorders (Geraert et al., 1996), oxidative stress (Star et al., 2008), suppression of the immune system (Quinteiro-Filho et al., 2010), and in severe cases death. Broilers subjected to these conditions can experience significant reductions in feed intake, weight gain, and feed efficiency (Sohail et al., 2012). Lara

and Rostagno determined that broiler breeders subjected to HS experience decreased egg production and livability (Lara and Rostagno, 2013). While high ambient temperatures and inefficient housing conditions are known to facilitate HS conditions, it has also been shown that high stocking densities can significantly increase HS related mortality (Pettit-Riley and Estevez, 2001). HS alone has been determined to cost the poultry industry $128 million, annually (St-Pierre et al., 2003). The significant economic losses for the poultry industry due to poor gut health, induced by challenging environmental conditions, exhibit a need for a mechanistic understanding of the effect of HS on growth and intestinal barrier integrity.

1.2 IMPORTANCE OF POULTRY PRODUCTION WORLDWIDE

Worldwide, the poultry industry's annual production increased by 3 percent from 2018 to 2019, marking the highest rate of growth the industry has seen in 5 years (USDA, 2019). Feed cost, increased consumption, and a growing global demand has allowed Brazil, the European Union (EU), and the United States to reach record levels of production (USDA, 2019). The United States poultry industry generated $46.3 billion in 2018, a record high. In that same year, 56.8 billion pounds of broilers were produced, accounting for 69% of the aforementioned $46.3 billion (USDA, 2019). These increases in production are necessary to meet the global demand as the world continues to consume more poultry and eggs. In the current decade (2018-2028), consumption of poultry is expected to grow by over 5 percent as many more countries turn to poultry for efficient and affordable protein (OECD/FAO, 2018). The ability to adequately feed the globe relies on the sustainable growth of poultry production infrastructure. Population growth suggests the world is on track to reach 9 billion people by 2050; therefore, there are increasing concerns with food security and the sustainable agriculture necessary to feed the growing world population (Tian et al., 2016). As the global population approaches 9 billion, there will be a

continued increase in demand for arable land and fresh water; finite resources required for food production. In a 2014 study, poultry were shown to require the least amount of land and water per Mcal (1000 kcals), and emit the least amount of greenhouse gasses when compared to beef, pork, and dairy (Eshel et al., 2014). The future rests on technology, innovation, and due diligence of the poultry industry to provide healthy, efficient, accessible, and affordable protein to the entire world.

1.3 RESEARCH LINE DEVELOPMENT

The Giant Jungle Fowl (JF) line represents a wild-type common ancestor of modern domesticated poultry. One JF male and five hens were brought to the University of Arkansas in 1951, alleged to have been brought over as fertilized eggs from Southeast Asia. Since this time, the JF has remained as a closed line, speculated to be inbred, but to what degree by inbreeding coefficient is unknown (Hayden, 2016).

The Athens Canadian Random Bred (ACRB) line represents the commercial broiler of the 1950s. This population is said to have been developed from the Ottawa Meat Control Strain (OMCS), developed by the Canada Department of Agriculture's research branch (Collins et al., 2016). The OMCS was derived from 3 commercial broilers available in the 19050s and one experimental strain of meat chicken. A subpopulation of the ACRB line then was moved to Athens, Georgia where 1806 pedigreed eggs were hatched. This population is still maintained at the University of Georgia and another subpopulation is housed at the University of Arkansas. It is possible that this line is the oldest pedigree meat-type chicken control strain in existence (Collins et al., 2016; Gyles et al., 1967).

The 1995 Arkansas Random Bred (95RAN) population used in this project is a random bred control broiler line established and maintained at the University of Arkansas by Dr. N.B. Anthony and students. This population represents the genetics of commercial parent stocks that were on the market in 1995. The development of this line was accomplished through an initial mating of 7 male (Avian 89, Ross SP, Hubbard HI-Y, Case, Cobb 500, Peterson Regular, and Shaver) and 6 female (Cobb 500, Ross 508, Arbor Acres Classic, Hubbard HI-Y, Case 573, and Shaver Yield B) parent stock sources. The intent of the second cross was to provide 25% contribution from the founder parent type, as well as to produce an equal number of offspring per mating combination. After this second cross, the population was paired at random with the exception of sibling mating. The 95RAN line has been maintained each generation at 24 males and 72 females at the University of Arkansas (Harford, 2014).

The Modern Random Bred (MRB) population was developed at the University of Arkansas by Dr. N.B. Anthony and students in 2015. This line is composed of four commercial broiler packages from 3 primary breeder companies: Cobb-Vantress, Aviagen, and Hubbard. The four packages included in development were Cobb MX x Cobb 500, Ross 544 x Ross 308, Ross Yield+ x Ross 708, and Hubbard HI-Y. Through five generations of random mating, with the exception of sibling mating, the MRB line represents a common commercial broiler from 2015.

1.4 FACTORS PREVENTING OPTIMAL GROWTH PERFORMANCE

Despite centuries of genetic progress and housing improvement, current commercial stock are still yet to meet their optimal performance due to the removal of sub-therapeutic antibiotics, changes in gut morphology and luminal environment, and their sensitivity to heat stress.

Antibiotics, many of which are produced by fungi or bacteria, have been used to prevent and treat infections in both humans and animals (Singer and Hofacre, 2006). For decades, antibiotics and increased biosecurity have been essential to the growth of the poultry industry by preventing disease. In combating these diseases, antibiotic use in the poultry industry has shown to improve feed conversion and growth in addition to disease prevention (Singer and Hofacre, 2006). These improvements are consequences of the control of gastrointestinal infections and microbiota modification in the intestinal tract of the bird (Torok et al., 2011). Use of growth promoting antibiotics are shown to have a positive effect on the microbiota, producing an optimal environment for growth (Dibner and Richards, 2005). While changes in the gut can influence overall immunity, there may be many factors that in turn affect the intestinal microbiota such as housing, pathogenic populations, diet composition. The presence of antibiotics have shown to prevent poor growth performance and disease (Gadde et al., 2017). The removal of antibiotics requires the poultry performance be made up in other areas of production.

The gut microbial environment plays a key role in the digestion and absorption of nutrients in poultry. Some dietary components are shown to influence the microbial environment in the chicken, leading to inflammation and possibly disease (Antonissen et al., 2016). Healthy gut structure and villi morphology also play a role in digestion of nutrients. The efficiency of nutrient absorption in the gut is increased with greater size and height of the intestinal villi (Samanya and Yamauchi, 2002). Villi are fingerlike projections of the small intestine with heights ranging from 0.5 to 1.5 mm and can increase the surface area of the small intestine by a factor of 10 (Gartner and Hiat, 2006). Growth rate has a strong correlation with changes in the size and height of the villi, allowing for the small intestine to have a larger surface area.

Selection for growth rate is positively associated with digestion and absorption of nutrients. (Marks, 1979). Gut morphology and the negative effects caused by dysbiosis in the gut play an important role in the efficiencies in modern broiler production.

1.5 INTESTINAL HEALTH IN POULTRY

The gastrointestinal tract exists, primarily, to break down food by way of digestion and absorb the nutrients and minerals released thereafter. The function of the gut is paramount in the uptake of necessary nutrients and minerals, and subsequently the expulsion of the remaining waste. Animal agriculture, an industry now centered on the ability to convert low energy grains into energy dense protein, relies on the overall health and efficiency of the gut to improve yield. As science and technology have progressed, there has become an increased interest in the gut. The gut is responsible for a number of functions, but its primary role is digestion. The GI serves an important role in that of immune function. When animals eat, drink, or breathe the gut can be exposed to potential pathogens in their environment. Because of this, the GI has developed a number of measures to serve as physical and chemical barriers, preventing pathogens from entering the circulatory system. This protection begins in the mouth, where enzymes and peptides in the saliva can kill bacteria, and protect the mouth from infection (Ramasundara et al., 2009). Protection also exists in the proventriculus, a harsh environment consisting of hydrochloric acid and pepsinogen, resulting in a very low pH which can kill potentially harmful bacteria (Hodges, 1974). As digestion continues to the small intestine (SI), the mucosal lining of the gut protects the body from pathogens due to a concentration of IgA antibodies and tight binding between cells of the intestinal epithelium (Wieland et al., 2004). Additionally, concentrations of symbiotic bacteria in the GI prevent excessive growth or transmission of pathogens (Gao et al., 2018). Despite these preventative measures, lapses in the homeostatic

environment of the GI can allow these pathogens to survive and thrive, causing a whole host of infections and disease.

Understanding how the gut develops is the first step in improving its overall function, and further preventing the presence of enteric pathogens. *In ovo,* the rate at which the weight of the SI increases is much greater than that of the overall weight of the bird. For 2 weeks post hatch, the SI continues increasing at a rate significantly higher than that of the overall body weight and is subjected to physical and enzymatic changes, maturing much like the SI of mammals, until fully formed (Uni et al., 1999; 2000). Nutritional and environmental factors during this delicate period are a function of later immune performance (Taha-Abdelaziz et al., 2018). Like other livestock, commercial poultry are intensively reared, which can serve as an additional stressor. Due to the presence of stress during early development, proper immune function is necessary to sufficiently protect the animal. Adverse immune responses in the GI can negatively affect feed efficiency, weight gain, and wellbeing (Habibian et al., 2015). Management of pathogens that can elicit immune response in the gut is accomplished by the floral environment, the chemical and enzymatic makeup, and the physical attributes of the GI.

The microflora of the gut is made up mostly of bacteria, in addition to commensal fungi and protozoa. The microflora of the GI depends on the chemical makeup of the gut, which is not only defined by the genetic parameter of the chicken, but also the type of the diet consumed (Apajalahti et al., 2004). Ideally, the commensal bacteria of the gut would prevent the culture of these harmful pathogens. However, many pathogenic microbes can harm GI health and integrity. As the bird consumes feed and water it not only consumes these nutrients, but also foreign material like litter and microbes, constantly exposing the GI to these pathogenic microbes. Once

these pathogens are in the system, and in the event that they overwhelm the commensal microflora, they can result in infection, necrosis, and inflammation (Williams, 2005).

Despite a presence of pathogens in the GI, the structure of the intestinal epithelium can still prevent the movement of harmful microbes into the abdominal cavity by selecting what can or cannot pass through the intestinal wall. Normally, the intestinal epithelium allows nutrients and minerals to pass through for transport and retains pathogens and waste; however, normal function of this selective permeability can be compromised by diet, texture and form of feed, or infectious agents (Yegani and Korver, 2008).

One problematic component of poultry diets are non-starch polysaccharides (NSPs). NSPs are a group of non-nutritional compounds that exist in a number of feed ingredients (Cardoso et al., 2018). They can be resistant to the bird's digestive enzymes and have a propensity for creating a viscous luminal environment. Wheat, rye, and barley all contain high levels of NSP which have shown to increase luminal viscosity, and decrease nutrient digestibility, feed efficiency, and growth rate (Bedford and Schulze, 1998). Additionally, the texture and formation of poultry feed can cause a dysfunction of the intestinal epithelium. A study conducted by Branton et al. (1987) supports a relationship between finely ground feed and increased mortality, as opposed to coarsely ground. These deaths were attributed to necrotic enteritis and coccidiosis, leading to an assumption that finely ground feed may aggravate the intestinal lining, inviting the aggregation of infectious bacteria (Branton et al., 1987). While the primary goal of the GI is to extract nutrients and minerals to be absorbed and delivered for its employment in growth and production, these processes are severely affected by the aggregation of pathogens. *Clostridium perfringens* is a common bacteria where poultry are raised and produced, causing necrotic enteritis. When the bird is faced with a subclinical infection of this

bacteria it often results in necrosis of the intestinal barrier, which decreases intestinal absorption, weight gain, and feed efficiency (Van Immerseel et al., 2004). *C. perfringens* normally composes a portion of the general gut flora in poultry, but will advance to a subclinical or acute stage when the immune system of the bird has been comprised, most notably by mucosal damage caused by coccidiosis (cocci). Cocci is also prevalent among areas where poultry are produced. Cocci proliferates in the GI resulting in a decrease in performance efficiency. Cocci and *C. perfringens* can function together to disable the immune system allowing both pathogens to take over, advancing *C. perfringens* to an acute stage causing severe necrotic enteritis and death (Van Immerseel et al., 2004).

While these issues have always existed to challenge the health and function of the intestinal barrier, they have become more prevalent as the poultry industry has continued to phase out the use of sub-therapeutic antibiotics. Studies show that antibiotic growth promoters (AGP) have improved animal health and efficiency (Coates et al., 1955; Miles et al., 2006); yet Graham et al. (2007) claims that the weight gained from these promoters is not substantial enough to offset the cost of the antibiotics (Graham et al., 2007). Despite these claims, the main reason for the removal of sub-therapeutic antibiotics has been attributed to poultry consumer preference.

The intestinal epithelium that lines the lumen offers protection, which separates the luminal contents from the abdominal cavity. The cells of the intestinal barrier are bound together by proteins called tight junctions, gap junctions, and adheren junctions (Groschwitz and Hogan, 2009). Tight junctions and adheren junctions are both transcellular proteins, which bind to the actin of the cytoskeleton. As the name suggests, tight junctions bind cells closely together, forming a physical barrier. Between this close binding of cells are the channels that exhibit an

extremely selective system of molecular transport. Under normal conditions, the regulation of this mechanism is highly specific; however, this regulation begins to break down if these tight junctions begin to fail. The dysfunction of tight junctions can be caused by an enteric pathogen with the ability to use tight junction proteins for the degradation of the epithelial lining (O'Hara and Buret, 2008). Tight junction proteins are transmembrane proteins which include claudins, occludin, junctional adhesion molecules, and tricellulin (Groschwitz and Hogan, 2014). These proteins make up the selectively-permeable intestinal barrier. Claudins are the primary molecule in tight junctions and establish the paracellular barrier, which serves as a gate regulating molecular passage through the intercellular space between cells of the intestinal epithelium. Some claudins are 'pore-sealing,' and some are 'pore-forming' (Awad et al., 2017). Occludin is a protein in the tight junction that supports barrier stability and function. This protein has the ability to move to a number of paracellular locations, altering intestinal permeability, acting as a turnstile of sorts. Zona occludens-1 (ZO-1), tight junction protein 1, is a key player in the formation of tight junctions and binds the tight junction to the cytoskeleton (Buckley and Turner, 2018). ZO-2 and ZO-3 provide assistance to the tight junctions, yet their primary role is not well defined. Together these proteins protect the body cavity by preventing the passage of pathogenic organisms. Other proteins key to intestinal function and selective permeability include villin, junctional adhesion molecule A (JAM-A), gap junction alpha-1 protein (GJA-1), protein associated to tight junctions (PATJ), cadherin, gap junction gamma-1 protein (connexin-45), lipocalin, and calprotectin. These proteins form a cooperative selective intercellular barrier between intestinal epithelial cells, preventing the passage of foreign molecules and pathogens. Despite the organizational structure of these tight junctions, microbes can stimulate secretion of pro-inflammatory cytokines, inducing phosphorylation of myosin light chain by myosin light

chain kinase which opens the tight junctions increasing permeability leading to leaky gut (Awad et al., 2017). This stimulation by microbes resulting in secretion of cytokines is aided by the existence of stress, heat stress being specifically know to elicit this kind of response (Song and Qian, 2013).

1.6 HEAT STRESS IN POULTRY

The environment in which poultry are produced has been thoroughly researched over the decades to provide living conditions conducive to high return on investment by way of improved feed efficiency and peak physiological performance. Unfortunately, maintaining these ideal environments requires up-to-date housing and a large amount of resources. One current challenge is providing a thermoregulated environment to keep the birds cool. In many areas of the world, either due to ambient temperature or lack of appropriate finances, this is not feasible. When the birds are not kept cool and comfortable they can become heat stressed. Heat stress has been defined as the inability to effectively thermoregulate in the presence of high temperature and humidity (Webster, 1983). Abidin et al. cite many in their review, stating that heat stress' effect on the economy is the result of the birds decrease in feed intake, weight gain, growth rate, egg quality, egg production, hatchability, immunity, livability, and carcass quality (Abidin et al., 2017). The impact that heat stress has on the performance of the bird is due to the behavioral, physiological, and immunological functions that are altered in the presence of this stress. When reared under a high ambient temperature, birds attempt to thermoregulate by panting, elevating their wings, limiting their movement, and reducing feed intake (Yahav et al., 2005). This practice can reduce the internal and external temperature of the chicken via thermal radiation, convection, and conduction (Zaboli et al., 2019). In this attempt of thermoregulation the bird suffers consequences. High rates of panting results in increased blood pH and CO_2 (Zaboli et al., 2019).

An adoption of this severe stress response can inhibit function of the immune system in the bird. Findings cited in a Lara and Rostagno's (2013) review suggest that under a condition of heat stress, the immune system of the chicken becomes extremely compromised. In laying hens, decreased weights of the thymus, spleen, and liver were observed (Lara and Rostagno, 2013). In broilers, reduced weights of the thymus, spleen, liver, and bursa were significant, in addition to decreased levels of circulating antibodies, and specific levels of IgM and IgG during the humoral response (Deng et al., 2012). Subsequently, in the gastrointestinal tract, a reduction of lymphocytes and IgA secretion was observed (Deng et al., 2012).

As the bird responds to the condition of heat stress through behavior methods and alterations in hypothalamic-pituitary-adrenal (HPA) function, the bird becomes susceptible to hyperthermia (Lambert et al., 2002). When hyperthermic, the blood from the visceral region moves to the extremities of the body in an attempt to further dissipate the heat, leaving the core of the body without sufficient amounts of blood, which then leads to improper tissue function and repair (Pearce et al., 2013). What follows is an increase in reactive oxygen species (ROS). ROS serve a role in apoptosis and can, in high concentrations, damage nucleic acids or oxidize lipids and proteins. In response to high concentrations of ROS the body undergoes oxidative stress, thus increasing the production of heat shock proteins (HSPs), chaperones that protect and repair damaged lipids and proteins. In chickens suffering from heat stress, higher concentrations of HSP70 were observed and highly upregulated 2-4 hours subsequent of heat exposure (Hao et al., 2012; Dokladny et al., 2006.)

Broilers and broiler breeders, both, are shown to have a deterioration in production traits when impacted by chronic heat stress. One study showed broilers chickens to exhibit a 16.4% reduction in feed intake, 32.6% reduction in weight gain, and 25.6% reduction in feed efficiency

(Sohail et al., 2012). These reductions where corroborated by a number of other studies, all showing significant reductions of performance traits under heat stress (Abu-Dieyeh, 2006; Ain Baziz et al., 1996; Rosa et al., 2007). A study also demonstrated an increase in fat deposition, thigh and drumstick yields in birds exposed to heat stress (Sohail et al., 2012). A reduction in breast yield, paired with an increase in fat deposition, thigh and drumstick yields may decrease post-process profit margin for the poultry industry as white meat sells for more per pound.

Feed conversion ratio, defined by the weight of feed intake per weight gained by the bird, and percent yield in the processing plant are essential for the poultry industry's ability to make a profit and provide high quality protein at an affordable cost. With their ability to provide a necessary service for a reasonable cost at stake, there is a need for understanding the mechanisms involved in HS that are causing reductions in the feed efficiency and parts yield of poultry, aside from reduced feed intake.

Heat stress has been shown to degrade the integrity of the GI, leading to a condition called 'leaky gut syndrome' (Singleton and Wischmeyer, 2006; Prosser et al., 2004; Lambert et al., 2002). This breakdown of the GI can lead to 'leaky gut,' which is indicative of increased intestinal permeability, allowing ingested pathogens to pass through the epithelial lining of the lumen. During heat stress, occludin has been shown to move from the tight junction aiding to a loss in barrier function (John et al., 2011). The bacteria that pass through can then enter circulation, infiltrating the bird's immune system and assisting in the development of a number of infections, including necrotic enteritis (Ducatelle et al., 2018), femoral head necrosis, and bacterial chondronecrosis (Wideman, 2016). Infection and disease in poultry is detrimental to growth performance, causing decreases in feed intake, feed efficiency, meat quality, and livability. Estimates suggest that the economic loss accrued from disease reaches approximately

20% of the value of total poultry production (Biggs, 1982). With increased intestinal permeability, or leaky gut, assisting in such an expense for the industry, it is critical that research be conducted to delineate the process in which GI health effects performance traits and animal well-being.

1.7 OBJECTIVES

Heat stress is detrimental to poultry production and sustainability due to its negative effects on the welfare, health, growth and mortality of chickens. Despite genetic improvements in the growth and performance efficiencies of the chicken they suffer from increased intestinal permeability, inducing 'leaky gut syndrome.' Determination of the effect of heat stress on gut function and barrier integrity is crucial to the industry, as extreme temperatures and an increasing need for poultry continues to demand production efficiency. The review at hand denotes a clear connection between heat stress, gut health, and growth rate. It is the intention of this review to inform on these connections and stimulate future ideas and research concerning these pressing issues.

Thus, the objectives of my master's research are to understand the effect of acute heat stress on the growth performance and intestinal barrier integrity of broiler chickens possessing different stages of genetic advancement.

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CHAPTER 2

Investigating Growth Performance and Intestinal Barrier Integrity in Heat-stressed Modern Broilers and Their Ancestor Jungle Fowl

2.1 ABSTRACT

Heat stress (HS) has a negative effect on poultry production sustainability due to its adverse consequence on bird welfare, health, growth, and mortality. Although modern broilers have greater gut mass and higher energy use efficiency than unselected birds, they are more vulnerable to HS that induces "leaky gut syndrome," or increased intestinal permeability. The aim of the current study was to determine the effect of HS on growth performance and gut barrier integrity in three modern broiler lines and their ancestor the Jungle Fowl. Four chicken populations including Giant Jungle Fowl (JF), Athens Canadian Random Bred (ACRB), 1995 Arkansas Random Bred (95RAN), and Modern Random Bred (MRB) were studied. Day-old male broiler chicks from each population were raised under thermoneutral (TN) conditions with feed intake, water intake, and temperature measured daily. On day 28 the birds were subjected to one of two environment conditions: TN (24°C) or acute HS (2 hrs at 36°C). After two hours, samples from each section of the small intestine were harvested from two birds per line per treatment and flash frozen in liquid nitrogen. Following 28 the remaining birds were grown out to 56, during which birds were subjected to either the TN condition or chronic cyclic HS (8 hrs a day at 36ºC). Growth performance, metabolite and blood hormone concentrations, and molecular data were analyzed by two-way ANOVA. These data show the significant effect HS had on growth performance and intestinal barrier integrity of the studied modern broilers. Acute HS was shown to decrease performance in the modern broilers and had significant effect on mRNA and protein expression of heat shock, tight junction, gap junction, and other intestinal barrier

associated proteins. These data provide evidence for a mechanistic understanding of gut barrier physiology and how it can be influenced by growth-rate and heat stress.

2.2 INTRODUCTION

As poultry continues to be a major commodity in the world market, the rising price of feed, reduction of subclinical antibiotic use, and environmental challenges have re-centered industry focus on the efficiency of rearing broilers in imperfect conditions (Nawab et al., 2018). The environment in which the poultry industry strives to raise their stock is still subject to disease, calling for more in-depth research to improve feed efficiency and poultry production sustainability, despite environmental challenges. An important factor in the overall performance, health, and general welfare of poultry is the gastrointestinal tract (GI). The GI functions to convert feed into nutrients for growth, and to serve as a primary defense against enteric disease (Broom and Kogut, 2018). With the poultry industry's decision to minimize the use of subclinical antibiotics as a result of consumer pressure, the GI now operates at a disadvantage. When damaged, the specific, selective intestinal barrier becomes more permeable leading to a condition called "leaky gut syndrome," a major contributor to poor gut health (Galarza-Seeber et al., 2016). One estimation places the cost of poor gut health at 11 cents per bird (Elvidge, 2016), or roughly $128 to $165 million for the U.S. poultry industry. Worldwide, poultry production is impacted by an increase in global temperatures and by inefficient housing conditions common to developing nations. These environmental conditions are subjecting the world's poultry to a condition known and documented as 'heat stress' (HS). HS has been shown to affect the health and well-being of poultry by causing metabolic disorders (Geraert et al., 1996), oxidative stress (Star et al., 2008), suppression of the immune system (Quinteiro-Filho et al., 2010), and in severe

cases death. Broilers subjected to these conditions can experience significant reductions in feed intake, weight gain, and feed efficiency (Sohail et al., 2012). Lara and Rostagno determined that broiler breeders subjected to HS experience decreased egg production and livability (Lara and Rostagno, 2013). While high ambient temperatures and inefficient housing conditions are known to facilitate HS conditions, it has also been shown that high stocking densities can significantly increase HS related mortality (Pettit-Riley and Estevez, 2001). HS alone has been determined to cost the poultry industry $128 million, annually (St-Pierre et al., 2003). The significant economic losses for the poultry industry due to poor gut health, induced by challenging environmental conditions, exhibit a need for a mechanistic understanding of the effect of HS on growth and intestinal barrier integrity.

2.3 MATERIALS AND METHODS

2.3.1 Populations

The broiler chickens involved in this trial were hatched from eggs collected at the University of Arkansas research farm and consist of four research lines; three of which represent the commercial broiler chicken of the 1950s, 1995, and 2015, and the fourth, indicative of the wild-type ancestor to the commercial broiler, the Jungle Fowl (JF). The JF population represents the South East Asian ancestor to the commercial broiler (Hayden, 2016). The Athens Canadian Random Bred (ACRB) line is indicative of the commercial broiler of the 1950s, a slow-growing broiler (Collins et al., 2016). The 1995 Random Bred (95RAN) line has the genetics of 7 male and 6 female commercial broiler lines available in the mid-1990s, a moderate-growing broiler (Harford, 2014). The Modern Random Bred (MRB) population is composed of broiler packages offered by three broiler genetics

companies and have been blended homogenously after many generations of random mating, representing the commercial broiler of 2015. All populations are maintained at the University of Arkansas research farm under close care and supervision, randomly mated each generation with the exception of full and half sibling pairings. All birds were raised and cared for under an animal use protocol approved by the International Animal Care and Use Committee at the University of Arkansas (Protocol #18083, Protocol #16084).

2.3.2 Bird rearing

Day-old broiler chicks from the four existing breeding populations were hatched at the University of Arkansas hatchery, individually wing-banded with a number and barcode, and vent-sexed prior to their placement at the University of Arkansas research farm. The male chicks were separated by line and placed into twelve environmental chambers with each chamber consisting of two equally sized pens allowing for triplication of the 4x2 factorial design. Twenty-five male chicks from one of the respective lines were placed per pen, 600 in total, and kept at an approximate density of 0.5 m^2 per bird in all pens. All birds had *ad libitum* access to feed and fresh water. During the first week, birds were provided with a '23 hour light: 1 hour dark' lighting program and subsequently a '20 hour light: 4 hour dark' lighting program throughout the remainder of the trial, day 8 to 56. Commercially available starter and finisher diets were fed from 0 to 28 days and 29 days through the remainder of the trial, respectively, which were formulated to meet or exceed NRC recommendations (NRC, 1994). Rearing temperature gradually decreased from 32ºC for days 1 to 3, 31ºC for days 4 to 6, 29ºC for days 7 to 10, 27ºC for days 11 to 14, and 24ºC for day 15 through day 28; on the morning of day 29 the birds were subjected to one of two environmental conditions: TN condition or cyclic heat stress (HS). Birds under the TN

condition experienced an ambient temperature of 24ºC for the remainder of the trial, days 28 through 56, whereas birds subjected to the cyclic HS experienced an increase in ambient temperature from 24ºC to 36ºC for 8 hours each day, 0800 to 1600, from day 28 to day 56.

2.3.3 Sampling protocol

Feed and water intake, body weight, feed conversion ratio (FCR), water conversion ratio (WCR), feed efficiency, mortality, core body temperature, and pen temperature and relative humidity were recorded on a pen by pen basis, with temperature measured instantaneously, feed intake, water intake, mortality, and humidity measured daily, and body weight measured weekly. Birds used for biological sampling were equipped with iButton sensors, which recorded core body temperature, one day prior to their sampling date. Blood samples were drawn from the leg of the birds one day prior to their sampling to measure blood chemistry, gases, and hematology. Two birds per pen, 6 birds per treatment, were sampled on day 29, 2 hours into the initial subjection of half of the birds to 36ºC, representing an acute heat stress. Another two birds per pen, 6 birds per treatment were, sampled on day 54, 2 hours into the 8 hour cyclic heat stress which they had experienced each day for over 3 weeks, representing a chronic heat stress. Following cervical dislocation, samples of each section of the small intestine (duodenum, jejunum, and ileum) were harvested from two birds in each pen. The tissues were then labeled with the band number of the bird from which they came, snap frozen in liquid nitrogen, and stored at -80ºC for future analysis.

2.3.4 Blood Chemistry, Gases, and Hematology

Blood pH, partial pressure of CO_2 (pCO_2), total CO_2 (TCO_2), partial pressure of O_2 (pO_2), bicarbonate (HCO_3^-), base excess (BE), O_2 saturation (sO_2), sodium (Na), potassium (K), ionized calcium (iCa), glucose, hematocrit (Hct), and hemoglobin (HB) were determined using i-STAT Alinity system (SN:801128; software version JAMS 8o.A.1/CLEW D36; Abaxis, Union City, CA, United States) with the i-STAT CG8+ cartridge test (ABBT-o3P77-25) according to manufacturer's recommendation. Analysis was performed at room temperature, immediately following blood draw, using the temperature correction function of the i-STAT Alinity system.

2.3.5 RNA isolation, reverse transcription, and quantitative real-time PCR

One μg of total RNA was extracted from the sampled tissues via Trizol reagent (Life Technologies) in accordance with the manufacturer's recommendations. Ribonucleic acids were then treated with DNAse, and reverse transcribed qScript cDNA SuperMix (Quanta Biosciences). Ribonucleic acid quality was assessed using 1% agarose gel electrophoresis and each sample was tested for concentration and purity by Take 2 micro volume plate reader using Synergy HT multi-mode microplate reader (BioTek). The cDNA was then amplified by real-time quantitative PCR (Applied Biosystems 7500 Real-Time PCR system) with Power SYBR green Master Mix (Life Technologies). Oligonucleotide primers used for chicken HSPs, inflammation, gap junction, and tight junction related genes are summarized in Table 1. Primer concentration of 0.5μL and volume of 1μL per sample was used. The qPCR cycling conditions were 50°C for 2 min, 95°C for 10 min followed by 40 cycles of a two-step amplification program (95°C for 15 s and 58°C for 1 min). At the end of the amplification, melting curve analysis was applied using the dissociation protocol from the

Sequence Detection system to exclude contamination with unspecific PCR products. The PCR products were also confirmed by agarose gel and showed only one specific band of the predicted size. For negative controls, no RT products were used as templates in the qPCR and verified by the absence of gel-detected bands. Relative expressions of target genes were determined by the $2^{-\Delta\Delta Ct}$ method (Schmittgen and Livak, 2008).

2.3.6 Western blot analysis

Total protein was extracted from the tissue samples, quantified via Bradford Assay, ran through a gradient bisTris gel (4-12%), transferred to a PVDF membrane, and analyzed via Western blot. Pre-stained molecular weight marker (Precision Plus Protein Dual Color) was used as a standard (BioRad). Membranes were blocked overnight in 5% TBST-Milk solution for non-specific binding. All primary antibodies used (1:1000) were purchased from Cell Signaling Technology, except for the anti-HSP70 and anti-HSP90 which were purchased from Pierce Thermo Scientific. The secondary anti-bodies were used (1:5000) for 1 hour at room temperature. The signal was visualized by enhanced chemiluminescence (ECL plus) (GE Healthcare Bio-Sciences) and captured by FluorChem M MultiFluor System (Protein simple). Image Acquisition and Analysis were performed by Alpha-View software (Version 3.4.0, 1993–2011, Protein simple).

2.3.7 Statistical analysis

Growth performance, feed intake (FI), water intake (WI), body weight gain (BWG), feed conversion ratio (FCR), water conversion ratio (WCR), plasma metabolite parameters (cholesterol, glucose, triglyceride, uric acid, LDH and creatine kinase), and heat stress/intestinal integrity related gene and protein expression data were analyzed by two-way

ANOVA, with chicken population (JF, ACRB, 95RAN, MRB) and environmental condition (thermoneutral, heat stressed) serving as the main effects and pen as the experimental unit. Body temperature data were analyzed using two-way repeated-measures ANOVA with time as the repeated measure and treatment (TN vs HS) as factors. Data are expressed as the mean ± SEM and analyzed using Graph Pad Prism software (version 8, La Jolla, CA). Interactions were deemed statistically significant at a P-value < 0.05, with means compared by Student Newman Keuls (SNK) multiple comparisons test.

2.4 RESULTS

2.4.1 Chamber temperature and bird core body temperature

During the study, as depicted in Figure 1, environmental temperature was manipulated in the experimental chambers. Data from sensors inside each chamber show a clear picture of the thermo-manipulation involved, and that it was successful. Shortly after 8:00 a.m., the chamber temperature of the HS treatment reached 36ºC as opposed to the 24ºC of the TN treatment. The figure also depicts the difference between the two treatments during the cyclic period and that it returned back to 24ºC after the cyclic period ended for the day at 4:00 p.m.

On day of sampling, 29, the core body temperature of birds subjected to the HS treatment was significantly increased ($P < 0.0001$), as seen in Figure 2. Between the start of the acute heat stress and the time of sampling, a 2 hour period, core body temperatures rose by up to ~1º C in heat-stressed birds. Under Tukey's multiple comparisons test, all comparisons were significant ($P < 0.0001$), except for 95RAN TN vs. MRB TN ($P = 0.9959$). This indicates that birds subjected to the HS treatment were effectively heat-stressed, as their core body temperature rose in accordance to the chamber temperature.

As the trial progressed, the HS chambers continued to cycle efficiently each day. In Figure 3, an existence of a summer heat wave can be detected in the final week of the trial as the TN chambers struggled to cool efficiently. Outdoor temperatures reached highs in the mid-30ºC and lows in the mid-20ºC proving difficult for the chambers to keep up. This returned to normal shortly after and there were no visible signs of heat stress in the TN birds. Overall, the chambers provided measureable consistencies in temperature.

However, the heat wave is corroborated in Figure 4 as the core body temperature of the birds in each treatment increased during the 6 day period. The trending increases seen in these data consist of the core body temperature of the HS birds each day during the cyclic HS period. Core body temperature data from the JF and ACRB are present two days prior to the second sampling on day 54, and show no significant difference in core body temperature between treatments ($P > 0.9424$), while the 95RAN and MRBs show a significant difference in core body temperature when combining between treatments ($P < 0.0001$). The core body temperature of JF and ACRB birds were not significantly higher under heat stressed conditions, while the core body temperature of the 95RAN and MRB were.

2.4.2 Growth performance

The modern broilers subjected to the HS treatment were significantly affected resulting in a decrease in growth performance and efficiencies. The MRB and 95RAN populations experienced the negative effects more consistently, likely due to their size and metabolic activity, while the ACRB and JF were seemingly unaffected at times. Both the MRB and 95RAN populations experienced a significant decrease in daily feed consumption and cumulative feed intake throughout the trial when subjected to the HS treatment ($P < 0.0001$) (Figure 5). The

MRB population under HS consumed significantly more water ($P < 0.0001$) when compared to the TN treatment, while the 95RAN, ACRB, and JF did not (Figure 5). Daily water intake and cumulative water intake were significantly increased all of the HS treatments, when compared to the TN ($P < 0.0001$) (Figure 5). With a decrease in feed intake, a decrease in body weight and weekly body weight gain was observed in the effected treatments of both the MRB and 95RAN broilers ($P < 0.0001$) (Figure 5). This became more evident as the birds continued to grow past the 5^{th} week. The negative effects of HS on the growth performance and efficiencies of the broilers is further supported by the FCR and WCR data (Table 2). A significant increase in FCR in the HS exposed chickens ($P < 0.0001$) (Table 2). The birds also consumed significantly more water when exposed to HS ($P < 0.0001$) (Table 2).

2.4.3 Blood parameters

Based on the circulating blood metabolite and hormone data, some blood and plasma parameters were significantly affected by the acute HS treatment. Sodium concentration in the HS treated birds was significantly ($P = 0.0006$) decreased, while glucose concentrations exhibited a significant environmental effect ($P < 0.05$). Parameters pH, HCO_3, BE, TCO_2, potassium, and glucose exhibited a line effect ($P < 0.05$). Glucose and iCa displayed a line x environment interaction ($P < 0.05$) (Table 3).

2.4.4 Gene and protein expression

Effect of HS on HSPs

Gene expression of HSP 70, HSP 60, and HSP 90 in the gut of HS birds was found to have significant differences compared to expression in the gut of TN birds (Figure 6, 9, and 12). HSP 70 expression in the duodenum was significantly upregulated by heat stress ($P = 0.0077$)

(Figure 6, 9, and 12). Additionally, HSP 60 expression in the jejunum exhibited a significant line x environment interaction (P = 0.0472) (Figure 6, 9, and 12). Heat shock protein 90 expression in the ileum displays a significant line effect (P = 0.0014) and approaches significance in the jejunum (P = 0.0518) (Figure 6, 9, and 12). In the ACRB, 95RAN, and MRB, HSP 90 exhibits an increase in protein expression under acute HS (Figure 6, 9, and 12).

Effect of HS on Tight Junction

Gene expression of tight junction protein-1, or zonula occludens-1, (ZO-1) showed significant line effect in all three sections of the gut: duodenum (P = 0.0018), jejunum (P = 0.0478), and ileum (P = 0.0023) (Figure 6, 9, and 12). Line effects also were observed in the jejunum (P = 0.0279) and ileum (P < 0.0001) for ZO-2 and in the duodenum for ZO-3 (P = 0.0111) (Figure 9, 12, and 7). Junctional adhesion molecule A (JAM-A) exhibited a significant line effect in all three sections of the gut as well (P = 0.0347, P = 0.0264, and P = 0.0214, in the duodenum, jejunum and ileum, respectively) (Figure 7, 10 and 13). Protein Associated to Tight Junctions (PATJ) expressed a significant line effect in the ileum (P = 0.0007) and an environmental effect in the duodenum (P = 0.0212) (Figure 7 and 10). Protein Associated to Tight Junctions appears to be upregulated in response to HS. Occludin expression in the jejunum was downregulated in response to HS; there was a significant effect (P = 0.0336) observed when comparing environmental treatments (Figure 6). In duodenal protein of the ACRB, 95RAN, and MRB, occludin is upregulated during HS, contrary to that of the JF where it seems to be downregulated (Figure 6).

Effect of HS on Gap Junction and other intestinal barrier mechanisms

Gap junction protein alpha 1 (GJA-1) exhibits a trend of upregulation in the presence of HS with a line x environment interaction in the ileum ($P = 0.0044$) (Figure 12). Connexin, another gap junction protein, appears to be upregulated in the duodenum during HS (Figure 13). A significant line effect ($P = 0.0015$, $P = 0.0005$, and $P = 0.0002$, in the duodenum, jejunum and ileum, respectively) is present in all sections of the small intestine in respect to expression of connexin with an environmental effect in the duodenum ($P = 0.0499$) and a line by environment interaction in the ileum ($P = 0.0147$) (Figure 7, 10, and 13). Villin expression has significant line effect in all three section of the small intestine ($P = 0.005$, $P = 0.0203$, and $P < 0.0001$, in the duodenum, jejunum and ileum, respectively) and an environmental effect in the ileum ($P=0.0440$) (Figure 7, 10, and 13). Cadherin expression exhibits significant line interaction in the ileum ($P = 0.0006$) (Figure 14). Lipocalin and calprotectin expression in the jejunum and ileum shows significant line effect ($P < 0.05$) and an environmental effect among calprotectin expression in the jejunum ($P = 0.0015$) (Figure 11 and 14).

2.5 DISCUSSION

Heat stress negatively affects poultry performance through reductions in feed intake, feed efficiency and body weight gain (Lara and Rostagno, 2013; Pettit-Riley and Estevez, 2001; Sohail et al., 2012; St-Pierre et al., 2003). Many physiological (Geraert et al., 1996), molecular (Star et al., 2008), and immunological (Quinteiro-Filho et al., 2010) processes can experience improper function due to HS induced effects, resulting in improper function of the GI and an increase in the permeability of the gastrointestinal barrier, though these mechanisms are not completely and appropriately defined. As commercial broilers are subjected to HS, the inability to efficiently thermoregulate can cause oxidative stress (Pearce et al., 2013), an influx of reactive

oxygen species (Hao et al., 2012), a decrease in proper immune function (Broom and Kogut, 2018), and the redirection of blood from the visceral region to the exterior of the bird (Pearce et al., 2013). This can cause damage to the intestinal wall, increasing permeability and the ability of opportunistic bacteria to colonize (Ducatelle et al., 2018). Notably, a deactivation of proper immune function and ability of bacteria to pass through a now highly permeable gastrointestinal wall is called, "leaky gut" (Prosser et al., 2004; Lambert et al., 2002). Increased intestinal permeability is becoming a focus in the poultry industry as more is understood concerning the implications that improper gut function can have on efficiencies and intestinal barrier selectivity.

Increases in core body temperature indicate an animal's subjection to HS and can lead to the aforementioned conditions. Increased core body temperature under a HS condition results in a decrease in FCR and BWG (Rosa et al., 2007; Abu-Dieyeh, 2006). The BWG of the 95RAN and MRB modern broilers was significantly, negatively affected by the environmental treatment (Figure 5). The size and high metabolic rate of these modern broilers may play a role in the inability of the bird to withstand the negative impact imparted by HS conditions. Despite the negative effect had on the efficiencies of the 95RAN and MRB, they outperformed the JF and ACRB birds, due to decades of performance driven selection. Figure 5 depicts a clear distinction between the 95RAN and MRB from each other and from the JF and ARCB, whereas the JF and ARCB performed strikingly similar. This depicts the genetic progress that has been made since the 1950s, when the ACRB line was established. The 95RAN and MRB birds, pound for pound, were as efficient with water conversion as the JF and ACRB, yet more efficient with feed conversion and weight gain. Nonetheless, when each specific line was compared across the two environmental treatments the HS 95RAN and MRB broilers were less efficient than their TN

equivalent, lending themselves at a disadvantage to the JF and ACRB which were not as affected.

When subjected to an acute HS broilers begin to breathe more rapidly, or hyperventilate, and biological processes can be negatively affected (Teeter et al., 1984). Birds begin to drink more water leading them to become hyponatremic, having low blood sodium concentration (Yamashiro et al., 2013). The sodium concentration of the blood samples taken from the JF, ACRB, 95RAN, and MRB birds were lower in that of the HS treatment than the TN treatment. Heat stress may indirectly induce hyponatremia in chickens as they attempt to cool themselves with increased water consumption, subsequently leading to lethargy, anorexia, or death. This lack of activity paired with a decrease in appetite negatively impacts the efficiencies of broiler chickens and may also have a negative effect on the integrity of the intestinal barrier, although more research is needed to determine such.

Heat shock proteins serve a major function in the GI, acting as chaperones that protect and repair damaged proteins during bouts of stress (Hao et al., 2012; Dokladny et al., 2006.). In this experiment, expression of HSPs in the GI were shown to be upregulated under HS conditions. As HS is known to denature and unfold proteins, it is intuitive that an upregulation of HSPs during HS is the result of increased activity needed to protect and repair proteins that have been damaged. This upregulation of HSPs during HS in GI tissue further suggests that HS induces increased intestinal permeability, due to damage caused to tight junction, gap junction, and other intestinal barrier proteins.

Damage to these junctional and intestinal barrier proteins increase the intestinal permeability of the chicken, negating the function of a once specific, selective barrier separating the lumen from the body cavity (Groschwitz and Hogan, 2009). In this experiment, significant

changes in the intestinal barrier were recorded. Upregulation of PATJ during an acute HS suggests a mechanistic response to the breakdown of the intestinal barrier. While this mechanism is not well defined, it could be proposed that junctional protein activity is increased due to a feedback mechanism, prompting more junctional connections to be made as the current barrier begins to degrade. The downregulation of occludin in response to HS suggests a structural change in gut morphology under HS, as gene activity is being significantly affected. Gap junction proteins GJA-1 and connexin-45 experienced upregulation in GI tissue. GJA-1 and connexin-45 activity in the gap junctions of the intestinal epithelium during HS is being amplified as the bird's body attempts to protect the intestinal barrier integrity and prevent infection via increased intestinal permeability. During an acute HS, tight and gap junction proteins function together to provide protection to the physiological barrier of the GI to provide the body with a physical enteric defense system.

Villin and lipocalin, both structural proteins of the small intestine are differed among the lines used in the study, suggesting that over many decades the structural integrity of the gut has changed. Over time, with increased selection pressure placed on commercial birds, some may argue that there has been a decrease in the quality and health of the intestinal barrier; however, with feed conversion and efficiency as primary selection traits, it may be that the quality, health, and efficiency of the intestinal barrier has improved over time. While it cannot be said for sure, selection pressures for growth efficiency may go hand in hand with intestinal health.

The research presented assesses the growth performance and intestinal barrier function of 3 broiler populations and their Giant Jungle Fowl ancestor under heat stress conditions. Data collected during the study provide evidence for heat stress' role in negatively affecting growth performance and efficiencies in modern broilers. Additionally, heat stress conditions are shown

to negatively affect the structural proteins involved in managing, maintaining, and repairing intestinal barrier integrity. Delineating the interaction heat stress with the growth and intestinal health of modern broilers and their ancestor will serve to open and expand areas of research involving broiler performance and health, and how the effect of heat stress on such can be mitigated. Further study into the mechanistic function of the intestinal barrier may provide more insight into the current state of the gut as compared to commercial broilers of generations past and their ancestor Jungle Fowl. The dramatic improvements in performance efficiencies due to genetic selection may have served to benefit the overall performance of the gastrointestinal tract.

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2.6 Figures and Tables

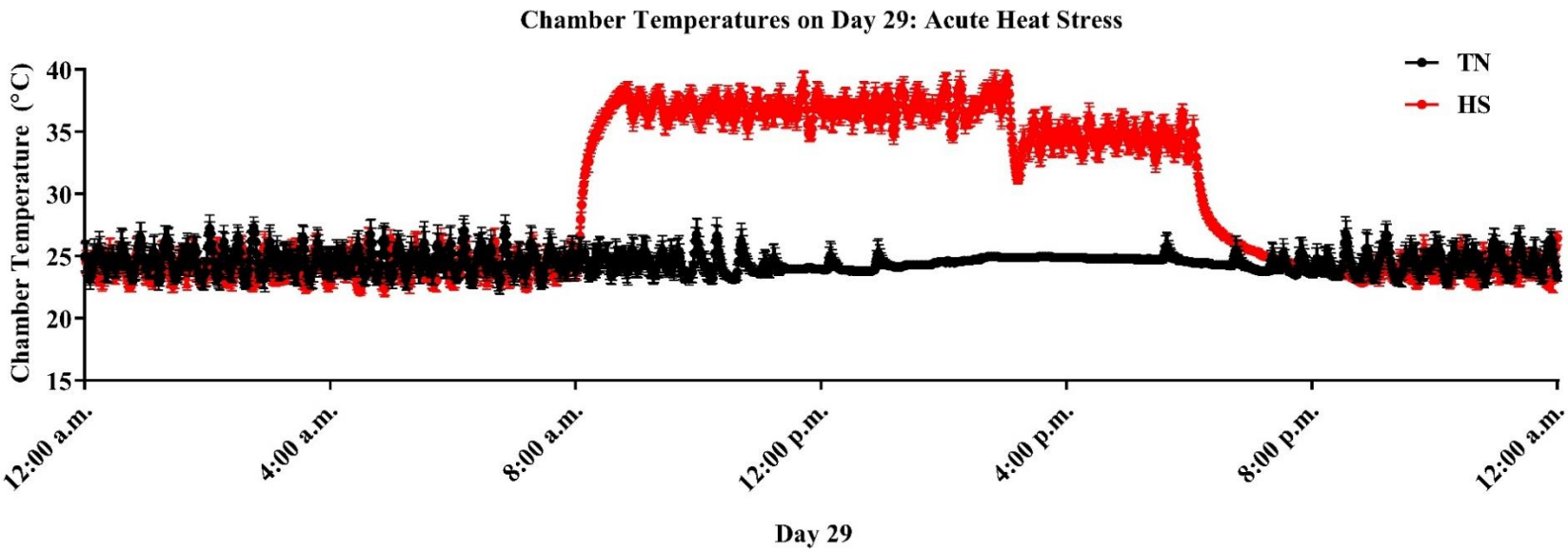

Figure 1. Ambient chamber temperatures on day 29 during which, environmental treatments commenced 2 hours prior to sampling. Thermoneutral (TN) and heat stress (HS) chambers showed a significant difference in chamber temperature, as heat stress chambers reached 36 ºC by 10:00 p.m. at the time of sampling. Thermostats on the HS chambers were turned up to 36 ºC at 8:00 a.m. and turned back down to 24 ºC at 4:00 p.m.; the daily schedule for cyclic chronic HS.

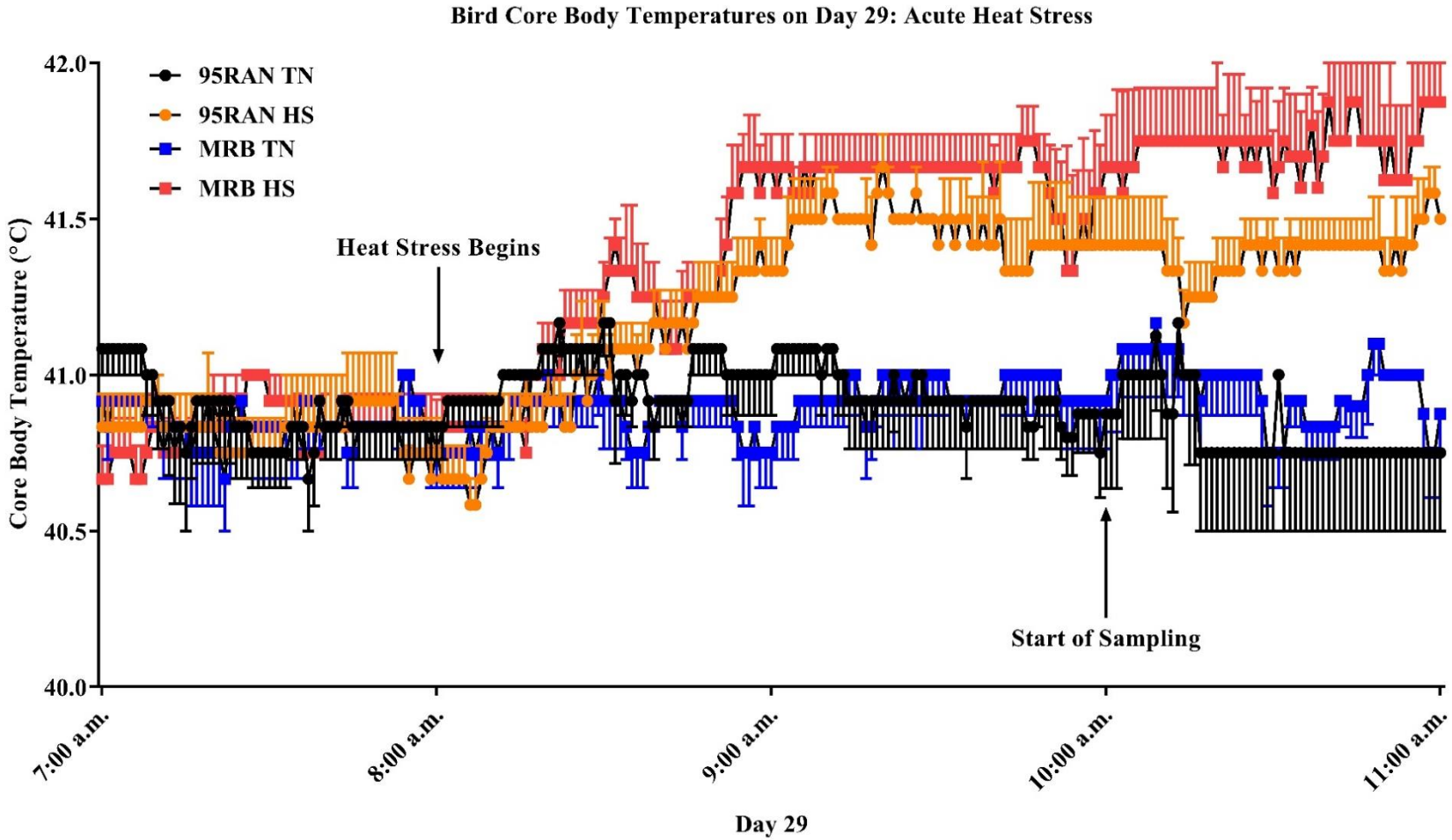

Figure 2. Bird core body temperature on day 29 was recorded during the birds' exposure to acute HS. Between 8:00 a.m., when the chamber temperature began to rise, and 10:00 a.m., when sampling occurred, the core body temperature of the 95RAN HS and MRB HS birds was significantly higher than that of the 95RAN TN and MRB TN. In the 2 hours of acute HS, the core body temperature of the 95RAN HS and MRB HS rose by as much as 1 ºC.

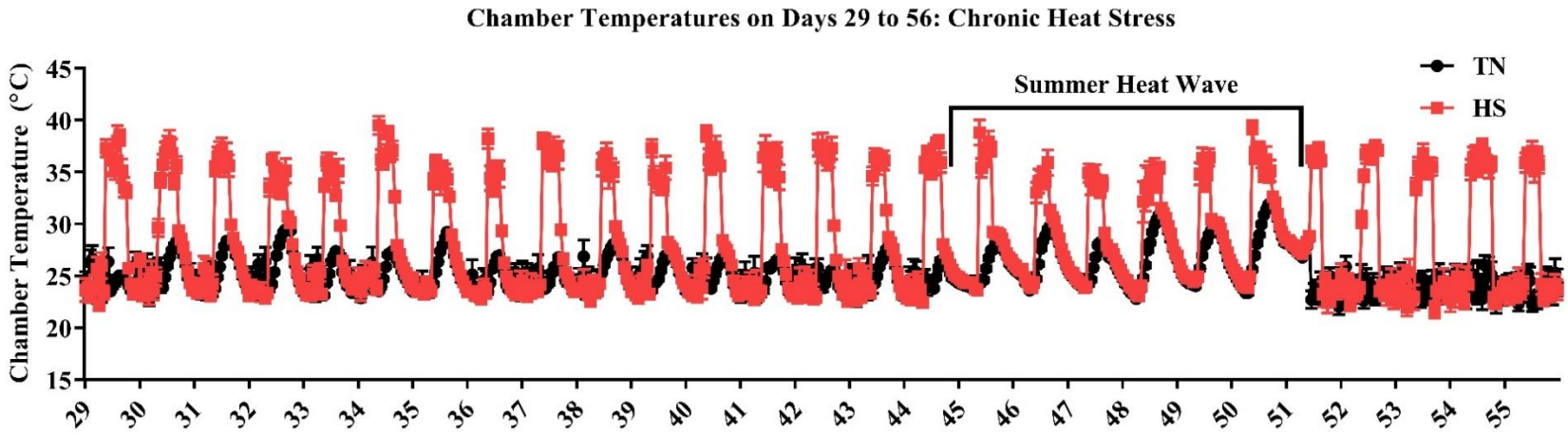

Figure 3. Eight hour cyclic HS was used to induce chronic HS in the study. From day 29 to 56, HS chambers were brought up to 36 ºC at 8:00 a.m. and brought back down to the TN temperature of 24 ºC at 4:00 p.m. Daily increases in the temperature of the HS chambers suggests an induction of a chronic HS condition. For most of the trial, chamber temperatures of both environmental treatments were fairly consistent, with the exception of a heat wave during the mid-summer months. This heat wave resulted in the HS chambers becoming somewhat cooler and the TN chambers warmer. This had no visible effects on the TN birds, as they were not panting or participating in behaviors endemic to HS.

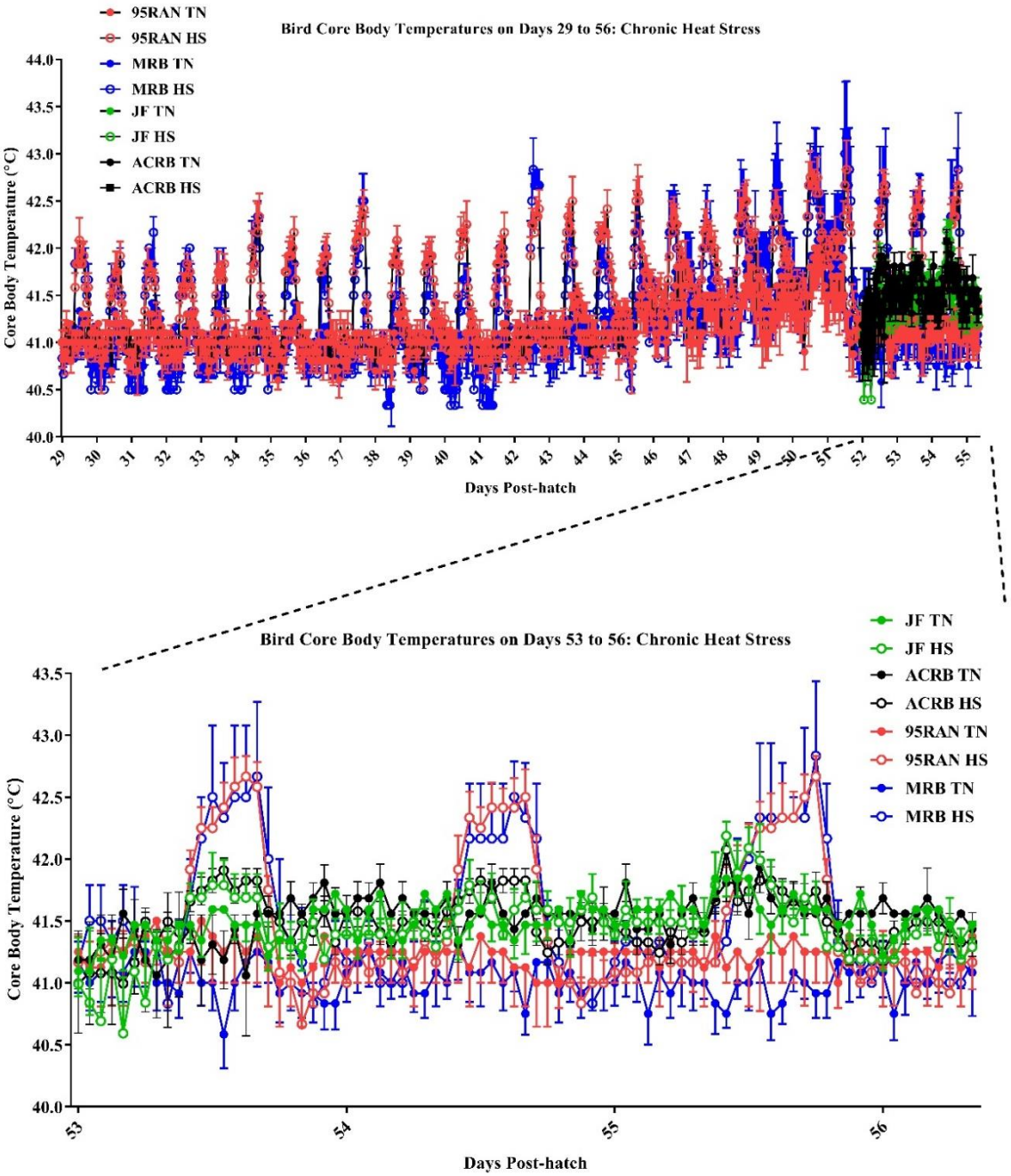

Figure 4. Bird core body temperature on days 29 to 56 with a specific look into the last 3 days of the trial. When looking at the top half of the figure, a trend exists similar to that seen in Figure 3. As temperature in the HS chambers rose and fell each day, so did the core body temperature of the HS birds. Significant increases in core body temperature were experience by the 95RAN and MRB birds under HS conditions, in addition to the ACRB, which seemed behaviorally unaffected for most of the trial, prior to day 53.

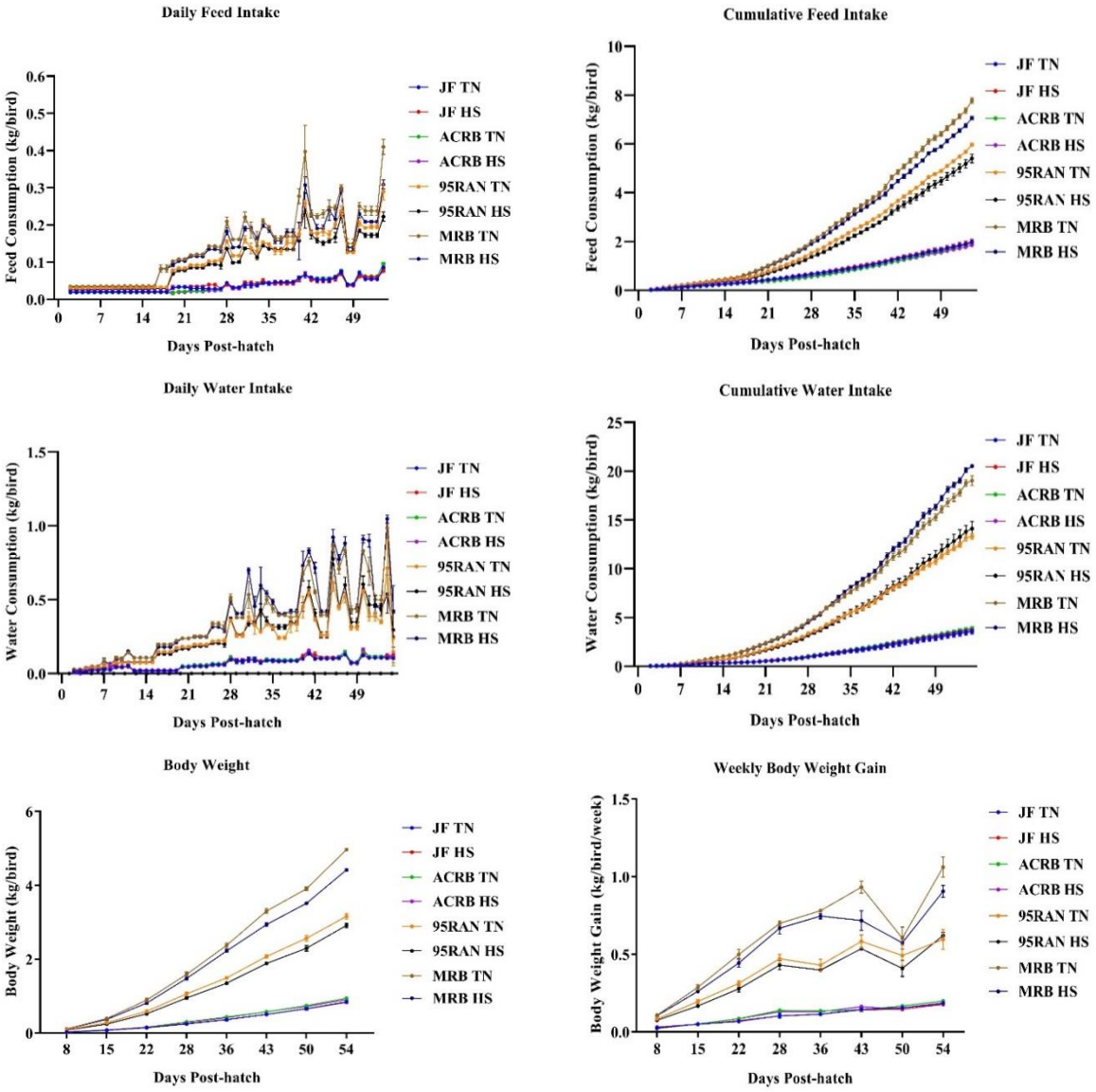

Figure 5. Effects of HS on feed intake, water intake, and growth in modern broilers and their ancestor Jungle Fowl. The HS treatment reduced daily and cumulative feed intake, while increasing daily and cumulative water intake. This resulted in a reduction of body weight and body weight gain. Cumulative feed intake, body weight and body weight gain were significantly reduced among all broiler lines. Data are presented as mean +/- SEM (n=600).

Duodenum

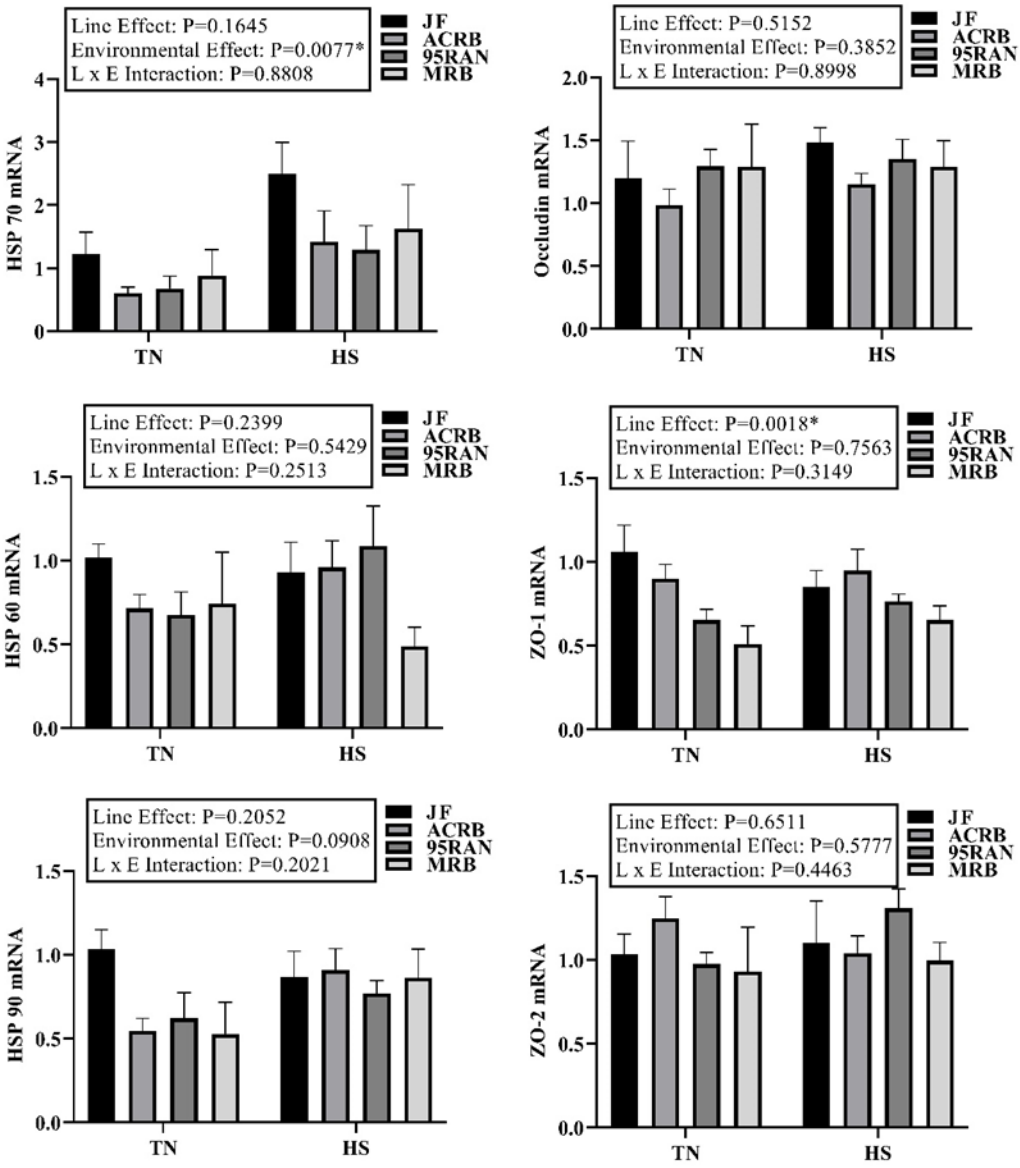

Figure 6. Effect of heat stress (HS) on duodenal heat shock protein (HSP) and tight junction protein mRNA expression in heat stressed (HS) broilers and those maintained under thermoneutral (TN) conditions. Data are presented as mean ± SEM (n = 6/group). Different letters indicate significant difference at $P < 0.05$.

Duodenum (Cont.)

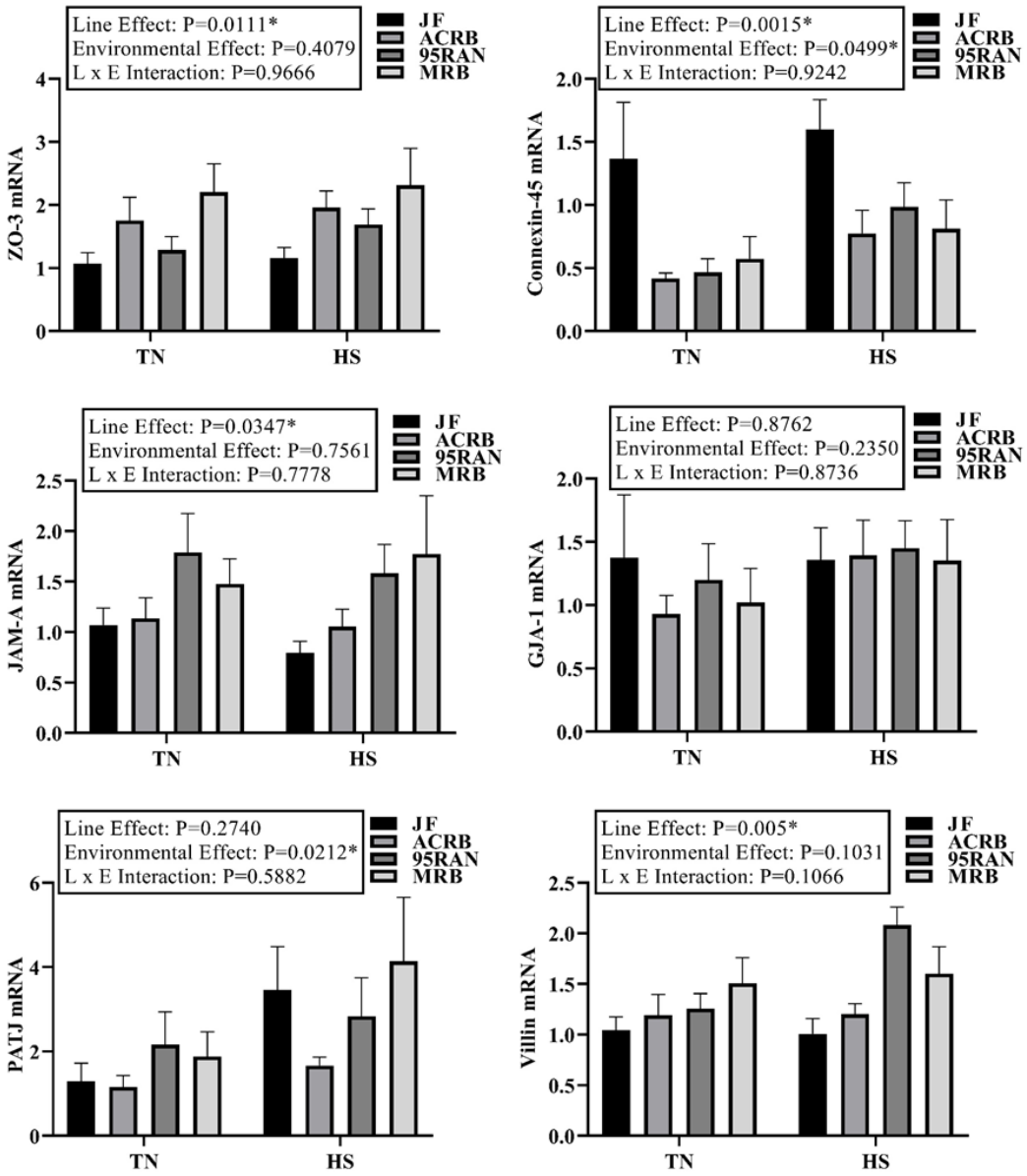

Figure 7. Effect of heat stress (HS) on duodenal tight junction, gap junction, and other intestinal barrier related proteins mRNA expression in heat stressed (HS) broilers and those maintained under thermoneutral (TN) conditions. Data are presented as mean ± SEM (n = 6/group). Different letters indicate significant difference at $P < 0.05$.

Duodenum (Cont.)

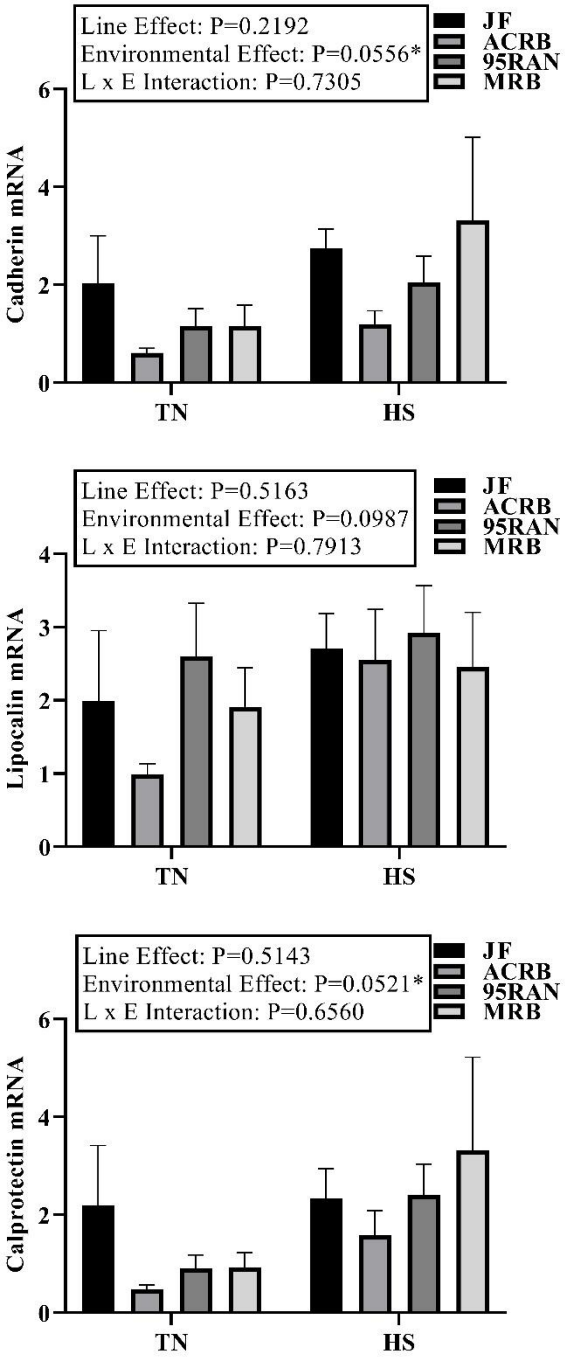

Figure 8. Effect of heat stress (HS) on duodenal intestinal barrier related proteins' mRNA expression in heat stressed (HS) broilers and those maintained under thermoneutral (TN) conditions. Data are presented as mean ± SEM (n = 6/group). Different letters indicate significant difference at $P < 0.05$.

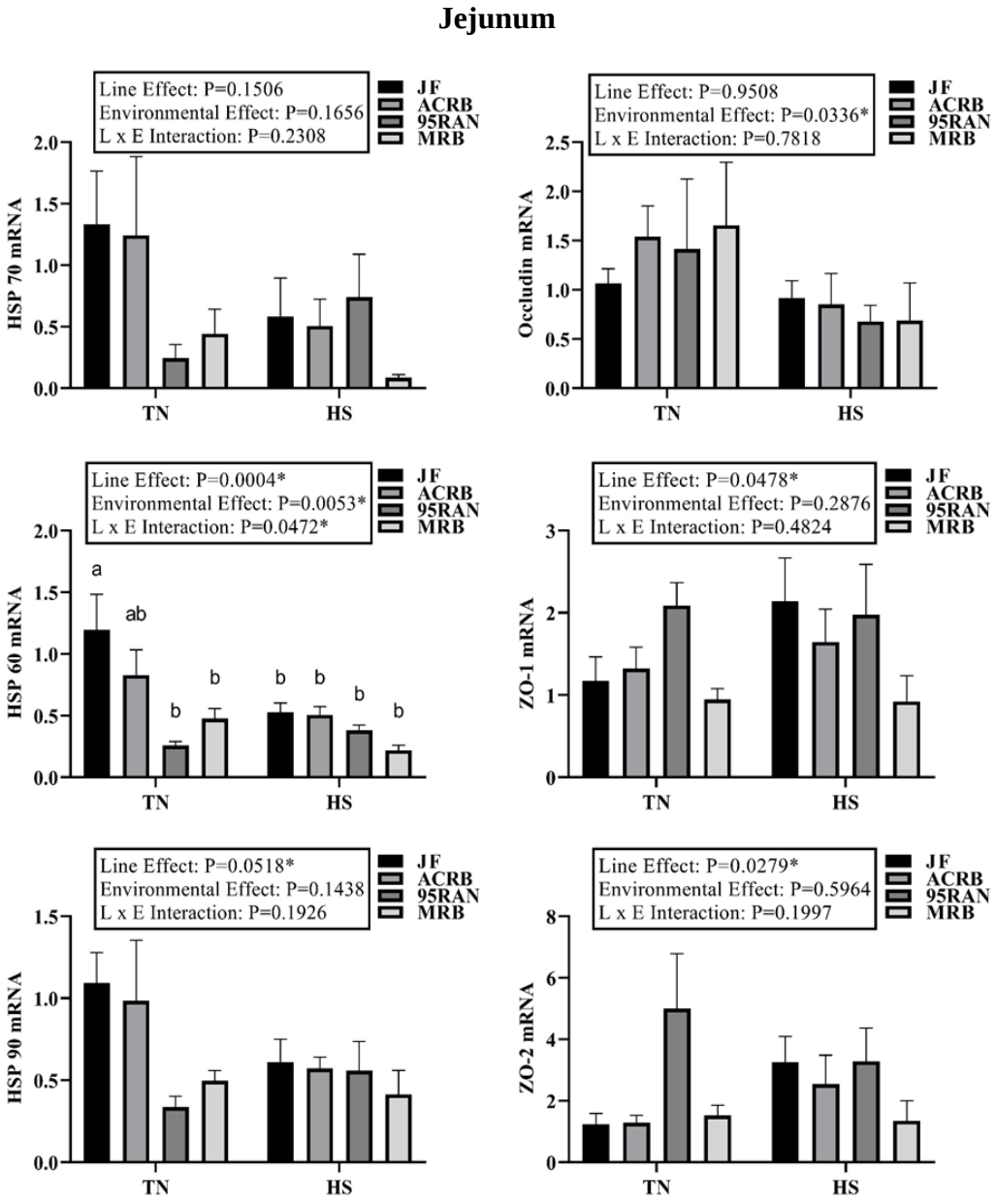

Figure 9. Effect of heat stress (HS) on jejunal heat shock protein (HSP) and tight junction protein mRNA expression in heat stressed (HS) broilers and those maintained under thermoneutral (TN) conditions. Data are presented as mean ± SEM (n = 6/group). Different letters indicate significant difference at $P < 0.05$.

Jejunum (Cont.)

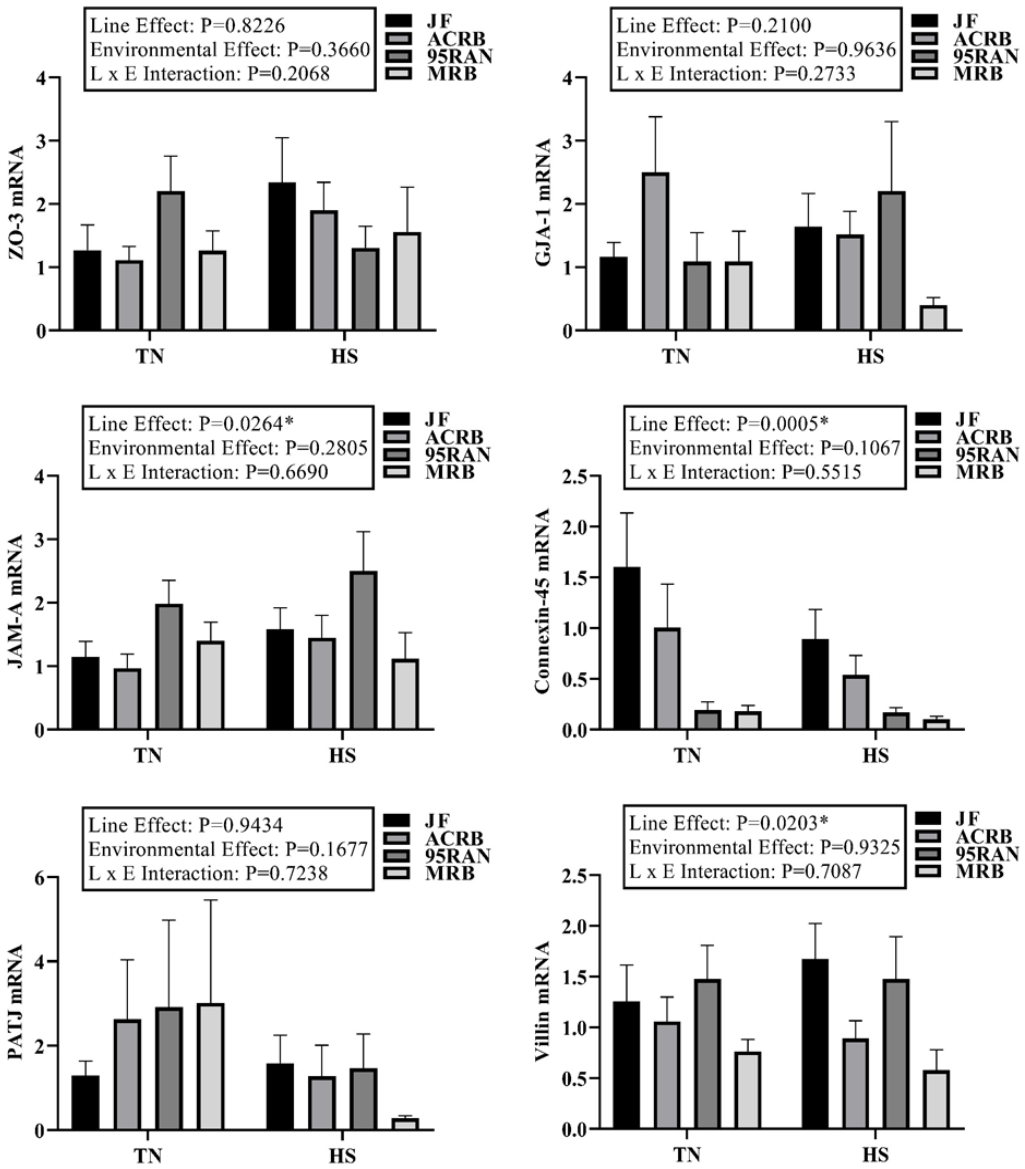

Figure 10. Effect of heat stress (HS) on jejunal tight junction, gap junction, and other intestinal barrier related proteins mRNA expression in heat stressed (HS) broilers and those maintained under thermoneutral (TN) conditions. Data are presented as mean ± SEM (n = 6/group). Different letters indicate significant difference at $P < 0.05$.

Jejunum (Cont.)

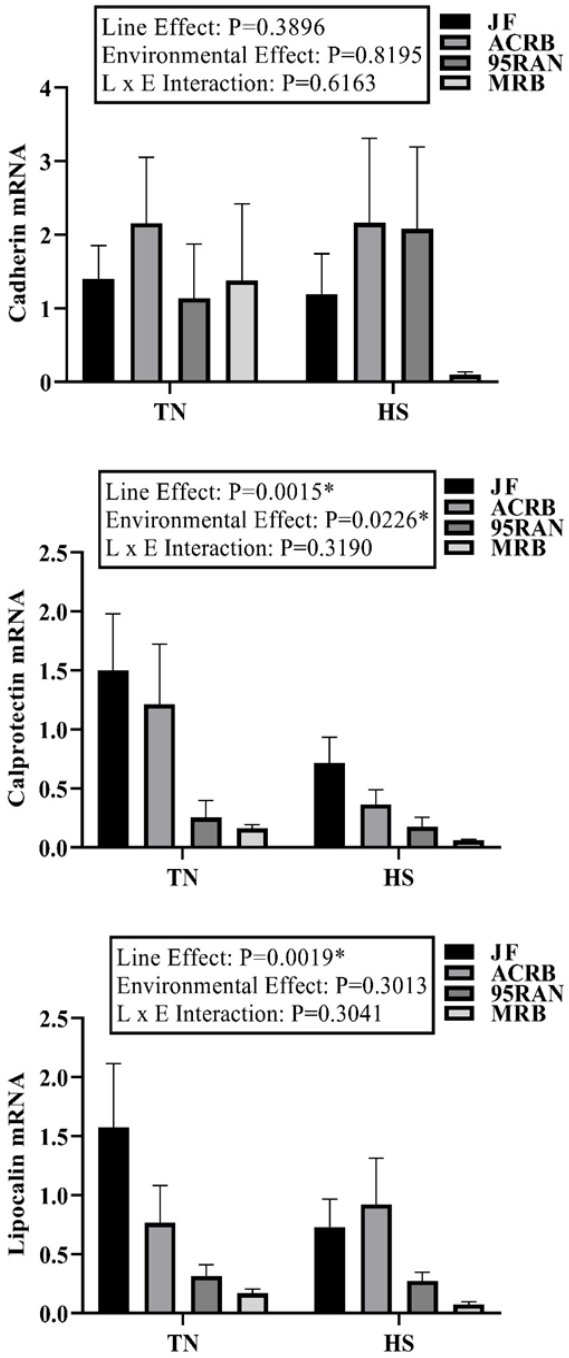

Figure 11. Effect of heat stress (HS) on jejunal intestinal barrier related proteins' mRNA expression in heat stressed (HS) broilers and those maintained under thermoneutral (TN) conditions. Data are presented as mean ± SEM (n = 6/group). Different letters indicate significant difference at $P < 0.05$.

Ileum

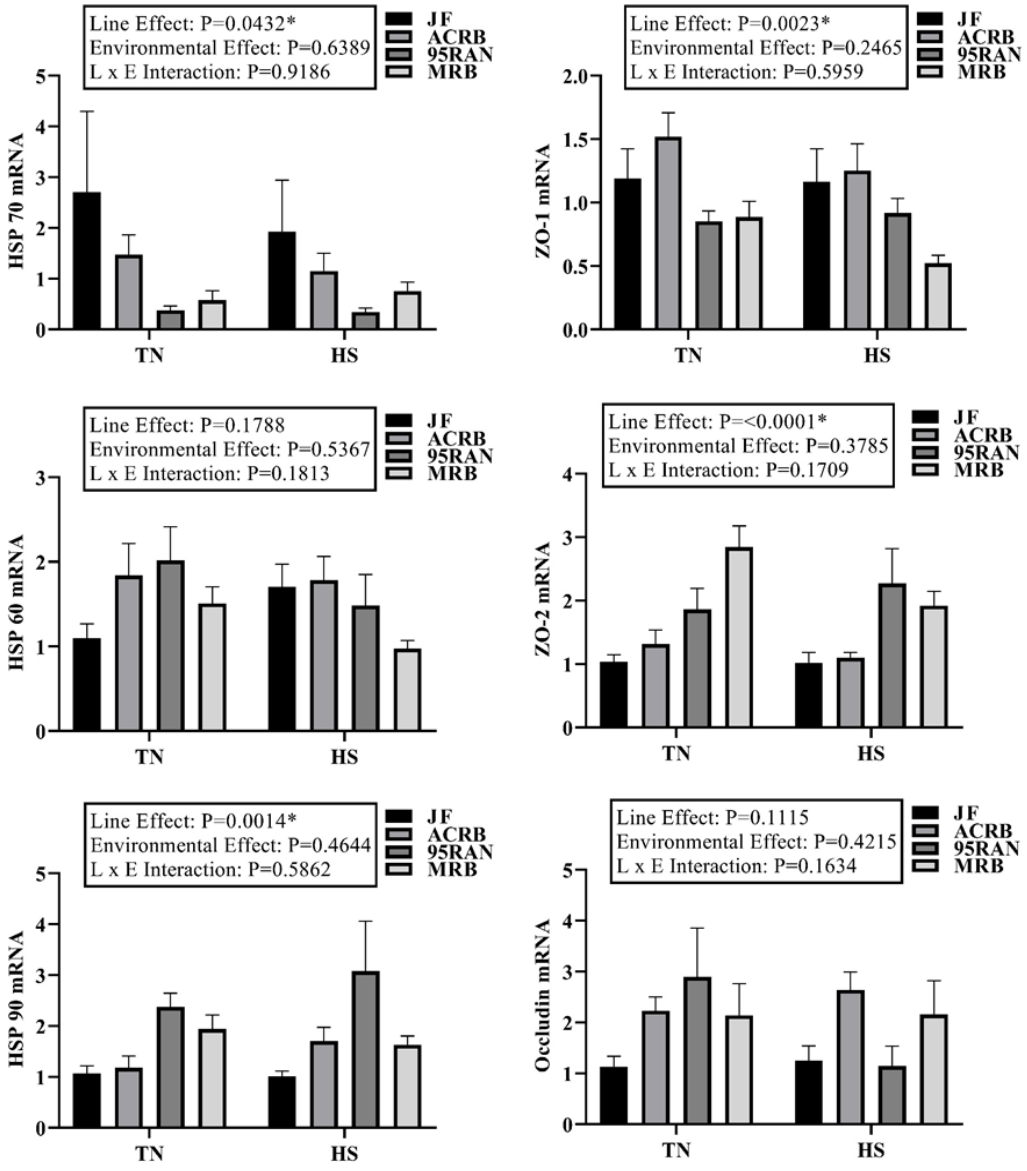

Figure 12. Effect of heat stress (HS) on ileac heat shock protein (HSP) and tight junction protein mRNA expression in heat stressed (HS) broilers and those maintained under thermoneutral (TN) conditions. Data are presented as mean ± SEM (n = 6/group). Different letters indicate significant difference at $P < 0.05$.

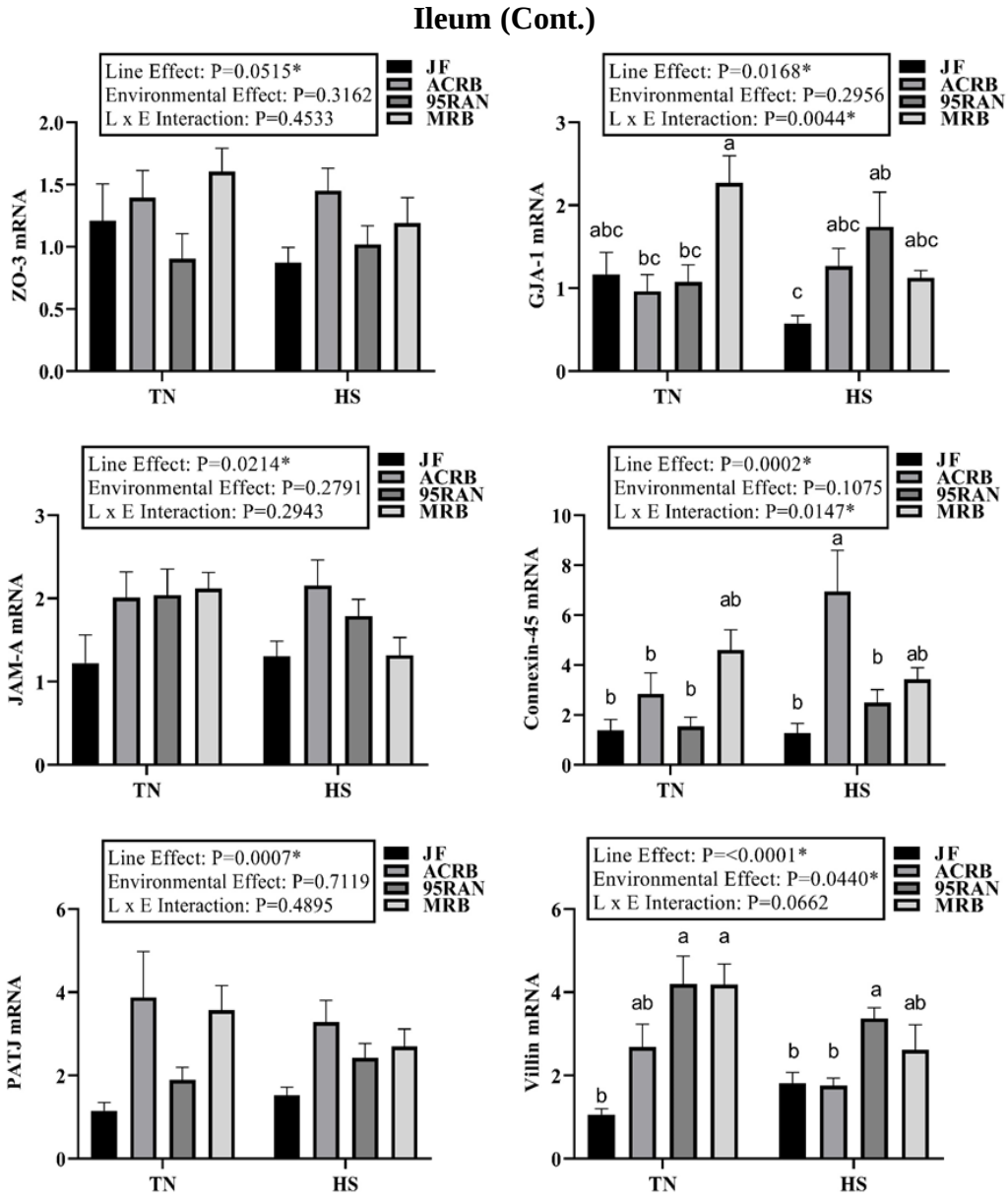

Figure 13. Effect of heat stress (HS) on ileac tight junction, gap junction, and other intestinal barrier related proteins mRNA expression in heat stressed (HS) broilers and those maintained under thermoneutral (TN) conditions. Data are presented as mean ± SEM (n = 6/group). Different letters indicate significant difference at $P < 0.05$.

Ileum (Cont.)

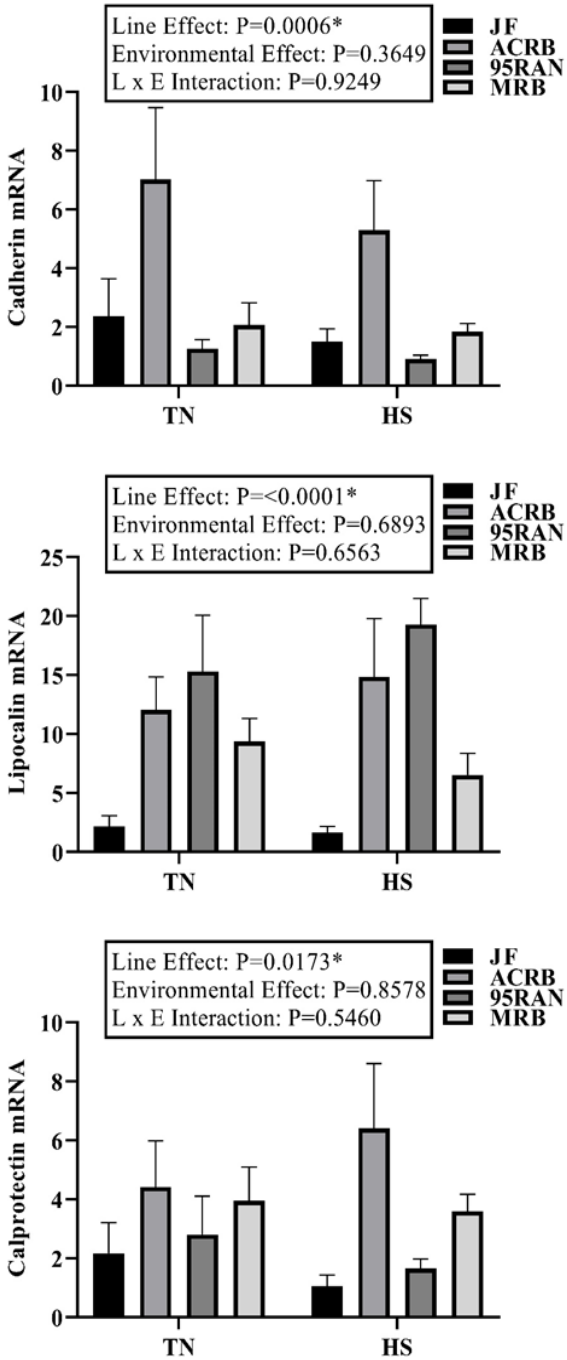

Figure 14. Effect of heat stress (HS) on ileac intestinal barrier related proteins' mRNA expression in heat stressed (HS) broilers and those maintained under thermoneutral (TN) conditions. Data are presented as mean ± SEM (n = 6/group). Different letters indicate significant difference at $P < 0.05$.

Figure 15. Effect of heat stress (HS) on ileac heat shock protein (HSP) and tight junction protein expression in heat stressed (HS) broilers and those maintained under thermoneutral (TN) conditions. Protein compared to housekeeping glyceraldehyde 3-phosphate dehydrogenase GAPDH protein. Data are presented as mean ± SEM (n = 6/group). Different letters indicate significant difference at $P < 0.05$.

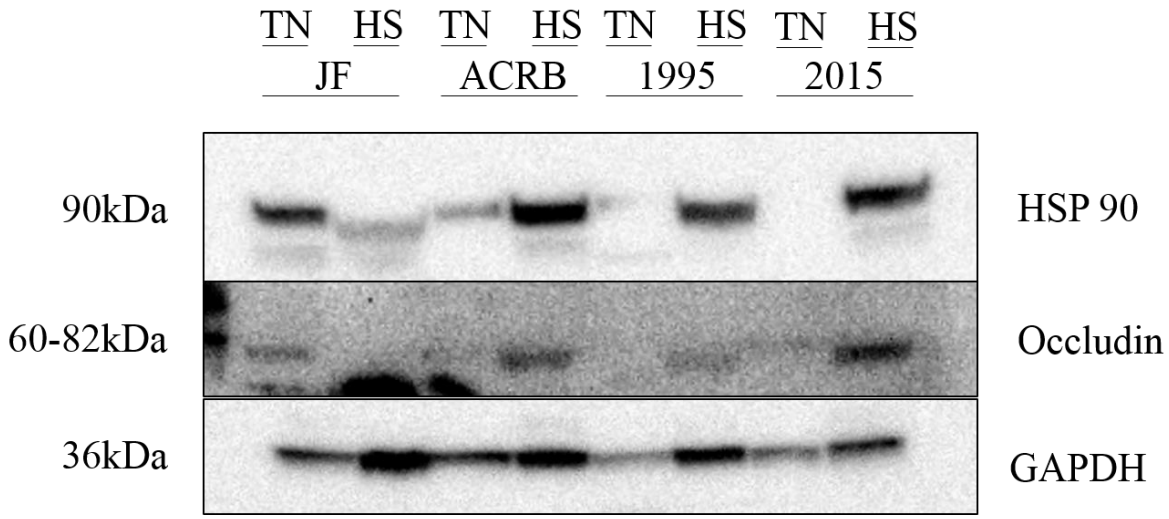

Table 1. Gene specific Oligonucleotide primers used for quantitative real-time PCR.

Gene	Primer sequence (5' → 3')	Orientation	Base Pairs
18s	TCCCCTCCCGTTACTTGGAT	Forward	60
	GCGCTCGTCGGCATGTA	Reverse	
HSP 70	GGGAGAGGGTTGGGCTAGAG	Forward	55
	TTGCCTCCTGCCCAATCA	Reverse	
HSP 60	CGCAGACATGCTCCGTTTG	Forward	2076
	TCTGGACACCGGCCTGAT	Reverse	
HSP 90	TGACCTTGTCAACAATCTTGGTACTAT	Forward	2187
	CCTGCAGTGCTTCAATGAAA	Reverse	
Claudin-1	CCCACGTTTTCCCCTGAAA	Forward	2578
	GCCAGCCTCACCAGTGTTG	Reverse	
Occludin	CGCAGATGTCCAGCGGTTA	Forward	1975
	GTAGGCCTGGCTGCACATG	Reverse	
ZO-1	GGGAACAACACACGGTGACTCT	Forward	7074
	AGGATTATCCCTTCCTCCAGATATTG	Reverse	
ZO-2	GCAATTGTATCAGTGGGCACAA	Forward	4438
	CTTAAAACCAGCTTCACGCAACT	Reverse	
ZO-3	CAAAGCAAGCCGGACATTTAC	Forward	4153
	GTCAAAATGCGTCCGGATGTA	Reverse	
Villin	TGC CGG TGC CCA CTA AAA	Forward	2387
	TCG ACA GCA GCA CGT AGC A	Reverse	
JAM-A	TCACCTCGGAGACAAAGGAAGT	Forward	979
	ACGCAGAGCACGGGATGT	Reverse	
GJA-1	TGGCAGCACCATCTCCAA	Forward	1558
	GGTGCTCATCGGCGAAGT	Reverse	
PATJ	GGATCCAGCAACGTGTCCTATT	Forward	8520
	GCATCCAGTGGAGTGTCTTTCC	Reverse	

Table 1. (Cont.)

Gene	Primer sequence (5' → 3')	Orientation	Base Pairs
Cadherin	GGG AGC GCG TTG CCT ACT A	Forward	4862
	GAG GGC TGC CCA GAT CTG A	Reverse	
Connexin	TCCACCTTCGTTGGCAAAA	Forward	1945
	TCAGAACGATCCGAAAGACGAT	Reverse	
Lipocalin	TGCAGCTTGCAGGGAGATG	Forward	727
	GCTTCTTGTCCTTGAACCAGTTG	Reverse	
Calprotectin	GCTGGAGAAAGCCATTGATGTC	Forward	593
	CCCCTCCCGTCTCGAGTAC	Reverse	

Table 2. Effect of heat stress (HS) on feed conversion ratio (FCR) and water conversion ratio (WCR) of modern broilers and their ancestor Jungle Fowl exposed to environmental conditions: TN or HS. Data are presented as mean ± SEM (n = 6/group) with pen as the experimental unit. Asterisks indicate significant difference at $P < 0.05$.

	JF	ACRB	95RAN	MRB
FCR				
Thermoneutral	2.34 ± 0.053	2.08 ± 0.012	1.89 ± 0.045	1.565 ± 0.031
Heat Stress	2.401 ± 0.140	2.059 ± 0.046	1.855 ± 0.140	1.598 ± 0.006
WCR				
Thermoneutral	4.069 ± 0.247	4.023 ± 0.165	4.166 ± 0.095*	3.781 ± 0.104*
Heat Stress	4.327 ± 0.027	4.123 ± 0.251	4.744 ± 0.367*	4.549 ± 0.043*

Table 3. Effect of heat stress (HS) on blood chemistry, gases, and hematology in modern broilers and their ancestor Jungle Fowl exposed to environmental conditions: thermoneutral (TN) or HS. Blood pH, partial pressure of CO2 (pCO2), total CO2 (TCO2), partial pressure of O2 (pO2), bicarbonate (HCO3-), base excess (BE), O2 saturation (sO2), sodium (Na), potassium (K), ionized calcium (iCa), glucose, hematocrit (Hct), and hemoglobin (HB) were determined using i-STAT Alinity. Data are presented as mean ± SEM (n = 6/group). Asterisks indicate significant difference at $P < 0.05$.

	JF	ACRB	95RAN	MRB	L. Effect	E. Effect	Interaction
pH					0.0009*	0.0659	0.7643
TN	7.428 ± 0.050	7.395 ± 0.016	7.407 ± 0.033	7.441 ± 0.022			
HS	7.416 ± 0.012	7.386 ± 0.019	7.374 ± 0.029	7.429 ± 0.039			
pCO2					0.0778	0.9454	0.1338
TN	35.817 ± 2.920	36.300 ± 4.215	37.033 ± 6.755	38.433 ± 4.010			
HS	34.225 ± 1.335	35.900 ± 4.640	42.183 ± 3.831	35.620 ± 4.958			
pO2					0.6960	0.0787	0.3210
TN	58.500 ± 8.713	66.000 ± 6.455	61.000 ± 6.633	58.667 ± 9.638			
HS	66.250 ± 8.584	64.500 ± 7.932	62.333 ± 8.882	67.200 ± 2.926			
HCO3					0.0096*	0.0590	0.0955
TN	23.017 ± 2.289	21.467 ± 2.191	22.250 ± 2.297	25.400 ± 3.240			
HS	21.200 ± 0.308	20.600 ± 1.920	23.400 ± 1.248	22.420 ± 1.093			
BE					0.0006*	0.1341	0.0857
TN	-0.333 ± 3.037	-2.667 ± 2.055	-1.667 ± 1.886	2.333 ± 3.590			
HS	-2.250 ± 0.433	-3.400 ± 1.744	-0.050 ± 1.500	-0.400 ± 1.020			
sO2					0.1930	0.7081	0.2489
TN	83.500 ± 7.320	87.500 ± 3.354	84.167 ± 4.845	83.333 ± 5.406			
HS	87.250 ± 4.763	86.400 ± 3.929	80.500 ± 7.136	86.600 ± 2.653			
TCO2					0.0147*	0.0604	0.0630
TN	24 ± 2.380	22.333 ± 2.494	23.000 ± 2.236	26.500 ± 3.304			
HS	22.000 ± 0.000	21.600 ± 1.744	24.333 ± 1.374	23.200 ± 1.662			

Table 3. (Cont.)

	JF	ACRB	95RAN	MRB	L. Effect	E. Effect	Interaction
Na					0.3928	0.0006*	0.6000
TN	148.500 ± 3.403	149.500 ± 4.113	150.167 ± 1.572	149.167 ± 2.267			
HS	146.000 ± 0.000	145.667 ± 2.134	147.167 ± 0.687	148.000 ± 2.530			
K					<0.0001*	0.7972	0.1230
TN	5.017 ± 0.211	5.333 ± 0.629	4.783 ± 0.121	4.617 ± 0.203			
HS	4.825 ± 0.030	5.483 ± 0.302	5.117 ± 0.441	4.420 ± 0.160			
iCa					0.3815	0.2823	0.0488*
TN	1.123 ± 0.155	1.147 ± 0.164	0.977 ± 0.172	1.140 ± 0.101			
HS	1.150 ± 0.090	1.167 ± 0.063	1.178 ± 0.117	1.048 ± 0.085			
Glucose					0.0193*	0.0472*	0.0385*
TN	189.333 ± 11.397	196.000 ± 18.886	173.833 ± 15.826	190.833 ± 11.393			
HS	196.750 ± 20.104	209.500 ± 10.844	198.167 ± 9.703	180.000 ± 14.546			
Hct					0.1022	0.5538	0.4548
TN	21.833 ± 3.848	21.500 ± 0.500	21.000 ± 2.449	19.000 ± 2.160			
HS	21.250 ± 1.639	23.333 ± 1.599	20.000 ± 3.162	20.600 ± 4.079			
Hb					0.0980	0.5644	0.4553
TN	7.433 ± 1.31	7.300 ± 0.200	7.140 ± 0.833	6.450 ± 0.741			
HS	7.225 ± 0.576	7.933 ± 0.547	6.800 ± 1.075	6.980 ± 1.391			

UNIVERSITY OF ARKANSAS

Office of Research Compliance

To:	Nicholas Anthony
Fr:	Craig Coon
Date:	February 9th, 2018
Subject:	IACUC Approval
Expiration Date:	February 1st, 2021

The Institutional Animal Care and Use Committee (IACUC) has APPROVED your protocol # **18083:** *General Rearing of Selected chicken and Quail Populations*.

In granting its approval, the IACUC has approved only the information provided. Should there be any further changes to the protocol during the research, please notify the IACUC in writing (via the Modification form) prior to initiating the changes. If the study period is expected to extend beyond February 1st, 2021 you must submit a newly drafted protocol prior to that date to avoid any interruption. By policy the IACUC cannot approve a study for more than 3 years at a time.

The following individuals are approved to work on this study: Nicholas Anthony, Sara Orlowski, and Joseph Hiltz. Please submit personnel additions to this protocol via the modification form prior to their start of work.

The IACUC appreciates your cooperation in complying with University and Federal guidelines involving animal subjects.

CNC/tmp

18083

UNIVERSITY OF ARKANSAS

Office of Research Compliance

MEMORANDUM

To:	Sam Dridi
From:	Craig Coon, IACUC Chair
Date:	July 08, 2016
Subject:	IACUC Approval
Expiration Date:	July 7, 2019

The Institutional Animal Care and Use Committee (IACUC) has APPROVED your protocol 3 16084 "Regulation of energy homeostasis and fat metabolism in avian species."

In granting its approval, the IACUC has approved only the information provided. Should there be any further changes to the protocol during the research, please notify the IACUC in writing (via the Modification form) prior to initiating the changes. If the study period is expected to extend beyond July 7, 2019 you must submit a newly drafted protocol prior to that date to avoid any interruption. By policy the IACUC cannot approve a study for more than 3 years at a time.

The IACUC appreciates your cooperation in complying with University and Federal guidelines involving animal subjects.

CNC/aem
cc: Animal Welfare Veterinarian

Button

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December 13, 2019

To whom it may concern,

Approval and training were granted to Travis Tabler to study under IACUC Protocol 16084 and 18083 until their expiration on July 7, 2019 and February 1, 2021 respectively. If you have any questions regarding this please feel free to contact me at nanthony@uark.edu or by phone (479) 601-3271.

Sincerely,

Dr. Nicholas B. Anthony
Professor Emeritus
Poultry Center of Excellence
University of Arkansas
Fayetteville AR, 72701